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ROLE OF PHENOLIC COMPOUNDS FROM BRAZILIAN NATIVE FRUITS ON OBESITY-INDUCED INSULIN RESISTANCE IN SKELETAL MUSCLE

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SÃO PAULO
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ÉRIKA VICÊNCIA MONTEIRO PESSOA

Role of phenolic compounds from Brazilian native fruits on obesity-induced insulin resistance in skeletal muscle

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RESUMO

PESSOA, Érika Vicência Monteiro. **Papel dos compostos fenólicos de frutos nativos brasileiros sobre a resistência à insulina do músculo esquelético induzida por obesidade.** 54p. Faculdade de Ciências Farmacêuticas – Universidade de São Paulo, São Paulo, 2021.

O músculo esquelético é um tecido metabólico importante na captação de glicose e, portanto, na homeostasia glicêmica. Evidências sugerem que compostos fenólicos podem exercer efeitos benéficos à saúde contra distúrbios metabólicos associados à obesidade incluindo o seu quadro de resistência à insulina. O objetivo deste trabalho foi investigar o papel dos compostos fenólicos presentes em dois frutos nativos brasileiros, cambuci (*Campomanesia phaea* Berg.) e jaboticaba (*Plinia jaboticaba* (Vell.) Berg), na resistência à insulina no músculo esquelético de camundongos obesos alimentados com dieta rica em gorduras e sacarose (HFS, high-fat high-sucrose diet). Para tal, foram utilizados dois protocolos experimentais independentes para cada fruto, onde foram usados camundongos machos C57BL/6J alimentados com dieta HFS para indução à obesidade. Uma vez instaurado o quadro de obesidade, os animais passaram a receber a administração diária, por gavagem, de extratos enriquecidos em compostos fenólicos obtidos a partir dos frutos, em doses atingíveis através da dieta. Ao final do período experimental os animais foram eutanasiados e seus tecidos e órgãos coletados. Os animais tratados com os extratos de jaboticaba e cambuci, independente da dose, apresentaram menor ganho de massa corporal em relação ao grupo HFS. Os resultados para glicemia de jejum semanal e a tolerância à glicose dos animais que receberam os extratos fenólicos de ambos os frutos demonstraram melhora na homeostase glicêmica, mesmo alimentados com a dieta deletéria HFS. No músculo gastrocnemius dos animais foi demonstrado que os extratos de cambuci e jaboticaba aumentaram significativamente o conteúdo da proteína transportadora de glicose 4 (GLUT-4) e da proteína quinase ativada por AMP (AMPK-Thr172), que possui um papel amplo na regulação metabólica. No que tange à inflamação, a administração dos extratos de ambos os frutos favoreceu a diminuição da fosforilação e ativação do fator nuclear-κB (NF-κB) e a expressão de alguns genes como IL-6, TNF-α, IL-1β, e JNK cujo aumento tem sido associado com a resistência à insulina. Deste modo, este estudo sugere que os fenólicos presentes em ambos os frutos nativos podem ser agentes terapêuticos importantes na atenuação da resistência à insulina muscular e da inflamação associada à obesidade.

PALAVRAS-CHAVES: compostos fenólicos, jaboticaba, cambuci, obesidade, diabetes *mellitus* tipo 2, gastrocnemius.

ABSTRACT

PESSOA, Érika Vicência Monteiro. **Role of phenolic compounds from Brazilian native fruits on obesity-induced insulin resistance in skeletal muscle.** 54p. Faculty of Pharmaceutical Sciences – University of São Paulo, São Paulo, 2021.

Skeletal muscle is an important metabolic tissue in glucose uptake and thus in glycemic homeostasis. Evidence suggests that phenolic compounds may exert beneficial health effects against metabolic disorders associated to obesity including its state of peripheral insulin resistance. The objective of this work was to investigate the role of phenolic compounds present in two Brazilian native fruits, cambuci (*Campomanesia phaea* Berg.) and jaboticaba (*Plinia jaboticaba* (Vell.) Berg), on the insulin resistance in the skeletal muscle of obese mice fed a high-fat-sucrose diet (HFS). For this, two independent experimental protocols were used for each fruit, where male C57BL/6J mice fed the HFS diet for the induction to obesity were used. Once the condition of obesity was established, animals started to receive daily oral administration (by gavage) of extracts enriched in phenolic compounds obtained from each fruit, in doses reachable through the diet. At the end of the experiments, the animals were euthanized and their tissue and organs collected. The animals receiving extracts of jaboticaba and cambuci, regardless of the dose, presented lower body weight gain in relation to the HFS group. The results for weekly fasting glycemia and glucose tolerance of the animals that received the phenolic extracts of both fruits showed an improvement in glycemic homeostasis even when fed with the deleterious diet. In the gastrocnemius muscle of the animals was demonstrated that cambuci and jaboticaba extracts significantly increased the content of glucose transporter protein 4 (GLUT-4) and AMP-activated protein kinase (AMPK-Thr172), which has a broad role in metabolic regulation. Regarding inflammation, the administration of extracts from both fruits favored the reduction of phosphorylation and activation of the nuclear factor-κe (NF-κB) and the expression of some genes such as IL-6, TNF-α, IL-1β, and JNK, whose increase has been associated with insulin resistance. In conclusion, this study suggests that the phenolics present in both native fruits may be important therapeutic agents in the reduction of muscle insulin resistance and inflammation associated with obesity.

Keywords: phenolic compounds, jaboticaba, cambuci, obesity, diabetes mellitus type 2, gastrocnemius.

FIGURE LIST

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LIST OF ABBREVIATIONS AND ACRONYMS

AKT	Protein kinase B;
AMPK	Activated protein kinase;
FOXO1	Forkhead box O1
GLUT-4	Glucose transporter type 4;
IKK	IKB protein kinase;
IL-6	Interleukin 6;
IRS-1	insulin receptor substrate 1;
JNK	c-Jun N-terminal kinases;
MCP-1	Monocyte chemoattractant protein-1;
mTORC1	Mammalian target of rapamycin complex 1;
mTORC2	Mammalian target of rapamycin complex 2;
NF-κB	Nuclear transcription factor kappa B;
PI3K	Phosphatidylinositol 3-kinase;
PKC	Protein kinase C;
TNFR1	Tumor necrosis factor receptor 1;
TNFR2	Tumor necrosis factor receptor 2;
TNF-α	Tumor necrosis factor-alpha;
TRL-4	Toll-like receptor type 4;
S6K	Ribossomal protein S6 kinase;

SUMMARY

1. INTRODUCTION

Obesity is a major and growing public health problem worldwide. High-fat, high-sugar diets drive to excessive accumulation of body fat, contributing to the development of other diseases, such as type 2 diabetes mellitus (T2DM). T2DM is a metabolic disorder that can be characterized by hyperglycemia, resulting from failures in insulin action, insulin secretion, or both. Skeletal muscle is considered the main site of insulin resistance, as it is the main tissue responsible for approximately 75% of whole-body glucose uptake under insulin action (JAGANATHAN; RAVINDRAN; DHANASEKARAN, 2018; LATHA; DAISY, 2011).

The prevalence of obesity tripled since 1975. In 2016, more than 1.9 billion people over 18 years were overweight. Of these, about 650 million were obese. In addition, more than 350 million children and adolescents aged between 5 and 19 years were overweight or obese (WHO, 2018). In Brazil, obesity is also a serious public health problem. The Brazilian Ministry of Health estimated that 52.5% of the population were overweight or obese in 2016. Among children from 0 to 8 years old, 22.7% were obese, from 9 to 12 years old, 17.2% and above 12 years old, 12.3% (BRASIL, 2017).

The obesity condition appears together with immune response alterations since a low-grade, chronic inflammatory process is caused. This inflammatory process is related with an excessive increase in adiposity, increased macrophage infiltration in the adipose tissue and production of proinflammatory cytokines such as interleukin 6 (IL-6), resistin, nuclear transcription factor kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), which are related to insulin resistance favoring the decrease of glucose uptake by myocites (JUNG; CHOI, 2014; SOARES et al., 2013; VERMA; HUSSAIN, 2017).

Skeletal muscle characterizes the main place for glucose uptake. Thus, this organ becomes a primary strategy for controlling diabetes by reducing insulin resistance. Lifestyle habits such as regular physical exercise and healthy eating behavior, especially fruit-and vegetable-enriched diets, reduce the occurrence of insulin resistance and type 2 diabetes mellitus (BOSTRÖM et al., 2012; SAMUEL, 2016). The role of diet in human health has recently attracted attention. Studies suggest that bioactive compounds from fruit and vegetables (e.g., phenolic compounds

and carotenoids) can reduce the risk of developing non-communicable chronic diseases (CUI et al., 2007; DEL RIO et al., 2013).

Phenolic compounds have shown biological activities in the prevention of obesity and related chronic diseases by various mechanisms, including anti-inflammatory and anti-diabetic activities, regulating insulin signaling pathways, or by reducing oxidative stress and dyslipidemia (GOTHAI et al., 2016; SILVA et al., 2010). A recent study demonstrated the therapeutic effects of phenolic compounds from cambuci (*Campomanesia phaea* (O. Berg.)) on obesity-induced insulin resistance. These phenolic compounds stimulated glucose uptake and reduced inflammation in obese mice (DONADO-PESTANA et al., 2021).

Additionally, Moura et al. (2021) also showed that phenolic compounds from Sabara jaboticaba (*Plinia jaboticaba* (Vell.) Berg) decreased insulin resistance in white adipose tissue, skeletal muscle, and liver; however, the molecular mechanism and the cellular signaling pathways involved in these biological effects are still unknown. Here, we aimed to investigate the effect of phenolic compounds from Sabara jaboticaba on specific targets in the glucose uptake and inflammatory pathways in skeletal muscle, elucidating mechanisms of amelioration on the insulin resistance in diet-induced obese mice.

1.1 OBESITY AND TYPE 2 DIABETES MELLITUS

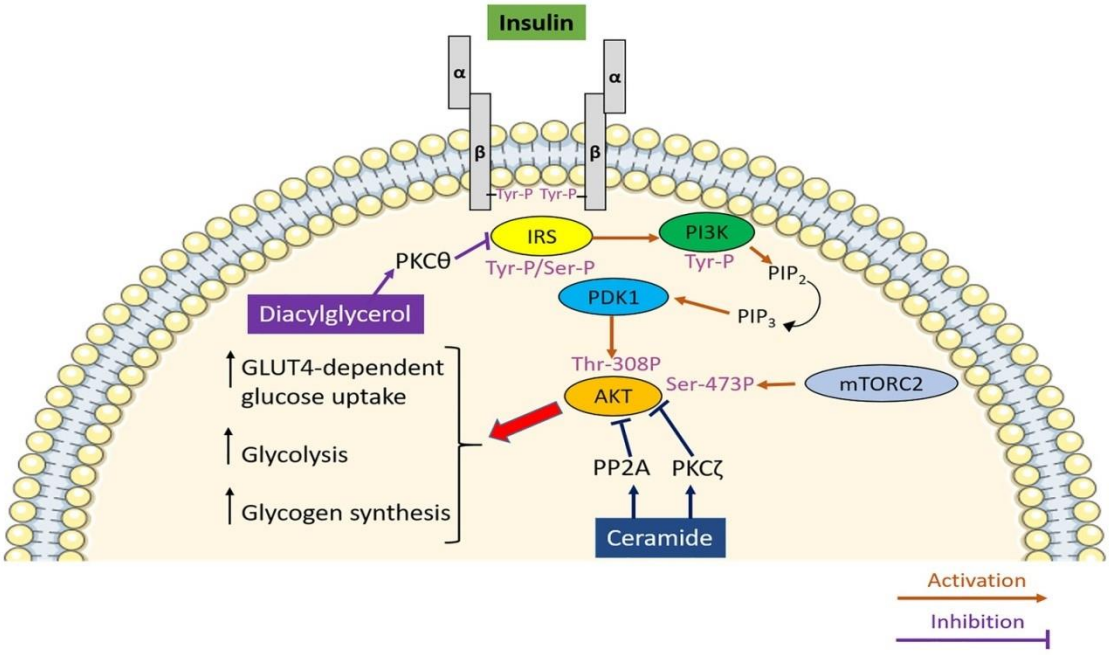
Obesity presents a low-grade chronic inflammation, which results in harmful health consequences such as metabolic disorders including cardiovascular diseases, liver steatosis, insulin resistance, and type 2 diabetes mellitus (HEBEBRAND et al., 2017). Increased fat deposition in obesity results from an imbalance between the caloric intake and energy expenditure and this further caloric overload leads to the fat accumulation in ectopic tissues (liver, skeletal muscle, and heart) and the visceral adipose depots, an event commonly termed as “lipotoxicity” (MUNIESA et al., 2017; OLIVEIRA et al., 2019).

The increase of circulating fatty acids and mitochondrial dysfunction causes a reduction in metabolic oxidation, and these events impair insulin signaling and promote insulin resistance (SAMOCHA-BONET et al., 2012). Excessive consumption of nutrients in the diet can also induce excessive production of mitochondrial reactive

oxygen species (ROS) production, which induces insulin resistance through oxidative damage to mitochondrial DNA (BURGOS-MOR et al., 2019).

Diacylglycerol (DAG) is primarily responsible for insulin resistance induction by activating distinct isoforms of protein kinase Cθ (PKCθ) inhibiting the activity and phosphorylation of tyrosine kinase from the insulin receptor. Ceramides prevent insulin signaling through dephosphorylation of Akt by protein phosphatase 2A (PP2A) and also by phosphorylation of Akt in the pleckstrin homology domain by PKCζ, as shown in **Figure 1** (SERGI et al., 2019).

Figure 1 Lipotoxitciy and insulin resistance.



