U.PORTO

MESTRADO EM CONTROLO DE QUALIDADE ESPECIALIDADE EM FÁRMACOS E PLANTAS MEDICINAIS

Screening of flavonoids and determination of scaffold structures for the development of new effective molecules in the treatment of obesity

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2023

U.PORTO
FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

Screening of flavonoids and determination of scaffold structures for the development of new effective molecules in the treatment of obesity

Rastreio de flavonoides e determinação de estruturas modelo para o desenvolvimento de novas e mais eficazes moléculas para o tratamento da obesidade

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Controlo de Qualidade

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Setembro 2023

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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(assinatura do autor)

Acknowledgments

Mais uma etapa do meu percurso académico se encontra na sua reta final e várias são as pessoas que se tornaram fundamentais para a sua realização e às quais estou verdadeiramente grata, tendo a sua presença tornado a realização desta etapa bem mais fácil.

Começo por agradecer à Doutora Ana Rufino, para quem não há agradecimentos suficientes. Agradeço pela sua disponibilidade e paciência, por todos os ensinamentos que me transmitiu e que contribuíram para o meu enriquecimento a nível científico. Obrigada por toda a confiança que depositou em mim e por ser sempre a primeira a encorajar-me e a mostrar-me que sou capaz. As suas palavras de carinho foram sempre uma grande ajuda ao longo desta etapa. Muito obrigada!

Não posso deixar de agradecer à Professora Doutora Eduarda Fernandes, por me ter dado a oportunidade de realizar este trabalho neste seu grupo tão especial que é a FRAU. Obrigada por toda a preocupação que sempre teve para comigo e por todo interesse que demonstrou ao longo deste percurso. Obrigada por todos os conhecimentos que me transmitiu que certamente levarei comigo, bem como por todo o apoio. Muito obrigada!

Agradeço à FRAU, por serem um grupo tão especial, onde nos é permitido sentir em família. Obrigada por estarem sempre disponíveis a ajudar, sem a vossa ajuda a elaboração deste trabalho teria sido bem mais difícil. Guardarei para sempre os momentos de descontração e convívio que me proporcionaram. Um especial agradecimento à Carina pela ajuda que me deu na manipulação das células e pelos ensinamentos que também me transmitiu. Cada um de vocês teve um papel importante no meu crescimento científico. Obrigada a todos!

Um especial agradecimento ao Jorge, por ser um dos meus maiores suportes e por sempre acreditar em mim e nas minhas capacidades. Obrigada por todo o carinho que demonstraste ao longo desta caminhada que fizemos lado a lado e por me incentivares cada dia a ser melhor. Obrigada!

Não posso deixar de agradecer às pessoas mais importantes, aos meus pais, às minhas irmãs, aos meus avós e à restante família por terem sido fundamentais para me tornar naquilo que sou hoje. Aos meus pais por serem o suporte de que preciso todos os dias e por me terem ajudado ao longo deste percurso, encorajando me sempre a seguir os meus sonhos. Às minhas irmãs por toda a amizade e apoio. Aos meus avós por terem sido fundamentais na transmissão dos valores que me moldam enquanto pessoa e por toda a educação que sempre me deram. Obrigada!

Este trabalho recebeu apoio financeiro de fundos nacionais PT (FCT/MCTES, Fundação para a Ciência e Tecnologia e Ministério da Ciência, Tecnologia e Ensino Superior) através do projeto UIBD/50006/2020 e fundos nacionais (FCT) através do projeto EXPL/MED-QUI/0815/2021.

Abstract

Obesity is a global health problem which the prevalence and incidence has increased over the years leading the World Health Organization classifying it as an epidemic of the 21st century. Obesity is associated with fat accumulation and expansion of the adipose tissue mainly because of the deregulation of two complementary processes, adipogenesis, which is the process in which the fibroblast-like preadipocytes turn into mature adipocytes and the lipogenesis which is the process of generation of new lipid molecules. The treatment of obesity should combine pharmacotherapy and lifestyle changes, however the currently available drugs used in the treatment of obesity are associated with low efficacy and/or undesirable side effects. For this reason, the search for new and safer molecules that can be used in the treatment of obesity is of great importance. Flavonoids, natural occurring compounds, are associated with diverse biological properties that have been studied by the scientific community over the years, such as their antidiabetic, antioxidant, anti-inflammatory and antirheumatic properties. Their anti-obesogenic activity has been described in the recent years which make this class of polyphenols interesting for their potential use as anti-obesity molecules.

The aim of this work is the evaluation of the capacity of a panel of hydroxylated flavonoids in the modulation of the lipidic metabolism. For that, a model of adipogenesis was established using an *in vitro* model of preadipocytes (3T3-L1 cell line). Cytotoxic assays were also performed to uncover the maximum concentration that did not affect the viability of the cells. The capacity of flavonoids to reduce the lipid content of the cells was evaluated by Oil Red O Staining assay and by the measure of the triglyceride content with a commercial kit. The expression of lipogenic and adipogenic proteins (ATGL, FAS and PPAR γ) was also evaluated.

The results obtained reveal that from the tested flavonoids, Quercetin and Myricetin at the concentration of 12.5 μM demonstrated ability to reduce the lipid content of the cells and Myricetin also demonstrated ability to downregulate the protein expression of ATGL, FAS and PPAR γ.

With this work it was suggested that flavonols with the presence of a catechol and pyrogallol group at the B-ring together with the presence of two hydroxyl groups (-OH) at the A-ring are promising anti-obesity structures and these particular substitutions seem to contribute to the observed effects. This conclusion serves to the basis for the design and development of other structures to obtain the most active flavonoid scaffold to be used as anti-obesity molecules.

Keywords: Obesity; Flavonoids; Lipogensis; Adipogenesis

Resumo

A obesidade é um problema de saúde pública cuja prevalência e incidência tem aumentado ao longo dos anos, levando a Organização Mundial de Saúde a classificá-la como uma epidemia do século XXI. A obesidade está associada à acumulação de gordura e à expansão do tecido adiposo, principalmente devido à desregulação de dois processos complementares, a adipogénese que é o processo em que os pré-adipócitos se transformam em adipócitos maduros, e a lipogénese, que é o processo de geração de novas moléculas lipídicas. O tratamento da obesidade deve combinar a farmacoterapia com alterações no estilo de vida, no entanto, os fármacos atualmente disponíveis para o tratamento da obesidade estão associados a baixa eficácia e/ou efeitos secundários indesejáveis. Por esta razão, a procura por novas e mais seguras moléculas que possam ser utilizadas para o tratamento da obesidade é de grande importância. Os flavonoides, compostos de ocorrência natural, estão associados a diversas propriedades biológicas que têm sido estudadas pela comunidade científica ao longo dos anos, tais como, as suas propriedades antidiabéticas, antioxidantes, anti-inflamatórias e antirreumáticas. A sua atividade anti-obesogénica tem sido descrita nos últimos anos, o que torna esta classe de polifenóis de interesse para o estudo da sua potencial utilização como moléculas anti obesidade.

O objetivo deste trabalho é a avaliação da capacidade de modulação do metabolismo lipídico por um painel de flavonoides hidroxilados. Para tal, foi estabelecido um modelo de adipogénese utilizando um modelo *in vitro* de pré-adipócitos (linha celular 3T3-L1). Foram também realizados ensaios citotóxicos para descobrir a concentração máxima que não afetava a viabilidade celular. A capacidade de reduzir a acumulação lipídica das células pelos flavonoides foi avaliada através de ensaios de Oil Red O Staining e da medição do conteúdo de triglicerídeos através de um kit comercial. Foi também avaliada a expressão de proteínas lipogénicas e adipogénicas (ATGL, FAS e PPAR γ).

Os resultados obtidos revelaram que, dos flavonoides testados, a Quercetina e Miricetina, na concentração de 12,5 μM, demonstraram capacidade para reduzir o teor lipídico das células, e a Miricetina demostrou ainda capacidade para regular negativamente a expressão proteica de ATGL, FAS e PPAR γ.

Com este trabalho, sugere-se que os flavonols que apresentam um grupo catecol ou pirogalol no anel B, juntamente com a presença de dois grupos hidroxilo (-OH) no anel A, são estruturas anti obesidade promissoras e que estas substituições específicas parecem contribuir para os efeitos observados. Esta conclusão serve de base para o design e desenvolvimento de outras estruturas para obter a estrutura do flavonoide que é mais ativa para ser utilizada como molécula anti obesidade.

Palavras-Chave: Obesidade; Flavonoides; Lipogénese e Adipogénese.

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List of abbreviations

4-AF- 4-Aminophanazone
4CL- 4-Coumarate: CoA ligase
ADD1 – Adipocyte determination and differentiation dependent factor
AMPS - Ammonium persulfate
ATGL – Adipose triglyceride lipase
BAT- Brown adipose tissue
BMI- Body mass index
CDC- Center for Disease Control and Prevention
C/EBP α, β or δ – CCAAT/ Enhancer-binding protein alpha, beta or delta
CoA – Coenzyme-A
CVD- Cardiovascular disease
C4H- Cinnamic acid-4-hydroxylase
DMEM- Dulbecco's Modified Eagle Medium
DMSO – Dimethyl sulfoxide
DPBS- Dulbecco's phosphate- buffered saline
EDTA- Ethylenediaminetetraacetic acid
EMA- European Medicines Agency
FAS- Fatty acid synthase
FBS- Fetal bovine serum
FCS- Fetal calf serum
FDA- Food and Drugs Administration
FFAs – Free fatty acids
G3P- Glycerol-3-phosphate
GK- Glycerol kinase
GLP-1 – Glucagon like peptide -1
GPO - Glycerolphosphate dehydrogenase
HSL - Hormone sensitive lipase
IBMX- 3-Isobutyl-1-methylxanthine
IL-6 – Interleukin-6
LDL - Low-density lipase
LDLR- Low-density lipase receptor
LPL - Lipoprotein lipase
MTT- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

PAL - Phenylalanine ammonia lyase

POD -Peroxidase

PPAR γ – Peroxisome proliferator-activated receptor γ

PVDF - Polyvinylidene fluoride

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

SDS - Sodium dodecyl sulfate

SEM- Standard error of the mean

TEMED - N,N,N',N'-Tetramethylethane-1,2-diamine

TGs - Triglycerides

TNF-α – Tumour necrosis factor alpha

VLDL- Very-low density lipoprotein

WAT- White adipose tissue

WHO- World Health Organization

General considerations

This work was carried out in the context of the Master's Degree in Quality Control of the Faculty of Pharmacy of the University of Porto. The theme was chosen based on the growing concern around obesity, which was considered by the World Health Organization as an epidemic of the twenty first century. The currently used pharmacotherapy for obesity is associated with low efficacy and undesirable side effects, so the search for new and safer molecules with capacity to be used for the treatment of obesity is of great importance. Flavonoids have already been recognised for their diverse biological properties, including anti-obesogenic properties. Thus, the aim of this thesis was to evaluate the anti-obesogenic activity of a panel of hydroxylated flavonoids, using an *in vitro* model of preadipocytes (3T3-L1 cell line).

Structure of the dissertation

This dissertation is divided into six chapters. The first chapter, **Introduction**, refers to a theorical framework with a characterization of obesity, an approach to its pathophysiology and the associated consequences. In this chapter it is also possible to find a characterization of the flavonoids. The general and specific objectives of this dissertation are also present in this chapter. In the second chapter, **Material and Methods**, is described all the experimental aspects related to the *in vitro* activity of the panel of chosen flavonoids in the lipidic metabolism of the cells. In the third chapter, **Results**, are present all the results related to the *in vitro* activity of the chosen panel of flavonoids in the lipidic metabolism of azthe cells. In the **Discussion**, there is an integrated discussion of all the obtained results. The chapter five, **Conclusions**, summarizes the main conclusions of the performed work. In the last chapter, **References**, there is the bibliographical references that supported the development of this work.

1. INTRODUCTION

1.1. Obesity

Obesity is a global health problem and according to the World Health Organization (WHO), an individual is considered overweight when their body mass index (BMI) (the weight in kilograms divided by the height in meters squared) is between 25 and 29.9 kg/m² and obese when this BMI is equal or greater than 30 kg/m². For children and adolescents aged between 2 and 18 years old, rather than the BMI a percentile scale based on the sex and age of the child is used. In the case of children, overweight is considered when it is between 85th and 94th percentile and obesity from 95th percentile onwards (1, 2). According to the Center for Disease Control and Prevention (CDC), obesity is frequently subdivided into categories considering the BMI. Obesity class I is considered when the BMI stands between 30 and 34.9 kg/m², and obesity class II when the BMI is between 35 and 39.9 kg/m². Finally, severe obesity (obesity class III) when the BMI is equal or greater than 40 kg/m² (3, 4) (Figure 1).

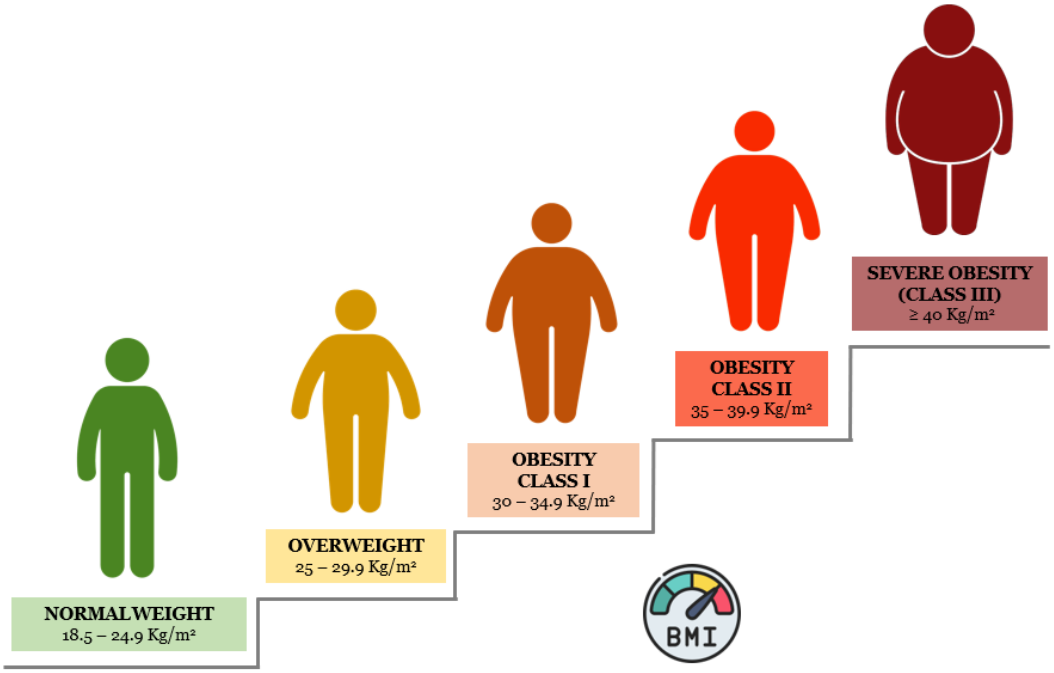


Figure 1- Weight categories according to body mass index (BMI).

The incidence and prevalence of obesity has increased significantly in the last years, propelling WHO to classify it as the epidemic of the 21st century (5). According to the WHO, worldwide, obesity has nearly tripled since 1975. In 2016, 39 % of the adults aged 18 years and older were overweight and 13 % were obese. When it refers to children, an increase in

the prevalence of obesity has also been shown. While just under 1 % of children and adolescents aged 5 to 19 years old were obese in 1975, more than 124 million (6 % of girls and 8 % of boys) were obese in 2016 all over the world (6). More recent data is also provided by the World Obesity Federation, which refers that in 2020 15 % of the population were obese and it is expected that in 2035, that number increases to 24 % of the population. In relation to children, in 2020 10 % of the boys and 8 % of the girls were obese and it is expected that in 2035 20 % of the boys and 18 % of the girls will be obese (Figure 2) (7).

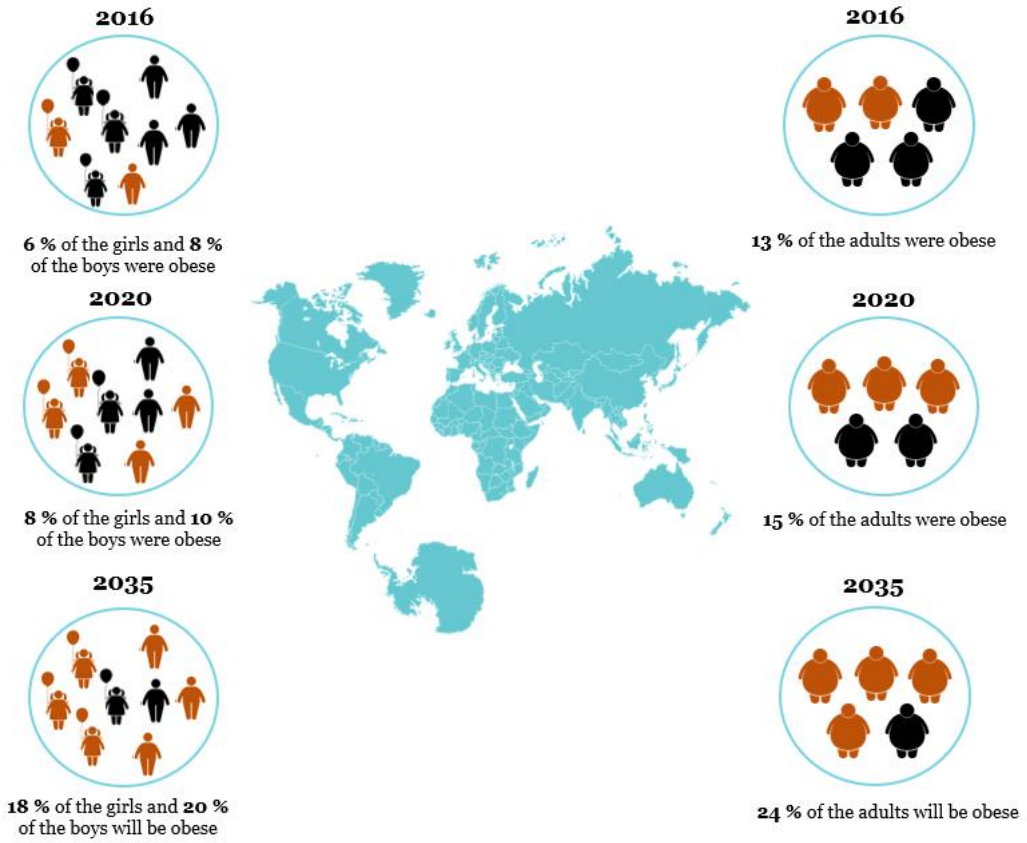


Figure 2- Data regarding the prevalence of obesity in the world according to World Health Organization (WHO) and World Obesity Federation.

