Study of the action of commercial plant mixtures in the biochemistry of a cell line

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“It is a scientific fact that your body
will not absorb cholesterol
if you take it from another person's plate.”
Dave Barry

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Abstract

The main goal of this thesis was to evaluate the effect of commercial mixtures of plants, sold for the purpose of lowering cholesterol in circulation, in the cholesterol transporters present in HepG2 cells (human liver carcinoma cell line), as well as it's expression in different culture conditions. The transporter studied was NPC1L1. It was also studied the influence of the mixtures in the metabolism of the cell line in order to check if there are changes in metabolites when the cells are in contact with the herbal mixture.

The variation in the expression of the transporter NPC1L1 as well as it's presence in HepG2 cells grown in contact with the herbal mixture was observed performing an electrophoresis and western blot using a polyclonal antibody to identify the transporter. A 2D electrophoresis was also performed to analyze the variation of the proteins. The study of the variation of cell metabolites and the major compounds of the extract was done by an analysis in a high performance chromatography. It was also studied the variation of protein content of HepG2 cells grown in contact with the extract by the Bradford method.

With the different studies and analysis it was possible to verify the presence of the transporter in the cultured cells with the lowest extract concentration which is consistent with the previous results on the effect of the extract in the reduction of cholesterol in circulation. It was also concluded that the extract leads to a reduction in the protein content as also in the expression of some proteins. In the metabolites analysis the differences among the cells grown in different conditions were minimal. When compared with the extract used it is concluded that the cells probably metabolize these compounds.

Key words: cholesterol, NPC1L1, HepG2 cells

Resumo

O objectivo desta tese foi avaliar o efeito das misturas de plantas comerciais, vendidas com o propósito de baixar o colesterol em circulação, nos transportadores, presentes nas células HepG2 (células cancerígenas do fígado humano), envolvidos no processo bem como a sua expressão nas diferentes condições de cultura das células. O transportador em estudo foi o NPC1L1. Foi ainda estudada a influência das misturas no metabolismo da linha celular indicada de modo a verificar se ocorrem alterações nos metabolitos quando as células se encontram em contacto com as misturas.

A variação na expressão do transportador bem como a sua presença nas células semeadas em contacto com o extracto foi verificada a partir de uma electroforese e western blot utilizando um anticorpo policlonal para identificação do transportador. Foi também realizada uma electroforese 2D de modo a analisar a variação das proteínas. O estudo da variação dos principais compostos e dos metabolitos das células foi feito a partir de uma análise por cromatografia de alta eficiência do conteúdo celular. Foi ainda realizado um estudo da variação do conteúdo proteico das células HepG2 cultivadas em contacto com o extracto através do método de Bradford.

Através dos estudos realizados foi possível verificar a presença do transportador nas células cultivadas com uma concentração mais baixa de extracto o que está de acordo com os resultados obtidos anteriormente sobre o efeito do extracto na redução do colesterol em circulação. Conclui-se ainda que o extracto tem um efeito de redução do conteúdo proteico bem como na expressão de algumas proteínas. Relativamente aos metabolitos as diferenças são mínimas entre as células semeadas em diferentes condições. Quando comparados com o extracto utilizado conclui-se que estes são provavelmente metabolizados pelas células.

Palavras-chave: colesterol, NPC1L1, células HepG2

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List of Abbreviations

ABC1 - ATP-binding cassette
ABCG5/8 - ATP-binding cassette hemi-transporter Gx
ACAT – Acyl CoA cholesterol acyltransferase
APS – Ammonium persulfate
BC – Biliary cholesterol
BSA – Bovine Serum Albumin
CHOL – Cholesterol
CO_2 – Carbon dioxide
CHD – Coronary Heart Disease
CVD – Cardiovascular disease
DC – Dietary cholesterol
DMEM – Dulbecco's Modified Eagle Medium
DTT – Dithiothreitol
EDTA-Na_2.2H_2O – Ethylene Diaminetetra acetic acid Disodium-Calcium Salt
ERC – Endocytic recycling compartment
FBS – Fetal bovine serum
HCl – Hydrochloric acid
HDL – High density lipoprotein
HMG-CoA – 3-hidroxy-methylglutaryl coenzyme A
LDL – Low density lipoprotein
LDL-C – Low density lipoprotein cholesterol
MeOH – Methanol
MTP – Microsomal triglyceride transfer protein
NaCl – Sodium chloride
NADPH – Nicotinamide adenine dinucleotide phosphate
Na_2CO_3 – Sodium carbonate
NPC1L1 – Niemann Pick C1 Like 1
PBS – Phosphate buffered saline
PS – Phytosterols
SDS – Sodium dodecyl sulfate
SDS-PAGE – Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis
SREBP – Sterol regulatory element-binding protein
SSD – Sterol sensing domain
SR-B1 – Scavenger receptor B1
TFA – Trifluoroacetic acid
TEMED – Tetramethylethylenediamine
Tris – Tris(hydroxymethyl)aminomethane
V – Volt

1. State of the art and objectives

Cholesterol is a controversial topic. While some researchers and doctors say that cholesterol brings health problems others claim that advertisements about diets and studies on the harms of diets rich in cholesterol are only studies commissioned by major pharmaceutical and food industries (Epide- et al. 1994).

The effects of the cholesterol and the diets are different between organisms since the response of the body is not the same. Even the mechanism to uptake of cholesterol and the regulatory response are not similar. This affects the studies since it can't be fully understand and generalized the way cholesterol acts on the human body (Manuscript & Transport 2014).

For the mammals, cholesterol is a structural component of cell membranes essential for maintaining membrane integrity, permeability and fluidity, a signaling molecule and also the precursor for the synthesis of steroid hormones and bile acids (Lecerf & Lorgeril 2011). The conversion of the cholesterol in bile acids had become very important since it was the font to energy acquisition and survival through the time since the daily diet was not sufficient (Olson 1998). This conversion is also critical for the intestinal absorption of cholesterol.

The main process to acquire cholesterol from animal sources is by absorption in the intestinal lumen, through some transporters localized in the membrane, from the dietary intake and biliary secretion but it can also be synthesized from simple substrates in order to maintain a stable pool when dietary intake is low. However this process requires a high amount of energy and therefore has evolved a physiological process to absorb the available cholesterol in the gut lumen (Lecerf & Lorgeril 2011). The daily intake of cholesterol in humans with a Western diet is approximately 300 to 500 mg and the daily secretion of cholesterol into bile is approximately 800 to 1200 mg (Wang 2007).

A high level of cholesterol is one of the major risks for cardiovascular diseases and atherosclerotic cardiovascular disease (CVD) in the world. In 2014 Europe had the highest prevalence of high cholesterol in the world with 54 % for both men and women. This factor contributes to 2.6 million deaths per year worldwide (Organization 2015). In the United States the American Heart Association accounted for 34.3 % of all deaths caused by CVD in 2006. The Europe is the continent where the levels of cholesterol in the population are the highest. In the five biggest countries in EU – Germany, France, Italy, Spain and UK, 133 million people suffer from this (Organization 2015). Therefore for all the disagreements and lack of knowledge in this subject, there are doubts in the process.

The use of medicinal plants, an ancient practice and the first way to treat some diseases, was always a complementary treatment along with the pharmaceutical drugs and also the source of the compounds with action in some diseases. However the benefits and even the way medicinal plants act in the human body remain unknown or at least not fully understand and therefore the research in this field becomes important to guarantee the credibility of them. This subject divides the population and doctors since the lack of knowledge creates doubts about the interference of the plants with the drugs taken in some treatments. There are cases of intoxication and even dead because of the high amount

of plants taken which in high concentrations are toxic for the cells. This is the main reason for the doctors disapprove this use. However in the last years it was proven that the plants have benefits and compounds that are anti-oxidant and have an important role in some cancer cases (Maciel et al. 2002).

For the case of cholesterol it can be found some commercial herbal mixtures that are sold with the aim of lowering the cholesterol in circulation in the human body. These mixtures are prepared according to the population knowledge and therefore it's not proved the purpose effect.

The understanding of the herbal mixtures role becomes important in order to prevent the humans from being intoxicated and verifies another way of treatment since the drugs commercialized nowadays, based in statins, have second's effects in the human body.

The purpose of this work is to verify the influence of one commercial herbal mixture reductive of the total cholesterol in circulation in the transporters localized in the HepG2 cell membrane and in the metabolism of them. This work corresponds to a continuation of a study done before where it was studied the inhibitory activities of herbal mixture in the synthesis and cholesterol absorption (appendix B and C). Since the results were positive and it was proved the ability of the herbal mixture in lowering the absorption of cholesterol and inhibition of the action of the enzyme responsible for the synthesis, it became interesting to verify the influence in the expression of the transporter as well as the influence in the expression of the proteins produced by the cells and their metabolism.

HepG2 cells belong to the human liver carcinoma. Since the presence of the transporters are also found in the membrane cells contituting the liver, and is where the cholesterol is synthesized, the HepG2 cells were chosen to avaliate the points in interest .

Therefore, this work is divided in chapters where is presented the information about the cholesterol and his mechanism, the results and discussion and the final conclusion and future perspectives.

2. Introduction

2.1. Discovery of cholesterol

Cholesterol was first discovered in the bile and gallstones by Poulletier de la Salle in 1769 (Dam 1958) and then rediscovered in 1815 by Chevreul who named it as cholesterine. Only a few years later was found in the blood. Only in the 20th century Heinrich Wieland identified the structure of cholesterol. In 1964 Konrad Bloch and Feodor Lynen discovered the mechanisms and regulation of cholesterol and fatty acid metabolism. The investigators received the Nobel Prize in Physiology or Medicine for the work in this topic (Gibbons 2003).

A few years later the researchers found the lipoproteins – protein-lipid complexes, transporters of cholesterol in the blood, and divided it into groups. In 1929 Michel Macheboeuf isolated from horse serum a stable and water-soluble lipoprotein that contained 59 % protein and 41 % lipid, which consisted of 18 % cholesterol and 23 % phospholipid. This lipoprotein could be dissolved again in water and form a clear solution. Later it was shown that this lipoprotein was a α-globulin and had the same composition as the α-lipoprotein that is known today as HDL (high density lipoprotein) (Olson 1998).

During the Second World War, Cohn (Olson 1998) isolated a variety of proteins from the human plasma. Since the lipoproteins were needed to the wounded soldiers the purpose of the work was to fractionate the human plasma into five protein families. This was made by gradual variations in pH, ionic strength and ethanol concentration at low temperatures (from 0 to 5 °C). With this work they discovered two fractions containing plasma lipids – fractions III and IV. Later was isolated a lipoprotein – β-globulin from the fraction III and the chemical analysis showed that the lipoprotein had 23 % protein, 30 % phospholipid, 8 % free cholesterol and 39 % cholesterol esters. The content of this lipoprotein is very similar to the LDL (low density lipoprotein) know nowadays (Olson 1998). The fraction IV was very similar to the HDL discovered before (Olson 1998). During the following years the research on the lipoproteins continued and therefore the understanding of what was the cholesterol and the structures involved in the process. Nowadays the research focuses on the transporters of cholesterol in the cells and new development of drugs to lower the cholesterol main cause of the some cardiovascular diseases.

2.2. Definition of cholesterol

Cholesterol ($C_{27}H_{46}O$, M=386.65 g.mol^{-1}) is an organic molecule more specifically a sterol or modified steroid also called cholest-5-en-3β-ol (figure 1). It's a lipid molecule and is biosynthesized by all mammals in order to maintain a stable pool when dietary intake is low. It is absent in plants, which have other sterols or phytosterols such as sitosterol, campesterol, stigmasterol, among others. The human body does not synthesize those sterols. The main functions of cholesterol are as a support compound of the membrane given it fluidity, integrity and permeability and as the precursor of vitamin D (synthesized in the skin), adrenal and gonadal steroid hormones and bile acids. The presence of cholesterol in the membranes enables animal cells to not need a cell wall (like plants and bacteria), to

protect membrane integrity/cell-viability and thus be able to change shape and move (unlike bacteria and plant cells which are restricted by their cell walls) (Lecerf & Lorgeril 2011).

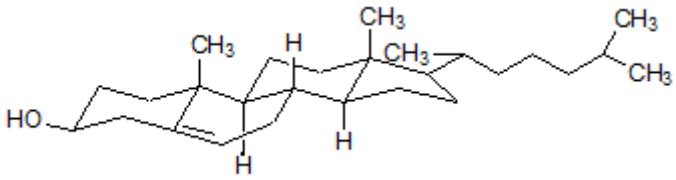

Figure 1 – Structure of cholesterol (James Hutton Institute (and Mylnefield Lipid Analysis) 2014).

Since cholesterol is required in the human body different mechanisms are available to provide the cholesterol needed. The mechanisms are the intestinal uptake, enterohepatic bile acid and cholesterol reabsorption cycles. In contrast phytosterols are poorly absorbed and rapidly excreted.

The cholesterol molecules derive from the novo synthesis and intestinal absorption. They are carried in the blood into lipoproteins as said before forming a protein-lipid complex and delivered to the cells for utilization via the circulation. Cholesterol can bind to low-density-lipoproteins or high-density-lipoproteins (Manuscript & Transport 2014). When it binds to LDL are called “bad cholesterol” and linked to HDL is called “good cholesterol” (Lecerf & Lorgeril 2011). High levels of total cholesterol in circulation and low-density-lipoprotein (LDL-C) cause atherosclerotic cardiovascular disease the leading cause of death in developed countries (Manuscript & Transport 2014) on the other hand HDL carries cholesterol from other parts of the body back to the liver. The liver removes the cholesterol from the body (Lecerf & Lorgeril 2011). The excess of cholesterol is disposed of in the feces through biliary and maybe direct intestinal secretion (Manuscript & Transport 2014).

The body can’t break cholesterol therefore when the dietary intake of cholesterol is too high, biliary and intestinal excretion will be intensified therefore when the dietary intake is low the synthesis and absorption are up-regulated to guarantee the amounts of cholesterol needed (Lecerf & Lorgeril 2011). The dietary cholesterol content doesn’t have influence in the plasma cholesterol values. These values are regulated by different genetic and nutritional factors and are the main causes to differences in cholesterol absorption or synthesis. In this sense mammals can be separated into two groups hyper-absorbers and others as hyper-responders leading to the need of creating new therapeutic issues to understand and create ways to regulated the different responses (Lecerf & Lorgeril 2011).

The efficient transport of cholesterol in the body depends on many proteins. The defects on the transport-related proteins and in proteins involved in cholesterol biosynthesis and its regulation disturb the body cholesterol homeostasis leading to the appearance of diseases.

2.3. Cholesterol biosynthesis

The synthesis of cholesterol begins with the formation of the enzyme 3-hidroxy-methylglutaryl coenzyme A (HMG-CoA). This molecule is formed by hydration of one molecule of acetoacetyl-CoA and acetyl CoA in the cytosol and catalyzed by the HMG-CoA synthase. This reaction is presented in figure 2.

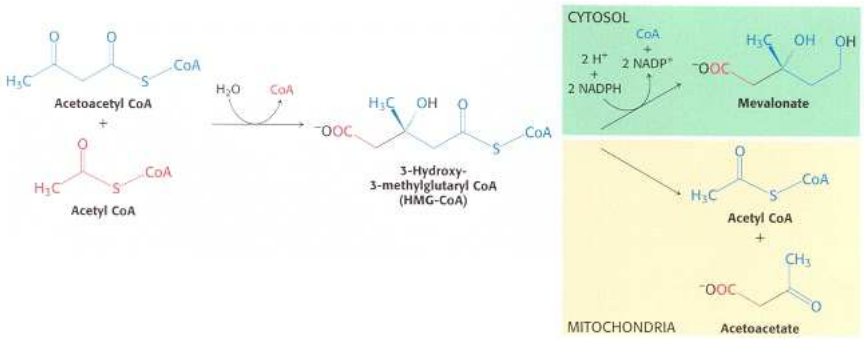

Figure 2 – First step of cholesterol synthesis.

Mechanism to produce HMG-CoA. It can be converted into two different products when is located in the cytosol – mevalonate or in mitochondria – acetyl CoA and acetoacetate. In the mitochondria HMG-CoA is used to form ketone bodies (Company. 2002).

The next procedures since the enzymes are membrane-bound and located in the endoplasmic reticulum occur in this organelle. The molecule of HMG-CoA is reduced to mevalonate by the enzyme HMG-CoA reductase that is an integral membrane protein in the endoplasmic reticulum. This step is regulated by several factors, rate limiting and irreversible in the cholesterol and sterol synthesis. The statins drugs taken to decrease the levels of cholesterol synthesized operate in this step since they are HMG-CoA reductase competitive inhibitors (Company. 2002).

After the formation of mevalonate three reactions requiring ATP take place to convert the compound in 3-isopentenyl pyrophosphate by decarboxylation (figure 3). After this reaction the molecules are condensate to form two farnesyl pyrophosphate (Company. 2002). In figure 4 is presented the complete mechanism to produce the farnesyl pyrophosphate (figure 4).

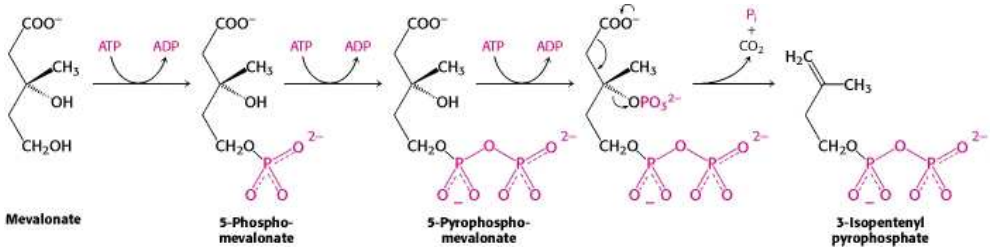

Figure 3 – Second step of cholesterol synthesis.

Synthesis of Isopentenyl pyrophosphate in three steps with the formation of intermediate compounds. The final step is the decarboxylation to form 3- Isopentenyl pyrophosphate (Company. 2002).

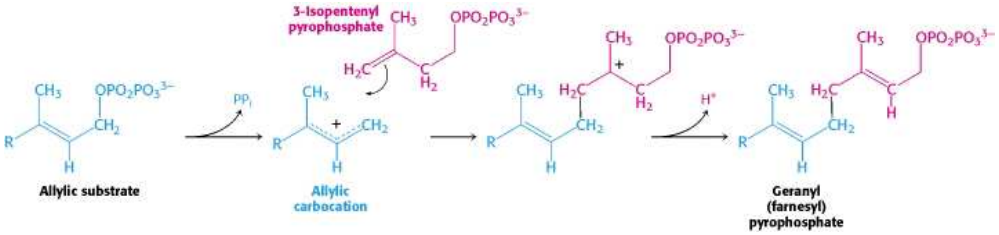

Figure 4 – Third step of cholesterol synthesis.

Reaction of condensation for the production of farnesyl pyrophosphate using an intermediate allylic substrate (Company. 2002).

The compound produced by the mechanism in figure 4, farnesyl pyrophosphate is then condensate to form squalene (figure 5). This last reaction takes place in the endoplasmic reticulum.

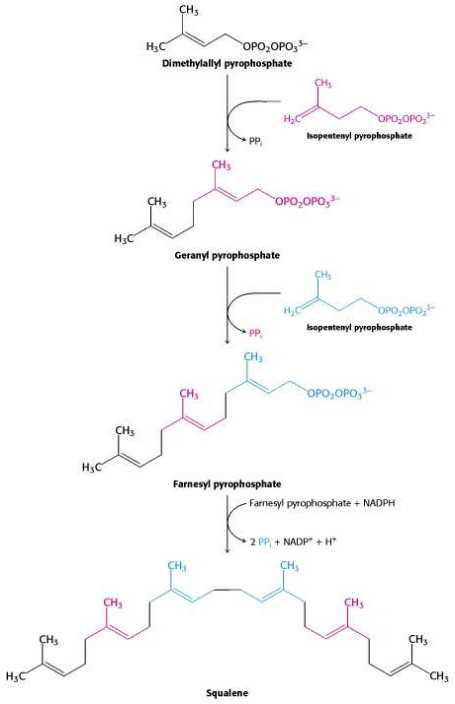

Figure 5 – Fourth step of cholesterol synthesis.

One molecule of dimethyallyl pyrophosphate and two molecules of isopentenyl pyrophosphate condense to form farnesyl pyrophosphate. The coupling between two molecules of farnesyl pyrophosphate forms one molecule of squalene (Company. 2002).

Squalene, produced by the reaction described in figure 5, is cyclized forming lanosterol (figure 6) which origins cholesterol after a process involving 19 steps (Company. 2002). The final step to form cholesterol is presented in figure 7.