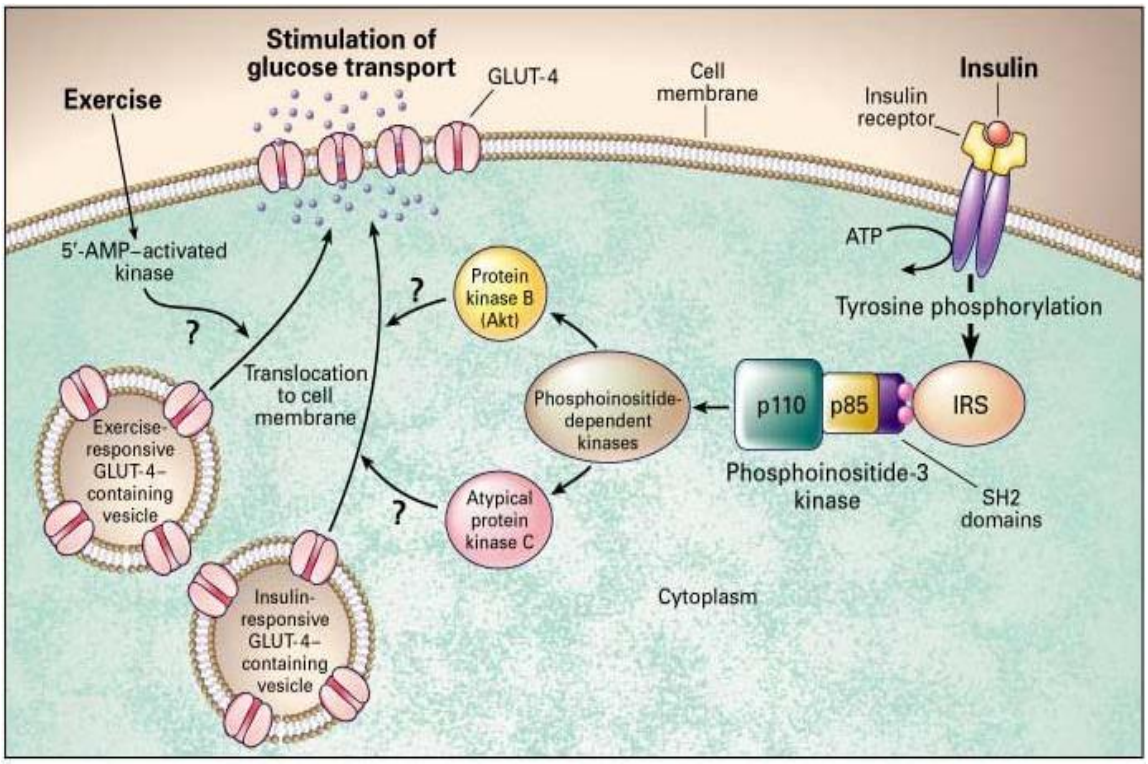
Source. (SERGI et al., 2019).

Insulin is an anabolic hormone produced by activated pancreatic β-cells after meals, through increased circulating glucose and amino acids. Among its actions are the increase of glucose absorption, predominantly in the muscular tissue, increase of the protein , fatty acids, and glycogen synthesis, as well as inhibition of hepatic glucose production, lipolysis and proteolysis (GARZILLI; ITZKOVITZ, 2018; JOUVET; ESTALL, 2017).

Intracellular signaling of insulin begins with its binding to a specific membrane receptor called insulin receptor (IR). IR activation results in tyrosine phosphorylation, including insulin receptor substrates 1 and 2 (IRS-1 and IRS-2). Phosphorylation of IRS proteins promotes the activation of another cytosolic protein, denominated

phosphatidylinositol 3-kinase (PI3K). The p85 regulatory subunit activation of phosphatidylinositol 3-kinase (PI3K) results in increasing serine phosphorylation of protein kinase B (Akt), and this allows the glucose transport to muscle and adipose tissue through the translocation of GLUT-4 to the cell membrane. The translocation of GLUT-4 to the membrane permits the entrance of circulating glucose by facilitated diffusion. Exercise stimulates glucose transport by pathways that are independent of phosphoinositide-3 kinase and that may involve AMP-activated protein kinase (AMPK), as shown in **Figure 2** (CHOI; KIM, 2010; HUA et al., 2012).

Figure 2 Insulin signaling pathway.



Source. (SHEPHERD; KAHN, 1999).

AMPK (**Figure 2**) has been evidenced by its regulatory role in cellular energy homeostasis. AMPK is a serine-threonine kinase that is activated by increases in the AMP/ATP. It was discovered as a switch regulating the oxidation of fatty acids in the heart and skeletal muscle. Recently, studies have shown that AMPK is an important regulator of glucose metabolism (HUA et al., 2012; PENG; SUN; PARK, 2019).

According to Cignarelli et al., (2019), in obesity, both concentration and activity of the IR receptor, tyrosine phosphorylation of IRS-1 and IRS-2, and activity of phosphatidylinositol 3-kinase (PI3K) protein are reduced. Some inflammatory proteins

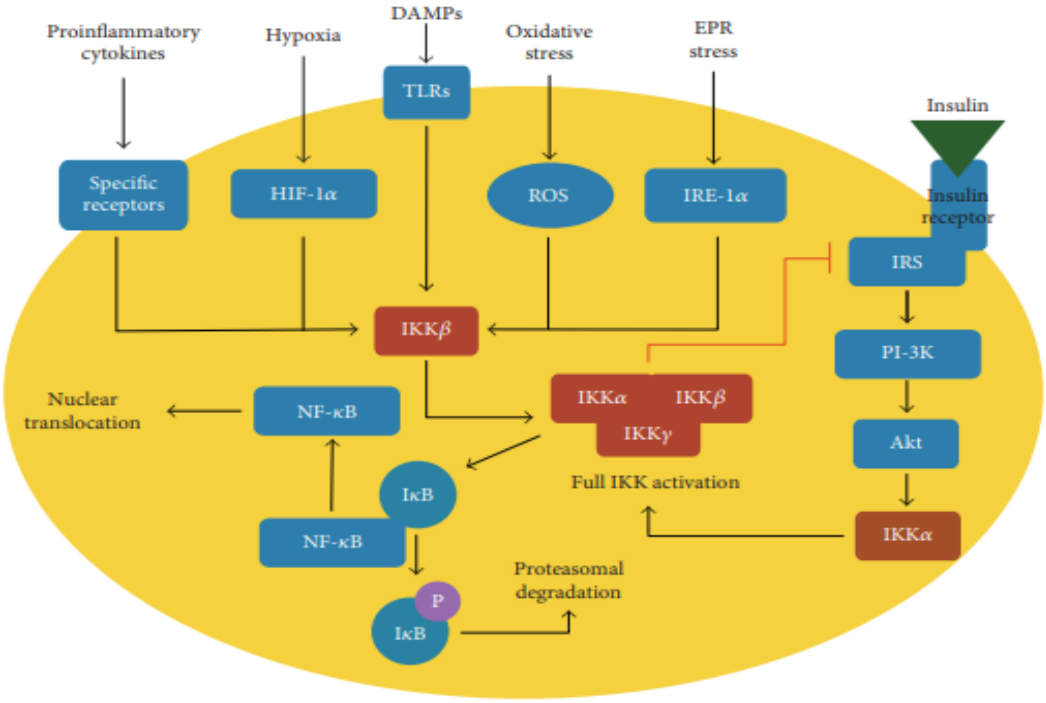
may promote alterations compromising the activity of PI3K and translocation of GLUT4 to the plasma membrane. In addition, the hypertrophic adipose tissue expansion, an common event in obesity, secretes proinflammatory adipokines such as TNF-α, which in turn, bind to its receptors on the plasma membrane, tumor necrosis factor receptors 1 and 2 (TNFR1 and TNFR2), activating other inflammatory proteins such as IkB protein kinase (IKK) and c-Jun N-terminal kinases (JNK), which promote insulin resistance (WOJCIK et al., 2018).

The serine phosphorylation of the insulin receptor impairs the glucose uptake cascade in the skeletal muscle. This event is associated with the increase of inflammatory cytokines (including TNF-α and IL-1β), activating JNK and IKK/NF-kB pathways through mechanisms mediated by classical receptors. Non-esterified fatty acids also promote insulin resistance by binding to the Toll-like receptor type 4 (TLR-4) and activate NF-κB pathway in an autocrine manner.

On the other hand, studies indicate that mTOR complex 2 (mTORC2) is negatively related to insulin signaling by activation of ribosomal protein S6 kinase (S6K), which in turn influences IRS-1 phosphorylation in serine residues, compromising the translocation of GLUT-4.

Events such as cellular hypoxia, endoplasmic reticulum (ER)stress, and oxidative stress also induce inflammation that leads to insulin resistance, as shown in **Figure 3** (BOUCHER; KLEINRIDDERS; KAHN, 2014; HOTAMISLIGIL; ERBAY, 2008; REHMAN; AKASH, 2016; VOGT; BRÜNING, 2013)

Figure 3 Mechanism of insulin resistance in obesity.



Source: (STAFEEV et al., 2017)

1.2 THE ROLE OF SKELETAL MUSCLE IN GLYCEMIC HOMEOSTASIS

Skeletal muscle is an important tissue in insulin-stimulated glucose uptake, actively participating in the maintenance of postprandial glycemia and recognized as the main site of insulin resistance in type 2 diabetics (PETERSEN; SHULMAN, 2006). This tissue corresponds to about 40% of the total body mass, and is responsible for 30% of energy expenditure and the uptake of approximately 75% of circulating glucose under insulin-dependent conditions. Insulin is one of the key regulators in the process of glucose uptake by skeletal muscle and insulin signaling dysregulation leads to impairment of its metabolic effects (HUANG et al., 2018; MARTINS et al., 2012).

Some obese individuals regularly develop a status of insulin resistance, an event defined by the reduced ability of insulin to stimulate glucose uptake by peripheral tissues of the body, including skeletal muscle. The transport of glucose from the extracellular to the intracellular medium by the muscle cells is the critical point of this metabolic pathway. The proteins that mediate these processes belong to the class of glucose transporters (GLUTs) and are responsible for transferring the glucose in favor of its concentration gradient by facilitated diffusion. GLUTs are essential for the maintenance of glycemic homeostasis, and in muscle tissue, this action is mediated

by GLUT1 and GLUT4 (CHENG et al., 2014; NAVALE; PARANJAPE, 2016). The GLUT4 is an insulin-sensitive isoform, is stored in intracellular vesicles and, after stimulation, is translocated to the plasma membrane. This is a mechanism of fundamental importance in the regulation of glucose uptake (MEIER; NAUCK, 2010).

Even when glucose is not readily available from the diet, muscle is capable of producing energy for muscle contraction due to its ability to store glucose in the form of glycogen. Other organs besides muscle possess this ability to store energy, such as kidney and liver, which helps the body to obtain essential energetic substrate during fasting or starvation. The skeletal muscle can undergo changes in response to physical adaptations, nutrient intake, diseases, and stress (ARGILÉS et al., 2016).

Elevated levels of circulating free fatty acids can perform a key role in developing insulin resistance in skeletal muscle. Fatty acids may affect the action of insulin due to the accumulation of intracellular lipid derivatives (diacylglycerol and ceramides), oxidative stress, gene transcription modulation, and inflammation (GUARINO et al., 2013; MARTINS et al., 2012). An increased accumulation of lipids promotes a marked reduction in IRS-1 phosphorylation in tyrosine, reduction in PI3K activity and phosphorylation of Akt. These actions are explained by the activation of several kinases such as IKK, JNK, and protein kinase C (PKC), which are responsible for catalyzing IRS-1 phosphorylation in serine, inhibiting its activity and directing it to degradation (BOUCHER; KLEINRIDDERS; KAHN, 2014; SOLINAS; BECATTINI, 2017).

1.3 PHENOLIC COMPOUNDS AND BIOLOGICAL EFFECTS ON INSULIN RESISTANCE

The bioactive compounds present in foods are important in promoting health, reducing the risk for the development of chronic non-communicable diseases. These compounds interfere with specific physiological pathways such as antioxidant defense system and inflammatory and mutagenic processes. The bioactive compounds can be derived from animal products (i.e. omega-3 fatty acids), plant (i.e. carotenoids, phytosterols, phenolic compounds, among others) or microorganisms (i.e. probiotic strains) (RODRIGUEZ-MATEOS et al., 2014).

In plants, phenolic compounds, also called polyphenols, are produced by secondary metabolism of plants and are widely distributed in nature, with more than 8000 compounds already identified. This large and complex group provides natural defense against pathogens, ultraviolet rays, and may act as agents to attract pollinators (SILVA et al., 2010). These compounds may have simple or complex structures, derived from the amino acids phenylalanine and tyrosine, which have at least one aromatic ring or more with hydroxyl groups (-OH) in their chemical structure (RODRIGUEZ-MATEOS et al., 2014; TSAO, 2010).

Phenolic compounds can be divided into two groups: flavonoids and non-flavonoids. Flavonoids have a basic structure formed by two aromatic rings bonded by three carbons (C6-C3-C6) and are the most common in the plant kingdom. Among the flavonoids, the main subclasses of these compounds are flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins. The most important non-flavonoids are phenolic acids which, in turn, can be classified into hydroxybenzoic or hydroxycinnamic acids and derivatives. Gallic and ellagic acids are the main hydroxybenzoic acids and are precursors of gallotannins and ellagitannins, also known as hydrolyzable tannins. Hydroxycinnamic acids occur conjugated to other compounds and are collectively referred as chlorogenic acids. Another minor class of non-flavonoids are stilbenes with resveratrol being the compound more representative (DEL RIO et al., 2013).

Several studies have attributed to phenolic compounds the ability to modulate metabolic inflammatory responses by inhibiting pathways activated by both TNF-α and lipopolysaccharides (LPS), resulting in decreased gene expression and proteins involved in insulin resistance (BASTOS; ROGERO; ARÊAS, 2009; DONADO-PESTANA; BELCHIOR; GENOVESE, 2015). Signaling pathways have been suggested as being involved in anti-inflammatory effects mediated by phenolic compounds such as NF-κB, and JNK (ZHANG et al., 2014).

In addition, phenolic compounds may also be associated with mechanisms of hyperglycemia regulation acting as inhibitors of enzymes that hydrolyze carbohydrates, mainly α-amylase and α-glycosidase (GONÇALVES; LAJOLO; GENOVESE, 2010). On the other hand, a study demonstrated that tannins may induce GLUT-4 expression through the activation of the insulin-mediated signaling pathway in adipocyte cells (LIU et al., 2005). Gallotannins demonstrated a health beneficial effect,

increasing the expression of the IRS-1, GLUT-4 and PI3K proteins in L6 cells (KANAUJIA et al., 2010).