The obesity epidemic has been boosted by social, economic, and technological advances. Nowadays, tasty, and high caloric food, the known fast food, is available at low price. However, this has not always been the scenario, with several nutrition transitions occurring over the years. Nutritional transition is a phenomenon defined by changes in diet and physical activity, especially in their structure and composition, promoted by culture, technical changes, and economic growth. According to researchers, there are five patterns of nutritional transition (8). The first pattern, also called Paleolithic pattern, is related to

hunger-gatherer societies, during which a very healthy diet existed, but the life span was short. The second pattern corresponds to the time modern agriculture and famine emerged and the nutritional status worsened. The third pattern corresponds to a decrease in hunger, and the fourth pattern is characterized by changes in diet and activity patterns, leading to the emergence of new diseases. In the fifth and last pattern, changes in behaviour begin to reverse the negative tendences of the previous patterns and allow a process of healthy aging. The fourth pattern is the one that most contributes to the increase in obesity because it is represented by a diet high in fat and sugar associated with an increase in sedentary lifestyle and is the one where the majority of world population remain, so the transition to the fifth pattern is of great importance (9). Noteworthy, the first patterns are not only restricted to the period in which they first appeared, since they continue to characterize certain geographic and socioeconomic populations (8, 10). In addition, the presence of an increased number of electronic devices at home has promoted sedentariness and led to a reduction in physical activity, especially among children (11, 12). Several other factors may influence the development of obesity, such as gender, age, race, and socio-economic status. Obesity seems to have a higher prevalence in women, older people, members of minority races and people of lower socio-economic status (13).

The causes of obesity are still not well explained, but there is a relationship between biological, behavioural, and psychosocial factors. Apart from the changes on nutritional behaviour already presented, the development of obesity has been associated with factors such as microorganisms, epigenetics, increased maternal age, lack of sleep, endocrine disorders, intrauterine and intergenerational effects. Certain comorbidities and their treatment may also be a factor in the development of obesity, such as hypothyroidism, Cushing's syndrome, polycystic ovarian syndrome and some neurological problems (2). The increasing numbers, the huge number of involved factors, the complexity and the unknown about the mechanisms of this disease turns its treatment and prevention a massive challenge, with multiple possible approaches and still limited outcomes.

The treatment and prevention of obesity is complex. Diet and exercise promote weight loss at least 5 % reduction in weight from the baseline level, however, the long-term adherence to lifestyle modifications is low. Most patients with obesity lose only modest weight with non-pharmacological interventions alone. Even if the weight loss is significant, approximately one third, more than a half and all the population with obesity regain the weight loss within one year, two years and five years, respectively (14). For this reason, guidelines for obesity treatment suggest that effective strategies should combine diet and

exercise, considering the emotional and genetic factors that contribute to obesity, with pharmacotherapy, as this combination has been shown to result in greater weight loss and weight maintenance than lifestyle changes alone (15). Nowadays, bariatric surgery is considered the most effective at achieving long weight loss and is the one with the best outcomes, however bariatric devices and surgical procedures are extremely invasive and expensive which make them the last resources to combat obesity (2, 16). Despite pharmacotherapy being much less invasive and much less restrictive than the other forms of treating obesity the drugs used for the treatment of obesity cannot act alone and must always be associated with behavioural changes. Most of the available drugs, act by decreasing appetite and reducing fat absorption, thus promoting weight loss. Currently, orlistat is one of the few drugs approved by European Medicines Agency (EMA) for the treatment of obesity, being an inhibitor of pancreatic lipase, the key enzyme for the digestion of triglycerides (TGs) from diet, is capable to block the absorption of 25 % to 30 % of the ingested calories from diet. In addition to the anti-obesity capacity of this drug it is also capable of moderately reduce blood pressure, improve oral glucose tolerance, and prevent the onset of type II diabetes. Clinical trials have shown a total weight loss of 2.4 % for orlistat after 4 years (14). However, orlistat has been associated with some adverse effects such as oily stool, faecal urgency, faecal incontinence, and flatus with discharge (17, 18) which led to frequent treatment withdrawal. More recently, in 2022, semaglutide was approved by EMA for obesity treatment (19). Semaglutide is a long-acting glucagon like peptide-1 (GLP-1) that can be administered once a week. It not only regulates blood sugar similar to native GLP-1 but also plays a role in reducing energy intake, increasing satiety, and reducing hunger. Some studies showed that the administration of 1 mg of semaglutide once a week for 12 weeks resulted in a weight loss of 5 kg (20). The liraglutide, which is similar to semaglutide in the mechanism of action, was firstly approved in United States by Food and Drugs Administration (FDA) in 2014. In contrast to semaglutide, liraglutide is a short-acting GLP-1 analogue that should be administrated daily (14, 21). In the United States, other molecules are approved for the treatment of obesity by the FDA, like phentermine, locaserin, phentermine + topiramate and naltrexone + bupropion (22). Except for liraglutide, all this drugs act at the level of the hypothalamus arcuate nucleus neurons to promote satiety (14). In summary, phentermine is an adrenergic agonist that increases resting energy expenditure and supresses appetite, while topiramate influences caloric intake through modulation of γ-aminobutyric acid receptors, inhibition of carbonic anhydrase and antagonism of glutamate. Since the regulation of appetite involves multiple pathways, targeting different mechanisms simultaneously can have an addictive effect on

body weight, so the combination of phentermine and topiramate is one of the used pharmacological treatments. On its turns, locaserin is a selective serotonin receptor agonist, it reduces the appetite and increases the satiety by binding to the 5HT-2C receptor on anorexigenic pro-opiomelanocortin neurons in the hypothalamus. Bupropion is a dopamine and norepinephrine reuptake inhibitor, and naltrexone is an opioid antagonist and together they have effect in two different areas of the brain involved in the regulation of food intake, in the arcuate nucleus of the hypothalamus and in the mesolimbic dopamine reward circuit (Table 1) (14, 18). Despite the potential of this molecules to treat obesity, the use of these drugs has demonstrated serious side effects, like gastrointestinal alterations, palpitations, addiction, increased blood pressure, hallucinations, anxiety and depression (23), reason why EMA considered that the benefits do not supplant the risks of their use.

Table 1- Resume of the pharmacotherapy approved in United States by FDA and in Europe by EMA for the treatment of the obesity.

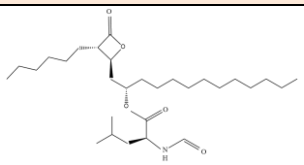
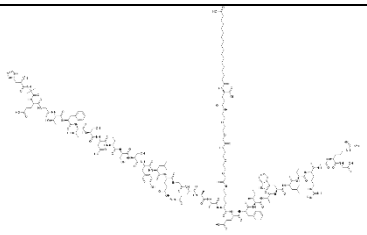
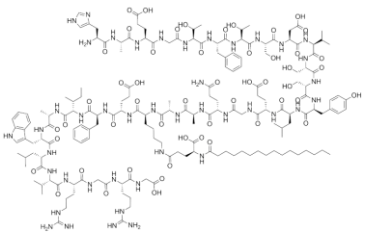
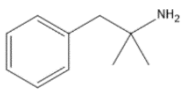
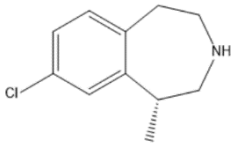
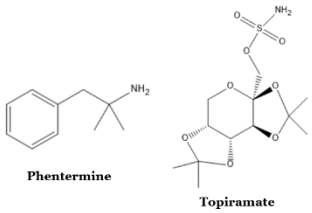
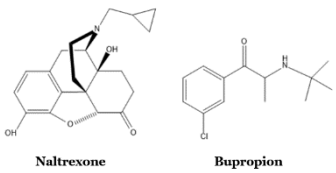
Drugs	Structure	Mechanism of action	Approved
Orlistat		Inhibitor of pancreatic lipase, capable of block the absorption of 25 % to 30 % of the ingested calories from diet.	EMA and FDA
Semaglutide		Long-acting GLP-1 analogue that is administered once a week. It is capable of reduce food intake, increase satiety, and reduce hunger.	EMA and FDA
Liraglutide		Short-acting GLP-1 analogue that is administered daily. It is capable of reduce food intake, increase satiety, and reduce hunger.	EMA* and FDA
Phentermine		Adrenergic agonist that increases resting energy expenditure and supresses appetite.	FDA

Table 1 – Resume of the pharmacotherapy approved in United States by FDA and in Europe by EMA for the treatment of the obesity.

Drugs	Structure	Mechanism of action	Approved
Locaserin		Is a selective serotonin receptor agonist, it reduces the appetite and increases the satiety by binding to the 5HT-2C receptor on anorexigenic pro-opiomelanocortin neurons in the hypothalamus.	FDA
Phentermine + Topiramate	 Phentermine Topiramate	<u>Phentermine:</u> Adrenergic agonist that increases resting energy expenditure and supresses appetite. <u>Topiramate:</u> influences caloric intake through modulation of γ-aminobutyric acid receptors, inhibition of carbonic anhydrase and antagonism of glutamate.	FDA
Naltrexone + Bupropion	 Naltrexone Bupropion	<u>Bupropion:</u> is a dopamine and norepinephrine reuptake inhibitor. <u>Naltrexone:</u> is an opioid antagonist. Together they have effect in 2 different areas of the brain, in the arcuate nucleus of the hypothalamus and in the mesolimbic dopamine reward circuit.	EMA* and FDA

*If a loss of 5 % of initial body weight was not observed within 12 weeks (liraglutide) or 16 weeks (naltrexone + bupropion) treatment should be discontinued (24, 25) .

1.1.1. Pathophysiology

Obesity is mainly associated with fat accumulation and expansion of the adipose tissue (26). Adipose tissue is an heterogenous mixture consisting of adipocytes, stromal preadipocytes, immune cells and endothelium. It can work as a complex endocrine organ with numerous diverse actions (27, 28). The adipose tissue responds to afferent signals from central nervous systems and can also express and secret factors with important endocrine functions (29), named adipokines, including leptin and adiponectin, cytokines, including interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α) and others, plasminogen

activator inhibitor-1, proteins of the renin-angiotensin system, resistin and complement components. These molecules act in the adipose tissue itself and in other tissues, and are capable of regulate food intake, energy balance and insulin sensitivity (29). The adipose tissue is also a major site for the metabolism of sex steroids and glucocorticoids (30, 31).

The adipocytes themselves are fundamental in the establishment of obesity because of their ability to store fat and to participate in the regulation of metabolic homeostasis. There are two ways of adipose tissue growth, the adipocyte hyperplasia, which correspond to the formation of new adipocytes through differentiation of resident preadipocytes, and the adipocyte hypertrophy, which correspond to the increase in adipocyte size (26, 32). Obesity can be classified according to these phenomena's, being mild obesity associated with an increase in adipose cell size, named hypertrophic obesity, and more severe obesity associated with both, an increase in cell size and in cell number, named hypertrophic/hyperplastic obesity (33). These differences are curious, because hyperplastic adipocytes are considered healthy and adaptative, as they maintain the proper vascularization, insulin sensitizing and anti-inflammatory levels, maintaining their metabolic and responsive abilities, while the hypertrophic adipocytes are associated with an increase in hypoxia because of their expanded size leading to an unhealthy fat storage (Figure 3). The decrease space among cells, the increased lipolysis and the secretion capacity, such as that of inflammatory cytokines (TNF-α, IL-6) and adipokines as leptin on one side and the reduced secretion of anti-inflammatory adipokines, such as adiponectin, on the other side lead hypertrophic adipocytes to a mechanical and hypoxic stress (26).

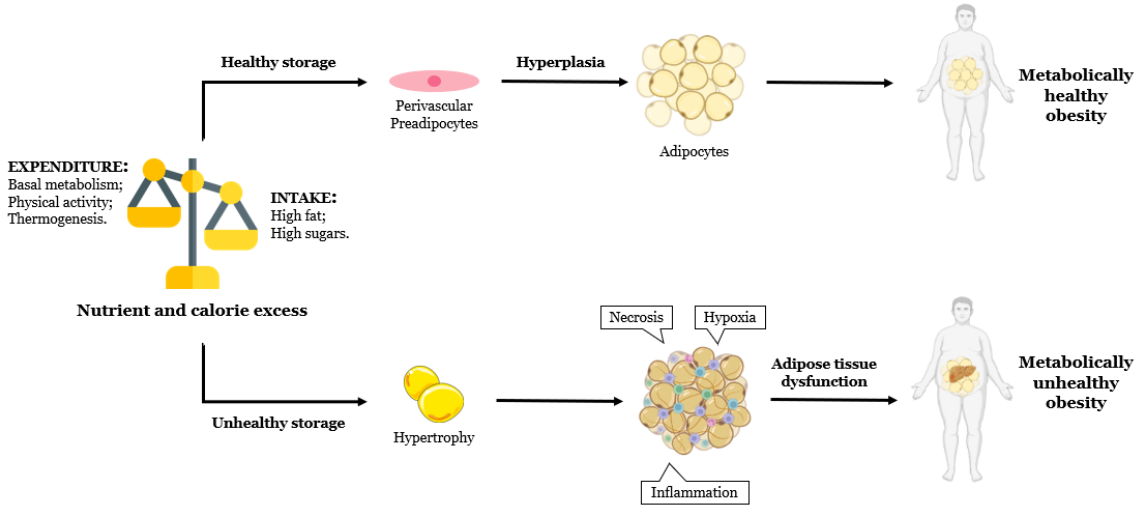


Figure 3- Mechanisms of adipose tissue expansion. Adapted from (26).

There are two types of adipose tissue, the brown adipose tissue (BAT), and the white adipose tissue (WAT). The BAT, despite existing in a small amount in human adults, is responsible for thermogenesis and temperature regulation. It is quite rich in mitochondrial and is an extremely vascularised tissue (34, 35). In its turn the WAT is the most abundant in human adults and is an important energy accumulator, helping to maintain homeostasis, which occurs through the balance between the storage and degradation of TGs (35, 36).

The adipocytes respond to external signals. In a moment of nutrients deprivation, the blood glucose levels decrease, and the adipocytes are stimulated by hormones, such as glucagon and noradrenaline which lead to the activation of the hormone sensitive lipase (HSL) and the adipose triglyceride lipase (ATGL) to release non-esterified fatty acids and glycerol from lipid droplets into circulation to be used by the organs. An opposite situation can also appear in a moment of nutrient excess, the blood glucose and an increase in insulin levels supress HSL and ATGL and lead to glucose uptake and *de novo* lipogenesis to generate lipid droplets and to the storage of the excess nutrients (26, 37).

Diverse metabolic processes are involved in the uptake, transport, and storage of lipids. When a meal containing fat is ingested, TGs suffer lipolysis into the intestinal lumen, generating free fatty acids (FFAs) and glycerol. Then, they are taken up by the enterocytes and cholesterol is transformed into cholesterol-esters and the FFAs and glycerol are assembled into TGs again. The TGs, specific proteins and the cholesterol-esters are packed together into the form of chylomicrons. The transformation of these chylomicrons into chylomicron remnants and of very-low-density lipoproteins (VLDL) into low-density lipoproteins (LDL) is promoted by lipoprotein lipase (LPL), with the release of FFAs into the circulation. VLDL are synthesized in the liver after postprandial TGs and FFAs uptake. Chylomicrons and VLDL deliver TGs and FFAs to the heart, muscles, and WAT in a process stimulated by insulin and glucose and mediated by LPL activity. This process is called lipolysis (38). The chylomicron remnants are captured by the liver due to an interaction with LDL receptors (LDLR). Once in the liver, when the nutrients are present in excess fatty acid synthase (FAS) is responsible for convert the excess of food consumed into lipids for storage in a process named *de novo* synthesis of FFAs by catalysing the condensation of acetyl-coA and malonyl-coA to generate long-chain fatty acids in the cytoplasm (39-41).

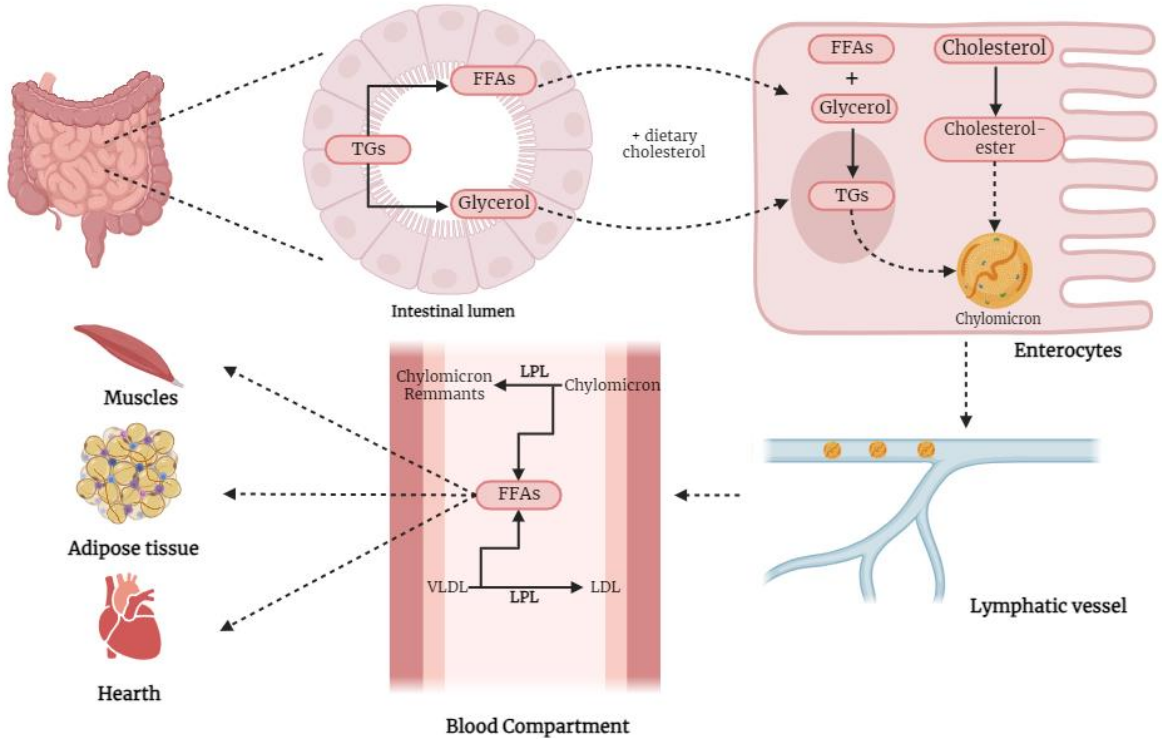


Figure 4- Schematic representation of the lipid metabolism. Triglycerides suffer lipolysis into the intestinal lumen and originate free fatty acids and glycerol. These are taken up by the enterocytes together with the dietary cholesterol via specific transporters. Into the enterocytes, the cholesterol is transformed into cholesterol-esters and the free fatty acids, and the glycerol are assembled into triglycerides again. The triglycerides, the cholesterol-esters and specific lipoproteins are packed together origination chylomicrons. The transformation of chylomicrons into chylomicron remnants and of very-low density lipoproteins into low-density lipoproteins is promoted by the lipoprotein lipase with the release of free fatty acids into the circulation. The triglycerides are delivered to the muscles, the adipose tissue and hearth in a process called lipolysis stimulated by insulin and glucose. TGs- Triglycerides; FFAs- Free Fatty Acids; LPL- Lipoprotein Lipase; VLDL- Very-low density lipoprotein; LDL – Low-density lipoprotein.

