UNIVERSIDAD AUTÓNOMA METROPOLITANA

UNIDAD IZTAPALAPA

"Study In vitro and *in vivo* of anti-diabetic and anti-obesity active principles of *Curcubita ficifolia* Bouche and *Vaccinium angustifolium* Ait"

TESIS

QUE PARA OBTENER EL GRADO DE

DOCTORA EN BIOLOGIA EXPERIMENTAL

PRESENTA

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Ciuidad de México, 2017

El Programa de Doctorado en Biología Experimental de la Universidad Autónoma Metropolitana pertenece al Programa Nacional de Posgrados de Calidad (PNPC) del CONACYT, registro 001481, en el Nivel Consolidado, y cuenta con apoyo del mismo Consejo, clave DAFCYT-2003IMPTNNN0020”

Este trabajo fue realizado en el laboratorio de Natural Health Products and Metabolic Diseases Laboratory and CIHR Team in Aboriginal Anti-diabetic Medicines, del Departamento de Farmacología y Fisiología de la Facultad de Medicina en la Universidad de Montréal, Quebec, Canadá y en el laboratorio de Farmacología del Departamento de Ciencias de la Salud la Universidad Autonóma Metropolitana Unidad Iztapalapa de la Ciudad de México, México.

El presente trabajo se realizó gracias al apoyo proporcionado por el CONACYT a través de la beca con Número de registro 270963 y al apoyo financiero brindado por Atlantic Food and Horticulture Research Center, Agriculture and Agrifood Canada, Nova Scotia, Canada.

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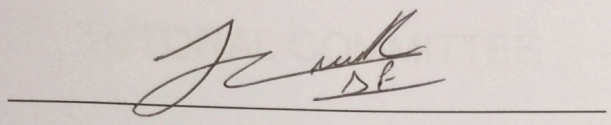

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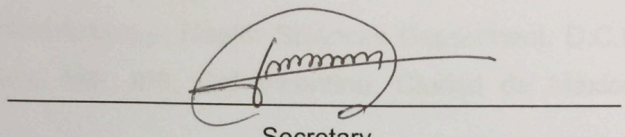

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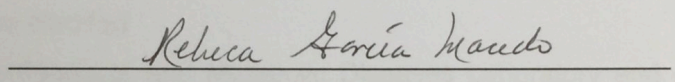

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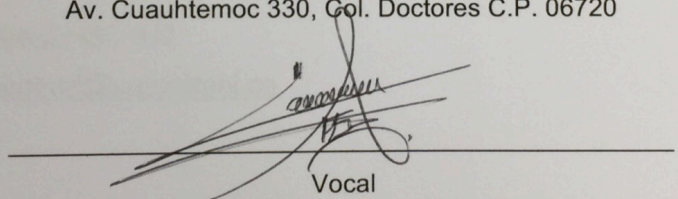

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Aknowlegment

I thank Consejo Nacional de Ciencia y Tecnología (CONACyT) from Mexican goverment to have provided the funds for this PhD research Project (scholarship no. 270963). I also acknowledge Agriculture and Agri-food Canada and the DIAP (Developing Innovative Agrifood Products) program for the funds to support a large part of this research project. My gratefulness goes to my co-supervisor Dr Francisco Javier Alarcón Aguilar for his unconditional support, encouragement and advice. To my co-supervisor Dr Pierre Haddad, my infinite thanks whose constantly encouragement and guidance that believed in me and made it possible providing valuable advice and support during difficult moments. I would like to express my great appreciation to Dr Ma. Del Carmen Escobar Villanueva for her unconditional support, advice in all moments at any time an honor received the guidance and supervise from all of you.

Special thanks to all my collegues (UAM-I and UM) for helping me to get through the difficult times, and for all the support, and caring they gave along this project.

DEDICATIONS

To my best friend, my daughter Camila, for her love, support and company all over allong this challenge. Thanks to make me so happy!!

To Edtson my husband and Yolita, Gaby y Emilio for the unconditional support in all moment, thousand thanks.

To my parents: Mitos y el Pollo, my sisters: Mabe y Allo and brother: Jaime, and my nieces: Paolita, Mixtli, Cintli y Mamoy and my nephew: Ligui for the huge support advise and bunch of love thousand thanks.

I also would like to dedicate part of this work to Rosita “Pink” and my uncle Gil that were in my life since I remember. These two persons touched many lifes and many lifes touched them. In their memory.

Resumen

Las propiedades antidiabéticas y antiobesidad de *V. angustifolium* y *C. ficifolia* han sido reportadas *in vivo* e *in vit*ro, por lo que ambas plantas ya cuentan con antecedentes importantes en la investigación de la diabetes y la obesidad. Sin embargo, sus mecanismos de acción y/o principios activos aún no están claros. Los tratamientos con el extracto fermentado FBE (F1), Fenoles (F2) y ácido clorogénico (CA) (F3-2) de *V. angustifolium* (Fermented Blueberry Extract) mostraron inhibición significativa en la acumulación de triglicéridos de hasta 70-75% en concentraciones de 5 μg/ml. El fraccionamiento y purificación permitió la obtención de otro compuesto puro, el catecol (CAT), que inhibió la adipogénesis hasta en un 75%. Ambos compuestos mostraron un papel importante en la disminución de trigliceridos. En los tratamientos que se llevaron a cabo *in vivo*, FBE mostró reducción significativa en la glucemia de ratones “Diet-induced models of obesity” DIO con respecto al control DIO y Chow. Sin embargo, no tuvo ningún efecto sobre el peso corporal. En la prueba de tolerancia a la glucosa intraperitoneal sólo se observaron diferencias significativas entre los tratamientos del extracto no fermentado con el control DIO en los primeros 15 y 60 min. Por analisis de Western Blot, también se investigó el papel de p-Akt, p-AMPK, SREBP1c y PPAR gamma en células 3T3-L1 tratadas con NBE, FBE, 7 fracciones y cuatro compuestos puros de *V. Angustifolium*. Con NBE, F1, F3-2, CAT y CA se observó un decremento para p-AKT, así como una tendencia para incrementar p-AMPK, especialmente en los tratamientos de F1, F2 y F3-2. Para la expresión de SREBP1-c no se observó ningun cambio significativo.

En contraste, PPARγ mostró un decremento significativo en todos los grupos experimentales que mostraron efecto inhibitorio en la acumulación de trigliceridos. Estos resultados demuestran que el extracto fermentado de blueberry contiene compuestos con actividad antiadipogénica, la cual podria servir para la estandarización de preparaciones nutracéuticas, asi como para el desarrollo de nuevos compuestos con propiedades antiobesidad. Para *C. ficifolia* se observó un efecto inhibitorio en una concentración de 20μg/ml sobre el contenido de triglicéridos con el extracto completo de hasta un 70% con respecto al control. En el analisis de Western Blot, las celulas 3T3-L1 tratadas con el extracto de *C. ficifolia* y fracciones primarias presentando una disminución en el proceso de adipogénesis. Se analizaron tambien las vias de señalización de p-AMPK, donde se observó tendencia a incrementar la forma fosforilada de AMPK en las celulas tratadas con el extracto de *C. ficifolia* con respecto al control DMSO; sin embargo, no hubo diferencias significativas en la activación de AMPK. *C. ficifolia* no presentó actividad positiva en el transporte de glucosa en comparación con el control en concentración óptima de 20μg/ml. Para la actividad enzimatica de glucosa-6-fosfatasa se observó inhibición de la enzima en celulas H4IIE de hepatocitos tratados con 40 μg/ml del extracto de *C. ficifolia*.

La fermentación de *V. angustifolium* presentó propiedades antidiabéticas y antiobesidad novedosas y potentes *in vitro*.

Esto confirma lo observado en investigaciones anteriores, por lo que estos resultados confieren un paso más en la investigación referente a los posibles mecanismos de acción y las vias de señalización por las cuales estos compuestos ejercen su acción. El fraccionamiento primario de *C. ficifolia* tambien representa un adelanto para el desarrollo de nuevos agentes terapéuticos para el tratamiento de la diabetes mellitus tipo 2, además de contribuir en la investigación relacionada a la identificación de componentes responsables de sus efectos antidiabético y de antiobesidad.

Abstract

Anti-diabetic and anti-obesity properties of *C. ficifolia* Bouche and *V. angustifolium* Aiton have been reported in *in vivo* and *in vitro* assays and thus are relatively well studied. However, their mechanisms of action and/or active principles still remain unclear. The inhibitory effect of Fermented Blueberry Extract (FBE) on triglyceride (TG) accumulation in adipocytes was attributed to fractions FBE (F1), phenolics (F2) and chlorogenic acid (F3-2) (70%) at a dose of 5 μg/ml. Further fractionation and purification yielded other pure compound, Catechol (CAT) that inhibited adipogenesis by 75%. *In vivo* FBE had a tendency to reduce glycaemia in DIO mice but did not have any effect on body weight with a significant difference on concentration 7.6 ml/kg of FBE respect to control DIO and CHOW. In Intraperitoneal glucose tolerance test only significant differences were observed between treatments from NBE extract and control DIO from the first 15 and 60 min. Examination of intracellular signalling components (p-Akt, p-AMPK) and transcriptional factors (SREBP-1c and PPARγ) was carried out by Western blot analysis. Treatment with NBE, F1, F3-2, CAT and CA decreased p-AKT, whereas p-AMPK tended to increase with F1, F2 and F3-2. The expression of SREBP1-c was not significantly modulated. In contrast, PPARγ decreased in all experimental groups inhibiting adipogenesis. These results demonstrated that FBE contains compounds with anti-adipogenic activity, which can serve to standardize nutraceutical preparations from fermented blueberry juice and to develop novel compounds with anti-obesity properties.

Fermentation conferred novel and potent *in vitro* anti-diabetic and anti-obesity properties to blueberry extract. Fermented blueberry extract (FBE) may represent a complementary therapy and a source of novel therapeutic agents against type 2 diabetes mellitus. In addition, this is one of the first steps in the identification of the principles responsables of its anti-diabetic and anti-obesity effects.

The optimal concentration of *C. ficifolia* crude extract (20 µg/mL) was tested in C2C12 murine myoblasts for glucose transport assay since skeletal muscle is an important insulin-target tissue in glucose homeostasis. However, it did not induce glucose transport when compared to control. *C. ficifolia* crude extract was also tested at the optimal concentration (20µg/ml) on adipogenesis process using 3T3-L1 cells, showing 70% of inhibition in the intracellular content of triglycerides (TG). Preliminary results from *C. ficifolia* extract and primary fractions showed effect in F16 and F17 with significance in respect to control DMSO. For the activity of glucose 6-phosphatase, H4IIE hepatocytes treated with 40 µg/ml of extract displayed approximately 60% enzyme inhibition. The primary fractionation of *C. ficifolia* crude extract also represents an advance for the development on the phytochemistry from the plant that might allow us to identify the active components responsible for its potential anti-diabetic and anti-obesity for the development of new therapeutic agents for the treatment of type 2 diabetes and obesity.

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I. Introduction

Plants have been used since early times with different purposes (Lawrence and Bennett, 1995; Evans, 2009) and medicinal herbs are being increasingly studied by pharmacological research (Sinclair, 1998). According to the World Health Organization (WHO) more than 80% of the world's populations rely on traditional medicine for their primary healthcare, majority of which use plants or their active principles (Gupta et al., 2005).

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. It involves disorders of the metabolism of carbohydrates, lipids and proteins. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is a deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia (ADA, 2009).

Pathogenic processes like destruction of the β-cells of the pancreas lead to insulin deficiency as in type 1 diabetes mellitus (T1D) whereas abnormalities that result in resistance to insulin action are often observed in type 2 diabetes (T2D) (ADA, 2008) can be observed. T2D is a global public health crisis that threatens the

economies of all nations, particularly developing countries. Fueled by rapid urbanization, nutrition transition, and increasingly sedentary lifestyles, the epidemic has grown in parallel with the worldwide rise in obesity (Frank, 2011).

Of the two types of diabetes mellitus, most of this growth has been driven by the increasing prevalence of T2D, whereas the prevalence of T1D has remained relatively stable (Smyth and Heron, 2006). The WHO attributes this growth to population growth, aging, unhealthy diets, obesity and increasingly sedentary lifestyles (WHO, 2015). The prevalence of overweight has continued to increase at an alarming rate, especially in younger age groups, and is a major risk factor for chronic diseases. It plays a central role in the ‘‘insulin resistance’’ or ‘‘metabolic’’ syndrome, which includes hyperinsulinemia, hypertension, hyperlipidemia, glucose intolerance, and an increased risk of atherosclerotic cardiovascular disease (Roya, 2007).

Metabolic dysfunction in T2D is also the product of reduced glucose effectiveness or the ability of glucose to be transported itself by a mass action effect (facilitated glucose transport). Central to this metabolic condition is altered glucose and lipid metabolism resulting from the combined effects of insulin resistance in skeletal muscle, hepatic, renal, and adipose tissue. The resulting hyperglycemia is the primary cause of the secondary complications associated with T2D (Basu et al., 1997).

There are two specific pathways: first, the one that encourage glucose transport activity in skeletal muscle, 5′-monophosphate-activated protein kinase (AMPK)-dependent mechanisms and second an insulin-dependent pathway activated via

upregulation of serine/threonine protein kinase Akt. Both could be good targets of research for the treatment of T2D and obesity (Mackenzie and Elliott, 2014).

Another key enzyme in T2D is glucose-6-phosphatase (G6Pase), an enzyme found mainly in the liver and the kidney that plays the important role of providing glucose during fasting. Glucose homeostasis is the result of a balance between glucose production by the liver (gluconeogenesis), its storage as glycogen (liver and muscle), and its uptake by peripheral tissues, notably insulin-responsive skeletal muscle and adipose tissue. Indeed, insulin, a hormone secreted by the beta pancreatic cells, works by decreasing glucose production in the liver, stimulating its uptake by skeletal muscle and peripheral tissues and enhancing its storage as glycogen (Arion et al., 1972).

1.1 Diabetes classification (AQUI ME QUEDE)

1.1.1 Type 1 diabetes

Type 1 diabetes (T1D) involve β-cell destruction, usually leading to absence of insulin, and it is considered as an immune-mediated diabetes. This form of diabetes, which accounts for only 5–10% of those with diabetes, was previously named as insulin-dependent diabetes, type I diabetes, or juvenile-onset diabetes (ADA, 2009). Markers of the immune destruction of the β-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2β. At least one and usually more of these antibodies are present in 85–90% of individuals when fasting hyperglycemia is initially detected. Also, the disease has strong HLA associations, with linkage to the DQA and DQB genes and is

influenced by the DRB genes. These HLA-DR/DQ alleles can be either predisposing or protective (ADA, 2009).

In this form of diabetes, the rate of β-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Some patients, particularly children and adolescents, may present ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly change to severe hyperglycemia and/or ketoacidosis in the presence of infection or other stress. Still other, particularly adults, may retain residual β-cell function sufficient to prevent ketoacidosis for many years; such individuals eventually become dependent on insulin for survival and are at risk for ketoacidosis. At this latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide. Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age, even in the 8th and 9th decades of life (ADA, 2009).

Autoimmune destruction of β-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined. Although patients are rarely obese when they present with this type of diabetes, the presence of obesity is not incompatible with the diagnosis. These patients are also prone to other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, and pernicious anemia (ADA, 2009).

1.1.2 Type 2 diabetes mellitus

Type 2 diabetes (T2D) ranges from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance. This form of diabetes, which accounts for ~90–95% of those with diabetes and was, previously referred to as non-insulin-dependent diabetes, type II diabetes, or adult-onset diabetes, encompasses individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency. At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive. There are probably many different causes of this form of diabetes. Although the specific etiologies are not known, autoimmune destruction of β-cells does not occur, and patients do not have any of the other causes of diabetes listed above or below (ADA, 2009).

Most patients with this form of diabetes are obese, and obesity itself causes some degree of insulin resistance. Patients who are not obese by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region. Ketoacidosis seldom occurs spontaneously in this type of diabetes; when seen, it usually arises in association with the stress of another illness such as infection. This form of diabetes frequently goes undiagnosed for many years because the hyperglycemia develops gradually and at earlier stages is often not severe enough for the patient to notice any of the classic symptoms of diabetes. Nevertheless, such patients are at increased risk of developing macrovascular and microvascular complications (ADA, 2009).

Whereas patients with this form of diabetes may have insulin levels that appear normal or elevated, the higher blood glucose levels in these diabetic patients would be expected to result in even higher insulin values had their β-cell function been

normal. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. Insulin resistance may improve with weight reduction and/or pharmacological treatment of hyperglycemia but is seldom restored to normal. The risk of developing this form of diabetes increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior gestational diabetes mellitus (GDM) and in individuals with hypertension or dyslipidemia, and its frequency varies in different racial/ethnic subgroups. It is often associated with a strong genetic pre-disposition more so than is the autoimmune form of T1D (ADA, 2008).

1.1.3 Idiopathic diabetes

Some forms of T1D have no known etiologies. Some of these patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of autoimmunity. Although only a minority of patients with T1D falls into this category, of those who do, most are of African or Asian ancestry. Individuals with this form of diabetes suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes. This form of diabetes is strongly inherited, lacks immunological evidence for β-cell autoimmunity, and is not HLA associated. An absolute necessity for insulin replacement therapy in affected patients may come and go (ADA, 2009).

1.2 Obesity

Obesity is an abnormal accumulation of body fat, usually 20% or more over an individual's ideal body weight and it is associated with an increased risk of illness, disability, and death. Twenty to forty percent over ideal weight is considered mildly obese; 40-100% over ideal weight is considered moderately obese; and 100% and more over ideal weight is considered severely, or morbidly, obese. More recent guidelines for obesity use a measurement called body mass index (BMI). BMI= kg/m^2 where a person's weight in kilograms is divided by their height in metres squared. A BMI of 25.9-29 is considered overweight; BMI over 30 is considered obese (Beers and Berkow, 2004).

The rising prevalence of overweight and obesity in a number of countries has been described as a global pandemic (Stevens et al., 2012; Finucane et al., 2011; De Onis et al., 2010; Wang and Beydoun, 2007; Rennie and Jebb, 2005). In 2010, overweight and obesity already were estimated to cause 3.4 million deaths, 3.9% of years of life lost and 3.8% of disability adjusted life years (DALYs) globally (Roth et al., 2004; Popkin et al., 2012; Swinburn et al., 2011; Lim et al., 2012).

Several factors have now been shown to predict the development of obesity in individuals, such as a family history of obesity, lifestyle, diet and socio-economic factors. Prevalence is higher where there is deprivation and in individuals with lower levels of educational achievement (NICE Public Health Guideline, 2014**)**

Individuals suffering from overweight and obesity are at a much higher risk of developing T2D. There are more than 500 million obese adults worldwide and

almost 1.5 billion who are overweight. Around 700 million adults are projected to be obese by 2015 (Han et al., 2013; WHO, 2013).

Obesity is a major health crisis, and diabetes is one of its most serious sequelae. Obesity is associated with a state of chronic systemic inflammation that is a primary etiologic factor in the development of insulin resistance and diabetes. This inflammatory state is based in adipose tissue and mediated in large part by tissue macrophages and their cytokine and adipokine products. Recent research has identified specific molecular mediators of the link between inflammation and insulin resistance in obesity. Study of these mediators and the specific mechanisms underlying inflammation and insulin resistance in obesity holds the promise for novel pharmacotherapy for obesity-related metabolic disease (O'Rourke, 2009).

1.2.1 Diabetes and obesity in Mexico

Mexico has been the subject of an epidemiological transition: in two decades, Mexico's disease profile has transformed from malnutrition, communicable infectious and parasitic diseases to a country dominated by obesity, diabetes and other nutrition-related noncommunicable diseases (NR-NCDs) (Rivera et al., 2002; Barquera et al., 2003; Barquera et al., 2006**).** Mexico has experienced some of the most rapid shifts in dietary and physical inactivity patterns and ultimately obesity ever recorded (Popkin, 2007; Baquera et al., 2010). Between 1988 and 2006, Mexico's annual prevalence rate of obesity (body mass index [BMI] ≥ 30 kg/m2) increased among adults by approximately 2% per year, the largest increase documented worldwide. Between 1988 and 2006, Mexico's annual prevalence rate of obesity (body mass index [BMI] ≥30 kg/m^2) increased among adults by

approximately 2% per year, the largest increase documented worldwide (Murillo et al., 2016).

From 1980 to 2000 researchers documented an alarming 47% increase in diabetes mellitus mortality rates: in 1980 diabetes mellitus was the ninth cause of mortality and ascended to the third by 1997 (Barquera et al., 2003). Based on nacional mortality statistics, after disaggregating cardiovascular disease, diabetes has been the primary cause of death among women and men since 2000 followed by coronary heart disease. In 2009, diabetes was responsible for 77,699 deaths, representing 13.76% of all deaths (Rull et al., 2005). Which therefore represents a cause of great concern for public health.

1.2.2 Diabetes and obesity in Canada

Similar to many countries, Canada has experienced a substantial increase in the prevalence of T2D and obesity (Sassi et al., 2012; Finucane et al., 2011; Flegal et al., 2010). A report by the Organization for Economic Co-operation and Development suggested that in some countries, including Canada, the prevalence of these diseases will continue to rise at a predicted rate of 4%–5% per year (Sassi et al., 2012). First Nations, Inuit and Métis people in Canada have much higher rates of obesity and diseases associated with obesity such as diabetes, hypertension and heart disease. Rates of obesity are even higher for First Nations, Inuit and Métis children compared to the Canadian born non-Aboriginal population of children. (Willows et al., 2012).

Considering that obesity is closely associated with T2D, Indigenous communities have also registered the highest age-adjusted obesity rates in Canada (37.8%

compared to 23% for the whole Canadian population). The problem is compounded by First Nations´ weak compliance to western medicine, which is attributed, at least partially, to the lack of culturally relevant interventions that take into account the holistic nature of Indigenous traditional medicine ant its different concepts of health and illness (Yu and Zinman, 2007).

1.3 Pharmaceutical approaches

Therapeutic approaches for T2D include sulphonylureas, which increase insulin release; metformin, which reduces hepatic glucose production; peroxisome proliferator-activated receptor-gamma activators (thiazolidinediones), which increase insulin sensitivity; alpha-glucosidase inhibitors, which interfere with glucose absorption and insulin itself. More recent oral hypoglycemic drugs include incretins and inhibitors of sodium coupled glucose transporter 2 (SGLT-2). **Metformin** is unequivocally the first-line treatment in patients with T2D. It belongs to the biguanide class of drugs and acts by increasing hepatic insulin sensitivity. It also increases the uptake of glucose into peripheral cells, reduces hepatic glucose production and aids weight loss. Caution is advised when used in patients with impaired renal function or other conditions that may increase the risk of lactic acidosis, such as acute heart failure or shock. However, a review of trials that included patients on metformin suggests that the potential risk of developing lactic acidosis is often overstated (UK Prospective Diabetes Study group, 1998; British National Formulary, 2015; Salpeter et al., 2010). **Sulfonylureas** (e.g. gliclazide, glimepiride, meglitinides) are commonly used as second-line agents in patients with T2D, but can also be used as an alternative first-line treatment instead of

metformin if the patient is not overweight, or is unable to tolerate metformin. Sulfonylureas can also be added to metformin if glycaemic control is inadequate. Sulfonylureas act by binding to a specific receptor on pancreatic beta cells, leading to increased secretion of endogenous insulin. The main side effects of sulfonylureas are weight gain and hypoglycaemia (Tran et al., 2015).

Thiazolidinediones act via the peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear transcription factor to decrease insulin resistance and have been shown to lead to a significant reduction in glycated haemoglobin (HbA1C), both as a monotherapy and when used in combination with other oral agents such as metformin and/or sulphonylureas. Thiazolidinediones are associated with an increased fracture risk (Lyssenko et al., 2013) and in some patients may have led to heart failure (Tran et al., 2015) However, all possess several side effects. Hence, the search for novel pharmaceuticals for the treatment of diabetes mellitus, with the least number of undesirable effects, remains pertinent and timely. Natural products and their derivatives have been extensively used as remedies for various diseases and a significant proportion of contemporary drugs are either natural compounds or their analogs (Cheng and Fantus, 2005).

Insulin replacement therapy will eventually be required in the majority of patients with T2D. Ideally, this would mimic the normal pattern of insulin secretion, where a background level of insulin is supplemented by higher release of insulin to match the glucose load following a meal. Oral agents are usually continued in patients who are starting insulin, but this may need to be reviewed if hypoglycaemia is a problem. Broadly, there are three strategies of insulin replacement; a basal insulin

alone, twice daily biphasic insulin containing a mix of rapid-acting and long-acting insulins and a basal bolus regimen with a long-acting insulin, with additional doses of a short acting insulin given at meal times. Patients with T2D who are already taking oral agents may initially only need a long acting basal insulin to improve their glycaemic control. Alternatively, a twice daily premixed insulin can be used to provide both a long-acting and short-acting insulin. This is potentially more convenient, but is less flexible than other regimens and may be better for patients with regimented mealtimes. A basal bolus regimen is more flexible but has the disadvantage of requiring four injections each day. All insulin regimens are associated with an increased risk of hypoglycaemia and can cause weight gain. In addition, fear of injections, perceived complexity of the treatment regimens, and concern about failure to self-manage the disease can form barriers to starting insulin treatment (Fonseca et al., 2014). Currently Sodium-glucose cotransporter-2 (SGLT-2) inhibitors are used in patients with T2D. These agents lower the renal glucose threshold, resulting in an increased amount of glucose being excreted in the urine. Nevertheless, traditional medicine using extracts to treat diabetes mellitus and related diseases have been known and used during long time, however their mechanism of action has not yet been studied in detail.