A previous study noted that epigallocatechin gallate (EGCG), the major phenolic compound of green tea, improved insulin-stimulated glucose uptake, in a dose-dependent manner, by enhancing GLUT-4 translocation to the plasma membrane of rat muscle cells. These biological effects were attributed to increased phosphorylation of AMPK (ZHANG et al., 2010). In the same order, resveratrol (present in red wine and grapes) increased the glucose uptake in muscle cells of the C2C12 lineage by activating AMPK. In the absence of insulin, resveratrol favored glucose uptake by activation of AMPK without PI3K pathway involvement. In the presence of insulin, resveratrol had a potentiating effect on the uptake by AMPK activation, but also, activating the PI3K-Akt signaling pathway (PARK et al., 2007).

Extracts obtained from grape seeds containing procyanidins favored the modulation of the insulin receptor, inducing its autophosphorylation and consequently leading to an increase in glucose uptake via the Akt pathway (MONTAGUT et al., 2010). In addition, phenolic extracts of seaweed improved glucose metabolism in mice with insulin resistance induced by high fructose diet, increasing the content of AMPK and Akt proteins (KANG et al., 2018).

1.4 BRAZILIAN NATIVE FRUITS AS SOURCES OF PHENOLIC COMPOUNDS: CAMBUCI (*Campomanesia phaea* Berg.) AND JABOTICABA (*Plinia jaboticaba* (Vell.) Berg) .

In Brazil there is an extensive genetic biodiversity, counting with more than 55,000 species catalogued from an estimated total between 350,000 and 550,000 species. However, less than 10% of these plants have been evaluated under biological aspects, and no more than 5% under chemical and functional aspects until the mid-1990s. Thus, there is a set of species, mainly native, that are still a source important for the discovery of new biologically active substances (FERRERA et al., 2016).

Brazilian native fruits grow under adverse environmental conditions, such as floods, droughts, and intense exposure to sunlight and heat. These tropical and sub-tropical conditions induce, in part, to a response of secondary metabolites involved in plant defense, especially in the synthesis of biologically active phenolic compounds. Species including jaboticaba (*Plinia jaboticaba (Vell.) Berg*), cambuci (*Campomanesia phaea* Berg.), cagaita (*Eugenia dysenterica* DC), camu-camu (*Myrciaria dubia HBK*

Mc Vaugh), and cupuaçu (*Theobroma grandiflorum Willd. Shum*), among others, are distributed in different Brazilian biomes such as Cerrado, Atlantic Forest, and Amazon. These species are an important source of phenolic compounds and previous studies have reported antioxidant, anti-inflammatory and anti-obesity biological activities (DONADO-PESTANA et al., 2018).

The cambuci or cambucizeiro is one of the primitive trees of the Atlantic Forest. It has a natural occurrence in the states of São Paulo and Minas Gerais (BALBI et al., 2016). The cambuci fruit is a source of vitamin C, sodium, potassium, phosphorus, magnesium and calcium (VALLILO et al., 2005). In addition, fruits have high levels of tannins, mainly ellagitannins and proanthocyanidins, which may be associated with the astringency and sour taste of the fruit (SANCHES AZEVEDO et al., 2017).

Cambuci presents high concentrations of phenolic compounds in comparison to other native fruits, and is related to a high *in vitro* antioxidant capacity (GENOVESE et al., 2008; HAMINIUK et al., 2011; SANCHES AZEVEDO et al., 2017). The cambuci phenolic compounds, in in vitro enzymatic assays, demonstrated potent inhibitory capacity of enzymes that hydrolyze carbohydrates (α-amylase and α-glycosidase) (GONÇALVES; LAJOLO; GENOVESE, 2010). The inhibition of these enzymes suppresses glucose uptake, which can be a therapeutic approach to prevent postprandial hyperglycemia common in diabetes. In addition, a previous study has suggested that cambuci phenolic compounds attenuate risk factors in obesity and the metabolic syndrome by reducing glucose intolerance, fasting hyperglycemia, inflammation, and hyperinsulinemia, as evidenced by the lower expression of proinflammatory cytokines such as TNF-α , IL-6, and monocyte chemoattractant Protein-1 (MCP-1) in the adipose tissue of mice fed diets high in fats and carbohydrates (DONADO-PESTANA et al., 2015).

On the other hand, the jaboticaba-Sabará is a Brazilian native fruit, of the family *Myrtaceae*, characteristic of the Atlantic Forest biome. The fruit is rich in proanthocyanidins, anthocyanins and ellagitannins, such compounds with anti-inflammatory and antioxidant properties (ALEZANDRO et al., 2013). Research has reported beneficial effects of extracts rich in phenolic compounds obtained from jaboticaba fruits, pulp, and peel have high antioxidant activity in addition to other important biological activities, such as anti-diabetes, and benefits in controlling obesity (WU; LONG; KENNELLY, 2013).

Ingestion of jabuticaba bark attenuated oxidative stress, as it increases its antioxidant defenses, protecting against damage caused by lipid peroxidation in the liver, kidneys and brain, and also the development of obesity-related diseases (BATISTA et al., 2014). Meals containing jaboticaba peel increased plasma antioxidant capacity and decreased circulating insulin and glucose levels in healthy adults (PLAZA et al., 2016). The daily administration of jaboticaba extract was able to prevent high concentrations of fasting blood glucose and attenuate hyperinsulinemia. In addition, prevented high levels of total cholesterol in a murine model of diet-induced obesity (MOURA et al., 2018). Furthermore, phenolic compounds from jaboticaba attenuated insulin resistance by reducing inflammation through modulation of key proteins and cytokines in signaling inflammatory pathways, including IL-1, IL-6, and NF-κB (DRAGANO et al., 2013).

2. GENERAL OBJECTIVE

To investigate the role of phenolic compounds of cambuci (*Campomanesia phaea* Berg.) and jaboticaba (*Plinia jaboticaba* (Vell.) on insulin resistance in the skeletal muscle of obese mice fed high-fat-sucrose diet.

2.1 SPECIFIC OBJECTIVES

- To study the effects of phenolic compounds from cambuci and jaboticaba fruits in regulating key proteins involved in the skeletal muscle insulin signaling pathway

- To determine the role of phenolic compounds from cambuci and jaboticaba on the inflammatory pathways in the skeletal muscle of obese mice with excessive exposure to fat and carbohydrate in the diet.

3. MATERIAL AND METHODS

3.1 MATERIAL

The cambuci frozen pulp was obtained from a local producer (Sítio do Bello, Paraibuna, São Paulo, Brazil) and the Jaboticaba Sabará fruit was obtained from a local market (‘Companhia de Entrepostos e Armazéns Gerais de São Paulo’,CEAGESP). The Jaboticaba fruits were sanitized, frozen in nitrogen, lyophilized and stored at -20 ºC.

3.2 METHODS

3.2.1 Extraction of phenolic compounds

The phenolic-rich extracts from jaboticaba (PEJ) and cambuci (PEC) were obtained by hydromethanolic extraction. For this, a methanol/water/acetic acid solution (70:30:0.5 v/v/v) was used for extraction (1:25 p/v) for 2 hours under shaking at 4 °C. The extract was vacuum filtered with Whatman nº 1 filter paper, and the residue re-extracted twice more, for 30 minutes, under the same conditions. The extract was then concentrated on a rotatory evaporator (Rotavapor R-210; Büchi, Switzerland) at ≤ 37 °C and resuspended in distilled water.

The phenolic-enriched extracts of both fruits were obtained by solid-phase extraction (SPE) using octadecylsilane (C18) columns (Supelclean ™ LC-18, Supelco), which were manually manufactured by adding the adsorbent in polypropylene syringes. The aqueous extract was passed into the preconditioned column with 20 mL of methanol and 60 mL of distilled water per gram of adsorbent. Next, column was washed with distilled water and the elution of the phenolic compounds made with methanol. Finally, the methanolic extract was evaporated to dryness as described above, and resuspended in water, thus obtaining PEJ and PEC.

3.3 ANIMALS AND EXPERIMENTAL DESIGN

The experiments were carried out in accordance with Brazilian legislation and approved by the Ethical Committee for the Animal Research (CEUA 522 to jaboticaba

protocol and CEUA 378 to cambuci protocol) of the Faculty of Pharmaceutical Sciences of the University of São Paulo.

One independent experiment was conducted for each fruit. In the two experimental protocols, eight-week-old C57BL/6 male mice were used and submitted to an adaptation period of one week to the experimental environment, receiving a standard diet and water *ad libitum*. At the beginning of the experiment, mice received a standard diet or a high-fat high-sucrose diet (HFS) for the induction to obesity. The standard diet used during the experiment followed the AIN 93M protocol providing 3.8 kcal/g, with 14.1% of this value provided by proteins, 18% from lipids and 81% from carbohydrates. The HFS diet was prepared manually according to Lemieux et al. (2003) yielding 4.6 kcal/g, 20% provided by proteins, 39% from lipids and 41% from simple carbohydrate (sucrose).

3.3.1 Cambuci protocol

Forty male C57BL/6J mice were purchased from IQ/FCF bioterium and housed in a controlled animal facility (12/12 h light/dark, 20 °C). After the adaptation period, mice were randomly distributed into two dietary groups, remaining for 7 weeks with the following distribution:

- HFS group (n = 30)*:* animals fed a HFS diet and water *ad libitum*.
- Chow group (n = 10): animals fed a standard diet and water *ad libitum*.

After this period, animals from the HFS group were redistributed in three different groups for another 7 weeks as follow:

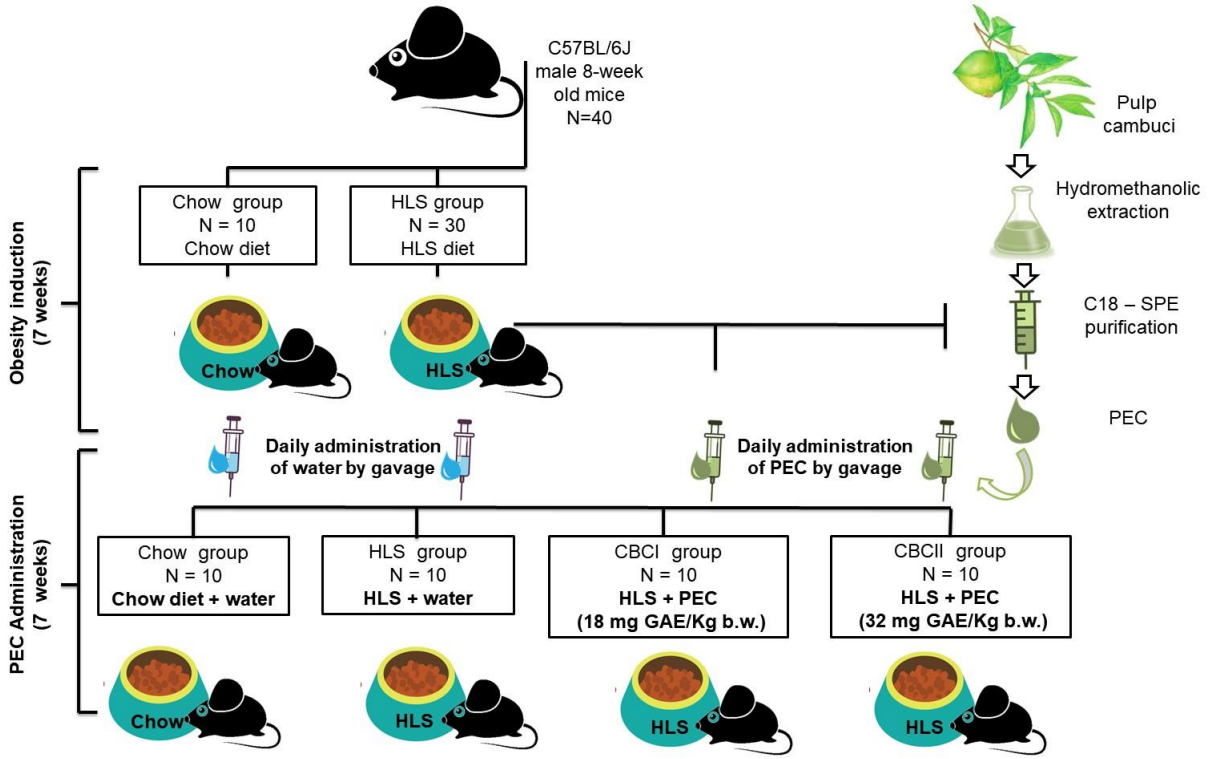
- HFS group (n = 10): fed with HFS diet and receiving water by daily gavage.
- CBCI group (n = 10): fed with HFS diet and receiving PEC at the dose of 18 mg gallic acid equivalent (GAE)/kg of body weight by daily gavage.
- CBCII group (n = 10): fed with HFS diet and receiving PEC at a dose of 32 mg GAE/kg of BW. by daily gavage.

The animals in the chow group were kept on the standard diet.

- Chow group (n = 10): Fed with standard diet and receiving water by daily gavage.

Figure 4 shows the schematization of the experimental design. After 14 weeks, the animals were euthanized by exsanguination under anesthesia. Serum was separated by centrifugation at *3000 g* at 4 °C for 20 min. The organs and tissues were removed, the gastrocnemius muscle was weighed and stored at - 80 °C for further analysis.

Figure 4 Summary of the experimental design for cambuci fruit.



HFS – high-fat high-sucrose diet; PEC – Phenolic-rich extract form cambuci; GAE – Gallic acid equivalent; BW. – body weight. SPE – solid-phase extraction; C18 – Octadecylsilane resin.
Source.: The author (2021).

3.3.2 Jaboticaba protocol

Forty mice were purchased from IQ/FCF bioterium and housed in a controlled animal facility (12/12 h light/dark, 20 °C), fed with standard diet (NUVILAB CR-1, Nuvital Nutrientes S/A, PR, Brazil) and water *ad libitum*, for 2 weeks before the beginning of the experimental protocol.

Mice were randomly distributed into two groups, remaining for 14 weeks with the following distribution:

- HFS group *(*n = *30):* animals fed a HFS diet and water *ad libitum*.
- Chow group (n = 10): animals fed a standard diet and water ad libitum.