As said, obesity is associated with fat accumulation and expansion of the adipose tissue through hypertrophy of mature adipocytes and differentiation of preadipocytes. This process is called adipogenesis and is characterized by the process in which the fibroblast-like preadipocytes turn into mature adipocytes capable of producing and storing TGs (42). In order to achieve a successful transformation into mature adipocytes, preadipocytes undergo marked changes in morphology and gene expression. Adipogenesis can be considered the consequence of preadipocytes proliferation and adipocytes differentiation,

being a key regulatory step of fat deposition into adipose tissue in which a sequence of events leads the path from proliferation to differentiation (16). The adipocytes differentiation consists in a process of morphological modifications in the adipocyte precursors containing four main phases: Growth arrest, mitotic clonal expansion, early differentiation, and terminal differentiation. At the mitotic clonal expansion phase, after the growth arrest at confluence, the preadipocytes received a combination of mitogenic and adipogenic signals to continue through the next differentiation steps (43). The early differentiation phase is characterized by the accumulation of lipid droplets while the terminal differentiation is characterized by the lipid droplets enlargement and coalescence (43). The stages of adipocyte differentiation are triggered by Pref-1 and CCAAT/enhancer-binding protein beta and delta (C/EBP β and δ) transcription factors and induce the expression and activity of peroxisome proliferator-activated receptor gamma (PPAR γ) which is a key regulatory factor of the adipocyte differentiation (44). PPAR γ is responsible for the induction of other target genes, particularly the expression of CCAAT/enhancer-binding protein alpha (C/EBP α) which binds to the promoter region of PPAR γ leading to a positive feedback (44). The stages of early and terminal differentiation depend on the activation of PPAR γ and C/EBP α and expression of lipogenic enzymes, fatty-acid binding proteins, including adipocyte protein 2 and FAS (43). At the end of the differentiation process, the mature adipocytes express all the markers mentioned above, peptide hormones, such as adiponectin and leptin, and high levels of lipid-droplets associated protein, perilipin 1, which is responsible for coat lipid droplets and protect them from the natural lipases (26, 45) (Figure 5).

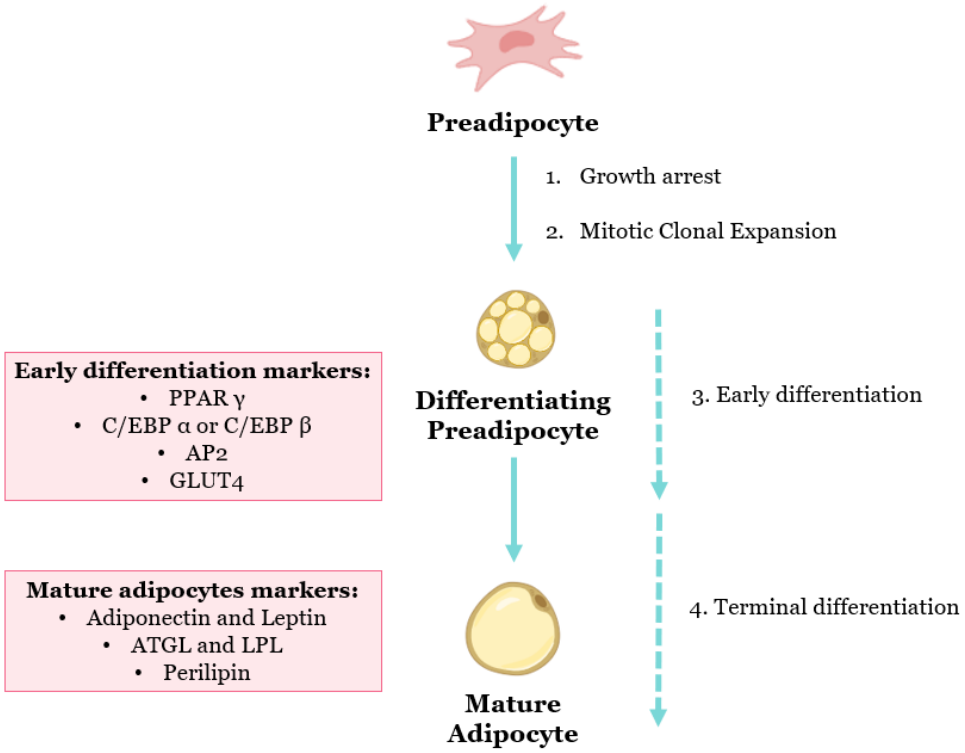


Figure 5- Schematic representation of the adipocyte differentiation. This process allows preadipocytes to develop into mature adipocytes capable of producing and storing triglycerides. Adipocyte differentiation is a process with four main phases: growth arrest, mitotic clonal expansion, early differentiation, and terminal differentiation. In the different stages of differentiation different markers are expressed.

The differentiation process, as mentioned earlier, involves the interaction and regulation of the transcription factors PPAR γ and C/EBP α. Different pathways including the adipocyte determination and differentiation dependent factor (ADD1)/SREBP1 potentiate the activity of PPAR γ through the endogenous expression of ligands for PPAR γ. After the ligand activation, the expression of C/EBP α and of other adipocyte genes is induced by PPAR γ. Through a positive feedback PPAR γ expression is maintained by C/EBP α. Together they corporate to promote the adipocyte differentiation, including adipocyte gene expression and insulin sensitivity (46, 47) as demonstrated in figure 6.

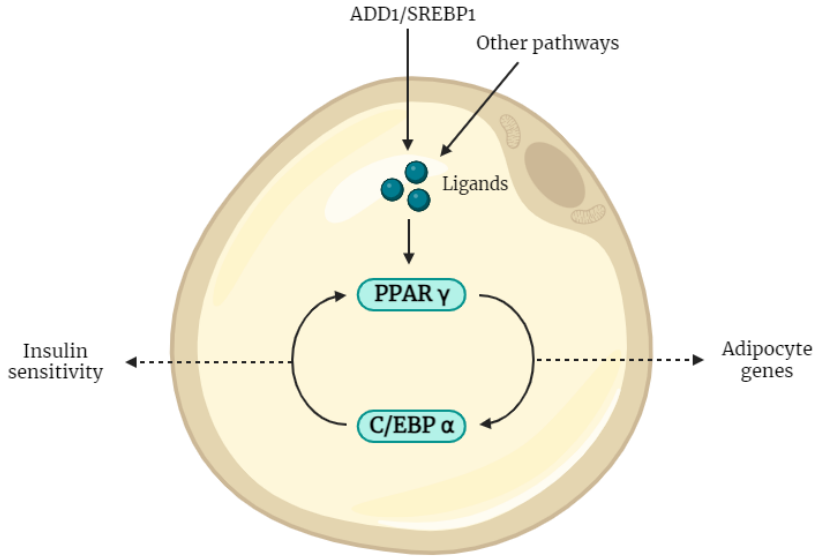


Figure 6-Schematic representation of the regulatory process of adipogenesis. ADD1/SREBP1 potentiate the activity of PPAR γ probably due to a production of endogenous ligands. The expression of C/EBP α and adipocyte genes is induced by PPAR γ. Through positive feedback the expression of PPAR γ is maintained by C/EBP α. Together they corporate the adipocyte differentiation including the expression of adipocyte genes and insulin sensitivity. Adapted from (46).

1.1.2. Consequences of obesity

Obesity is associated with an increased risk of develop several diseases that can lead to high levels of morbidity and mortality (48), for every five units increase in BMI above 25 kg/m², overall mortality increases by 29 %, vascular mortality by 41 % and diabetes related mortality by 210 % (2). This condition is associated with an increase in the prevalence of several diseases, as shown in figure 7, such as cardiovascular disease (CVD) (49), hypertension (50), dyslipidemia (38), sleep apnea (51), liver and gall bladder disease (52), neurological diseases (53), osteoarthritis (54), various cancers (55), and type II *diabetes mellitus* (T2DM) (56, 57). The association of obesity with an increased risk of development of T2DM is associated with the increased abdominal and intra-abdominal distribution and intrahepatic and intramuscular triglyceride content, characteristic of obesity, which causes insulin resistance and β cell dysfunction (56). The relationship between obesity and cancer risk may be due to diverse mechanisms, including pathways related to insulin resistance, inflammation and sex hormones (55).

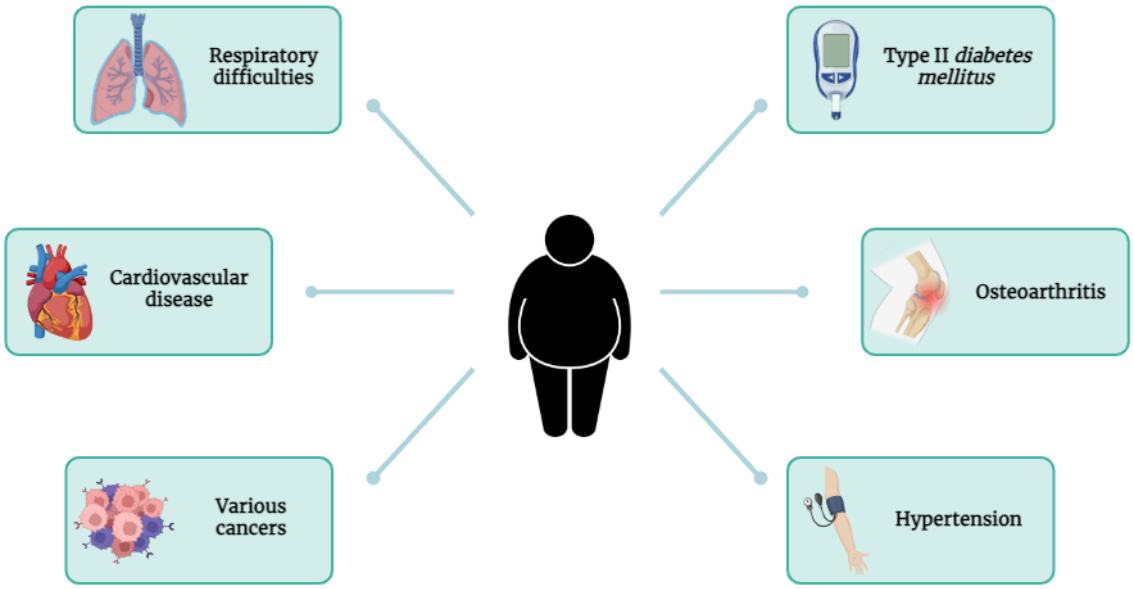


Figure 7- Examples of health consequences associated with obesity.

The excess of lipid accumulation into the adipose tissues, which characterises obesity stimulates them to release inflammatory mediators, like TNF-α and IL-6, and lead to the reduction of adiponectin, predisposing the tissue to a pro-inflammatory state and oxidative stress. These alterations, in particular the increased levels of IL-6 stimulate the secretion of C-reactive protein by the liver. Inflammation is an incorporated mechanism for the development of CVD and non-cardiovascular diseases such as cancer and renal diseases. The reduction in the adiponectin level is associated with impaired fasting glucose which lead to T2DM development (58, 59).

In the case of children, associations can also be made between obesity and internalizing disorders, periodontal disease, poor school performance, altered pre-pubertal hormones, and attention-deficit hyperactivity disorder (48).

Psychosocial consequences can also result from obesity. Obese population suffer from a social stigma that contribute to higher rates of anxiety, depression and for a lower self-esteem (48). Beside the health and psychosocial consequences, obesity has also economic consequences, as all its aspects including being a risk factor for other disease and its

treatment and make it a very expensive health problem and a burden on health services (60).

The consequences associated with the development of obesity and the fact that the pharmacotherapy currently used for the treatment of obesity is associated with low efficacy and undesirable side effects, as mentioned earlier, lead to the utmost need for the search for new and safer molecules for the treatment of obesity.

1.2. Flavonoids

1.2.1. Characterization and structure

Polyphenols are natural chemical compounds and correspond to plant secondary metabolites. They have been studied over the last years for their biological activities and can be found in fruit, vegetables, coffee, chocolate, wine and tea (61).These compounds present a structure with one or more aromatic rings, and they can occur linked with other chemical groups or molecules, such as amines, carboxylic, and organic acids, lipids or other phenol groups (62). There are more than 8000 polyphenolic structures, which are divided in different groups, including the flavonoids, the phenolic acids, the stilbenes and the lignans (63). Phenolic acids are subdivided into benzoic acids with a C1-C6 skeleton and the cinnamic acids with a C3-C6 skeleton. The stilbenes have a C6-C2-C6 structure and the lignans have a C6-C4-C6 structure (63).

Flavonoids are one of the largest groups of polyphenols. They are characterized by a basic-15-carbon structure, made up of two benzene rings (C6) joined by a linear-3-carbon chain (C3), which may be represented by C6-C3-C6 structure (16) (Figure 8).

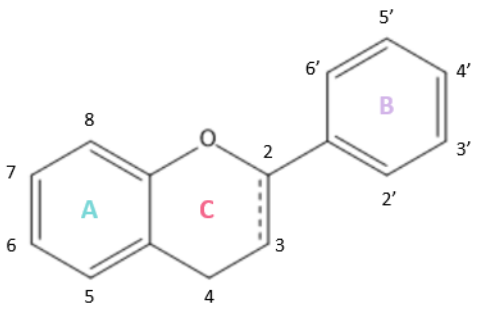
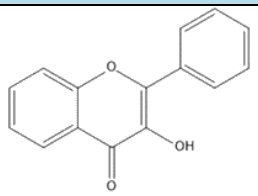
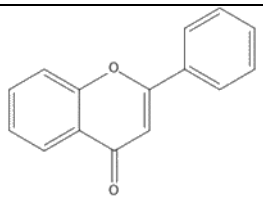
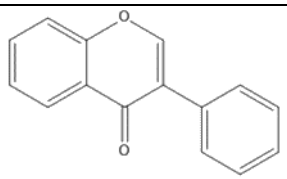
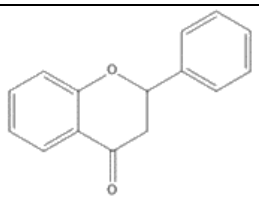
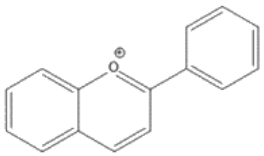


Figure 8-Representation of the chemical structure and numbering system of the flavonoids.

This class of phenolic compounds is usually subdivided into five subclasses according to their structure and oxidation levels. Those subclasses are, flavones, flavanones, isoflavones, anthocyanins and flavonols (64) as presented in table 2.

Table 2- Different flavonoids subclasses with the corresponding basic structure and examples of naturally occurring compounds belonging to each class.

Structure	Flavonoid subclass	Examples
	Flavonols	Quercetin, Kaempferol, Myricerin, Herbacetin and Robinetin
	Flavones	Chrysin, Apigenin, Luteolin and Rutin
	Isoflavones	Genistein and Diadzein
	Flavanones	Naringin, Hesperidin and eriodictyol
	Anthocyanins	Cyanidin, malvidin and apigenidin

1.2.2. Biosynthesis

The biosynthesis of flavonoids occurs through the phenylpropanoid pathway, in which the phenylalanine is converted into *p*-coumaroyl-CoA through the activity of different enzymes (65). The first three steps are related to the general phenylpropanoid pathway. The first step is related to the deamination of phenylalanine to *trans*-cinnamic acid catalysed by

phenylalanine ammonia lyase (PAL). In the second step, the cinnamic acid-4-hydroxylase (C4H) catalyse the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid. Lastly, the third step of this pathway, the 4-coumarate: CoA ligase (4CL) catalyse the formation of *p*-coumaroyl-CoA by the addition of a coenzyme A (CoA) unit to the *p*-coumaric acid (66).

The entry of the *p*-coumaroyl-CoA into the biosynthesis lead to the formation of specific flavonoids, which begins with the chalcone formation, which are considered the flavonoid precursors. In the next step, one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA generate naringenin-chalcone through the action of chalcone synthase or with the simultaneous action of chalcone synthase and chalcone reductase generates the isoliquiritigenin (66, 67) as demonstrated in figure 10.

Chalcone isomerase is responsible to catalyse the cyclization of chalcones to form flavanones, leading to the formation of the heterocyclic C-ring in the flavonoid pathway. After that, different groups of enzymes can modify the basic skeleton of flavonoids, originating their different subclasses (67).

