EVALUATION OF ANTHOCYANIN EXTRACT OF THAI *MORUS ALBA* L., ON BLOOD GLUCOSE LEVEL IN DIABETIC RATS AND PREDIABETES PATIENTS

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DOCTOR OF PHILOSOPHY

IN

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Ariya Sarikaphuti

Dissertation Title	Evaluation of Anthocyanin Extract of Thai *Morus alba* L., on Blood Glucose Level in Diabetic Rats and Prediabetes Patients
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Degree	Doctor of Philosophy (Anti-Aging and Regenerative Science)
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ABSTRACT

Mulberry (*Molus alba* L.) contains abundant anthocyanin (ANC), a natural antioxidant. The purpose of this study was to determine the ANC composition of Thai *Morus alba* L. fruits, and to assess its effect on blood glucose and insulin levels in ZDF rats and pre diabetes patients. In this study the major components of ANC were identified by high-performance liquid chromatography–electrospray ionization–mass spectrometry. ZDF and lean rats were administered with 125 or 250 mg ANC/kg body weight, or 1% carboxymethylcellulose (CMC) twice daily for 5 weeks. Neither ANC dose affected body weight. After 5 weeks of treatment, glucose levels increased from 105.5 ± 8.7 to 396.25 ± 21 mg/dl ($P < 0.0001$) in CMC-treated ZDF rats, but levels were significantly lower in rats treated with 125 (228.25 ± 45 mg/dl) or 250 (131.75 ± 10) mg/kg ANC ($P < 0.001$ vs. CMC). Administration of 250 mg/kg ANC normalized glucose levels in ZDF rats towards those of lean littermates. Insulin levels decreased significantly in ZDF rats treated with CMC or 125 mg/kg ANC ($P < 0.0001$), but not in rats treated with 250 mg/kg ANC. Histologically, 250 mg/kg ANC prevented islet degeneration compared with islets in

CMC-treated rats. We confirmed that ANC was well tolerated and had effective antidiabetic properties in male leptin receptor-deficient Zucker diabetic fatty (ZDF) rats.

In human study, the effectiveness of freeze dried mulberry fruit of Thai *Morus alba* L., on fasting blood sugar and HbA1C in pre diabetes patients were evaluated. Participants included 18 pre diabetes patients. They were divided into 2 groups (n=9). Subjects ingested either a capsule containing ANC or gelatin 750 mg. for 2 times a day. Fasting blood sugar (FBS), haemoglobin A1C (HbA1C), body weight, and body mass index (BMI) were assessed before and at intervals of 4 weeks for 12 weeks. After 12 weeks of treatment, FBS decreased significantly in pre diabetes patients treated with ANCs from 112.8 $\pm$ 5.5 mg/dl to 93.0 $\pm$ 10.45 mg/dl (p=0.0001), but not in the patients treated with gelatin (p= 0.1). Moreover, HbA1c also decreased in pre diabetes patients treated with freeze dried mulberry from 5.87 $\pm$ 0.13 % to 5.46 $\pm$ 0.37 (p=0.006) but not in the patients treated with gelatin (p=0.43). Here, we show for the first time that ANCs extracted from Thai *Morus alba* L. are an important antidiabetic agent. Furthermore, the ANC extract appeared to prevent the pathogenic lesions in diabetic islets by suppressing islet degeneration. We conclude that ANC may represent a promising class of therapeutic compounds that can be useful in type 2 diabetes prevention.

Keywords: Mulberry fruit/*Molus alba* L./Anthocyanin/ZDF rats/Glucose level/ Diabetic rats/Prediabetes patients/Islet/HbA1C/Fasting blood sugar

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CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes is a large and growing global health problem. The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. As in the World Health Organization (King, Aubert & Herman, 1998) Global Burden of Disease Study, it is projected that the number of individuals with diabetes will rise from an estimated 135 million in 1995 to a projected 300 million in 2025 Age-specific prevalence remains constant, the number of people with diabetes in the world is expected to approximately double between 2000 and 2030, based solely upon demographic changes (Wild, Roglic, Green, Sicree & King, 2004) . The estimated national prevalence of diabetes in Thai adults was 9.6% (2.4 million people), which included 4.8% previously diagnosed and 4.8% newly diagnosed (Aekplakorn et al., 2011).

Type 2 diabetes is preceded by the inability of β cells to secrete sufficient insulin to overcome insulin resistance or reduced insulin sensitivity, combined with reduced insulin secretion. Degeneration of the islets of Langerhans with β-cell loss is secondary to insulin resistance and is regarded as the pathophysiology of type 2 diabetes (Jun et al., 1999). Oral hypoglycemic agents directly stimulate insulin release from β-cells to overcome insulin resistance and normalize blood glucose level. However, these drugs can induce some adverse effects such as hypoglycemia (Rosak, 2002; Jennings, Wilson & Ward, 1989) Consumption of anthocyanins (ANCs) was reportedly associated with a reduced risk of degenerative diseases, such as atherosclerosis (Xia et al, 2006), cardiovascular diseases (Wallace, 2011), cancer (Wang & Stoner, 2008), and diabetes (Grace et al., 2009). ANCs extracted from *Calendula officinalis* fruits enhanced insulin

release from pancreatic β cells *in vitro* (Jayaprakasam, Vareed, Olson & Nair, 2005) . Mulberry leaves and fruits have been used to treat many diseases (Duthie & Crozier, 2000; Kaewkaen et al., 2012; Andallu, Kumar & Varadacharyulu, 2012; Musabayane, Bwititi & Ojewole, 2006). Mulberry fruit (*Morus alba L.*, Family Moraceae) has been used as medicine since ancient times. Mulberry fruit contains abundant ANCs, sugars, organic acids, free amino acids, vitamins, micronutrients, and other components. Mulberry fruit contains ANCs, which can scavenge reactive oxygen species (Du, Zheng, & Xu, 2008), have anti-obesity effects, and inhibit low-density lipoprotein oxidation (Peng et al., 2011). Water extract of Mulberry decreases serum lipids and has anti-obesity effect and ability to inhibit hepatic lipogenesis in high fat diet-fed hamsters. The predominant ANCs in mulberry, cyanidin 3-rutinoside and cyanidin 3-glucoside, dose-dependently inhibited the migration and invasion of highly metastatic A549 human lung carcinoma cells (Chen et al., 2006). Furthermore, it was recently reported that the cyaniding 3-O-β-D-glucopyranoside fraction from mulberry fruit protects against bladder dysfunction in streptozotocin-induced diabetic rats (Ha et al., 2012). However, it is not clear whether ANCs in mulberry can significantly contribute to lower blood glucose levels and useful in the treatment of pathogenesis of type 2 diabetes.

The evolution of diabetes in male leptin receptor-deficient Zucker diabetic fatty (ZDF) rat (ZDF/CrlCrlj) has made it a popular model for preclinical studies of type 2 diabetes because these rats exhibit disrupted islet architecture, β cell degranulation, and increased β cell death (Clark, Palmer & Shaw, 1983; Mega et al., 2011). Therefore, we used ZDF male rats, as a rodent model of type 2 diabetes in this study.

Prediabetes is diagnosed when blood glucose is higher than normal but not high enough for a diagnosis of type 2 diabetes. For people with prediabetes, the development of type 2 diabetes could be prevented or delayed through lifestyle changes Prevalence of prediabetes is increasing worldwide and experts have projected that more than 470 million people will have prediabetes by 2030. Prediabetes is associated with the simultaneous presence of insulin resistance and β-cell dysfunction-abnormalities that start before glucose changes are detectable (Tabák, Herder, Rathmann, Brunner & Kivimäki, 2012). Three types of blood tests are used to diagnose diabetes and prediabetes. The HbA1C is a measure of average blood glucose over the previous two to three months. The fasting plasma glucose (FPG) tests your blood glucose level on an empty stomach. The

oral glucose tolerance test (OGTT) measures blood glucose before and two hours after drinking a syrupy glucose solution (Grundy, 2012). In this study, prediabetes patients were evaluated for the effect of ANC on the fasting blood sugar and HbA1C levels compare with control subjects.