1.4 Biological pathways involved in metabolic homeostasis and metabolic dysfunction

Pathways are required for the maintenance of homeostasis within an organism and the flux of metabolites through a pathway is regulated depending on the needs of the cell and the availability of the substrate. The end product of a pathway may be used immediately, initiate another metabolic pathway or be stored for later use.

The metabolism of a cell consists of an elaborate network of interconnected pathways that enable the synthesis and breakdown of molecules (anabolism and catabolism). The biological pathways involved in maintaining energy homeostasis have been targeted for pharmacological manipulation to combat insulin resistance (IR) and metabolic dysfunction caused by chronic nutrient excess (Ruderman and Prentki, 2004). Each metabolic pathway consists of a series of biochemical reactions that are connected by their intermediates: the products of one reaction are the substrates for subsequent reactions, and so on. Metabolic pathways are often considered to flow in one direction. Although all chemical reactions are technically reversible, conditions in the cell are often such that it is thermodynamically more favorable for flux to flow in one direction of a reaction. For example, one pathway may be responsible for the synthesis of a particular amino acid, but the breakdown of that amino acid may occur via a separate and distinct pathway. One example of an exception to this "rule" is the metabolism of glucose (Weckwerth, 2006).

Insulin-dependent and -independent mechanistic studies revealed enhanced phosphorylation of Akt (protein kinase B) and AMP-activated protein kinase (AMPK). These kinases are reliable indicators of stimulation of the major pathways controlling G6Pase activity in hepatocytes (Figure 1), namely, the insulin-dependent phosphatidylinositol 3-kinase (PI3- K) pathway and the insulin-independent AMPK pathway, respectively. On the other hand, stimulation of hepatic glycogen synthase (GS), the enzyme that stores excess glucose as glycogen, was positively correlated with increased phosphorylation of glycogen synthase kinase-3 (GSK-3). GSK- 3 is a serine/threonine kinase and an inhibitor of

glycogen synthesis in liver and muscle. The phosphorylation of this enzyme by the plant extract relieves its inhibitory effect on GS and promotes glycogen synthesis, a mechanism similar to that of insulin in glycogenesis (Nachar et al., 2013).

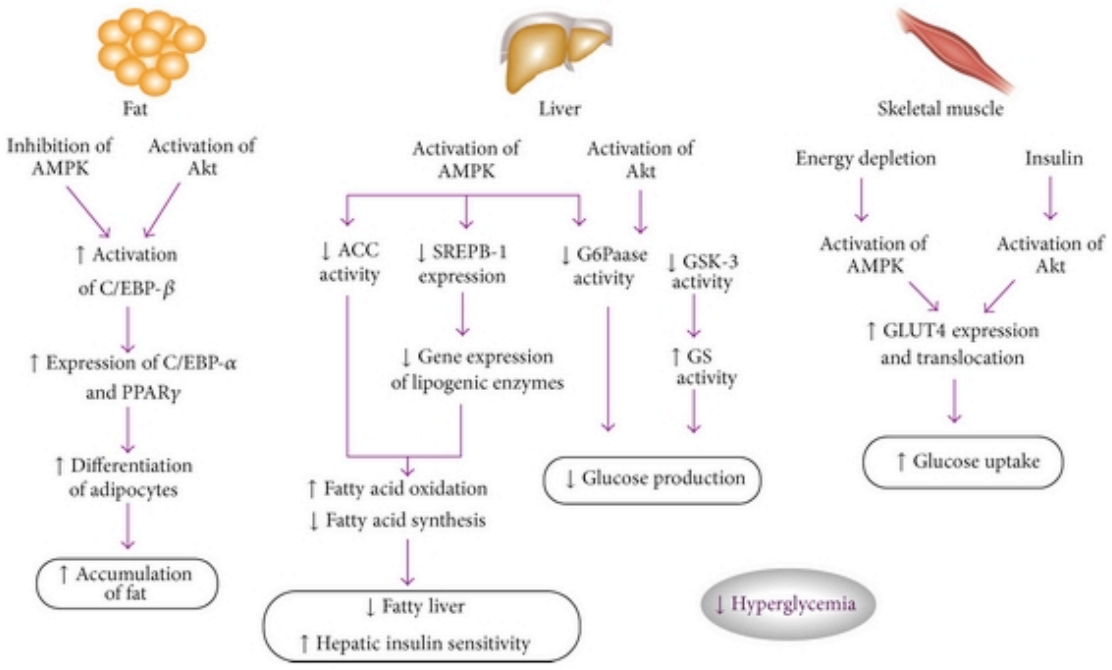


Fig. 1. Insulin-dependent and –independent regulation of glucidic and lipidic metabolism. In skeletal muscle, insulin and insulinin dependent pathways (Eid and Haddad, 2014).

1.4.1 AMP-activated protein kinase (AMPK)

One such pathway is that of AMP-activated protein kinase (AMPK), an enzyme that has come to be known as a master regulator of metabolism. This nutrient-sensing serine/threonine kinase is activated when cellular energy levels are low (Ruderman and Prentki, 2004). Upon activation, AMPK signals through its downstream substrates to restore normal energy levels by stimulating processes that generate ATP (such as fatty acid [FA] oxidation) and inhibiting those that use ATP (such as

triglyceride and protein synthesis). Overall, AMPK activation improves insulin sensitivity and glucose homeostasis, making it an attractive target for T2D and metabolic syndrome (Coughlan et al., 2014).

AMPK is a phylogenetically conserved serine/threonine kinase. It exists as a heterotrimer, consisting of a catalytic α-subunit and regulatory β- and γ-subunits. Each subunit has multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3), making a total of 12 possible heterotrimer combinations. Whether there are functional differences between the different isoforms remains unclear; however, some isoforms are tissue-specific. For example, heterotrimers containing the α1 isoform predominate in the liver and adipose tissue, whereas those containing α2 predominate in the brain, heart, and skeletal muscles (O'Neill HM, 2013; Steinberg and Kemp, 2009).

AMPK activation has effects on a multitude of tissues. In skeletal muscle, its activation stimulates glucose uptake, FA oxidation, glucose transporter (GLUT) type 4 translocation, and mitochondrial biogenesis, while inhibiting protein and glycogen synthesis (Steinberg and Kemp 2009), FA oxidation, and glycolysis (Srivastava et al., 2012). AMPK stimulates glucose uptake and FA oxidation in liver, while inhibiting gluconeogenesis, as well as cholesterol, FA, and protein synthesis (Ruderman et al., 2013). In adipose tissue, it stimulates FA oxidation and reduces FA synthesis and lypolysis (Figure 2) (Coughlan et. al., 2014).

AMPK inhibits insulin secretion from pancreatic β-cells (Steinberg et al., 2009), and it signals to increase food intake in the hypothalamus. (Minokoshi et al., 2004). Nearly all of the physiological effects of peripheral AMPK activation would be beneficial for T2D. For this reason, the pharmacological activation of AMPK has

been a seemingly promising target for drug discovery and development during the past 2 decades (Coughlan et al., 2014).

Activation of AMPK in the liver stimulates fatty acid oxidation and inhibits expression of genes encoding lipogenic enzymes (fatty acid synthase and ACC) (Viollet et al., 2003). The action of AMPK on the lipogenic genes is mediated by reduction of transcription activators namely carbohydrate responsive element binding protein (ChREB) and sterol regulatory element-binding protein 1c (SREPB-1c). In addition, AMPK inhibits the synthesis of cholesterol via the suppression of 3-hydroxy-methyl-glutaryl-CoA reductase (HMGR). AMPK also decreases hepatic glucose production mainly by inhibiting the expression of gluconeogenic genes such as phosphoenolpyruvate carboxylase (PEPCK) and glucose 6-phosphate (G-6-Pase). The action of AMPK on the expression of these genes involves regulation of transcription factors including cAMP-response element-binding protein (CREB), hepatocyte nuclear factor-4α (HNF4-α), Forkhead box O1(FOXO1), and the orphan nuclear receptor small heterodimer partner (SHIP). The metabolic actions of AMPK are summarized in Figure 2.

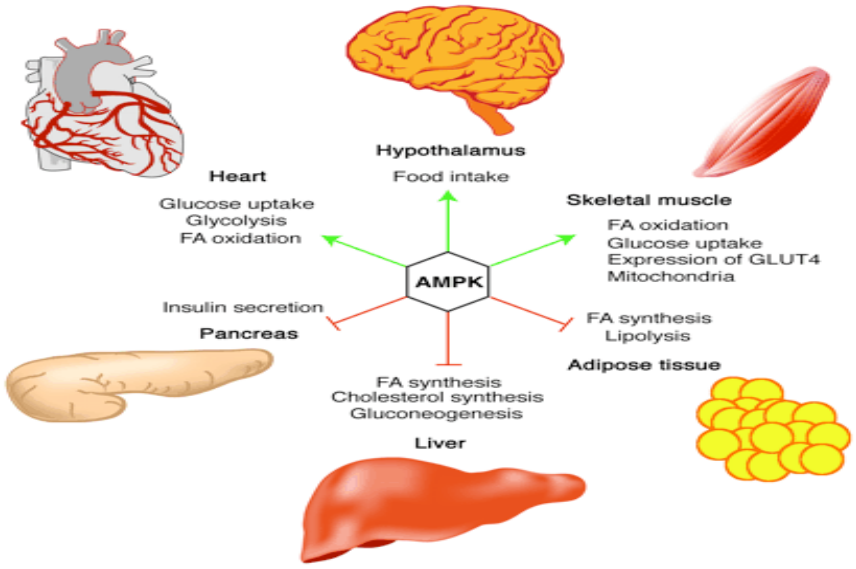


Fig 2. Roles of AMPK in the control of whole-body energy metabolism. Activation of AMPK stimulates the energy-generating pathways in several tissues while inhibiting the energy-consuming pathways (red lines). In skeletal muscle and heart, activation of AMPK increases glucose uptake and fatty acid oxidation. In the liver, AMPK activity inhibits fatty acid and cholesterol synthesis. Lipolysis and lipogenesis in adipose tissue are also reduced by AMPK activation. Activation of AMPK in pancreatic β-cells is associated with decreased insulin secretion. In the hypothalamus, activation of AMPK increases food intake **(Coughlan et. al., 2014).**

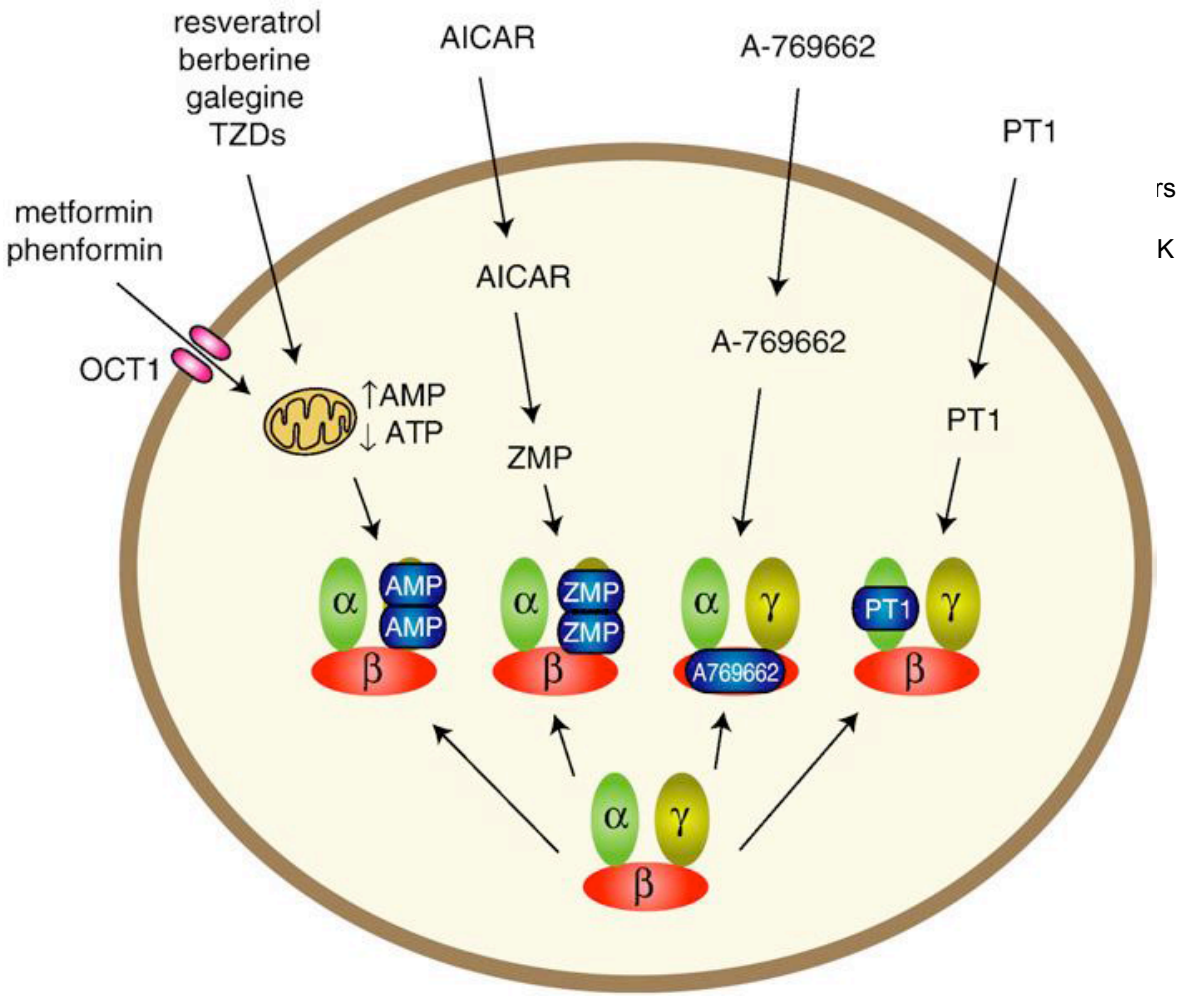
1.4.2 AMPK and Type 2 Diabetes

The regulation of AMPK is of great interest in the study of T2D and metabolic syndrome due to accumulating evidence suggesting that the dysregulation of AMPK plays an important role in the development of IR and T2D, and that AMPK activation (either physiological or pharmacological) can prevent and/or ameliorate some of the pathologies of associated with IR and T2D (Ruderman et al., 2013). Multiple animal models with a metabolic syndrome phenotype have exhibited

decreased AMPK activity in muscle (Ruderman and Prentki, 2004), and evidence exists that AMPK activity is diminished in the skeletal muscle or adipose of humans with T2D and/or obesity (Bandyopadhyay et al., 2006; Xu et al., 2012).

Activation of AMPK stimulates the pathways that increases energy production, such as, glucose transport and fatty acid oxidation, and switches off pathways that consume energy, such as lipogenesis. It is well-established that AMPK regulates the expression of sterol regulatory element-binding proteins (SREBPs) and its target genes such as adipocyte fatty acid binding protein (aP2) and fatty acid synthase (FAS) (Morrison et al., 2000; Rosen and McDougald, 2006).

It has been reported that the insulin-sensitizing drugs thiazolidinediones and biguanides, though chemically unrelated, exert part of their effects through regulation of the activity of AMPK (Fryer et al., 2002). Biguanides such as metformin and phenformin are reported to be transported by the organic cation transporter-1 (OCT1) which is highly expressed in liver. The detailed mechanisms of these agents will be discussed under treatment section. On the other hand, the synthetic nucleotide analogue nucleoside 5-aminoimidazole-4-carboxamide riboside (AICAR) has been widely used to study the effect of AMPK in animals. Inside the cell, AICAR is metabolized to the monophosphorylated derivative ZMP, which mimics the effect of AMP on AMPK activation. Interestingly, direct activators of AMPK such as A-769662 and PT1 have been recently developed. The action of these agents does not seem to be mediated by AMP. Activation of AMPK by A-769662 appears involve the β-subunit (Fogarty and Hardie, 2010).



1.4.3 Serine/threonine-specific protein kinase (Akt)

Akt, also known as PKB, is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes, including cell growth, survival, proliferation, and metabolism (Gonzalez and McGraw, 2009). Its activation depends on PI3 kinase, following upstream stimulation by the insulin receptor substrate and causes recruitment of phosphatidylinositol (4,5)-bisphosphate (PIP2) and production of the second lipid messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3).

PIP3 then binds to the pleckstrin homology domain of Akt/ PKB, allowing for its translocation and binding to the cell membrane. Following this membrane translocation, Akt/PKB can then be phosphorylated and activated by two further

kinases, PDK1 and mTOR complex 2. These phosphorylations occur, within the T-loop of the catalytic domain (Thr308) and the carboxyl terminal hydrophobic domain (Ser473) of Akt, respectively, resulting in the phosphorylation of many downstream targets involved in cellular growth and metabolism (Gonzalez and McGraw, 2009).

It is clear that Akt signaling plays a central role in insulin- stimulated glucose uptake in both muscle and adipose tissue while inhibiting glucose release from hepatocytes (Steinberg and Kempt, 2009). The effect of insulin on glucose uptake in peripheral tissue via Akt/ PKB is through its ability to translocate GLUTs to the cell membrane, thereby facilitating glucose uptake. Binding of insulin to its cell surface protein receptors causes subsequent tyrosine phosphorylation, resulting in phosphorylation of insulin receptor substrates on specific tyrosine residues and activation and recruitment of PI3 kinase and its downstream target Akt/PKB (Mora et al., 2004).

Akt phosphorylates and regulates a large number of substrates involved in a diverse array of biological processes (Manning and Cantley, 2007), many of which could contribute to the role of Akt in driving adipocyte differentiation. For instance, the differentiation defect in mouse embryo-derived fibroblasts (MEFs) and 3T3-L1 preadipocytes lacking Akt1 stems from an inability to induce peroxisome proliferator-activated receptor γ (PPARγ) expression at the initiation of the adipogenesis program (Peng et al., 2003; Baudry et al., 2006; Xu and Liao et al., 2004). The PPARγ transcription factor is the master regulator of adipocyte differentiation, and like Akt1, its activation is both necessary and sufficient for differentiation (Rosen and Spielgeman, 2001). Supporting an essential role for

PPARγ induction downstream of Akt1, forced expression of PPARγ in Akt-deficient MEFs was found to rescue their severe adipogenesis defect (Peng et al., 2003).

Among its many roles, Akt appears to be common to signaling pathways that mediate the metabolic effects of insulin in several physiologically important target tissues. Refining our understanding of those pivotal molecular components that normally coordinate insulin action throughout the body is essential for a full understanding of insulin resistance in diabetes mellitus and obesity and ultimately the successful treatment of these diseases (Whiteman et al., 2002).

1.4.4. Glucose-6-Phosphatase

Glucose-6-phosphatase is one of several prominent membrane-bound enzymes that catalyze the removal of the phosphate group from glucose-6-phosphatase to form free glucose and inorganic phosphate. To understand the importance of this phosphatase, we need to appreciate both the way in which liver cells store glycogen and the reason and mechanism for its subsequent breakdown (Becker et al., 2000). Glucose is formed from gluconeogenesis in the liver and is also available from glycogen. Gluconeogenesis and glycogen breakdown ultimately result in the formation of glucose-6-phosphate (Glc-6-P), which has to be hydrolysed by glucose-6-phosphatase (G6Pase) before being liberated as glucose into the circulation. G6Pase plays thus a critical role in blood glucose homoeostasis (Becker et al., 2000).

Gluconeogenesis is the major metabolic pathway through which the liver produces glucose from precursors such as amino acids, lactate, glycerol, and pyruvate. This process includes several linked enzymatic reactions and is mainly activated after a

fast and in diabetic patients (Watford, 2005). Insulin normally reduces the activity of these enzymes to help normalize blood glucose. It does so through the activation of the signaling kinase Akt and the subsequent phosphorylation of transcription factors controlling the expression of G6Pase. The phosphorylated transcription factors are then expelled from the nucleus, the expression of the enzyme is inhibited, and the production of glucose in the liver is eventually reduced (Puigserver et al., 2003; Herzig et al., 2001) (Fig. 4).

In diabetic patients, hepatic insulin resistance interferes with these events, resulting in an increased hepatic glucose production, a major contributor to fasting hyperglycemia (Rosella et al., 1993; Mues et al., 2009). G6Pase is an endoplasmic reticulum enzyme responsible of the final release of glucose into the circulation (Gonzalez-Mujica, et al., 2005), and is considered to represent the rate-limiting step of gluconeogenesis (Schmoll, et al., 2000). Moreover, an inactivatingmutation in thegeneof this enzyme leads to hypoglycemia, but an increase in its expression is followed by hyperglycemia and the onset of diabetes (Hutton and O'Brien, 2009).

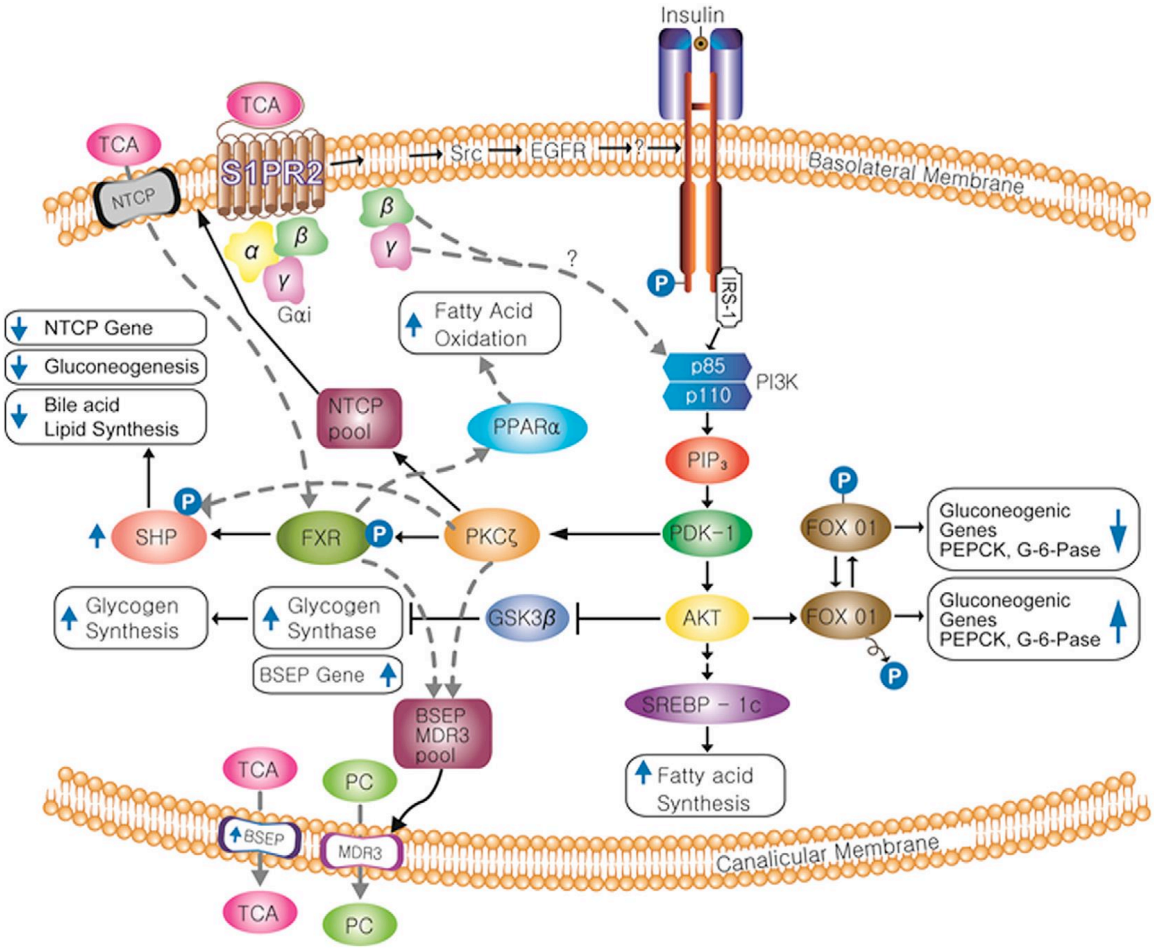


Fig. 4. Insulin pathway in regulating hepatic nutrient metabolism. G6Pase, glucose-6-phosphatase; PDK1, phosphoinositide-dependent protein kinase 1; AKT, protein kinase B; SREBP, sterol regulatory element-binding protein **(Yunpeng et al., 2015)**

2. Background

C. ficifolia has shown acute hypoglycemic activity in transiently hyperglycemic rabbits, in alloxan-diabetic rabbits, and in T2D diabetes patients (Acosta-Patiño et al., 2001; Roman-Ramos et al., 1995; Roman-Ramos et al., 1991). Another study based on the popular use and preparation of *C. ficifolia* to treat diabetes mellitus in traditional Mexican medicine showed that the aqueous extract of *C. ficiflia* also diminishes the possibility that the effect on insulin levels reported by Xia and Wang, 2006, is mediated through the KATP channels (Banderas et al.,2012)

Cucurbita ficifolia Bouché (Cucurbitaceae) and D-chiro-inositol modulate the redox state and inflammation in 3T3-L1 adipocytes (Fortis et al., 2013). More recently has been observed that Cucurbita ficifolia (Cucurbitaceae) modulates inflammatory cytokines and IFN-γ in obese mice (Fortis et al., 2016), exciting findings of *C. ficifolia* phytochemistry is a chemical fingerprint where observed five majoritarian compounds: p-coumaric acid, p-hydroxybenzoic acid, salicin, stigmast-7,2,2-dien-3-ol and stigmast-7-en-3-ol (Garcia et al., 2017). On the other hand, *V. angustifolium* has been fermented with *Serratia vacinii* to produce biotransformed blueberry juice or fermented blueberry extract (FBE) and this product was evaluated in various diabetes-related assays, including glucose uptake, adipogenesis, and the signaling pathways that regulate glucose transport in muscle cells and adipocytes (Martineau et al., 2005; Vuong et al., 2007). Fermented extract, but not unfermented juice, was found to potentiate glucose uptake in C2C12 myotubes and 3T3-L1 adipocytes in the presence or absence of insulin (Voung et al., 2007).