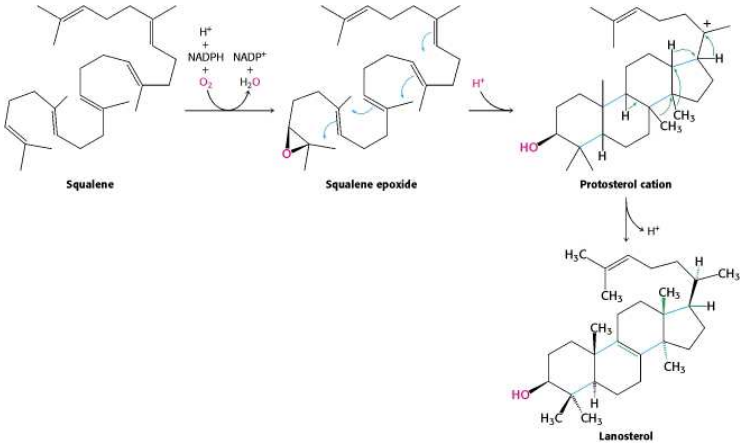

Figure 6 – Mechanism to produce lanosterol.

Squalene forms squalene epoxide which forms an intermediate that is protonated and forms a carbocation that cyclizes originating a tetracyclic structure. This structure after rearrangement forms lanosterol (Company. 2002).

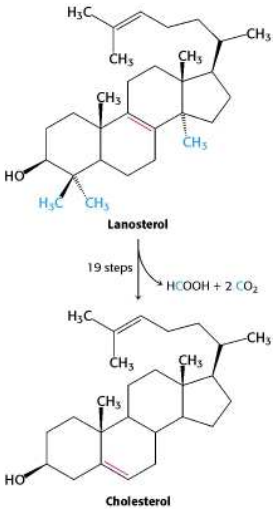

Figure 7 – Last step of cholesterol synthesis.

Mechanism to produce cholesterol. This mechanism is a multistep process that begins with the conversion of lanosterol by the removal of three methyl groups, the reduction of one double bond by NADPH and the migration of the other double bond (Company. 2002).

The biosynthesis of cholesterol is common in all animal cells depending on the cell type and organ function. The main production of the cholesterol happens in the liver however it can be also produced in the intestines, adrenal glands and reproductive organs (Lecerf & Lorgeril 2011).

2.4. Cholesterol absorption

Besides the biosynthesis of cholesterol the higher amounts of cholesterol come from the absorption in the small intestine. This mechanism maintains its homeostasis. The cholesterol entering the intestinal tract is typically from two sources: food intake and biliary secretion into the duodenum. In recent studies was found another source of intestinal cholesterol in mice. A significant part of excess of cholesterol is excreted directly via the intestine upon an activation of the liver X receptor. This information indicates an existence of an important alternative route for cholesterol disposal. However this mechanism is unclear in the case of humans (Lecerf & Lorgeril 2011).

Taking into account the values presented for the intake of cholesterol from the daily dietary it can be assumed that approximately 1000 – 2000 mg of cholesterol reach the lumen of small intestine. This cholesterol is mostly esterified and therefore it needs to be hydrolyzed by intestinal pancreatic enzymes originating also fatty acids and non-esterified cholesterol. Since the biliary cholesterol is non-esterified the bile, which is rich in bile acids, emulsifying phospholipids (lecithin) and cholesterol, form micelles via a detergent effect with the cholesterol. The emulsion formed enables the solubilization of the cholesterol (Lecerf & Lorgeril 2011). Some studies suggest that biliary cholesterol is absorbed slightly more when compared with dietary cholesterol due to its inherent association with bile acids (Wilson & Rudel 1994). However this difference has a little impact on overall cholesterol balance (Samuel & McNamara 1983).

The absorption of cholesterol in the intestine is an integrated process that include three major steps: solubilization in micelles, transport across the apical membrane to absorptive enterocytes,

mobilization to chylomicrons for the secretion into the lymph and blood through the basolateral membrane of enterocytes (Lecerf & Lorgeril 2011).

The biliary cholesterol carried by chylomicrons travels to the liver through the portal vein and the bile acids are reabsorbed in the ileum by the apical Na-dependent bile acid transporter. The bile acids-binding agents such as cholestyramine limit the resorption leading to higher synthesis of LDL receptors in order to internalize plasma LDL. This resorption causes a higher use of cholesterol in the liver in order to synthesize bile acids measured by the activity of the cholesterol 7-α-hydroxylase. In the case of the non-esterified cholesterol it is incorporated into mixed micelles, containing bile acids, phospholipids and hydrolytic products of triglycerides, in order to facilitate the diffusion of the compound through the boundary layer and then the brush border of the intestinal mucosa mainly in the duodenum and proximal jejunum (Lecerf & Lorgeril 2011). The step is critical for the diffusion of cholesterol across the unstirred water layer that lies between the bulk water phase and the intestinal brush border membrane (Manuscript & Transport 2014).

The transport of the non-esterified cholesterol and the biliary cholesterol is guarantee by the transporters located in the small intestine (Lecerf & Lorgeril 2011). The pathway of the cholesterol from the lumen to the lymph is presented in figure 8. In figure 9 is presented the full pathway of the cholesterol from the small intestine to the liver.

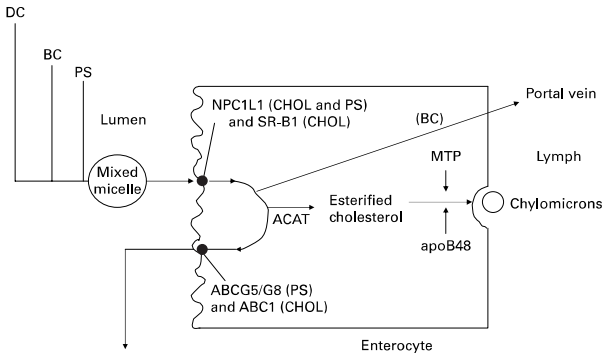

Figure 8 – Mechanism of the absorption of sterols and cholesterol from the lumen.
The abbreviations correspond to NPC1L1 - Niemann–Pick C1-like 1, SR-B1 - scavenger receptor B1, ACAT - acyl CoA cholesterol acyltransferase, ABC Gx - ATP-binding cassette hemi-transporter Gx, ABC - ATP-binding cassette, MTP - microsomal transfer protein, DC - dietary cholesterol, BC - biliary cholesterol, PS – phytosterol and CHOL-cholesterol (Lecerf & Lorgeril 2011).

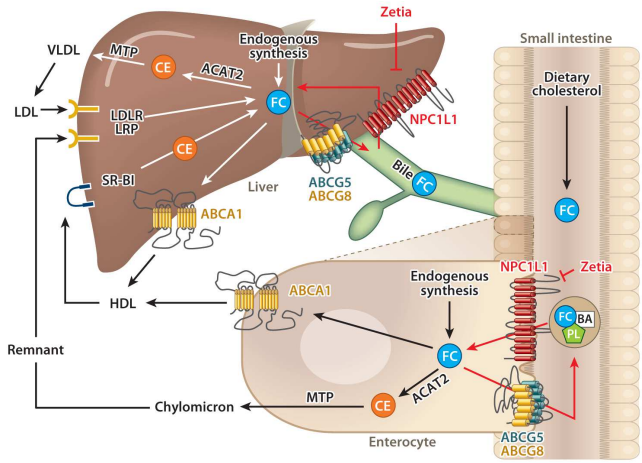

Figure 9 – Transport of the cholesterol in the small intestine and liver.
The image presents also the conversions described before for the diffusion of cholesterol through the transporters and membrane and therefore to reach the liver (Manuscript & Transport 2014).

The majority of cholesterol is esterified in the endoplasmic reticulum by ACAT2 (figure 9) and is incorporated into the chylomicrons as said before. The intracellular assembly of this nascent chylomicron requires microsomal triglyceride transfer protein (MTP), which is a protein that also facilitates the formation of very low density lipoprotein (VLDL) in the hepatocyte. The mature chylomicrons are secreted to the lymph across the basolateral membrane of enterocytes and transported to the bloodstream. In the circulation lipoprotein lipase hydrolyzes triglycerides in the core of chylomicrons for utilization and storage by peripheral tissues such as fat and muscle, whereas the majority of cholesterol in the chylomicron remnant is delivered to the liver (Manuscript & Transport 2014).

The transporters involved in the uptake of cholesterol are described in the next point.

2.5. Cholesterol transporters

2.5.1. Scavenger receptor B1

The transporter scavenger receptor class B type I is localized at the apical and basolateral membrane of enterocytes, with different expression levels along the length of small intestine (REF3). It mediates the cellular cholesterol uptake. This transporter recognizes the lipoproteins containing apoAI and therefore is also called by HDL receptor. However the process by which the lipoproteins are transported is not clearly defined (Sané et al. 2006).

SR-BI binds to the HDL particles present in the lumen and transports selectively the esterified cholesterol into the cell. The protein component of the lipoprotein is left in the extracellular space or plasma to be re-used or cleared by the kidney. The esterified cholesterol liberated by SR-BI is hydrolyzed by a neural cholesteryl esterase and enters directly into the metabolically active pools. SR-BI also act bidirectionally to promote cellular cholesterol efflux out of the cells and facilitated the selective uptake of cholesteyl ester from the HDL also promoting the efflux of cholesterol (Brown et al. 2007).

2.5.2. ABC1

The transporter ABC1, an ATP-binding cassette transporter, promotes cellular phospholipid and cholesterol efflux in the lipid removal pathway (Wang et al. 2001). It's localized in the basolateral membrane of enterocytes (Kruit et al. 2006). This protein belongs to a large family of evolutionary conserved transmembrane proteins, which transport a wide variety of substrates such as drugs, peptides, among others across the membranes (Hobbs & Rader 1999). ABCA1 has two transmembrane domains; each is formed by alpha helices and two intracellular nucleotide binding domains. The efflux of free cholesterol from the peripheral cells is transferred to the lipo-poor apoA1 through the ABCA1 transporter (Lawn et al. 1999). This transporter plays a central role in the regulation of cellular cholesterol homeostasis and in the formation of HDL (Lawn et al. 1999). In image 10 is presented the localization of the membrane transporter (Kruit et al. 2006).

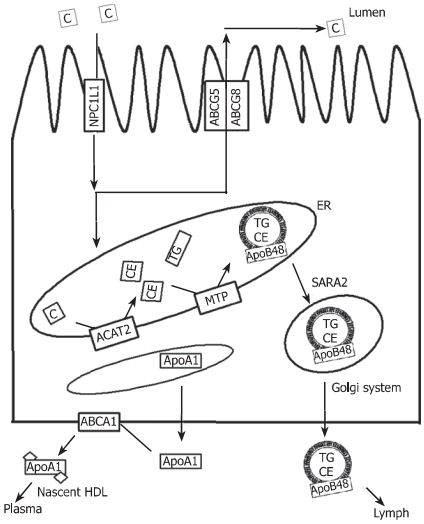

Figure 10 – Route for the uptake of cholesterol.

The transporter ABCA1 is connected to the apoA1 protein both responsible for the cholesterol efflux (Kruit et al. 2006).

The mutations on the gene ABC1 lead to the development of Tangier's Disease and familial high-density lipoprotein deficiency (Lawn et al. 1999). The Tangier Disease is responsible for elevated amounts of fat in the blood and disturbances in nerve function, among other pathologies (Lawn et al. 1999). The accumulation of cholesterol in Tangier disease appears to be primarily in macrophages and other cells of the reticuloendothelial system which leads to the conclusion that other cells types are better able to control the lipid entry and are not dependent on the efflux to guarantee that there is no accumulation (Hobbs & Rader 1999).

2.5.3. ABCG5/G8

The transporters ABC are two ATP-binding cassette hemi-transporters G5 and G8 act as functional heterodimers and are responsible for the efflux of cholesterol and critical in control of sterol

absorption (Kruit et al. 2006). Therefore they excrete the phytosterol (plant sterols) to the intestinal lumen. Since the phytosterol is not a good substrate for acyl CoA cholesterol acyltransferase it needs to be eliminated in order to protect the body against its accumulation in the blood and tissues. The presence of phytosterol also decrease the absorption of cholesterol since it competes with it at the micelle level and by the induce of the expression of ABCA1 and or ABCG5/G8 hemi-transporters it may lead to the efflux of cholesterol from the enterocytes to the intestinal lumen (Lecerf & Lorgeril 2011). The accumulation of phytosterol in the body origins a disease called sitosterolemia an autosomal recessive condition.

The transporters are localized at the canalicular membrane of hepatocytes and at the brush border membrane of enterocytes (Kruit et al. 2006). Studies demonstrated that the transporters mediate the hepatobiliary secretion of unesterified cholesterol (Manuscript & Transport 2014).

2.5.4. NPC1L1

This transporter NPCL1 is abundantly expressed in the small intestine and in the liver (Manuscript & Transport 2014). It is a homolog of Niemann-Pick C1 (NPC1) protein the amino acids sequence of NPC1L1 share 51 % similarity and 42 % identity with the amino acids from NPC1. NPC1L1 also shares topological similarity with the resistance-nodulation-division family if prokaryotic permeases. These permeases mediate the efflux of lipophilic drugs, detergents, fatty acids, bile acids, metal ions and dyes from the cytosol of bacteria, which leads to hypothesis that NPC1L1 is a lipid transport. The mutations in Niemann-Pick C1 cause a disease characterized by the lysosomal accumulation of cholesterol and other lipids. NPC1L1 has 1,332 amino acids. It has a typical signal peptide, an N-terminal NPC1 domain and extensive N-glycolysation sites (Altmann et al. 2004) (Davies et al. 2000). NPC1L1 has 13 transmembrane domains in which 5 constitute a sterol-sensing domain (SSD). This region is conserved in several polytopic transmembrane proteins involved in cholesterol metabolism and regulation including NPC1, HMG CoA reductase, sterol regulatory element-binding protein (SREBP) cleavage activating protein – SCAP and patched. The SCAP protein controls the transport and proteolytic activation of SREBPs, which are membrane-bound transcription factors that regulate the synthesis of cholesterol, when the cellular cholesterol content is low and other lipids (van der Velde et al. 2007) and the patched is a membrane receptor for the cholesterol-linked signaling peptide Hedgehog.

In figure 11 is presented the predicted structure of NPC1L1.

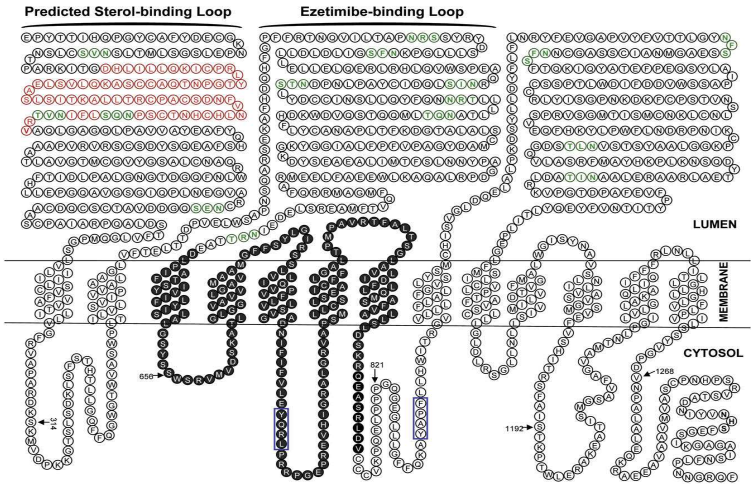

Figure 11 – Predicted topological structure of human Niemann-Pick C1 Like 1 and the amino acid sequence.

The conserved N-terminal NPC1 domain corresponds to the red residues. The residues in dark correspond to the sterol-sensing domain and the ones in green are the extensive N-linked glycosylation sites. The N-terminal 21 amino acids are assumed to be the signal peptide and are not shown in this figure (Betters & Yu 2010).

In animals the transporter NPC1L1 resides mainly at the apical membrane of enterocytes and in the canalicular membrane of hepatocytes. This region is exposed to unesterified free cholesterol. In the case of hepatoma cells NPC1L1 is localized in both plasma membrane and endocytic recycling compartment (ERC). The subcellular localization of the transporter is regulated by the cellular cholesterol availability (van der Velde et al. 2007). When the cells are enriched with cholesterol NPC1L1 is localized predominantly in the endocytic recycling compartment in contrast when there is a depletion of cholesterol the transporter moves to the plasma membrane. The SSD may regulate NPC1L1's intracellular itineraries by sensing membrane cholesterol content (Manuscript & Transport 2014).

The transcriptional regulators of NPC1L1 remain unclear. Some regulators such as peroxisome proliferator-activated receptor (PPAR)α, PPARδ, liver X receptor (LXR) and retinoid X receptor (RXR) have been implicated in the regulation od intestinal cholesterol absorption but the way they regulate the expression of NPC1L1 is not very elusive (Manuscript & Transport 2014).

Some studies suggest that NPC1L1 may be transcriptionally regulated by cellular cholesterol content through the SREBP-2, which is a membrane-bound transcription factor that increases the expression of cholesterol synthetic genes when the content is low (Brown & Goldstein 2009).

The animal studies done established the necessity of NPC1L1 in the mediation of intestinal cholesterol absorption and the localization of the transporter varies from the ERC to the cell membrane. In conditions of depletion of cholesterol, NPC1L1 increases its endogenous location in the cell surface of HepG2 (Yu et al. 2006). The same is verified for other cell lines as Caco-2, McArdle RH7777, among others (Yamanashi et al. 2007). The transporter is seen in the plasma membrane and in the intracellular vesicular compartments in cultured cells and in the small intestine as said before. NPC1L1 can facilitate the uptake of cellular cholesterol when it is localized in the cell surface disappearing from the membrane to reappear in the intracellular compartments (Yu et al. 2006). It also has an important role in the pathogenesis of metabolic disorders (Betters & Yu 2010).

2.5.4.1. Mechanism of NPC1L1- cholesterol mediated uptake

Some studies suggest that the mechanism for the uptake of cholesterol through the NPC1L1 that is regulated by clathrin-mediated endocytosis. The evidences for this conclusion are the different locations of NPC1L1 between the cell surface and the intracellular compartments in a cholesterol-dependent manner; the translocation of the transporter can be blocked by potassium depletion, a treatment that inhibits clathrin-mediated endocytosis, the caveolae is not the cellular basis for the NPC1L1 cholesterol uptake since the caveolin-1, a structural protein of caveolae, is dispensable for intestinal cholesterol absorption, and the co-immunoprecipitates with the μ2 subunit of the adaptor protein complex AP2 with the clathrin heavy chain. These two proteins are necessary for he clathrin-dependent endocytosis (Betters & Yu 2010). The proposed model of NPC1L1 is presented in figure 12.

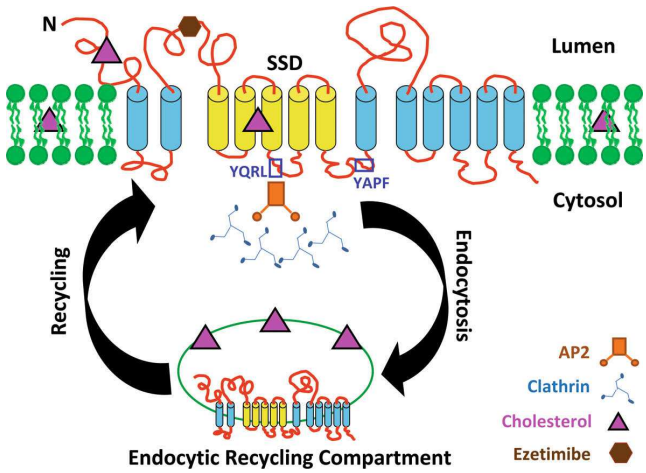

Figure 12 – Proposed model for the NPC1L1 cholesterol uptake.
Two potential YXXØ motifs known to facilitate the clathrin-mediated endocytosis of many plasma membrane proteins via interaction with the l subunit of AP2 are highlighted in blue squares. The recycling of NPC1L1 involve an endocytic recycling triple complex consisting of the microfilament-interacting motor myosin Vb, the small GTPase Rab11a, and the adaptor Rab11- FIP2 (Chu et al. 2009).

NPC1L1 recruits extracellular free cholesterol through its N-terminus to the cell membrane location, which creates a RAFT-like plasma membrane microdomain. The cholesterol in this micro domain is sensed by the sterol sensing domain (SSD) from the NPC1L1. The SSD somehow senses cholesterol content in this microdomain through conformational changes, which may cause subsequent interactions of NPC1L1's YXXØ (Manuscript & Transport 2014). When the cholesterol is enriched to a threshold in this membrane region, the microdomain is endcytosed to facilitate the cholesterol uptake (Betters & Yu 2010).

2.5.4.2. Hepatic NPC1L1

The liver plays a central role in the body cholesterol homeostasis. It contributes to a significant proportion of cholesterol via synthesis; it produces and takes up lipoprotein cholesterol, converts cholesterol to bile acids and therefore regulates intestinal absorption efficiencies of lipids and vitamins (Manuscript & Transport 2014). It secretes unesterified and cholesterol-derived bile acids into bile. The

reason why the liver express NPC1L1 remains unclear (Betters & Yu 2010). However the deficiency on NPC1L1 disrupts the enterohepatic circulation of endogenous cholesterol causing a substantial loss of endogenous cholesterol and a dramatic feedback upregulation of endogenous cholesterol synthesis. Therefore it is temping to speculate that hepatic NPC1L1 prevents excessive loss of cholesterol by retrieving ABCG5/ABCG8-derived cholesterol from the canicular bile (Manuscript & Transport 2014).