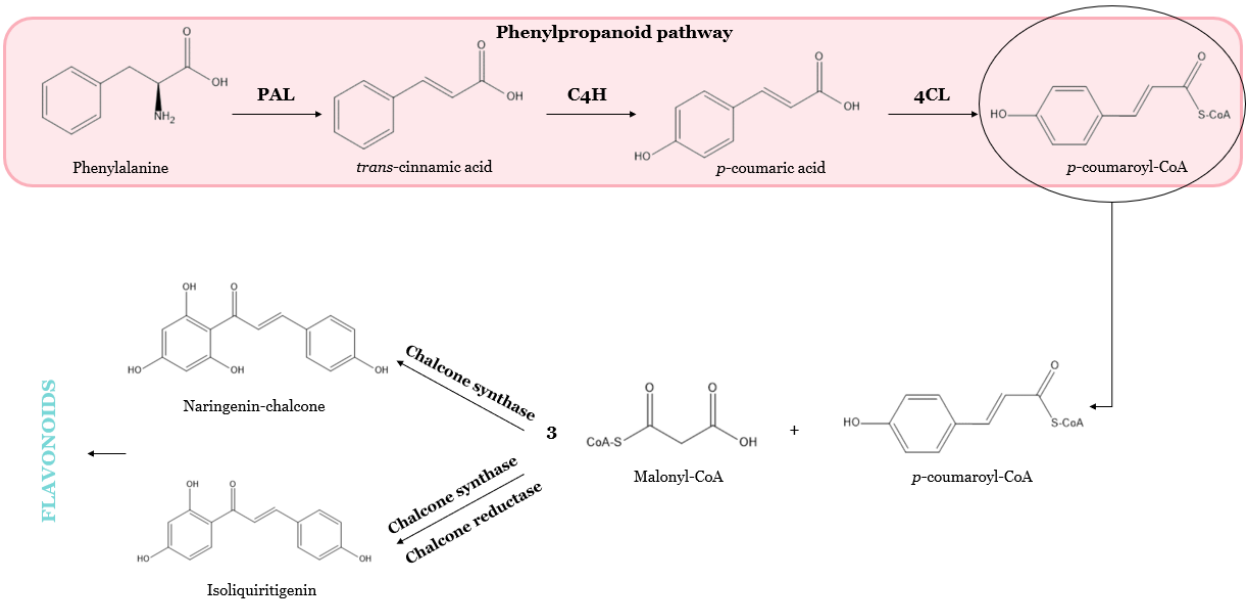


Figure 9- Steps of the flavonoid's biosynthesis. Starting with the phenylpropanoid pathway in the pink box, where the phenylalanine origin p-coumaroyl-CoA with the intervention of diverse enzymes. After the phenylpropanoid pathway one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA give origin to chalcones (naringenin-chalcone and isoliquiritigenin) which are flavonoid precursors. PAL- phenylalanine ammonia lyase; C4H- cinnamic acid-4-hydroxylase; 4CL- 4-coumarate: CoA ligase.

1.2.3. Occurrence and metabolism

Flavonoids are natural compounds that occur in nature in different parts of the plants and play important roles in the pigmentation of flowers and in the protection of flowers from UV irradiation and oxidative stress (64, 68). Flavonoids can be found in food of plant origin, such as fruits, vegetables, teas, wine, and cocoa (69).

Despite the flavonoids can be found in a wide range of food, their bioavailability is influenced by their different structures. The molecular weight, glycosylation, esterification, metabolic conversion and interaction with colonic microflora are factors that affect the absorption and consequently the bioavailability of the flavonoids (70). The flavonoids are substrates for conjugating and hydrolysing enzymes in the small intestine, liver, and colon and all are conjugated with O-methyl esters, sulfate esters and O-glucuronides. This conjugation occurs firstly in the small intestine, then in the liver where they are further metabolised. The produced glucuronides and sulfate derivatives facilitate the excretion via urine and bile. The flavonoids that are not absorbed in the intestinal tract reach the colon where they can be extremely metabolised by the colonic microflora (71).

Absorption and metabolism of flavonoids are crucial for their activity as therapeutic agents, since their therapeutic potential depends not only on the local of action, directly in the intestine, but also on the way in which the absorbed part and its metabolites can actuate in the body.

1.2.4. Biological properties

In the last years the biological properties of the flavonoids have been extensively studied. Flavonoids are associated with diverse biological activities, particularly their antioxidant and anti-inflammatory activities (72). Flavonoids molecules have the ability to disable the progression of reactive oxygen species (ROS) and reactive nitrogen species (RNS) production by the inhibition of the oxidases, activation of antioxidant enzymes or direct scavenging of ROS and RNS by hydrogen atom donation (72). The neuroprotective (73), anticarcinogenic (74), antiviral (75), antirheumatic (76) and antidiabetic (77, 78), activities were also described for flavonoids.

In addition to the effects already described for the flavonoids, in the recent years their anti-obesity effects are under study, being described their effects on the reduction of food

intake and appetite control, reduction of the intestinal fat absorption, stimulation of energy expenditure by inducing the nonshivering thermogenesis, modulation of adipogenesis, lipolysis and β-oxidation and in gut microbiota dysbiosis (79) (Figure 9).

These biological properties of the flavonoids make them compounds of great interest for be further studied for their use as potential anti-obesity molecules.

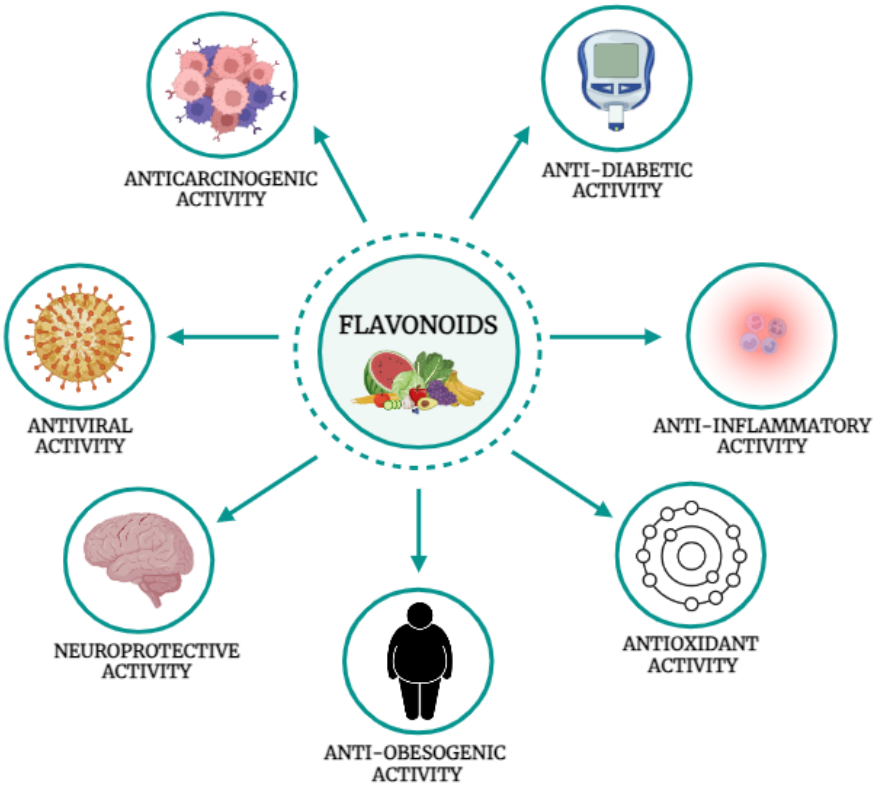


Figure 10- Biological activities of the flavonoids.

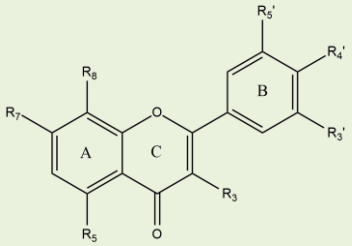
1.3. General and specific objectives of the dissertation

The main objective of this dissertation was to evaluate the anti-obesogenic effect of a panel of hydroxylated flavonoids and evaluate their capacity to modulate lipid metabolism using for that an *in vitro* model of preadipocytes, the 3T3-L1 cell line. This work can contribute for the search for new and safer molecules for the treatment of obesity.

The following specific objectives were established:

- Optimization of the chemical differentiation of 3T3-L1 cells, in order to establish an *in vitro* model of adipogenesis;
- Evaluation of the effect of flavonoids on cells differentiation using Oil Red O staining assays and a kit for TGs measurement;
- Evaluation of the modulatory effect of flavonoids in proteins involved in the lipid metabolism through western blot analysis.

Table 3- Chemical structure of the flavonoids panel tested.

Flavonoid 	**$R_{3'}$**	**$R_{4'}$**	**$R_{5'}$**	**R_3**	**R_5**	**R_7**	**R_8**
Apigenin	-	-OH	-	-	-OH	-OH	-
Luteolin	-	-OH	-OH	-	-OH	-OH	-
Kaempferol	-	-OH	-	-OH	-OH	-OH	-
Herbacetin	-	-OH	-	-OH	-OH	-OH	-OH
Quercetin	-	-OH	-OH	-OH	-OH	-OH	-
Myricetin	-OH	-OH	-OH	-OH	-OH	-OH	-
Robinetin	-OH	-OH	-OH	-OH	-	-OH	-

2.MATERIALS AND METHODS

2.1. Chemicals

The following reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO): Apigenin, Quercetin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Pantothenic acid, Biotin, Insulin, Dulbecco's phosphate-buffered saline (DPBS), Oil Red O, Hematoxylin, Ammonium persulfate (AMPS), N,N,N',N'-tetramethylethane-1,2-diamine (TEMED), Glycine, 2-mercaptoethanol, Formalin, 2- Propanol, methanol, Bovine calf serum (FCS), sodium dodecyl sulfate (SDS), β-actin antibody and human insulin. Myricetin, Robinetin, Herbacetin and Kaempferol were obtained from Extrasynthese (Z.I Lyon Nord). Clarity western ECL substrate, Laemmli sample buffer, 10x Tris/ Glycine buffer, Precision plus protein standards dual colour, DC protein assay kit, blot filter paper, goat anti-rabbit antibody and TGX Stain-Free FastCast Acrylamide Solutions were obtained from BioRad (California, USA). 3-isobutyl-1-methylxanthine (IBMX) and Dexamethasone were purchase from Abcam (Cambridge, UK). Luteolin were obtained from Alfa Aesar (Massachusetts, USA). PPAR γ and anti-mouse antibodies were obtained from Santa Cruz Biotechnology (Texas, USA). FAS and ATGL antibodies were obtained from Cell Signaling Technology (Massachusetts, USA). Triglycerides colorimetric determination assay kit were obtained from Spinreact (Girona, Spain). 3T3-L1 Mouse embryonic Fibroblasts (CL-173) were purchase from ATCC (Virginia, USA). Dulbecco's Modified Eagle Medium (DMEM) High glucose were obtained from VWR Chemicals (Pennsylvania, USA). Dimethyl sulfoxide (DMSO), Sodium Chloride and polyvinylidene fluoride (PVDF) membranes were obtained from Thermo Fischer Scientific (Hampton, USA). PhoSTOP ™ and cOmplete ™ mini phosphatase and protease inhibitor cocktails, respectively, were obtained from Roche (Basel, Switzerland). Pen Strep and Fetal Bovine Serum (FBS) were obtained from Gibco (Hampton, USA).

2.2. Equipment

Colorimetric readings were performed using a microplate reader (Synergy HT, BIO-TEK). The microscopic images were obtained using a microplate reader with a built-in microscopy system (Cytation 5, BIO-TEK). Electrophoresis was performed using a Mini PROTEAN® Tetra Cell system (BioRad, California, USA) and transfer using a Criterion Blotter (BioRad, California, USA). The image acquisition was performed in a Chemidoc Imaging System (BioRad, California, USA).

2.3. Cell culture and differentiation

2.3.1. Principle of the method

To achieve the propose of this work it was necessary to create an adipogenesis model, using the pre-adipocytic cell line 3T3-L1. Since literature presents several approaches for the differentiation of these cells into mature adipocytes, capable of producing and storing TGs (Figure 11), it was necessary to optimise the differentiation conditions (80-82). Different constitutions of the differentiation and adipocyte medium and different time of differentiation were tested.

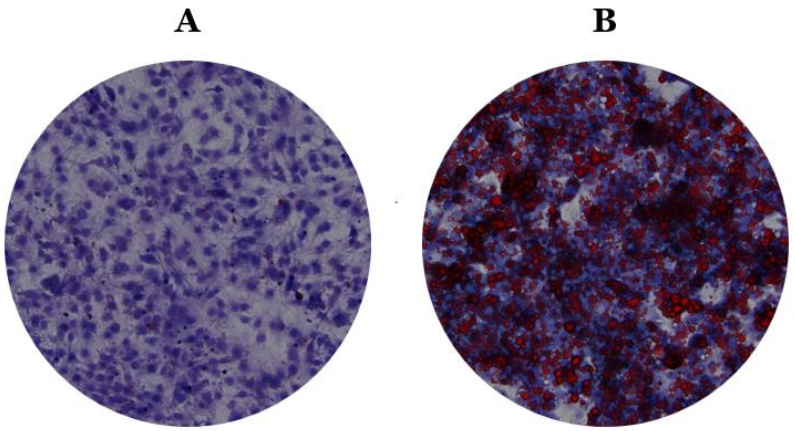


Figure 11-Microscopic image of 3T3-L1 cells in different stages. **A**- preadipocytes (before differentiation); **B**- adipocytes (after differentiation).

2.3.2. Experimental procedure

Before differentiation procedures, 3T3-L1 preadipocytes were maintained in DMEM High Glucose with stable Glutamine and Sodium Pyruvate containing 10 % of Bovine Calf Serum (FCS) and 1 % PenStrep and passaged at low confluence rates. Cells were maintained at 37 ˚C in a humidified 5 % CO_2 atmosphere. The cells were used for differentiation until passage 8.

For the differentiation process the different approaches were schematically represented in figure 12. In a first approach, cells were seeded in a 6-well plate at a density of 8 x 10^4 cells/well. Four days after confluence (day 0) the cells were stimulated with the

differentiation medium containing DMEM supplemented with 10 % FBS, 1 % PenStrep, 1.0 µM of Dexamethasone, 0.5 mM IBMX and 1.0 µg/mL of Insulin for 2 days. On day 2, the differentiation medium was replaced for DMEM with FBS and 1.0 µg/mL of Insulin. The adipocyte medium was changed every two days until the day the cells were used. In the other approach cells were seeded in a 6- well plate at a density of 8 x 10^4 cells/well. Four days after confluence (day 0) the cells were stimulated with the differentiation medium containing DMEM supplemented with 10 % Fetal Bovine Serum (FBS), 1 % PenStrep, 1.0 µM of Dexamethasone, 0.5 mM IBMX and 10.0 µg/mL of Insulin. On day 2, the differentiation medium was replaced for DMEM with FBS and 10.0 µg/mL of Insulin. On day 4, the medium was replaced for DMEM with FBS and 0.2 µg/mL. The medium was changed every two days until the day cells were used. Both the differentiation and adipocyte medium contain 8 µg/mL of Biotin and 4 µg/mL of Pantothenic acid. The differentiation time was also tested, being tested the 8 days differentiation and 10 days differentiation (Figure 12). Subsequently, the use of 12-well plate was tested, seeding the same ratio of cells (4 x 10^4 cells/well).

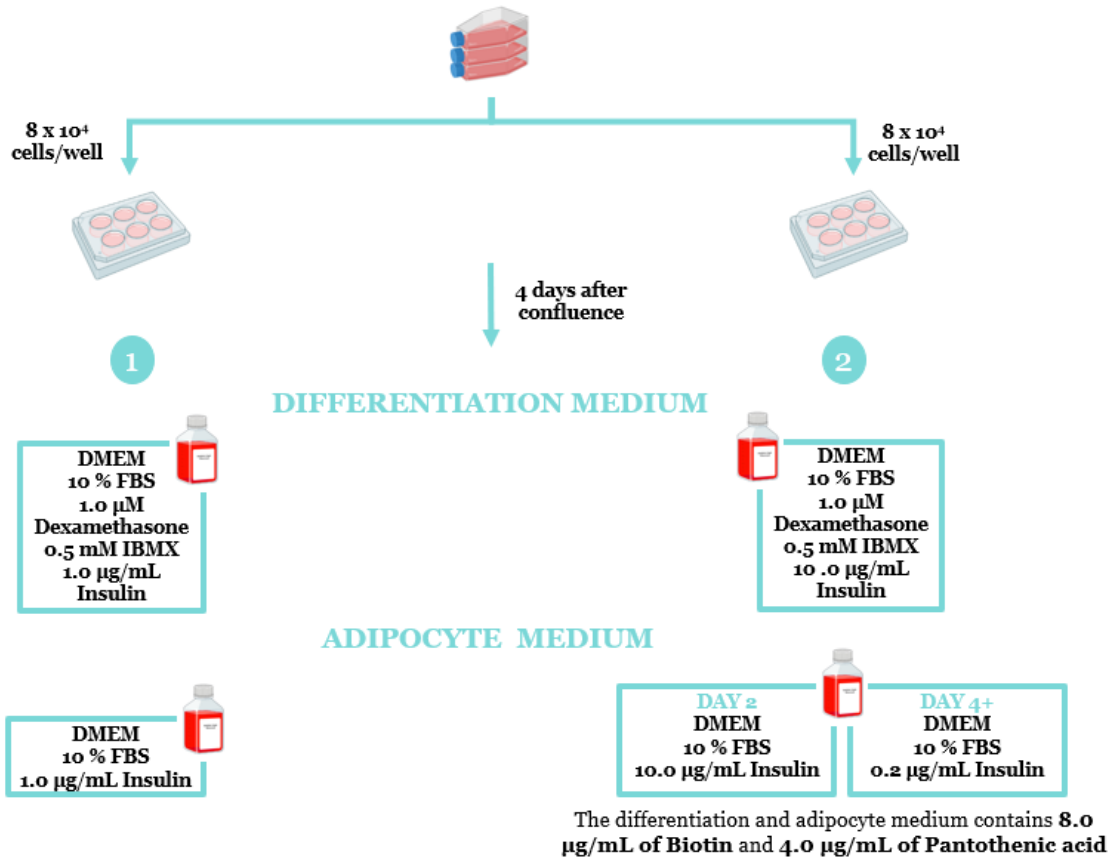


Figure 12- Schematic representation of the optimisation of cells differentiation conditions.

After being established the optimal conditions for 3T3-L1 preadipocytes differentiation into mature adipocytes, the next step is to check the effect of the chosen compounds. For that, the cells were incubated with the compounds from the day 0 to day 8 at different concentrations. The referred concentrations were chosen after the cytotoxicity/viability assays that will be described thereafter. Controls were treated with the maximum DMSO concentration used for the flavonoid's treatments. As referred before, the medium was changed every two days and the compounds were placed in the medium every time it is changed. On day 8, the culture supernatant was removed, and the cells were used for the subsequent assays (Figure 13).