1.2 Research Objectives

1.2.1 To identify anthocyanins composition in Thai *Morus alba L*.

1.2.2 To measure the level of anthocyanins in Thai *Morus alba L*.

1.2.3 To evaluate the cytotoxic effect of anthocyanin extract from Thai *Morus alba L*. on murine macrophage and rat kidney cell culture

1.2.4 To assess the of anthocyanin extract fromThai *Morus alba L*. on insulin secretion and blood glucose level in diabetic rats

1.2.5 To evaluate the stage of organ damage following Type 2 diabetes and the cytotoxic effect of anthocyanin extract on these internal organs

1.2.6 To evaluate the effectiveness of freeze dried mulberry fruit of Thai *Morus alba L.*, on fasting blood sugar and HbA1C in prediabetes patients.

1.3 Hypothesis

1.3.1 Anthocyanin extract of *Thai Morus alba thai* L., may enhance insulin secretion and reduce blood glucose level in diabetic rats.

1.3.2 Anthocyanin extract of Thai *Morus alba* L., may reduce blood glucose and HbA1C levels in prediabetes patients.

CHAPTER 2

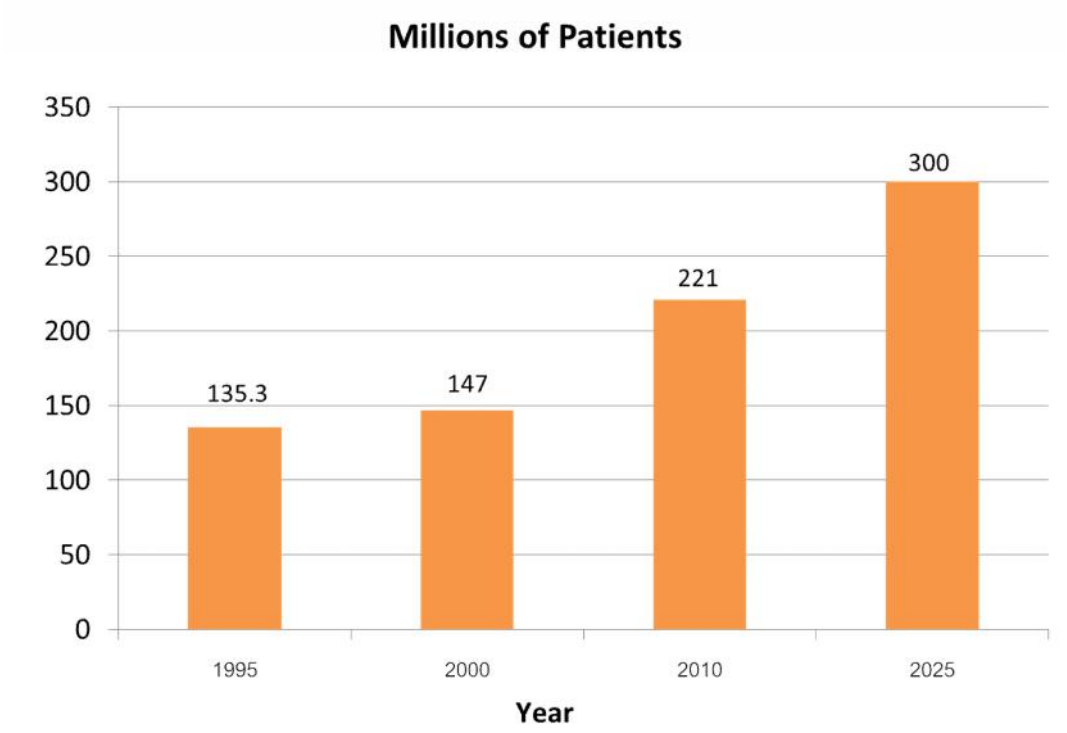
LITERATURE REVIEW

2.1 Diabetes Mellitus

2.1.1 Global Prevalence of Diabetes and mechanism of the development of diabetes mellitus

Diabetes is a large and growing global health problem. The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. Age-specific prevalence remains constant, the number of people with diabetes in the world is expected to approximately double between 2000 and 2030, based solely upon demographic changes (Wild et al., 2004). As in the World Health Organization (WHO) Global Burden of Disease Study, it is proposed that the number of individuals with diabetes will rise from an estimated 135 million in 1995 to a projected 300 million in 2025 (Figure 2.1) (King et al., 1998). The estimated national prevalence of diabetes in Thai adults was 9.6% (2.4 million people), which included 4.8% previously diagnosed and 4.8% newly diagnosed. (Aekplakorn et al., 2011) Diabetes mellitus is chronic hyperglycemia due to insulin insufficiency. This disorder is considered to be caused by the combination of some genetic factors and lifestyle factors. In particular, in insulin non-dependent diabetes mellitus (type 2), which accounts for more than 90% of all cases of diabetes mellitus, blood glucose is increased by the combination of decreased insulin secretion from pancreatic β cells and aging or lifestyle factors that decrease insulin sensitivity, such as overeating, high fat diet, insufficient exercise, and obesity. The estimated overall mean fasting plasma glucose level for Thai adults aged more than 35 years was 100.0 ± 1.0 mg/dl. Fasting plasma glucose was slightly higher in men (101.0 mg/dl) compared with women (99.1 mg/dl) and substantially greater in the residents of urban (106.6 mg/dl) compared with rural (97.0

mg/dl) areas. Levels were higher in older compared with younger individuals peaking in the 55- to 64-year age-group (Table 2.1). An estimated additional 5.4 ± 0.6% had impaired fasting glucose levels. The proportion of diabetes that was diagnosed did not significantly differ between women and men (53 vs. 47%) or between urban and rural areas (57 vs. 46%), but was substantially greater for older people than younger people (63% for ≥55 years vs. 37% for less than 54 years) (Table 2.1) (Aekplakorn et al., 2011).



From King, H., Aubert, R. E. & Herman, W. H. (1998). Global Burden of Diabetes, 1995–2025: prevalence, numerical estimates, and projections. **Diabetes Care, 9**(21), 1414-1431.

Figure 2.1 It is Estimated that by the Year 2025, the Number of People in the World with Diabetes Will Approximate 300 Million, Which is Considered a Global Epidemic

Table 2.1 Mean Fasting Plasma Glucose Levels (mg/d) Overall and by Age, Sex, and Area of Residence

Age-group	Population group				
	Men	**Women**	**Urban**	**Rural**	**Total**
All ages	101.0 ± 1.5	99.1 ± 1.4	106.6 ± 1.4	97.0 ± 1.1	100.0 ± 1.0
35–44 years	98.2 ± 2.6	93.0 ± 1.7	101.9 ± 2.0	92.5 ± 1.5	95.5 ± 1.6
45–54 years	101.7 ± 2.5	102.1 ± 2.5	108.4 ± 1.5	98.8 ± 1.8	101.9 ± 1.8
55–64 years	102.8 ± 2.6	105.7 ± 3.3	113.9 ± 3.5	100.3 ± 1.9	104.3 ± 2.2
≥65 years	105.7 ± 2.3	102.6 ± 2.3	109.4 ± 1.5	101.7 ± 2.0	104.0 ± 1.7

Note. Data are means ± SEM.