Fermented extract dramatically inhibited triglyceride accumulation during adipogenesis of 3T3-L1 cells, which could constitute a potential anti-obesity action. Examination of the insulin-dependent and insulin–independent intracellular signaling components by Western blot analysis showed that fermented blueberry extract significantly enhanced insulin-independent AMP kinase, an action similar to the common oral hypoglycemic drug metformin. Thus, fermentation confers novel and potent *in vitro* antidiabetic and anti-obesity properties to FBB extract (Martineau et al., 2005; Vuong et al., 2007).

As mentioned, fermented blueberry extract thus holds great promise as a novel complementary therapy and as a source of therapeutic agents against T2D. Another important model was male C57BL/6J DIO (diet induce obesity) a high-fat induced obese mouse model considered a gold standard that closely mimicks the progression of human diabetes development and the interaction of nutritional components with genetic variables. Numerous studies have demonstrated that the C57BL/6J DIO mouse is a suitable animal model for examining novel therapeutic interventions and can also illustrate how diverse anti-diabetic drugs exert *in vivo* efficacy (Haddad et al., 2012).

Therefore, the major performance indicator will be the results of studies examining the efficacy and systemic mode of action of selected wild blueberry product extract in the C57BL/6J DIO mice. This study is highly relevant since the anti-obesity and anti-diabetic potential of fermented blueberry juice have already been evaluated using *in vivo* in mouse models (KKAy and C57BL/6J) (Voung et al., 2009), as was also done for the anti-diabetic properties of *C. ficifolia* (Alarcon et al., 2007). Such studies would also represent an opportunity to develop novel products in the field of obesity and diabetes. Indeed, some of the bioactive components identified can also hold potential for the development of pharmacological agents and the pharmaceutical industry growth could be even more significant.

Adipocytes are highly specialized cells that play a critical role in regulating lipid metabolism and energy balance, and they are associated with adipose tissue mass (Otto et al., 2005). Adipogenesis is the process by which an undifferentiated preadipocyte is converted to a fully differentiated adipocyte (Cristancho et al.,

2011). The accumulation of lipid increases the adipogenic process and it is regulated by either genetic and growth factors (Peng et al., 2003). The insulin-signalling pathway is a major regulator of energy homeostasis in the cell. Insulin and Akt signaling have been found previously to be essential for adipogenesis. During differentiation of the adipocyte the action of the ser/thr kinase Akt plays an essential role because the lack of Akt1 causes an inability of differentiation into adipocytes, as has been observed in mouse embryonic fibroblasts (MEFs) (Yun et al., 2008; Sundararajan et al., 2009).

The Akt kinase acts downstream of the growth factor receptors to regulate numerous cellular processes including proliferation, differentiation and cell death. Knockout models of the different Akt isoforms (Akt1 and Akt2), demonstrate their importance in regulating glucose metabolism in the body (Green and Kehind 1976). Thus, Akt is necessary to drive adipogenesis. Its role includes phosphorylation and regulation of a large number of subsrates that are involved in a diverse array of biological processes (Peng et al., 2003). Adipocyte differentiation is also mediated by temporally regulated expression of PPARγ that is another a key regulator of this transcriptional program, and is induced before the transcriptional activation of most adipocyte-specific genes (Baudry et al, 2006). In addition to Akt and insulin, another factor involved in fat metabolism is AMP-activated protein kinase (AMPK). Recently, AMPK has been implicated in the control of adipose tissue content (Dagon et al., 2006).

Skeletal muscle is another insulin-target tissue responsible for maintenance of whole body glucose homeostasis. Insulin stimulation of glucose uptake in skeletal

muscle cells is mediated through translocation of GLUT 4 (Klip and Ishiki, 2005). When there is some defect in glucose transport efficiency and GLUT4 activity, this results in insulin resistance (Petersen and Shulman, 2006). However, the mechanism regulating the translocation of GLUT4 can still be further elucidated. *In vitro* model systems are essential for a deeper understanding of glucose uptake that involved GLUT4 in muscle cells and to continue providing answers concerning different pathways involved in these actions.

Glucose homeostasis is the result of a balance between glucose production by the liver (gluconeogenesis), its storage as glycogen (liver and muscle), and its uptake by peripheral tissues, notably insulin-responsive skeletal muscle and adipose tissue. Indeed, insulin, a hormone secreted by the beta pancreatic cells, works by decreasing glucose production in the liver, stimulating its uptake by skeletal muscle andperipheral tissues and enhancing its storage as glycogen. Therefore, in T2D, beta pancreatic cell insulin deficiency is combined with insulin resistance, thus contributing to a state of hyperglycemia through an increased hepatic glucose production and a reduced peripheral glucose disposition (Gonzalez et al., 2006).

Gluconeogenesis is the major metabolic pathway through which the liver produces glucose from precursors such as amino acids, lactate, glycerol, and pyruvate. This process includes several linked enzymatic reactions and is mainly activated after a fast and in diabetic patients (Watford, 2005). Hepatic gluconeogenesis is controlled at three major points, namely, the reactions catalyzed by phosphoenolpyruvate carboxykinase (PEPCK), by fructose-1,6-biphosphatase, and glucose-6-phosphatase (G6Pase). Insulin normally reduces the activity of these enzymes to

help normalize blood glucose. It does so through the activation of the signaling kinase Akt and the subsequent phosphorylation of transcription factors controlling the expression of PEPCK and G6Pase.

The phosphorylated transcription factors are then expelled from the nucleus, the expression of enzymes is inhibited, and the production of glucose in the liver is eventually reduced (Puigserver et al., 2003; Herzig et al., 2001). In diabetic patients, hepatic insulin resistance interferes with these events, resulting in an increased hepatic glucose, a major contributor to fasting hyperglycemia (Rosella et al., 1993; Mues et al., 2009). G6Pase is an endoplasmic reticulum enzyme responsible of the final release of glucose into the circulation (Gonzalez et al., 2005) and is considered to represent the rate-limiting step of gluconeogenesis (Schmoll et al., 2000).

Aside from the hormonal regulation involving insulin, hepatic glucose homeostasis is also regulated by factors implicated in the control of energetic balance. A key kinase in this context is called AMP-activated protein kinase (AMPK), which is activated after metabolic stress (increase of the ratio AMP/ATP in the cell) (Twoler et al., 2007). Once phosphorylated, active AMPK inhibits anabolic pathways (decrease of the fatty acids synthesis, decrease of the gluconeogenesis, and increase of the storage of glucose as glycogen) and simultaneously increases catabolic pathways (increase of muscle glucose uptake, increase of the fatty acid oxidation, and increase of the glycolysis). Moreover, AMPK is activated by metformin, one of the most common oral hypoglycemic drugs used worldwide for glycemic control in diabetes and metabolic syndrome (Violet et al., 2007). Previous

studies from Dr. Haddad research team probed liver cell cultures for a potential action of Cree plants on AMPK, in relation to their effects on liver glucose metabolic enzymes (Nachar et al., 2013).

3. Problem and Justification

Diabetes imposes a large economic burden on the individuals, national healthcare systems and economies. Healthcare expenditures on diabetes accounted for 10.8% of the total healthcare expenditure in the world in 2013. By 2035, estimated global healthcare expenditures to treat and prevent diabetes and its complications are expected to total at least $627 billion of dollars (Parvez et al., 2007).

Due, the cases of diabetes and obesity are growing at an average rate of 400, 000 new cases per year. The vast majority of these cases (90%) are of the T2D etiology. Therefore, it is important to continue research on new anti-diabetic agents. In particular, traditional knowledge and related medicines still exist and represent an alternative health care approach for people from México and Canada. Traditional medicine is an important resource for health care because it is generally inexpensive and easily accessible. Thus, the use of medicinal plants needs to be encouraged and man-plant relationships enhanced and analyzed, from the anthropological, ecological, botanical, pharmacological and medicinal points of view (WHO 2016).

The current medical use of *C. ficifolia* includes the treatment of T2D and obesity. In this case, the local healers recommend the ingestion of a macerate in water of the fresh fruit (Aguilar et al., 1994). The hypoglycaemic effect of this plant has been demonstrated (Roman-Ramos et al., 1995; Acosta-Patino et al., 2001; Alarcon-Aguilar et al., 2002). The plants of the family Cucurbitaceae play an important role in health care for the treatment of various ailments. Some plants of the family

Cucurbitaceae have shown anti-diabetic activity (Sharma and Arya, 2011). However, in order to a better approach of adequate characterization of this activity has not yet been done.

In addition, fermented blueberry extract that significantly enhanced insulin-independent AMPK, an action similar to the common oral hypoglycaemic drug metformin, speaks to its important anti-diabetic and anti-obesity properties. It thus holds strong potential as a good alternative or complementary therapy against T2D. For this reason it is essential to pursue research, notably to determine which compounds are specifically responsible its beneficial actions.

Moreover, *V. angustifolium* and *C. ficifolia* are relatively well study and present a potential promising antidiabetic and anti-obesity. However, their mechanisms of action and/or active principles still remain unclear. Further studies are therefore fully warranted in order to facilitate their safe and efficacious use in the tretmeant of T2D and obesity.

For instance, to examine *V. angustifolium* and extracts effects, an *in vivo* model of metabolic syndrome and T2D could be used. As mentioned, the male C57BL/6J DIO high-fat induced obese mouse model is considered a gold standard in that it closely reflects the progression of human diabetes development and the interaction between of nutritional components and genetic variables (Reuter, 2007). Numerous studies have demonstrated that the C57BL/6J DIO mouse is a suitable animal model for examining novel therapeutic interventions and can also illustrate how diverse anti-diabetic drugs exert *in vivo* efficacy (Ahren et al., 2000; Kelly-Sullivan et al., 2003).

The next essential step is to determine the mechanism of action by which bioactive components are exerting their effect and, specifically the compounds responsible for the beneficial actions of fermented wild blueberry juice. To identify the latter, bioactivity directed fractionation and *in vitro* testing are appropriate approaches.

The aim of this project is thus to elucidate which compounds are specifically responsible for the beneficial actions of the antidiabetic and anti-obesity properties using a bio-directed fractionation with *in vitro* and *in vivo* assays from the crude extract of *C. ficifolia* and specific fractions of FBB extract from *V. angustifolium*.

4. Hypothesis

If there is a number of specific compounds with anti-obesity and anti-diabetic properties presents in the extracts of *C. ficifolia* and *V. angustifolium* then one or more of these could inhibited the accumulation of fatty acids into triglycerides during adipogenesis of 3T3-L1 pre-adipocytes and stimulate glucose uptake activity in C2C12 muscle cells. If such biological activities are expressed, then the phosphorylated forms of AMPK and AKT will be expressed and/or transcriptional factors involved on insulin pathways will be activated.

5. General Objective

Identify which compounds are specifically responsible for the anti-diabetic and anti-obesity beneficial actions of semi-purified fractions of fermented juice of *V. angustifolium* and to chemically characterize fractions of antioxidant compounds,

and flavonoid type from *C. ficifolia* as well as to study their influence on molecular markers of hyperglycemic and anti-adipogenic actions.

5.1 Particular Objectives

1. To determine the effect of fermented *V. angustifolium* extract and semi-purified fractions on adipogenesis process using 3T3-L1 cells and on phosphorylate forms of AMPK, AKT, SREBP1-c and PPARgamma.
2. To test the anti-diabetic and anti-obesity active principles of fermented *V. angustifolium* extract identified in objective 1 *in vivo*, using an animal model of obese and insulin resistant mice (C57BL6) treated with high-fat diet and plant extract.
3. To determine the effect of *C. ficifolia* extract and primary fractions on adipogenesis process and on phosphorylated forms of AMPK and PPAR gamma using 3T3-L1 cells.
4. To determine the anti-diabetic effect of *C. ficifolia* extract and its primary fracions on glucose uptake activity and on phosphorylated forms of AMPK in C2C12 muscle cells.
5. To characterizate the chemical general profile of *C. ficifolia* crude extract and test its primary fractions namely on objective 3 *in vitro* on triglyceride content using 3T3-L1 cells and on phosphorylated form of AMPK.
6. To determine the inhibition of G6-phosphatase on 3T3-L1 cells treated with *C. ficifolia* crude extract and on phosphorylated form of AMPK.

6. Materials and Methodology

6.1 Chemicals and biochemicals

Dexamethasone (DXM), bovine pancreatic insulin, 3-isobutyl-1methylxanthine (IBMX) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (city and state). Rosiglitazone came from Alexis Biochemicals (Hornby, ON). Dulbecco's Modified Eagle Medium was from Wisent Inc. (St-Bruno, QC). For the measurement of triglyceride content, we used the AdipoRed reagent purchased from Lonza Walkersville Inc. (Walkersville, MD). For revealing membranes from blots Western Lightning ECL was used from Perkin Elmer (Waltham, MA USA). AICAR 5-Aminoimidazole-4-carboxamide ribonucleotide (positive control for AMPK) was purchased from Toronto Research Chemicals (Toronto, ON,). The measurement of protein was carried out with an assay kit from Bio-Rad (Mississauga, Ontario, Canada).

6.2 Preparation of extracts

Blueberry extract *V. angustifolium* was prepared by blending wild blueberry fruit (*Vaccinium angustifolium* Aiton) (100 g) with an equivalent quantity (100 g) of Minimal Broth Davis without dextrose (MM) (Difco Laboratories, Detroit, MI). The preparation was centrifuged to remove insoluble particles. The resulting extract was sterilized using 0.22 µm Express Millipore filter (Millipore, Etobicoke, ON) and fermented with *Serratia vaccinii* bacteria as described (Martineau and Matar, 2005). Normal blueberry extract (NBE) was processed in an identical manner but

was not fermented with the bacteria. Portions of NBE and the fermented blueberry extract (FBE) were freeze-dried and used for chemical analysis.

***C. ficifolia* extract**. Mature fruits with a diameter of 18–20 cm and an approximate weight of 3.5 kg were gathered in the Acolman municipality, State of Mexico during the winter of 2011-2012. After being gathered, they were cut in halves and dried whole fruits without seeds were grounded using an electric extractor; this yielded 446 g of material, which was macerated with water at 4°C during 24 h. The precipitate was sterilized by filtration and evaporated to dryness at room temperature. Crude extract was maintained at 4°C until testing.

6.3 Phytochemical fractionation of FBE

Organic solvents were purchased from Fisher Scientific Canada (Ottawa, ON). Fractions were prepared by a multi-step process (Table 1) that started with material that was either NBE or NBE after fermentation with *Serratia vaccinii* to produce FBE. As a first fractionation step, FBE (identified as F1; Table 1) was loaded in batches of 500 ml onto 29.5 cm x 5 cm chromatography columns (pre-conditioned with 1 column volume of methanol then 2 column volumes of water) containing Waters preparative C18 resin (125 Å, 55-105 µm), and washed with 2 column volumes of water to remove sugars and organic acid (discarded). Phenolic compounds were eluted from the column using 1.2 column volumes of 100% ethanol containing 13 mM trifluoroacetic acid (Sigma Aldrich, ON). This ethanol eluent, which contained all the phenolics from the FBE starting material, was dried using rotary evaporation and freeze dried. This total phenolic fraction from FBE was called F2.

For the second fractionation step of the FBE, one portion of F2 was dissolved in water and applied to another preconditioned C18 column. From this was isolated Fraction F3-1 that contained low MW phenolic compounds. This was done by selective elution using 4 column volumes of aqueous 2.06 M (12.5%) ethanol containing 0.16 M HCl (Ricca Chemical Company, Arlington, TX). F3-1 compounds recovered in this step were identified by HPLC by comparing retention times and UV-Vis profiles of the peaks to pure standards. The next fraction, called F3-2, was produced by passing through the same C18 column an additional 2 column volumes of aqueous 2.06 M (12.5%) ethanol containing 0.16 M HCl. This F3-2 fraction was rich in chlorogenic acid, which was confirmed by HPLC. The remaining bound materials were eluted using 0.16 M HCl and 13.7 M (80%) ethanol in water to produce fraction F3-3 (Table 1). The F3-1, F3-2 and F3-3 fractions were dried using rotary evaporation and then freeze dried. To produce three more fractions one portion of F3-3 was dissolved in 4.28 M (25%) ethanol and applied to a 34.5 cm x 5 cm column of Sigma-Aldrich lipophilic Sephadex LH-20 (25-100 μm). The first fraction from Sephadex LH-20 was obtained by washing with 7 column volumes of 4.28 M (25%) ethanol. This fraction, called F4-1, was enriched in anthocyanins. The same Sephadex LH20 column was then washed with 3 column volumes of 8.56 M (50%) ethanol to yield a fraction enriched in phenolic heteropolymers and called F4-2. The last Sephadex LH20 fraction was eluted using 3 column volumes of 9.53 M (70%) acetone. This fraction was enriched in proanthocyanidins and called F4-3. The three fractions obtained from LH20 (F4-1, F4-2 and F4-3) were dried using rotary evaporation and freeze dried. These fractions were examined on HPLC and four pure compounds of interest

were identified, namely catechol (CAT), protocatechuic acid (PA), gallic acid (GA) and chlorogenic acid (CA), which were subsequently purchased from Sigma-Aldrich (Oakville, ON, Canada).

6.4 Obtention of primary fractions from *C. ficifolia* aqueous extract

The fractionation from direct *C. ficifolia* plant aqueous extract was performed by collecting the primary fractions on an open column containing common silica 8-100 as the stationary phase, under atmospheric pressure. For the mobile phase, we used (acetone/ chlorofom and ethanol/chloroform) 95:5 85:15, 90:10, 85:15 and 80:20 (ethanol/chloroform). This procedure yielded 27 primary fractions from aqueous *C. ficifolia* extract that consequently were analyzed on certain bioassays.

6.5 Phytochemistry of *C. ficifolia* aqueous extract

The process was performed in a Waters chromatograph with a photodiode array detector (PDA) 996, quaternary pump, manual injector, Empower 2 software. Alltima reverse phase column C18 3u (particle size), dimensions 53 x 7 mm (length and diameter), Alltec brand. In this type of columns, the polar compounds eluted first (retention times are smaller), and, as the polarity decreases, the compounds are retained longer.

In order to obtain an analytical chromatogram of the *C. ficifolia* aqueous extract using high performance liquid chromatography (HPLC), an organic fraction of it was generated as next described:

1. 1.38 g of the aqueous extract was dissolved in 20 ml of HPLC grade deionized water.

2. A defatting process was conducted using a separation funnel. For this process, 20 ml of grade reactive n-hexane were added to the aqueous solution three times. The organic phases were discarded each time, as they contain components that could interfere with the analysis.

3. The defatted extract was further partitioned using grade reactive ethyl acetate (40 ml, three times). The organic phases were collected and filtered through anhydrous sodium acetate to eliminate residual water.

4. Then, the organic fraction of ethyl acetate was evaporated under reduced pressure; 3.5 mg of dry ethyl acetate fraction were obtained.

6. One mg of this fraction was dissolved in 400 ml of grade reactive ethyl acetate; this was further partitioned with HPLC grade deionized water (200 ml). The organic fraction was dried filtered through anhydrous sodium acetate and dried under reduced pressure. The resulting ethyl acetate fraction (0.5 mg) was dissolved in 300 ml of an acetonitrile: water mixture (HPLC grade, 50:50), and 20 ml were injected in the HPLC equipment.

7. The mobile phase consisted of a gradient water (A) and acetonitrile (B) as follows: 30% B (0-10 min) and 100% B (10-30 min), at a flow rate of 0.4 ml/min.

8. UV detection was conducted from 205 nm to 354 nm.

6.6 Cell culture (3T3-L1 pre-adypocyte)

Murine 3T3-L1 pre-adypocyte cells were obtained from ATCC (Manassas, VA). They were grown in Dulbecco's modified Eagle's medium (DMEM), containing 10% bovine calf serum until confluent at 100%. Two days after confluence (Day 0), the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS), 500 nM insulin, 1 µM Dexamethasone and 250 µM IBMX for 2 days (Day 2) (Short Term Medium).

Cells were maintained in 10% FBS/DMEM with 500 nM insulin for another 4 days (Long Term Medium) at which time >90% of wells showed mature adipocytes with accumulated fat droplets. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

6.7 Cell Culture (C2C12 murine myoblasts)

C2C12 muscle cells grew in Dubelco's Modificatied Eagle's Medium IX, WISENT INC_medium (DMEM) supplemented with 10% Fetal Bovine Serum, Horse Serum 10% and penicillin 0.5 % in a humidified atmosphere of 5% CO_2/95% air at 37°C. The cells were cultured in Petri dishes with proliferation medium replaced every two days until cells got 80% of confluence. Cells were re-seeded into 12-well plates. Once myoblasts reached 60-70% of confluence for fully differentiated myotubes, they were serum-starved in DMEM medium containing 2% of horse serum (HS) and 0.5% of pen-strep prior to experiments until day 6 of differentiation (treatment). Cells used in this study were from passage 4 to 8.

6.8 Cell Culture of H4IIE (rat hepatoma)

Cells were provided by American Type Culture Collection (ATCC). H4IIE cells grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 0.5% antibiotics (PS: Penicillin 100 U/mL, Streptomycin 100 µg/mL). The cells were incubated at 37◦C, 5% CO_2 until reaching 90% confluence.

6.8 Cell viability

Cell viability was assed through a Cytotoxicity Detection Kit that was purchased from Roche (South San Francisco, CA). 3T3-L1 pre-adipocytes were seeded in 24-well plates and cultured to 100% confluence in culture medium and treated for 7

days as described above. After treatments with various different concentrations (5, 10 and 15 µg/ml of NBE, FBE/F1, specific fractions and pure compounds for *V. angustifolium*), *C. ficifolia* crude extract was tested at (10, 20, 40, 60, 80 and 100 µg/ml concentrations) and the latter plant for C2C12 murine myoblasts at (5, 20, 40 and 60 µg/ml), H4IIE liver cells were treat with *C. ficifolia* crude extract at (10, 20, 40, 60, 80 and 100 µg/ml), toxicity was measured as a ratio of LDH released in the medium to the total LDH (intracellular and extracellular) according to manufacturer's specifications. Cell culture media for each condition (by duplicate and/or triplicate) were collected separately (released LDH) and then cells were lysed with culture medium containing 1% Triton X-100, for 10 min (intracellular LDH). All samples were collected in Eppendorf tubes and lyses samples were centrifuged at 250xg at 4ºC for 10 minutes. Fluorescence was measured (Wallac Victor2, Perkin-Elmer, Waltham, MA) at an emission wavelength of 590 nm. Results were expressed as the ratio of released LDH to total LDH, normalized to values obtained from cells treated with the vehicle control (0.1% DMSO). The optimal concentration was determined for each tested component and used for bioassays.

6.9 Glucose uptake activity (*C. ficifolia*)

Starting differentiation of C2C12 murine myoblasts at 60-70 % (day 0), cells were used on day 6. They were treated during 18 hrs with 20 µg/ml optimal concentration of *C. ficifolia* extract dissolved in DMEM medium, Vehicle (control), positive controls Metformine (400 µM) and insulin (100 nm) and negative control of cytochalasin B. Krebs, HEPES (KH) buffer (1X) containing: NaCl, 4.8 mM KCl,

1.85 mM $CaCl_2$ and 1.3 mM $MgSO_4$ at pH 7.4 was used to washed the cells 3 and incubated at 37ºC in a humidified 5% CO_2 atmosphere for 30 min, followed by an additional incubation period of 10 min with 500 μl of [3H] 2-deoxy-D-glucose (2DG) and cytochalasin B. The cells were washed 3 times with ice-cold KH buffer, and lysed by adding 500 μl of 0.1 M NaOH and then transferred to vials with scintillation cocktail. The GLUT4 specific uptake of [3H] 2-deoxy-D-glucose into C2C12 cells was measured on day 7. Incorporated radioactivity was measured in a scintillation counter.