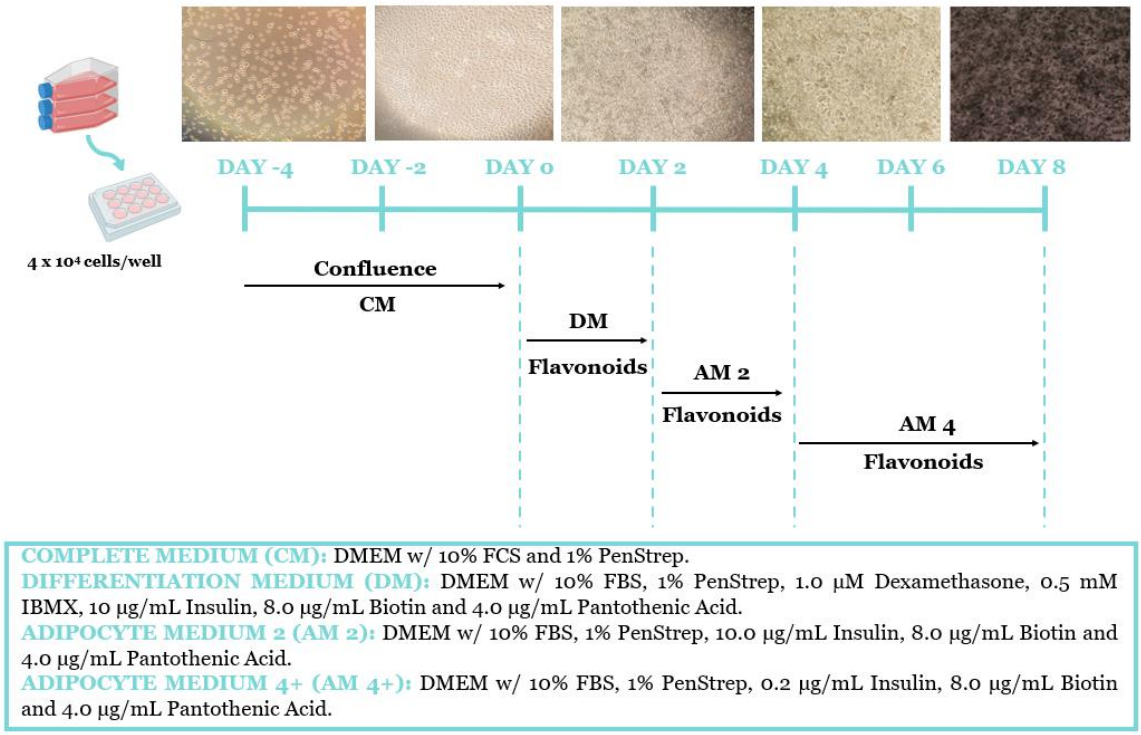


Figure 13- Schematic representation of cell differentiation and treatment process.

2.4. Cell viability

2.4.1. Principle of the method

Cell viability was accessed by MTT assay in preadipocytes to disclose the maximum concentrations that did not affect the viability of our cells and it was also performed during the differentiation to confirm if the maximum concentrations continued not to affect the

cells viability during the 8 days of the differentiation process. The MTT assay is a colorimetric assay based on the reduction of the yellow tetrazolium salt (3-(4,5-dimethylthyazol -2-yl)-2,5-diphenyl tetrazolium bromide) into purple formazan crystals by the NADPH-dependent oxidoreductase enzymes present in the metabolic active cells (Figure 14). The insoluble formazan crystals were dissolved using DMSO and the resulting-coloured solution is quantified by measuring the absorbance at 570 nm in the microplate reader.

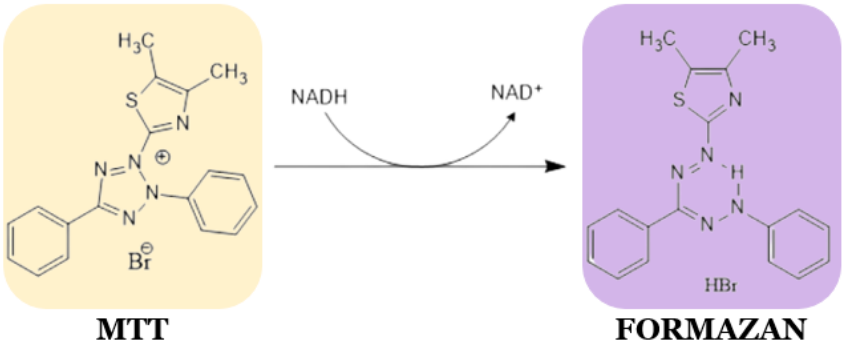


Figure 14- MTT reduction reaction.

2.4.2. Experimental procedure

The 3T3-L1 cells were grown in 96-well plates at a density of 5 x 10^3 cells/well in the complete medium (DMEM supplemented with 10 % FCS and 1 % PenStrep) and incubated for 24h until reach the full confluence. After reaching confluency, different concentrations of flavonoids (0-50 µM) were added to the medium. The cells used as control were treated with the maximum amount of DMSO used (50 µM). After 48h, 20 µL of 5 mg/mL MTT were added to the 200 µL medium in each well for 2h. The solution was removed and DMSO (200 µL/well) was added for 30 minutes to dissolve the formazan crystals. After the incubation period 100 µL/well were removed and placed in other empty wells and the absorbance was measured at 750 nm in a microplate reader.

To perform this assay during the differentiation process, the cells of the 96-plate were treated as described above in the differentiation section. At the day 8, 20 µL of 5 mg/mL MTT were added to the 200 µL medium in each well for 2h. After the reaction period, the MTT solution was removed and DMSO (200 µL/well) was added for 30 minutes to dissolve

the formazan crystals. After the dissolution time, 100 µL/well were removed and placed in other empty wells and the absorbance was measured at 750 nm in a microplate reader (Figure 15).

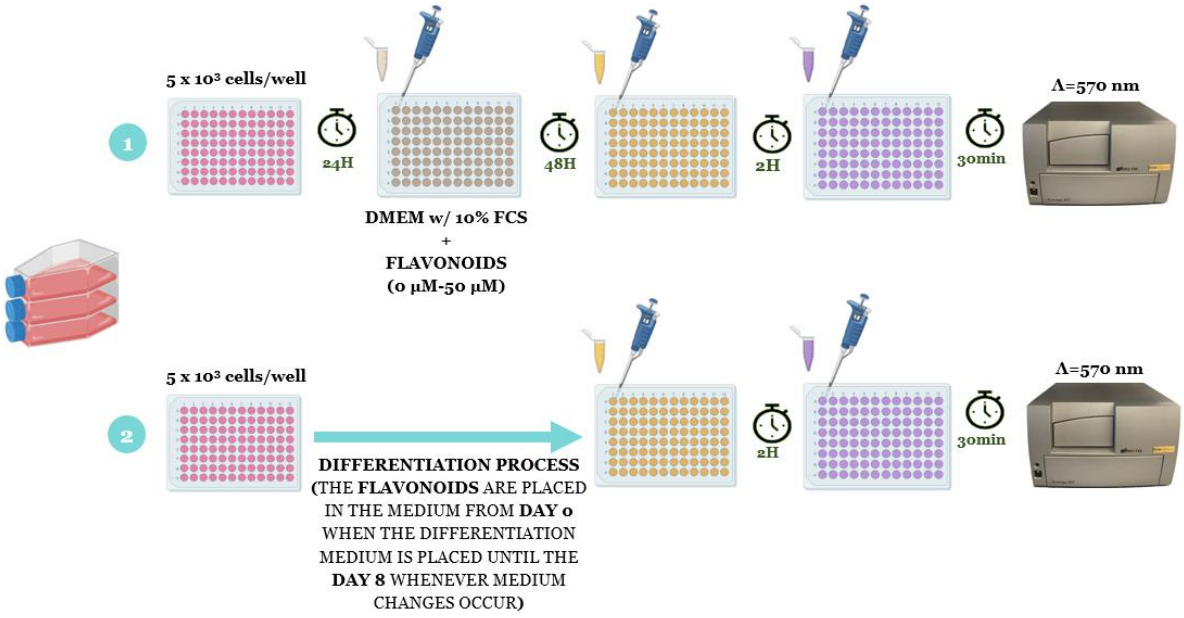


Figure 15– Schematic representation of the MTT assay.1- MTT assay in preadipocytes. 2- MTT assay during differentiation.

2.5. Oil Red O Staining and Quantification

2.5.1. Principle of the method

The lipid accumulation of the 3T3-L1 cell line is accessed by Oil Red O staining assay. Oil Red O is a fat-soluble dye used to stain neutral lipids in red. The hematoxylin stains the nuclei of the cells in purple and was used as a counterstaining dye (Figure 16). The assay allows both a qualitative and quantitative analysis of the lipid accumulation. To obtain a quantity measure of the lipids the cells stained with Oil Red O were washed in isopropanol 100 % to dilute the dye and the absorbance was read at 510 nm.

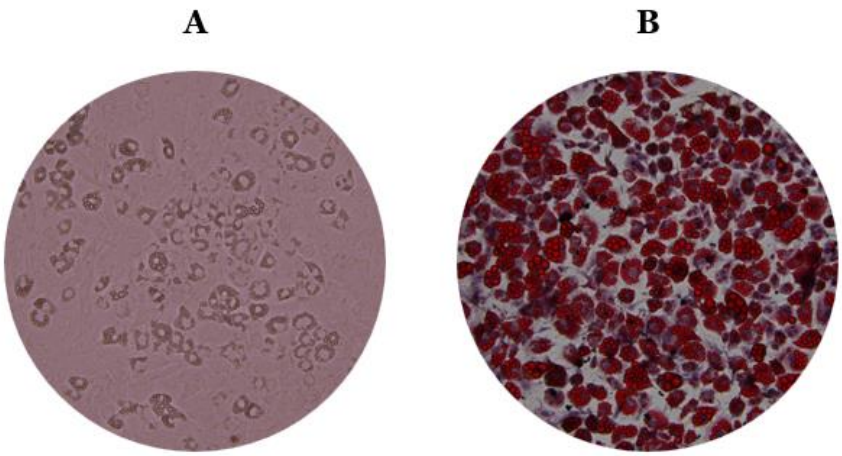


Figure 16- Microscopic image of 3T3-L1 cells after differentiation. **A**- non stained cells; **B**- cells stained with Oil Red O.

2.5.2. Experimental procedure

After day 8, the differentiated 3T3-L1 cells were washed twice with PBS and fixed in 10 % Formalin until use. At the staining time, the formalin was removed, the fixed cells were washed twice with distilled water and incubate thereafter with 60 % isopropanol for 5 minutes at room temperature. Then, the cells were stained with Oil Red O working solution (3 parts of Oil Red O Stock Solution and 2 parts of distilled water) for 20 minutes at room temperature. After the 20 minutes the staining solution was removed, and the cells were washed four times with distilled water. Hematoxylin was added to the cells for 1 minute for counterstaining. After that, the cells were washed four times with distilled water. Microscopic images were taken to visualize stained lipid droplets in differentiated 3T3-L1 cells in Cytation 5, BIO-TEK. To quantify the lipid content of the cells, 100 % isopropanol was used to dilute the Oil Red O Staining and incubated with gently agitation for 10 minutes. Thereafter, 200 µL of the Oil Red O were transferred to a 96-well plate and the absorbance was measured at 510 nm in a microplate reader (Figure 17).

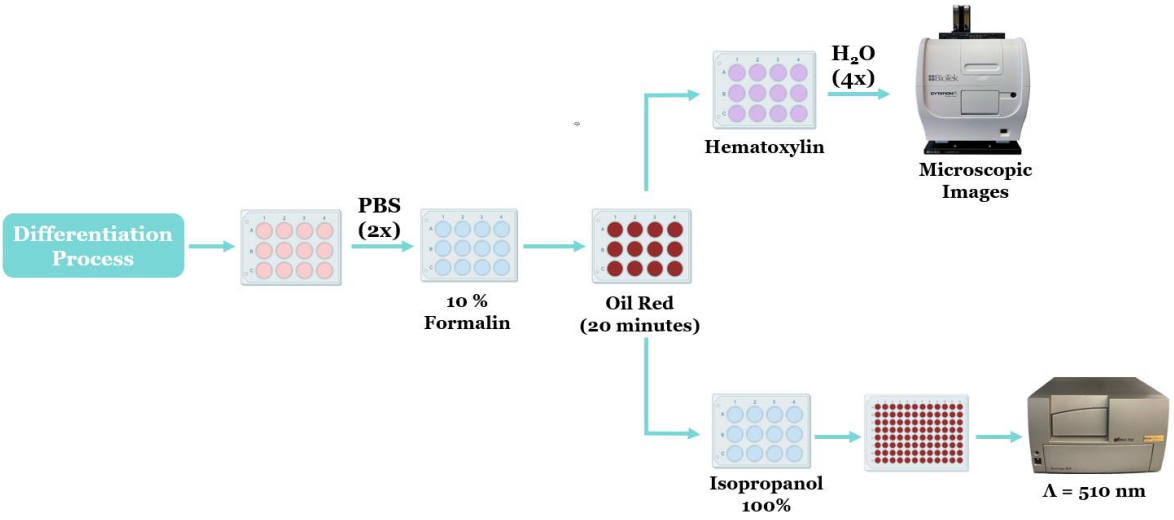


Figure 17- Schematic representation of the Oil Red O Staining assay.

2.6. Quantitative determination of triglycerides

2.6.1. Principle of the method

Triglycerides when incubated with lipoprotein lipase (LPL) release glycerol and free fatty acids. Glycerol is phosphorylated by glycerolphosphate dehydrogenase (GPO) and ATP in the presence of glycerol kinase (GK) to produce glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP). G3P is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2) by GPO. At the end, the H_2O_2 reacts with 4-aminophanazone (4-AF) and *p*-chlorophenol in a reaction catalysed by peroxidase (POD) resulting in a red colour (83) as demonstrated in the equations present in the figure 18. The intensity of the formed colour is proportional to the triglyceride's concentration present in the sample.

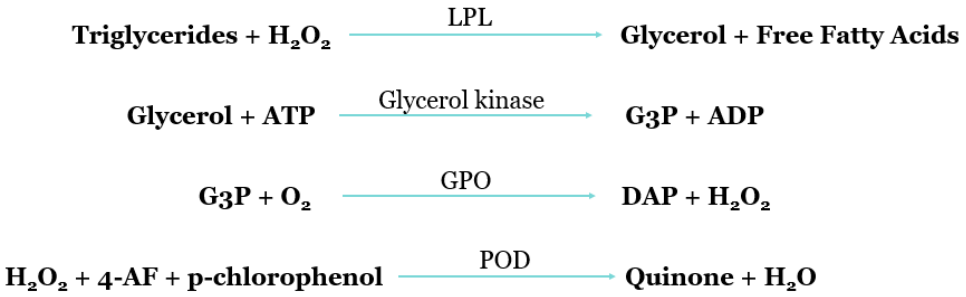


Figure 18- Equations of the triglyceride's quantitative determination kit.

2.6.2. Experimental procedure

After day 8 of the differentiation, the medium was removed, and the cells were washed with PBS twice. The cells were suspended in 200 µL of 10 mM Tris-HCl pH 7.4, 150 mM NaCl and 1 mM EDTA buffer and sonicated on ice with one 10 s burst in a Vibra cell CV17 Sonicator. The triglyceride content was measured according to the instructions of the commercial kit. Protein measurements were performed using the Lowry method. Triglyceride content values were obtained as the ratio (mg triglycerides/mg protein) (Figure 19).

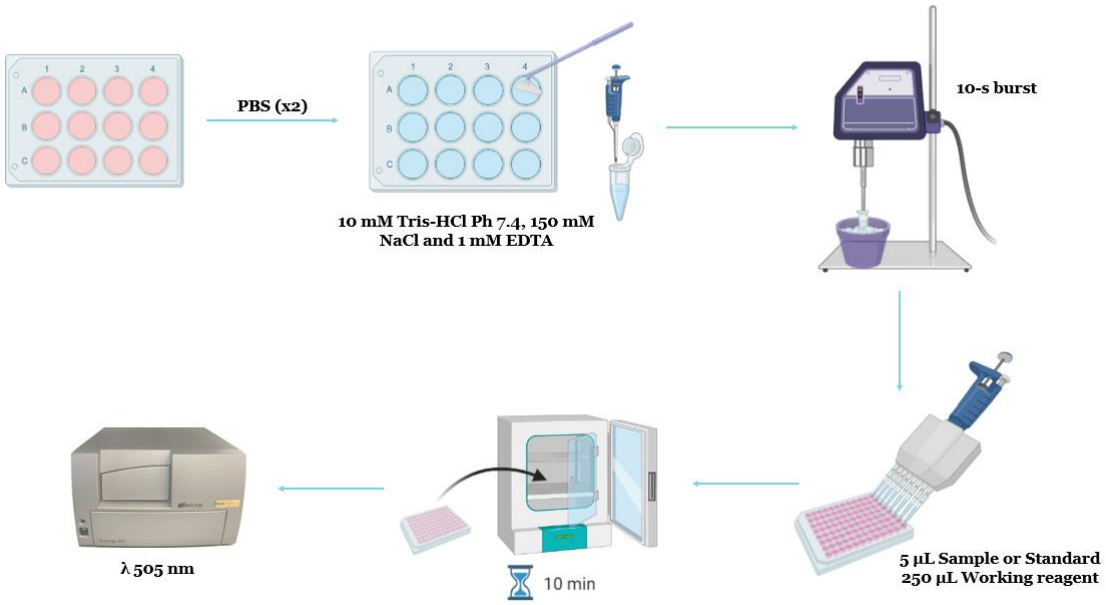


Figure 19- Schematic representation of the quantitative determination of triglycerides.

2.7. Western Blot

2.7.1. Principle of the method

Western blot is used to separate and identify proteins. It allows the separation of proteins based on their molecular weight and type through SDS-PAGE electrophoresis which were then transferred to a PVDF membrane. The membrane allows the immunoblot, where it was probed with specific antibodies to the protein of interest. The protein bands were revelled using chemiluminescence. The thickness of the obtained band corresponds to the amount of protein present in the sample and the difference between the bands are usually proportional to the effect of each condition/treatment on that protein.