After this period, animals in the HFS group were redistributed in three different groups for another 14 weeks:

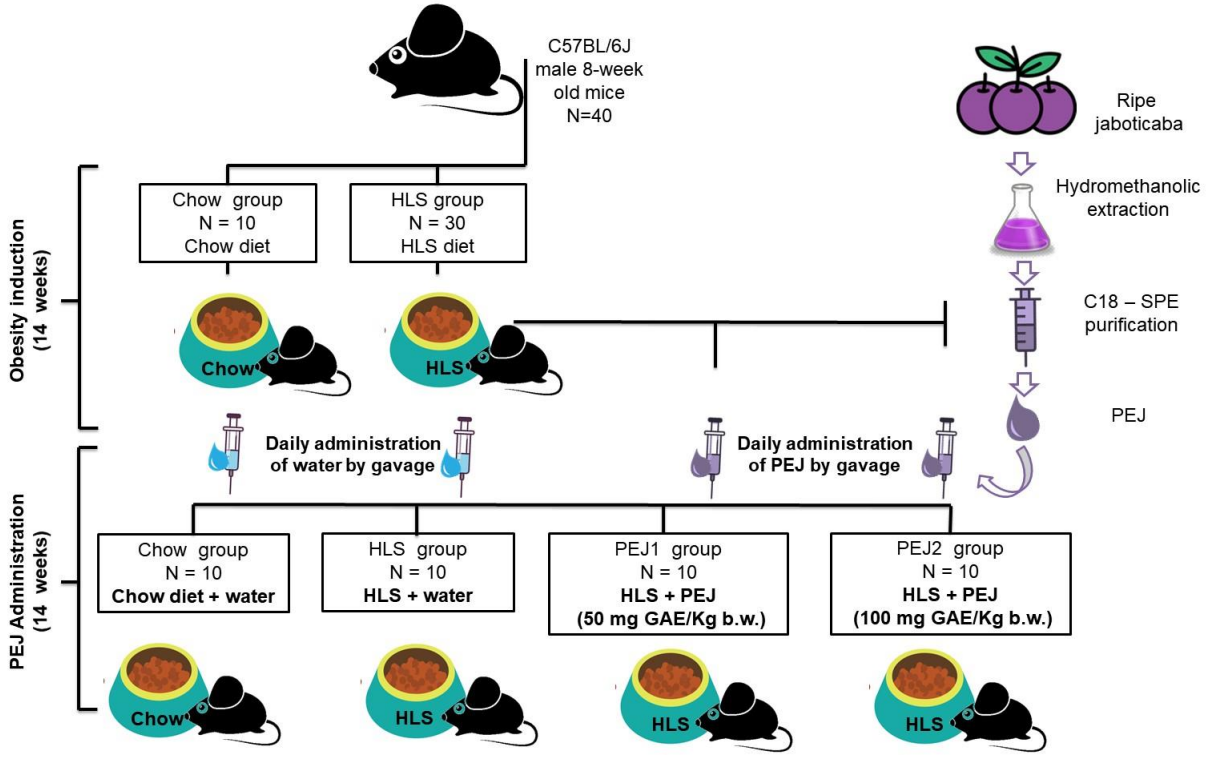
- HF Group (n = 10): fed with HFS diet and daily water gavage.
- PEJ1 group (n = 10): fed with HFS diet and daily gavage from PEJ at the dose of 50 mg GAE/kg of BW.
- PEJ2 group (n = 10): fed with HFS diet and daily gavage of PEJ at a dose of 100 mg GAE/kg of BW.

The animals in the chow group were kept on the standard diet.

- Chow group (n = 10): fed with standard diet and receiving water by daily gavage.

Figure 5 shows the schematization of the experimental design. After 28 weeks, the animals were euthanized by exsanguination under anesthesia. Serum was separated by centrifugation at 3000*g* at 4 °C for 20 min. The organs and tissues were removed, the gastrocnemius muscle was weighed and stored at -80 ° C for further analysis.

Figure 5 Summary of the experimental design for jaboticaba fruit.



HFS – high-fat high-sucrose diet; PEC – Phenolic-rich extract form cambuci; GAE – Gallic acid equivalent; BW. – body weight. SPE – solid phase extraction; C18 – Octadecylsilane resin.
Source.: The author (2021).

3.3.3 Fasting glycemia and oral glucose tolerance test (oGTT)

Glycemia was measured weekly after 6 or 4 hours of fasting (to cambuci and jaboticaba protocol, respectively) using an Accu-Chek Performa glucosimeter (Roche, Mannheim, Germany) from blood drawn from the caudal vein. For the oGTT, a glucose solution was administered to the 6 or 4-h fasted animals (1.0 g / kg BW.) per gavage. Glucose concentration was determined from blood drawn from the caudal vein prior to gavage and at 15, 30, 45, 60 and 90-minute intervals after administration of the glucose load. Blood samples were collected at 0, 30, 60 and 90 min, and the serum separated by centrifugation at 3000*g* for 20 min at 4 °C for insulin determination (Rat/Mouse Insulin ELISA Kit, Millipore, Missouri, USA).

3.3.4 Protein analysis of skeletal muscle by immunoblotting.

A fraction of gastrocnemius muscle (45-55 mg tissue) was homogenized (T10, Ultra-Turrax®) in 400 µL of ice-cold extraction in lysis buffer (1% Triton-X 100, 100 mM Tris (pH 7.4), pyrophosphate 100 mM sodium fluoride, 10 mM EDTA, 100 mM sodium

orthovanadate, 2 mM PMSF and 0.01 mg/mL aprotinin). The samples were centrifuged at 12000*g* for 20 min at 4 °C. The concentration of protein in the supernatant was determined by a commercial kit (Pierce BCA, Thermo Scientific, Rockford, USA). The proteins were denatured by heating in Laemmli buffer and then loaded and separated on 12% SDS-PAGE and transferred to PVDF membranes (Merck Millipore, Massachusetts, USA). Then, membranes were incubated with primary antibodies against phosphorylated AMP-activated protein kinase (pAMPK T172), AMPK-Total, glucose transporter type 4 (GLUT-4), Akt-total, pAkt Ser473, pAkt Thr308, Toll-like receptor type 4 (TLR-4), ribosomal protein S6 kinase (pS6 Ser240/244), S6 total, nuclear transcription factor-kappa B (pNF-κB ser 536), NF-κBTotal, forkhead box O1 (FOXO1), proliferator-activated receptor-γ co-activator 1α (PGC-1α), tubulin and β-actin. Antiboides were purchased from Cell Signaling Technology, Beverly, USA, Thermo Scientific, Rockford, USA, and Santa Cruz Biotchnology, Dallas, TX, USA.. Membranes were washed and incubated with peroxidase-conjugated secondary antibody and revealed by chemiluminescence (ECL, GE Healthcare, USA). The densitometry of the bands was determined using the Image J program (National Institute of Health, Illinois, USA).

3.3.5 Gene expression analysis by quantitative PCR

RNA from skeletal muscle samples was extracted using Trizol (Life Technologies, Thermo Scientific, Waltham, MA, USA). mRNA transcript levels were determined on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The synthesized cDNA was mixed with the primer sequences of targets of interest (Table 1) and the reactions were performed with SYBR Green PCR Master Mix (Sigma Aldrich, St. Louis, MO) on a Rotor-Gene (Qiagen, Valencia, CA). The quantification of the gene expression was performed using the ΔΔCt method and expressed relative to the housekeeping gene (VEGF), which was selected because no significant variation in its expression was observed between the studied groups.

Table 1 Primer sequences for real-time PCR.

Primer name	Forward	Reverse
IRS	TTCGATGTCCACCCCAGCTC	GCTATTTGGCACCGAACGGG
PI3K	TGCTGAGAAGGACACGTGGG	TGTCCTCCATCAACGGGGTG
PKCα	TCGCCAACAGGGAAGGGTAAG	GGGCAGGTTTGATTGGCAGC
AMPK	GCAAAGTGAAGACTACCAGGTG	CGCGCTTCCACCTCTTCAAC
MAPK	GACCCAAGTGATGAGCCCATTG	AGTCCTCTGAGCCCTTGTCCTG
NF-κB	TCAGAACTCTGCAGGTGAGACC	CAGAACTCTGCAGGTGAGACC
TNF-α	GGGCAGTTAGGCATGGGATG	TACCTACGACGTGGGCTACAG
JNK	TCAGAAGCAGAAGCCCCACC	ACGGCTGCCCTCTTATGACTC
iNOS2	TTCTCAGCCACCTTGGTGAAG	ACTCCGTGGAGTGAACAAGACC
IL-1β	GCCACCTTTTGACAGTGATGAG	TGATCTGCTGCTGCGAGATT
F480	GCCACGGGGCTATGGGATGC	TCCCGTACCTGACGGTTGAGCA
CD86	CCCAGCAACACAGCCTCTAA	ACTCTGCATTTGGTTTTGCTGA
VEGF-α	ACTGGACCCTGGCTTTACTG	TGAACTTGATCACTTCATGGGAC

IRS, Insulin substrate receptor, PI3K, phosphoinositide 3-kinase, PKC, protein kinase C, AMPK, AMP-actvated protein kinase, MAPK, mitogen-activated protein kinase, NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells, TNF-α, tumor necrosis factor alpha, JNK, c-Jun N-terminal kinase, iNOS, inducible nitric oxide synthase 2, IL-1β, interleukin 1 beta, F4/80, CD86, cluster of differentiation 86, vascular endothelial growth factor alpha (VEGF-α).

3.3.6 Enzyme-linked immunosorbent assay (ELISA)

Skeletal muscle homogenates (45-55 mg) were prepared as described above (Subsection 3.3.5). Protein content of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) were measured by ELISA (DuoSet ELISA®, R&D Systems, Minneapolis, MN, USA) following the supplier's recommendations.

3.4 STATISTICAL ANALYSIS

Results were expressed as mean ± standard deviation or mean ± standard error. Data were analyzed by the Shapiro-Wilk test to determine the nature of their distribution. For the parametric data, the t-test followed by the Walch test or t-test followed by Man-Whitney for non-parametric data was used. Analysis of variance (ANOVA) with Tukey test (normal distribution) or Kruskal-Wallis with Dunn test (non-parametric) was used to determine significant differences between treatments. The analysis was performed using GraphPad Prism software (GraphPad Software, version 6.0, La Jolla, CA, USA).

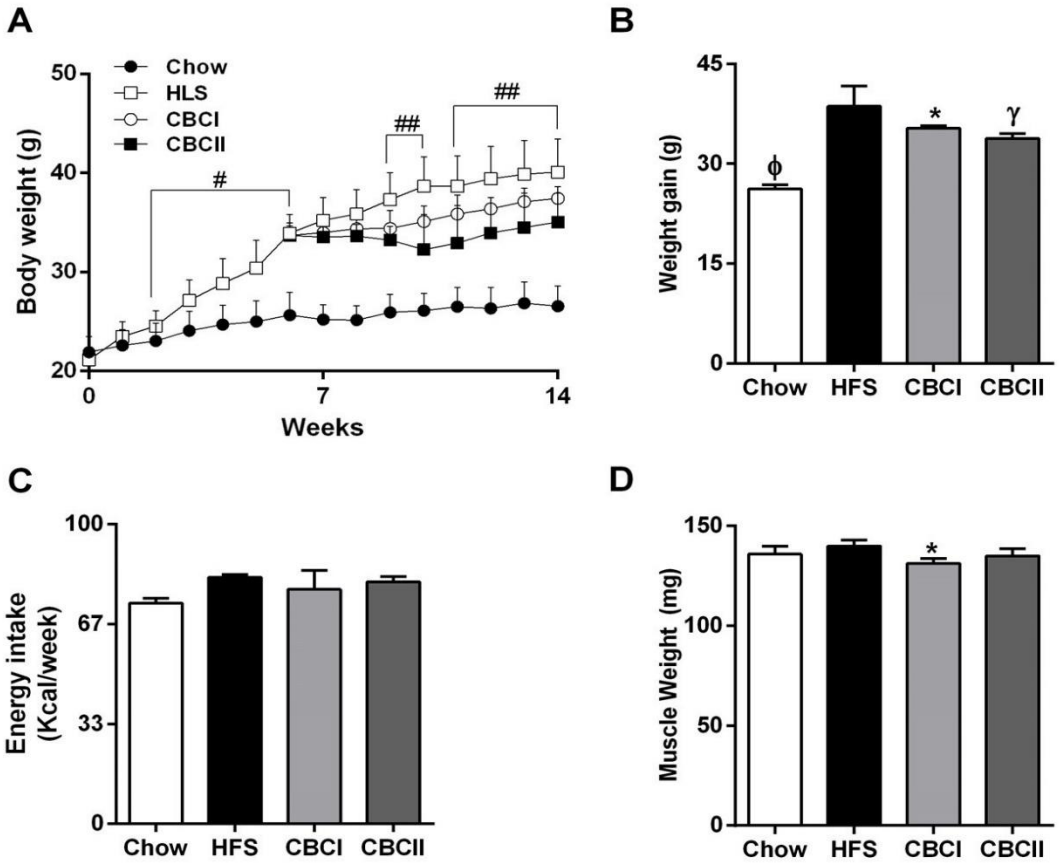
4. RESULTS AND DISCUSSION

4.1 Effect of Cambuci phenolics on body weight, energy intake, and glucose metabolism of obese mice.

4.1.1 Body weight and energy intake

As expected, mice fed an HFS diet developed marked obesity when compared to mice fed a Chow diet. However, daily administration of PEC in two doses protected the animals against excessive weight gain, showing a significant reduction in body weight when compared to the HFS group (**Figures 6 A and B**). . The energy intake was similar for all groups (**Figure 6 C**). In addition, CBC reduced gastrocnemius muscle (only CBC I) masses (**Figure 6 D**).

Figure 6 Body weight, weight gain, energy intake and gastrocnemius muscle weight of mice fed on high-fat high-sucrose (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from cambuci at two doses (CBCI and CBCII) by gavage for 7 weeks.