6.10 Adipogenesis assay with *V. Angustifolium*

NBE, FBE (F1), specific fractions (5 µg/mL) and four pure compounds at 5 µg/ml (except catechol at 3 µg/ml) as well as rosiglitazone (10 µM; positive control) were dissolved in DMSO and added to the cells as of day 0 of differentiation. The final concentration of DMSO was kept at 0.1% throughout the differentiation period. Adipogenesis was assessed in the well-characterized 3T3-L1 cell model by measuring the accumulation of triglycerides upon differentiation after treatments as described previously (Spoor et al., 2006; Harbilas et al., 2009), using the AdipoRed reagent according to the manufacturer's instructions. Briefly, after washing each well twice with phosphate-buffered saline (PBS: 8.1 mM Na_2HPO4, 1.47 mM KH_2PO_4, 137 mM NaCl, and 2.68 mM KCl; pH 7.4), 2 ml of PBS 1X containing 60 µl of AdipoRed reagent were added to each well and incubated for 10 min at room temperature. Fluorescence was measured (Wallac Victor2, Perkin-Elmer, Waltham, MA) at 485 nm excitation and 572 nm emission wavelengths. Results are reported as percentage of the value obtained for the vehicle control (0.1% DMSO).

For each experiment, the mean fluorescence value obtained from the negative control condition was considered as background and subtracted from all other readings.

6.11 Adipogenesis assay with *Cucurbita ficifolia*

Pre-adipocyte 3T3-L1 cells were grown to confluency in DMEM containing 10% BCS (proliferation medium). 24 h post-confluence (day 0), cell were induced to differentiate with high-glucose DMEM supplemented with 10% FBS, 1M dexamethasone, 250 µM 3-isobutylmethylxanthine (IBMX) and 500 nM insulin. Cells were treated with *C. ficifolia* extract at 20 µg/ml and the same concentration has been assessed in 27 fractions from *C. ficifolia* extract (maximal non-toxic concentration) as well as rosiglitazone (10 µM; positive control) were dissolved in DMSO (0.1% final concentration) and added to the cells at day 0 of differentiation. After 48 h, the media was replaced with DMEM containing 10% FBS and 500 nM Insulin. Cells were differentiated for a total of 7 days with media change every 2 days. After treatments as described previously (Spoor et al., 2006; Harbilas et al., 2009), using the AdipoRed reagent according to the manufacturer's instructions. Cells were washing each well twice with phosphate-buffered saline (PBS: 8.1 mM Na_2HPO4, 1.47 mM KH_2PO_4, 137 mM NaCl, and 2.68 mM KCl; pH 7.4), 2 ml of PBS 1X containing 60 µl of AdipoRed reagent were added to each well and incubated for 10 min at room temperature. Fluorescence was measured (Wallac Victor2, Perkin-Elmer, Waltham, MA) at 485 nm excitation and 572 nm emission wavelengths. Results are reported as percentage of the value obtained for the vehicle control (0.1% DMSO). For each experiment, the mean fluorescence value

obtained from the negative control condition was considered as background and subtracted from all other readings.

6.12 Glucose 6 phosphatase activity

Glucose-6-phosphatase activity was assessed in the H4IIE cell line. Briefly, cells (90% confluent in 12-well plates) were treated for 18 h with negative control (0.1% DMSO vehicle), positive control (Insulin, 100 nM), and *C. ficifolia* crude extract at the optimal nontoxic concentration (40 μg/ml). After 18 h, cells were washed then lysed in 15 mM phosphate buffer containing 0.05% triton X-100 and 1.3 mM phenol (pH = 6.5). Cell lysates were incubated in glucose-6-phosphate-containing buffer (200 mM) for 40 min at 37°C where the G-6-P serves as a substrate for endogenous glucose-6-phosphatase to yield glucose. Quantification of the glucose generated in this reaction was measured using Wako AutoKit Glucose colorimetric assay (Wako Chemicals USA Inc., Richmond, VA, USA), according to manufacturer´s instructions. Results are presented relative to vehicle control (0.1% DMSO).

6.13 *In vivo* study

The Diet Induced Obesity (DIO) mouse model was used to obtain a state of obesity associated with insulin resistance and mild hyperglycemia. Briefly, male non-diabetic C57BL/6 mice were obtained from Charles River Laboratories (Saint-Constant, QC, Canada). After a minimum adaptation period of 7 days, the mice were divided into groups of 7 mice each. One group (Chow control) was given a standard diet purchased from Charles River (Chow: 3.84 kcal/g). Another group

(DIO control) were given a high fat diet acquired from Bio-Serv (Frenchtown, NJ, USA; DIO: 5.78 kcal/g; 35% of calories from fat). DIO experimental groups were divided into four different subgroups that received FBE, NBE of *V. angustifolium* and catechol (pure compound). Experimental groups received the high fat diet to which was incorporated the specific plant extracts at the relevant concentrations (as determined by preliminary studies) during 8 weeks. Freeze dried of Normal or fermented fruit juice of *V. angustifolium* was added to the food in concentrations namely as 1.9 ml/kg and 7.6 ml/kg of FBE and 7.6 ml/kg of NBE and one pure compund at 10 μg/kg equivalent of 20 and 80 of FBE and 80 of NBE of ml/kg per day according to previous studies (Vuong et al., 2009). All mice were housed individually in cages with hardwood chip bedding and maintained on a 12 h light/dark cycle in a temperature-controlled animal room (22 ºC). All animals were allowed ad libitum access to solid food and water. The animal experimentation ethics committee of the University of Montreal approved all experimental protocols were carried out in full respect of the guidelines from the Canadian Council for the Protection of animals.

6.14 Acute and Chronic Treatment

Control mice received normal Chow whereas DIO mice were divided into three groups (n=7) and received the respective plant extract and pure compound (powder into the food) at the appropriate concentrations (1.9 ml/kg and 7.6 ml/kg of FBE and 7.6 ml/kg NBE, 10 μg/kg of CAT). The non-fasting blood glucose concentration was measured using a standard glucometer every other day after the respective plant products were administered. Food and water intake were also

measured every other day. Upon sacrifice of the animals, a maximal amount of blood was drawn from the renal artery. Samples of liver, skeletal muscle and epididymal fat were collected and frozen in liquid nitrogen. They were kept at -80°C for further analysis. Plasma samples were kept at -80°C for further analysis.

6.15 Intraperitoneal glucose tolerance test (IPGTT)

Mice were fasted for approximately 6 ho prior to the test. They had access to drinking water at all times. Mice were weighted before starting the test. Glucose solution used for IP injection was as follows: the volume of 20% w/v glucose solution required to deliver 1g of glucose/kg body mass was injected into the abdominal cavity of the animal. Glucose level (t = 0) was obtained from a skin puncture on the tail of each animal and recorded in the experiment record sheet. After intraperitoneal injection of the glucose solution, blood glucose levels were measured at 15, 30, 60 and 120 minutes (t = 15, t = 30, t = 60 and t = 120) with a glucometer.

6.16 Western blot analysis

Cells were cultured, treated and differentiated as described above for the adipogenesis assay (either for *C. ficifolia* and/or *V. Angustifolium*). Cultured cells were homogenized in lysis buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl 5 mmol/L EGTA, 2 mmol/L $MgCl_2$, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (2 mmol/L PMSF and Complete Mini-EDTA-free protease inhibitor cocktail tablets; ROCHE, Laval, QC) and phosphatase inhibitors (0.5 mmol/L NaF, 2 mmol/L Na_3VO_4, 1 mmol/L $Na_4P_2O_7$). Protein

concentration of lysate was assessed by the Bradford colorimetric assay and 40 µg of total protein were loaded onto a 12% acrylamide gel. Samples were electrotransferred to nitrocellulose membrane (Bio-Rad Laboratories Hercules, CA). Membranes were incubated overnight with primary antibodies to Akt and p-Akt, (1:250, Cell Signalling Technology, Danvers, MA) as well as p-AMPK, AMPK, SREBP-1c and PPARγ (1:250, 1:500 (total AMPK) Santa Cruz Biotechnology Inc., Dallas, TX; and Cell Signalling Technology Inc, Danvers, MA) and beta-actin (1:1000, Cell Signalling Technology Inc, Danvers, MA). Incubation with anti-rabbit HRP-conjugated secondary antibody (Cell Signalling Technology Inc., Danvers, MA) was then carried out at 1:20000 dilution in TBST (1X) (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.5% Tween 20) + 5% non-fat dried milk for 1 h at room temperature. Membranes were then washed with TBST 3X for 5 min and the blots were revealed using the Western Lightning ECL enhanced chemiluminescence (Enhanced Chemiluminescence Substrate for 1000 cm^2 of membrane). Densitometry analysis was performed with General Electric Image Quant LAS 4000 mini scanner and Image J software (GE Healthcare Bio Sciences, Baie d'Urfé, QC).

6.17 Statistical analysis

Results are presented as mean ± SEM of 3 independent experiments carried out in triplicate. Statistical analysis was performed with Prism GraphPad software (La Jolla, CA). The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's and/or Tukey's multiple comparison test for statistically significant differences between groups, as applicable. Statistical significance was set at a level of $P < 0.05$.

7. Results

7.1 Fractionation of FBBE

Phytochemical fractionation scheme and selection of pure compounds was obtained by using a C18 column with varying ethanol concentrations; sugars and organic acids were removed and several fractions were obtained as illustrated in Figure 1 and listed in Table 1. This fractionation scheme was used previously to study the effect of NBE components on cardiomyocyte integrity (Louis et al., 2014) and was applied to FBE as a practical approach. Based on HPLC profiles (Figure 1), fractions containing phenolic compounds (F2), whose subfractions contained gallic acid (GA), catechol (CAT) and protocatechuic acid (PA) (Fraction F3-1), as well as chlorogenic acid (CA) (Fraction 3-2).

Table 1 Fractionation of fermented blueberry extract (F1). The major component(s) in each fraction (F) are indicated.

Fraction	Major Component (s)	Starting Material	Column Resin	Eluant
F1 (FBE)	Sugars, organic acids, growth media, phenolics			
F2	phenolics	F1	C_{18}	EtOH
F3-1	Gallic acid, catechol, protocatechuic acid	F2	C_{18}	12% EtOH
F3-2	Chlorogenic acid	F2	C_{18}	12% EtOH
F3-3	Flavonoids	F2	C_{18}	80% EtOH
F4-1	Anthocyanins	F3-3	LH20	25% EtOH
F4-2	Heteropolymers	F3-3	LH20	50% EtOH
F4-3	Proanthocyanidins	F3-3	LH20	70% Acetone

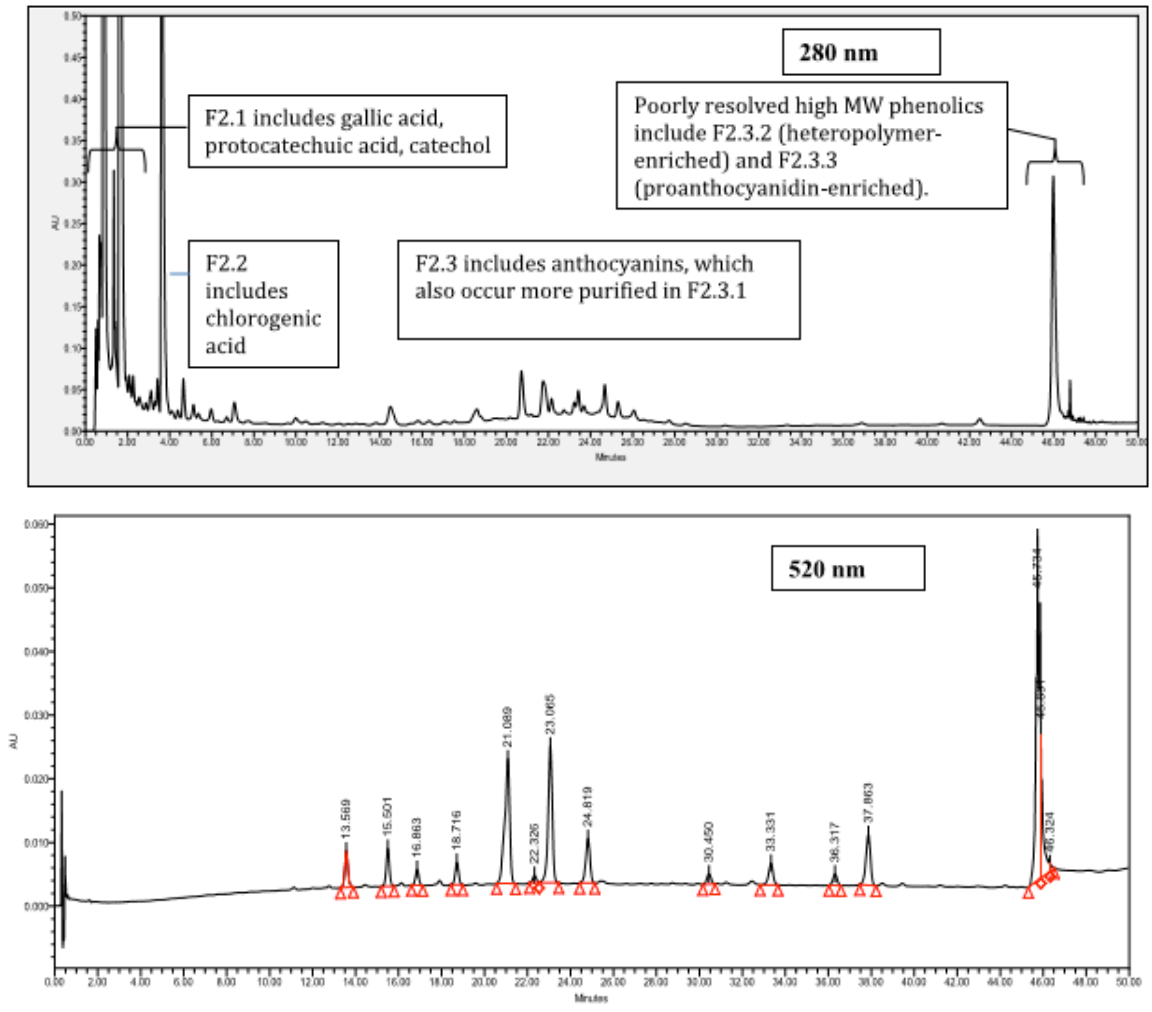


Figure 1 HPLC profile (280 nm) of fermented blueberry extract indicating the major components contained in fractions (see also Table 1).

7.2 Obtention of primary fractions from C. *ficifolia* crude extract

Through an open column chromatography method based on a stationary phase and a mobile phase, we obtained 27 primary fractions from *C. ficifolia* crude extract by using several elution times and two different solvent combinations (acetone/ chlorofom and ethanol/chloroform). From the polar fractions obtained from *C. ficifolia* crude extract, it seemed that the extract might contain terpens, phytoesterols (Garcia et al., 2017) and phenolic compound which are characterizated by large amounts of anti-oxidants, especially flavonoids that are well known as a primary content on *C. ficifolia* fruit (Dezsi et al., 2015), whose characteristics include anti-inflammatory activity.

7.3 Cell viability

Figure 2 presents the results of the LDH cytotoxicity assay. NBE and FBE as well FBE fractions and pure compounds were tested at 5, 10 and 15 µg/ml. As can be appreciated, NBE and FBE crude preparations induced LDH leakage that was similar to the DMSO control at concentrations of 5 and 10 µg/ml. In contrast, the optimal concentrations of other fractions and pure compounds were 5 µg/ml (except catechol at 3 µg/ml). In order to compare the various preparations at similar per weight contents, we also tested NBE and FBE at 5 µg/ml. Cells did not exhibit damage in response to such concentrations of test substances and were morphologically comparable to cells treated with DMSO control (data not illustrated).

On Fig. 3 is shown the results of *C. ficifolia* cytotoxicity evaluation tested on 3T3-L1 pre-adipocytes at concentration of 20, 40,60, 80 and 100 µg/ml of crude extract. As it is appreciated there is lack of toxicity on optimal contration of 20 µg/ml; H4IIE liver cells presents lack of toxicity on 40 µg/ml in (Fig. 5), and in C2C12 murine cells did not show any citotoxicity at 20 µg/ml in response to the crude extract (Fig. 4). Primary fractions from *C. ficifolia* crude extract were tested on 3T3-L1 preadipocytes with lack of toxicity at optimal contration of 20 µg/ml (Table 2). Therefore, this optimal concentration was used for each test indicated in our methodological approach.

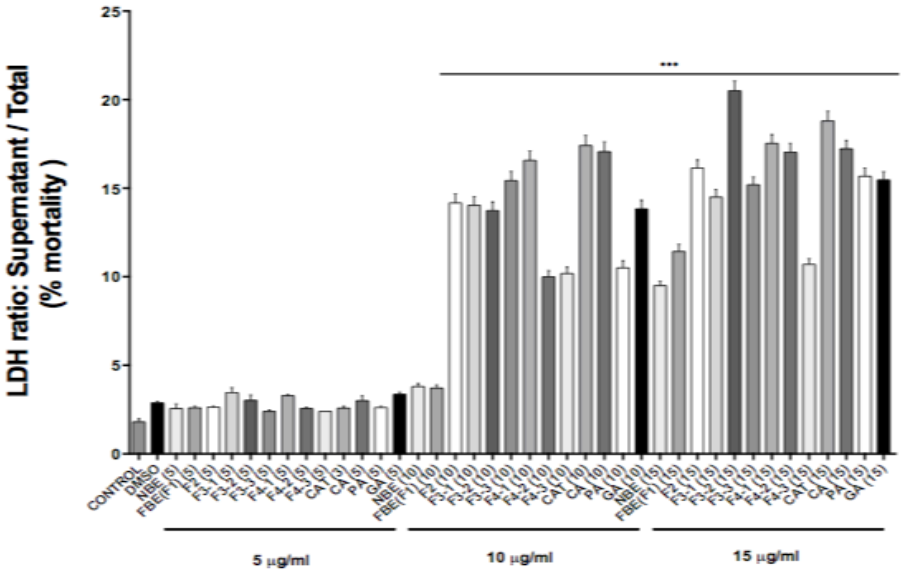


Figure 2. Lack of toxicity of optimal concentration (5_µg/ml), of fermented blueberry extract and components. 3T3-L1 pre-adipocytes were seeded at a density of 2 × 104 cells, cultured to 100% confluence and treated for 7 days with treatments as described above. Cytotoxicity was measured by LDH leakage. Medium LDH activity was expressed as a percentage of total enzyme activity (medium + lysate). The results are shown as the mean ± SEM. n=9.

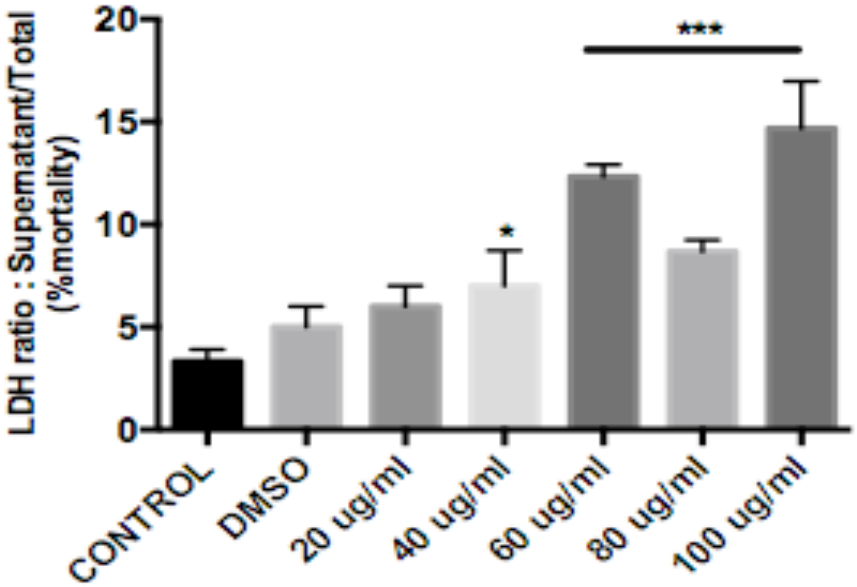


Figure 3. Lack of toxicity of optimal concentration (20 µg/ml), of *C. ficifolia* crude extract. 3T3-L1 pre-adipocytes were seeded at a density of 2 × 104 cells, cultured to 100% confluence and treated for 7 days with treatments as described above. Cytotoxicity was measured by LDH leakage. Medium LDH activity was expressed as a percentage of total enzyme activity (medium + lysate). The results are shown as the mean ± SEM. n=6.

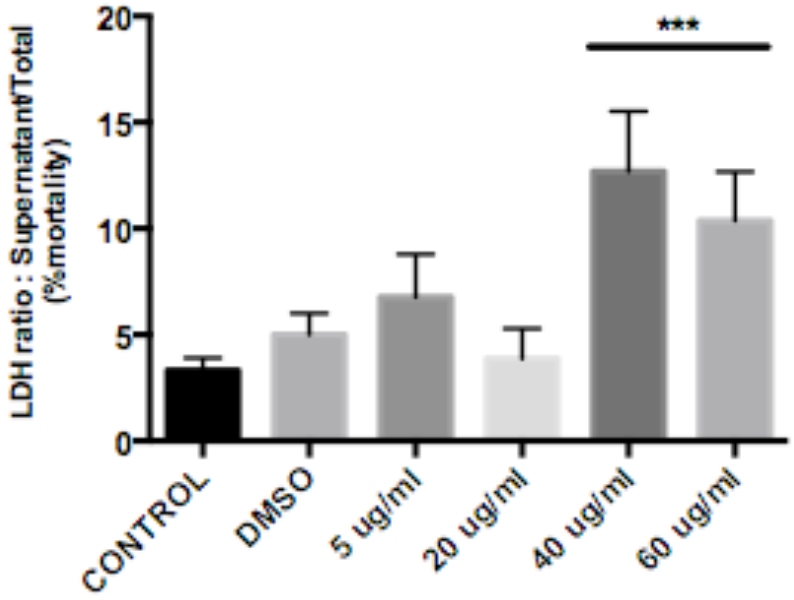


Figure 4. Lack of toxicity of optimal concentrations (20 μg/ml), of *C. ficifolia* crude extract. C2C12 muscle cells were seeded at a density of 2 × 104 cells, cultured to 100% confluence and treated for 7 days with treatments as described above. Cytotoxicity was measured by LDH leakage. Medium LDH activity was expressed as a percentage of total enzyme activity (medium + lysate). The results are shown as the mean ± SEM. n=6.

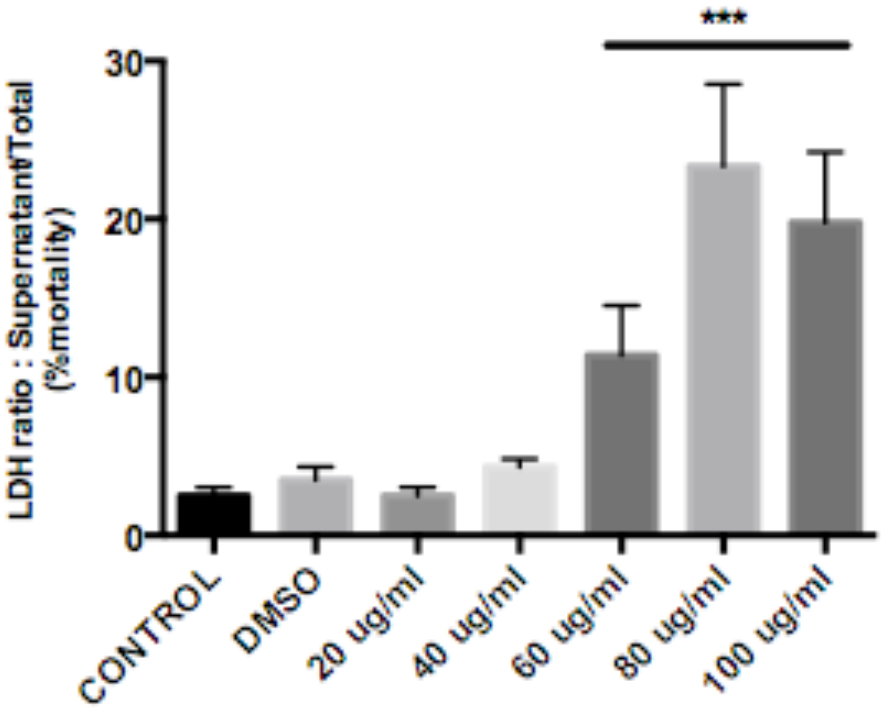


Figure 5. Lack of toxicity of optimal concentrations (40 μg/ml), of *C. ficifolia* crude extract. H4IIE liver cells were seeded at a density of 2 × 104 cells, cultured to 100% confluence and treated for 7 days with treatments as described above. Cytotoxicity was measured by LDH leakage. Medium LDH activity was expressed as a percentage of total enzyme activity (medium + lysate). The results are shown as the mean ± SEM. n=6.