2.7.2. Experimental procedure

After 8 days of differentiation the total protein of the 3T3-L1 cells was isolated from differentiated 3T3-L1 adipocytes using 100 μL of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 Mm EDTA, 1 % Triton X-100, 0.5 % DOC, 0.1 % SDS and phosphatase and protease inhibitors) and centrifugated (14.000 rpm, 10 minutes, 4 ºC) to remove membranes and other cellular residues. Protein concentration was determined by Lowry method and the lysates were denatured in sample buffer (Laemmli sample buffer with 2-mercaptoethanol). Total protein was separated (25 μg/well in the case of FAS and ATGL and 30 μg/well in the case of PPAR γ) by SDS-PAGE in a polyacrylamide gel (10 %) and transferred to PVDF membranes. The membranes were blocked in 5 % casein for ATGL and FAS and in 5 % skim milk powder for PPAR γ for 1 hour, at room temperature. Subsequently, the membranes were incubated with the primary antibodies Rabbit anti-FAS (1:1000), rabbit polyclonal anti-ATGL (1:1000) and mouse polyclonal anti-PPAR γ (1:500) overnight at 4 ºC. After, membranes were incubated with anti-mouse and anti-rabbit-HRP conjugated secondary antibodies (1:5000) for 1 hour and 30 minutes, respectively, at room temperature. Mouse anti-β-actin monoclonal antibody was used to detect β-actin as the loading control. The bound antibodies conjugates were detected by Clarity western ECL substrate using a Chemidoc Imaging System and the bands were analysed using QLICS software (version 7.0, Total Lab), schematically represented in figure 20.

The results were normalized by calculating the ratio between the intensities of the bands corresponding to the protein of interest and the actin used as the loading control.

Taking into account the fact that PPAR γ and actin used as a loading control in other proteins have similar molecular weight and do not allow adequate band separation for detecting PPAR γ, were used TGX Stain-Free gels and the results were normalized by total protein method.

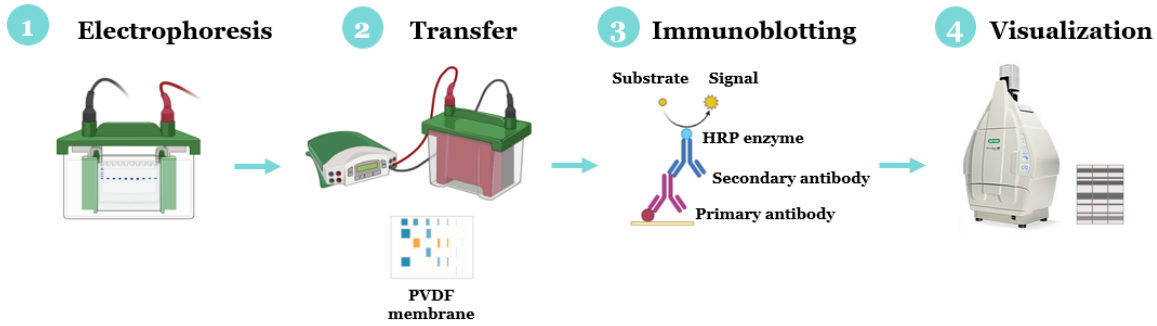


Figure 20- Schematic representation of the western blot procedure.

2.8. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and correspond to at least three independent experiments. The statistical analysis was performed using GraphPad Prism (version 9.0). An unpaired t-test was used for comparison of each condition with the respective control. Results were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Optimization of the chemical differentiation process

The different approaches to the cell differentiation process present in the literature were evaluated based on microscopic images obtained. In figure 21, are present the images of the two approaches.

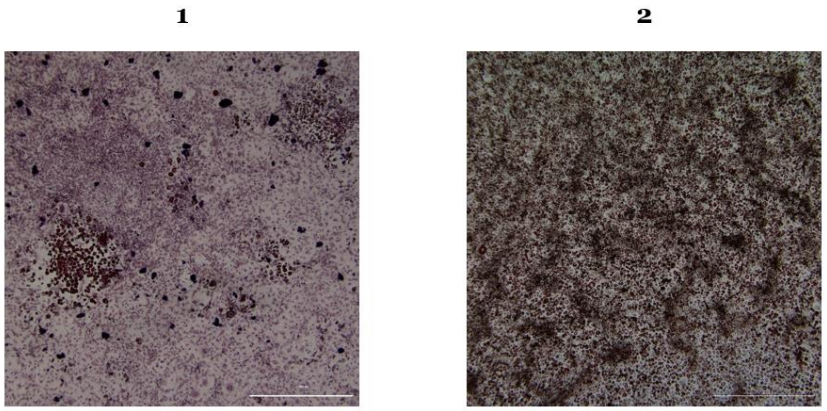


Figure 21- Microscopic image of adipocytes differentiated from 3T3-L1 cells. The images represent a capture at 4 x after 8 days of differentiation. **1**- Result of the first approach for chemical differentiation of the cells, where the differentiation medium contains 1.0 µM of Dexamethasone, 0.5 mM of IBMX and 1.0 µg/mL of Insulin and the adipocyte medium contains 1.0 µg/mL of Insulin; **2**- Result of the second approach for chemical differentiation of the cells, where the differentiation medium contains 1.0 µM of Dexamethasone, 0.5 mM of IBMX and 10.0 µg/mL of Insulin and the first adipocyte medium contains 10.0 µg/mL of Insulin and the second 0.2 µg/mL . In this case both the differentiation and adipocyte mediums contain pantothenic acid and biotin.

Observing these images, is possible to observe a higher accumulation of lipids reason why the second approach was the one chosen. After being established the protocol, the differentiation time was also tested. The 8 days and 10 days differentiation were tested, and the results are present in the figure 22. The use of a 6-well plate and 12-well plate was also tested, but the ratio of seeded cells was the same, however, cells demonstrated a better behaviour when seeded in a 12-well plate.

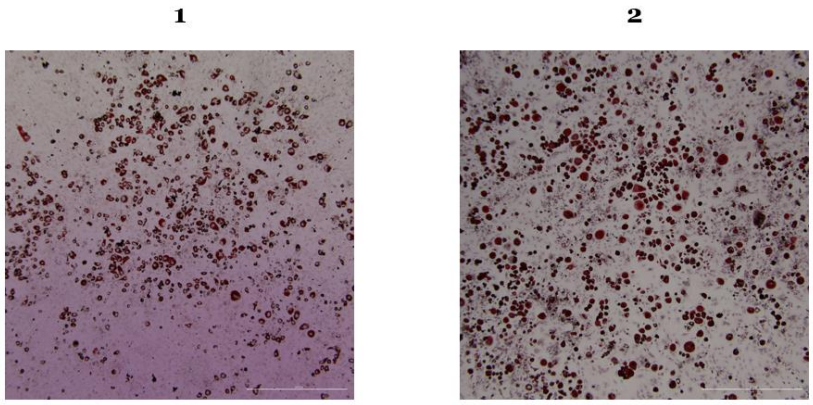


Figure 22- Microscopic image of adipocytes differentiated from 3T3-L1 cells. The images represent a capture at 4 x. **1**- Result of the 8 days differentiation; **2**- Result of the 10 days differentiation.

Observing the images, both the 8 days and the 10 days differentiation do not show much difference between them, so the less time possible was chosen (8 days differentiation), in order to saving resources and obtaining results more quickly.

During the course of this work, it was also possible to see that as the cell passage became higher, the differentiation capacity seemed to decrease, as we can see in figure 23. For this reason, we tried to use lower passages (until passage 8) whenever possible.

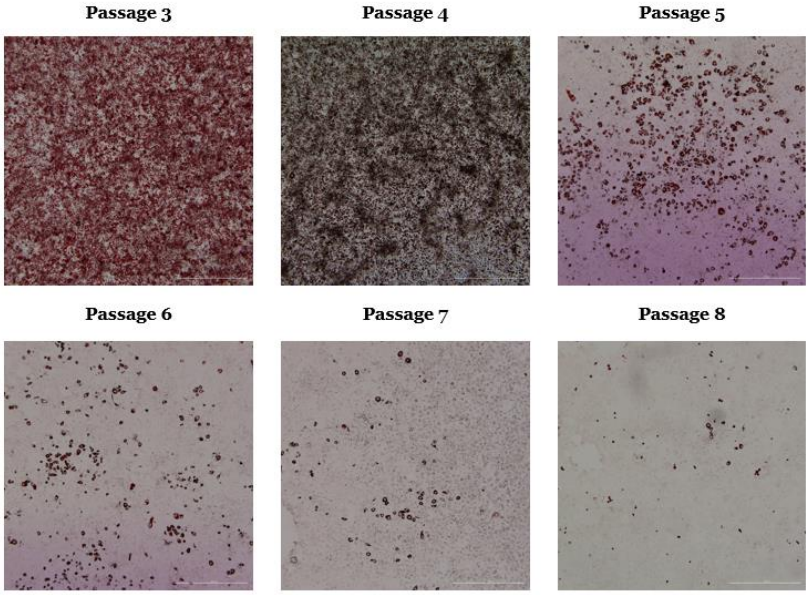


Figure 23- Microscopic image of adipocytes differentiated from 3T3-L1 cells in different cell passages. The images represent a capture at 4 x.

3.2. Cell viability

In order to disclose which are the non-cytotoxic concentrations of the flavonoids to the 3T3-L1 cell line, MTT assays were performed. These assays were performed both in preadipocytes (before inducing the chemical differentiation of the cells) to understand which are the maximum concentration to which cells can be exposed during the chemical differentiation and during the differentiation process to verify if the maximum concentration used continued not to affect the viability of the cells during the long exposure period.

The preadipocytes were exposed to concentrations up to 50 µM. As shown in figure 24, for all the compounds tested (Herbacetin, Kaempferol, Myricetin, Apigenin, Robinetin and Quercetin), except for Luteolin, the maximum concentration that did not significantly affect the viability of the cells were 12.5 µM. For luteolin the maximum non-toxic concentration was 6.25 µM.

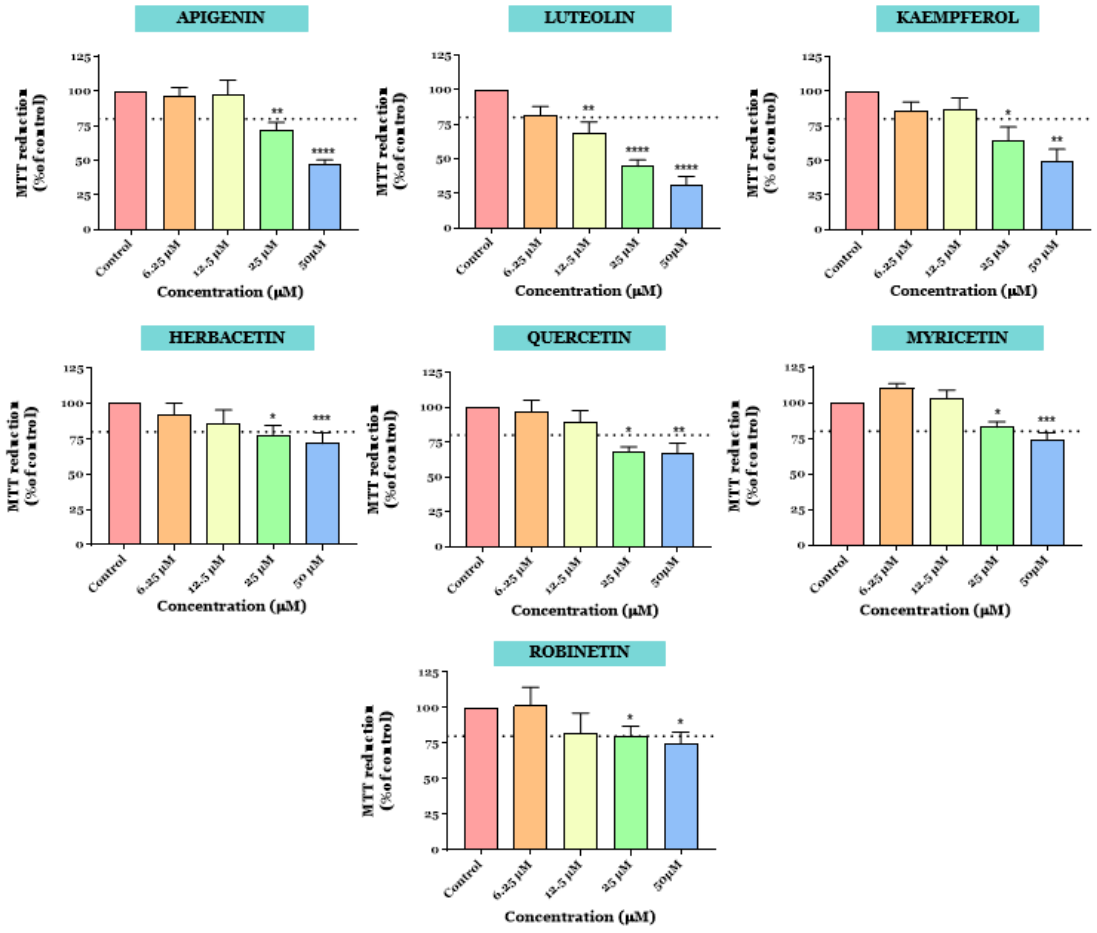


Figure 24- Representative graphs of the MTT reduction in % control of the preadipocytes treated with the flavonoids in concentrations up to 50 µM. Control corresponds to cells treated with the vehicle (DMSO) alone. Values are given as mean ± SEM (n≥3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ relative to the corresponding control.

During the differentiation process the concentrations up to 12.5 µM for Herbacetin, Kaempferol, Myricetin, Apigenin, Robinetin and Quercetin and up to 6.25 µM for Luteolin continued not to affect the viability of the cells as shown in figure 25.

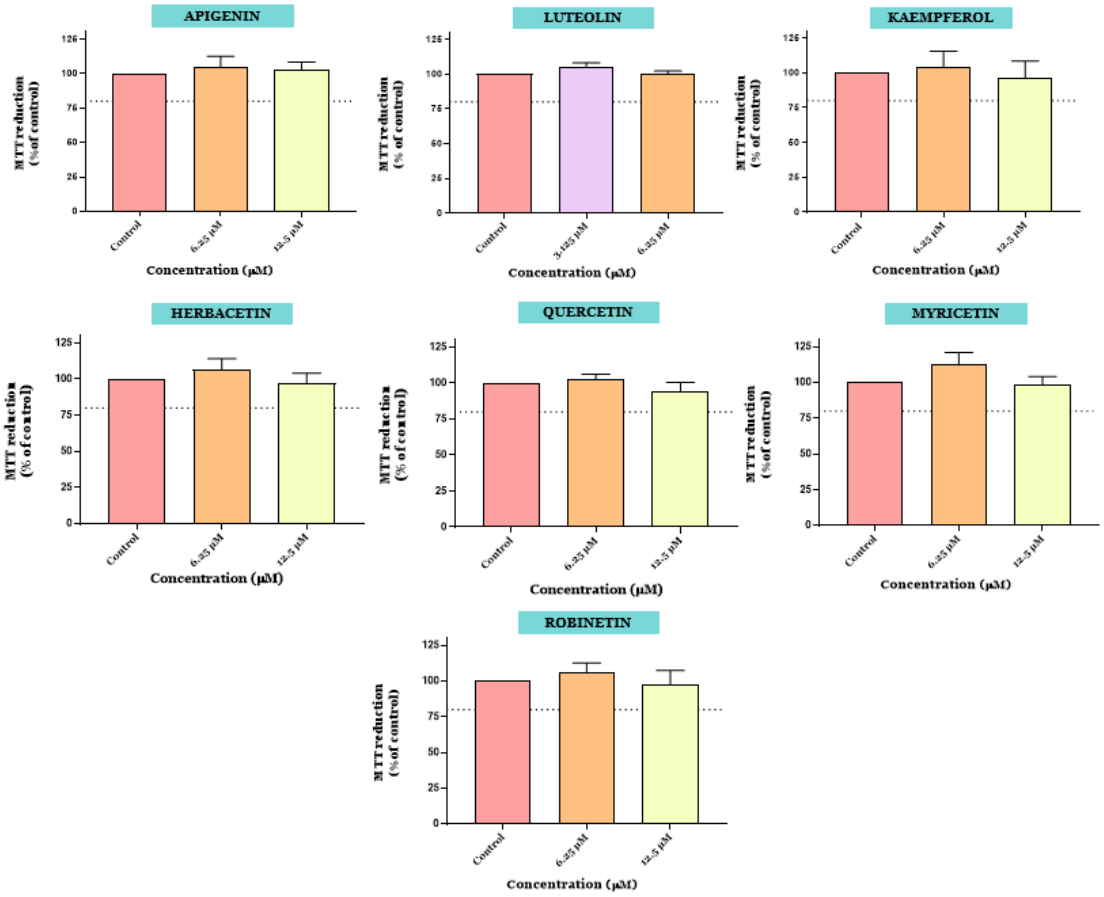
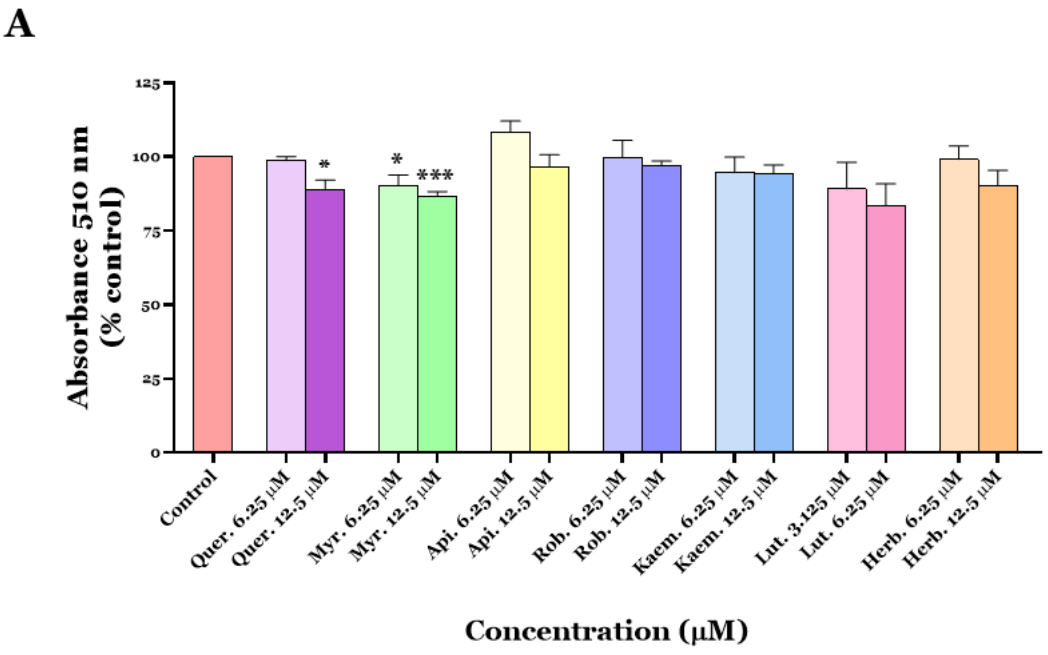


Figure 25- Representative graphs of the MTT reduction in % control of the adipocytes during the differentiation process treated with the flavonoids in concentrations up to 12.5 µM and 6.25 µM for Luteolin. Control corresponds to cells treated with the vehicle (DMSO) alone. Values are given as mean ± SEM (n≥3).