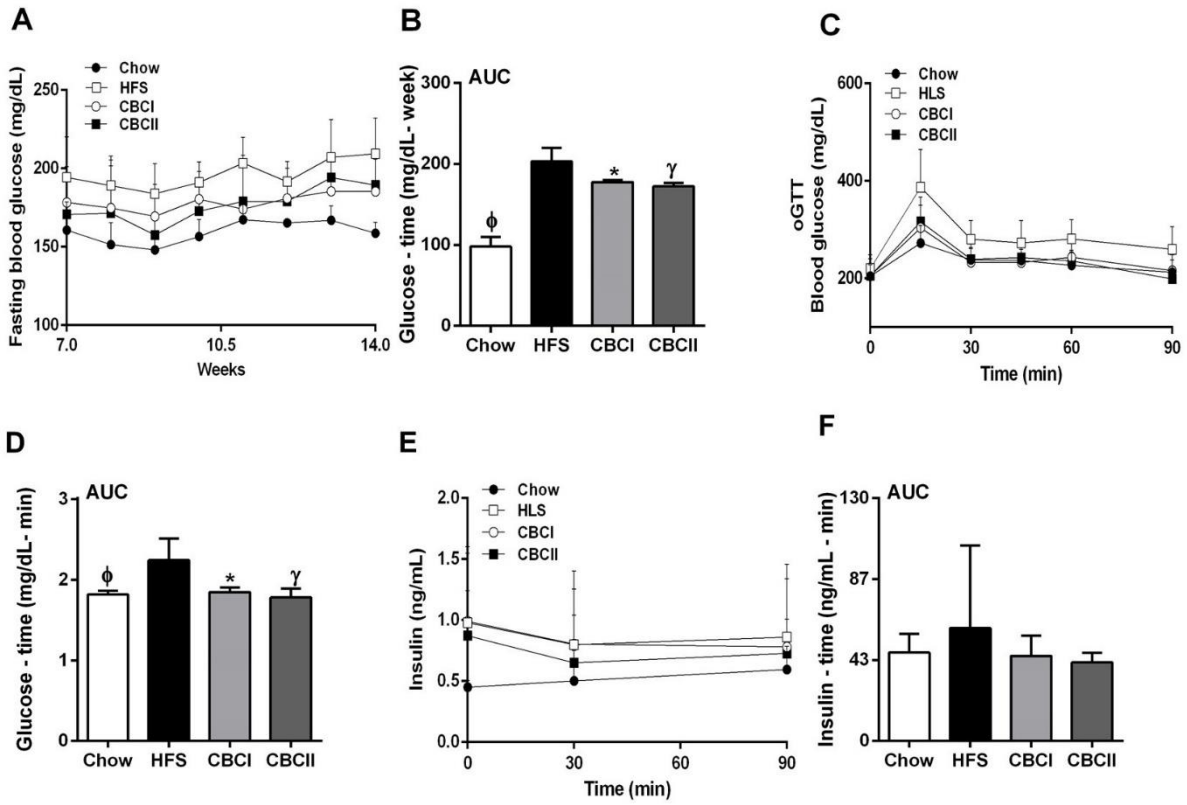


Results are expressed as mean ± SD. #(*p< 0.05*) Chow *vs* HFS. ##(*p< 0.05*) HFS *vs* CBCI and CBCII. Values are means ± SD. Figure A (N=11-15), figure B (N=7-8) and figure C (N=6-8).

4.1.2 Glucose metabolism

Fasting glycemia presented a reduction in the animals that received administration of PEC, compared to the HFS group (**Figures 7 A and B**). The oGTT showed statistical difference for the area under the curve between the HFS group and the groups that received PEC, demonstrating an improved glucose homeostasis for the CBCI and CBCII animals (**Figures 7 C and D**). Concerning serum insulin, there was no significant difference between the groups, but a slight decrease was observed for the CBCI and CBCII groups in relation to the HFS group (**Figures 7. E and F**).

Figure 7 Fasting blood glucose, oral glucose tolerance test, and plasma insulin of mice fed on high-fat high-sucrose (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from cambuci at two doses (CBCI and CBCII) by gavage for 7 weeks.

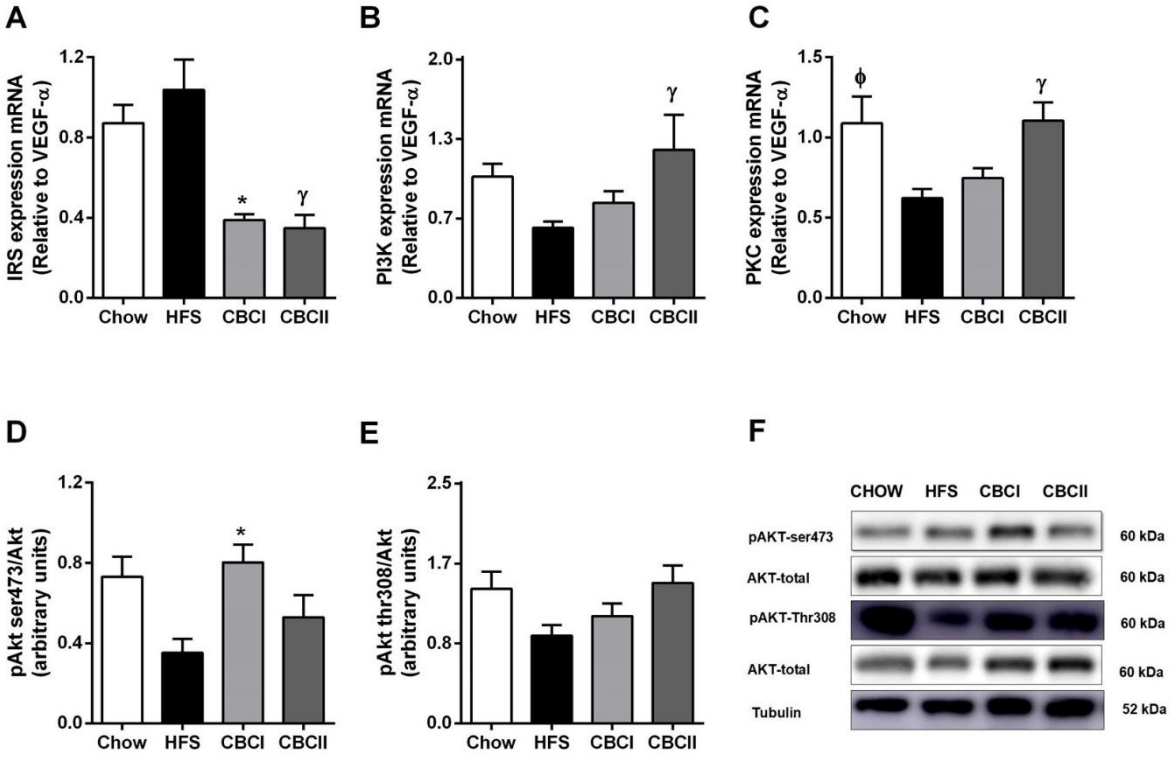


Results are expressed as mean ± SD. ϕ (*p< 0.05*) HFS vs. Chow, * (*p< 0.05*) HFS vs. CBCI, γ (*p< 0.05*) HFS vs. CBCII. A, B (N=6-9), figure C, D (N=8-9) and D, E (N=3-4).

To understand the mechanism of action of phenolic compounds from cambuci on glycemic levels, we investigated the pathway of glucose uptake in the skeletal

muscle evaluating protein modulation, including phosphorylation of protein kinase B PKB/AKT in Thr308 and Ser473, and the expression of the IRS, PI3K and PKC genes (**Figure 8**). It was found that PEC promoted reduction of IRS gene expression in both doses (CBCI and CBCII), when compared to the HFS group, and a significant increase in the PI3K and PKC gene expression of the animals that received the highest dose (CBCII) (**Figures 8 A, B and C)**. There was also a significant in the increase protein content of Akt phosphorylated in Ser 473 (**Figure 8 D)**. However, no significant difference in the content of Akt phosphorylated in Thr308 was noted (**Figure 8 E)**.

Figure 8 Gene expression of IRS , PI3K and PCK and immunoblots of Akt (Ser473 and Thr308) from the gastrocnemius muscle of mice fed high-fat high-sucrose (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from cambuci at two doses (CBCI and CBCII) by gavage for 7 weeks.

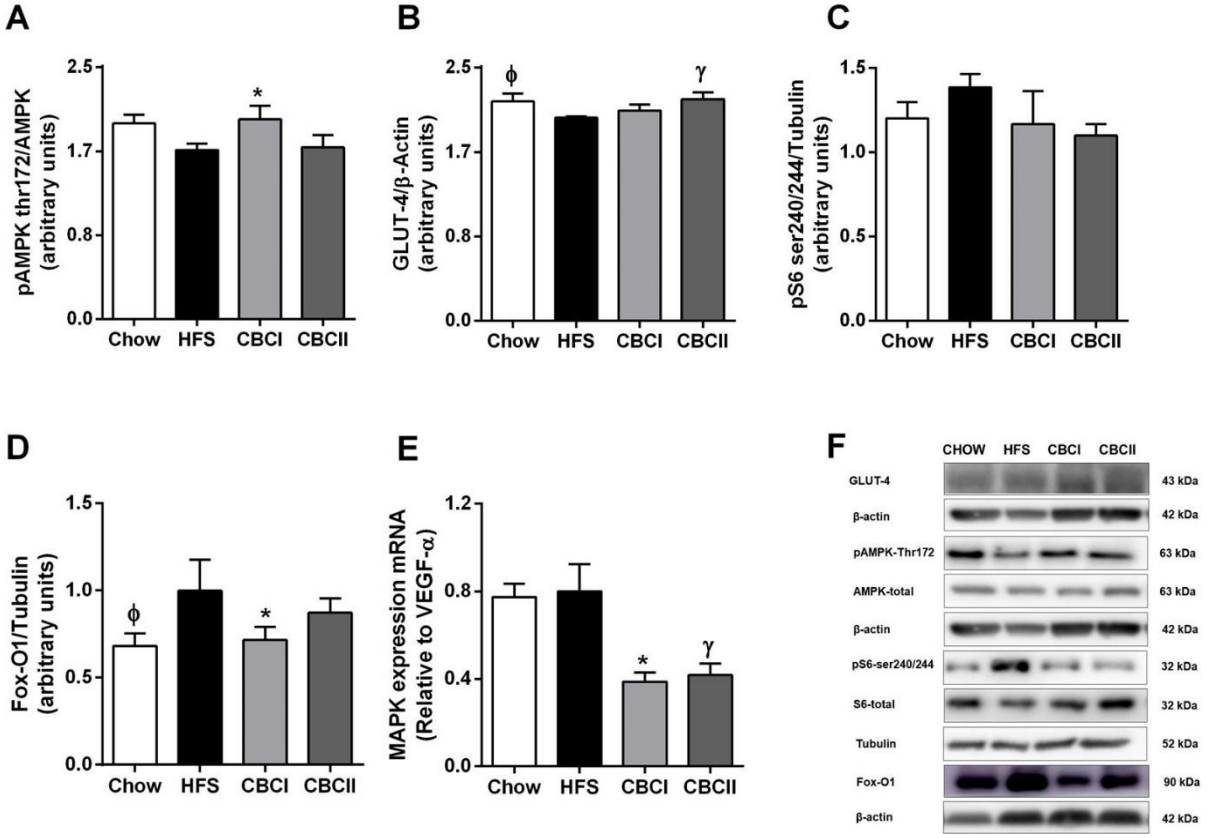


Gene expression IRS (A), PI3K (B) and PKC (C). Phospho-Akt Ser473 and Thr308 (D and E) representative immunoblots (F); with (n= 5-6) of all targets of obese mice fed high-fat/high-sucrose or chow diets and receiving water (Chow and HFS groups) or phenolic-rich extract from cambuci at two doses (CBCI and CBCII) by daily gavage. Data are means ± SEM (n=5-6). HFS vs. Chow, * ($p< 0.05$) HFS vs. CBCI, γ ($p< 0.05$) HFS vs. CBCII.

Our results showed that PEC significantly increased the content of the AMPK protein that acts as a nutrient sensor and is also a promoter of GLUT-4 translocation (**Figures 9 A and B**). On the other hand, phosphorylation of S6 protein did not show a marked change after CBC administration (**Figure 9 C**). There was a significant

reduction in the Fox-O1 content and MAPK gene expression for animals that received CBCI or CBCII (**Figure 9 D and E**).

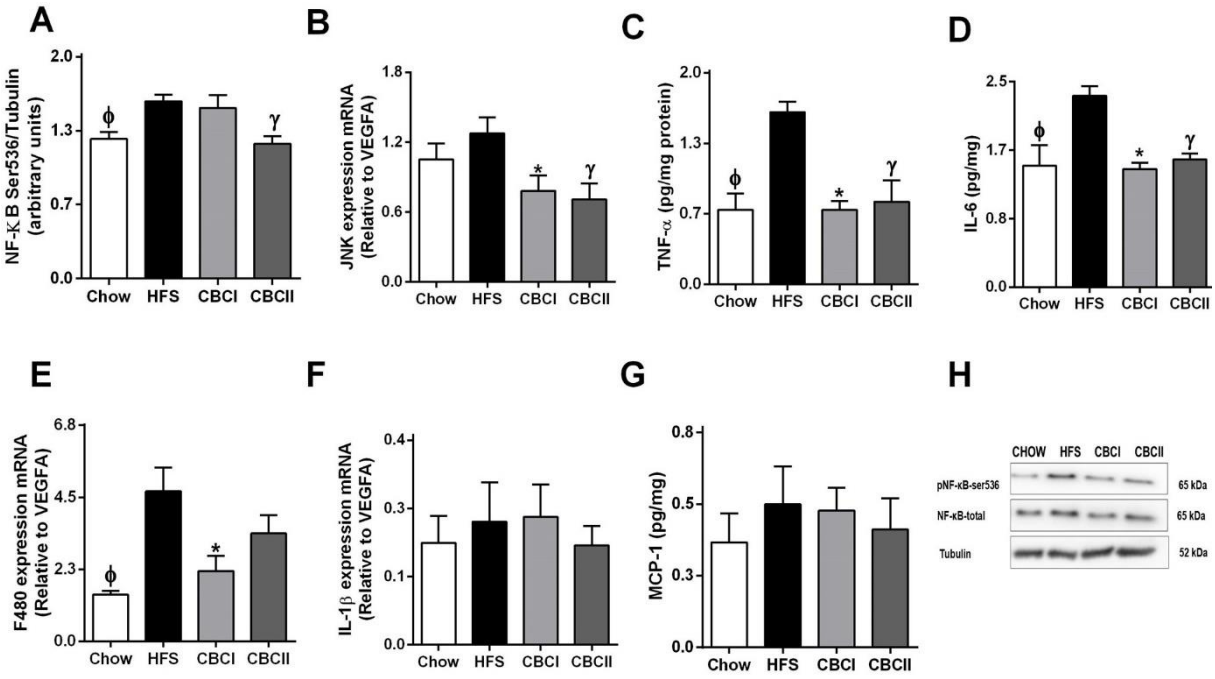
Figure 9 Immunoblots of AMPK (Thr172), GLUT-4, S6 (Ser240/244) and Fox-O1 and gene expression of MAPK from the gastrocnemius muscle of mice fed on high-fat high-sucrose (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from cambuci at two doses (CBCI and CBCII) by gavage for 7 weeks.