2.6. Causes and treatment

The levels of plasma cholesterol bring different responses by the human body. As said before each organism has their own metabolism and therefore the response is not the same for each one (section 2.7). High levels of plasma cholesterol and low density lipoprotein, also called as hypercholesterolemia, contributes to the development of Coronary Heart Disease (CHD), the leading cause of death in the Western countries (Organization 2015). On the other, although the research on this is very limited, the low values of plasma cholesterol contribute to hypocholesterolemia, however this condition is associated with illness or genetic factors (Biggerstaff & Wooten 2004).

The hypercholesterolemia contributes to the development of atherosclerosis a condition in which occurs the accumulation of white blood cells, which include cholesterol (Durrington 2003) in the arteries. This condition affects the blood circulation contributing to CHD.

The increased concerns about CHD led to the development of statins with the aim of inhibit the cholesterol biosynthesis (Manuscript & Transport 2014). Despite this drugs became a way to lower the cholesterol levels a major amount of cholesterol comes from the absorption. This led to the hunt for other agents to aggressively lower the cholesterol levels (Manuscript & Transport 2014) such as Ezetimibe. Ezetimibe, or commercially known as *Zetia*, inhibits the cholesterol absorption, lower plasma cholesterol levels and inhibits the development and progression of atherosclerosis (Betters & Yu 2010), but for many years the way of action was not fully understood. Nowadays it become clear that one of the targets of Ezetimibe is NPC1L1 (Manuscript & Transport 2014). The co-administration of Ezetimibe with statins significantly reduces the levels of LDL (Betters & Yu 2010).

However the treatment with Ezetimibe has proven to improve many metabolic disorders besides hypercholesterolemia such as the reduction of hepatic steatosis, attenuate weight gain and insulin resistance.

The inhibition of NPC1L1 or NPC1L1-dependent intestinal cholesterol absorption could be a potential preventive and a therapeutic way for some diseases such as non-alcoholic fatty liver disease, insulin resistance, type 2 diabetes and central obesity (Betters & Yu 2010).

2.7. Medicinal plants

For a long time the humans used the medicinal plants to treat a wide variety of diseases. This filed of Science is called Ethnobotany. The medicinal plants still represent the only therapeutic method for some ethnic groups and communities. The selection of the plants was based in intuition and

discoveries. After verifying the effects in person treated, the plants were selected. However, this analysis is based only on the outer effects of the treatment and the opinion of the people and so it is not scientifically proven its efficiency or role in the human body. However the knowledge and selection of the plants for each disease has passed through the years and remained in the communities' culture without the correct knowledge about the active compounds of the plants, many people got intoxicated since they represent complex molecules and the human body is not capable of metabolize some of their compounds (Maciel et al. 2002).

Nowadays, the use of the plants remains in a lot of cultures, either as a tea or a mixture to apply in the skin in the developed countries. For many cultures, the traditions and treatments remain the same since their ancestry. The pharmaceutical industries have an increasing interest in the medicinal plants to discover and develop other drugs based in the population knowledge and use. However, since the plants represent complex molecules it's necessary to be aware of all the effects they may cause in the human body (Veiga et al. 2005).

According to *World Health Organization* herbal medicines correspond to plant-derived materials, which contain either raw or processed ingredients from one or more plants. There are three kinds of herbal medicines: raw plant materials, processed plant materials and medicinal herbal products (Organization 2015).

2.7.1. Plant compounds

The polyphenols are micronutrients present in the diet and they have a role in the prevention of degenerative diseases such as cancer and cardiovascular diseases. These compounds have also antioxidant proprieties and modulate the activity of a range of enzymes and cell receptors. They constitute the active substances in many medicinal plants (Manach et al. 2004).

These compounds are secondary metabolites of plants and are involved in defense against ultraviolet radiation or aggression by pathogens. They are classified into different groups by the number of phenol rings and the structural elements that bind the rigs to each other. The more abundant group is the phenolic acids, which have two classes: derivatives of benzoic acid and derivatives of cinnamic acid. The derivatives of benzoic acid have a content generally low. The caffeic acid is generally the most abundant phenolic acid and represents between 75% and 100% of the total hydroxycinnamic acid content of most fruit however is found in other parts of the plants (Manach et al. 2004).

The teas represent other sources of phenols since are prepared from mixtures of plants.

As said in the article present in the appendix C, the major phenolic compounds reach the human organs acting as inhibitors of the cholesterol synthesis and as inhibitors in the cholesterol uptake in the intestinal lumen representing a possible way of treatment that can be used along with pharmaceutical drugs.

3. Materials and Methods

3.1. Plant extract and preparation

The herbal infusions selected are commercial mixtures sold in the supermarkets with the aim of lower the cholesterol.

The composition of the herbal mixture is shown in Table 1. The extracts were prepared at a ration of 1 tea bag for each 100 ml. These amounts are recommended by the manufacturers to accomplish the desired effect. The mixture was frozen and then lyophilized (Heto Power Dry LL3000) to get the solid extract.

Table 1 – Composition of the commercial mixture used.

Name	Composition		%
Extract	Cavalinha	*Equisetum arvense L.*	30
	Espinheiro branco (flor)	*Crataegus monogyna Jacq.*	25
	Urtiga verde	*Urtica dioica*	25
	Canela (casca)	*Cinnamomum*	20

3.2. Cell culture

The cell line used was HepG2 ATCC No. 8065 HB. Cells were maintained in a DMEM medium (Dulbecco's Modified Eagle's Medium, Lonza) containing 4.5 $g.l^{-1}$ glucose supplemented with 5 ml of glutamine (200 mM in 0.85 % NaCl solution, Lonza), 50 ml of FBS (Heat inactivated, Lonza) and 5 ml of pen-strep (10.000 U $Penicillin.ml^{-1}$, 10.000 $Streptomycin.ml^{-1}$, Lonza) in a humidified 5 % CO_2 incubator at 37 °C (CO_2 incubator, Sanyo). The medium was changed every 48 to 72 hours. In some cases the cells were passed every 72 hours but since they took a long time to reach confluence the following passages were done at each 4 to 5 days.

3.3. Preparation of cell culture for electrophoresis

For protein extraction to use in electrophoresis, the HepG2 cells were seeded at a density of 5×10^4 cells/cm^2 in 12-well plates with 2.3 cm diameter (Polystyrene, BD Falcon) and in T25 flasks (4.5×6.3 cm^2, VWR and Orange Scientific) seeded with the same density of cells (with a volume of 7 ml). To seed the cells it is necessary to remove them from the T-flask where they grow. For this, the cells are washed with a solution of PBS 1x. The PBS with the unviable cells was removed and it was added 1 ml of trypsin 1x in the flask. The flask was incubated for 5 minutes in the incubator at 37 °C (CO_2 incubator, Sanyo). It's important to not exceed 15 minutes when the cells are in contact with trypsin because they can collapse. After this time, the cells detached are collected with a pipette. Its necessary to make sure that all the cells are collected from the flask. The cells collected are mixed in

a falcon (50 ml or 15 ml, Orange Scientific and VWR) with 1 ml of medium to inactivate the action of the trypsin. The solution of trypsin is prepared at a ratio of 1:10 with trypsin 10x (with Versene, Lonza) and a solution of PBS 1x. The PBS 1x is prepared from a dilution of PBS 10x (Lonza) and Mili-Q water (Millipore, Darmstadt). All of those solutions are sterilized in an autoclave (Uniclave 88, ajc), except the ones already sterilized from the supplier, and the procedures carried out in a laminar flow chamber (Class II Biohazard Safety Cabinet, ESCO) previously sterilized by UV light. To ensure that the seeded cells into the plates are at the density desired, its necessary to count the cells in the solution after the contact with trypsin. For this, is taken 100 µl of the cell solution and mixed with 100 µl of trypan blue and placed in a Neubauer chamber (Marienfeld, Germany). The concentration of cells in the samples was calculated by the average of number of cells counted in each quadrant (equation 1) and taking into account the dilution. After that the volume necessary to seed on the flasks and plates was determined.

$$Concentration\ of\ cells = \frac{\sum Number\ of\ cells/ml}{\sum quadrants} \times 2 \qquad \text{(Equation 1)}$$

3.4. Application of the compounds in the cell lines

After the cells are seeded in the 12-well plates or T25 flasks, they grow for 2 days and it's applied the compounds to test. In the case of the T25 the cells grow for 1 week and then the compounds are seeded. The compounds are the herbal mixture and cholesterol (99 %, 386.66 $g.mol^{-1}$, Sigma). The cholesterol is applied at a concentration of 50 µg/ml and the herbal mixture in a range of concentrations from of 1 mg/ml and 0.1 mg/ml. Both solutions are diluted with DMEM medium with the same formula but without FBS because FBS interacts with the compounds, which don't have the correct interaction with the cells. In each well were added 2 ml of the solutions leaving one column that represents the control (only medium). In the T25 flask the volume needed is 7 ml and each T25 has different conditions. The plate or flask is left to grow for 24 hours at the same conditions in the incubator. After the 24 hours the cells are lysed.

3.5. Lysis

To begin the lysis process, the cells growing in 12-plates or T25 flasks are washed with PBS to remove the remaining medium (3 ml for two times).

During the process of optimization of the lysis method, various lysis buffers were tested to get the best results in the protein extraction. Fist the cells were lysed with 4 % (v/v) Igepal (Electrophoresis reagent, Sigma), 2 % (w/v) DTT (154.25 $g.mol^{-1}$, 99 %, Fluka), 6 M Urea (60.06 $g.mol^{-1}$, high purity grade, Sigma) adding 500 µl in each well of the 12-plate. The cells were scrapped and at the end were added another 500 µl to homogenate and recover the rest of the cells present in the plate. After the lysis, the solutions went to the ultrasound bath to help lysing the remaining cells. The solution was then centrifuged at 9000 rpm for 5 minutes and the supernatant and pellet kept for

the electrophoresis. Since this lysis buffer interferes with Bradford method, another lysis buffers were tested. The selected lysis buffers are presented in Table 2.

Table 2 – Composition of the different lysis buffers used.

Lysis buffer				
	Buffer A	**Buffer B**	**Buffer C (or RIPA)**	**Buffer D**
Composition	Distilled water, methanol (32.04 $g.mol^{-1}$, Ultragradient, Carlo Erba)	4% (w/v) Chaps (615 $g.mol^{-1}$, Merck) and 8 M Urea (60.06 $g.mol^{-1}$, high purity grade, Sigma)	150 mM NaCl (58.44 $g.mol^{-1}$, 99.5 %, Sigma), 1 % (v/v) triton-X100 (Fluka Chemicals), 0.5 % (w/v) sodium desocycolate (414.56 $g.mol^{-1}$, 98%, Fluka), 0.1 % (w/v) SDS (288.38 $g.mol^{-1}$, 99 %, Sigma) and 50 mM Tris (121.14 $g.mol^{-1}$, 99.5 %, Merck) pH 8	50 mM Tris-HCl (121.14 $g.mol^{-1}$, 99.5 %, Merck) pH 7.5, 100 mM NaCl (58.44 $g.mol^{-1}$, 99.5 %, Sigma), 1 mM (w/v) DTT (154.25 $g.mol^{-1}$, 99 %, Fluka) and 5 % (v/v) glycerol (92.10 $g.mol^{-1}$, 99 %, AnalaR, BDH Chemicals)

For all the methods the protocol used was *abcam* protocol (Electrophoresis & References n.d.). In the case of the first lysis buffer the method was different. The cells were scrapped with water (500 µl in each well) and collected to an eppendorf, then was added MeOH (500 µl) to precipitate the cells. The lysed solution was centrifuged in a mini spin (Eppendorf) according to the *abcam* protocol (Electrophoresis & References n.d.). For the other lysis buffers were also added to each well 500 µl of buffer. The pH was adjusted with HCl 37 % (HCl fuming 37 %, 36.5 $g.mol^{-1}$, Merck) using pH meter (WTE, Inolab).

To ensure that the protein of interest was extracted the pellet was resuspended with another lysis used at the beginning of the work. Another step was added to the protocol with the aim of helping the disruption of the membranes so after collecting the lysed cells they were put in the ultrasound bath in cycles of 30 seconds till the solution was clear since it was not present in the *abcam* protocol (Electrophoresis & References n.d.). The quantities and the procedure was the same as described.

The cells growing in the T25 were collected with PBS (500 µl) and centrifuged for 5 minutes at 1500 rpm. The time in the centrifuge varied because in some cases the 5 minutes weren't enough to create the pellet of cells. The supernatant (almost PBS) was removed and 100 µl of the lysis buffer were added to each eppendorf. The cells were kept in the ice for 10 minutes. After they were taken to the ultrasound bath and the rest of the protocol was the same as described before. The lysis buffer had two changes, the elimination of DTT from the lysis used in the first step and then the NaCl. The elimination of DTT was because of the presence of DTT in the second step and it could be eliminated. The elimination of NaCl was due to the interest of using the samples to perform electrophoresis 2D. The elimination of DTT was kept for the next lysis. It was also tested the effect on doing just a total lysis. For this study the cells were lysed using RIPA without SDS to quantify the protein since SDS interferes with Bradford reagent.

The supernatant and pellet are kept for further analysis and use in electrophoresis.

To prepare the samples for the electrophoresis 2D the cells were lysed with a solution of 8 M Urea (60.06 $g.mol^{-1}$, high purity grade, Sigma) and 4 % (w/v) Chaps (615 $g.mol^{-1}$, Merck). The procedure was the same for cells growing in T25. Since the lysis was total it was only done one centrifugation and the samples were kept at -80 °C for further analysis (Panasonic).

3.6. Bradford method

For the calibration curve of Bradford method was prepared a solution of 1:10 µg of BSA (66 kDa, 98 %, Sigma) in distilled water. The curve is calculated measuring the absorbance of the solution: BSA and Bradford reagent (Protein assay, Bio-Rad) in different concentrations of BSA at 595 nm in a UV spectrometer (UV/VIS spectrometer, Jasco V-560). For the measurement were prepared two cuvettes, one cuvette with distilled water (800 µl) and Bradford reagent (200 µl), to remove the interference of Bradford in the solution, and the other with the solution of BSA (range of 10 to 100 µl), water (range of 790 to 700 µl) and Bradford reagent (200 µl).

To analyze the samples were prepared two cuvettes, one with distilled water and Bradford reagent in the same amounts as before and the other cuvette with the sample (5 µl), distilled water (795 µl) and Bradford reagent (200 µl). The absorbance was measured at the same wavelength in the same spectrometer. In order to have the cuvettes more similar was made a change in the protocol. Therefore in the cuvette with distilled water and Bradford reagent was added lysis buffer at the same quantity as the sample (5 µl). The other cuvette remained with the same amounts.

3.7. Electrophoresis

To perform the electrophoresis was used a horizontal system (Gel Box, Amersham, GE Healthcare) with an electrical source (Power Source 300 V, VWR) using a gel 4-12 % of acrylamide (Amersham ECL, GE Healthcare, 10 wells).

Before starting the electrophoresis was added 90 ml of running buffer (Tris-glycine 25 mM 192 mM), prepared by dilution of a TG buffer 10X (Ready-pack (Tris-glycine 25 mM 192 mM), Amresco) with distilled water and the addition of 0,1 % (w/v) SDS (288.38 $g.mol^{-1}$, 99 %, Sigma) to each tank of the electrophoresis system till the tanks were full. The gel inside the cassette, without the tapes in the two legs of the cassette, was placed in the box with the well side in the cathode side (-) and the end of the cassette in the anode (+). It's important to be sure that the legs don't have remains of gum that will slow down the electrophoresis. It was made a pre-running of the gel for 10 minutes at 160 V.

After the pre-running, the lip from the wells was removed to make them available for sample loading and added running buffer till the wells were full. In two wells were loaded 3 to 5 µl of protein marker (Bluestep, Protein MW Marker, Broad Range, Prestained, Amresco) and in the others the samples mixed before with loading buffer (Protein Loading Buffer 2x, Amresco) in a ratio of 1:1. The samples are loaded at a maximum of 30 µl in each well. The electrophoresis ran for 1.30 hours at 120 V. When the electrophoresis finished, the cassette was opened by inserting the edge of the comb in

the slot opposite the sample wells and twisted. The gel was cut approximately 2 cm downstream of the wells and the stacking gel in the end. To visualize the proteins the gel was stained with Blue Bandit^{TM} Protein Stain (Amresco) overnight in a shaker (Rocking Platform, VWR). After, the gel was washed with distilled water and photographed in the machine (ImageQuant LAS 500, GE Healthcare). The gels were stained with Coomassie: 40 % distilled water, 10 % (v/v) acetic acid (Acetic acid glacial 100 %, 60,05 g.mol^{-1}, Merck), 50 % (v/v) methanol (32.04 g.mol^{-1}, Ultragradient, Carlo Erba) and 0.25 % by weight of Coomassie Brilliant Blue R-250 (826 g.mol^{-1}, Fluka). Before staining the gel, it was treated with a solution of 40 % distilled water, 10 % (v/v) acetic acid and 50 % (v/v) methanol to prevent the diffusion of proteins. After the staining the gel was washed with a solution containing 67.5 % (v/v) distilled water, 7.5 % (v/v) acetic acid and 25 % (v/v) methanol in a shaker, until the excess of Coomassie was removed.

To denature the samples, they were boiled at 95 – 100 °C for 5 minutes or 5 to 10 minutes at 70 °C. This procedure was done to all the samples, but after some gels, the heating step was only done to the samples without Urea in the lysis buffer. Before and after the heating step, the samples were mixed by vortexing.

It was also used the loading buffer called Laemmli buffer (2x) containing 4 % (w/v) SDS (288.38 g.mol^{-1}, 99 %, Sigma), 10 % (v/v) 2-mercaptoethanol (78.13 g.mol^{-1}, Merck), 20 % (v/v) glycerol (92.10 g.mol^{-1}, 99 %, AnalaR, BDH Chemicals), 0.004 % (w/v) bromophenol blue (669.99 g.mol^{-1}, Merck) and 0.125 M Tris-HCl ((121.14 g.mol^{-1}, 99.5 %, Merck) pH of 6.8. Since it was necessary to minimize the dilution of the samples, the Laemmli buffer was prepared at 6X.

The migration buffer or also called running buffer had to be prepared with the following amounts 25 mM Tris (121.14 g.mol^{-1}, 99.5 %, Merck), 190 mM glycine (75.07 g.mol^{-1}, 99.7 %, AnalaR, BDH and 75.07 g.mol^{-1}, 99 %, Sigma), 0,1 % (w/v) SDS (288.38 g.mol^{-1}, 99 %, Sigma) and a pH of 8.3. The final procedure to the electrophoresis is in appendix A.

To compare results and buffers, it was prepared a different loading buffer to see the results in the resolution of the gel. The loading buffer contained 0.5 M Tris-HCl (121.14 g.mol^{-1}, 99.5 %, Merck), 10 % (w/v) SDS (288.38 g.mol^{-1}, 99 %, Sigma), 2 ml of glycerol (92.10 g.mol^{-1}, 99 %, AnalaR, BDH Chemicals), 2 mg bromophenol blue (669.99 g.mol^{-1}, Merck) and 310 mg of DTT (154.25 g.mol^{-1}, 99 %, Fluka).

The electrophoresis was also done using gels made in the lab. First was prepared a separation gel (7,5 %) with 40 % of acrylamide/Bis-acrylamide. The reagents necessary to the gel were added gradually in the following order: 54.7 % distilled water, 25 % 1.5 M Tris-HCl pH 8.8 (121.14 g.mol^{-1}, 99.5 %, Merck, HCl fuming 37 %, Merck), 18.8 % of acrylamide/Bis 40 % (Acryl/Bis 29:1, Amresco), 1 % (v/v) of 10 % SDS (288.38 g.mol^{-1}, 99 %, Sigma), 0.5 % (v/v) of 10 % APS (98 %, Sigma) and 0.05 % (v/v) TEMED (116.21 g.mol^{-1}, VWR). The solution of APS and SDS were prepared before and the volume necessary for the gel was added to the mixture. After adding the TEMED is important to place the mixture as fast as possible because the mixture begins to solidify. This mixture was placed between two glasses to solidify and added distilled water on the top; the gel was put in a vertical support (Biorad) to solidify. Once the gel has solidified, was added the mixture to prepare the concentration gel 4 % with 40 % of acrylamide/Bis. To prepare this mixture the reagents were also

added sequentially in the following order: 3.18 ml of distilled water, 1.26 ml of 0.5 M Tris-HCl pH 6.8, 500 µl of 40 % of acrylamide/Bis, 50 µl of 10 % SDS, 25 µl of 10 % APS and 5 µl of TEMED (116.21 $g.mol^{-1}$, VWR). This mixture was placed on the top of the separation gel already solidified. On the top of the concentration gel was placed a comb to create the wells in the gel.

To run the electrophoresis using this gel is necessary to use the vertical system (15 W, Mini-Protean, Biorad) and the same migration buffer described before. The electrophoresis ran for 1.30 hour almost and the gel was cut on the well side and kept to stained with Coomassie.

The markers used were three, one already described, the other two were from A (HMW-SDS calibration kit, Pharmacia Biotech) and B (NZY Blue Protein Marker, NZY Tech).

In the gels were also loaded peptide (Blocking Peptide, Novus Biologicals) and tubulin mixed with loading buffer in a ratio of 1:1.