From Thai National Health Examination Survey IV Study Group. (2011). Prevalence and management of diabetes and metabolic risk factors in Thai adults: The Thai National Health Examination Survey IV, 2009. **Diabetes Care, 34**(9), 1980-1985.

Environmental factors have drawn particular attention because of the rapidity of the increase in type 2 diabetes mellitus (Hu et al., 2001). Most notable is the increase in the prevalence of obesity that has paralleled the rise in type 2 diabetes mellitus and the interrelated aspect of sedentary lifestyles (Mokdad, 1999; 2001). Obesity is the main risk factor of diabetes mellitus. Not all persons with obesity or physical inactivity develop diabetes mellitus, but these factors constitute part of the risk for type 2 diabetes mellitus because of their strong effect to induce insulin resistance. Study of the insulin dose-response of suppression of hepatic glucose output supports this concept (Revers, Fink, Griffin, Olefsky & Kolterman, 1984). After consuming a food containing carbohydrate, there will be an increase in blood glucose levels. The mechanism is occurred by α-amylase enzyme which acts at random locations along the starch chain and breaks down

long-chain carbohydrates to be glucose molecule. In type 2 diabetes mellitus, fasting blood glucose is raised in direct proportion to hepatic glucose output (Revers et al., 1984; Bogardus, Lillioja, Howard, Reaven & Mott, 1984; DeFronzo, Gunnarsson, Björkman, Olsson & Wahren, 1985) and decreased insulin secretion or increased peripheral insulin resistance, and increased hepatic production of glucose make insulin levels inadequate to normalize plasma glucose levels. Insulin production then falls, further exacerbating hyperglycemia. The disease generally develops in adults and becomes more common with age and appears to be a result of lack of exercise, overeating and obesity (Figure 2.2).

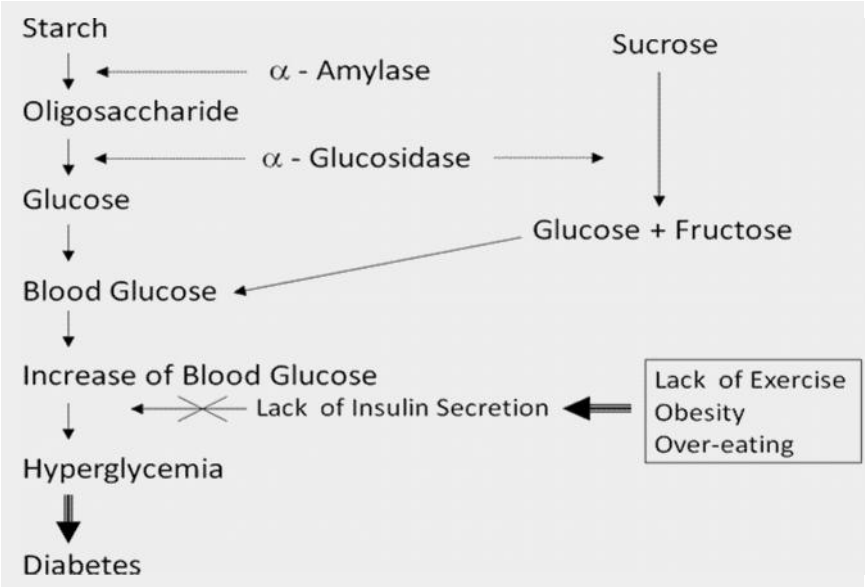


Figure 2.2 Association Between Digestion/Absorption of Carbohydrates and Type 2 Diabetes Mellitus

2.1.2 Pathways Leading to Diabetic Microvascular Complications

Diabetes greatly increases the risks of macro- and microvascular complications (Figure 2.3). (Brownlee, 2001) There have been four hypotheses how glucose might lead to the long-term complications.

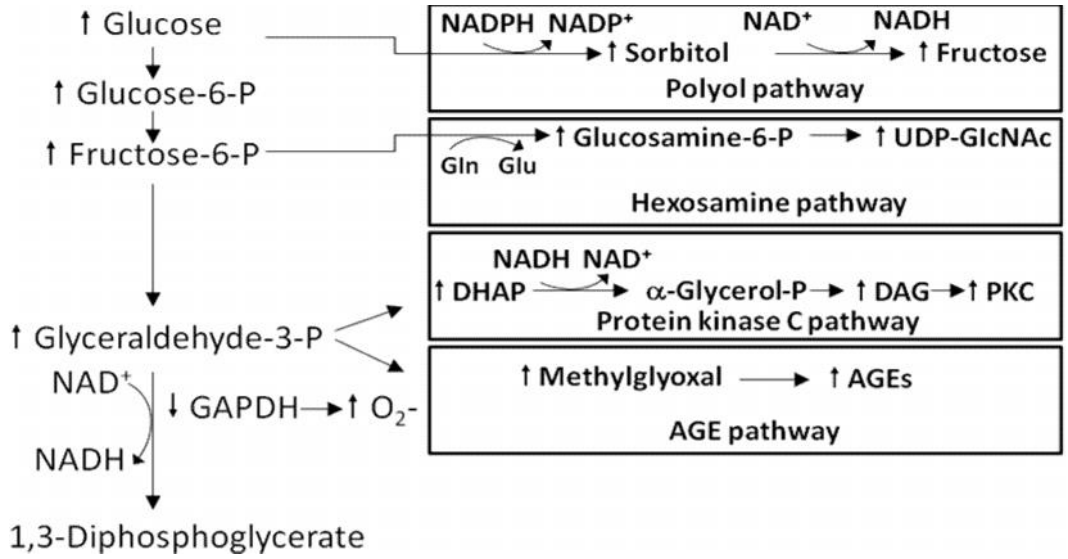


Figure 2.3 Potential Mechanism by Which Hyperglycemia-induced Mitochondrial Superoxide Overproduction Activates Four Pathways of Hyperglycemic Damage

2.1.2.1 The polyol pathway

The polyol pathway. Endothelial metabolic/dysfunction can be a consequence of the polyol pathway. The polyol pathway is clearly been studied for a long time. Under hyperglycemia, aldose reductase reduces glucose to sorbitol (a polyol or sugar alcohol), which is later oxidized to fructose. In this process, the aldose reductase consumes cofactor NADPH. Therefore, the hyperglycemic polyol pathway consumes NADPH and hence results in the depletion of gluthathione (GSH). This increases intracellular oxidative stress and chronic accumulation of intracellular sorbitol (Figure 2.4), which may induce tissue susceptibility to chronic diabetic complications. An increasing in the expression of aldose reductase has been implicated as the critical link between chronic glucose toxicity and tissue damage. (Burg & Kador, 1988; Dent et al., 1991)

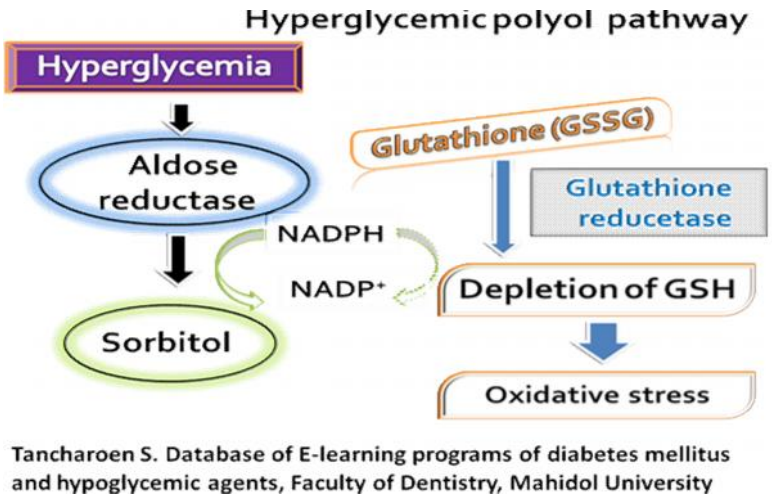


Figure 2.4 The Polyol Pathway

2.1.2.2 The hexosamine pathway

The hexosamine pathway (Figure 2.5) relates to insulin resistance and may be important in diabetic complications. The hyperglycemic hexosamine pathway starting from the glycolytic intermediate, D-Fructose-6-Phosphate (F-6-P), which is converted by Glucosamine: fructose-6-phosphate aminotransferase (GFAT) to glucosamine-6-P and eventually to UDP-N-acetylglucosamine (UDPGlcNAc), an O-linked N-acetylglucosamine (O-linked GlcNAc). Intracellular glycosylation by adding GlcNAc moieties to serine and threonine residues of proteins (e.g. transcription factors) is catalysed by O-linked N-acetylglucosaminyltransferase (OGT). Increased glycosylation of transcription factors, such as Sp1, AP2 and CREB, often at phosphorylation sites, increases the expression of cytokines and enzymes, including TGF- b1, PAI-1, and induce vascular and capillary occlusion (Du et al., 2000).