Representative immunoblots (F) of AMPK Thr172 (A) GLUT-4 (B), S6 Ser240/244 (C), and Fox-O1 (D), and gene expression of MAPK (E) of obese mice fed on high-fat/high-sucrose or chow diets and receiving water (Chow and HFS groups) or polyphenol-rich from cambuci extract at two doses (CBCI and CBCII) by daily gavage. Data are means ± SEM (N=5-6). HFS vs. Chow, * (*p< 0.05*) HFS vs. CBCI, γ (*p< 0.05*) HFS vs. CBCII.

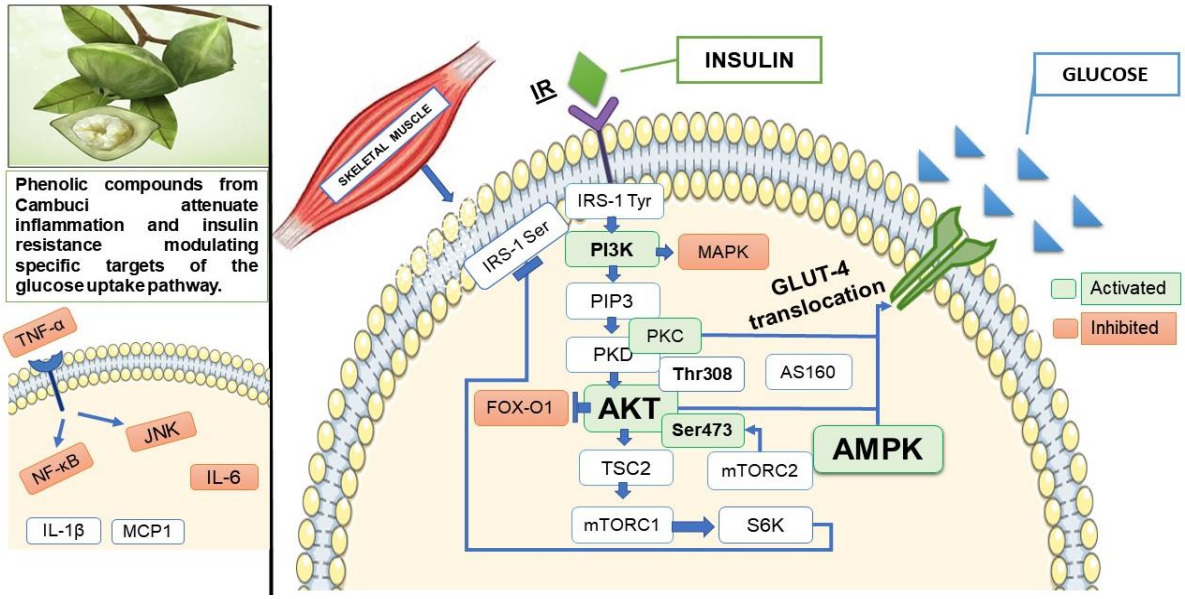
To further understand the influence of cambuci phenolic compounds on inflammatory responses, we measured the levels of IL-6, TNF-α, NF-κB, JNK, F4/80, IL-1β, and MCP1 in skeletal muscle. We observed that cambuci phenolic compounds reduced significantly levels of NF-κB, JNK, TNF-α, IL-6, and F4/80 which are pro-inflammatory cytokines, preventing the development of obesity-associated metabolic disorders, such as insulin resistance. (**Figure 10. A, B, C, D and E**) . There was no significant difference in the expression of IL-1β and MCP1 (**Figure 10 F and G**).

Figure 10 Representative immunoblots of NF-κB ser536 and, gene expression of JNK, F480, and IL-1β and levels of IL-6, TNF-α and MCP-1 from the gastrocnemius muscle of mice fed on high-fat high-sucrose (HFS) or chow diet (Chow) and receiving water or phenolic- rich extracts from cambuci at two doses (CBCI and CBCII) by gavage for 7 weeks.



Representative immunoblots (H) of NF-κB Ser536 (A), gene expression of JNK (B), F480 (E), and IL-1β (F) and levels of TNF- α (C), IL-6 (D) and MCP1 (G) of obese mice fed on high-fat/hight-sucrose or chow diets and receiving water (Chow and HFS groups) or polyphenol-rich from cambuci extract in two doses (CBCI and CBCII) by daily gavage. Data are means ± SEM (N=5-6). HFS vs. Chow, * ($p< 0.05$) HFS vs. CBCI, γ ($p< 0.05$) HFS vs. CBCII.

Figure 11 Schematic representation for the potential action of the phenolic compounds of cambuci on the glucose uptake pathway in gastrocnemius muscle of obese mice.



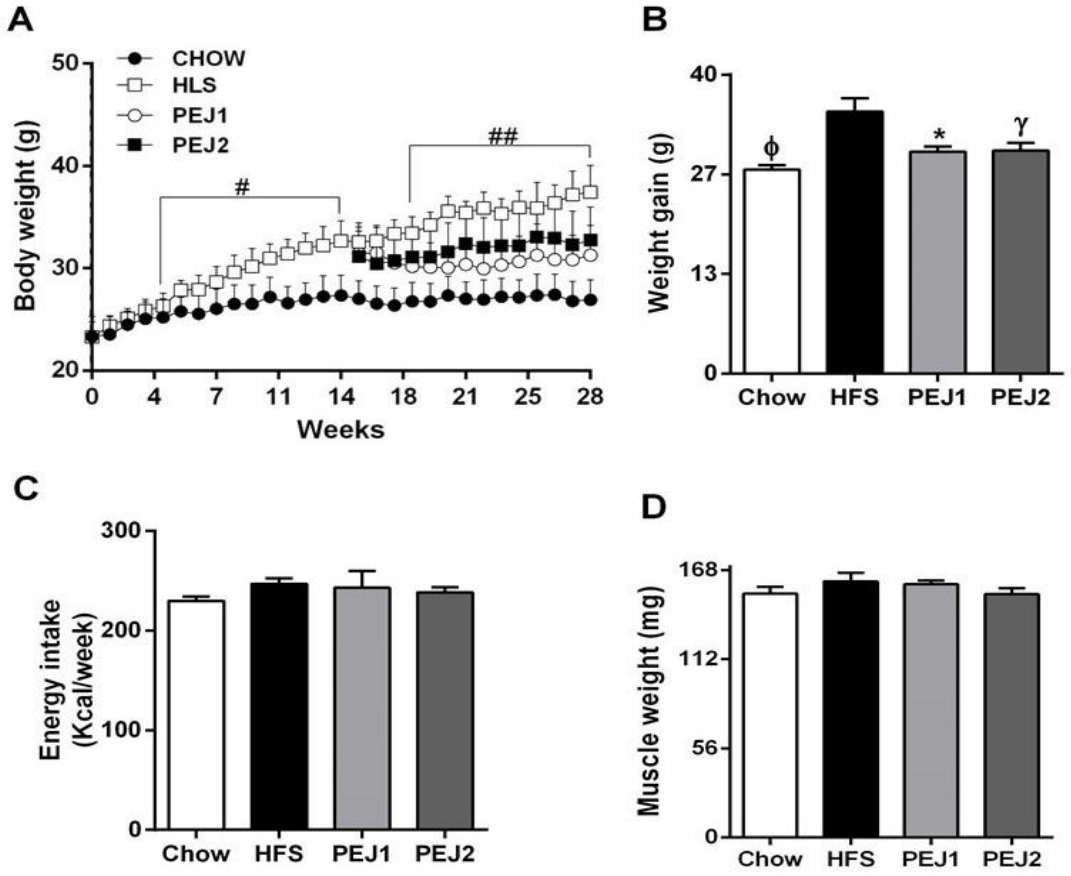
The proteins obtained by Western Blotting, RT-PCR, and ELISA are highlighted by green (Activation or increase in levels) and red (Inhibition or decrease in levels) color.
Source. The author, 2021.

4.2 Effect of Jaboticaba phenolics on body weight, energy intake, and glucose metabolism of obese mice.

4.2.1 Body weight and energy intake

As expected, after 5 weeks the body weight gain was significantly higher for mice fed the HFS diet, compared to the control diet. Mice receiving the administration of the phenolic-rich extract from jaboticaba at two doses, PEJ1 and PEJ2 (50 and 100 mg/kg body weight), showed a significant reduction in body weight (**Figure 12 A and B**). The energy intake did not differ among the groups fed Chow or HFS diets, receiving or not the phenolic extract of jaboticaba (**Figure 12.C).** In relation to the gastrocnemius muscle mass did not show a marked change after CBC administration (**Figure 12 D**).

Figure 12 Body weight, weight gain, energy intake and gastrocnemius muscle weight of mice fed on high-fat high-sucrose (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from jaboticaba at two doses (PEJ1 and PEJ2) by gavage for 14 weeks.

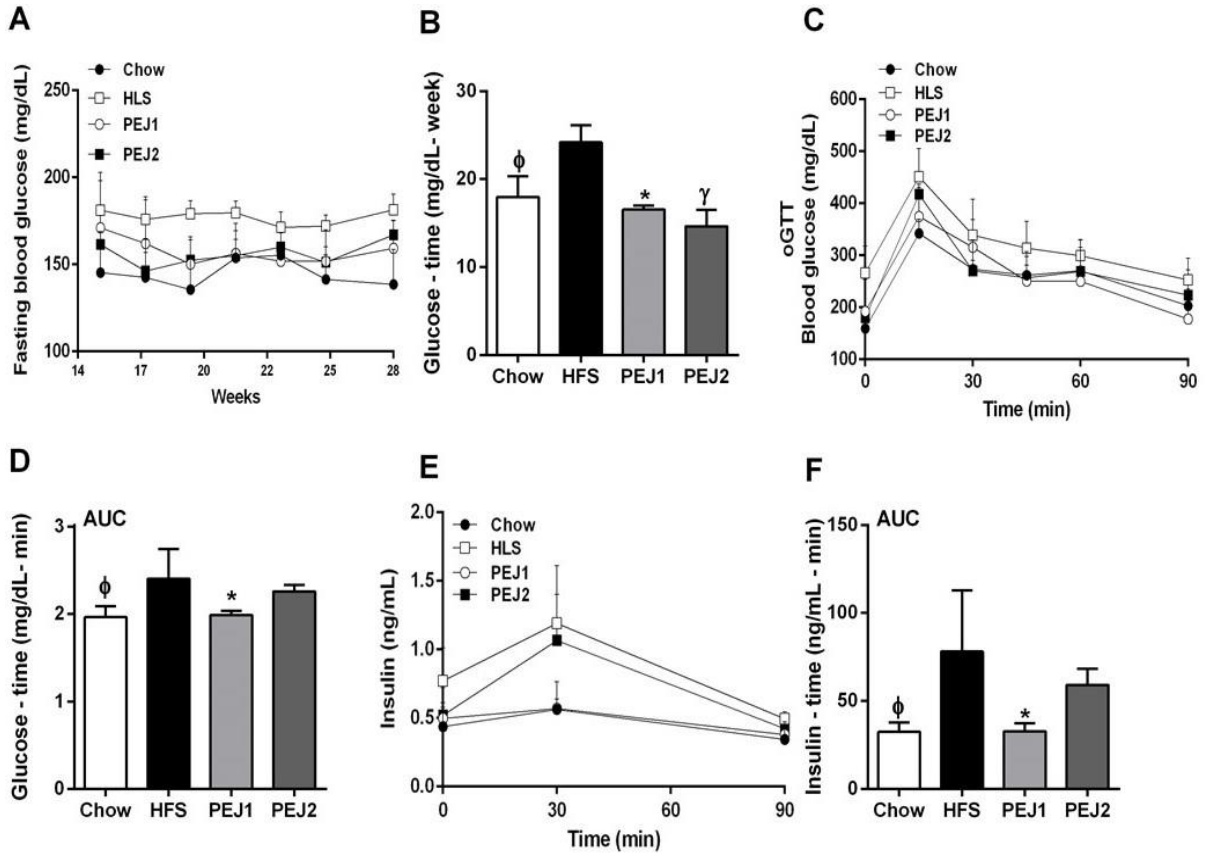


Results are expressed as mean ± SD. #(p<0.05) Chow *vs* HFS. ##(p<0.05) HFS *vs* PEJ1 and PEJ2. Figure A (N=10-14) and figure B (N=10-14).