3.8. Electrophoresis 2D

To perform the electrophoresis 2D was followed the protocol in Falé et al. 2012. First was necessary to prepare the gel for the second step of the electrophoresis. The gel was made as described before but more concentrated. To prepare the separation gel (10 %) with 40 % of acrylamide/Bis-acrylamide the reagents were added gradually in the following order: 48.5 % distilled water, 25 % 1.5 M Tris-HCl pH 8.8 (121.14 $g.mol^{-1}$, 99.5 %, Merck, HCl fuming 37 %, 36.5 $g.mol^{-1}$, Merck), 25 % of acrylamide/Bis 40 % (Acryl/Bis 29:1, Amresco), 1 % of 10 % SDS (288.38 $g.mol^{-1}$, 99 %, Sigma), 0.5 % of 10 % APS (98 %, Sigma) and 0.05 % TEMED (116.21 $g.mol^{-1}$, VWR). This mixture was placed between two glasses to solidify and added distilled water on the top. The gels made had a thickness of 1.5 mm. Once the gel has solidified, was added the mixture to prepare the gel concentration 4 % with 40 % of acrylamide/Bis. The procedure was the same as described before.

To prepare the samples to apply in the strips were mixed 125 µl of the samples (with lysis buffer) with 2.5 µl of IPG buffer at pH 3-11 NL (GE Healthcare), 1.25 mg of DTT (154.25 $g.mol^{-1}$, 99 %, Fluka) and 0.002 % of bromophenol blue (669.99 $g.mol^{-1}$, Merck), to ensure that the content of protein was approximately 50 µg in each sample. This mixture stayed for one hour under agitation at room temperature. The samples were applied to a 7 cm Immobiline DryStrip pH 3-11 NL (GE Healthcare) with 1 ml of oil (Plus One Dry Strip Cover Fluid, Amersham Biosciences) to prevent dry strip. The isoelectric focusing was performed at 30 V over 26.32 hours with the final values for the run of 1150 V, 17359 V per hour. The strips were then kept overnight at -80 °C (Panasonic).

The focused strips were incubated in an equilibrium buffer (6 M Urea, 75 mM Tris pH 8.8, 29.3 % (v/v) glycerol, 2 % (w/v) SDS, and 0.002 (w/v) bromophenol blue) with 1 % (w/v) DTT for 15 minutes at room temperature with gentle agitation. After the strips were transferred to equilibrium buffer containing 2.5 % (w/v) iodoacetamide (184.96 $g.mol^{-1}$, SigmaUltra, Sigma) for 15 minutes at room temperature with gentle agitation.

The second dimension was performed in the gel described before, the strip was placed with the gel side facing the front of the glass support and added 4 µl of molecular marker (NZY Blue Protein Marker, NZY Tech) on a piece of filter paper in one end of the gel. On top of the strip was

added a solution of agarose with bromophenol blue and waited till the mixture solidified. To prepare the agarose solution was added 0.5 g of agarose, 200 µl of 1 % (m/v) bromophenol blue (669.99 $g.mol^{-1}$, Merck) and 20 ml of 5 X running buffer. The agarose was pre cooked in the microwave before the mixture. The running buffer used was the same as the one in the electrophoresis point but 5 times more concentrated than the other.

The electrophoresis ran in a vertical system (15 W, Mini-Protean, Biorad) for 1.30 hours at 100 V, to prevent diffusion, and more 30 minutes at 120 V. The tank was full with migration buffer.

The gels were stained with Coomassie and registered the results by photographing the gel (LAS 500, ImageQuant LAS 500, GE Healthcare). The gels were also stained with silver using the PlusOne silver staining kit (Healthcare n.d.). The procedure followed was the one with the kit (Healthcare n.d.) with some differences. The silver nitrate to prepare the solution 2.5 % was made with one from Merck (167.87 $g.mol^{-1}$, 99.8 %, Merck); Na_2CO_3 from Merck (105.99 $g.mol^{-1}$, 99.9 %, Merck) and EDTA-$Na_2.2H_2O$ was replaced for the EDTA solution since it had sodium in its composition (Disodium- calcium salt, 374.3 $g.mol^{-1}$, 100 % purity based on H_2O 2.3 $mol.mol^{-1}$, Sigma). The fixation step was performed overnight and the developing for 6 minutes. The results were registered using the LAS 500 machine (LAS 500, ImageQuant LAS 500, GE Healthcare).

3.9. Western blot

To perform the wet transfer was used a transfer system (TE22 Mighty Small Transphor Unit, Hoefer). The buffer used to the wet transfer also called transfer buffer was prepared by the addition of 20 % (v/v) methanol (2.04 $g.mol^{-1}$, Ultragradient, Carlo Erba) and Mili-Q water (Millipore, Darmstadt) to the solution of Tris-glycine described in the beginning of the electrophoresis chapter.

First it was necessary to cut the membrane. The membrane used was nitrocellulose (NC) membrane (Hybond ECL Nitrocellulose membrane, Amersham, GE Healthcare). The membrane was pre-wet in distilled water for 5 minutes and then in transfer buffer for 10 minutes. The membrane should have, approximately, the size of the gel. The transfer unit was filled with pre-chilled transfer buffer.

To mount the cassette, the anode side (white) was placed down in a tray with transfer buffer. On top of the cassette was placed foam sponge (3 mm), two pre-wet papers and on top the pre-wet membrane. The gel, previously cut, was placed on top of the membrane making sure that there were no bubbles. The gel was covered with two pre-wet papers and the sponge. The cassette, closed, was placed into the tank with the white side to the red wire. The transfer ran for 1.30 hours at 100 V under agitation.

After the transfer finished, the membrane was removed from the cassette and wet in Ponceau S Stain (1 X ready-to-use solution, Amresco) for 2 to 10 minutes. The membrane was washed with distilled water and the results were photographed in LAS 500 machine (ImageQuant LAS 500, GE Healthcare). The membrane was incubated with 2 % blocking solution (1 g of blocking powder (ECl Prime Blocking Agent, Amersham, GE Healthcare) and 50 ml of transfer buffer with 0.1 % Tween-20 (M)) on an orbital shaker for 1 hour at room temperature. After this, the membrane was washed two

times with wash buffer (transfer buffer with 0.1 % Tween-20) and incubated with the primary antibody (Anti-NPC1L1, polyclonal, Rabbit IgG, Sigma) overnight in an orbital shaker at 4 °C. The primary antibody was diluted in transfer buffer (with methanol) at first (5.4 µl antibody and 1994.7 µl of wash buffer). After this, the membrane was washed two times with wash buffer and then 4 to 6 times for 5 minutes in an orbital shaker. The membrane was then incubated with the secondary antibody (ECL Anti-Rabbit IgG Horseradish Peroxidase-Linked Whole Antibody (from Donkey), Amersham, GE) for 1 hour at room temperature on an orbital shaker. The secondary antibody was diluted in the same solution as the primary antibody with the amounts 0.27 µl antibody and 1999.73 µl transfer buffer with Tween-20.

After this, the membrane was briefly washed 2 times with wash buffer and 4 to 6 times for 5 minutes on an orbital shaker. The membrane was then incubated with the detection solutions. The detection solutions were Luminol and Peroxide (ECL Prime Western Blotting Detection Reagent, Amersham, GE Healthcare). The mixture was made by the addition of the two solutions in a ratio of 1:1. The mixture was applied in the surface of the membrane and incubated for 5 minutes at room temperature. The excess was drained and the results were captured using LAS 500 machine (ImageQuant LAS 500, GE Healthcare).

Since the antibodies should not be mixed with a solution containing methanol, the wash buffer was replaced by the Tris Buffered Saline solution (TBS). This solution (10 X) was prepared with 24.23 g of Tris (121.14 $g.mol^{-1}$, 99.5 %, Merck), 80.06 g of NaCl (58.44 $g.mol^{-1}$, 99.5 %, Sigma) diluted in Mili-Q water and with a pH 7.6. To prepare the wash buffer with Tween-20 (High purity, Amresco), the solution was diluted in a ratio of 1:10 and added 1 ml of Tween-20 (0,1 % (v/v)).

The primary antibody used was replaced for another one NOVUS (Niemann-Pick type C1 Like-1, polyclonal, from Rabbit, Novus Biologicals). It was diluted in a solution of 50 mM Tris-HCl pH 7-8 (121.14 $g.mol^{-1}$, 99.5 %, Merck).

The transfer buffer was prepared as described before for the migration buffer with the addition of methanol (20 %) and without SDS.

The complete protocol for western blot is in the appendix A.

3.10. HPLC-DAD analysis of metabolites

The HPLC analysis was carried out in an Elite LaChrom® VWR Hitachi Liquid Chromatograph equipped with a Column Oven L-2300 and Diode Array Detector L-2455 (VWR, USA). The column used was a LiChroCART® 250-4 LiChrospher® 100 RP-8 (5 µm).

First it was necessary to equilibrate the HPLC by passing the reagents used to analyse the samples in the tubes and column and do the purge to be sure that there were no waste of previous solutions.

To analyse the samples were injected 25 µl with an auto injector. The samples contained lysed cells (control) and lysed cells grown in contact with the extract. The analysis was carried out between 200 and 500 nm with a diode array detector for 30 minutes (each sample). The analysis was done using a gradient composed of solution 0.05 % TFA, prepared with Mili-Q water (A) (114.02

g.mol^{-1}, 99.8 %, Uvasol, Merck) and methanol (B) (32.04 g.mol^{-1}, Ultragradient, Carlo Erba): 0 min - 80% A and 20% B; 20 min 20% A and 80% B; 25 min - 20% A and 80% B, 28 min up to 30 min – 80 % A and 20 % B. The flow rate was 1 ml.min^{-1} and the pressure between 0 and 392 and the temperature at 30 °C.

4. Results and discussion

4.1. Study of influence of the compounds in cell growth

As demonstrated in previous work the extract in contact for 24 hours is not toxic at concentrations up to 2 mg.ml^{-1} for the cells HepG2. However, it was confirmed the influence of compounds in the extract on cell growth. To analyze this influence, the cells after being in contact with the extract for 24 hours were counted using the Neubauer chamber. The cells were counted and the concentration was calculated using the equation 1. The results are presented in Table 3.

Table 3 – **Results of the HepG2 cell concentration.**
The sample called control corresponds to the cells, Extract (1 mg.ml^{-1}) and Extract (0.5 mg.ml^{-1}) to cells growing in a solution of extract at different concentrations. The samples correspond to cells grown during one week at the same conditions.

Sample	Concentration
Control	$(2.68 \pm 1.68) \times 10^6 \, number \, of \, cells/ml$
Extract (0.5 mg.ml^{-1})	$(3.72 \pm 3.09) \times 10^6 number \, of \, cells/ml$
Extract (1 mg.ml^{-1})	$(2.54 \pm 1.99) \times 10^6 number \, of \, cells/ml$

Taking into account the results present in Table 3 it can be verified that the values are in the same order of magnitude. This is consistent with the results obtained before and therefore it is not expected to have significant variations between the concentrations of cells in the different conditions.

4.2. Study of protein quantification

To quantify the content of proteins in the cells HepG2 it was done the Bradford method. As explained before the lysis buffer used for the first samples contained urea, which interferes with Bradford reagent. However it was made a quantification of the concentration of proteins. These values were not take into account to understand what happens to the proteomics of the cells but contributed to verify if the cells were growing and were in an amount sufficient to get protein to use in electrophoresis.

To quantify the protein content it was necessary to obtain a calibration curve represented by the variation of BSA concentration and the absorbance. For the first protein quantification was used a previous curve (equation 2) and for the second was obtained a new calibration curve to determine the protein concentration.

$$y\,(Abs) = 0.0721x\left(\frac{\mu g}{\mu l}\right) + 0.003 \qquad \text{(Equation 2)}$$

The calibration curve is represented in figure 13. The dilutions were prepared from a solution of BSA 0.1 mg.ml^{-1}. The calibration curve (equation 3) was calculated taking in account the volume

present in the cuvette and therefore it was necessary to multiply the values by the dilution factor (5:1000 µl) to obtain the value of concentration in the samples.

$$y\,(Abs) = 37.8x\left(\frac{\mu g}{\mu l}\right) - 0.0215 \qquad \text{(Equation 3)}$$

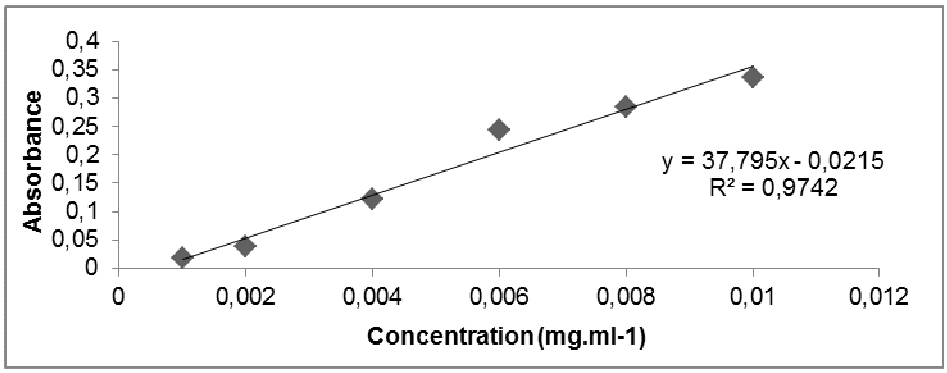

Figure 13 – Calibration curve obtained for the different dilutions of a BSA solution 0.1 mg.ml^{-1}.

Since it was necessary to verify if the Bradford reagent maintained the same values for the calibration curve, it was made another one. The dilutions were prepared from a solution of BSA 0.2 mg.ml^{-1} to increase the limit of the calibration curve. The calibration curve (equation 4) is represented in figure 14. The concentration calculated with equation 3 takes into account the dilution factor (5:1000 µl).

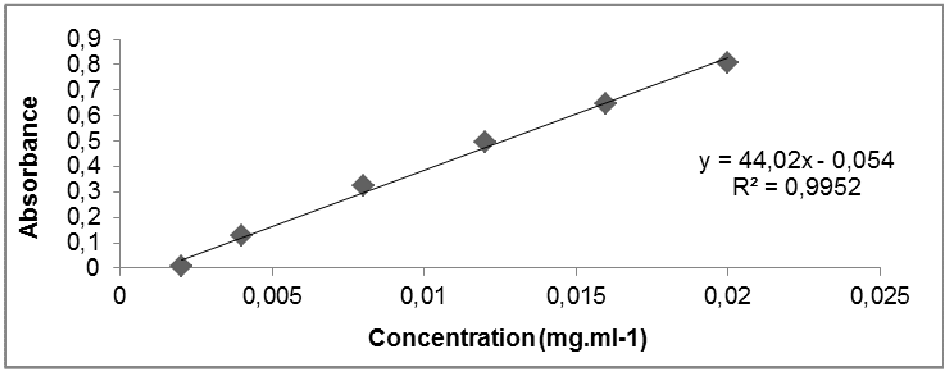

Figure 14 – Calibration curve obtained for the different dilutions of a BSA solution 0.2 mg.ml^{-1}.

$$y\,(Abs) = 44.02x\left(\frac{\mu g}{\mu l}\right) - 0.054 \qquad \text{(Equation 4)}$$

For the two samples lysed with the buffer containing Urea, Igepal and DTT the quantification was calculated taking into account the first two equations presented before. For the assay A was obtained using the equation 2 and the other ones equation 3. The results are presented in Table 4.

Table 4 – **Results for the protein quantification in the samples containing lysed HepG2 cells.**
The sample called control correspond to normal cells, Cholesterol to cells growing in a solution of cholesterol with a concentration of 5 mM, Extract (0.5 $mg.ml^{-1}$) and Extract (1 $mg.ml^{-1}$) to cells growing in a solution of extract with different concentrations. The results for A were obtained for cells grown in 12-well plates and quantified using equation 2 and for B were calculated using equation 3.

Assay	Samples	Absorbance	Protein concentration
Assay A	Control	0.403	5.55 $mg.ml^{-1}$
	Cholesterol	0.388	5.33 $mg.ml^{-1}$
	Extract (0.5 $mg.ml^{-1}$)	0.985	13.62 $mg.ml^{-1}$
	Extract (1 $mg.ml^{-1}$)	0.951	13.14 $mg.ml^{-1}$
Assay B	Control	0.334	1.88 $mg.ml^{-1}$
	Cholesterol	0.150	0.908 $mg.ml^{-1}$
	Extract (0.5 $mg.ml^{-1}$)	0.761	4.14 $mg.ml^{-1}$
	Extract (1 $mg.ml^{-1}$)	0.713	3.89 $mg.ml^{-1}$

The results presented in Table 4 show that in the case of cholesterol the value for the concentration of protein is lower than in the other cases. This may be due to the amount of cholesterol concentration in the cell medium. However, the values are not acceptable because the values in the extract are superior to the values of the control cells, thus demonstrating that it is not possible to quantify the protein content correctly using this lysis buffer since the Bradford reagent is very sensitive with other reagents (Systems & Additives n.d.). The Bradford reagent reacts with a large range of reagents including urea, detergents and protease inhibitors. However to extract membrane proteins is necessary to add a detergent or urea to the buffer since these proteins needs to be solubilized and therefore the protein quantification needs to be performed in another way.

To overcome this problem, other lysis buffers used for membrane proteins extraction were tested. The buffers chosen are described in the methods section. The results for the different buffers were analyzed taking into account the amounts of protein quantification and electrophoresis results (point 4.3). The results obtained for the protein quantification are presented in Table 5. The samples tested were cells HepG2 growing in 12-plates. The absorbance was measured using two cuvettes as explained in the methods section, with the one cuvette containing Bradford reagent and lysis buffer (800 µl) and the other containing water and Bradford to see the difference in the absorbance. Using equation 2 was calculated the protein concentration.

Table 5 – **Results protein concentration for the different lysis buffers tested.**
The protein concentration was calculated using equation 3 taking into account the dilution factor.

Sample	Protein concentration ($mg.ml^{-1}$)	
	Blank (water and Bradford)	Blank (lysis buffer and Bradford)
Buffer A	0.977	0.976
Buffer B	-	1.21
Buffer C	-	1.08
Buffer D	0.453	0.494

The results in Table 5 show that the lysis buffer D had a lower protein concentration. The lysis buffers containing urea or detergents are more effective in protein extraction however it is not possible to quantify correctly the protein concentration. Since the analysis was only using Bradford method, the lysis buffer D was the chosen one.

In Table 6 are presented the values of protein quantification for cells grown in 12-well plated and 24 hours in contact with the compounds. The assays C and D (Table 6 and 7) were obtained by performing the lysis with buffer D. For the assay D was added another step in the lysis protocol as explained in the methods section. The samples cholesterol and extract (0.5 $mg.ml^{-1}$) from assay C were the only quantified. The other two samples had a value of absorbance negative probably due to an experimental error.

The results for the assay E, presented in Table 7, were obtained in the same way as the ones from D however the calibration curve used to quantify the protein concentration was the equation 4.

Table 6 – **Values for the protein concentration for the assay C.**
The cells grew 2 days in the plate and 24 hours in contact with the compounds. The values were calculated taking into account the different blank cuvette.

Results	Sample	Protein concentration ($mg.ml^{-1}$)	
		Blank cuvette (water and Bradford)	Blank cuvette (lysis buffer and Bradford)
Assay C	Cholesterol	0.160	0.247
	Extract (0.5 $mg.ml^{-1}$)	0.213	0.310

Table 7 – **The values for the protein concentration were obtained for the two steps of the lysis.**
The protein concentration of the lysed cells was obtained for the sample after the ultrasound bath step and the first supernatant after the centrifugation. The value for the pellet was calculated by difference between the lysed cells and the first supernatant. The concentration of protein was calculated taking into account the dilution factor.

Results	Sample	Protein concentration ($mg.ml^{-1}$)		
		Lysed cells	First supernatant	Pellet
Assay D	Control	0.600	0.250	0.350
	Extract (1 $mg.ml^{-1}$)	0.530	0.486	0.044
	Extract (0.5 $mg.ml^{-1}$)	0.414	0.348	0.066
Assay E	Control	0.402	0.228	0.174
	Extract (1 $mg.ml^{-1}$)	0.220	0.0718	0.148
	Extract (0.5 $mg.ml^{-1}$)	0.285	0.0738	0.211

Analyzing the values presented in the previous tables it is possible to verify some differences in the concentration of proteins for the different samples. The results for the assay E (Table 7) show that the protein concentration was dependent with the concentration of the extract present in the cell medium. However the results are not consisting since the samples with the higher concentration of extract in cell medium had more concentration of protein (assay D). The difference between protein concentration and extract concentration in cell medium became more consisting with the next results

and it became clear that higher extract concentration led to less proteins produced by the cells. These values are presented next. In the case of cholesterol the protein concentration was always less than in the other cases. It may be due to high cholesterol concentration leading to stress of cells or the concentration may inhibit some production of proteins although it was already done the study of toxicity and concluded that the concentration used in this work was not toxic to the cells. However, the conditions of the cells were not the same and therefore it would be necessary to do the study of the influence in cell growth and the toxicity of cholesterol. In the electrophoresis results can also be verified the conclusions taking into account the protein quantification.