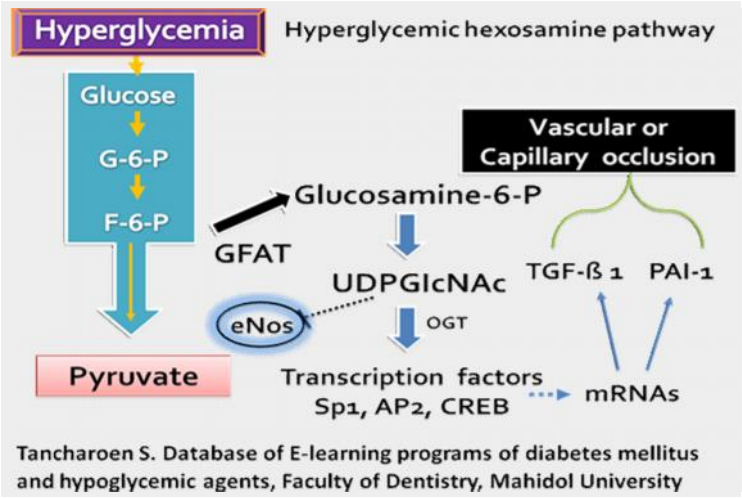


Figure 2.5 Hyperglycemic Hexosamine Pathway

2.1.2.3 The protein kinase C (PKC) pathway

There is also the protein kinase C (PKC) pathway (Figure 2.6). The pathogenic consequences of hyperglycemic PKC through activating transcription factors for a wide range of proteins, including cytokines. Many transcription factors, such as NF-kB, are activated through hyperglycemia-induced PKC activation, resulting in oxidatives stress, increased vasopermeability, angiogenesis,vascular occlusion, capillary occlusion, and abnormal blood flow (Clarke & Dodson, 2007).

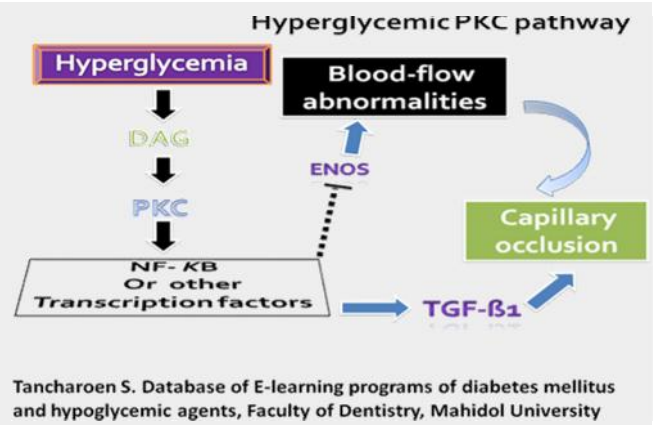


Figure 2.6 The Protein Kinase C (PKC) Pathway

2.1.2.4 The advanced glycation end-product pathway

The advanced glycation end-product pathway (Figure 2.7). The hyperglycemia-induced intracellular AGE precursors induce pathological consequences in diabetes retinophathy. The intracellular AGEs precursors can diffuse out of the cell and modify nearby cells (even the same cell itself), extracellular matrix, such as Bruch's membrane and choroidal capillary membranes. The alterations may compromise cell-matrix signaling (e.g. integrin-laminin between retinal pigment epithelium [RPE]-Bruch's membrane) and cause physiological dysfunction of the eyes (Aisenbrey et al., 2006).

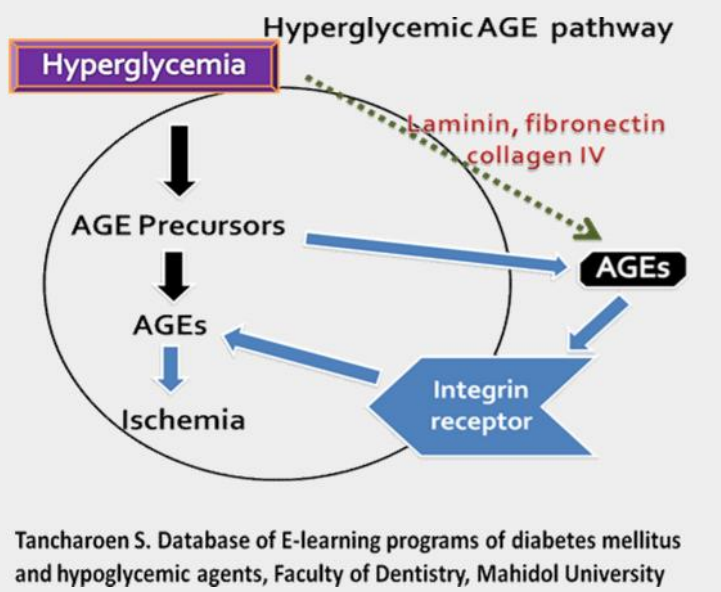


Figure 2.7 The Advanced Flycation End-product Pathway

2.1.2 Current Status of Diabetes Treatment: Side Effects of Oral Antidiabetic Agents

Use of oral hypoglycemic agents can induce some side effects. Drugs that directly stimulate insulin release from β-cells such as sulfonylurea (SU)-based drugs has shown that in type 2 diabetic patients, insulin secretion from the islets can be elevated sufficiently to overcome peripheral insulin resistance and the normalize the blood glucose level. However, One disadvantage of this use of SU-based drugs is that it fails to control

normal blood glucose levels (Looker et al., 2003) . These drugs also adversely affect the ability of β-cells to secrete insulin, and they can cause weight gain Other adverse effects consist of digestive manifestations (nausea, epigastric pain, liver pain) and of haematological manifestations (pancytopenia, autoimmune hemolytic anemia, thrombocytopenia) (Spranger et al., 2003).

The main side effect of Biguanide derivative agents is digestive manifestations, especially epigastric pain and diarrhea, occur in approximately 20% of cases and it is requiring a dose reduction or even quitting to this class of oral antidiabetic medication. Lactic acidosis is another adverse effect. Metformin is widely used in treatment of diabetes mellitus type 2. Metformin's major side effects are seen in the gastrointestinal tract, with nausea, cramps, and diarrhea. These side effects occur in 30% of patients but are usually transient and can be ameliorated or avoided by prescribing metformin to be taken after meals and starting with a low dose that is built up slowly. Even though many side effects of metformin can be ameliorated if it is taken in the slow-release form, but the most important and serious side effect is lactic acidosis. So, metformin is contraindicated in renal insufficiency patients (Clifford, 1992).