3.3. Effect of flavonoids on lipid accumulation

Lipid accumulation is a crucial indictor of adipogenesis, and thus an indicator of the role of the tested compounds through the process. It was accessed by Oil Red O Staining. During the chemical differentiation of 3T3-L1 cell line the cells were expose to the different non-toxic concentrations of the flavonoids. The absorbance of the Oil Red O Staining was measured at 510 nm and demonstrated that among the panel of seven flavonoids tested two of them, Myricetin and Quercetin were capable to reduce the lipid accumulation. Specifically, Myricetin reduce the lipid accumulation in 13.4 ± 1.6 % at the concentration of 12.5 μM and in 9.7 ± 3.5 % at the concentration of 6.25 μM while Quercetin reduced lipid accumulation by 11.1 ± 3.2 % at the concentration of 12.5 μM. The other tested flavonoids did not significantly affect the lipid accumulation of 3T3-L1 cells as shown in Figure 26A. The images present in the Figure 26B, representing the Oil Red O Staining of lipids corroborate the results shown in the graph.



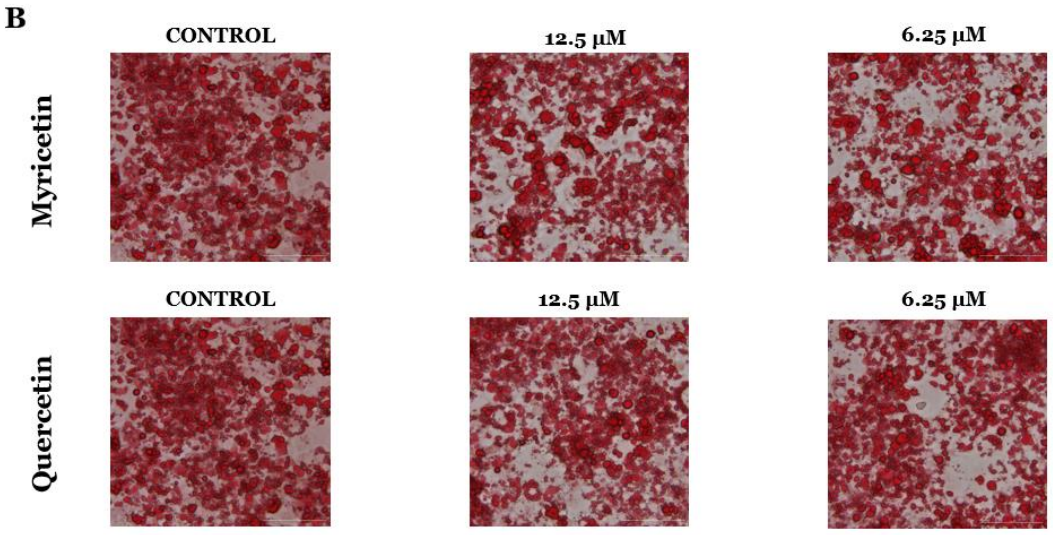


Figure 26- Effect of the tested flavonoids on lipid accumulation in adipocytes differentiated from 3T3-L1 cells. The values represent the absorbance of Oil Red O extracted from the cells with isopropanol 100 % measured at 510 nm (**A**). The data is expressed as percentage of control. Values are given as mean ± SEM (n≥3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared with the corresponding control. The images present in (**B**) are representative cell images after the chemical differentiation process treated with the flavonoids that demonstrated ability to significantly reduce the lipid content of the cells. In the first line the images related to cells treated with Myricetin in concentrations of 12.5 and 6.25 µM and in the second line the cells treated with Quercetin in concentrations of 12.5 and 6.25 µM. Control corresponds to cells treated with the vehicle (DMSO) alone. The images represent a capture at 20 x.

To confirm the obtained results with the lipid staining, the triglycerides accumulation was also accessed. The two highest non-toxic concentrations were tested for each compound, (12.5 µM and 6.25 µM, and 6.25 µM and 3.125 µM for Luteolin).

Of the tested flavonoids, only Myricetin and Quercetin demonstrated to significantly reduce the triglycerides accumulation at the concentration of 12.5 µM. When compared with the control, Quercetin reduced the triglyceride content in 15.6 ± 0.9 % at the concentration of 12.5 µM and Myricetin reduced it in 34.8 ± 2.7 %. At the concentration of 6.25 µM, none of the flavonoids tested demonstrated ability to reduce the triglyceride content. When compared the result obtained for Myricetin 12.5 µM and for Quercetin 12.5 µM, they have demonstrated to be statistically different. The results are graphically present in the Figure 27.

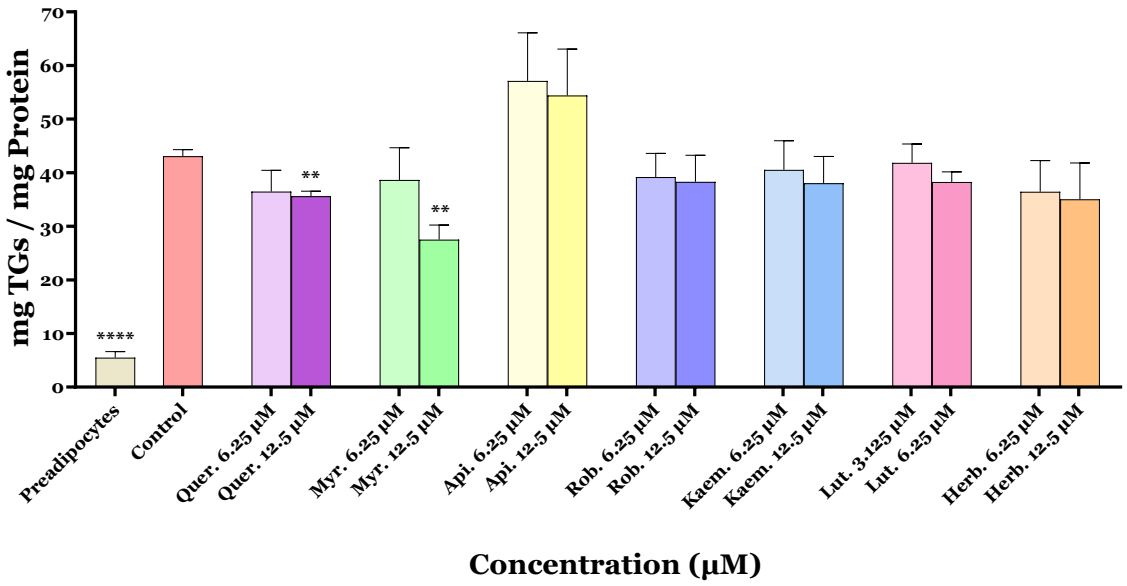
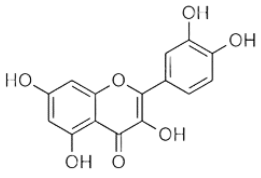
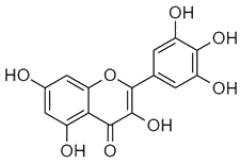


Figure 27- Effect of the tested flavonoids on triglyceride content in adipocytes differentiated from 3T3-L1 cells. Values are given as mean ± SEM (n≥3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the corresponding control and the values with # are significantly different from Quercetin 12.5 µM (# $p < 0.05$, ## $p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$).

Table 4- Resume of the results of the lipid and triglycerides accumulation.

Compound	Concentration	% Reduction of lipid accumulation in the Oil Red O assay	% Reduction of the triglyceride accumulation in the Triglycerides assay
 **Quercetin**	**6.25 µM**	1.3 ± 1.4	8.9 ± 9.6
	12.5 µM	11.1 ± 3.2 *	15.6 ± 0.9 *
 **Myricetin**	**6.25 µM**	9.7 ± 3.5 *	10.6 ± 13.3
	12.5 µM	13.4 ± 1.6 *	34.8 ± 2.7 * #

The values with * are significantly different from the corresponding control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) and the values with # are significantly different from the Quercetin 12.5 µM (#$p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$).

3.4. Effect of the flavonoids on adipogenic and lipogenic protein expression

The process of lipid accumulation greatly depends on the adipocyte differentiation and synthesis of new lipids. Accordingly, to better understand the mechanism by which Myricetin and Quercetin were able to reduce the lipid content and TG accumulation, the levels of different proteins were evaluated. FAS is a lipolytic enzyme involved in the process and is responsible for the synthesis of new fatty acids. Our results, graphically expressed in figure 28, showed that Myricetin in a concentration of 12.5 µM was able to significantly reduce the FAS expression when compared to the control (mature adipocytes untreated). The reduction was of 38.7 ± 22.8 %. Moreover, this reduction has driven the FAS expression levels to a no significantly different value from that of preadipocytes (cells in which chemical differentiation was not induced). Surprisingly, a similar analysis for Quercetin showed that for the concentration of 6.25 µM the expression of FAS increased when compared whit the control, while the concentration of 12.5 µM did not significantly affect the FAS expression.

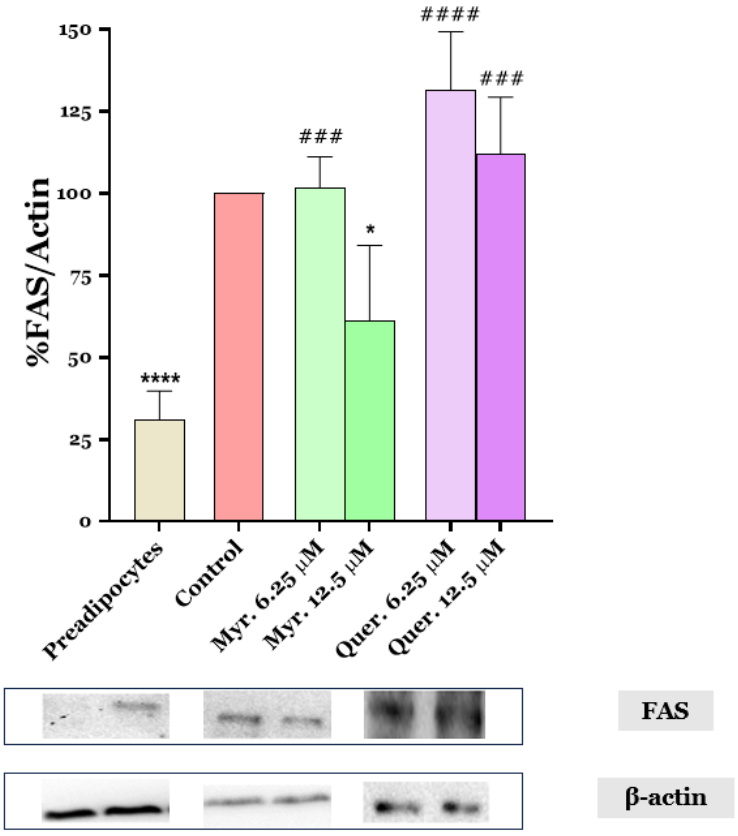


Figure 28- Effect of Quercetin and Myricetin on FAS protein expression in adipocytes differentiated from 3T3-L1 cells. Values are expressed as mean ± SEM (n≥3). * $p < 0.05$, ** $p <$

*0.01, *** p < 0.001, **** p < 0.0001* when compared to the corresponding control and the values with # are significantly different from the preadipocytes (*# p < 0.05, ## p < 0.01, ###p < 0.001, ####p < 0.0001*).

Furthermore, ATGL, is an enzyme responsible for the degradation of the TGs into diglycerides and has its expression increased in mature adipocytes, being, because of that an interesting target to understand the mechanism of action of the flavonoids in the adipogenic and lipogenic process. The obtained results showed that Myricetin at the concentration of 6.25 µM demonstrated ability to reduce the ATGL expression into 15.4 ± 6.2 % when compared with the control (Figure 29). Conversely, Quercetin at both concentrations tested (6.25 and 12.5 µM) demonstrated to increase the expression of ATGL.

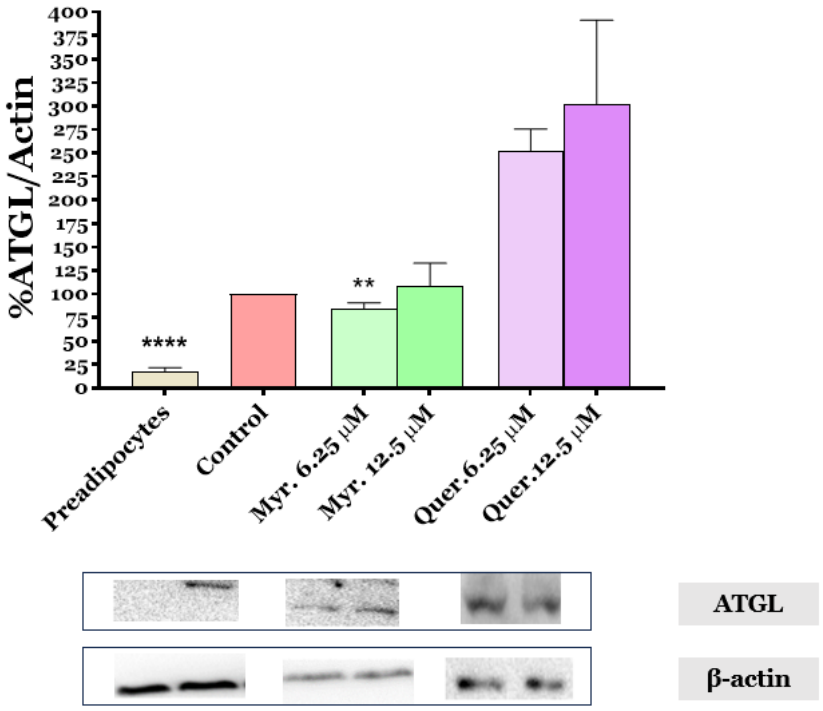


Figure 29- Effect of Quercetin and Myricetin on ATGL protein expression in adipocytes differentiated from 3T3-L1 cells. Values are expressed as mean ± SEM (n≥3). * *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001* when compared to the corresponding control.

PPAR γ is a transcription factor that play a regulatory role in lipid metabolism, and is essential for adipose tissue differentiation, both *in vivo* and *in vitro*. Quercetin, similarly, to FAS and ATGL, shown a neglectable effect through PPAR γ expression at the tested

concentrations. On the other side, Myricetin at the concentration of 12.5 µM demonstrated ability to reduce the PPAR γ expression in 36.0 ± 6.6 % when compared with the control and to a value not statistically different from that of preadipocytes (Figure 30).

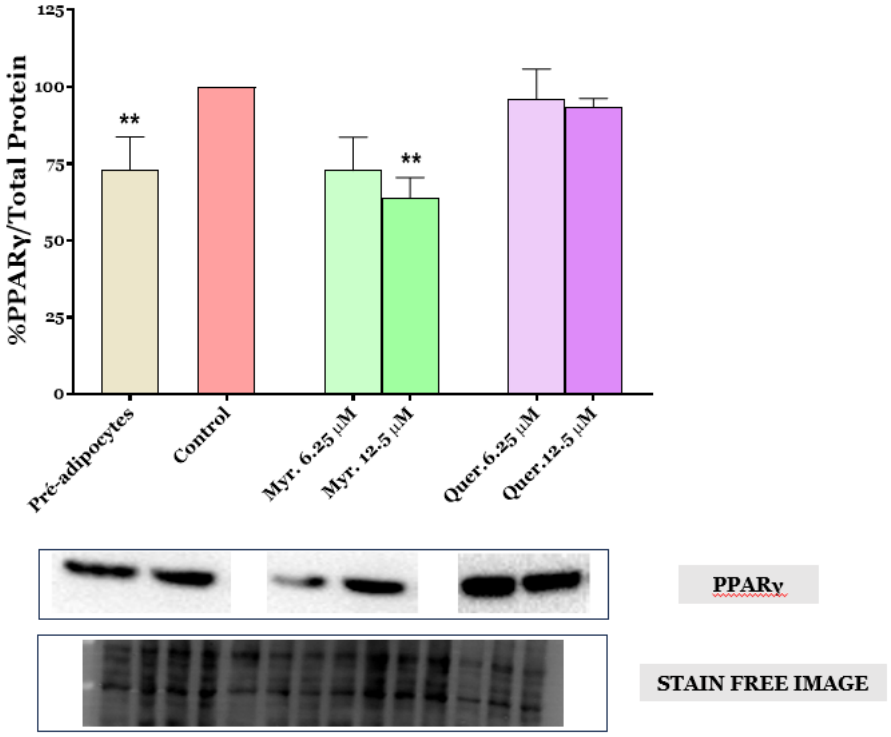


Figure 30- Effect of Quercetin and Myricetin on PPAR γ protein expression in adipocytes differentiated from 3T3-L1 cells. Values are expressed as mean ± SEM (n≥3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the corresponding control.

4.DISCUSSION

The concern about the prevalence of obesity worldwide and the current insufficient strategies that allow body weight lowering, including the pharmacotherapy, lead to the search for new and safer molecules for the treatment of this condition. Obesity is generally associated with fat accumulation and expansion of the adipose tissue (26). There are diverse processes and enzymes that can be used as targets for obesity control, including, adipogenesis, lipogenesis, respectively the process during which fibroblast-like preadipocytes develop into mature adipocytes and the process that correspond to the process of generation of new lipid molecules (26) and also pancreatic lipase, α-amylase, and α-glucosidase (84, 85) enzymes from the nutrients digestion.