The values presented in the previous tables show the correct variation on the protein concentration for the samples collected at different stages of the lysis and the variations between the samples of cells cultivated in different conditions.

The next results were obtained for the cells growing in T25. The cells from each assay grew for one week due to the long duplication time. Since the area was higher than in 12-well plates, the protein concentration was also higher. This resolved the problem of low protein concentration verified before and also in the electrophoresis gels (point 4.3). The cells were lysed in two steps as explained in the methods section. The protein concentration was calculated using equation 4 and the results for the protein concentration of each assay are presented in Table 8.

Table 8 – Values for the protein concentration for the assays F to I.
The protein concentration of the lysed cells was obtained for the sample after the ultrasound bath step and the first supernatant after the centrifugation. The value for the pellet was calculated by difference between the lysed cells and the first supernatant. The concentration of protein was calculated taking into account the dilution factor. The assay H was obtained by total lysis.

Results	**Sample**	**Protein concentration ($mg.ml^{-1}$)**		
		Lysed cells	First supernatant	Pellet
Results F	Control	2.12	1.76	0.36
	Extract (0.5 $mg.ml^{-1}$)	1.95	1.65	0.3
	Extract (1 $mg.ml^{-1}$)	0.872	0.791	0.081
Results G	Control	7.38	5.83	1.55
	Control 2	6.11	4.97	1.14
	Extract (0.5 $mg.ml^{-1}$)	6.97	5.97	1.00
	Extract (1 $mg.ml^{-1}$)	6.29	4.83	1.46
Results H	Control	7.11	-	-
	Extract (0.5 $mg.ml^{-1}$)	6.79	-	-
	Extract (1 $mg.ml^{-1}$)	6.65	-	-
Results I	Control	1.51	0.682	0.828
	Extract (0.1 $mg.ml^{-1}$)	2.03	1.96	0.07
	Extract (0.5 $mg.ml^{-1}$)	1.08	0.400	0.68
	Extract (1 $mg.ml^{-1}$)	0.967	0.318	0.649

Analyzing the results in Table 8 it can be concluded that the concentration of extract in cell medium influences the concentration of proteins produced by the cells. Cells grown for one week in T25 due to the long time to multiply in the flask this may be due to the fact that the number of passages of cells into flasks should not be too high since the cells lost the capacity to attach and multiply leading to higher duplicate times. In the case of results I the cells cultivated with extract in a concentration of 0.1 $mg.ml^{-1}$ had more protein concentration than the control cells. This result doesn't correspond to the expected since the presence of the extract would decrease the protein concentration verified in the assays done before. This didn't happen maybe because the cells in the flask had more cells leading to higher protein concentration or an experimental error when the Bradford method was performed.

To conclude and looking at figure 15 the values for the protein concentration (from assay B to I) are different when the cells are grown in different conditions. The main differences for the different samples correspond to the lysed cells and first supernatant since the proteins present in these fractions are essentially proteins from the cytosol. For the pellet the values are similar although they are always smaller than the control cells except in some cases that were due to experimental errors in the quantification or the different concentration of cells in the flasks that result in higher protein concentrations in the cells cultivated with the extract. This variation is also verified in the results from electrophoresis 2D (section 4.5).

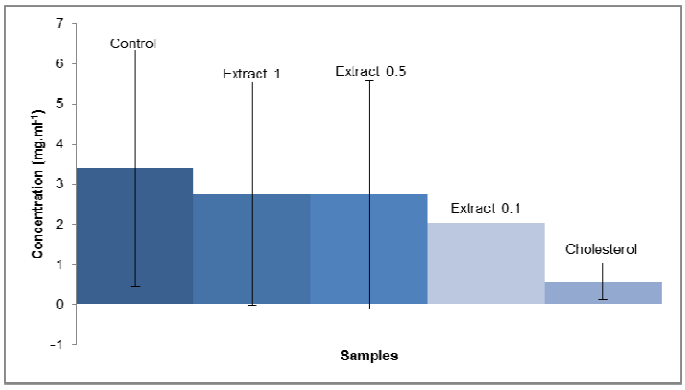

Figure 15 – Average of the concentration of the different samples taking into account the values of protein concentration presented before.

Despite the values for the protein concentration for the cells cultivated with the extract at a concentration of 0.5 $mg.ml^{-1}$ were almost always higher than in the case of the extract at 1 $mg.ml^{-1}$ the average was a little higher because the number of results for each sample was not the same. But the figure shows the difference between the control cells and cells cultivated in different conditions.

4.3. Study of the electrophoresis optimization and analysis of protein bands

The two first gels obtained after performing the electrophoresis were done using the protocol established before. This protocol worked for the electrophoresis; however it had problems during the transfer for western blot. It was also impossible to quantify the protein concentration since the lysis buffer contained urea. The gels represented in image 16 show the proteins bands for HepG2 cells lysed with buffer containing Urea, Igepal and DTT (Gel A) and also the protein bands for cells HepG2

growing in medium containing cholesterol (50 mM) and extract in two different concentrations (1 $mg.ml^{-1}$ and 0.5 $mg.ml^{-1}$) (gel B).

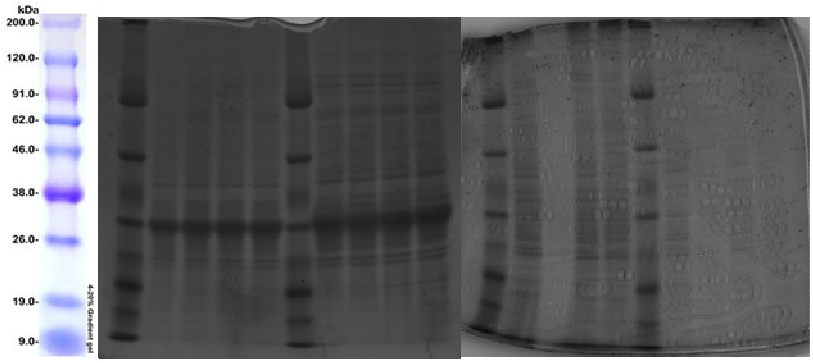

Figure 16 – **Electrophoresis gels representing protein bands from HepG2**.
In the gel A the bands correspond to the supernatant obtained after centrifugation. Lanes 7 to 10 loading buffer containing a ratio of 1 volume DTT stock (1 M) to 4 volumes of Protein Loading (Protein loading buffer 2x, Amresco). In the gel B the lanes have the following sequence: control, cholesterol, extract 1 $mg.ml^{-1}$ and extract 0.5 $mg.ml^{-1}$ with lanes 2-5 representing the pellet and 7 to 10 the supernatant. In all lanes were loaded 20 µl from a solution with 20 µl sample and 20 µl loading buffer (lanes 2-5) and 25 µl sample and 25 µl loading buffer (lanes 7-10).

The proteins presented in figure 16 correspond to the proteins from the supernatant and therefore belong to the cytosol. In the case of gel A the cells weren't washed with PBS and therefore the protein band in the middle of the gel and the bigger one might correspond to proteins present in the cell medium. This conclusion makes sense since for the gel B prepared in the same way the protein band doesn't have the same intensity. Looking for the protein bands on the side that had DTT in the loading buffer there are some proteins between the molecular weight of 200 to 120 kDa that may be the protein from NPC1L1 since the protein has a molecular weight of 146 kDa (Xie et al. 2012). However to be sure that the protein is the one of interest it would be necessary to perform the western blot to see if the antibody recognize the protein band. The addition of the DTT in the loading buffer is to reduce oxidation damage and keep the protein in solution (Laboratory 2015).

In the case of gel B there are some considerations that can be made. On the cholesterol lane there are no protein bands. Besides it was said that in a concentration of 50 mM the cells didn't die and taking into account the values of protein concentration, it can be assumed that the cells were not viable and the concentration for the cholesterol had to be lower. However the cell line was the same, the conditions of the previous work couldn't be the same and therefore the cells didn't respond the same way. In the case of the supernatant the protein bands are not very clear this may be due to insufficient time in the ultrasound bath or a dilution to high taking into account the protein concentration. In this gel it's not clear the protein bands localized at the top of the gel and therefore it cannot be concluded if it was possible to extract the protein from the membrane. Since the samples were boiled, and the lysis buffer contained urea, it may contribute to the denaturation of the samples and therefore the protein bands are not well defined comparing to with the ones in gel A.

To verify the difference in the protein bands to complete the lysis study, was performed an electrophoresis with the cells HepG2 lysed with the buffers tested (figure 17). Taking into account the results from protein quantification, it's expected to have more protein bands and more defined for the

buffers more efficient. In the gel is represented the protein bands for the pellet (first lanes) and the supernatant obtained in the lysis process (last lanes). In the case of the pellet to obtain more volume to treat the sample and load in the gel the samples were mixed with 10 µl distilled water and 20 µl loading buffer.

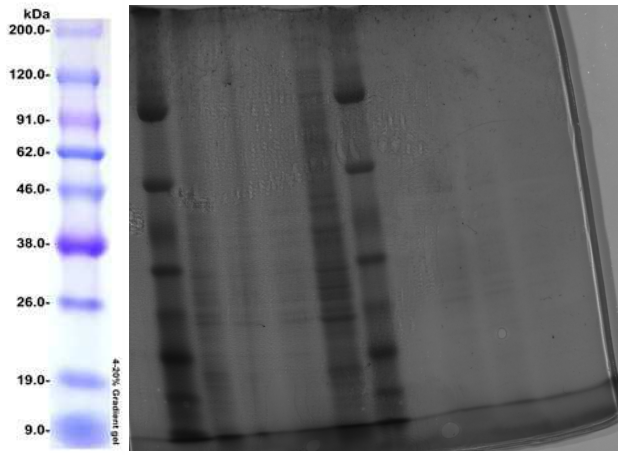

Figure 17 – Electrophoresis gels representing protein bands from HepG2 lysed with buffers (A, B, C and D respectively.

In lane 1 and 6 was loaded the molecular marker: 5 µl (Bluestep, Protein MW Marker, Broad Range, Prestained, Amresco). Buffer A: lane 2 (20 µl) and 7 (0,01 mg), Buffer B: lane 3 (20 µl) and 8 (0,012 mg), Buffer C: lane 4 (20 µl) and 9 (0,011 mg), Buffer D: lane 5 (20 µl) and 10 (0,005 mg). The samples from supernatant were mixed in a ratio of 1:1 with loading buffer (Protein loading buffer 2x, Amresco).

Analyzing the results from figure 17 it was concluded that the lysis buffer D, despite the lower values in the protein quantification got a better protein bands separation. Although the cells didn't grow similarly in the 12-well plates, the samples correspond to three wells collected. It can be assumed that the four samples got the same amount of material. This is also proved by the values of protein concentration that are not too different from each other. Just in the case of buffer D but looking at the gel the results in separation of proteins were good.

The supernatant had a low quantity of material. Only in the case of buffers B and C it can be visualized protein bands. However these samples, containing urea, were boiled and it may denature the proteins even more and led to the lost of some material. The volume of lysis buffer may also contribute to the dilution of the samples and therefore the bands are not too clear. Looking at the mass applied it also verified that the value for Buffer D is low and therefore it should be applied a higher mass of the sample.

After the choice in the lysis buffer taking into account the values of protein concentration and the results in the electrophoresis gels, the cells were lysed with buffer D but it was tested other steps in the process of lysis as the addition of a second step in the method and longer cycles in the ultrasound bath and the samples treated in an iced environment.

The results in figure 18 were obtained performing the same procedure as before with the addition of one step. The samples were treated in a cold system and using the lysis buffer cold. This was done due to the fact that the difference in temperature causes a thermal shock in the cells helping her lysis (Laboratory 2015).

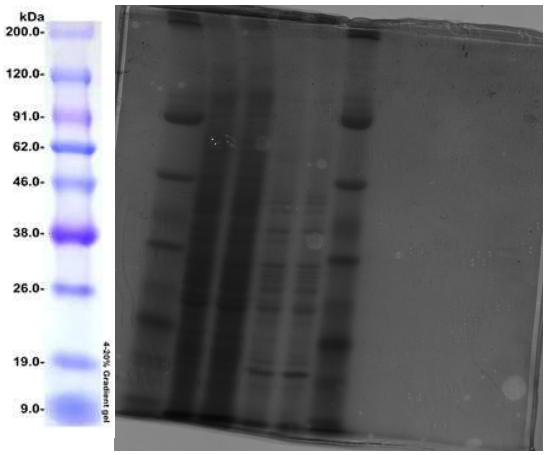

Figure 18 – Electrophoresis gels representing protein bands from HepG2 in contact with the compounds (cholesterol 50 µM, extract 1 mg.ml^{-1} and extract 0.5 mg.ml^{-1} respectively).

In the lanes 1 and 6 were 5 µl of molecular weight (Bluestep, Protein MW Marker, Broad Range, Prestained, Amresco). The gel represents the pellet (left side) (20 µl sample and 20 µl loading buffer) and the supernatant (20 µl sample and 20 µl loading buffer) both mixed with the same Laemmli buffer 2x in a ratio 1:1. The samples were loaded 30 µl in each well.

Analyzing figure 18 it can be concluded that the protein bands in the pellet for the control and cholesterol are not clear which means that the extraction was not sufficient to extract the proteins. The samples may needed more time in the ultrasound bath or the quantity of lysis buffer should be higher. To resolve this point and because maybe the protein from the membrane were not being extracted, the pellet were ressuspended in another lysis buffer as explained in the methods section.

In the case of the supernatant there are no protein bands, which contribute to the fact that the extraction was not enough since it was expected to have protein bands in the supernatant from the cytosolic that are the first proteins extracted.

The cells containing cholesterol in the medium were viable and at the concentration tested were able to see protein bands. However for this sample the protein bands are not defined as explained before. To overcome this problem it should be used more lysis buffers in this case and also the control cells or let the samples remain more time in the ultrasound bath.

Looking at the top of the gel it can be seen two protein bands too bright in the case of the control cells and the cells cultivated with cholesterol that may be the protein NPC1L1, however to be sure it would be necessary to perform the western blot to see if the antibody connect in that protein band.

The results presented in figure 19 were obtained after the addition of a second step in the lysis process. The pellet was ressupended with another lysis buffer containing urea, Igepal and DTT. The figure shows the supernatant obtained after centrifuging the sample from the ultrasound bath containing the pellet with the second lysis buffer (100 µl). To verify if the extraction of the transporter NPC1L1 was efficient was loaded in the gel the peptide (Blocking Peptide, Novus Biologicals) of NPC1L1. This gel was performed with the same amount of sample as the gel prepared to do wet transfer. This was made to see the efficiency in the transfer process.

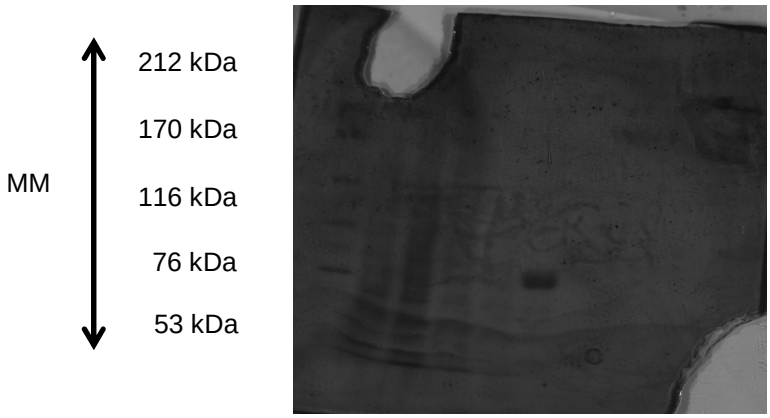

Figure 19 – Electrophoresis gels representing protein bands from HepG2 in contact with the compounds (cholesterol 50 mM, extract 0.5 mg.ml^{-1} and extract 1 mg.ml^{-1} respectively).

In the lanes 1 was loaded 5 µl of molecular marker (HMW-SDS calibration kit, Pharmacia Biotech) and in lane 6 4 µl of peptide. In the first five lanes (A) were loaded 30 µl of each sample (20 µl sample and 20 µl loading buffer) in the other lanes (B) the same amount of samples from another assay. The loading buffer used was Laemmli loading buffer 2x.

The results B in the gel are not visible since they were from other samples prepared before. The pellet kept in the freezer was mixed with the lysis buffer mentioned but since there are no protein bands and the samples didn't had proteases inhibitors to keep the proteins intact when they were saved the protein may be denatured in the process. The results from lanes A are not very clear. This may be due to the dilution of the samples. In fact, since the protein was not quantified it's not possible to know how much proteins were present in the first step of the lysis. It can't be seen also if there are proteins located at the higher values of molecular weight at least to admit that the extraction of NPC1L1 was successful. The presence of the peptide (control peptide) in the gel is to confirm the effective connection of the antibody since the peptide is specific for the antibody used. The NPC1L1 does not appear at 46 kDa.

The concentration used for the cells growing in cholesterol was 50 mM. Despite this concentration led to unviable cells before in this case the cells were viable and it was obtained protein bands in the gel. However as said before the protein bands are not very clear which may be due to low volume of lysis buffer and more time in the ultrasound bath to help lysing the cells. These samples were boiled and this may contribute to denature more the proteins and therefore the material content is too low and the signal in the gel was weak.

4.4. Study of western blot and variation in proteins bands stained with Ponceau red

After the method for the lysis and the electrophoresis it was possible to perform the western blot to see the presence of the proteins from the transporter NPC1L1. The membranes obtained were also analyzed to compare the intensity of the protein bands from each sample. Since the conditions were not always the same, the concentration of antibody in contact with the membranes was also optimized.

4.4.1. Study of the difference in the intensity of the protein bands stained with Ponceau Red

The variation in the protein bands in the membrane after wet transfer is divided in the results for the cells growing in 12-well plates and the T25. The variation in the protein bands was already explained in the electrophoresis section 4.3 and the results presented in this point are just a confirmation of what has been said before.

In figure 20 are presented three membranes obtained from the wet transfer. The samples loaded in the membrane A belong to cells grown in a 12-well plate and the protein concentration of each sample is presented in table 7 (assay D). The first lanes present in the membrane correspond to the samples from the first step of the lysis method (lanes 2 to 4), using buffer D, and the other lanes to the second supernatant (lanes 5 to 10).

The samples for the membranes B and C were obtained using the optimized lysis method. The protein concentration was quantified and presented in table 8 belonging to the assay F and I respectively. The first lanes correspond to the first supernatant and the other lanes to the second supernatant for each sample.

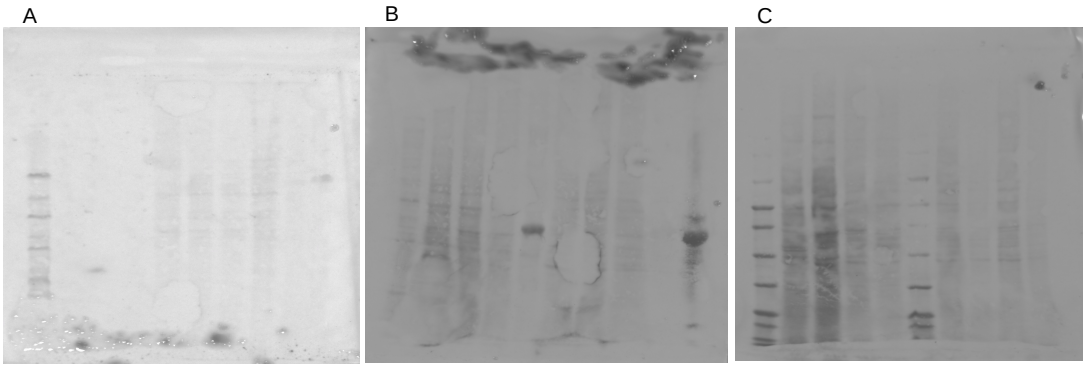

Figure 20 – Membranes obtained after the wet transfer.

Membrane A lane 1: molecular weight marker (marker C, methods), lanes 2, 5 and 8: control cells 0.003 mg and 0.0035 mg (lane 5 and 8), lanes 3, 6 and 9: extract 1 $mg.ml^{-1}$: 0.004 mg and 0.0004 mg respectively, lanes 4 and 7 extract 0.5: 0.003 mg and 0.0007 mg respectively. The samples were mixed in a ratio of 1:1 with Laemmli loading buffer 2x.
Membrane B: lane 1, molecular weight (marker A, methods), lanes 2 and 6 (control cells): 0.037 mg and 0.008 mg respectively, lanes 3 and 7 (extract 1 $mg.ml^{-1}$): 0.016 mg and 0.002 mg respectively, lanes 4 and 8 (extract 0.5 $mg.ml^{-1}$): 0.034 mg and 0.006 mg respectively, lane 5: 4 µl and peptide and lane 9: 4 µl tubulin (loading control).
Membrane C: lane 1 and 6, molecular weight (marker C, methods), lanes 2 and 7 (control cells): 0.017 mg and 0.021 mg respectively, lanes 3 and 8 (extract 0.1 $mg.ml^{-1}$): 0.049 mg and 0.002 mg respectively, lanes 4 and 9 (extract 0.5 $mg.ml^{-1}$): 0.010 mg and 0.017 mg respectively, lane 5 and 10 (extract 1 $mg.ml^{-1}$): 0.008 mg and 0.016 mg respectively.