The major side effect of a-glucosidase inhibitors is flatulence. This occurs when undigested carbohydrate enters the large bowel, where it is digested by colonic bacteria resulting in gas formation. At high doses or in the presence of renal decompensation, hepatic necrosis may occur due to high serum a-glucosidase levels. Alpha glucosidase inhibitors can cause a slight weight gain, abdominal bloating, flatulence, abdominal discomfort, diarrhea, and rarely can cause liver test abnormalities (Gerich, 1989). The major problems with the thiazolidinediones are those of fluid retention, weight gain, and a normochromic, normocytic, dilutional anemia. The weight gain that occurs with the thiazolidinediones is in excess of that which would be associated with better glycemic control; it is mostly due to formation of new adipocytes in the subcutaneous fat, where it is not associated with an increase in insulin resistance (Ovalle & Bell, 2001) The weight gain that occurs with the thiazolidinediones may also be associated with fluid retention. The mechanisms proposed for the fluid retention are excess production of the cytokine vascular endothelial growth factor (VEGF), which causes increased permeability in the microcirculation, closure of calcium channels, and excess stimulation of the peroxisome proliferator-activated receptor (PPAR) gamma receptors (Olefsky, 2000). However, the

usual edema associated with thiazolidinediones does not respond to loop diuretics, thiazide diuretics, or spironolactone, and only responds to reduction or discontinuance of the drug. It is necessary to control liver enzymes (AST, ALT in particular) before and during treatment with thiazolidinediones. Other side effects are mild edema of the lower limbs, through the loss of elimination of salt and water, which, on the one hand, may decrease hemoglobin, with the appearance of anemia, and on the other hand, requires to be administered with caution to patients with type 2 diabetes and heart failure (Bell & Ovalle, 2001).

2.2 Anthocyanins

2.2.1 Consumption of Anthocyanins in Associated with a Reduced Risk of Several Diseases

In people with type 2 diabetes, insulin resistance and defects in b-cell function contribute to the development of hyperglycemia and glucose intolerance. (Rosak, 2002) The consumption of a diet low in fat and rich in antioxidants reduces the risks of developing obesity and insulin-resistance (Blakely et al., 2003). Anthocyanins, anthocyanidins, and proanthocyanidins are a large water-soluble pigment group found in a large number of fruits, vegetables and flowers. particularly grapes and berries. These pigments give plants their brilliant colors ranging from pink through scarlet, purple and blue. Scientists have identified several hundred different anthocyanins, including pelargonidin, malvidin, delphinidin, peonidin, and cyanidin, all attached to a sugar molecule. Anthocyanidins are similar to anthocyanins but they do not have a sugar molecule and provide powerful antioxidant properties (Giusti & Wrolstad, 2001).

Consumption of anthocyanins in associated with a reduced risk of several degenerative diseases, such as retinal impairment, atherosclerosis, cardiovascular diseases, cancer, and diabetes (Jayaprakasam, Zhang & Nair, 2004). These compounds are well-known free-radical scavengers which can trap free radicals that damage biomolecules and are potential chemopreventive agents (Burns et al., 2000). The chemical basis for these desirable properties of flavonoids is believed to be related to one anthocyanin, cyanidin 3-glusoside (Tsuda, Horio, Uchida, Aoki & Osawa, 2003). The serum antioxidant capacity is increased by the consumption of strawberries, cherries, and

red wine. (Van Velden, Mansvelt, Fourie, Rossouw & Marais, 2002; Kang, Seeram, Nair & Bourquin, 2003) Further, a recent study demonstrated that cyanidin 3-glusoside, reduced high fat diet-induced obesity in mice (Tsuda et al., 2003). The anthocyanin pigments of Bilberries (Vaccinium myrtillus) have long been used for improving visual acuity and treating circulatory disorders. There is experimental evidence that certain anthocyanins and flavonoids have anti-inflammatory properties, and there are reports that orally administered anthocyanins are beneficial for treating diabetes and ulcers and may have antiviral and antimicrobial activities. In addition, there is intense interest in the anthocyanin content of foods and nutraceuticals because of possible health benefits such as reduction of coronary heart disease (Bridle & Timberlake, 1996) improved visual acuity (Timberlake & Henry, 1988) antioxidant activities (Yamakami, Yamada, Yamamoto, Nakamura & Takeuchi, 1994; Wang, Rivas, Parrado, Cai & Flair, 1997) and anticancer activities (Karaivanova & Getov, 1990; Yuasa et al., 1995). Because anthocyanins are so widely consumed, any additional biological activities of these compounds are of great interest.

Table 2.2 shows anthocyanin content of some common fruits and vegetables (Giusti & Wrolstad, 2001).

Table 2.2 Anthocyanin Content of Some Common Fruits and Vegetables

Source	**Pigment content (mg/100 g fresh weight)**
Apples	10
Bilberries	300-320
Black currants	83-326
Black berries	130-400
Blueberries	25-495 *
Red cabbage	25
Black chokeberries	260

Table 2.2 (continued)

Source	Pigment content (mg/100 g fresh weight)
Cherries	4-450 *
Cranberries	60-200
Elderberry	450
Grapes	6-600 *
Kiwi	100
Red onions	7-21
Plum	2-25
Red rasdishes	11-60
Black raspberries	300-400
Red raspberries	20-60
Strawberrries	15-35
Tradescantia pallid (leaves)	120

Note. *Anthocyanin contents could be varied depending on the solvent and method extraction.

From Giusti, M. M. & Wrolstad, R. E. (2001). Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy. **Current Protocols in Food Analytical Chemistry**. doi 10.1002/0471142913.faf0102s00

2.3 *Morus alba* L.

2.3.1 Chemical Composition of *Morus alba* L.

Mulberry fruit changes the colour from green to black purple through red with maturity. Some varieties introduced from mid Asia have white fruits. The pH-dependent

conformational rearrangement of the anthocyan molecule has been studied for anthocyanins bearing a sugar on C3. The conformation predominates depends upon pH. At neutral pH, anthocyans occur as chalcones with an open C ring. Under mild acidic conditions, the ring is closed to form a carbinol pseudo base. In strong acid (pH 2), ring C acquires aromaticity involving a flavylium cation, which imparts intense colour on the molecule. In alkali, oxidation of ring A generates a quinoid structure with the elimination of the positive charge, this species is also coloured. The ring-opened chalcone can be reformed at neutral pH (Figure 2.8). Figure 9 shows the molecular structures of cyanidin 3-O-rutinoside (1), cyanidin 3-O-glucoside (2), pelargonidin 3-O-glucoside (3), pelargonidin 3-O-rutinoside (4), cyanidin (5), and pelargonidin (6) which contain in *Morus alba* L.