Flavonoids are natural occurring phenolic compounds, that has aroused the interest of the scientific community due to its low toxicity and anti-obesogenic potential (16).

Some studies have demonstrated the flavonoids anti-obesogenic activity via modulation of adipogenesis and lipogenesis processes (80, 86-88). Despite the studies already existent, the comparison of the results found in the literature is complicated and consequently, the establishment of a structure-activity relationship, due to the different *in vitro* and *in vivo* models used (e.g., different cell lines or different animal models), different origin of the flavonoids (isolated from natural sources, chemical synthesized, or commercially acquired), the flavonoid concentrations tested, and the different methods used.

Considering the wide and disperse information about the model's establishment this work begins with the establishment and optimization of a differentiation model that could be used to evaluate the capacity of a panel of seven structurally-related hydroxylated flavonoids to modulate the lipidic metabolism, through Oil Red O Staining assays and measure of the TGs content of adipocytes obtained from the pre-adipocytic 3T3-L1 cell line. Afterwards, it was also evaluated the modulatory effect of the most active flavonoids against adipogenic and lipogenic proteins. A structure-activity relationship was performed whenever possible.

To achieve the main goals of this work, it has been divided into some interrelated sections. As said, the first corresponds to the establishment of the *in vitro* model of adipogenesis. For that an optimization of the differentiation conditions of the pre-adipocytic cell line 3T3-L1 was performed, considering different approaches present in the literature (80-82). Different concentrations of insulin, the presence of biotin and pantothenic acid, and different differentiation times were tested.

The differentiation medium containing insulin (10.0 μg/mL), pantothenic acid as well biotin, dexamethasone and IBMX and the adipocyte medium with decreasing insulin concentrations (at day 2 a medium containing 10.0 μg/mL was replaced on day 4 by a decreased concentration of insulin - 0.2 μg/mL), pantothenic acid and biotin revealed to promote a better differentiation profile of the cells. Pantothenic acid and biotin have already been described as capable to increase lipid metabolism and cell proliferation (89). Moreover, biotin was also described as a coenzyme for carboxylases regulating lipid and amino acid metabolism in adipose tissue (89, 90). Dexamethasone, a synthetic glucocorticoid, and IBMX, a phosphodiesterase inhibitor, on their turn, were shown to increase the expression of C/EBP β and lead to the subsequent PPAR γ expression and adipocyte differentiation (43, 91). Insulin, besides its role on the induction of several transcription factors that govern differentiation of preadipocytes into mature adipocytes also stimulates cells to take up glucose and storage in the form of TGs (91, 92). This information helps to understand the relevance of each component in the medium and the better differentiation rate obtained when all these components are present in the medium. Despite, it is well described the possibility of differentiation of 3T3-L1 cells with medium supplemented with calf serum only, the presented substances hasten differentiation without being necessary for maintaining the fully differentiated state. Their use also offers valuable insights into the biochemical pathways potentially involved in adipocyte differentiation (43) and allows to understand the huge amount of possible differentiation protocols found in literature. Even though their importance in the culture medium to potentiate the differentiation state of adipocytes, we cannot exclude the possibility of their presence to interfere and/or influence the final response of the compounds/flavonoids tested, which make the interpretation of the results more delicate. The optimal differentiation time was established at 8 days, as the results of 10 days and 8 days differentiation comparison did not reveal significant difference observed between the two timepoints. So, a less time differentiation was chosen to promote a lower expenditure of resources and quicker results. Another interesting information observed was that as the passage of cells increased, their ability to differentiate seemed to decrease, which can be explained by the loss of multipotency and/or senescence of the cells with the increased passage numbers (93).

After being established the optimal conditions for 3T3-L1 preadipocytes differentiation, it is needed to understand which are the maximum concentrations of the flavonoids, intended to be studied in this work, that did not significantly affect the viability of the cells. For that, a cytotoxic assay was performed. This assay was performed in preadipocytes (cells

before the induction of chemical differentiation) to understand which are the maximum concentration that can be placed into the cells during the differentiation. The results showed that for **Herbacetin**, **Kaempferol**, **Myricetin**, **Apigenin**, **Robinetin** and **Quercetin** the 12.5 μM was the maximum concentration that showed not to significantly affect the viability of the cells. For **Luteolin** this concentration was of 6.25 μM. A similar assay was performed in during the differentiation process which confirmed that the chosen concentrations continued not to affect the viability of the cells during the long period of time and confirming that the concentrations up to 6.25 μM for luteolin and up to 12.5 μM for the other compounds were safe to proceed with the subsequent assays.

The work proceeds with the evaluation of the effect of the flavonoids in the lipid accumulation of the 3T3-L1 preadipocytes. For that, were performed two different assays, the Oil Red O Staining assay, and a measure of the TGs content of the cells using a commercial kit. Matsuda *et al* (94) have previously performed a study to evaluate the effect of diverse flavonoids in the development of adipogenesis. This study revealed that the hydroxylated flavonoids, as those included in the panel studied, did not promote adipogenesis, which helps validating the compounds choice and the results of the present work. In the Oil Red O Stanning assay, our results showed that from the panel of flavonoids tested, **Quercetin** have demonstrated capacity to reduce the lipid accumulation at the concentration of 12.5 μM and **Myricetin** have demonstrated capacity to reduce the lipid accumulation at the concentrations of 12.5 μM and 6.25 μM. These results were corroborated by the measurement of the TGs content of the cells, in which **Quercetin** and **Myricetin** reduced the TGs content at the concentration of 12.5 μM. Also Mosqueda-Solís *et al* (80) have already demonstrated the capacity of Quercetin to reduce the TGs accumulation on 3T3-L1 cells at the concentration of 10 μM. On the other hand, *Wang et al* (95) verified the capacity of Myricetin to suppress the differentiation of 3T3-L1 preadipocytes by Oil Red O Staining assays. The fact that the Myricetin at a concentration of 6.25 μM have shown ability to reduce lipid accumulation in the Oil Red O Staining assay but not in the measure of TGs content, may be due to the fact that this reduction in the case of Oil Red, being relatively low, becoming indetectable in the TGs measurement.

The observation of the chemical structures of the chosen flavonoids and their proven activity it is possible to notice that both the active compounds (Quercetin and Myricetin) present two hydroxyl (-OH) groups at the A-ring and two or three -OH groups at the B-ring, respectively. When we look to the chemical structure of other studied compounds, such as Kaempferol, is observed a chemical structure similar to Quercetin and Myricetin, but with

only one -OH group at the B-ring. Robinetin chemical structure is also similar to that of Myricetin, but with only one -OH group at the A-ring. In the case of Luteolin, we see a structure similar to that of Quercetin but without a -OH group at the C-ring, making it a flavone. Considering that in the presented work, these three flavonoids, Kaempferol, Robinetin and Luteolin, have shown not to affect the lipid accumulation it suggests that the presence of two -OH groups on the A-ring, conjugated with the presence of a catechol (two -OH groups) and pyrogallol (three -OH groups) groups at the B-ring, promotes activity to the compounds. Both the active compounds belong to the class of flavonols, which are a class of flavonoids with a -OH group at the position 3 of the C-ring, considering that from the group of flavonoids chosen were the only ones showing activity also suggests that this specific organization and pattern of substitution might be important for the observed activity. When comparing Quercetin with Myricetin it is possible to notice that the greater number of -OH groups in the B-ring, the greater the ability to reduce lipid accumulation, since quercetin shows lower reduction values than those observed for myricetin, and in the case of the TGs measure kit, this value is more than a half lower. Our results are in line with other, already reported effects of flavonols in lipid metabolism. Their potentialities have been shown, for example into the cardiovascular system, where they showed the capacity to regulate the lipid metabolism promoting a diminish of the atherosclerosis and hyperlipidemia (96). Quercetin also showed effects on steatosis, which is a way to manifest an imbalance in fatty acid metabolism, which lead to increases FFAs availability and synthesis, showing alterations in fatty acid degradation, in the PPAR signalling pathway and in the biosynthesis of unsaturated fatty acids. Quercetin is also related to lipolysis of TGs in the adipose tissue (97).

Following this, became appealing to understand possible mechanisms or processes that being modulated by Quercetin and Myricetin, might contribute to the observed lipid-accumulation lowering effect. In line with the already described effects only Quercetin (6.25 μM and 12.5 μM) and Myricetin (6.25 μM and 12.5 μM) were tested.

A close look on FAS, which is a lipolytic enzyme responsible for the synthesis of new fatty acid molecules shows that only **Myricetin** at the higher concentration tested (12.5 μM) reduced the expression of this protein. When the expression of FAS in the condition of Myricetin 12.5 μM is compared with the preadipocytes (cells in which chemical differentiation was not induced) the values are not statistically different, which suggest that the levels of FAS expression in cells treated with Myricetin 12.5 μM became similar to the ones of the non-differentiated preadipocytes, being this fact of great interest. This result

seems to indicate that the results obtained in the previous assays, might partially occur via inhibition of FAS by Myricetin. Despite the result obtained, the literature is inconsistent with this observation with Akindehin *et al* (98), using adipocytes differentiated from C3H10T1/2 cells at a concentration of 10 μM reporting that Myricetin did not affect the expression levels of FAS. The observed differences can be result of the different model and concentrations used and is, again, indicative of the need to carry out more consistent and uniform studies that would otherwise be difficult to compare to provide meaningful results, for the scientific community.

In the case of ATGL, which is responsible for the degradation of TGs into diglycerides, surprisingly only **Myricetin** at the lowest concentration (6.25 μM) demonstrated ability to reduce its expression. Even though this is not a frequent situation, sometimes lower concentrations can produce more significant effects than higher concentrations and it can be explained by some factors. The hormesis effect, is the term used to described the beneficial effect of lower doses of one substance that would not be beneficial at higher doses, and can suggest an explanation for what was observed for the regulation of ATGL by Myricetin, in which the lower concentration (6.25 μM) demonstrated the effect, while the higher concentration (12.5 μM) did not (99). Sometimes, flavonoids can also have different relation between the dose and the non-linear effect, following, instead a U-shaped curve, which means that at lower concentrations no effect or a minimal effect is observed, at moderate concentrations a significant effect is observed but at higher concentrations the effect can reach the *plateau* or decline, which can suggest another explanation for the observed effect of Myricetin in ATGL expression (100, 101). Finally, sometimes lower concentrations of flavonoids can be more selective in targeting specific cellular pathways, leading to more pronounced effects than higher concentrations, that can sometimes affect multiple pathways attenuating the impact, not to mention the already described possibility of flavonoids interfering with the compounds used to stimulate the differentiation process.

Besides the lipid metabolism also PPAR γ, as a representative essential transcription factor for the adipocyte differentiation was evaluated. **Myricetin** at the concentration of 12.5 μM demonstrated ability to significantly reduce the expression of PPAR γ. The values of reduction of PPAR γ expression obtained for Myricetin demonstrated not to be significantly different from the ones obtained for preadipocytes, which suggest that Myricetin (12.5 μM) completely reversed the expression levels of PPAR γ. *Wang et al* (95) have already demonstrated a reduction of PPAR γ protein levels with Myricetin treatment in 3T3-L1 cells. In a study carried out by Aranaz *et al* (102) Myricetin is shown to interact

with PPAR γ during the early and latest stages of adipocytes differentiation. Other study suggested that Myricetin downregulate the PPAR γ expression in high fat diet-fed mice (103). These studies help to corroborate the results obtained in this work.

Another interesting point of view for the analysis of our work is the interrelation between the studied proteins that helps to understand the potential mechanism of action of myricetin. In one hand in the literature is described that ADD1 contribute for the expression of PPAR γ and for the production of endogenous ligands, and subsequently this transcription factor can activate the expression of a variety of adipocyte-specific genes associated with adipogenesis and lipogenesis, like FAS (81). On the other hand, ATGL has been identified to be potentially transactivated by PPAR γ (104). The study of Lodhi *et al* (105) demonstrated a relationship between FAS and PPAR γ expression, since is suggested that inhibiting a lipogenic pathway initiated by FAS lead to a reduction of the activation of PPAR γ. FAS in the liver is part of a lipogenic pathway which is suggested to be involved in the generation of ligands for PPAR γ. The results observed in the present work demonstrating a reduction in PPAR γ and FAS expression as shown in the study mentioned of Lodhi *et al* (105) makes it more likely that the effect observed for Myricetin occurs due to modulation of this pathway. Moreover, Kershaw *et al* (106) verified that PPAR γ upregulated the ATGL mRNA and protein expression in mature adipocytes *in vitro* and in adipocytes *in vivo*, however the direct mechanism of this process is still unclear. On other study it was verified that PPAR γ activation increases the lipolysis in WAT, and that the PPAR γ agonism (rosiglitazone) increase mRNA levels of ATGL (107). The information provided by these articles helps to understand the results of this work, by highlighting the relationship between PPAR γ and FAS or ATGL, increases the possibilities that the reduction of PPAR γ observed in this work and promoted by Myricetin lead also to a decrease in FAS and ATGL. It is also suggested that reducing the activity of PPAR γ can be an effective strategy for the treatment of obesity.

Even though, **Myricetin** demonstrated ability to reduce the lipid content of the cells and to down-regulate the FAS, ATGL and PPAR γ expression, there are diverse mechanisms that regulate adipogenesis, so the reduction of lipid accumulation by Myricetin and also Quercetin can be due to other non-explored factors. One example is the inhibitory effect of Pref-1 in adipocyte differentiation, as described in the literature, the expression of Pref-1 in 3T3-L1 cells prevents the lipid accumulation and expression of adipocyte transcription factors such as PPAR γ and FAS (108). Adenosine Monophosphate-Activated Protein Kinase (AMPK) also as an effect on adipogenesis, playing a role in the inhibition of adipogenesis in

3T3-L1 cells, by inhibiting the expression of adipogenic markers, such as FAS and transcription factors, like PPAR γ (109). These premises remind us that the action mechanism may be complex and will require further exploration to understand exactly how Myricetin influences the production and accumulation of lipids in these cells and specially to understand on how **Quercetin** even though not being able to reduce PPAR γ, ATGL or FAS significantly affected lipid accumulation. In fact, on the contrary, Quercetin increased FAS and ATGL expression. This phenomenon can occur and is not expectable or described in the literature frequently (110) and should be further analysed in order to understand how Quercetin affected the expression of these enzymes.

Finally, another analysis of structure activity relationship gives a completely different overview from that described for the ability of the two molecules to reduce lipid accumulation and on the importance of the specific substituents in the mechanisms of action of the molecules. Even though the similar structures presented similitudes in the lipid lowering effects, this doesn't seem to have any impact on the mechanism of action by which this happens. In this respect, the third hydroxyl group in Myricetin seems to be the most important in terms of its ability to modulate PPAR γ and consequently the expression of FAS and ATGL while the catechol group in the B-ring not. Finally, meaning that even though both compounds might present important anti-obesogenic properties the exact mechanism of action is yet to be disclosed and further studies would be needed to clarify the question.

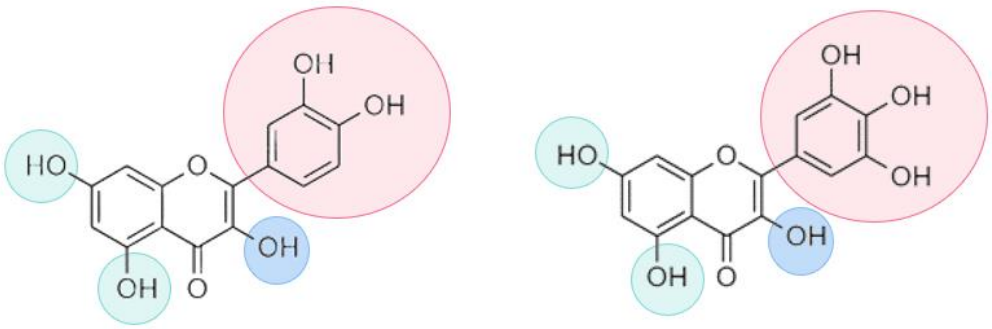
5. CONCLUSIONS

The results obtained in the present work allowed us to draw some conclusions about the influence of a panel of hydroxylated flavonoids in the modulation of lipidic metabolism of an *in vitro* model of preadipocytes. Firstly, we conclude that concentrations up to 12.5 μM for all the compounds except for Luteolin did not affect the viability of the cells used in this study (the pre-adipocytic cell line 3T3-L1), this means that the active compounds can be considered jointly potent for the mechanisms of action they modulate. For Luteolin this result was observed for concentrations up to 6.25 μM and we cannot exclude the possibility that it is possible that the no observed effect is caused on the high toxicity to cells and not because of its inactivity on the expected mechanisms of action. It was also observed that of the tested flavonoids, Quercetin and Myricetin were able to reduce the lipid content of the cells. In addition, Myricetin was also capable of downregulate the expression of adipogenic and lipogenic markers, such as FAS, ATGL and PPAR γ.

Observing these results, it was suggested that on flavonols, the presence of a catechol and pyrogallol group conjugated with two -OH groups on the A-ring were important to confer modulatory activity of the lipid metabolism to the compounds. Comparing the results obtained for Quercetin and Myricetin, we can conclude that Myricetin is the compound with more interest, since it has been shown not only to reduce the lipid content of our cells, but also to modulate the expression of important adipogenic and lipogenic markers. The complete mechanism of action is yet to be disclose for quercetin.

This study provided important considerations about the flavonoids scaffold and their anti-obesogenic effect. These results may also contribute to the design of novel molecules that can be used in the management of obesity.

The vast literature relative to the *in vitro* models for assessing the activity of diverse compounds in adipocytes reveal that different conditions must be taken into account, including compounds that potentiate differentiation (such as insulin, dexamethasone, IBMX, pantothenic acid and biotin) and the exposure time. We cannot exclude the possibility that the presence of these compounds in the culture medium may interfere with the influence of the final response of the compounds/flavonoids tested, which make the interpretation of the results more delicate. Even so, the work allowed an optimization of the *in vitro* model being the presence of lipids significant after the exposure to the compounds.



Quercetin Myricetin

Figure 31- Potential promising substitutions of the flavonoids which contributes to the modulation of lipidic metabolism of adipocytes differentiated from 3T3-L1 cells.

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