Analyzing the membranes obtained after the wet transfer and stained with Ponceau Red there are some considerations that can be made.

For the membrane A the samples used were defrosted and the samples represented are the supernatants from each step of the lysis process. The first supernatant is not visible in the membrane, which can be explained by the fact that the samples didn't contain proteases inhibitors and the proteins after defrosting were denatured and it also may be due to the dilution of the samples. Also in this membrane is not very visible the difference in the intensity of the protein bands due to the conditions on cell growth and therefore it can't be visualized the difference that was shown in the protein quantifications (assay D).

In the membrane B the sample from the control cells have the protein bands more intense than in the other cases. Taking into account the results for the samples from cells with the extracts in the cell medium is possible to verify the difference between the intensity of the bands and the extract concentration. In the case of the second supernatant the intensity is not very different and it can be concluded that the difference in the proteins from the first supernatant and the second due to the presence of compounds is most pronounced. The difference in the intensity of the proteins can be explained assuming that the extract affects more strongly the cytosolic proteins since these are the first to be extracted in the lysis process compared to the membrane proteins that because they have higher molecular weight are extracted essentially in the second step of the lysis process. These results are supported by the values in the protein quantification (assay F).

In the case of membrane C the different protein bands are separated. However for the sample of extract 0.1 $mg.ml^{-1}$ in the first supernatant the intensity in the protein bands was higher than in control cells. In fact it was made the same conclusion when was analyzed the values for the protein concentration (table 8). It was expected to see a slight difference when compared to the control cells since the presence of the extract would reduce the protein concentration as seen before however it didn't happened maybe because of a bad dilution of the extract or because the flask with this condition had a higher concentration of cells than the other three or also because of an error in the Bradford method. For the extract with a concentration of 0.5 and 1 $mg.ml^{-1}$ the intensity was different than in the case of the control cells as predicted and concluded with the previous results. In the case of the second supernatant the protein bands are not to clear due to the dilution of the sample during the treatment for the electrophoresis gel.

To improve the results in the transfer method some conditions could be tested. Since the large proteins, such as the protein of interest, tend to precipitate the amount of methanol and the addition of SDS in the transfer buffer could be analyzed. The addition of SDS to a final concentration of 0.1 % helps avoiding the precipitation of the proteins; however methanol tens to remove it from the proteins and thus the percentage of methanol can be decrease to 10 % or less. The decrease of the methanol percentage promotes the swelling of the gel allowing large proteins to transfer more easily. The time of the transfer process can be increased and performed at 4 °C (Electrophoresis & References n.d.).

The other membranes obtained during the optimization of the process are presented in the appendix D.

4.4.2. Study of the antibody bands

After the optimization of the processes to extract and visualize the proteins from the transporter in study was possible to perform the western blot and search for the presence of the transporter by the identification of the antibody. However it was not possible to see the antibody bands in all of the membranes presented in the point before. In some cases the antibody was diluted in solutions containing methanol, the protein was not effectively extracted and the antibody may lost the affinity in some cases due to the fact that was re-used.

However the antibody band was identified in two of the membrane obtained. The antibody band was verified with the information on the product (Biologicals 2015). The bands from the antibody are presented in figure 22. This information is presented in the information on the product from the supplier (Biologicals 2015).

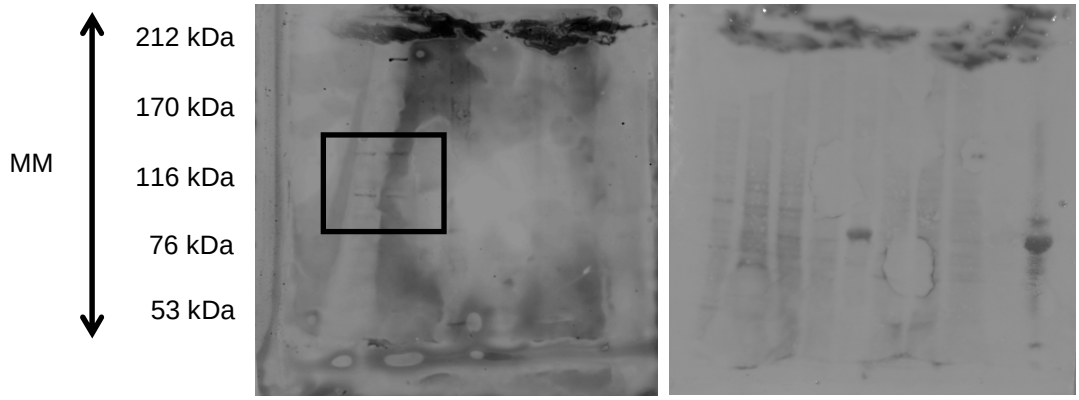

Figure 21 – Membranes obtained after the wet transfer (right side) and incubation with the antibody (left side).
The membrane on the right was stained with Ponceau Red and is the same as presented in figure 20. In the left side the membrane was incubated with the first antibody (Niemann –Pick type C1 Lyke-1, polyclonal, from Rabbit, Novus Biologicals) and after with the second antibody (ECL Anti-Rabbit IgG Horseradish Peroxidase-Linked Whole Antibody (from Donkey), Amersham, GE) detected with the detections reagents. The membrane on the left side was obtained after 1 minute of exposure with the detection solutions.

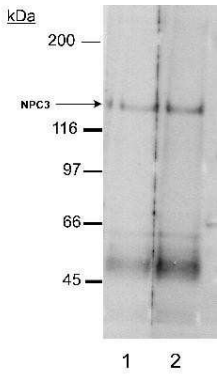

Figure 22 – Western Blot: Niemann-Pick type C1 Like-1 Antibody [NB400-127].
Detection of NPC3 in rat small intestine membrane preparations (20 µg) using NB 400-127. Lane 1: 2 $\mu g.ml^{-1}$ and Lane 2: 4 $\mu g.ml^{-1}$. ECL: 5 minutes exposure (Biologicals 2015).

Analyzing figure 21 and taking into account the information for the antibody (figure 22) it can be concluded that the bands signalized in the figure with the box are antibody connections to the proteins of NPC1L1 at approximately 140 kDa (band representing NPC1L1). Despite the molecular marker is not too visible in the membrane, the location of the protein bands it's approximately similar to the one in figure 21. It was expected to see the protein in the second supernatant since its more effective in the extraction of membrane proteins, however the extraction of the membrane proteins were effective in the first step and therefore it was possible to see the proteins in the first one. The other two bands present in the membrane are not considered as proteins from the transporter and it may be due to some nonspecific interaction of the antibody since it's not expected to see bonds with

protein with that molecular weight. The other bands present in the membrane represent non-specific connections that normally occur when using polyclonal antibodies (Portable & Laser 2014).

The bands visualized in the figure correspond to the sample control cells and cells in contact with extract at 0.5 $mg.ml^{-1}$. In the case of the cells in contact with extract at 1 $mg.ml^{-1}$ there are no bands from the antibody. This may be due to an insufficient extraction of the protein since there are no certainties in the effect of the plant mixture in the expression of NPC1L1. Since it was verified that the mixture decreased the absorbance of cholesterol (Manuscript & Transport 2014) it would led to the increase of endogenous NPC1L1 in the cell surface of HepG2 (Manuscript & Transport 2014) and therefore It was expected to see the connection of the antibody with the protein.

In the figure 23 it's possible to see the band of the antibody linked to the peptide. The peptide is specific for the antibody in use and therefore it was expected that the connection appeared in all the membranes incubated with the antibody. There is no other band of the antibody, which may be due to the fact that the protein was not extracted correctly. As explained in the point before, this extraction was performed without salt (NaCl) in the lysis buffer and the volumes used where not sufficient to lyse the amount of cells. Probably the membrane proteins were not correctly extracted since it was found the presence of the proteins in the membrane before which means that at least the control cells have the presence of the protein in their cellular structure or the for this membrane the concentration of the peptide was higher and the antibody only connected with it.

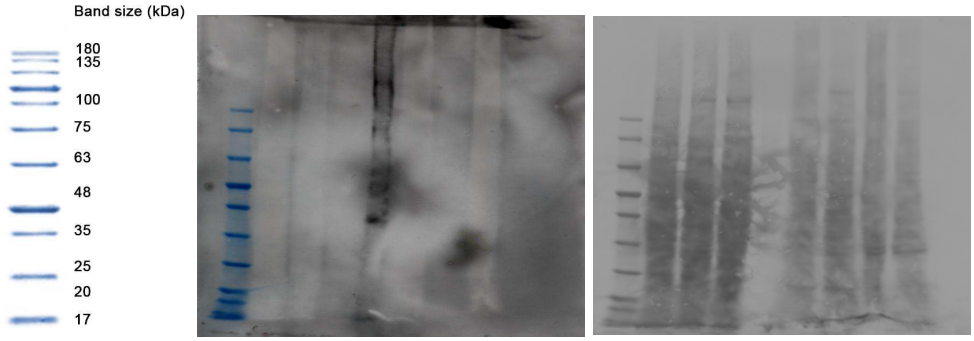

Figure 23 – Membranes obtained after the wet transfer (right side) and incubation with the antibody (left side).
The membrane on the right was stained with Ponceau Red. In the left side the membrane was incubated with the first antibody (Niemann –Pick type C1 Lyke-1, polyclonal, from Rabbit, Novus Biologicals) and after with the second antibody (ECL Anti-Rabbit IgG Horseradish Peroxidase-Linked Whole Antibody (from Donkey), Amersham, GE) detected with the detections reagents. The membrane on the left side was obtained after exposing for 5 minutes with the detection solutions.

Looking to membrane in figure 23 it's possible to see the antibody protein linked to the peptide since the antibody recognized the peptide. The other bands are not visible because of what was explained before. The blocking peptide binds specifically with the antibody because the peptide resembles the epitope recognized by the antibody. The antibodies linked to the blocking peptide do not connect with the epitope of the protein in interest and therefore the amount of peptide needs to be low to ensure the connection in the protein of study (Required n.d.).

For the other membranes, to achieve better results, the membranes should be more time in contact with the detections solutions and more time in the machine since they had a weak signal. However the problem could be due to the high amount of sample and the unspecific bound of the

antibody with the proteins due to much quantity of antibody. To overcome the unspecific bounds the antibodies could be diluted in the blocking agent however this option can result in a weaker signal. This procedure needs to be optimized to ensure that the conditions were the best to study the differences in the NPC1L1 transporter.

4.5. Study of the difference in the protein bands obtained in an Electrophoresis 2D

To verify if the difference in the protein concentration was performed a 2D-gel electrophoresis to see if there were differences in the cell proteome. This electrophoresis was performed according to the information present in (Falé et al. 2012)

In figure 24 is presented the gels obtained for the control cells and cells in contact with extract at 1 $mg.ml^{-1}$.

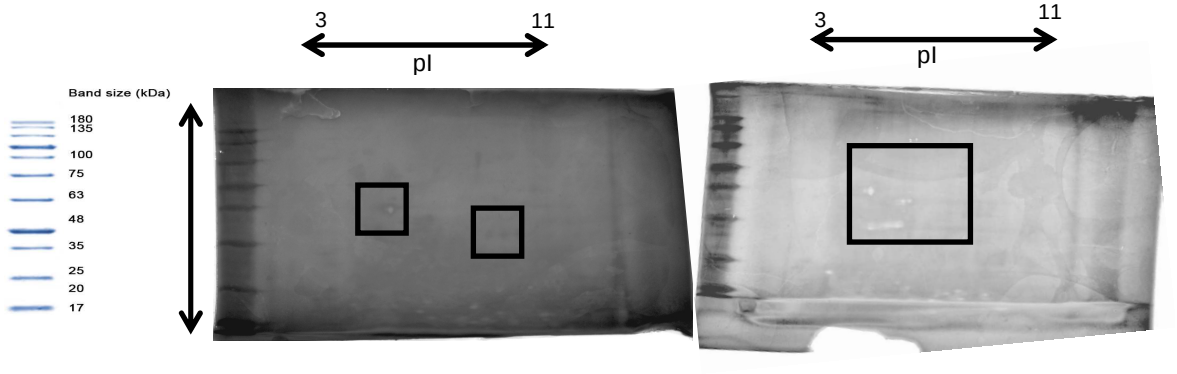

Figure 24 – Gel obtained after the second step of the electrophoresis 2D.
The gel on the left (A) corresponds to the control cells and cells in contact with extract at 1 $mg.ml^{-1}$ (B). The gels were stained with silver nitrate. The marker was loaded at a quantity of 4 µl (NZY Blue Protein Marker, NZY Tech).

The two gels represent the soluble fraction (supernatant) from the lysed cells after the centrifugation. This fraction represents the cytosolic proteins, which have different expression when the proteins from the control cells are compared with the cells that grew in the presence of the extract. These results corroborate the results from the protein quantification.

Analyzing the proteins inside the black squares in the gel A it's visible that some proteins lost their expression when they were in contact with the extract and other increased their expression (black square in the image on gel B).

Taking in consideration what was concluded in (Falé et al. 2012)it can be hypothesized that some of the cells that changed their expression may correspond to adhesion cells or cadherins (Goodwin & Yap 2004) responsible for cell-cell adhesion and recognition. These changes may result in the detachment of the cells when they are cultivated with the compounds.

4.6. Study of the metabolites of extracts from HepG2 cells in contact with the extracts

To confirm if the presence of the compounds in the cell medium affects the metabolism of them was made an analysis of the cell content after the lysis. The content was analyzed in an HPLC-DAD and the chromatograms were compared with the chromatogram of the extract to verify if the main compounds of the extract were found in the cell content.

The results in figure 25 were obtained by the two-step lysis and the results for the sample lysed cells and supernatant and in figure 26 the pellet obtained in the process. In the figure 27 are presented the results for the samples obtained by performing a total lysis.

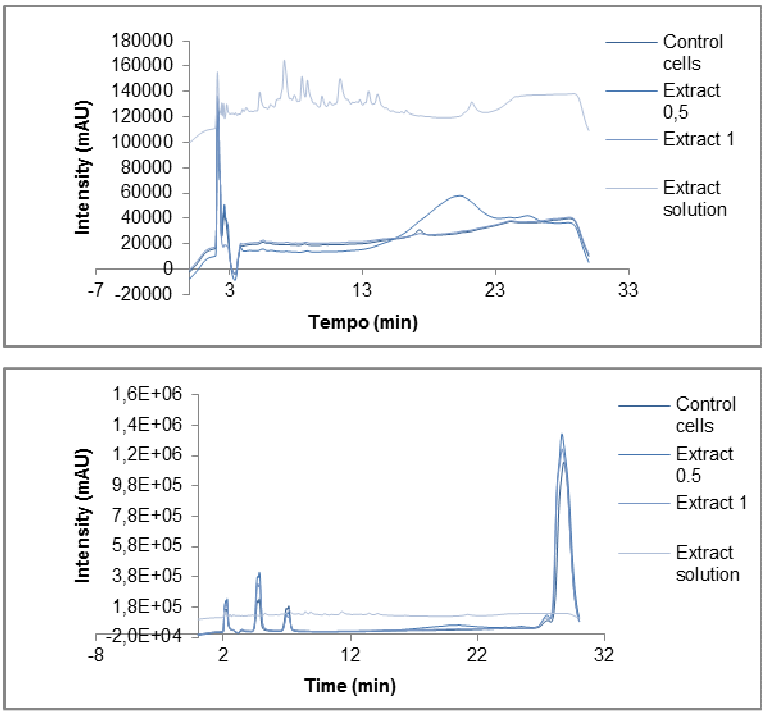

Figure 25 – Chromatograms obtained by the average of the wavelength 200 to 500 nm.
The different lines correspond to the samples tested before: control cells, cells in contact with extract at 0.5 mg.ml^{-1} (extract 0.5), extract 1 mg.ml^{-1} (extract 1) and extract solution that correspond to the extract diluted in distilled water and with a concentration of 1 mg.ml^{-1}. The first chromatogram corresponds to the samples collected after the first step of the lysis (supernatant from the centrifugation), the second figure to the samples after the second step of the lysis (supernatant from the second centrifugation). The extract solution was moved up to see the peaks.

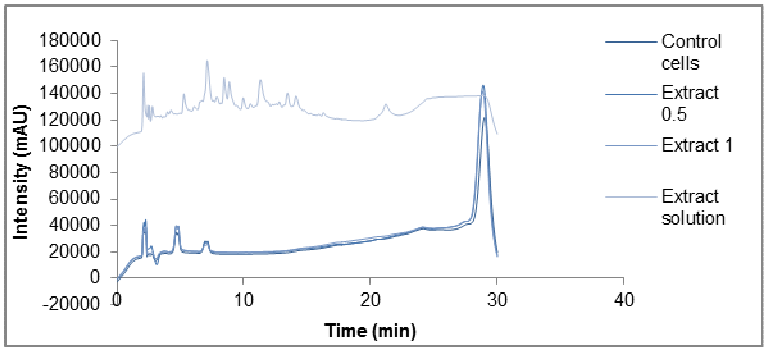

Figure 26 – Chromatogram obtained by the average of the wavelength 200 to 500 nm.
The different lines correspond to the samples tested before: control cells, cells in contact with extract at 0.5 mg.ml^{-1} (extract 0.5), extract 1 mg.ml^{-1} (extract 1) and extract solution that correspond to the extract diluted in distilled water and with a concentration of 1 mg.ml^{-1}. The chromatogram corresponds to the remaining pellet after the second step of the lysis process. The extract solution was moved up to see the peaks.

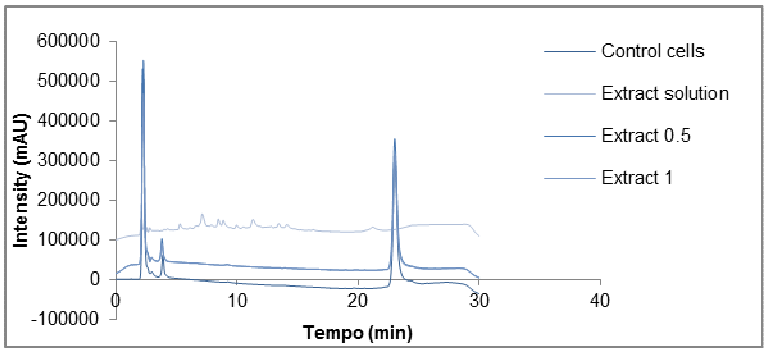

Figure 27 – Chromatogram obtained by the average of the wavelength 200 to 500 nm.
The different lines correspond to the samples tested before: control cells, cells in contact with extract at 0.5 $mg.ml^{-1}$ (extract 0.5), extract 1 $mg.ml^{-1}$ (extract 1) and extract solution that correspond to the extract diluted in distilled water and with a concentration of 1 $mg.ml^{-1}$. The chromatogram corresponds to the supernatant obtained after performing a lysis in one step. The extract solution was moved up to see the peaks.

The analysis of the difference in the chromatograms was made by tanking into account the maximum value present in the chromatograms belonging to the major components of the herbal mixture (appendix B). Those major compounds are essentially phenolic acids and flavonoid glycosylate. In Table 7 are presented the values for the chromatograms presented in figure 25 and 26. It can be concluded that there are some decreases in the major compounds when the extract sample is compared with the samples obtained from the cells.

It's also possible to analyze the chromatograms presented in the three figures presented before by comparing each sample with the one for the control cells obtained in the same conditions. Despite the decrease in the maximum values for the samples, the chromatogram is very similar and therefore it can be concluded that the cells didn't change the metabolism when they grew in contact with the extract and therefore maintained the mechanism. It's tempting to admit that the proteins, which changed their expression in contact with the extract, weren't proteins involved in critical pathways necessary to the survival of the cells.

Table 9 – Values for the absorbance of the main components of the herbal mixture identified in appendix B.
These values correspond to the chromatograms presented in figures 16 and 17. The major compounds are: caffeoylquinic acid (5.48 min), kaempferol-3-O-sophoroside-7-O-glucoside (7.34 min), chlorogenic acid (8.71 min), A-type procyanidin trimer (9.12 min), chryoeriol 6.8-di-C-hexoside (9.94 min), rhamnosylvetexin (11.67 min), quercetin glucoside (13.94 min) and acetyl-rhamnosylvitexin (14.15 min).