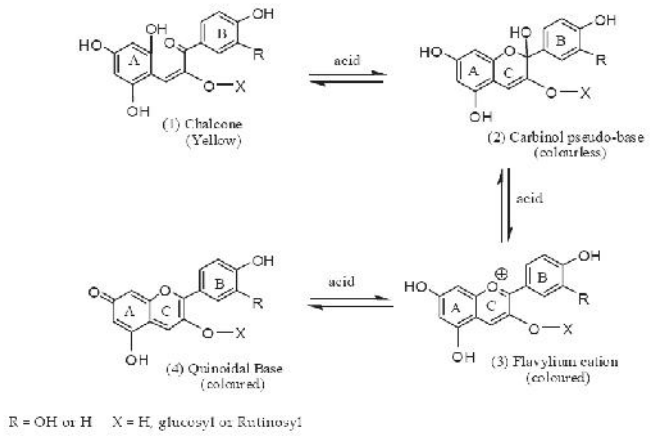
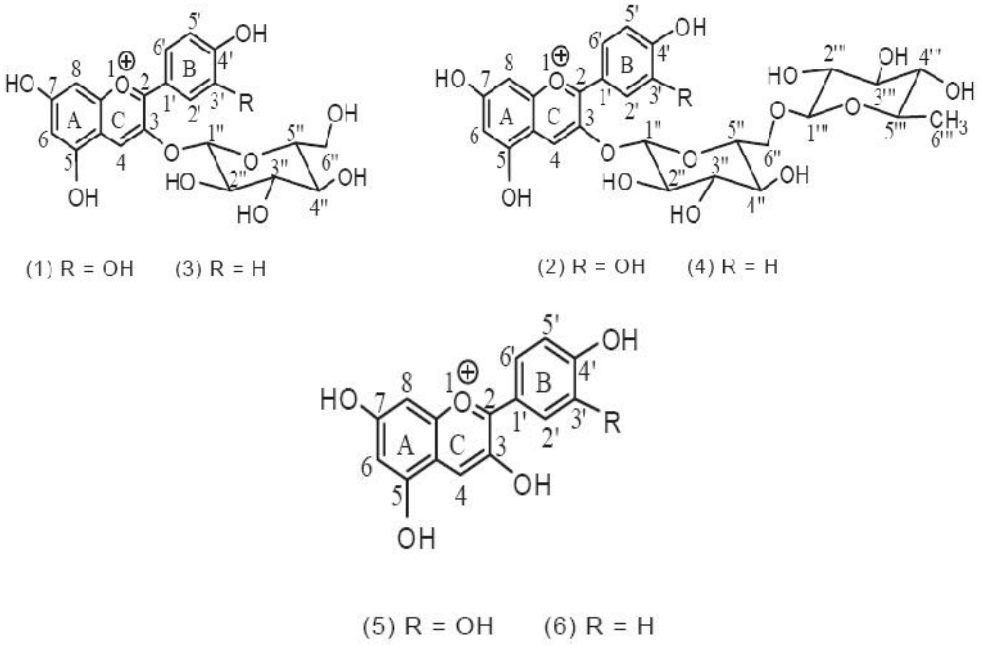


Figure 2.8 Chemical Composition of *Morus alba* L.



Note. (1), Cyanidin 3-O-Glucoside (2), Pelargonidin 3-O-Glucoside (3), Pelargonidin 3-O-Rutinoside (4), Cyanidin (5), and Pelargonidin (6)

Figure 2.9 The Molecular Structures of Cyanidin 3-O-Rutinoside

2.3.2 Pharmacological Activity of *Morus alba* L.

Mulberry fruit has been used as medicine since ancient times. Mulberry fruit contains anthocyanins (Wang & Stoner, 2008) sugars, organic acids, free amino acids, vitamins, micronutrients, and other components . Mulberry fruit has also been used as important medicinal materials in China (Grace et al., 2009) It has been found that mulberry fruit extract has an anti-oxidative property (Duthie & Crozier, 2000) and antiradical capacity (Kaewkaen et al., 2012).

2.3.2.1 Atherosclerosis

Mulberry water extract has been reported to be able to scavenge reactive oxygen species, inhibit low-density lipoprotein oxidation in vitro, prevent platelet aggregation and decrease serum lipids in cholesterol-fed rabbits (Chen et al., 2005). The levels of triglyceride, cholesterol and low-density lipoprotein cholesterol (LDL-C) were

lower in the serum of rabbits fed high cholesterol diet plus mulberry than in the serum of rabbits fed high cholesterol diet. Feeding with mulberry water extract (0.5 or 1% in the diet) to rabbits significantly reduced severe atherosclerosis in the aorta by 42-63%. Another study published in 2011 states that water-based mulberry extracts can lower the levels of cholesterol and triglycerides in the blood and thereby, prevent atherosclerosis, or plaque formation. Mulberry water extract also had the ability of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging for reducing the formation of free radicals mediated by copper ions. Furthermore, mulberry water extract 0.5-1.0 mg/mL could decrease macrophage death induced by oxLDL. In addition, mulberry water extract also could inhibit the formation of foam cells (Xia et al., 2006).

2.3.2.2 Cancer

Anthocyanins extracted from mulberry plants also inhibit the movement and invasion of lung cancer cells in the laboratory. Cyanidin 3-rutinoside and cyanidin 3-glucoside (extracted from *Morus alba* L.) exerted a dose-dependent inhibitory effect on the migration and invasion, of highly metastatic A549 human lung carcinoma cells in absence of cytotoxicity. The results showed that cyanidin 3-glucoside and cyanidin 3-rutinoside treatments could decrease the expressions of matrix matalloprotinase-2 (MMP-2) and urokinaseplasminogen activator (u-PA) in a dose-dependent manner and enhance the expression of tissue inhibitor of matrix matalloprotinase-2 (TIMP-2) and plasminogen activator inhibitor (PAI) (Chen et al., 2006). However, these benefits on cancer have not been proven in the clinical patients.

2.3.2.3 Alzheimer's Disease

Laboratory animals of six-month old SAMP8 and SAMR1 mice fed with basal diet supplemented with 0.18% and 0.9% mulberry extracts rich in phenolics and anthocyanins for consecutive 12 weeks, demonstrated significantly less amyloid β protein, improved memory and learning and, lower levels of free-radical mediated damage. Treatment with mulberry extracts decreased the levels of serum aspartate aminotransferase, alanine aminotransferase, triglyceride and total cholesterol that increase with ageing. The hepatoprotective effect of mulberry extracts appeared to occur through a mechanism related to regulation of the mitogen-activated protein kinases and activation of the nuclear factor-erythroid 2 related factor 2, where the latter regulates the induction

of phase 2 antioxidant enzymes and reduction of oxidative damage. The extracts may thereby, benefit Alzheimer's patients (Shih, Chan, Liao, Wang & Yen, 2010).

2.3.2.4 Obesity

Water extract of mulberry decreases serum lipids and has anti-obesity effect and ability to inhibit hepatic lipogenesis in high fat diet-fed hamsters. After 12 weeks of treatment with water extract of mulberry, it can lowered high-fat diet (HFD)-induced body weight and visceral fat, accompanied with hypolipidemic effects by reducing serum triacylglycerol, cholesterol, free fatty acid, and the low-density lipoprotein (LDL)/high-density lipoprotein (HDL) ratio. Water extract of mulberry decreased hepatic lipids, thus protected livers from impairment. The hepatic peroxisome proliferator-activated receptor α and carnitine palmitoyltransferase-1 were elevated, while fatty acid synthase and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase were reduced by water extract of mulberry (Ovalle & Bell, 2001).

2.3.2.5 Diabetes

Mulberry leaves enriched with the chemical 1-deoxynojirimycin can help lower blood sugar levels after meals and promote secretion of insulin. Healthy volunteers received 0, 0.4, 0.8, and 1.2 g of DNJ-enriched powder (corresponding to 0, 6, 12, and 18 mg of DNJ, respectively), followed by 50 g of sucrose. A human study indicated that the single oral administration of 0.8 and 1.2 g of DNJ-enriched powder significantly suppressed the elevation of postprandial blood glucose and secretion of insulin, revealing the physiological impact of mulberry DNJ. This study suggests that DNJ-enriched powder can be used as a dietary supplement for preventing diabetes mellitus (Kimura et al., 2007).