Table 2. Optimal concentrations of plant *C. ficifolia* primary fractionsextracts used to treat 3T3-L1 pre-adipocytes.

CELL LINE	PLANT EXTRACT AND ABREVATION USED	CONCENTRATIONS IN µg/mL AND PERCENTAGE OF VIABILITY
3T3-L1 pre-adipocytes	*C.ficifolia* extract primary fractions	Optimal concentration were observed at 20 µg/ml more than 80% of viability

7.4 Adipogenesis assay

7.4.1 *V.angustifolium*

The quantity of accumulated triglycerides was measured on day 7 in 3T3-L1 cells treated with the specified concentrations of NBE, F1 and its specific fractions, as well as CAT, CA, PA and GA (7 fractions and 4 pure compounds). As illustrated in Figure 6, only certain fractions of F1 were able to significantly inhibit TG accumulation. Notably, fractions F2 and F3-2 reduced triglyceride accumulation to 25 and 30% as compared to the vehicle control (set at 100%), respectively. Similarly, only two of the four pure compounds exerted a significant anti-adipogenic effect. Indeed, CA and CAT yielded adipogenic values that were 75 and 70% lower than DMSO (Fig 6).

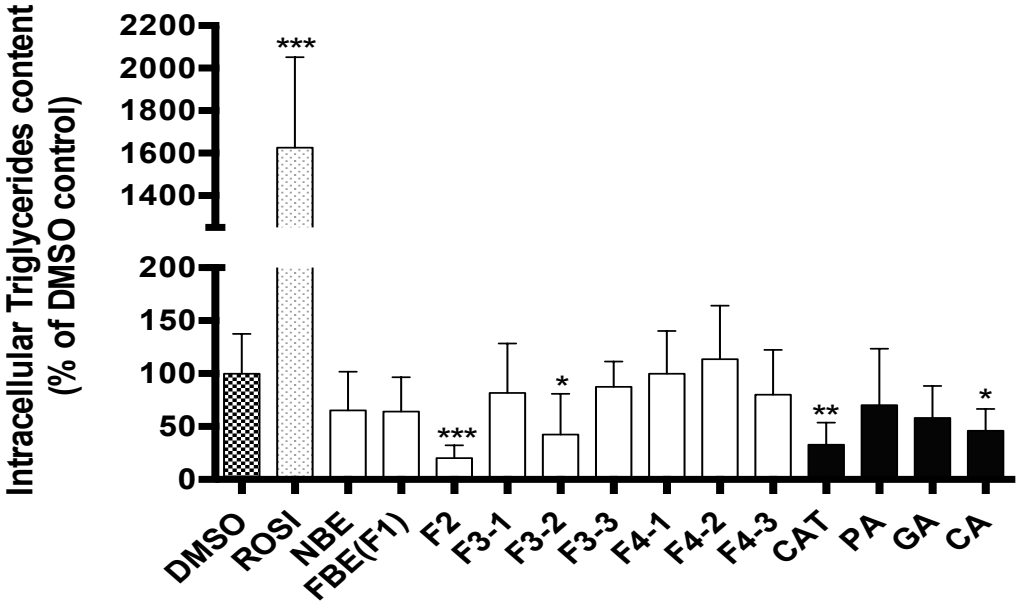


Figure 6. Fermented blueberry extract and fractions inhibit adipogenesis. 3T3-L1 pre-adipocytes were seeded at a density of 2 × 104 cells, cultured to 100% confluence and treated for 7 days with indicated treatments as described in Materials and Methods. Rosiglitazone was used as a positive control inducing differentiation (10 μM). DMSO 0.1% was used as vehicle control. The results are shown as the mean ± SEM. Significantly different compared to DMSO control DMSO (one way ANOVA: post hoc analysis Dunnett`s multiple comparison test; P< 0.05). *Statistically significant from vehicle control (p<0.05); **statistically significant from vehicle control (p<0.01). ***statistically significant from vehicle control (p< 0.001). n=9.

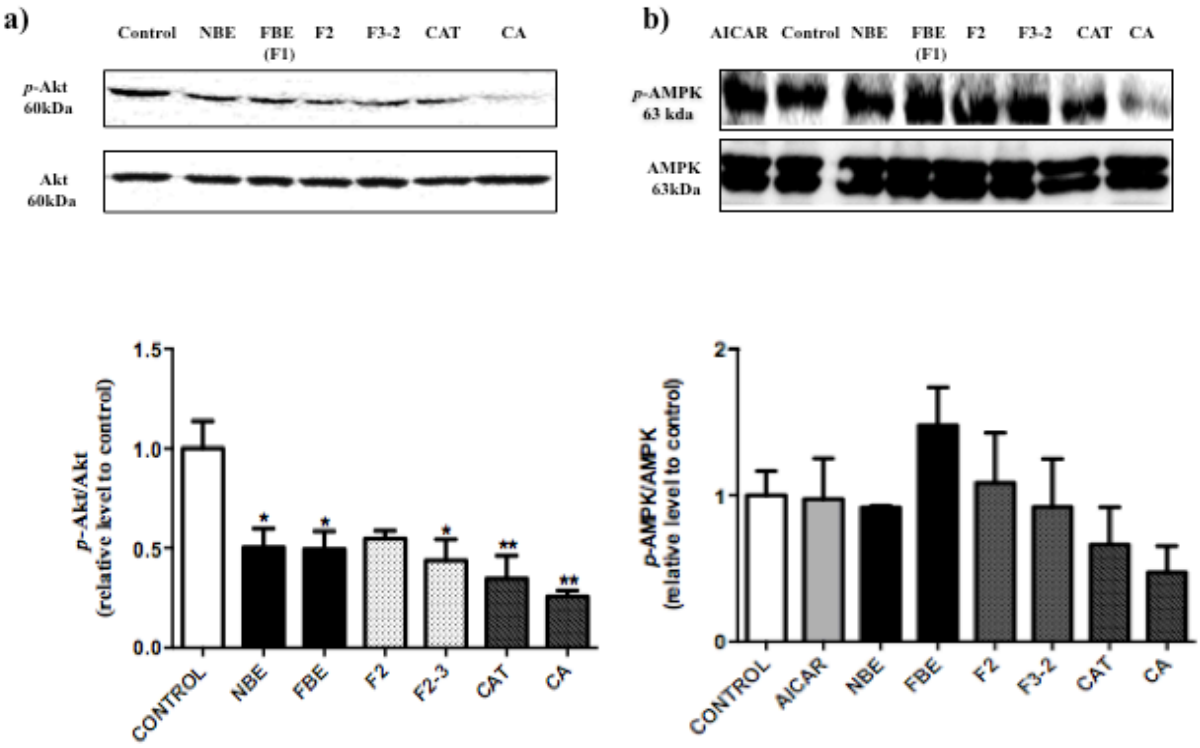
7.5 Western blot analysis

7.5.1 *V. angustifolium*

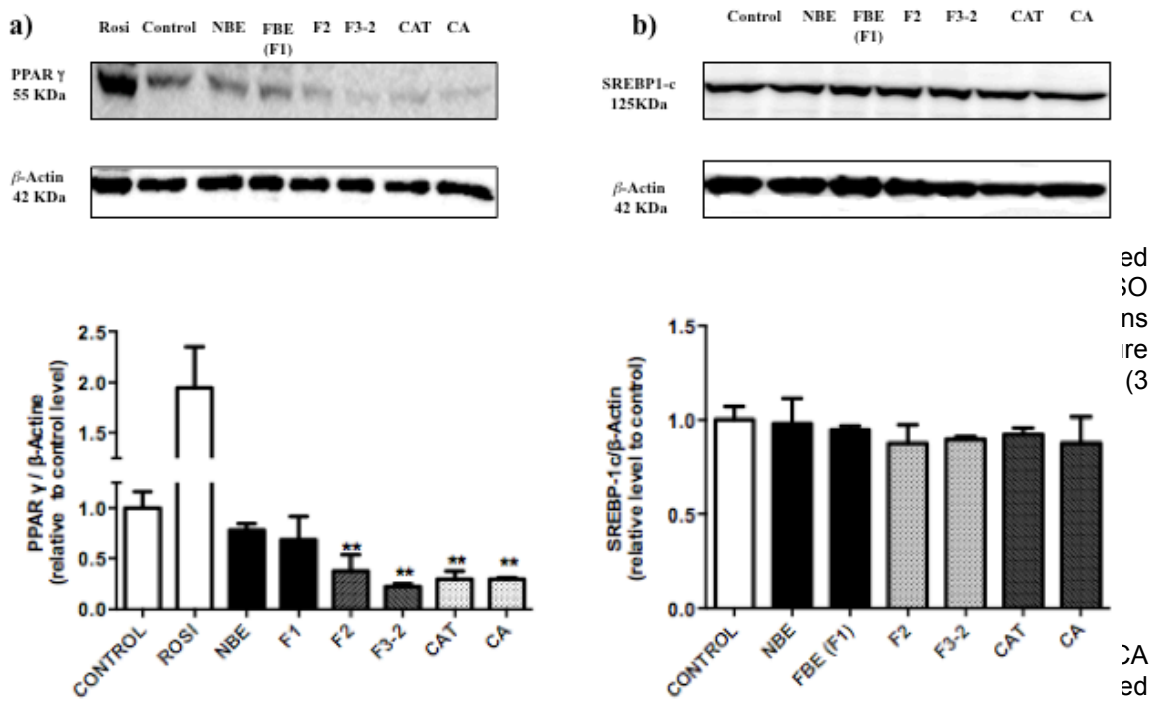
Fractions and pure compounds with significant anti-adipogenic effect, namely F2, F3-2, CAT and CA, were analyzed by Western blot for cellular components involved in the control of adipogenesis and were compared with NBE and FBE (F1). As shown in Figure 7 a), all treatments that inhibited adipogenesis also decreased the phosphorylation of the insulin-dependent kinase Akt, with CA having the most prominent effect. In contrast, the phosphorylation of the insulin-independent kinase AMPK was not significantly affected by any of the treatments,

albeit F1, F2 and F3-2 had a tendency to increase it relative to vehicle control (Figure 7 b).

We next examined two selected key adipocyte-associated transcription factors. As illustrated in Figure 8 b, SREBP-1c was not significantly affected by any of the treatments. Conversely, all treatment conditions that significantly reduced adipogenesis, namely F2, F3-2, CAT and CA, markedly decreased the expression of PPARγ as compared to the vehicle control (Figure 8 a). As expected, the positive control rosiglitazone significantly increased PPARγ expression (Figure 8 a).



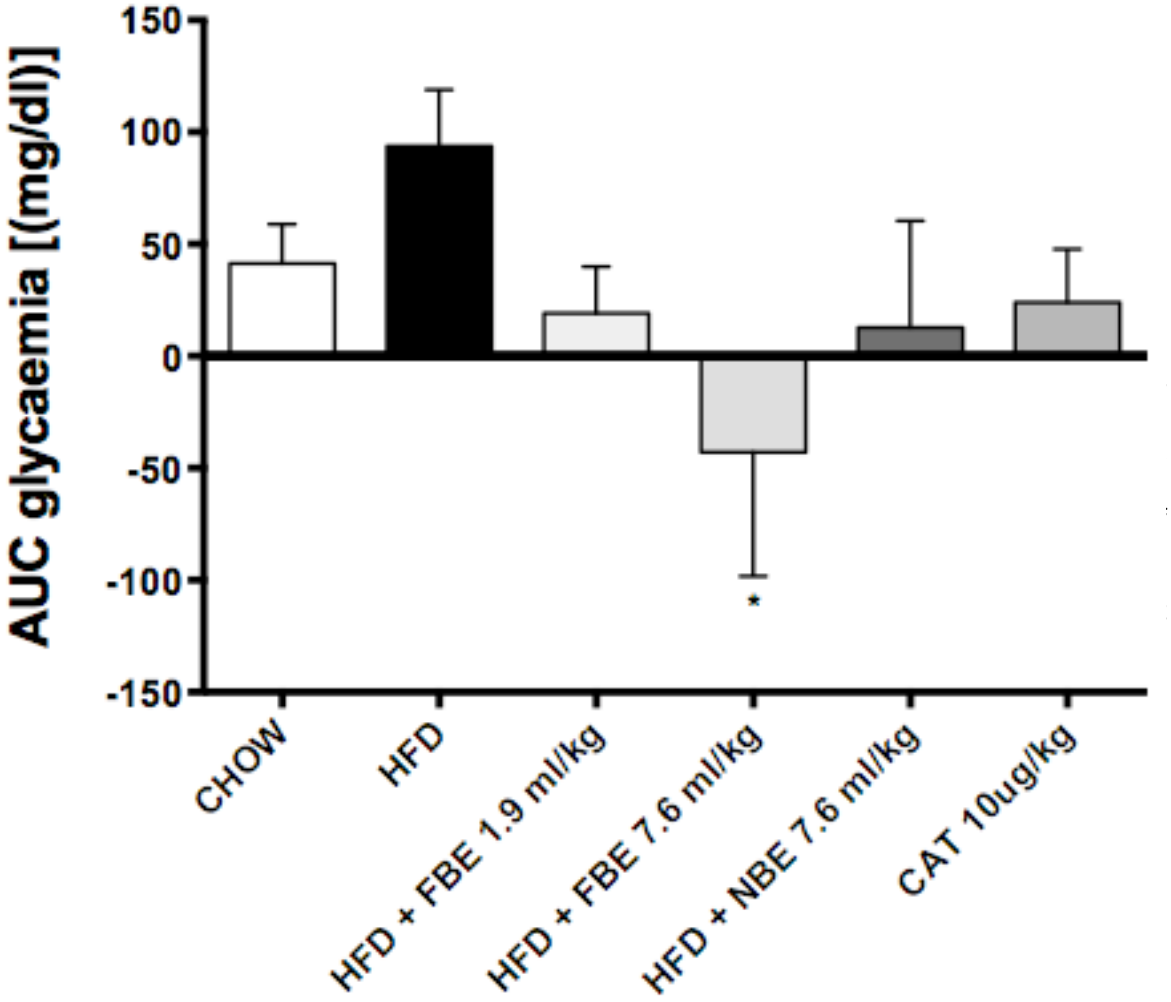
signalling proteins were measured after treatments by Western blot analysis; p-Akt, Akt (a), p-AMPK, AMPK (b). The results are shown as the mean ± SEM. Significantly different compared to DMSO control *p < 0.05, **p < 0.01, n=3.



to differentiate. PPARγ (a) and SREBP-1c (b) expression levels were examined by Western blot. Protein expressions were normalized using β-actin as a reference. The results are shown as the mean ± SEM. Significantly different compared to DMSO control *p < 0.05, **p < 0.01. n=3.

7.6 *In vivo* study

In vivo FBE showed a significant reduction on glycaemia in DIO mice for treatment with 1.9 ml/kg of FBE compared with high fat diet (HFD) and Chow control without effect on body weight during 8 weeks of treatment exposed (Figs. 9 and 10). IPGTT test showed significant differences between HFD control and NBE during the first 15 and 60 minutes (Fig. 11). Other treatments groups shown significant difference with CHOW control but not with HFD control. In contrast, there was not significant difference on weight with mice treated with either NB or FBE on high fat diet DIO treatment (Fig. 10).


AUC glycaemia [(mg/dl)]
150
100
50
0
-50
-100
-150
*
CHOW
HFD
HFD + FBE 1.9 ml/kg
HFD + FBE 7.6 ml/kg
HFD + NBE 7.6 ml/kg
CAT 10ug/kg

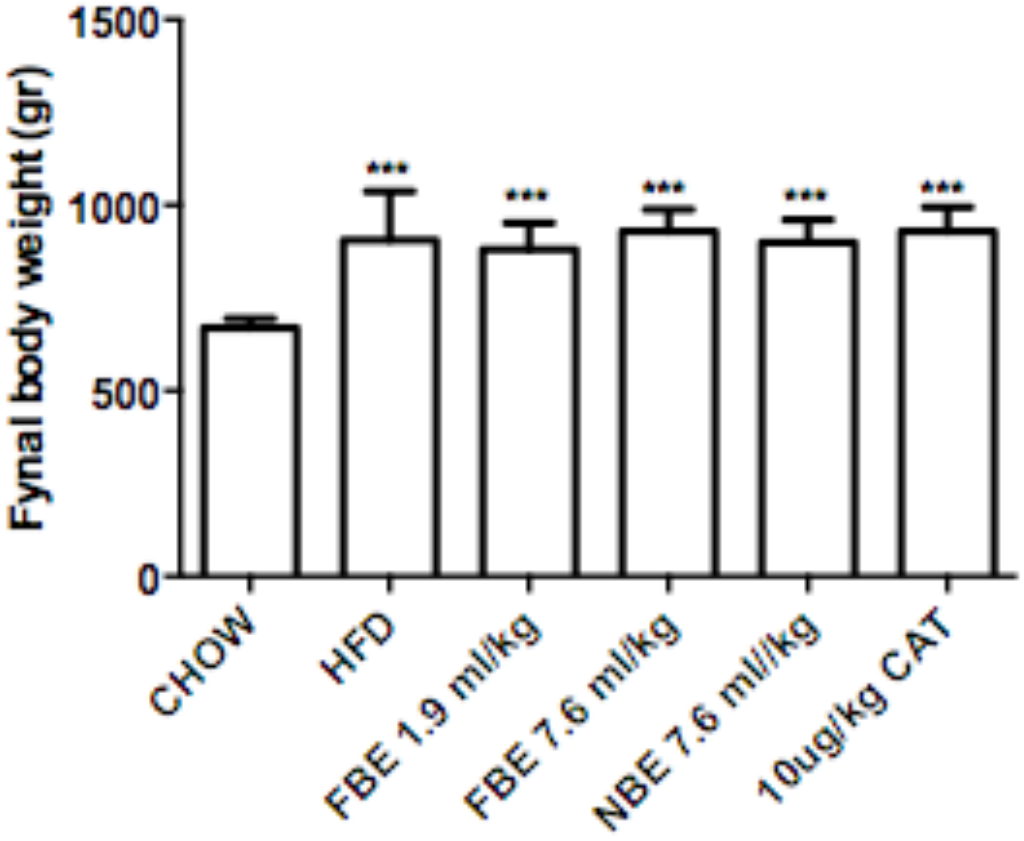
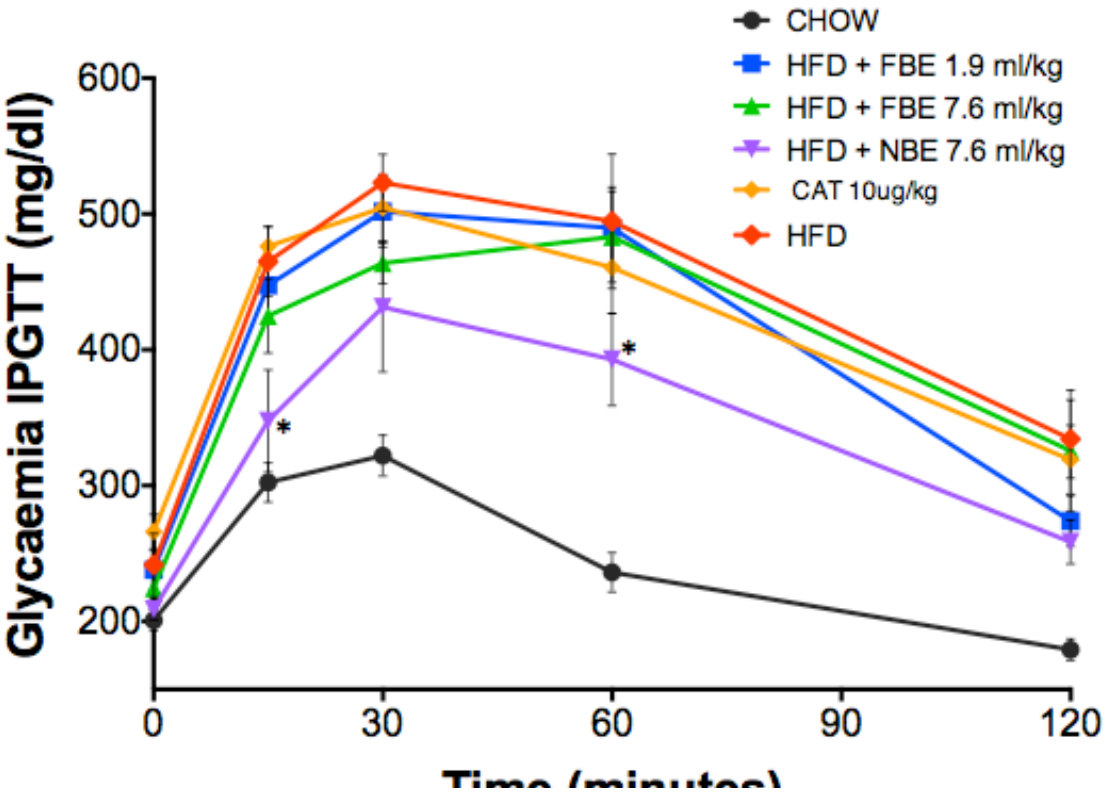


Fig10. Cumulative body weight (CBW) in C57BL/6 mice treated with Chow control, HFD (DIO), or Fermented Blueberry Extract (FBE) at 1.9, 7.6 ml/kg and Normal Blueberry Extract (NBE) at 7.6 ml/kg and one pure compound CAT 10 μg/kg which was incorporated in food for 8 weeks in the treatment protocol. All values are mean ± SEM. Number of animals/group: n=7. Values are mean ± SEM. One way ANOVA: post hoc analysis multiple comparison Dunnet test). *Statistically significant from controls (CHOW and HFD) ($p < 0.05$) n=7



1.9-7.6 ml/kg and Catechol CAT 10 μg/kg which was incorporated in the HFD for 8 weeks in the treatment protocol. Total AUC for blood glucose levels versus time in min was calculated. Values are mean ± SEM. Number of animals/group: n=7. One way ANOVA: post hoc analysis multiple comparison Dunnet test). *Statistically significant from controls (CHOW and HFD). ($p<0.05$) n=7

7.7 Glucose uptake (C2C12 murine myoblasts)

7.7.1 *C. ficifolia*

Muscle murine myoblasts are efficient as an *in vitro* system to show induction of glucose transport by insulin or a defect in glucose transport efficiency and involving the specific transporter GLUT4 activity that results from insulin resistance. However, *C. ficifolia* crude extract at maximal non-toxic concentration of 20 μg/ml did not induce glucose uptake (Fig 12), compared with positive controls, Metformin and Insuline.

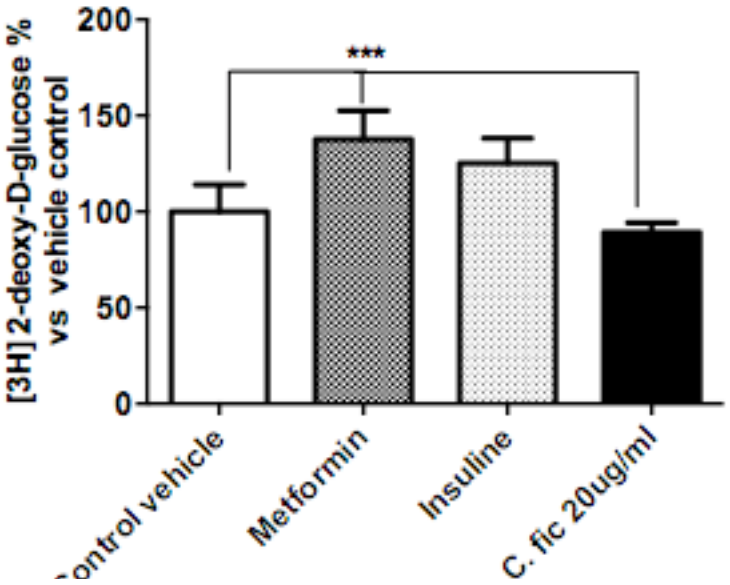


Fig. 12. ^{3} H-Deoxyglucose uptake into cells shown as a percentage of vehicle control after 18 h treatment of the differenciated murine myoblasts C2C12 at day 6 with *C. ficifolia* at 20 μg/ml concentration and Metformine at 400 mM for 18 h. Cyt B (cytocalasin B), and isulin (In) at 100 nM for 30 min. Values are mean ± SEM. One way ANOVA: post hoc analysis multiple comparison Dunnet *** statistically significant from vehicle control ($p < 0.001$). n=6.

7.8 Phytochesmistry of *C. ficifolia* aqueous fraction

7.8.1 Chromatrogram HPLC

In chromatographic analyses the highest concentration of *C. ficifolia* aqueous fraction was detected at 20-25 min of retention time.