Retention time (min)	**Absorbance**									
-	Samples									
-	Extract solution	**Control**			**Extract 0.5**			**Extract 1**		
		Lysed cells	First supernatant	Pellet	Lysed Cells	First supernatant	Pellet	Lysed Cells	First supernatant	Pellet
5.48	$2,95\times10^4$	$2,14\times10^4$	$2,50\times10^4$	$1,95\times10^4$	$1,57\times10^4$	$2,72\times10^4$	$2,09\times10^4$	$2,27\times10^4$	$2,87\times10^4$	$2,11\times10^4$
7.34	$4,56\times10^4$	$1,93\times10^4$	$5,12\times10^4$	$2,12\times10^4$	$1,39\times10^4$	$4,55\times10^4$	$2,15\times10^4$	$2,13\times10^4$	$5,05\times10^4$	$2,17\times10^4$
8.71	$3,58\times10^4$	$2,00\times10^4$	$2,07\times10^4$	$1,83\times10^4$	$1,41\times10^4$	$1,66\times10^4$	$1,95\times10^4$	$2,13\times10^4$	$2,06\times10^4$	$1,98\times10^4$
9.12	$3,52\times10^4$	$1,89\times10^4$	$2,04\times10^4$	$1,82\times10^4$	$1,37\times10^4$	$1,63\times10^4$	$1,95\times10^4$	$2,05\times10^4$	$2,04\times10^4$	$1,96\times10^4$
9.94	$3,65\times10^4$	$1,92\times10^4$	$2,03\times10^4$	$1,82\times10^4$	$1,37\times10^4$	$1,60\times10^4$	$1,96\times10^4$	$2,06\times10^4$	$2,03\times10^4$	$1,97\times10^4$
11.67	$3,53\times10^4$	$1,95\times10^4$	$2,04\times10^4$	$1,86\times10^4$	$1,42\times10^4$	$1,69\times10^4$	$1,98\times10^4$	$2,11\times10^4$	$2,10\times10^4$	$2,01\times10^4$
13.94	$3,13\times10^4$	$2,16\times10^4$	$2,20\times10^4$	$2,03\times10^4$	$1,80\times10^4$	$2,03\times10^4$	$2,15\times10^4$	$2,29\times10^4$	$2,35\times10^4$	$2,18\times10^4$
14.15	$3,74\times10^4$	$2,20\times10^4$	$2,24\times10^4$	$2,07\times10^4$	$1,89\times10^4$	$2,11\times10^4$	$2,19\times10^4$	$2,32\times10^4$	$2,38\times10^4$	$2,22\times10^4$

Table 10 – Values for the absorbance of the main components of the herbal mixture identified in appendix B.

These values correspond to the chromatogram in image 18. The major compounds are: caffeoylquinic acid (5.48 min), kaempferol-3-O-sophoroside-7-O-glucoside (7.34 min), chlorogenic acid (8.71 min), A-type procyanidin trimer (9.12 min), chryoeriol 6.8-di-C-hexoside (9.94 min), rhamnosylvetexin (11.67 min), quercetin glucoside (13.94 min) and acetyl-rhamnosylvitexin (14.15 min).

Retention time (min)	**Absorbance**			
-	**Samples**			
-	**Extract solution**	**First supernatant**		
		Control cells	**Extract 0.5**	**Extract 1**
5.48	$2,95\times10^{4}$	$8,87\times10^{2}$	$4,22\times10^{4}$	$4,18\times10^{4}$
7.34	$4,56\times10^{4}$	$-3,60\times10^{3}$	$3,87\times10^{4}$	$3,81\times10^{4}$
8.71	$3,58\times10^{4}$	$-7,35\times10^{3}$	$3,54\times10^{4}$	$3,53\times10^{4}$
9.12	$3,52\times10^{4}$	$-7,34\times10^{3}$	$3,68\times10^{4}$	$3,63\times10^{4}$
9.94	$3,65\times10^{4}$	$-9,79\times10^{3}$	$3,32\times10^{4}$	$3,34\times10^{4}$
11.67	$3,53\times10^{4}$	$-1,34\times10^{4}$	$3,01\times10^{4}$	$3,06\times10^{4}$
13.94	$3,13\times10^{4}$	$-1,58\times10^{4}$	$2,78\times10^{4}$	$2,85\times10^{4}$
14.15	$3,74\times10^{4}$	$-1,62\times10^{4}$	$2,75\times10^{4}$	$2,82\times10^{4}$

Analyzing the values from Table 9 it's possible to conclude that the values in all the samples are lower than in the extract solution to each retention time. There are two values that are higher than in the case of the extract. These values belong to the samples from the first supernatant for the control cells and the cells cultivated with 1 $mg.ml^{-1}$ of extract. In the case of the control cells the value cannot be taken into account since it's just one case where it's verified that condition and therefore the value could be because of a bad dilution or some residues that could be present in the vial. In the second case since are the cells with extract it could be a residue from the extract present in the medium that led to a highest absorbance at that retention time.

Looking at the values in the Table 10 it's possible to conclude that the values of absorbance are lower than in the case of the control cells, which means that the cells don't produce the compounds present in the extract solution which is confirmed by literature since these compounds are essentially present in plants and fruits (Manach et al. 2004). Analyzing the values for the cells cultivated with extract all the values are lower than the values for the extract solution just in the case of the compounds with a retention time of 5.48 and 9.12 min the values are higher. These retentions times belong to caffeoylquinic acid and A-type procyanidin trimer. As said in the article (appendix C) these compounds are highly permeated in the Caco-2 monolayers going then to the liver. So the expected was that the difference between the values for these compounds in both samples was minimal since the cells don't produce those compounds present in the extract used. Therefore the difference may be due to an error in the dilution or presence of residues that absorb light at the same wavelength.

In general the values present in both table tend to decrease even for the cells cultivated with extract. The cases where the values are higher are not similar in both tables.

5. Conclusion and future perspectives

The research field regarding cholesterol is still a controversial subject with some points that are not yet clarified. In more than 50 years since the discovery of the importance of cholesterol, we still have doubts in how the body responds to the presence of dietary cholesterol and how he regulates it.

The consent among doctors and researches about it is far from being achieved and therefore this question will remain in discussion for a while. The future challenges go through the understanding of the mechanism and the effects of the cholesterol in the human body to achieve and develop better ways to control the cholesterol. The acceptance of other forms of treatment represents another challenge due to the lower credibility when compared with the pharmaceutical drugs.

The use of mixtures of herbs, very old practice, is a way of controlling cholesterol. However for these plants, used due to customs and popular knowledge, the action in the body and the diseases is unproven.

The main goal of this thesis was to evaluate the effect of these mixtures in cells and cholesterol transporters in order to verify the differences when using mixtures and if they block the action of transporters and therefore prove if they are a good way to reduce the cholesterol in circulation.

Despite the results presented in the first study that proved that the herbal mixture inhibit the permeation of cholesterol for Caco-2 cells, it was verified that the herbal mixtures led to differences in the protein concentration for the cells grown in contact with the extracts and therefore it would be necessary to identify these proteins and their role in the human body. The influence of the extract in the loading controls such as tubulin or actin should also be analyzed to guarantee that the extract doesn't inhibit or change the protein level of those proteins. It would be expected to see a difference in the expression of the transporter NPC1L1 since as said before the herbal mixture inhibits the absorbance. The transporter was found in the control cells and the cells gown in a medium containing extract at 0.5 $mg.ml^{-1}$. However the results presented for the inhibition were obtained for extract concentration of 2 $mg.ml^{-1}$ and therefore it was expected to see the transporter in the cells grown at a condition of 1 $mg.ml^{-1}$ of extract concentration. In the case of the proteins that varied their expression it would also be necessary to identify to ensure that the proteins that lost expression in the cells grown with extract aren't proteins essential in the human body.

Taking into account that the compounds from the herbal mixtures reach the organs during the simulation of the digestion, was also confirmed the presence of the compounds after 24 hours of contact with the cells. It's possible to hypothesize that the cells used the main compounds in their metabolic pathways. Comparing the metabolism of the cells HepG2 in the different conditions it can be concluded that besides the existence of some decreases in the maximum peaks, the chromatograms obtained are similar for all the samples. These peaks can be identified in order to know what are the main compounds produced in the samples used. This analysis could be done by a mass spectrometry.

Since the herbal mixtures introduce different compounds in the body, is necessary to ensure that they don't have unwanted reactions compromising the main effect that is lower and inhibit

cholesterol absorption and synthesis and become a substitute or a complementary treatment to the drugs sold.

For achieve better results and verify the influence of the conditions in the mechanism of the cholesterol uptake some improvements could be done. In the electrophoresis the use of protease inhibitors will prevent the degradation of the transporter, which will lead to specific connections with the antibody. The optimization of the western blot and the correct dilution of the antibody can also be improved. As said in the results, the adjustment of the reagents in the transfer will lead also to effective transfers to the membrane. A complementary study could also be done to compare the expression of the proteins and the transporter from cells grown with the herbal mixture and the drug Ezetimibe to conclude about the efficacy of the herbal mixtures and therefore find another way of treatment.

Finally, in order to verify the main differences when these herbal mixtures are consumed the range of concentrations should be higher. The proteins produced by the cells in the different conditions should be identified, using genomics and proteomics tools, to confirm the benefits of the mixtures. It could also be done a long-term medical trial to see the differences in the total cholesterol levels for individuals taking the herbal mixtures as tea and individuals treated with the drugs sold for the reduction in the cholesterol levels to evaluate the results in the human body. In this trail it would also be necessary to evaluate the effects in the human body to guarantee that the herbal mixtures don’t have unwanted reactions.

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Appendix

Appendix A – protocol for the preparation of samples to the Western Blot

Lysis Protocol

Lysis buffer

- 50mM Tris-HCl pH 7,5
- 100mM NaCl
- 1mM DTT
- 5% glycerol

Preparation of lysate from cell culture

1) Place the cell culture dish in ice and wash the cells with ice-cold PBS.
2) Drain the PBS, then add ice-cold lysis buffer (0.5 ml per □5x106 cells/60 mm dish/75 cm2 flask and 0,5 ml in one well to wash the remain cells after scratching).
3) Scrape adherent cells off the dish using a cold plastic cell scraper and then gently transfer the cell suspension into a pre-cooled eppendorf.
4) Put the eppendorf in an ultrasound bath in cycles of 30 seconds and switch with the vortex. (testar o melhor)
5) Take a sample to determine the protein concentration using Bradford method.
6) Centrifuge in a microcentrifuge at 4°C during 10 minutes at the maximum speed.
7) Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice.
8) Take a sample of the supernatant to determine the protein concentration.
9) Resuspend the pellet in the buffer containing:
   a) 4 % Igepal
   b) 2 % DTT
   c) 6 M Urea and distillated water
10) Take a sample to determine the protein concentration.
11) Put the eppendorf in an ultrasound bath in cycles of 30 seconds and switch with the vortex. (verificar)
12) Centrifuge during 5 minutes at 9000 rpm.
13) Remove the supernatant to other eppendorf and take a sample to protein concentration. Save the pellet.

Electrophoresis

1. Preparation of buffers
   i) Loading buffer:
      (1) 4 % SDS
      (2) 10 % 2-mercaptoethanol
      (3) 20 % glycerol
      (4) 0,004 % bromphenol blue
      (5) 0,125 M Tris-HCl pH 6.8
   ii) Migration buffer (1 x Tris-glycine)
      (1) 25 mM Tris base
      (2) 190 mM glycine
      (3) 0,1 % SDS, pH 8,3

2. Preparation before the run

   a) Add 90 ml of running buffer to each tank of the gel box.
   b) Cut open the gel package and gently remove the gel from the package.
   c) Rinse the gel cassette with distilled water. Peel off the tapes from the two legs of the cassette. Make sure that there is no gum in the legs since it slows the electrophoresis time.
   d) Place the gel in the box so that the well side of the cassette faces toward the cathode (-) and the other cassettes leg faces toward the anode (+).
   e) Place the safety lid on top of the box.
   f) Connect the box to the power supply and pre-run the gel for 12 minutes in 160 V.
   g) Once the pre-run is finished, switch off the power. Remove the safety lid.
   h) Wiggle the comb back and forth, and bring it straight up from the cassette to make the wells available for sample loading.

i) Add 6 ml of running buffer to the well container.

3. Preparation of the samples
   a) Mix the sample with the loading buffer in 1:1 ratio.
   b) Boil the mixture at 95 – 100 °C for 5 minutes.
   c) During protein sample treatment the sample should be mixed by vortexing before and after the heating step.
   d) Spin down the samples quickly in a microcentrifuge and load the samples directly into the wells in the gel.
   e) Place the safety lid on top of the box.
   f) Run the electrophoresis for 1.30 hours in 120 V.
4. Removing the gel from the cassette
   a) Once the run is completed, shut off the power, disconnect the electrodes, remove the safety lid and finally, remove the gel cassette from the box.
   b) Open the cassette by inserting the edge of the comb in the slot opposite the sample wells and twist.
   c) Remove the top plate from the gel cassette.
   d) Cut the stacking gel with the end of the top plate approximately 2 cm downstream of the wells. Repeat the procedure on the other side of the gel to remove the front.
5. Visualization of proteins in gels: Coomassie stain
   a) To prevent diffusion of proteins treat the gel with:
      i) 40 % distilled water
      ii) 10 % acetic acid
      iii) 50 % methanol
   b) To visualize the fixed proteins place the gel in the same mixture of water/acetic acid/methanol but with the addition of 0,25 % by weight Coomassie Brilliant Blue R-259. This solution can be kipped and re-used many times.
   c) Incubate for 4 hours to overnight at room temperature on a shaker.
   d) Transfer the gel to a mixture of:
      i) 67,5 % distilled water
      ii) 7,5 % acetic acid
      iii) 25 % methanol
   e) Place on a shaker and replace with fresh rinse mixture until the excess dye has been removed.

Western Blot

1. Preparation of buffer:
   a) Transfer buffer
      i) 25 mM Tris
      ii) 190 mM glycine
      iii) 20 % methanol, pH 8,3
   b) Washing buffer (TBS – T (1))
      i) Transfer buffer
      ii) 0,1 % Tween-20
   c) Tris buffered saline (TBS) (10 x):
      i) 24,23 g Trizma HCl
      ii) 80,06 g NaCl
      iii) Mix in 800 ml ultra pure water
      iv) pH 7,6 with pure HCl
      v) Top up to 1 L
   d) TBST
      i) 100 ml TBS 10 x
      ii) 900 ml of ultra pure water
      iii) 1 ml of Tween-20

2. Preparing the membrane
   a) Pre-wet the membranes. NitroCellulose (NC) membrane: pre-wet the membrane in water distilled for 5 minutes followed by 10 minutes in transfer buffer. The membrane should have, approximately, the size of the gel.
3. Preparing the transfer tank

a) Fill the transfer unit with pre-chilled transfer buffer.
b) Place the open transfer cassette with the anode side (+) (white side) down in a tray with transfer buffer and ensure that it is immersed in at least 3 cm of the transfer buffer.
c) Build the stack with the membrane closest to the anode side. The white part of the cassette is on the bottom.
d) Layer 1: place a 3 mm thick foam sponge in the transfer cassette and press gently to remove any air bubbles.
e) Wet the blotting paper in transfer buffer.
f) Layer 2: place the two pre-wetted papers on the sponge and press gently to remove any air bubbles.
g) Layer 3: place the pre-wetted membrane on layer 2 and remove all air-bubbles.
h) Equilibrate the gel in cold transfer buffer for 10 to 20 minutes.
i) Layer 4: place the gel on the membrane and ensure there are no air bubbles.
j) Layer 5: cover the gel with two pre-wetted sheets of blotting paper.
k) Layer 6: finally place a sponge of 3 mm and again press gently to remove any air bubbles.
l) Close the cassette and press lightly to lock the tabs.
m) Place the cassette into the tank.
n) Run the transfer for 1.30 hours at 100 V.
o) Blots are preferably used immediately but may be stored in PBS-T or TBS-T at 2 °C to 8 °C.
p) After the transfer is finished, take out the membrane and wet the membrane in Ponceau Red for 2 to 10 minutes. Wash with distilled water and see the results in the LAS 500 machine.

4. Blocking
   a) Prepare the 2 % blocking solution (1 g blocking powder and 50 ml of TBS-T).
   b) Incubate the membrane in a suitable blocking solution on an orbital shaker for 1 hour at room temperature or overnight at 2 °C to 8 °C.
   c) Briefly rinse the membrane with two changes of wash buffer.

5. Primary antibody incubation
   a) Dilute the primary antibody in PBS-T or TBS-T (5,3 µl antibody and 1994,7 µl of TBS-T).
   b) Incubate the membrane in the primary antibody solution on an orbital shaker for 1 hour at room temperature or overnight at 2°C to 8°C.
   c) Briefly rinse the membrane with two changes of wash buffer.
   d) Wash the membrane 4 to 6 times in wash buffer for 5 minutes each at room temperature on an orbital shaker.

6. Secondary antibody incubation
   a) Dilute the secondary antibody (HRP conjugated or biotinylated antibody) in PBS-T or TBS-T (0,27 µl antibody and 1999,73 µl TBS-T).
   b) Incubate the membrane in the secondary antibody solution for 1 hour at room temperature on an orbital shaker**.**
   c) Briefly rinse the membrane with two changes of wash buffer.
   d) Wash the membrane 4 to 6 times in wash buffer for 5 minutes each at room temperature on an orbital shaker.

7. Detection
   a) Allow the detection solutions to equilibrate to room temperature for 20 minutes.
   b) Mix detection solutions A (luminol) and B (peroxide) in a ratio of 1:1 to a working solution. The final volume of detection reagent required is 0,1 ml/cm^2 membrane (1 ml).
   c) Drain excess wash buffer from the washed membrane and place it protein side up in a suitable box or on a sheet of plastic wrap or other suitable clean surface. Add detection reagent onto the membrane and make sure it completely covers the membrane.
   d) Incubate for 5 minutes at room temperature.
   e) Drain off excess detection reagent by holding the membrane edge gently against a tissue.
   f) See the results in the LAS 500 machine.

Appendix B – Lab report for the study of the herbal mixture in lowering the total cholesterol in circulation

Some results and methods are not presented since they belong to the paper under review.

Materiais e Métodos

Materiais

I) Reagentes

Tabela 1 – Identificação dos reagentes utilizados e respectivas marcas.

Reagente	Marca
NADPH	Sigma (Barcelona, Spain)
HMG-CoA redutase	Sigma (Barcelona, Spain)
HMG- CoA	Sigma (Barcelona, Spain)
MTT	Sigma (Barcelona, Spain)
Água miliQ	Millipore (Darmstadt, Alemanha)
Metanol	Fisher Chemical
Metanol (HPLC)	Merck
DPPH	Sigma (Barcelona, Spain)
Pepsina	Fluka
Pancreatina	Sigma – Aldrich
HBSS	Lonza (Verviers, Belgium)
DMEM	Lonza (Verviers, Belgium)
Pen-Strep	Lonza (Verviers, Belgium)
PBS	Lonza (Verviers, Belgium)
FBS	Lonza (Verviers, Belgium)

II) Aparelhos

Tabela 2 – Identificação dos aparelhos utilizados e respectivas marcas.

Aparelho	Marca
Liofilizador	Heto PowerDry LL3000
Centrifugadora (miniSpin)	Eppendorf
HPLC	Elite LaChrom® VWR Hitachi Liquid Chromatograph equipado com Column Oven L-2300 e Diode Array Detector L-2455 (VWR, USA).
LC-MS	Surveyor Plus Modular LC system
Espectrofotómetro	Camspec M350 Double Beam UV-Visible Spectrophotometer
Millicell ERS-2 Volt-Ohm Meter	Millipore (Darmstadt, Alemanha)

Métodos

I) Preparação de infusões e composição do extracto das misturas de plantas comercializadas

Primeiramente prepararam-se as infusões, fervendo em 200 mL de água, 5 saquetas de cada uma das misturas de plantas em estudo. De seguida, procedeu-se à liofilização das mesmas, a uma temperatura de -80°C, durante quatro dias. Após este período, determinou-se a massa dos extractos.

De forma a determinar a composição química de cada extracto, realizou-se uma cromatografia em coluna líquida (HPLCC-DAD) e também uma espectrometria de massa (LC-MS).

A composição das misturas de plantas, fornecida pelo fabricante encontra-se na Tabela 3.

Tabela 3 – Composição das infusões em estudo.

Nome da infusão	Composição		%
A	Cavalinha	Equisetum arvense L.	30
	Espinheiro branco (flor)	Crataegus monogyna Jacq.	25
	Urtiga verde	Urtica dioica	25
	Canela (casca)	Cinnamomum	20
B	Cavalinha	Equisetum arvense L.	30
	Oliveira (folhas)	Olea europaea	30
	Espinheiro alvar (fruto)	Crataegus monogyna Jacq.	20
	Hortelã-Pimenta	Mentha piperita	20

II) Inibição da enzima HMG-CoA redutase

Este procedimento foi adaptado do protocolo fornecido pela marca Sigma (Barcelona, Espanha). Relativamente ao ensaio espectrofotométrico, que segue o decaimento de NADPH, ao comprimento de onda de 340 nm, o protocolo experimental não sofreu alterações.

A reacção enzimática foi também realizada como se encontra sugerido neste protocolo, uma vez que os ensaios com os extractos de plantas apresentaram uma absorvência inicial bastante elevada, ao comprimento de onda de trabalho.

A única modificação decorreu aquando da quantificação do NADPH. Neste ponto recorreu-se ao método descrito por Mozzicafreddo et al. (2010). Deste modo, retirou-se um volume de 900 µL de solução de tampão KH_2PO_4 (100 mM), previamente preparada, e adicionaram-se 10 µL de extracto, inibidor da enzima.
De seguida, retiraram-se cinco amostras durante 6 minutos, nos intervalos de 0, 1, 2, 4 e 6 minutos. Interrompeu-se a reacção, adicionando-se 50% de metanol, e mediu-se a quantidade de NADPH, recorrendo-se a uma análise por cromatografia líquida (HPLC-DAD). Este passo realizou-se em triplicado.