2.4 Zucker Diabetic Fatty (ZDF) Rat

2.4.1 Zucker Diabetic Fatty (ZDF) Rat Investigation of Type 2 Diabetes

A glucose tolerance test was used to indicate the response of insulin receptors to the elevation of exogenous glucose thus serving as a measure of sensitivity of insulin receptors to a glucose load. In the obese Zucker rat, the sensitivity of insulin receptors to glucose is significantly reduced (Lee, Lin, Hsiao & Shin, 2001). Therefore, it is a suitable animal model to test many medicines which may potentially improve sensitivity to insulin

receptors. Obese Zucker rats were very sensitive to exogenous glucose-induced hyperglycemia. Following glucose feeding to obese Zucker rats, glucose levels were increased by approximately 174%, however, lean Zucker rats displayed less sensitivity to the exogenous glucose-induced hyperglycemic effect, glucose levels increased by approximately 52% following glucose feeding at both time intervals. The leptin receptor deficient (*fa/fa*) Zucker diabetic fatty (ZDF) rat has become a popular model for the investigation of type 2 diabetes. Diabetes develops spontaneously in male rats at ages around 7 weeks, and is associated with the usual clinical symptoms of hyperphagia, polyuria, polydypsia, and impaired weight gain. β-cell mass fell by 51% from at ages 8–12 weeks (Finegood et al., 2001). Pick et al. attributed this failure of the b-cell mass to expand to an increased b-cell death at ages 6-12 weeks (Pick et al., 1998).

2.4.2 Zucker Diabetic Fatty (ZDF) Rat Strain Characteristics

Zucker, L.M. and Zucker, T. F. (1961; 1962; 1963), Zucker and Antoniades (1972)

2.4.2.1 Obesity

Obesity is evident at 5 weeks and may be noted as early as 3 weeks of age. By 14 weeks of age, their body composition is over 40 percent lipid and at 40 weeks of age, *fa/fa* rats weigh almost double as much as normal fa/+ littermates. Restricted feeding does not affect obesity. Feeding sucrose increases the level of lipogenesis in *fa/fa* rats. Obesity is unusual in the fact that fat cell proliferation occurs beyond the normal developmental period. It is of the hypertrophic-hyperplastic type involving an increase in both number and size of the adipocytes.

2.4.2.2 Hyperphagic

Significantly hyperphagic compared to lean littermates as early as 17 days of age and during periods of rapid growth. The obese Zucker rats' hyperphagia is not necessary for expression of the obese syndrome.

2.4.2.3 Hyperlipemia

Hyperlipemia found after weaning, and increases with age. Total fatty acid serum content of mutants reaches tenfold levels of non-obese fa/+ rats.

2.4.2.4 Mean arterial pressure

Mean arterial pressure for obese (fatty) Zucker rats is significantly greater than in the lean Zucker rat and the Lewis rat. The greater blood pressure in the obese rats was not dependent on hyperphagia or increased body weight per se since moderate caloric restriction, achieved by pair-feeding with lean rats, decreased weight gain but did not attenuate hypertension. Pair-fed obese rats retained less sodium than lean control rats, suggesting that greater blood pressure in the obese rats is not a consequence of increased renal retention of sodium. A unique feature of the Zucker strain is that the increased blood pressure appears to be specifically associated with the obese genotype. Fatty rats do not develop a premature atherosclerosis.

2.4.2.5 Hyperinsulinemic

Obese Zucker rats are hyperinsulinemic, both in the fed state and in response to oral glucose. Skeletal muscle of the Zucker *fa/fa* rat is highly insulin resistant, with depressed basal and insulin-stimulated glucose transport. Obese Zucker rats exhibit severe hepatic as well as peripheral insulin resistance. Moderate diabeteslike microvascular changes were reported consisting of decreased plantar muscle capillary density and increased basement membrane thickness in obese compared to lean Zucker rats.

2.4.2.6 Brain neuropeptide

Abnormal brain neuropeptide physiology related to a fa gene-dosage effect. Obese (*fa/fa*) and heterozygous lean (+/fa) Zucker rats have lower than normal brain insulin content when compared to homozygous lean (+/+) animals, particularly in the olfactory bulb.

Table 2.3 Summarize of Zucker Diabetic Fatty (ZDF) Rat Research Applications

Model used	References
Model of obesity, early-onset, hyperplastic-hypertrophic obesity and insulin resistance	Finegood et al., 2001; Unger, 1997
Model of human NIDDM	Blakely et al., 2003
Hypertension	Frisbee, 2005
Hyperlipidemia	Fujinami, Kojima, Aragane & Kusunoki, 2001

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Research Design

3.1.1 Prepare of Thai *Morus alba* L. freeze-dried crude extract and purify anthocyanin compound.

3.1.2 Identify anthocyanins composition and measure anthocyanins content in Thai *Morus alba* L. purchased from Kamnan Jul Farm, Petchaboon province, Thailand by high performance liquid chromatography- tandem mass spectrometry/ mass spectrometry (HPLC-MS/MS).

3.1.3 Study the effect of the concentration of anthocyanins from mulberry fruit pigment by UV-Visible Spectroscopy.

3.1.4 Thai *Morus alba* L. cytoxicity test in murine macrophage and rat kidney cell culture

3.1.5 Evaluate the effects of anthocyanin extract of Thai *Morus alba* L., on insulin secretion, blood glucose level and internal organs in diabetic rats.

3.1.6 Evaluate the effectiveness of freeze dried mulberry fruit of Thai *Morus alba* L., on fasting blood sugar and HbA1C in prediabetes patients.

3.2 Materials and Methods

3.2.1 Plant Material and Extraction

Mulberry fruits were obtained from Kamnan Jul Farm, Petchaboon Province, Thailand. The fruit was first extracted in ethanol-water (50/50, v/v%). The extract was then filtered through a Buchner funnel and filter paper (CHMLAB, BARCELONA,

Spain), and transferred to a 100 ml flask. The extract was collected and condensed at 40˚C using a Buchi B-490 rotary evaporator (BUCHI Labortechnik AG, Flawil, Switzerland) under a vacuum, and lyophilized with a freeze-dryer (Labconco Corp., USA).

3.2.2 Isolation and Purification of Mulberry ANCs

A C18 Sep-Pak cartridge (Waters Associates, Milford, US) was activated for 30 min with distilled water and high-performance liquid chromatography (HPLC)-grade methanol (Merck KGaA, Darmstadt, Germany). The ANC extract was then loaded onto the column. After successive washes with five volumes of distilled water (acidified with 0.01% HCl) and ethyl acetate (Fisher Scientific, UK), the ANCs were eluted with methanol containing 0.01% HCl. The ANC solution was then collected and condensed at 40˚C using a Buchi B-490 rotary evaporator under vacuum.

3.2.3 HPLC-Electrospray Ionization (ESI)-Mass Spectrometry (MS)

ANCs in the partially purified extracts were separated and quantified by reverse-phase HPLC using a Hypersil Gold C18 column (inner diameter, 5 μm; 4.6 × 250 mm; Thermo Fisher Scientific Inc., IL). The column was eluted with a mobile phase consisting of water, 3.75% formic acid (VWR International, Ltd., UK) and 15% methanol at a flow rate of 1 ml/min. The separated ANCs were detected and measured at 530 nm, and were identitified based on the retention times and ultraviolet (UV)-visible (Vis) wavelength spectra of pure authentic standards (cyanidin 3-O-glucoside, cyaniding-3-rutinoside, pelargonidin 3-glucoside, pelargonidin 3-rutinosid; Sigma, St. Louis, MO, USA). The identity of each peak was verified by LC-MS (Agilent 1100; Agilent Technologies, Santa Clara, CA, USA) using ESI and operating in a single quadrupole mode. The instrument was scanned over the *m/z* range of 200-1500 in the ESI positive ion mode. The LC-MS was eluted with acetonitrile (Fisher Scientific) and 0.5% ammonium hydroxide (90:10, v/v%).