A defatting process was performed with 20 ml of reagent grade hexane where 1.38 g of extract was dissolved in 20 ml HPLC grade deionized water. This step was performed to remove the fat components that were extacted and could interfere with the analysis. The extract was subjected to partitioning with 40 ml of ethyl acetate (three times). This represents an organic fraction of the aqueous extract. This type of extraction isolates components of medium polarity that are dissolved in the ethyl acetate phase (organic phase) and separated from the water. The organic fraction of ethyl acetate was filtered through anhydrous sodium sulfate, in order to remove residual water. In this process, we obtained 3.5 mg, which is below expected performance. This happened because some emulsions formed and this

decreases the performance of the organic fractions. We then took 1 mg of this fraction and dissolved it in 400 µl of ethyl acetate, which we subjected to partitioning with HPLC deionized water again (200 µl), and we dry over anhydrous sodium sulfate. This step was carried out because in the first tests we saw that there was a very polar part inteferíng with the rest of the analysis, perhaps pigments, sugars or polar fatty acids. We dissolved the resulting ethyl acetate organic fraction (0.5 mg) in 300 µl of acetonitrile: water mixture (both HPLC grade, 50:50) and proceeded to inject 20 µl on the HPLC.

In our experimental conditions first peaks in the chromatogram (from 0 to 5 min) corresponded to highly polar compounds (peak 1) Fig 13 a), then polarity decreases as retention time increases. From these data, we can deduct that the compounds we are seeing are most likely phytoestorols (Garcia et al., 2017), because the absorption maximum they have is characteristic of many compounds of this type (peaks 2,3,4) (Fig 13 a)). It may also be noted that there is a majority compo

The

differer

confirm

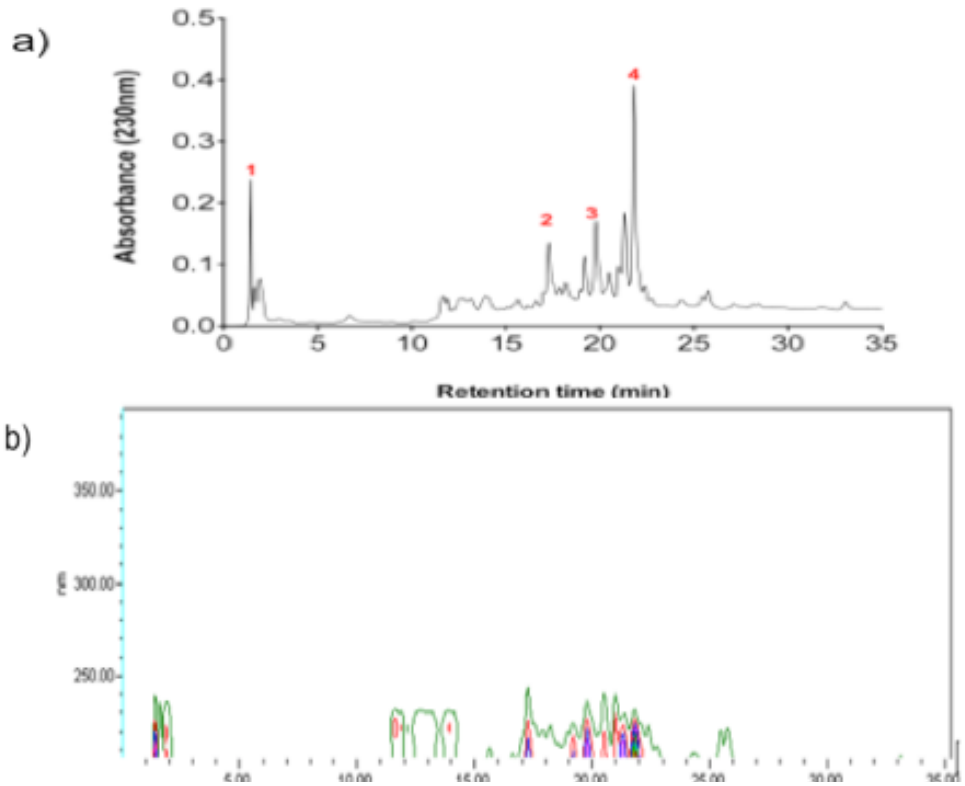


Fig. 13 RP-HPLC profiles of *Cucurbita ficifolia* crude extract (aqueous fraction) from wavelength (205-360 nm). The HPLC separation analysis was performed in a Waters cromatrograph in a PDA detector in a software Empower 2 (Fig. 13 a) and confirmed by UV (Fig. 13 b).

7.9 Adipogenesis assay

7.9.1 *C. ficifolia*

The quantity of accumulated triglycerides was measured on day 7 in 3T3-L1 cells treated with *C. ficifolia* extract and primary fractions. On figure 14 it is showed that *C. ficifolia* extract has a significant inhibitory effect on TG accumulation during the differentiation of the adipocyte (adipogenesis) compared with vehicle control. The anti-adipogenic effect represents a 70%. In respect to effects on adipogenesis from 27 primary fractions obtained from *C. ficifolia* extract, only crude extact and primary fractions F16 and F17 showed an inhibitory effect (70-75%) on TG content, with a statistically significant difference compared to control (Figs. 14 and 15).

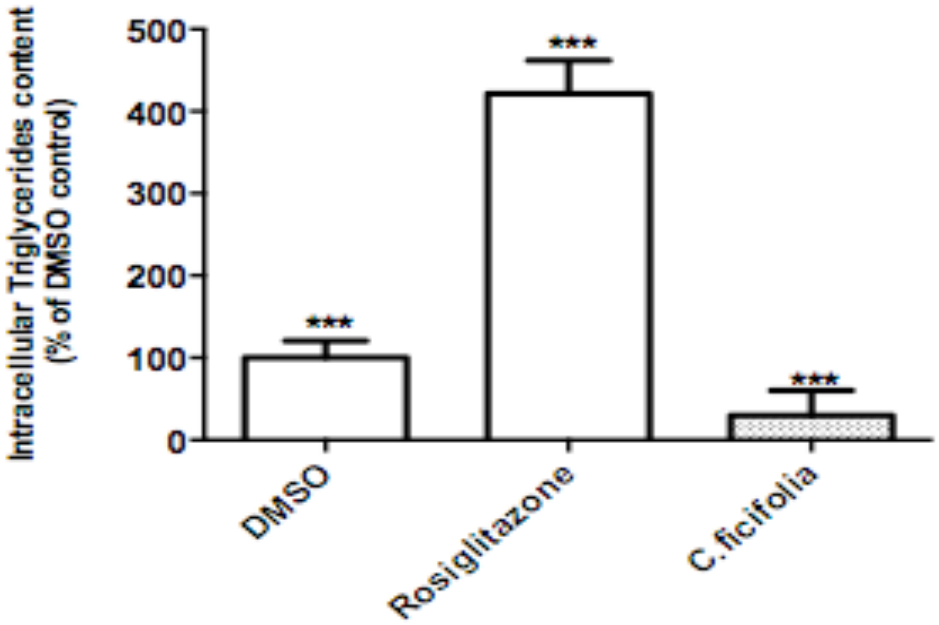


Fig 14. Anti-adipogenic activity of *C. ficifolia* extract at 20 μg/ml concentration. 3T3-L1 pre-adipocytes were seeded at a density of 2 × 104 cells, cultured to 100% confluence and treated for 7 days with indicated treatments as described in Materials and Methods. Rosiglitazone was used as a positive control inducing differentiation (10 μM). DMSO 0.1% was used as vehicle control. The results are shown as the mean ± SEM. Significantly different compared to DMSO control (one way ANOVA: post hoc analysis Dunnett`s multiple comparison test). *** Statistically significant from vehicle control ($p<0.001$). n=12.

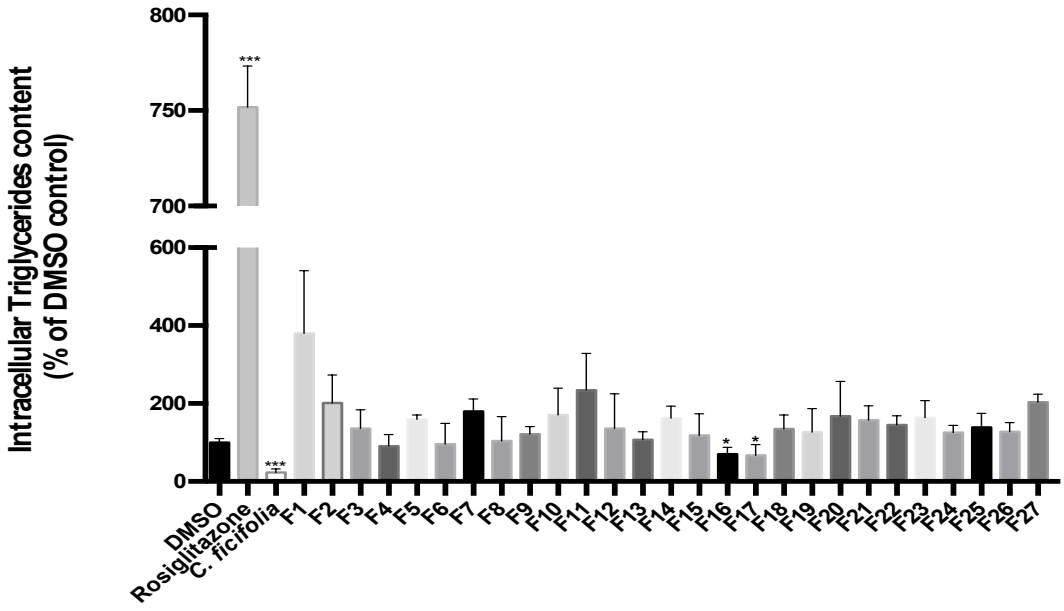


Fig 15. Adipogenic activity of *C. ficifolia* extract, 27 primary fractions obtained from *C. ficifolia* extract at 20 μg/ml. 3T3-L1 pre-adipocytes were seeded at a density of 2 × 104 cells, cultured to 100% confluence and treated for 7 days with indicated treatments as described in Materials and Methods. Rosiglitazone was used as a positive control inducing differentiation (10 μM). DMSO 0.1% was used as vehicle control. The results are shown as the mean ± SEM. Significantly different compared to DMSO control (one way ANOVA: post hoc analysis Dunnett`s multiple comparison test; * Statistically significant from vehicle control ($p<0.05$). *** Statistically significant from vehicle control ($p< 0.001$). n=9.

7.10 Western blot analysis

7.10.1 *C. ficifolia* on 3T3-L1 adipocytes

Inhibitory effect on TG content in 3T3-L1 adipocytes was analysed and confirmed by Western Blot. As shown, treatments (*C. ficifolia* crude extract) that decreased

adipognenesis slightly increased the phosphorylation of the insulin-independent kinase AMPK compare to DMSO control vehicle, however, this modulation was not statistically significant (Fig. 16).

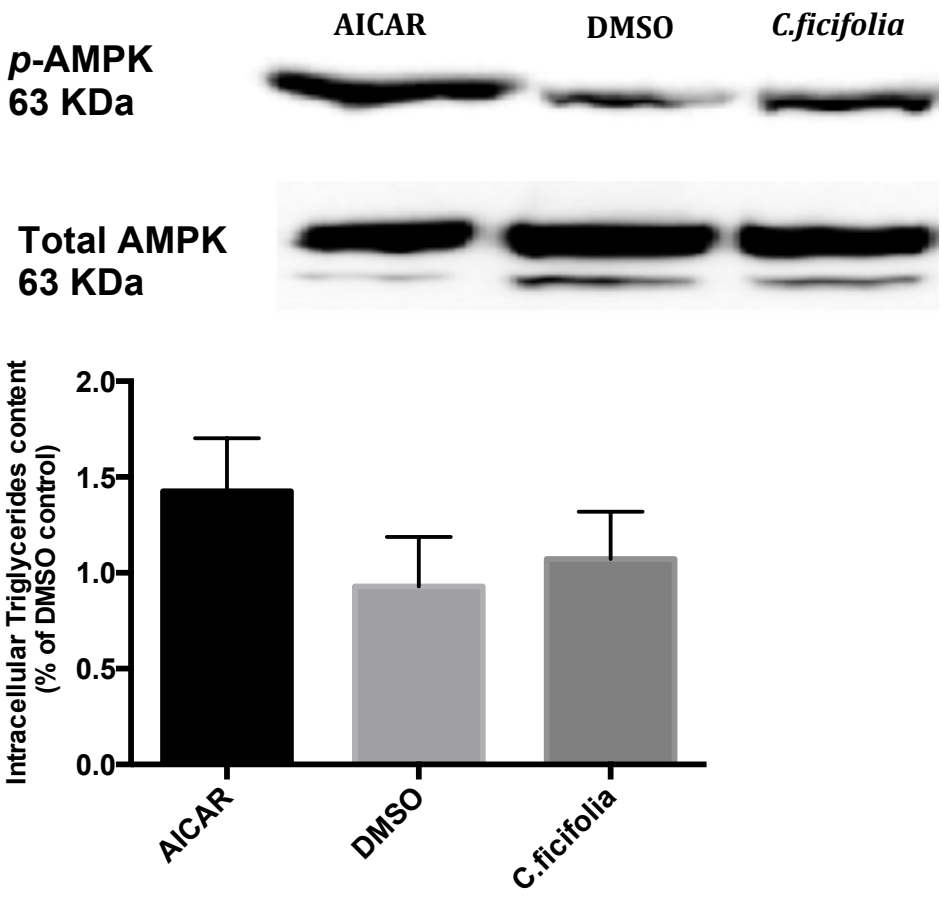


Fig. 16. Effects of *C. ficifolia* aqueous extract on the phosphorylation of insulin-independent AMPK. 3T3-L1 preadipocytes were incubated with DMSO (0.1%) or 20 µg/ml of *C. ficifolia*, induced to differentiate. The expression levels of key signalling proteins were measured after treatments by Western blot analysis p-AMPK, AMPK. The results are shown as the mean ± SEM. n=3.

7.11 Glucose-6-phosphatase

H4IIE cells (90% confluent in 12-well plates) were treated for 18 h with negative control (0.1% DMSO vehicle), positive control (Insulin, 100nM), and *C. ficifolia* crude extract at the optimal nontoxic concentration (40 µg/ml). *C. ficifolia* extract

decreased the enzyme activity by approximately 51.5% whereas insulin reduced it by 63%, both compared to DMSO vehicle control. The inhibition of glucose-6-phosphatase in H4IIE hepatocytes was slightly lower than the insulin positive control that was run in parallel (positive control). See figure 17.

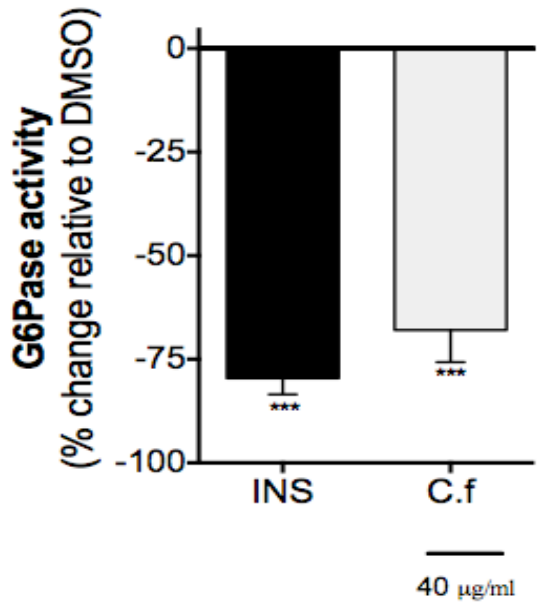
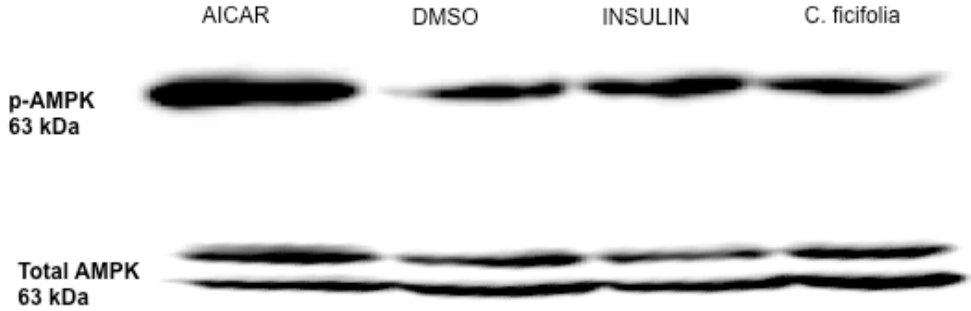


Figure 17. Effect of insulin and *C. ficifolia* plant extract on G6Pase activity. Results represent the change in G6Pase activity observed after overnight treatment of H4IIE cells with *C. ficifolia* extract (40 μg/ml) or with insulin (100 nM). They are expressed relative to DMSO (0.1%) and vehicle controls (0% inhibition). Assays were carried out in triplicate on three different cell cultures. ***p*<0.01 significantly different from DMSO vehicle control. ****p*<0.001 significantly different from DMSO vehicle control. n=3.



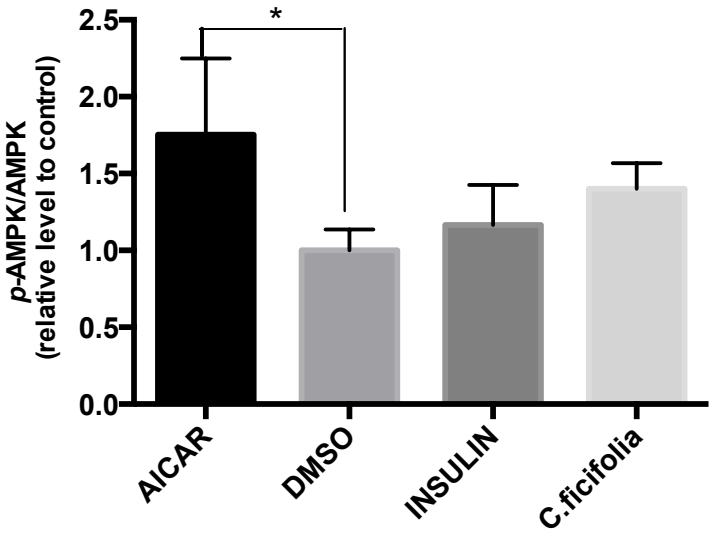


Fig. 18. Effects of *C. ficifolia* extract on the phosphorylation of insulin-independent AMPK. H4IIE adipocytes were incubated with vehicles controls DMSO (0.1%), Insulin (100 nM), AICAR (4mM) and treatment of 40 µg/ml *C. ficifolia* aqueous extract. The expression levels of key signalling proteins were measured after treatments by Western blot analysis p-AMPK, AMPK. The results are shown as the mean ± SEM. Significantly different compared to DMSO control *p<0.05. n=3.

The inhibition on G6pase on H4IIE hepatocytes treated with the aqueous *C. ficifolia* extract was confirmed by western blot. As mentioned above, H4IIE hepatocytes were incubated with *C. ficifolia* extract and tested for phosphorylated AMPK. As shown in Figure 18, *C. ficifolia* extract did not have significant modulation on p-AMPK compared to controls. Nevertheless, there was a tendency to increase its activations when treated with C. ficifolia extract compared with controls DMSO and insulin.

8. Discussion

Research in obesity and diabetes is increasingly focusing on adipose and muscle tissues as well as in adipogenesis and glucose uptake process, since these tissues

appears to be involved with the development of metabolic syndrome (Grundy, 2015). Food and medicinal plants are important sources of natural products that display great chemical diversity, making them excellent candidates for drug development, including for the treatment of obesity and other related diseases as T2D (Abdollahi and Afshar-Imani, 2003).

8.1 Potential anti-obesity and anti-diabetic actions of *V. angustifolium*

For *V. angustifolium,* we sought to determine the biologically active compounds that could underlie such anti-adipogenic effects. We used an HPLC-based fractionation scheme that provided us with several fractions focused primarily on phenolic components. We notably identified four major compounds, namely gallic acid, catechol, protocatechuic acid and cholorogenic acid, which we also tested. We used the 3T3-L1 cell line, one of the most reliable models for the study of adipogenesis (Ntambi et al., 2000)

In previous studies, we observed that the fermentation of the juice from wild lowbush blueberries (*Vaccinium angustifolium* Aiton), with a bacterium (*Serratia vaccinii)* found on the fruit skin, was able to greatly increase the total phenolic content (Martineau et al., 2005). This biotransformation also conferred it potent anti-diabetic properties (Vuong et al., 2007). In the latter study, we also uncovered an inhibitory action of fermented blueberry juice on 3T3-L1 adipogenesis, representing a putative anti-obesity action that was later confirmed *in vivo* (Vuong et al., 2009). One of the aims of this PhD project was to determine the biologically active compounds that could underlie such anti-adipogenic effects.

As a first step, we freeze-dried the fermented blueberry juice and the resulting extract, called F1, had a tendency to inhibit adipogenesis, albeit not to the extent that the intact fermented blueberry juice had done previously (Vuong et al., 2007). This may be explained by the different concentrations used in the two studies. In our previous study, intact fermented blueberry juice was administered to cells at a dose of total phenolics equivalent to 30 μM of gallic acid (GAE) (Vuong et al., 2007). In the present studies, we used FBE/F1 at the same weight-based concentration found to be optimal for the phytochemical fractions and pure compounds, in order to have a valid comparison. This concentration (5 μg/ml) amounts to 1.6 μM GAE, when the total phenolic content of FBE was taken into consideration. It is therefore almost 20 times less concentrated than that used in our previous study (Vuong et al., 2007). More surprisingly, when NBE was subjected to a similar freeze-drying step, it was found to also have a non-significant tendency to reduce adipogenesis. This was different than what was observed in our previous study, where NBE was without effect (Vuong et al., 2007). Further studies will be necessary to understand the potential effect of freeze-drying on the phytochemical composition of wild lowbush blueberry juice, notably in the context of adipogenesis. Indeed, other researchers, using cultivated highbush blueberry phenolics, have observed a concentration-dependent inhibition of adipogenesis in the 3T3-F44A cell line (Moghe et al., 2012).

Notwithstanding, we found that two sub-fractions of our fermented blueberry extract exhibited a significant inhibition of adipogenesis. The first, F2, was the fraction that contained all the phenolic compounds while the second, F3-2, was enriched in chlorogenic acid. This was confirmed by the corresponding activity of

the pure compound (chlorogenic acid). Of the other pure compounds, only catechol was found to also inhibit adipogenesis significantly. This implies that chlorogenic acid and catechol may contribute to the anti-obesogenic potential of fermented blueberry juice.

We next began assessing some of the potential molecular mechanisms that can underlie the anti-adipogenic activity of fermented blueberry juice. Adipogenesis involves a complex and coordinated transcriptional cascade that leads to lipogenesis and TG accumulation (Martineau et al., 2010; Sasisharan et al., 2011). Insulin-dependent Akt signalling can trigger this cascade (Altomare et al., 2012; Zhang et al., 2009; Nielsen et al., 2008). In contrast, insulin-independent AMPK signalling can inhibit adipogenesis (Vuong et al., 2007). In line with the inhibitory effect of fermented blueberry juice and its components, we found that F1 as well as sub-fractions F2 and F3-2 significantly inhibited the phosphorylation of Akt. This result is in line with the report by Song and collaborators who studied blueberry peel extracts and found an anti-adipogenic effect that was related to the inhibition of Akt phosphorylation (Song et al., 2013).

On the other hand, F1, F2 and F3-2 had a tendency to increase AMPK, although this effect failed to reach statistical significance. This implies that fermented blueberry products may diminish adipogenesis principally by reducing insulin-dependent signalling. Interestingly, our previous work had shown that fermented blueberry juice could stimulate AMPK in 3T3-L1 adipocytes, an effect that was implicated in the stimulation of glucose transport observed in the same cells (Voung et al., 2007). The implication of AMPK in the inhibition of adipogenesis by

fermented blueberry juice may be less critical at the concentration used in the present studies.

The adipogenic process is a complex and highly regulated program of gene expression that involves several key transcription factors that include SREBP-1c and PPARγ (Ntambi et al., 2000; Cristancho et al., 2011). To determine the protein content of these two transcription factors is an initial step to assess potential underlying molecular mechanisms. Although treatment with sub-fractions F2 and F2-3 as well as pure compounds CAT and CA failed to significantly modulate SREBP-1c protein expression, they all had a tendency to reduce it. However, SREBP-1c is activated early in the transcriptional cascade such that our assessment near the end of adipogenesis may explain the low variations in protein expression that we observed. More detailed studies, including time-course and mRNA assessments will be necessary to verify the potential role of SREBP-1c in the effects of FBE on adipogenesis.

These results clearly show that the same active components of fermented blueberry juice induced a strong inhibition of PPARγ. These results suggest that the negative modulation of PPARγ may be involved in the anti-adipogenic action of fermented blueberry juice. This is similar to what was observed by Song and collaborators, where blueberry peel extracts significantly reduced PPARγ (Song et al., 2013). It is well known that a high phenolic content is associated with the prevention of certain chronic diseases, whereas phenolic acids have demonstrated antioxidant and anti-inflammatory properties (Manach et al., 2004). Indeed, chlorogenic acid, a major component of green bean coffee extract, has been reported to reduce blood sugar levels and potentially exert an anti-diabetic effect

(Meng et al., 2013). It has also been implicated in weight loss in humans (Blum et al., 2007; Thom E. 2007) and recently shown to stimulate lipolysis in adipocytes in culture (Hsu and Yen 2008; Flanagan et al., 2014).