4.2.1 Glucose metabolism

The PEJ administration was effective in promoting a reduction in the HFS-induced fasting hyperglycemia (**Figure 13 A and B**). To analyze the glycemic response, an oral glucose tolerance test (oGTT) was performed, which presented a statistically significant difference, with AUC significantly different ($p < 0.05$) between the HFS group and the supplemented groups (**Figure 13 C and D**). Plasma insulin levels during the oGTT showed a significant reduction in the area under the curve (AUC) for the PEJ1 group, compared to the HFS group (**Figure 13 E and F**)

Figure 13 Fasting blood glucose (A), oral glucose tolerance test (C), and plasma insulin (E) of mice fed on high-fat high-sucrose (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from jaboticaba at two doses (PEJ1 and PEJ2) by gavage for 14 weeks

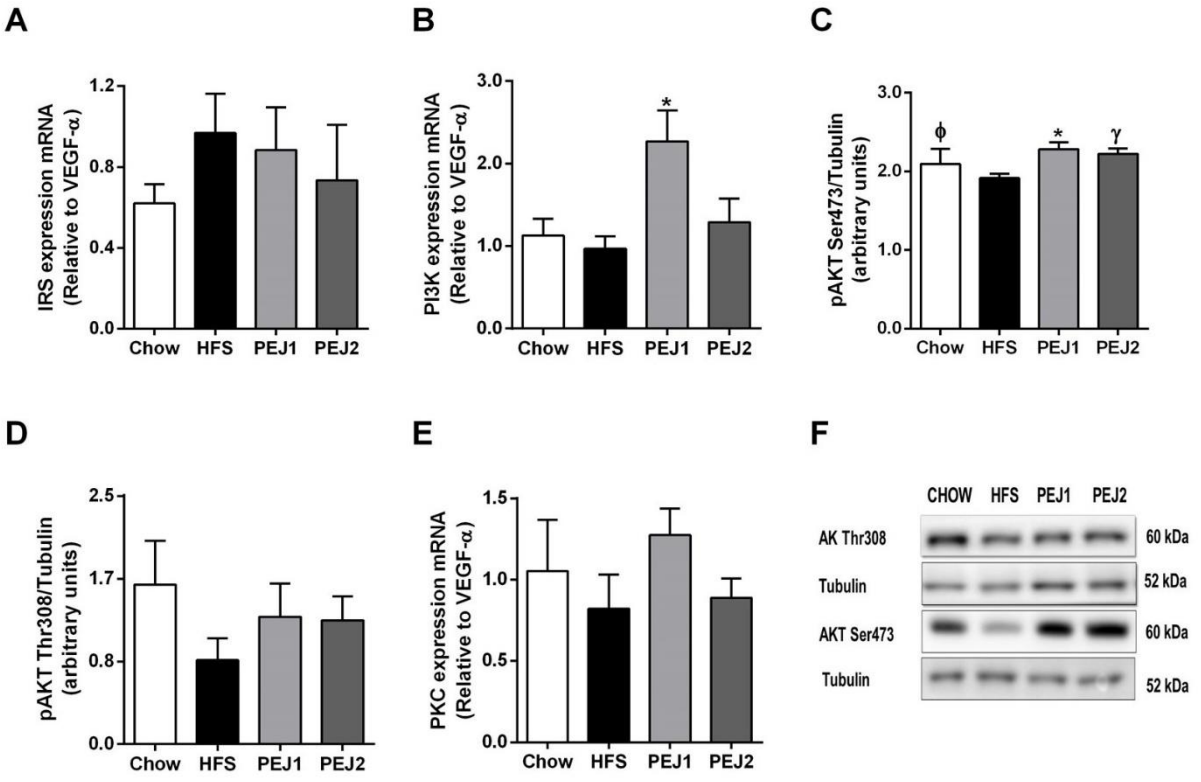


Statistical difference $p<0.05$. Values are means ± SD from. Figure A (N=7-15), figure B (N=6-9) and figure C (N=4-5).

Risk factors associated with obesity, like low-grade inflammation, can affect the expression of genes involved in insulin sensitivity across peripheral tissues (KANG et

al., 2019). When evaluating the action of the phenolic compounds of jaboticaba in the insulin signaling pathway of the skeletal muscle, it was observed that the PEJ promoted a slight reduction in the IRS-Ser gene expression, but there was no significant difference among both doses, when compared to the HFS group (**Figure 14 A**). Regarding the expression of the PI3K gene and the content of Akt Ser473 protein, there was a significant increase for animals receiving PEJ1 (PI3K) and both PEJ1 and PEJ2 (Akt Ser473) (**Figure 14 B and C**). No changes in the content of Akt phosphorylated in Thr308 and the expression of PKC gene were seen after PEJ administration(**Figure 14 D and E**).

Figure 14. Gene expression of IRS, PI3k and PKC, and immunoblots of phospho-Akt protein (Thr308) and (Ser473) from the gastrocnemius muscle of mice fed on a high-fat-sucrose diet (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from Jaboticaba at two doses (PEJ1 and PEJ2 groups).

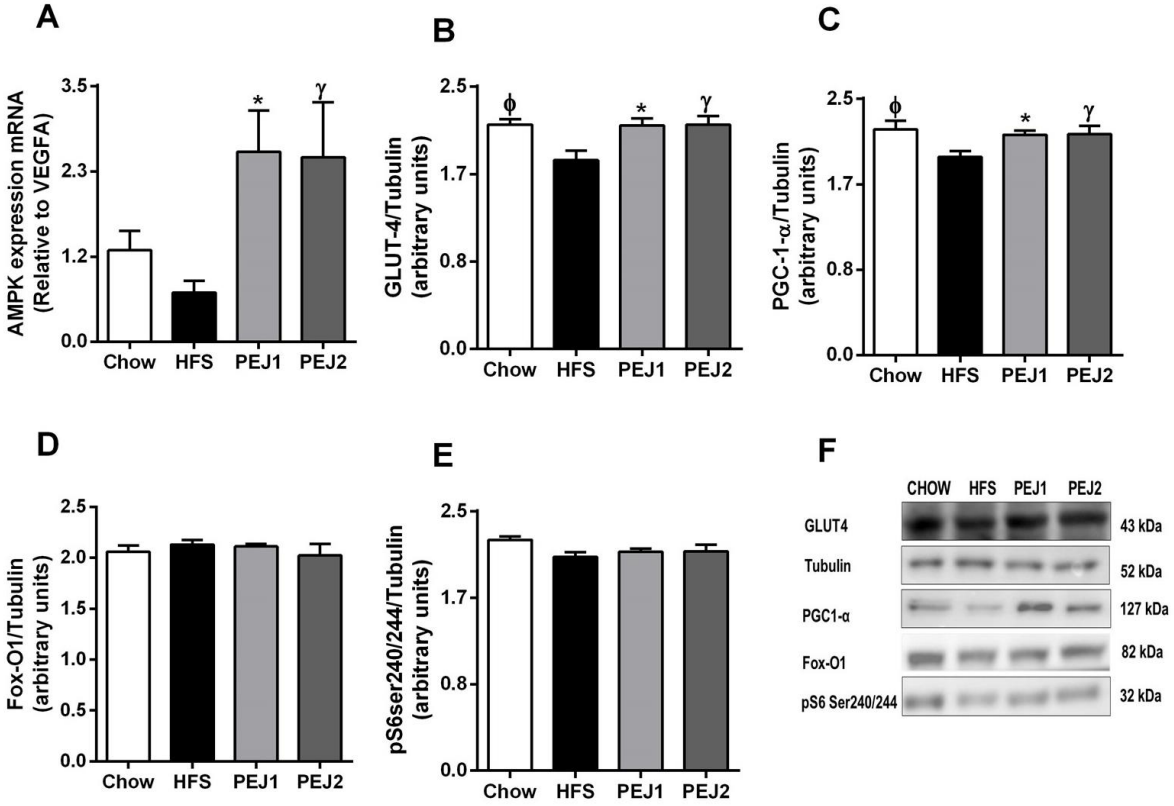


Gene expression IRS (A), PI3K (B), and PKC (E), and representative immunoblots (F), Phospho-Akt Ser473 and Thr308 (C and D) of obese mice fed on high-fat/high-sucrose or chow diets and receiving water (Chow and HFS groups) or polyphenol-rich jaboticaba extract in two doses (PEJ1 and PEJ2) by daily gavage. Data are means ± SEM from each treatment (n = 5-6). ϕ ($p< 0.05$) HFS vs. Chow, * ($p< 0.05$) HFS vs. PEJ1, γ ($p< 0.05$) HFS vs. PEJ2.

To better understand of the mechanisms by which jaboticada phenolic compounds improve insulin sensitivity, we evaluated other specific targets such as

AMPK, GLUT-4, PGC-1-α, Fox-O1 and S6. The results showed that PEJ at both doses significantly increased AMPK gene expression (**Figure 15.A**). There was also a significant increase in the content of GLUT-4 and PGC-1-α proteins in both groups that received PEJ administration (**Figure 15 B and C**). In relation to Fox-O1 and phospho-S6 Ser240 / 244 proteins, no significant differences were noted for the animals that received PEJ (**Figure 15 D and E**).

Figure 15 Gene expression of AMPK and representative immunoblots of the GLUT-4, PGC-1-α, Fox-O1 and phospho-S6 protein (Ser240/244) from the gastrocnemius muscle of mice fed on a high-fat-sucrose diet (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from Jaboticaba at two doses (PEJ1 and PEJ2 groups).

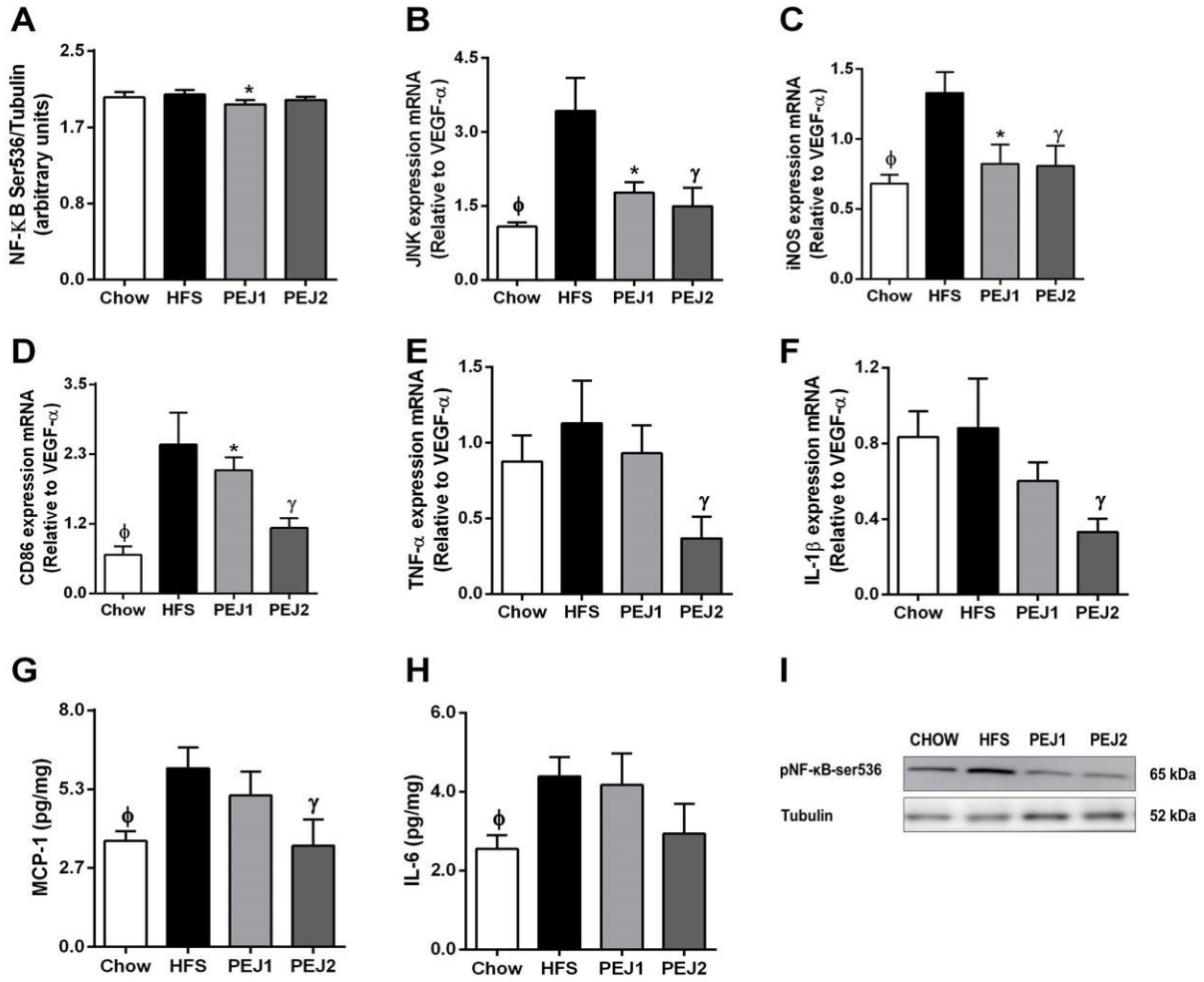


Gene exprerssion AMPK (A), and representative immunoblots (F) of GLUT-4 (B), PGC-1-α (C), Fox-O1 (D), and pS6 Ser240/244 (E) of obese mice fed on high-fat/high-sucrose or chow diets and receiving water (Chow and HFS groups) or polyphenol-rich jaboticaba extract in two doses (PEJ1 and PEJ2) by daily gavage. Data are means ± SEM from each treatment (n = 5-6). ϕ (*p< 0.05*) HFS vs. Chow, * (*p< 0.05*) HFS vs. PEJ1, γ (*p< 0.05*) HFS vs. PEJ2.