A actividade enzimática foi medida na presença de variadas concentrações de extracto de *A. cherimolla*, *rutina* e *sinvastatina*. A inibição determinou-se, em percentagem, através da comparação da actividade enzimática na presença e na ausência de extracto.

Calcularam-se, ainda, valores de IC_{50} através do quociente entre a inibição determinada e a concentração de inibidor utilizada.

III) Medição do poder antioxidante (DPPH)

De modo a caracterizar o poder antioxidante dos extractos em estudo, preparou-se uma solução com o reagente DPPH (Diphenylpicrylhydrazyl), dissolvendo-se 2mg deste reagente em 100 mL de metanol.

Mediu-se, de seguida, a absorvência desta solução no espectrofotómetro, de forma a verificar o sucesso da sua preparação. Para tal, o valor para a absorvência teria que rondar os 0,7.

As concentrações de solução de extracto a analisar no espectrofotómetro diferiram nas marcas em estudo. Assim, para um volume final de 0.1 mL, prepararam-se soluções de 8, 6, 4, 2 e 0 mg/mL, para a marca Diese, e de 2, 1.5, 1, 0.5, 0.1 e 0 mg/mL, para a marca Cem por Cento.

Após a medição das absorvências, foi necessário retirar a interferência do "branco", de forma a garantir que a absorvência medida fosse apenas do extracto. Deste modo, no eixo dos yy, encontra-se os resultados deste acerto: $y = 100 - \frac{100*Absorvência\ medida}{média\ da\ absorvência\ do\ branco}$

Substituindo, nas rectas obtidas, o y por 50, tornou-se possível auferir o valor do IC_{50}.

IV) Simulação da digestão: com suco gástrico e suco pancreático.

Sendo as infusões ingeridas oralmente, tornou-se necessário verificar a sua resistência ao processo da digestão e a sua eficácias nos órgãos alvo. Deste modo, sujeitaram-se os extractos à acção dos sucos gástrico e pancreático.

Para este ensaio seguiu-se o protocolo adaptado de Yamamoto et al. (1999). Efectuou-se, no entanto, uma alteração para extracto A. Assim, para o extracto B, pesaram-se 28 mg de extracto, que se diluíram em 7 mL de água. Para a preparação do suco gástrico, diluíram-se 32 mg de pepsina em 20 mg de NaCl, pH 1.2 (com HCl) .

Procedeu-se à preparação de dois tubos de digestão. Num dos tubos, colocou-se 2mL de suco gástrico e 2 mL de solução de extracto. No outro, o de controlo, colocou-se, à semelhança do anterior, 2 mL de suco gástrico, e 2 mL de água.

Colocaram-se as amostras num banho termostatizado a 37°C, retirando-se, a cada hora, 500 µL de digerido para um tubo eppendorf contendo 500 µL de metanol.

Relativamente ao extracto A, pesou-se 10 mg de extracto que se diluíram em 1 mL de água. A preparação

do suco não sofreu alterações. No entanto, aquando da preparação dos tubos de digestão, as quantidades utilizadas diferiram, uma vez que se colocaram 750 µL de suco gástrico e de solução de extracto. Também nos tubos eppendorf a relação digerido/metanol foi diferente, recolhendo-se a cada hora 200 µL de digerido para os tubos eppendorf contendo 800 µL de metanol.

Para o ensaio com o suco pancreático, seguiu-se, tal como para o suco gástrico, o protocolo adaptado de Yamamoto et al. (1999). Procedeu-se, no entanto, a alterações para ambas as marcas. Deste modo, pesaram-se 10 mg de extracto que se diluíram em 1 mL de água. Para a preparação do suco pancreático, diluíram-se 25 mg de pancreatina em 1 mL de tampão potássio-fosfato.

Neste caso, ambos os tubos de digestão apresentavam 0.5 mL de extracto e 0.5 mL de suco.

À semelhança do ensaio com suco gástrico, as amostras foram colocadas num banho termostatizado a 37°C. No entanto, os volumes retirados para os tubos eppendorf foram de 100 µL de digerido para 900 µL de metanol.

Após centrifugação, as amostras obtidas foram analisadas por HPLC e os respectivos cromatogramas analisados.

V) Ensaios de toxicidade com os extractos.

De forma a verificar se os compostos activos dos extractos eram ou não tóxicos, utilizaram-se dois tipos de células, Caco-2 e HepG2, que constituem modelos para as células do intestino e do fígado, respectivamente.

O procedimento utilizado foi o teste MTT (Mosmann, 1983). Neste procedimento, as células, contendo soluções de extracto a diferentes concentrações, foram incubadas durante 24 horas, com o composto MTT.

Após este período, mediram-se as absorvências das soluções em cada poço, num espectrofotómetro, ao comprimento de onda de 590 nm (632).

Por fim, traçaram-se as respectivas rectas e calculou-se o IC_{50} .

Resultados

I. Determinação da composição química das amostras em estudo

As duas infusões em estudo foram analisadas por HPLC-DAD e LC-MSn de forma a determinar os principais compostos de cada uma das infusões. Na tabela 4 a identificação dos picos.

Tabela 4 – Identificação dos picos de cada umas das infusões, obtidos por espectrometria de massa.

		RT	Name	MS	MS2	MS3
B	1	7.36	kaempferol-3-O -sophoroside-7-O -glucoside	771	609(100)	593(3) 489(9) 447(11) 429(100) 411(3) 393(5) 381(4) 369(3) 327(10) 309(4) 285(81) 255(17) 179(3)
	2	8.72	Chlorogenic acid	353	191(100), 161(1), 179(4), 353(3)	191(100), 93(5), 111(6), 127(7), 171(2), 173(5)
	3	9.93	Caffeic acid	179	179(100), 135(13), 180(3)	179(100), 135(57), 121(1)
	4	10.89	Eriodictyol rutinoside	595	287(100)	287(11) 269(5) 161(1) 151(100) 135(6) 125(3) 107(2)
	5	11.69		577	457(6), 414(5) 413(100) 341(1) 311(3) 293(12)	413(2) 293(100)
	6	13.63	Luteolin-glucoside-rahmnoside (different positions)	593	285(100),447(2),286(2)	285(100) 243(1) 241(3) 199(2) 175(1)
	7	14.17	Oleuropein	539	377(100), 275(18), 307(45), 345(6), 507(2), 403(2), 327(3), 359(1)	307(100), 275(40), 345(12), 149(5), 327(3), 195(1); [307] 307(4) 275(100) 255(2) 247(1) 165(2)
A	1	5.48	Caffeoylquinic acid	353	191(100) 179(4) 173(1) 135(1)	

2	7.34	Kaempferol-3-O -sophoroside-7-O -glucoside	771	609(100)	489(4) 461(2) 447(7) 429(100) 411(4) 393(3) 381(3) 351(2) 327(10) 309(6) 285(83) 257(8) 255(11) 239(2) 227(2) 211(2) 179(4)
3	8.71	Chlorogenic acid	353	191(100), 179(4), 173 (1), 135(1)	191(100), 127(9), 171(2), 155(2), 153(3), 122(2), 111(3), 109(2), 99(1), 93(4), 87(2)
4	9.12		863	737(1) 711(100) 693(15) 573(25) 559(14) 541(3) 531(8) 451(21) 407(1) 411(19) 299(3) 289(5) 185(2)	693(100) 675(3) 667(2) 657(2) 585(7) 573(3) 571(2) 570(3) 567(8) 559(99) 541(28) 463(5) 437(4) 425(11) 411(3) 407(14) 285(3) 282(2)
5	9.94		623	605(5) 587(8) 573(2) 551(7) 533(11) 517(5) 505(11) 503(100) 495(5) 425(5) 417(2) 413(25) 383(37) 325(8) 285(7) 231(9) 221(5)	485(5) 472(13) 443(3) 425(6) 413(6) 395(14) 390(8) 383(100) 345(11) 331(6) 313(4) 229(11) 225(7) 212(5) 170(5) 141(14)
6	11.67		577	457(6) 413(100) 341(1) 311(3) 293(13)	413(2) 293(100)
7	13.94	Quercetin glucoside	463	463(7) 343(3) 301(100) 271(2) 255(1) 179(2) 151(1)	301(100) 271(27) 255(18) 179(44) 151(24)
8	14.15		619	499(6) 413(100) 293 (14)	413(4) 293(100)

II. Inibição da enzima HMG-CoA redutase

Tal como foi descrito anteriormente, sobre as formas de diminuir o colesterol no sangue, foram realizados dois estudos tendo em conta a acção da enzima HMG-CoA redutase. Assim, obtiveram-se os valores apresentados na tabela 5 para a inibição do enzima por parte de cada uma das amostras, bem como a inibição ao nível das células intestinais.

Tabela 5 – Valores de IC 50% para cada uma das amostras, bem como os valores de inibição da absorção de colesterol nas células Caco-2. Os valores de toxicidade, obtidos para uma concentração de amostra de 2 mg/mL de amostra, foram calculados para as células HepG2 e Caco-2.

Infusão	**IC_{50} HMGR (µg/mL)**	**Toxicidade com 2mg/mL (%)**	
		HepG2	**Caco-2**
A	165.3±2.6	3.2±1.7	4.3±2.4
C	246.7±4.9	3.4±1.0	-0.5±0.9

Analisando os valores presentes na tabela 5, verifica-se que ambas as amostras inibem a absorção do colesterol nas células em mais de 50%, para uma concentração de 1 mg/mL de amostra. Os valores da inibição são próximos para ambas as amostras, no entanto a amostra da Diese apresenta um valor de inibição superior e como tal a amostra do 100% é a melhor para este caso.

Relativamente aos valores de inibição na permeação do colesterol ao nível das células intestinais, mais uma vez é a amostra do 100% que apresenta melhores resultados face à amostra da Diese.

III. Metabolismo gástrico e pancreático e toxicidade

a. Toxicidade das infusões nas linhas celulares: Caco-2 e HepG2

A toxicidade das infusões foi testada em cada uma das linhas celulares mencionadas anteriormente.

Analisando os valores da tabela 5, verifica-se que os valores da toxicidade de ambas as infusões são menores que 10% e por isso pode-se concluir que as infusões não são tóxicas para as células intestinais.

b. Metabolismo gástrico e pancreático in vitro

Após a realização dos metabolismos in vitro, as soluções foram analisadas por HPLC-DAD (350 nm), obtendo-se as figuras 1 e 2 para a digestão com suco gástrico e pancreático, para a infusão A, e as figuras 3 e 4 para a digestão com o suco gástrico e pancreático para a infusão B.

- A

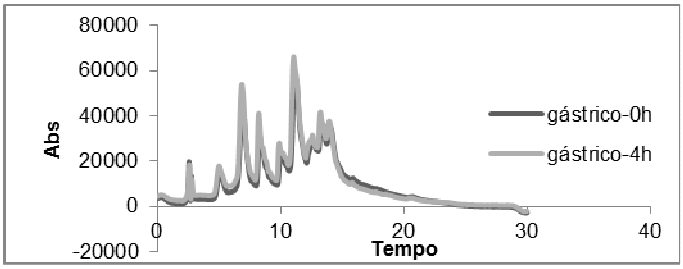

Figura 1 – Cromatograma da digestão gástrica da infusão A, para as 0 horas (inicio da digestão) e 4 horas (fim da digestão).

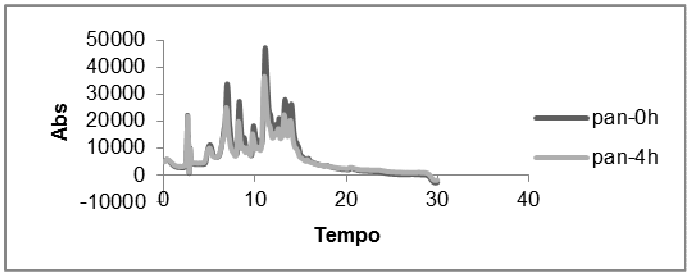

Figura 2 – Cromatograma da digestão pancreatica da infusão A, para as 0 horas (inicio da digestão) e 4 horas (fim da digestão).

- B

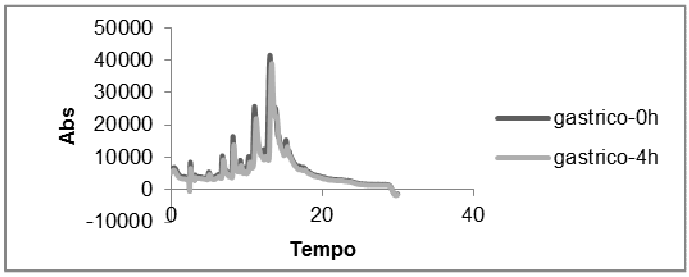

Figura 3 – Cromatograma da digestão gástrica da infusão B, para as 0 horas (inicio da digestão) e 4 horas (fim da digestão).

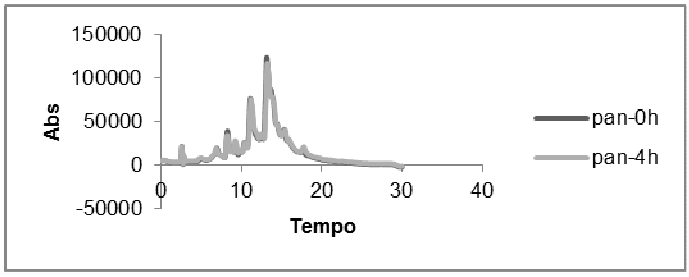

Figura 4 – Cromatograma da digestão pancreática da infusão B, para as 0 horas (inicio da digestão) e 4 horas (fim da digestão).

IV. Poder antioxidante das amostras

Para determinar o poder antioxidante das infusões, fez-se a reacção deste com o reagente DPPH. Desta reacção, e juntamente com os valores de absorvância, determinou-se os valores de IC 50% de cada uma das infusões, presentes na tabela 7.

Assim, verifica-se que os valores de IC são baixos e por isso assume-se que as infusões têm poder antioxidante.

Tabela 7– Valores de IC obtidos através da reacção com o reagente DPPH.

Amostra	**IC 50 (mg/ml)**
100%	1,874
Diese	3,654

Conclusão

É possível concluir que os métodos e as condições utilizadas para a elaboração deste estudo são apropriadas uma vez que foi possível obter resultados concordantes.

Assim, é possível afirmar que as infusões comercializadas com o propósito da redução do colesterol são eficazes, não apresentando efeitos nocivos para as células humanas. O tratamento do colesterol com a utilização destas infusões pode ter algum sucesso nas pessoas que possuem este problema de saúde.

Atendendo aos valores obtidos para cada umas das infusões estudadas, verifica-se que é para o extracto B que os valores obtidos são melhores e por isso esta infusão é mais eficaz no tratamento do colesterol.

Appendix C – Abstract from the paper about the work in appendix B.

The paper is under review.

Reduction in cholesterol intestinal absorption and HMG-CoA reductase inhibition by herbal infusions

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Abstract

Mixtures of herbs are commercialized to prepare infusions with the recommendation for the treatment of high cholesterol levels, however there are no scientific studies concerning the chemical composition or the activity of these mixtures. The herbs that compose these mixtures, namely *Equisetum arvense*, *Crataegus monogyna*, *Olea europaea*, *Mentha x piperita*, *Lithospermum diffusum, Salix alba*, *Cichorium intybus*, *Parietaria officinalis*, *Lamium album*, *Urtica dioica,* and *Cinnamomum*, were chosen by their traditional uses. Our aim was to determine the chemical composition of the herbal mixtures and correlate it with the activities in decreasing dietary cholesterol permeation and inhibiting HMG-CoA reductase activity. The stability of the components of the extracts in gastric and pancreatic juices was studied, as well as their permeation in Caco-2 cell monolayers, to predict if the compounds may reach the target organs. The infusions showed activity as inhibitors of cholesterol permeation in Caco-2 monolayers, approx. 60% with 1mg/mL, and as inhibitors of HMG-CoA reductase, with IC_{50} values between 165-247 µg/mL. The active components, flavonoids and caffeoylquinic acid derivatives such as chlorogenic acid and cynarins, were stable under gastric and pancreatic conditions and were able to permeate Caco-2 monolayers, and therefore may potentially act as HMG-CoA reductase inhibitors.

Keywords: cholesterol absorption, HMG-CoA reductase inhibition, phenolic compounds, Caco-2 cells, HepG2 cells.

Appendix D – Electrophoresis gel and membranes, from wet transfer, obtained during the process optimization

The samples, from the gel presented before (figure 19) were loaded in a vertical gel to confirm the existence of the proteins bands identified. However this gel didn't run to the end because the time was not sufficient.

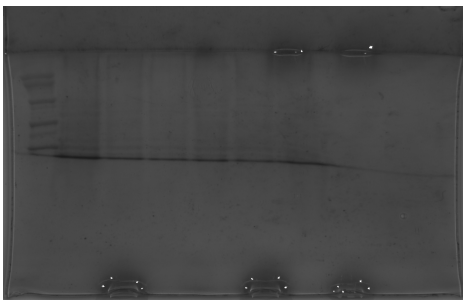

Figure 1 – Electrophoresis gels representing protein bands from HepG2 in contact with the compounds (cholesterol 50 mM, extract 0.5 mg.ml^{-1} and extract 1 mg.ml^{-1} respectively).
The samples in the right are repeated. The samples were boiled before the application in the gel and loaded 30 µl in each well. The molecular marker was loaded at a quantity of 5 µl (HMW-SDS calibration kit, Pharmacia Biotech) and the peptide at 4 µl mixed in a ratio of 1:1 with loading buffer.

The samples presented in the membrane in figure 2 were obtained by the two-step lysis method. Since the difference in the protein bands is not conclusive, the membrane is not presented in the results. However it was used to analyze the conditions of the method used.

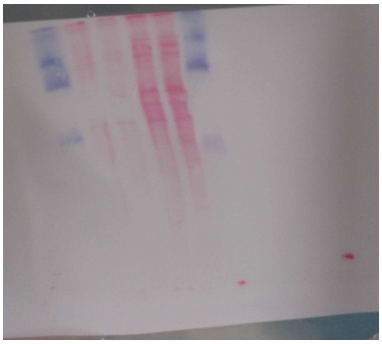

Figure 2 – Lane 1 and 6 molecular weight marker (marker A, methods), lane 2 and 7: control cells, lane 3 and 8: cholesterol (50 µM), lane 4 and 9: extract 1 mg.ml^{-1} and lane 5 and 10: extract 0.5 mg.ml^{-1}.

The membrane in figure 3 was obtained to analyze the difference in the protein bands from cells lysed with a buffer without salt (NaCl). This study was performed to evaluate the role of the salt in the lysis process and therefore decide if it could be removed to use the samples in the electrophoresis 2D. However, since the results were not conclusive and the membrane has an overloading of samples, the salt was not eliminated from the lysis buffer. It is important to have a salt in the lysis buffer to maintain the ionic strength.

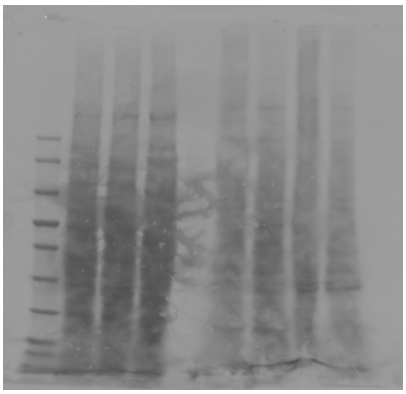

Figure 3 – Lane 1, molecular weight (marker C, methods), lanes 2, 7 and 9 (control cells): 0.146 mg, 0.039 mg and 0.029 mg respectively, lanes 3 and 8 (extract 1 $mg.ml^{-1}$): 0.121 mg and 0.037 mg respectively, lanes 4 and 6 (extract 0.5 $mg.ml^{-1}$): 0.149 mg and 0.025 mg respectively, lane 5: 4 µl and peptide.

The membrane in figure 4 was obtained by performing a total electrophoresis using the RIPA buffer. This study was performed to verify the presence of the transporter in the samples. However the incubation with the antibody was not visible and the conditions for the lysis process were maintained since the transporter was found in the previous membrane.

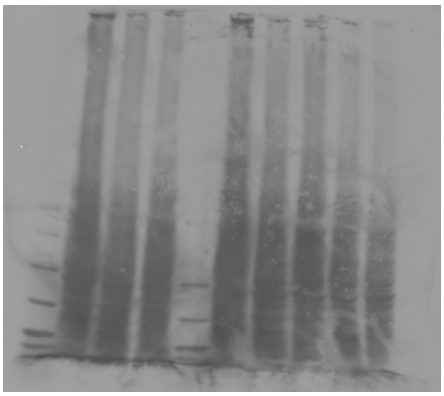

Figure 4 – Lane 1 and 5, molecular weight (marker C, methods), lanes 2, 6 and 9 (control cells): 0.148 mg (25 µl), 0.178 mg (30 µl) and 0.089 mg (15 µl) respectively, lanes 4, 8 and 10 (extract 1 $mg.ml^{-1}$): 0.139 mg (25 µl), 0.166 mg (30 µl) and 0.083 mg (15 µl) respectively, lanes 3 and 7 (extract 0.5 $mg.ml^{-1}$): 0.141 mg (25 µl) and 0.170 mg (30 µl) respectively.