Results suggest that chlorogenic acid may also exert its anti-obesity potential through the inhibition of adipocyte Akt phosphorylation and of the downstream transcription factor PPARγ, a major regulator of adipogenesis. On the other hand, catechol is a simple polyphenol found in a wide variety of plants. In the present studies, it was also found to significantly diminish adipogenesis through mechanisms involving an inhibition of Akt phosphorylation and a reduction in PPARγ. To our knowledge, this is the first instance where such biological activity has been reported for catechol.

For the *in vivo* study using diet-induced obese mice (C57BL/6J), our findings still show some discrepancy in the obtained results. Indeed, the treatment with FBE had a tendency to reduce glycaemia in DIO mice with a significant difference observed at 7.6 ml/Kg treatment as compared to control. However, this treatment did not have any effect on body weight compared with the high fat diet (HFD) control. In addition, in the IPGTT study, we observed a significant effect of NBE to reduce the area-under-the-curve as compared to HFD controls, whereas we failed to observe this effect with FBE as expected.

Several factors could explain these unexpected results. In a previous study, we incorporated fermented blueberry juice (FBJ) directly into the drinking water of young KKAy mice (Vuong et al., 2009). This partly diminished hyperphagia in these diabetic mice and significantly reduced their weight gain. Moreover, FBJ protected young KKAy mice against the development of glucose intolerance and T2D in a

manner that involved the adipokines pathway as indicated by the significantly increased adiponectin levels in these obese mice (Vuong et. al., 2009). Since KKAy mice have a defect in the melatonin pathway that involves Agouti-related peptide and modulates hunger and satiety, our results suggest that FBJ may act on such central mechanisms. In the present studies, we used the DIO model based on C57BL/6J mice, which have no hyperphagic defect. In addition, we incorporated the freeze-dried extract of FBJ (FBE) directly into the HFD. It is thus possible that FBE may not affect feeding behavior in that model and this could explain, at least inpart, why we failed to see effects on body weight. The reason why NBE had a significant effect on IPGTT, whereas FBE did not, will require further experimentation.

8.2 Potential anti-obesity and anti-diabetic actions of *C. ficifolia*

On the other hand, we carried out primary phytochemical fractionation of *C. ficifolia* crude extract as a preliminary step towards bioguided assay assessment of this plant in terms of anti-obesity and anti-diabetic effects. This it is especially relevant since the crude extract of *C. ficifolia* has shown promising effects in this context (Alarcon et al., 2002), *C. ficifolia* has shown to be a potential source for the development of novel and alternative nutraceutical preparation for the treatment of type 2 diabetes and related diaseases. Since adipogenesis is intricately related to adipocyte differentiation and maturation, inducing apoptosis and inhibiting

adipogenesis at various stages of the adipocyte life cycle may be a target for treating obesity.

In Mexico, traditional medicine uses different plants for the treatment of T2D, including *Cucurbita ficifolia* Bouché (Cucurbitaceae). The fruit juice prepared from this plant has a hypoglycaemic effect in animals with experimental diabetes (Roman et al., 1992; Alarcon et al., 2002), and in T2D patients (Acosta et al., 2001). Recently, it has been demonstrated that an aqueous fraction of *C. ficifolia* extract has antioxidant and anti-inflammatory effects, in addition to a glucose-lowering effect, in streptozotocin-induced diabetic mice. (Roman et al., 2012; Diaz et al., 2012). Apparently, the hypoglycaemic effect is related to the extract's content of D-chiro-inositol (DCI) (Xia and Wang 2006), which is part of the structure of inositol phosphoglycans. These compounds are mediators of the action of insulin (Kawa et al., 2003), and are activators of glycogen synthase and pyruvate dehydrogenase enzymes. In the current thesis, we demonstrated a clear inhibitory effect of *C. ficifolia* aqueous extract on adipogenesis, which represents a potential anti-obesogenic action that could also be beneficial in T2D.

C. ficifolia extract contains several compounds that recently exhibited effects on oxidative stress, including other components that have a very relevant role over hypoglycaemia and antioxidant effect, such as D-quiro-inositol (DCI), (±)-catechin, L-ascorbic acid, p-coumaric acid and gallic acid (Fortis et al., 2013).

As described in previous studies, a possible mechanism of action involved in insulin pathways is Akt, also known as PKB. It was recently demonstrated that after incubation of adipocytes with 25 mm glucose and *C. ficifolia* or DCI for 24 h, the activation of PKB tended to increase, although this change was not significant.

PKB was activated significantly only by DCI (30 min) in the absence of insulin stimulation (Fortis et al., 2013). These findings reinforce the fact that some components, such as DCI, are relevant for research on the potential role of enzymes involved in insulin pathways that could be directly and/or undirectly related to the process of adipogenesis, lipogenesis and gluconeogenesis.

Dietary bioactives derived from natural products have shown interesting effects on adipose tissue, like inducing apoptosis, decreasing lipid accumulation and inducing lipolysis. Since a number of complex interconnected cells signaling pathways are involved in regulating all the above-mentioned processes, treating adipocytes with multiple natural products can result in enhanced effects. This strategy can be achieved by exerting beneficial effects through additive or synergistic actions of several natural compounds acting at single or multiple target sites in the adipocyte life cycle associated with physiological processes like apoptosis, adipogenesis and lipolysis. In the case of *C. ficifolia* crude extract, we just began to explore different pathways that may provide clues as to possible mechanisms of action. Further research will be needed in order to clarify the potential effects of the plant on adipogenesis.

In our own studies, we did not observe any modulation of glucose transport in C2C12 muscle cells at the optimal concentration of 20 μg/ml despite the fact that other researchers have observed such activity with D-chiro-inositol. This compound is found in a proportion of approximately 30% in the fruit of *C. ficifolia* and was shown to be the responsible for its anti-diabetic properties by acting as an insulin mediator with antihyperglycaemic effects in rats (Xia and Wang 2006). In L6 myotubes, inositol derivatives stimulate glucose uptake; D-chiro-inositol inducing

glucose transport at higher concentrations of 0.1 mM (Yap et al. 2007). It may be that C2C12 myocytes respond differently to *C. ficifolia* or that D-chiro-inositol should be in higher concentrations and the concentration of this compound in the fruit it is not sufficiently high to induce the glucose transport.

Another relevant result obtained in our studies of *C. ficifolia* biological activity related to obesity and T2D is the inhibition of G-6-phosphatase activity. Indeed, it represents one of the principal endoplasmic reticulum enzymes responsible of the final release of glucose into the circulation (Gonzalez-Mujica et al., 2005) and is considered to represent the rate-limiting step of gluconeogenesis (Schmoll D. et al., 2000). Moreover, an inactivating mutation in the gene of this enzyme leads to hypoglycemia, but an increase in its expression is followed by hyperglycemia and the onset of diabetes (Hutton and O'Brien, 2009). To determinate the inactivation of G6Pase enzyme may allow us to probe another ability of *C. ficifolia* crude extract as for additional antidiabetic potential targeting the liver.

9. Future perspectives

One very promising avenue for research in obesity is to focus on adipose tissue and adipocyte development (adipogenesis) (Rosen et. al, 2006), since adipose tissue appears primarily involved in metabolic syndromes, with an important role in obesity. Obtaining knowledge about adipocyte differentiation provides an approach of a multitude of stimuli and coordinated regulation of cellular responses (Dodson et al, 211). Identification, purification and structural characterization of bioactive components will constitute major milestones of the research and open the possibility to therapeutic agents. Assessment of the protein expression of kinases such as Akt and AMPK should also yield important information on modes of action. Indeed, these proteins play an important role in energy metabolism. Akt is necessary to drive adipogenesis, its downstream targets being involved in regulating this differentiation (Peng et al., 2003). For instance, our examination of the insulin-dependent and insulin-independent intracellular signalling components by western blot analysis showed that fermented blueberry extract might significantly enhance p-Akt and hence its activity.

The bioassay-guided fractionation led us to approach the study of crude plant extracts and its fractions. Fractions were screened in pursuit of those that contain biologically active compounds. The analysis of bioactive compounds present in the plant extracts involves the applications of common phytochemical screening assays and chromatographic techniques such as HPLC. These applications confer

an advantage since natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Sasidhara et al., 2001).

Fermented blueberry and *C. ficifolia* extracts thus hold great promise as complementary therapies for obesity and T2D. These plants have been used since ancient times to treat anti-diabetic symptoms and current research related to these plants has shown very important advances in this context.

For instance, *C. ficifolia* has also shown a potential effect on glucose pathways (Fortis et al., 2011; Miranda et al., 2012). In this context, in our studies (Fig. 16-17), *C. ficifolia* crude extract exhibited promising anti-adipogenic and anti-hypoglycemic effects in other *in vitro* assays. Moreover, the preliminary results obtained with primary fractions from the aqueous *C. ficifiolia* extract can be considered as a first step toward elucidating the specific compounds responsible for the anti-diabetic and anti-obesity actions. Nevertheless, further research needs to be done in order to confirm our preliminary work, to isolate the specific compound(s), and to explore the relationship between their chemistry and the effects observed.

In regards to the possible mechanism of action of *C. ficifolia*, the modulation of AMPK was hinted at by the tendency of the plant extract to increase its phosphorylated form when compared to controls in both adipocytes and hepatocytes in culture. As is well known, G6Pase is a key enzyme implicated in hepatic gluconeogenesis, being essential for glucose release from hepatocytes (Schmoll D. et al., 2000). Insulin reduces the expression of the G6Pase enzyme through a great cascade of different transcription factors activated by the insulin

receptor signaling pathways (Yoon et al., 2001; Zhang et al., 2006). In contrast, our results suggest the implication of the insulin-independent serine/threonine kinase AMPK that is an implicated in energy balance (Towler and Hardie, 2007). It is involved in hepatic metabolic homeostasis (Zang et al., 2004), which includes the ability to decrease G6Pase activity through the phosphorylation of CREB (c-AMP-regulator element-binding protein) (He at al., 2009). It remains to be seen if *C. ficifolia* extract may also act through pathways similar to insulin and have different effects on insulin signaling pathways in different tissues. Indeed, in previous studies, the plant has shown a significant anti-inflamatory and anti-oxidant effect where the extract may act through these effects to improve insulin sensitivy (Fortis et al., 2013), because of possible insulin mimetic-effects or a possible synergistic effect. These observations might represent a novel path toward the pharmacological development of alternative and/or complementary drugs for the treatment of obesity and T2D. However, further studies are neccesary in order to confirm the possible(s) mechanisms of action of aqueous *C. ficifolia* extract.

The general HPLC profile of *C. ficifolia* extract that we obtained pointed clearly to flavononids and terpenoids as major components, based on peaks observed in the UV spectrum that are characteristic of many compounds of this type. This is also concordant with the retention times observed and recent results obtained from Dr Alarcon's research team. Similarly, to the pharmacological activity, future studies will be necessary to identify the principal compounds responsibles of the anti-inflimmatory, anti-obesity and anti-diabetic effects conferred to the extract. As well, a better interpretation of previous observations where some compounds may act synergistically (Fortis et al., 2013).

10. Conclusions

1. Fermented juice dramatically inhibited triglyceride accumulation during adipogenesis of 3T3-L1 cells and this could constitute a potential anti-obesity action.
2. Phytochemical fractionation was the approach that we used to identify the specific active principles from fermented blueberry extract. We identified chlorogenic acid and catechol as active components of fermented blueberry juice. These compounds can serve to further develop the potential of this fermented juice as a novel nutraceutical.
3. Both compounds can also serve as templates for the development of novel alternative therapeutic agents against obesity and related diseases.
4. *C. ficifolia* also showed an important inhibitory effect on adipocyte differentiation, which could be related to its anti-inflammatory potential (Fortis et al., 2013).
5. In addition, it inhibited hepatocyte G6Pase, a key enzyme contoling liver glucose procduction. *C. ficifolia* did not have any stimulation over the glucose transport on the optimal concentration for L6 myotubes muscle cells.
6. These results represent one more piece for the possible action of mechanism by which the aqueous extract of *C. ficifolia* may exert anti-obesity and anti-diabetic actions.

7. Preliminary results point to fractions enriched in flavononids and terpenoids in exerting anti-adipogenic activity of the *C. ficifolia* aqueous extract.
8. Future studies will need to provide more detailed analysis in order to confirm the specific compounds responsible for the biological actions of *C. ficifolia* as well to elucidate the possible signaling pathways and transcriptional factors involved.

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12. List of abbreviations

AICAR: aminoimidazole carboxamide ribonucleotide

AKT: serine/threonine-specific protein kinase

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ATP: Adenosine triphosphate

CA: Chlorogenic acid

CAT: Catechol

cm: Centimeter

DIO: high-fat induced obese

DCI: D-chiro-inositol

DMSO: Dimethyl sulfoxide

FFAs: Free fatty acids

GDM: Gestacional Diabetes Mellitus

G-6-Pase: Glucose 6-phosphatase

HbA1C: glycated haemoglobin

HPLC: high performance liquid chromatography

IPGTT: Intraperitoneal glucose tolerance

Kg: Kilogram

MEFs: mouse embryo-derived fibroblasts

min: Minute

mL: Milliliter

PDK1: 3-phosphoinositide-dependent protein kinase-1

PPAR: Peroxisome-proliferator–activated receptor

SREBPs: Sterol regulatory element-binding proteins

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

TNFγ: Tumor Necrosis Factor gamma

µg: Microgram

µl: Microliter

Sánchez-Villavicencio et al. BMC Complementary and Alternative Medicine (2017) 17:24
DOI 10.1186/s12906-016-1519-9

BMC Complementary and Alternative Medicine

RESEARCH ARTICLE Open Access

Fermented blueberry juice extract and its specific fractions have an anti-adipogenic effect in 3 T3-L1 cells

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Abstract

Background: Obesity and Type 2 diabetes have reached epidemic status worldwide. Wild lowbush blueberry (*Vaccinium angustifolium* Aiton) is a plant of the North American Aboriginal traditional pharmacopeia with antidiabetic potential, especially when it is fermented with *Serratia vaccinii*.

Methods: A phytochemical fractionation scheme was used to identify potential bioactive compounds as confirmed by HPLC retention times and UV–Vis spectra. 3 T3-L1 cells were differentiated for 7 days with either Normal Blueberry Extract (NBE), Fermented Blueberry Extract (FBE/F1), seven fractions and four pure compounds. Triglyceride content was measured. Examination of selected intracellular signalling components (p-Akt, p-AMPK) and transcriptional factors (SREBP-1c and PPARγ) was carried out by Western blot analysis.

Results: The inhibitory effect of FBE/F1 on adipocyte triglyceride accumulation was attributed to total phenolic (F2) and chlorogenic acid enriched (F3-2) fractions that both inhibited by 75%. Pure compounds catechol (CAT) and chlorogenic acid (CA) also inhibited adipogenesis by 70%. Treatment with NBE, F1, F3-2, CAT and CA decreased p-AKT, whereas p-AMPK tended to increase with F1. The expression of SREBP1-c was not significantly modulated. In contrast, PPARγ decreased in all experimental groups that inhibited adipogenesis.

Conclusions: These results demonstrate that fermented blueberry extract contains compounds with anti-adipogenic activity, which can serve to standardize nutraceutical preparations from fermented blueberry juice and to develop novel compounds with anti-obesity properties.

Keywords: Obesity, Adipogenesis, Insulin signaling, Fermented blueberry extract

Background

According to the WHO, global estimates of obesity rates in 2014 showed that more than 1.9 billion adults were overweight whereas 600 million were experiencing obesity [1]. Similar to many countries, Canada has experienced a substantial increase in the prevalence of obesity [2, 3]. Obesity and Type 2 diabetes have increased especially in Canadian Aboriginal populations. A report by the Organisation for Economic Co-operation and Development suggested that in some countries, including Canada, the prevalence of obesity will continue to rise at a predicted rate of 4–5% per year [2].

Adipocyte differentiation, or adipogenesis, implicates the accumulation of cellular lipid and is regulated by genetic and growth factors as well as hormones, notably by insulin [4]. Insulin is a major anabolic regulator of energy homeostasis and its signalling pathways implicate the serine/threonine-specific protein kinase Akt. Knockout models of the different Akt isoforms (Akt1 and Akt2) demonstrated their essential role in regulating adipogenesis [5] as well as glucose metabolism in the body [6]. Thus, Akt can drive adipogenesis. Its role includes the phosphorylation and regulation of a large number of substrates involved in several biological processes [7]. Adipocyte

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differentiation is also mediated by the temporally modulated expression of several transcription factors, notably PPARγ (peroxisome-proliferator-activated receptor gamma) that is a key regulator of this transcriptional program [8].

Another important enzyme involved in fat metabolism is the insulin-independent AMP-activated protein kinase (AMPK). It acts as a metabolic master switch regulating several intracellular systems, including the cellular uptake of glucose, the β-oxidation of fatty acids and mitochondrial biogenesis, which appear highly sensitive to energy status. Upon activation, AMPK increases cellular energy levels by inhibiting anabolic pathways (fatty acid synthesis, protein synthesis) and stimulating catabolic pathways (fatty acid oxidation, glucose transport) [9].

The use of natural health products as complementary or alternative approaches to existing medications is growing in popularity for the treatment and management of obesity and related diseases such as type 2 diabetes. Natural and synthetic agents can exert anti-obesity effects by increasing lipolysis in white adipocytes and by blocking adipocyte differentiation [10]. Notably, fermented blueberry extract was previously shown to inhibit triglyceride accumulation during adipogenesis of 3 T3-L1 cells [11], which could constitute a potential anti-obesity action. Such bio-transformed blueberry extract was also found to contain a much higher content in total phenolics and it was able to increase AMPK phosphorylation and glucose uptake in muscle cells and adipocytes [11, 12]. However, the compounds responsible for these effects still remain unclear, as does the participation of Akt in this process.

Consequently, the principal aim of this study was to identify potential bioactive components of fermented wild blueberry extract using 3 T3-L1 cells. For this purpose, we carried out phytochemical fractionation of fermented blueberry extract to generate semi-purified fractions and isolate active compounds that could be responsible for the observed inhibitory effect on adipogenesis. We also began addressing the potential molecular mechanisms that could underlie such an anti-adipogenic action by assessing the protein expression of selected key regulatory elements.

Methods

Chemicals and biochemicals

Dexamethasone (DXM), bovine pancreatic insulin, 3-isobutyl-1-methylxanthine (IBMX), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Rosiglitazone came from Alexis Biochemicals (Hornby, ON). Dulbecco's Modified Eagle Medium was from Wisent Inc. (St-Bruno, QC). For the measurement of triglyceride content, the AdipoRed reagent was used (Lonza Walkersville Inc., Walkersville, MD). We also used the Western Lightning ECL from Perkin Elmer (Waltham, MA). Aminoimidazole carboxamide ribonucleotide (AICAR) was purchased from Toronto Research Chemicals (Toronto, ON). The measurement of protein was carried out with an assay kit from Bio-Rad (Mississauga, ON).

Preparation of wild blueberry extract

Frozen wild blueberry fruit (*Vaccinium angustifolium* Aiton) was obtained from Oxford Frozen Foods Ltd (Oxford, NS). It represents a uniform blend of a large number of genotypes coming from several producers in Northeastern United States and Canada. In this respect, it accurately reflects the material that is normally used to prepare commercial wild blueberry juice. Notwithstanding, our laboratory analysis has confirmed that cultivar types do not affect the fermentation profile significantly.

The extract was prepared by blending such wild blueberry fruit (100 g) with an equivalent quantity (100 g) of Minimal Broth Davis without dextrose (MM) (Difco Laboratories, Detroit, MI). The preparation was centrifuged to remove insoluble particles. The resulting extract was sterilized using 0.22 μm Express Millipore filter (Millipore, Etobicoke, ON) and fermented with *Serratia vaccinii* bacteria as described [12]. Normal blueberry extract (NBE) was processed in an identical manner but was not fermented with the bacteria. Portions of NBE and the fermented blueberry extract (FBE) were freeze-dried and used for chemical analysis.

Phytochemical fractionation of FBE

Fractions were prepared by a multi-step process (Table 1) that started with material that was either NBE or NBE after fermentation with *Serratia vaccinii* to produce FBE. As a first fractionation step, FBE (identified as F1; Table 1) was loaded in batches of 500 ml onto 29.5 × 5 cm chromatography columns (pre-conditioned with 1 column volume of methanol then 2 column volumes of water) containing Waters preparative C18 resin (125 Å, 55–105 μm). These were washed with 2 column volumes of water to remove sugars and organic acid (discarded). Phenolic compounds were eluted from the column using 1.2 column volumes of 100% ethanol containing 13 mM trifluoroacetic acid (Sigma Aldrich, ON). This ethanol eluent, which contained all the phenolics from the FBE starting material, was dried using rotary evaporation and lyophilisation. This total phenolic fraction from FBE was called F2.

For the second fractionation step of the FBE, one portion of F2 was dissolved in water and applied to another preconditioned C18 column to generate Fraction F3-1 that contained low MW phenolic compounds. This was done by selective elution using 4 column volumes of aqueous 2.06 M (12.5%) ethanol containing 0.16 M HCl (Ricca Chemical Company, Arlington, TX). F3-1 compounds recovered in this step were identified by HPLC by comparing retention times and UV–Vis profiles of the peaks to pure standards. The next fraction, called

Table 1 Fractionation of fermented blueberry extract (F1). The major component (s) in each fraction (F) are indicated

Fraction	Major component (s)	Starting material	Column resin	Eluant
F1 (FBE)	Sugars, organic acids, growth media, phenolics			
F2	phenolics	F1	C_{18}	EtOH
F3-1	Gallic acid, catechol, protocatechuic acid	F2	C_{18}	12% EtOH
F3-2	Chlorogenic acid	F2	C_{18}	12% EtOH
F3-3	Flavonoids	F2	C_{18}	80% EtOH
F4-1	Anthocyanins	F3-3	LH20	25% EtOH
F4-2	Heteropolymers	F3-3	LH20	50% EtOH
F4-3	Proanthocyanidins	F3-3	LH20	70% EtOH

F3-2, was produced by passing through the same C18 column an additional 2 column volumes of aqueous 2.06 M (12.5%) ethanol containing 0.16 M HCl. This F3-2 fraction was rich in chlorogenic acid, which was confirmed by HPLC. The remaining bound materials were eluted using 0.16 M HCl and 13.7 M (80%) ethanol in water to produce fraction F3-3 (Table 1). The F3-1, F3-2 and F3-3 fractions were dried using rotary evaporation and then freeze dried.

To produce three additional and final fractions, one portion of F3-3 was dissolved in 4.28 M (25%) ethanol and applied to a 34.5 × 5 cm column of Sigma-Aldrich lipophilic Sephadex LH-20 (25–100 μm). The first fraction from Sephadex LH-20 was obtained by washing with 7 column volumes of 4.28 M (25%) ethanol. This fraction, called F4-1, was enriched in anthocyanins. The same Sephadex LH20 column was then washed with 3 column volumes of 8.56 M (50%) ethanol to yield a fraction enriched in phenolic heteropolymers and called F4-2. The last Sephadex LH20 fraction was eluted using 3 column volumes of 9.53 M (70%) acetone. This fraction was enriched in proanthocyanidins and called F4-3. The three fractions obtained from the Sephadex LH20 column (F4-1, F4-2 and F4-3) were dried using rotary evaporation and lyophilisation. All fractions were examined on HPLC and four pure compounds of interest were identified, namely catechol (CAT), protocatechuic acid (PA), gallic acid (GA) and chlorogenic acid (CA), which were subsequently purchased from Sigma-Aldrich (Oakville, ON).

Cell culture

Murine 3 T3-L1 pre-adipocyte cells were obtained from ATCC (Manassas, VA) and used between passages 5 and 8. They were grown in Dulbecco's modified Eagle's medium (DMEM), containing 10% bovine calf serum until confluent at 100%. Two days after confluence (Day 0), the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS), 500 nM insulin, 1 μM Dexamethasone and 250 μM IBMX for 2 days (Day 2) (Short Term Medium). Cells were maintained in 10% FBS/DMEM with 500 nM insulin for another 4 days (Long Term Medium) at which time >90% of wells showed mature adipocytes with accumulated fat droplets. Cells were maintained at 37 °C in a humidified 5% CO_2 atm and differentiated for a total of 7 days with media change every 2 days.

Evaluation of cytotoxicity

Cell viability was assed through a Cytotoxicity Detection Kit that was purchased from Roche (South San Francisco, CA). 3 T3-L1 pre-adipocytes were seeded in 24-well plates and cultured to 100% confluence in culture medium and treated for 7 days as described above with various component concentrations (5, 10 and 15 μg/ml of NBE, FBE/F1, specific fractions and pure compounds). Cell culture media for each condition were collected separately (released LDH) and then cells were lysed with culture medium containing 1% Triton X-100, for 10 min (intracellular LDH). All samples were collected in Eppendorf tubes and centrifuged at 250xg at 4 °C for 10 min. Fluorescence was measured (Wallac Victor2, Perkin-Elmer, Waltham, MA) at an emission wavelength of 590 nm. Results were expressed as the ratio of released LDH to total LDH (intracellular plus released), normalized to values obtained from cells treated with the vehicle control (0.1% DMSO). The optimal concentration was determined for each tested component and used for bioassays.