Since chronic activation of pro-inflammatory pathways may contribute to obesity-related insulin resistance in the skeletal muscle, we sought to evaluate whether PEJ could modulate the inflammation induced by high-fat/high-sucrose diet. Therefore, some targets such as NF-κB, TNF-α, JNK, iNOS, IL-1β, IL-6, MCP-1, and CD86 were investigated (**Figure 16).** There was also a significant reduction in the content of NF-

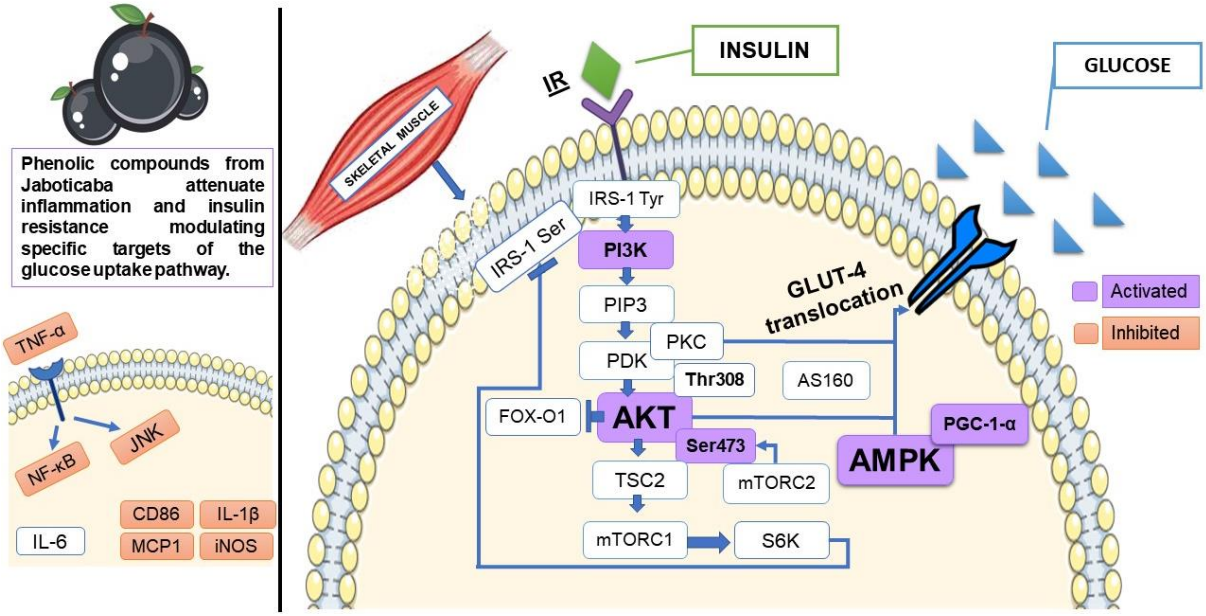
κB protein for animals supplemented with PEJ1 (**Figure 16 A**). Notably, the levels of JNK, iNOS and CD86 gene expression of the groups that received PEJ in both doses were significantly reduced (**Figure 16 B, C and D**). This effect was accompanied by a significant decrease in the levels of TNF-α and IL-1β gene expression of animals that received PEJ2 (**Figure 16 E and F**). In addition, the concentration of MCP-1 decreased in PEJ2 animals (**Figure 15 G**); however, concentrations of the proinflammatory cytokines IL-6 showed no significant difference among the groups (**Figure 16 H**).

Figure 16 Representative immunoblots of phospho-NF-κB (Ser536) and Gene expression of JNK, iNOS, CD86, TNF-α, and IL-1β, and levels of IL-6 and MCP-1, from the gastrocnemius muscle of mice fed on a high-fat-sucrose diet (HFS) or chow diet (Chow) and receiving water or phenolic-rich extracts from Jaboticaba at two doses (PEJ1 and PEJ2 groups).



Representative immunoblots (I) of NF-κB Ser536 (A), gene expression of JNK (B), iNOS (C), and CD86 (D), TNF-α (D) and IL-1β (F), and levels of MCP-1 (G) and IL-6 (H) of obese mice fed on high-fat/high-sucrose or chow diets and receiving water (Chow and HFS groups) or polyphenol-rich jaboticaba extract in two doses (PEJ1 and PEJ2) by daily gavage. Data are means ± SEM from each treatment (n = 5-6). ϕ ($p< 0.05$) HFS vs. Chow, * ($p< 0.05$) HFS vs. PEJ1, γ ($p< 0.05$) HFS vs. PEJ2.

Figure 17 Schematic representation of the potential action of the phenolic compounds of jaboticaba on the glucose uptake pathway in gastrocnemius muscle of obese mice.



The proteins obtained by Western Blotting, RT-PCR, ELISA are highlighted by purple (Activation or increase in levels) and red (Inhibition or decrease in levels) color.
Source. The author, 2021.

In this work, the effects of phenolic-rich extracts produced from Brazilian native fruits, namely cambuci and jabuticaba, through solid-phase extraction, were evaluated on an animal obesity model, in relation to body weight gain, energy intake, glucose metabolism, and inflammation.

Mice fed a HFS diet showed excessive weight gain during the experiment: however, PEC and PEJ administration provided protection against diet-induced weight gain.Previous studies have demonstrated that the intake of dietary phenolics may have beneficial health effects, including protection against obesity, reducing body weight gain in animals (DEL RIO et al., 2013; DONADO-PESTANA et al., 2015). Some reports have described that bioactive compounds of jaboticaba could play a role in the reduction of weight gain and metabolic changes in obese animals (LENQUISTE et al., 2012; MOURA et al., 2018).

Our findings demonstrasted that PEC and PEJ improved glucose homeostasis in obese mice, as evidenced by improved glucose tolerance and attenuated hyperglycemia. Furthermore, mice supplemented with PEJ also showed improvement in hyperinsulinemia.The protective properties of the cambuci and jaboticaba phenolic compounds may be associated with several glycemia regulatory mechanisms, involving diverse pathways. Evidence suggests that phenolic compounds may play a

key role in the inhibition of α-glucosidase and α-amylase. The inhibition of α-glucosidase and α-amylase reduces digestion and intestinal absorption of glucose, consequently controlling the postprandial glycemic response, which is key to the management of type 2 diabetes (GONÇALVES; LAJOLO; GENOVESE, 2010; ZHANG et al., 2015).

Previously, it was demonstrated that phenolic compounds from cambuci decreased fasting hyperglycemia and glucose intolerance in a preventive model of obesity (DONADO-PESTANA; BELCHIOR; GENOVESE, 2015). Antihyperglycemic effects of phenolic compounds commonly found in plant foods are related to an improvement in glucose metabolism through reduction of glycosylated hemoglobin (Hb), blood glucose level, and hepatic glycogen, and improvement of the antioxidant status in the pancreas (OYEDEMI et al., 2019).

Phenolic compounds may help improving glycemic homeostasis by modulating key proteins in the glucose uptake pathway. In skeletal muscle, translocation of the glucose transporter type 4 (GLUT-4) by insulin is important for glycemic homeostasis. The GLUT-4 translocation is regulated by a complex cascade of multiple protein kinases. In the insulin pathway, binding of insulin activates the tyrosine kinase activity of its receptor, which phosphorylates insulin receptor substrate 1 (IRS-1) followed by phosphorylation of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), that induces phosphorylation of Akt, and finally, regulating GLUT-4 translocation to the plasma membrane (UEDA-WAKAGI et al., 2015).

Our results demonstrated that PEC and PEJ were able to increase the PI3K gene expression; and PEC also increased the PKC gene expression, which may be related to the improvement in the insulin signaling pathway. In addition, there was a significant reduction of Fox-O1 protein in animals supplemented with PEC, whose overexpression is related to the accumulation of lipids in the liver, worsening insulin resistance. In the liver, insulin resistance is prominent in *ob/ob* mice and involves an impairment of the Akt protein and subsequent impairment of Fox-O1 phosphorylation, leading to increased expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). These changes may lead to an increase in the production of hepatic glucose and also to hepatic steatosis. This imbalance in the liver impairs insulin signaling in the skeletal muscle (LANGLET et al., 2017). A study with obese humans also demonstrated that a

profound endothelial resistance to insulin in adipose tissue improved with the inhibition of Fox-O1 (KARKI et al., 2015).

The insulin receptor is a protein that has a crucial role in controlling glucose homeostasis. Naturally, binding of insulin to the extracellular domain of the insulin receptor α subunit triggers autophosphorylation of the insulin receptor on the insulin receptor substrate type 1 (IRS-1) on tyrosine residues and this is required for insulin-stimulated responses (SCAPIN et al., 2018). On the other hand, phosphorylation of IRS-1 on serine residues may modulate its degradation, affecting insulin sensitivity. Interestingly, this study shows that the administration of extracts rich in cambuci phenolic compounds demonstrated a significant reduction of the IRS-1 gene expression. Studies have shown that inflammatory cytokines contribute to insulin resistance, increasing phosphorylation of IRS-1 in serine residues, which has been reported during insulin resistance (CHUANG et al., 2011).

On the other hand, Akt protein is key in the glucose uptake by the muscle. Akt is recognized as a serine/threonine protein kinase expressed primarily in insulin-sensitive tissues such as liver, muscle, and adipose tissue, being a fundamental mediator for GLUT-4 translocation and consequently, for glucose uptake and glycogen synthesis (RATHINAM; FITZGERALD, 2017; SAYEM et al., 2018). The administration of PEC and PEJ demonstrated a potential effect against insulin resistance in the skeletal muscle by increasing the content of Akt Ser473, as well as the GLUT-4 content available to eventual translocation, a key event in the improvement of glucose uptake by muscle. These findings corroborate a recent study by Moura et al. (2021), which showing that the PEJ improved glucose metabolism, by increasing the GLUT-4 content in skeletal muscle, regulating positively the AKT/mTORC pathway.

Animals receiving PEC and PEJ showed a increase of AMPK , which has a relevant role in metabolic and energy regulation. Evidence suggests that AMPK stimulates the translocation of glucose transporter GLUT4 to the plasma membrane increasing the entrance of glucose in the cell. A study by Peng; Sun; Park (2019) showed that chicoric acid, a phenolic compound belonging to the subclass of hydroxycinnamic acids, promoted insulin-independent glucose uptake and Akt phosphorylation by the regulation of AMPKα in cultured C2C12 myotubes, and improved glucose tolerance in a mouse model.

Polyphenols from cambuci, also a Myrtaceae fruit, such as jaboticaba, demonstrated beneficial health properties by improving insulin resistance through the

activation of both Akt and AMPK in the liver and skeletal muscle (DONADO-PESTANA et al., 2021). In the same way, with phenolic compounds from *Vernonia cinerea (Cyanthillium cinereum)* increased insulin sensitivity in obese mice by modulating the PI3K/Akt and AMPK pathways in the liver, skeletal muscle and adipose tissue (NAOWABOOT; WANNASIRI; PANNANGPETCH, 2018).

In parallel, AMPK also inhibits the activity of the mTORC1 protein (LIU et al., 2016). In the presence of nutrients, such as amino acids and glucose,mTORC1 functions in a mitogenic pathway downstream of PI3K and is activated by insulin. In addition, it has been shown that the activation of mTOR and S6K plays a role in insulin resistance through serine phosphorylation of IRS to attenuate signal flow to downstream effectors (SAXTON; SABATINI, 2017).

In a study by Kang et al. (2018), the anti-inflammatory and antidiabetic effects of two algae types, *Laminaria japonica* (LJ) and *Hizikia fusiforme* (HF), were evaluated in skeletal muscle of C57BL/6 mice. The results demonstrated that mice fed a high-fat diet supplemented with 5% of LJ or HF, containing high amounts of phenolic compounds, showed an improved glucose uptake partially due to the activation of Akt and AMPK in the muscle.

In skeletal muscle, reduced mitochondrial function may be related to the development of metabolic diseases such as T2DM (PINTI et al., 2021). PEJ increased the content of PGC1-α protein, which may be related to improved oxidative metabolism and mitochondrial biogenesis in the skeletal muscle. Corroborating our results, daidzein, a phenolic compound found in soybeans, was shown to regulate regulates mitochondrial biogenesis by modulating specific targets such as SIRT1, PGC1-a and AMPK in muscle, protecting against chronic diseases (YOSHINO et al., 2015) .

Obesity is currently characterized by a broad inflammatory response that induces the release of proinflammatory cytokines and immune cell infiltration. These events are closely associated with the development of insulin resistance through interactions with the insulin signaling pathway in peripheral tissues (ZATTERALE et al., 2020). It is known that changes in the secretion of chemokines and inflammatory cytokines observed in the framework of obesity are important factors for the development of insulin resistance (MAKKI; FROGUEL; WOLOWCZUK, 2013). This study showed that PEJ and PEC were able to down-regulate pro-inflammatory pathways involved in the obesity-induced chronic inflammation and the pathogenesis of insulin resistance. In obesity, adipose cells expand and recruit immune cells such

as macrophages leading to an increase in peripheral inflammation through pro-inflammatory cytokines, such as IL-6 and TNF-α. The inflammatory environment in the tissue plays a key role in the development of insulin resistance (WU et al., 2017). In fact, a recent study by Shabani et al., (2020), demonstrated that a decreased macrophage infiltration into skeletal muscle of HFD-fed mice leads to the polarization of macrophage to the M2 direction, as well as decreases the expression of pro-inflammatory cytokines including tumor necrosis factor α (TNF-α), interleukin 1 β (IL-1β) and interleukin 6 (IL-6).

The study by Dragano et al., (2013) that utilized anthocyanin-rich jaboticaba peel demonstrated that supplementation was effective in reducing insulin resistance, confirmed by enhanced signal transduction through IRS and Akt and by the attenuation of hepatic inflammation, as evidenced by lower IL-1β and IL-6 expression and decreased levels of phosphorylated IkB-α protein in mice supplemented with jaboticaba peel. In addition, raspberry phenolics also promoted an improvement in insulin signaling by decreasing levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in the skeletal muscle of mice fed a high fat diet (ZHAO et al., 2018)

In summary, both PEC and PEJ possess beneficial properties against obesity-induced insulin resistance in the skeletal muscle. These properties are related to improved signal transduction mediated by Akt Ser473, PI3K, and GLUT-4, which are essentials in insulin signaling pathway. Our results also demonstrated that the AMPK pathway can contribute to improving glucose metabolism by promoting GLUT-4 translocation. There was a reduction in the levels of Fox-O1 and MAPK for animals that received PEC, which in a state of metabolic disturbances as obesity have deleterious effects on glycemic homeostasis. In addition, it was observed that the phenolic compounds of both fruits significantly reduced inflammation in skeletal muscle, contributing to an improvement in glycemic metabolism (**Figure 11 and 17**).

5. CONCLUSIONS

The phenolic compounds present in jaboticaba and cambuci were able to attenuate body weight gain and improve glycemic homeostasis in a murine model of diet-induced obesity. Interestingly, phenolic extracts of both fruits enhanced the glucose uptake pathway through the PI3K/Akt/GLUT-4 and AMPK pathways and ameliorated obesity-associated insulin resistance in the skeletal muscle.. In addition to the improvement in the glucose uptake pathway, both extracts also had an anti-inflammatory effect reducing the levels of pro-inflammatory cytokines such as TNF-α, IL-6, as well as some markers including NF-kB and JNK. Thus, the findings of this study suggest that the phenolics present in both Brazilian native fruits may be important therapeutic agents in attenuating insulin resistance in skeletal muscle and obesity-associated inflammation.

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