Adipogenesis assay

NBE, FBE (F1), specific fractions (5 μg/ml), four pure compounds at 5 μg/ml (except catechol at 3 μg/ml), and rosiglitazone (10 μM; positive control) were dissolved in DMSO and added to the cells as of day 0 of differentiation. The final concentration of DMSO was kept at 0.1% throughout the differentiation period. Adipogenesis was assessed in the well-characterized 3 T3-L1 cell model by measuring the accumulation of triglycerides upon differentiation after treatments as described previously [13, 14], using the AdipoRed reagent according to the manufacturer's instructions. Briefly, after washing

each well twice with phosphate-buffered saline (PBS: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, and 2.68 mM KCl; pH 7.4), 2 ml of PBS 1X containing 60 μl of AdipoRed reagent were added to each well and incubated for 10 min at room temperature. Fluorescence was measured (Wallac Victor2, Perkin-Elmer, Waltham, MA) at 485 nm excitation and 572 nm emission wavelengths. Results are reported as a percentage of the value obtained for the vehicle control (0.1% DMSO).

Western blot analysis

Cells were cultured, treated and differentiated as described above for the adipogenesis assay. Cultured cells were homogenized in lysis buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl 5 mmol/L EGTA, 2 mmol/L $MgCl_2$, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (2 mmol/L PMSF and Complete Mini-EDTA-free protease inhibitor cocktail tablets; Roche, Laval, QC) and phosphatase inhibitors (0.5 mmol/L NaF, 2 mmol/L Na_3VO_4, 1 mmol/L $Na_4P_2O_7$). The protein concentration of lysates was assessed by the Bradford colorimetric assay and 40 μg of total protein were loaded onto a 12% acrylamide gel. Samples were electro-transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated overnight with primary antibodies to Akt and *p*-Akt, (1:250, Cell Signalling Technology, Danvers, MA) as well as *p*-AMPK, AMPK, SREBP-1c and PPARγ (1:250, Santa Cruz Biotechnology Inc., Dallas, TX) and beta-actin (1:1000, Cell Signalling Technology Inc., Danvers, MA). Incubation with anti-rabbit HRP-conjugated secondary antibody (Cell Signalling Technology Inc., Danvers, MA) was then carried out at 1:20000 dilution in TBST (1X) (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.5% Tween 20) plus 5% non-fat dried milk for 1 h at room temperature. Membranes were then washed with TBST 3X for 5 min and the blots were revealed using the Western Lightning ECL enhanced chemiluminescence (Enhanced Chemiluminescence Substrate for 1000 cm2 of membrane). Densitometry analysis was performed with General Electric Image Quant LAS 4000 mini scanner and Image J software (GE Healthcare Bio Sciences, Baie d'Urfé, QC).

Statistical analysis

Results are presented as the mean ± SEM of 3 independent experiments carried out in triplicate. Statistical analysis was performed with Prism GraphPad software (La Jolla, CA). The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's and/or Tukey's multiple comparison test for statistically significant differences between groups, as appropriate. Statistical significance was set at a level of $p < 0.05$.

Results

Phytochemical fractionation scheme and selection of pure compounds

Using a C18 column with varying ethanol and acid concentrations, sugars and organic acids were removed and several fractions were obtained as illustrated in Fig. 1 and listed in Table 1. This fractionation scheme was used

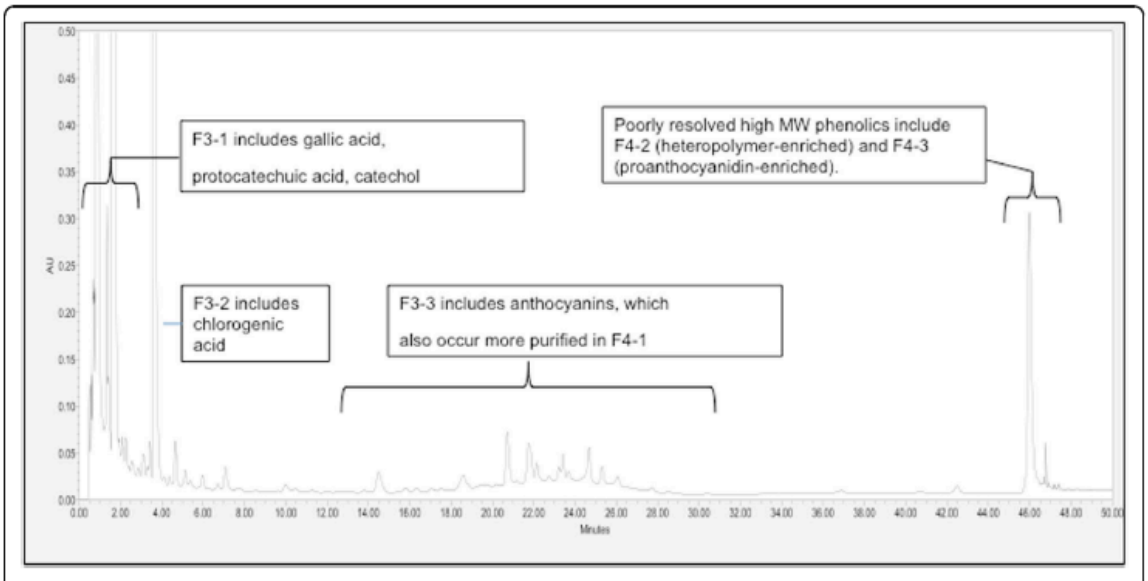

Fig. 1 HPLC profile (280 nm) of fermented blueberry extract indicating the major components contained in fractions (see also Table 1)

previously to study the effect of NBE components on cardiomyocyte integrity [15] and was applied to FBE as a practical approach. Based on HPLC profiles (Fig. 1), fractions containing phenolic compounds (F2), whose sub-fractions contained gallic acid (GA), catechol (CAT) and protocatechuic acid (PA) (Fraction F3-1) as well as chlorogenic acid (CA) (Fraction 3-2), were of greatest interest [15].

Cell viability

Figure 2 presents the results of the LDH cytotoxicity assay. NBE and FBE as well FBE fractions and pure compounds were tested at 5, 10 and 15 μg/ml. As can be appreciated, NBE and FBE crude preparations induced LDH leakage that was similar to the DMSO control at concentrations of 5 and 10 μg/ml. In contrast, the optimal concentrations of other fractions and pure compounds were 5 μg/ml (except catechol at 3 μg/ml). In order to compare the various preparations at similar per weight contents, we also tested NBE and FBE at 5 μg/ml. Cells did not exhibit damage in response to such concentrations of test substances and were morphologically comparable to cells treated with the vehicle control 0.1% DMSO (data not illustrated). These concentrations were thus used subsequently for all in vitro assays.

Adipogenesis assay

The amount of accumulated triglycerides was measured on day 7 in 3 T3-L1 cells treated with the specified concentrations of NBE, F1 and its specific fractions, as well as CAT, CA, PA and GA (7 fractions and 4 pure compounds). As illustrated in Fig. 3, only certain fractions of F1 were able to significantly inhibit TG accumulation. Notably, fractions F2 and F3-2 reduced triglyceride accumulation to 25 and 30% as compared to the vehicle control (set at 100%), respectively. Similarly, only two of the four pure compounds exerted a significant anti-adipogenic effect. Indeed, CA and CAT yielded adipogenic values that were 75 and 70% lower than DMSO.

Western blot analysis

Fractions and pure compounds with significant anti-adipogenic effect, namely F2, F3-2, CAT and CA, were analyzed by Western blot for cellular components involved in the control of adipogenesis and were compared with NBE and FBE (F1). As shown in Fig. 4a, all treatments that inhibited adipogenesis also decreased the phosphorylation of the insulin-dependent kinase Akt, with CA having the most prominent effect. In contrast, the phosphorylation of the insulin-independent kinase AMPK was not significantly affected by any of the treatments, albeit F1 had a tendency to increase it relative to vehicle control (Fig. 4b).

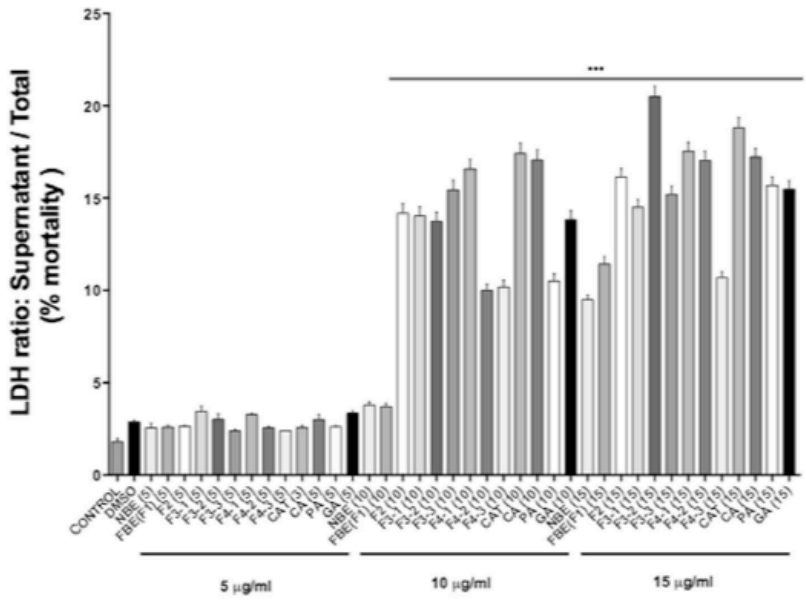

Fig. 2 Lack of toxicity of optimal concentrations of fermented blueberry extract and components. 3 T3-L1 pre-adipocytes were seeded at a density of 2×10^4 cells, cultured to 100% confluence and treated for 7 days with treatments as described above. Cytotoxicity was measured by LDH leakage. Medium LDH activity was expressed as a percentage of total enzyme activity (medium + lysate). The results are shown as the mean ± SEM. Significant differences compared to DMSO vehicle control were assessed by one way ANOVA; post hoc analysis with Dunnett's multiple comparison test. *** Denotes statistically significant from vehicle control ($p < 0.001$)

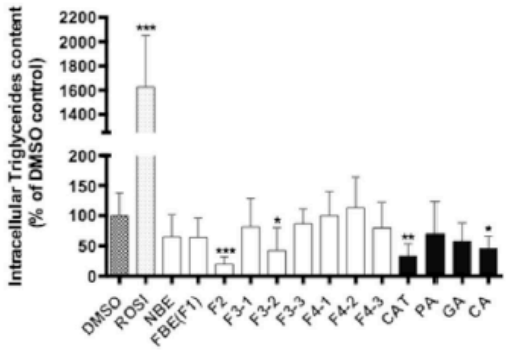

Fig. 3 Fermented blueberry extract and fractions inhibit adipogenesis. 3 T3-L1 pre-adipocytes were seeded at a density of 2×10^4 cells, cultured to 100% confluence and treated for 7 days with indicated treatments as described in Materials and Methods. Rosiglitazone was used as a positive control inducing differentiation (10 μM). DMSO 0.1% was used as vehicle control. The results are shown as the mean ± SEM. Significant differences compared to DMSO vehicle control were assessed by one-way ANOVA; post hoc analysis with Dunnett's multiple comparison test. *Denotes statistically significant from vehicle control ($p < 0.05$). **Denotes statistically significant from vehicle control ($p < 0.01$). ***Denotes statistically significant from vehicle control ($p < 0.001$)

We next examined two selected key adipocyte-associated transcription factors. As illustrated in Fig. 5b, SREBP-1c was not significantly affected by any of the treatments. Conversely, all treatment conditions that significantly reduced adipogenesis, namely F2, F3-2, CAT and CA, markedly decreased the expression of PPARγ as compared to the vehicle control (Fig. 5a). As expected, the positive control Rosiglitazone significantly increased PPARγ expression (Fig. 5a).

Discussion

Research in obesity is increasingly focusing on adipose tissue and adipogenesis. Indeed, adipose tissue appears to be involved with the development of the metabolic syndrome [16]. Food and medicinal plants are important sources of natural products that display great chemical diversity. This makes them excellent candidates for drug development, including for the treatment of obesity and other related diseases [17]. We previously observed that the fermentation of the juice from wild lowbush blueberries (*Vaccinium angustifolium* Aiton), with a bacteria (*Serratia vaccinii*) found on the fruit skin, was able to greatly increase the total phenolic content [12]. This biotransformation also conferred it potent anti-diabetic properties [11]. In the latter study, we also uncovered an inhibitory action of fermented blueberry juice on 3 T3-L1 adipogenesis, representing a putative anti-obesity action that was later confirmed in vivo [18]. In the present study, we sought to determine the biologically active compounds that could underlie such anti-adipogenic effects. We used an HPLC-based fractionation scheme that provided us with several fractions focused primarily on phenolic components. We notably identified four major compounds, namely gallic acid, catechol, protocatechuic acid and chlorogenic acid, which we also tested. We used the 3 T3-L1 cell line, one of the most reliable models for the study of adipogenesis [19].

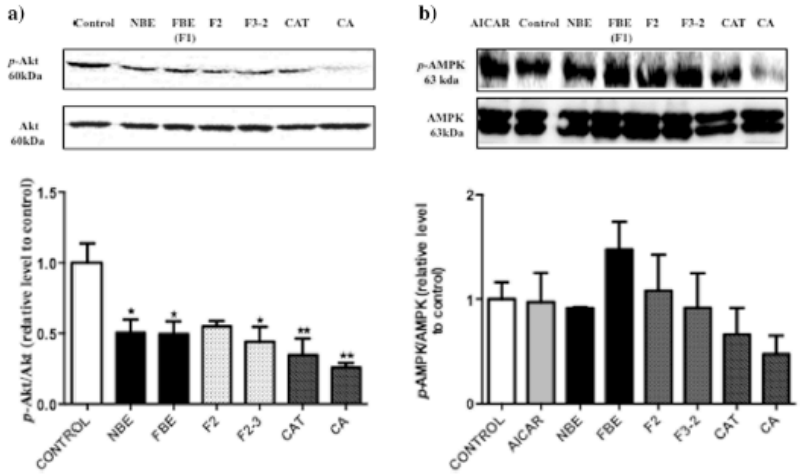

Fig. 4 Effects of FBE and specific fractions on the phosphorylation of insulin-dependent Akt **a** and insulin-independent AMPK **b**. 3 T3-L1 preadipocytes were incubated with DMSO (0.1%) or 5 μg/ml of NBE, fractions F1, F2, F2-3 and two pure compounds CAT (3 μg/ml), CA (5 μg/ml) and induced to differentiate. The expression levels of key signalling proteins were measured after treatments by Western blot analysis; *p*-Akt, Akt **a** *p*-AMPK, AMPK **b**. Results are shown as the mean ± SEM. Significantly different compared to DMSO control *$p < 0.05$, **$p < 0.01$

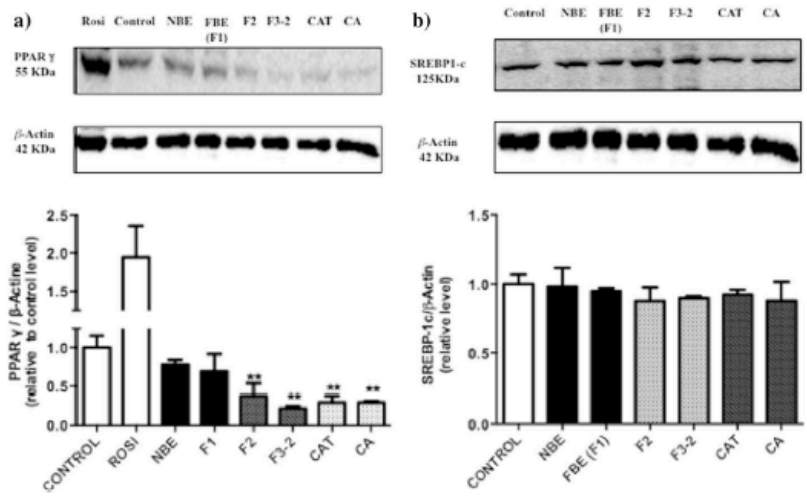

Fig. 5 Effects of FBE and specific fractions on the expression of adipogenesis-related transcription factors. 3 T3-L1 preadipocytes were incubated with DMSO (0.1%) or 5 μg/ml of NBE, fractions F1, F2, F2-3 and pure compounds CAT (3 μg/ml), CA (5 μg/ml) and induced to differentiate. PPARγ **a** and SREBP-1c **b** expression levels were examined by Western blot. Protein expressions were normalized using β-actin as a reference. Results are shown as the mean ± SEM. Significantly different compared to DMSO control $^{*}p < 0.05$, $^{**}p < 0.01$

As a first step, we freeze dried the fermented blueberry juice and the resulting extract, called F1, had a tendency to inhibit adipogenesis, albeit not to the extent that the intact fermented blueberry juice had done previously [11]. This may be explained by the different concentrations used in the two studies. In our previous study, intact fermented blueberry juice was administered to cells at a dose of total phenolics equivalent to 30 μM of gallic acid (GAE) [11]. In the present studies, we used FBE/F1 at the same weight-based concentration found to be optimal for the phytochemical fractions and pure compounds, in order to have a valid comparison. This concentration (5 μg/ml) amounts to 1.6 μM GAE, when the total phenolic content of FBE was taken into consideration. It is therefore almost 20 times less concentrated than that used in our previous study [11]. More surprisingly, when NBE was subjected to a similar freeze-drying step, it was found to also have a non-significant tendency to reduce adipogenesis. This was different than what was observed in our previous study, where NBE was without effect [11]. Further studies will be necessary to understand the potential effect of freeze-drying on the phytochemical composition of wild lowbush blueberry juice, notably in the context of adipogenesis. Indeed, other researchers, using cultivated highbush blueberry phenolics, have observed a concentration-dependent inhibition of adipogenesis in the 3 T3-F44A cell line [20].

Notwithstanding, we found that two sub-fractions of our fermented blueberry extract exhibited a significant inhibition of adipogenesis. The first, F2, was the fraction that contained all the phenolic compounds while the second, F3-2, was enriched in chlorogenic acid. This was confirmed by the corresponding activity of the pure compound (chlorogenic acid). Of the other pure compounds, only catechol was found to also inhibit adipogenesis significantly. This implies that chlorogenic acid and catechol may contribute to the anti-obesogenic potential of fermented blueberry juice.

We next began assessing some of the potential molecular mechanisms that can underlie the anti-adipogenic activity of fermented blueberry juice. Adipogenesis involves a complex and coordinated transcriptional cascade that leads to lipogenesis and TG accumulation [21, 22]. Insulin-dependent Akt signalling can trigger this cascade [5–7]. In contrast, insulin-independent AMPK signalling can inhibit adipogenesis [11]. In line with the inhibitory effect of fermented blueberry juice and its components, we found that F1 as well as sub-fractions F2 and F3-2 significantly inhibited the phosphorylation of Akt. This result is in line with the report by Song and collaborators who studied blueberry peel extracts and found an anti-adipogenic effect that was related to the inhibition of Akt phosphorylation [23].

On the other hand, F1 had a tendency to increase AMPK, although this effect failed to reach statistical significance. This implies that fermented blueberry products may diminish adipogenesis principally by reducing insulin-dependent signalling. Interestingly, our previous work had shown that fermented blueberry juice could stimulate AMPK in 3 T3-L1 adipocytes, an effect that

was implicated in the stimulation of glucose transport observed in the same cells [11]. The implication of AMPK in the inhibition of adipogenesis by fermented blueberry juice may be less critical at the concentration used in the present studies.

The adipogenic process is a complex and highly regulated program of gene expression that involves several key transcription factors that include SREBP-1c and PPARγ [19, 24]. We therefore chose to determine the protein content of these two transcription factors as an initial step to assess potential underlying molecular mechanisms. Although treatment with sub-fractions F2 and F2-3 as well as pure compounds CAT and CA failed to significantly modulate SREBP-1c protein expression, they all had a tendency to reduce it. However, SREBP-1c is activated early in the transcriptional cascade such that our assessment near the end of adipogenesis may explain the low variations in protein expression that we observed. More detailed studies, including time-course and mRNA assessments will be necessary to verify the potential role of SREBP-1c in the effects of FBE on adipogenesis.

In contrast, the results of the current studies clearly show that the same active components of fermented blueberry juice induced a strong inhibition of PPARγ. Our results thus suggest that the negative modulation of PPARγ may be involved in the anti-adipogenic action of fermented blueberry juice. This is similar to what was observed by Song and collaborators, where blueberry peel extracts significantly reduced PPARγ [23]. Nevertheless, more detailed studies, notably addressing the modulation of the gene expression of several components controlling adipogenesis, will be necessary to confirm this point and determine if other factors than PPARγ are involved.

It is well known that a high phenolic content in the diet is associated with the prevention of certain chronic diseases, whereas phenolic acids have demonstrated antioxidant and anti-inflammatory properties [25]. Indeed, chlorogenic acid, a major component of green bean coffee extract, has been reported to reduce blood sugar levels and potentially exert an anti-diabetic effect [26]. It has also been implicated in weight loss in humans [27, 28] and recently shown to stimulate lipolysis in adipocytes in culture [29, 30].

The present studies suggest that chlorogenic acid may also exert its anti-obesity potential through the inhibition of adipocyte Akt phosphorylation and of the downstream transcription factor PPARγ, a major regulator of adipogenesis. On the other hand, catechol is a simple polyphenol found in a wide variety of plants. In the present studies, it was also found to significantly diminish adipogenesis through mechanisms involving an inhibition of Akt phosphorylation and a reduction in PPARγ. To our knowledge, this is the first instance where such biological activity has been reported for catechol.

Conclusions

We identified chlorogenic acid and catechol as active components of fermented blueberry juice. These compounds can serve to further develop the potential of this fermented juice as a novel nutraceutical. Both compounds can also serve as templates for the development of novel alternative therapeutic agents against obesity and related diseases.

Abbreviations

AICAR: Aminoimidazole carboxamide ribonucleotide; AKT: Serine/threonine-specific protein kinase; AMP: Adenosine monophosphate; AMPK: AMP-activated protein kinase; CA: Chlorogenic acid; CAT: Catechol; cm: Centimeter; DMSO: Dimethyl sulfoxide; DXM: Dexamethasone; FBE: Fermented blueberry extract; HPLC: High performance liquid chromatography; IBMX: 3-isobutyl-1 methylxanthine; L: Liter; ml: Milliliter; NBE: Normal blueberry extract; PPAR: Peroxisome-proliferator–activated receptor; SREBPs: Sterol regulatory element-binding proteins; T2DM: Type 2 diabetes mellitus; μ g: Microgram; μ l: Microliter

Acknowledgements

We acknowledge Agriculture and Agri-food Canada and the DIAP (Developing Innovative Agrifood Products) (no 5025) program for the funds to support this research project. The PhD project of M.L.S.V. was supported by the National Council of Science and Technology (CONACyT) grant from Mexican government (no 270963) and PAPIIT and PAPIME projects from UNAM (no IN115615 and no. PES112716). The authors would like to thank Dr. Tri Vuong for the preparation of fermented blueberry juice and Dr. Edtson Emilio Herrera Valencia from Autonomous National University of Mexico for his valuable help in the structure of this paper.

Availability of data and materials

The data and materials are included within the article.

Authors' contributions

MLSV performed in vitro and Western blot experiments as well as data analysis. MVT, WK and CM carried out the fermentation process, prepared the plant extracts and did the phytochemical experiments. PSH, FJAA and MCEV participated in the conception and design of the study. All authors read and approved the final manuscript.

Competing interests

The authors declare no conflict of interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Received: 24 August 2016 Accepted: 14 December 2016
Published online: 06 January 2017

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Casa abierta al tiempo
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ACTA DE DISERTACIÓN PÚBLICA

No. 00093
Matrícula: 2121800460

STUDY IN VITRO AND IN VIVO OF ANTI-DIABETIC AND ANTI-OBESITY ACTIVE PRICIPLES OF Cucurbita ficifolia Bouche AND Vaccinium angustifolium Ait.

En la Ciudad de México, se presentaron a las 11:30 horas del día 22 del mes de junio del año 2017 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DR. JULIO CESAR ALMANZA PEREZ
DRA. MARIA DEL CARMEN ESCOBAR VILLANUEVA
DRA. REBECA GARCIA MACEDO
DRA. MARGARITA DIAZ FLORES

Bajo la Presidencia del primero y con carácter de Secretaria la última, se reunieron a la presentación de la Disertación Pública cuya denominación aparece al margen, para la obtención del grado de:

DOCTORA EN BIOLOGIA EXPERIMENTAL

DE: MAYRA LUZ SANCHEZ VILLAVICENCIO

y de acuerdo con el artículo 78 fracción IV del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

Aprobar

Acto continuo, el presidente del jurado comunicó a la interesada el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.

MAYRA LUZ SANCHEZ VILLAVICENCIO
ALUMNA

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