3.2.4 Quantification of ANCs by UV-Vis Spectroscopy

The ANCs were quantified by UV-Vis spectroscopy. The model reaction solution was diluted with 0.01% HCl in distilled water. The absorbance at 510 nm was compared

with those of known standard solutions using a Genesys 10 UV spectrophotometer (Thermo Spectronic, NY, USA).

3.2.5 Determination of Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteau reagent (FRC). Briefly, 2.5 ml of mulberry ethanol extract was mixed with 0.5 ml of FCR (Sigma) and 1.0 ml of 20 g/100 g solution of sodium carbonate. The mixture was then incubated for 2 h in the dark at 25˚C. The absorbance of the mixture was measured at 765 nm using a UV-Vis Genesys 10 UV spectrophotometer. A standard curve was plotted using gallic acid (0.07-10 mg/mL in methanol; Sigma) as a standard. The total phenolic content was expressed as gallic acid equivalents (GAEmM/Gfw). The assay was carried out in triplicate and the mean value was recorded (Figure A1).

3.2.6 Determination of Ferric Reducing Antioxidant Power (FRAP)

Briefly, FRAP reagent, which consisted of 0.3 M acetate buffer (pH 3.6) and 10 mM TPTZ (Fluka, Switzerland) in 40 mM HCl and 20 mM $FeCl.6H_2O$ at a ratio of 10:1:1 (v/v/v) was freshly prepared before each measurement. Then, 200 µL of mulberry extract was mixed with 1.3 ml of FRAP reagent and incubated for 30 min at 37˚C. The absorption was measured at 595 nm using a spectrophotometer (Epoch; Biotek, USA) with the Gen 5 Data analysis software interface. Aqueous or methanol solutions containing known Fe (II) concentrations were used to calibrate the FRAP assay. FRAP values, expressed as mmol of Fe (II) equivalents (FeFmM/gFW), were determined by comparing the change in absorption of the test mixture with those of the Fe (II) standards. The assay was carried out in triplicate and the mean value was recorded.

3.3 Methods ZDF Rat Model

3.3.1 Evaluation of the Antidiabetic Effects of ANCs in ZDF Rats

Five-week-old male ZDF ($Lepr^{fa}$/CrlCrlj) and age-matched lean rats ($Lepr^{fa}/\pm$) were used in this study. All rats were ordered as bred from Charles River Laboratories International (Wilmington, MA). All animal studies were conducted according to the

National Institutes of Health Guidelines for the Care and Use of Animals, and were reviewed and approved by the committee on Animal Experimentation of Kagoshima University, Japan. All rats were kept under pathogen-free conditions with a 12-h light–dark cycle (lights on at 7:00) at 22 ± 1˚C.

ZDF and lean ZDF rats were administered by gavage, twice daily, with 125 or 250 mg ANC/body weight dissolved in 1% carboxymethylcellulose (CMC; Sigma) in distilled water. The control groups received 1% CMC in distilled water alone.

After the rats were allocated to each experimental group, they were allowed to acclimatize and were fed with a control diet for 1 week. Then, food was withheld for 24 h and tail vein blood samples were collected after cutting the tip of the tail with a scalpel. The blood samples were centrifuged, and plasma was stored at -20˚C until assay. Blood glucose levels were monitored every week using a glucose meter (Accu-Chek® Active; Roche Diagnostics). After 5 weeks of treatment with ANC or CMC, the rats were killed by heart puncture using sterile needles and syringes under anesthesia with diethyl ether, and blood was collected. Plasma insulin levels were measured using an enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI, USA). All experiments were performed using conscious unrestrained rats.

After killing the rats, the pancreas was perfused with physiological saline and rapidly excised. The tissue samples were kept in 10% neutral-buffered formalin, dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin wax. Sections (4 μm thick) were stained with hematoxylin and eosin (H&E). For histological analysis, tissue sections were photographed using a high-resolution color digital camera mounted on an Olympus BX51 microscope, and the images were transferred to a computer. Four sections were examined from each animal in each treatment groups.

3.3.2 Cell Culture and Treatment

Murine macrophage-like cells (RAW 264.7) and rat renal tubular epithelial cells (NRK-52E) were obtained from the American Type Culture Collection (Manassas, VA, USA). RAW 264.7 cells were maintained in RPMI 1640 medium (GibCo BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 2 mmol/L glutamine (Hyclone Logan, UT). NRK-52E cells were grown in Dulbecco's modified Eagle's medium (DMEM; GibCo BRL) containing 7% (v/v%) fetal bovine serum and 2 mmol/L

glutamine (Hyclone Logan). RAW 264.7 cells (3.5×10^4 cells/well) and NRK-52E cells (4×10^4 cells/well) were cultured in serum-free OPTI-MEM I medium (GibCo BRL) and serum-free DMEM, respectively before stimulation with various concentrations (0, 2, 10, 30, 50, or 100 μg/mL) of mulberry extract for 24 h.

3.3.3 Methyl Thiazolyl-Diphenyl-Tetrazolium Bromide (MTT) Assay

Cell viability was assessed using a modified MTT assay. Briefly, after cells were exposed to the specified concentration of mulberry extract for 48 h, MTT solution was added to each well of the six-well plate. After 3 h, dimethyl sulfoxide (DMSO) was added and the plate was incubated for 24 h at 37˚C. Absorbance was measured at 570 nm using an automatic microplate reader (ImmunoMini NJ-2300; InterMed, Tokyo, Japan).

3.4 Methods Pre Diabetes Patients Model

3.4.1 Evaluation of the Antidiabetic Effects of ANCs in prediabetes patients Participants

Pre diabetes patients age between 35-60 years were divided into 2 groups (n=9). The inclusion criteria included

3.4.1.1 Gender : Male or Female

3.4.1.2 Not currently intake any medication

3.4.1.3 Fasting blood sugar level ranges between 100 mg./dl. and 126 mg./dl.

3.4.1.4 HbAIC level ranges between 5.7 % and 6.4 %

3.4.1.5 No history of liver and kidney disease

3.4.1.6 No history of high or low blood pressure

3.4.1.7 No history of hyperlipidemia

3.4.2 Materials

Mulberry fruits, Thai *Morus alba* L., were obtained from Kamnan Jul Farm, Petchaboon Province, Thailand. The fruits were freeze dried and capsulated at 750 mg./cap. Anthocyanin extraction was measured by HPLC. For the control group, gelatin,

brand Gelita, from Taweepol Bakery (Yaowarat, Bangkok) was capsulated at 750 mg./cap.

3.4.3 Methodology

Participants included 18 pre diabetes patients were divided into 2 groups. One group of 9 subjects ingested gelatin 750 mg/cap and the other group of 9 subjects ingested dry mulberry powder 750 mg/cap 2 times a day half an hour before breakfast and dinner. FBS, HbA1C, body weight, and BMI were assessed before and at intervals of 4 weeks for 12 weeks.

Fasting blood sugar (FBS) was measured by the hexokinase enzymatic method. HbA1c concentration was measured after hemolysis of the EDTA anti-coagulated whole blood specimen by the COBAS INTEGRA 800 (I800) system (Roche Diagnostic, Basel Switzerland). The concentration of HbA1c was determined by high performance liquid chromatography (HPLC) with Boronate Affinity method using PDQ from PRIMUS CORP.

3.4.4 Statistical Analysis

Data were analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Data are shown as means ± standard deviation. The significance of differences between two groups was assessed by Student's *t*-test, and differences between multiple groups were assessed by one-way analysis of variance (ANOVA) followed by the Scheffé's multiple range test. Values of $P < 0.05$ were considered statistically significant.

CHAPTER 4

RESULTS

4.1 Analysis of Mulberry ANCs

The ANC composition of mulberry fruit was determined by HPLC-ESI-MS. The ANC extract was purified using a C-18 Sep-Pak cartridge, and the resulting chromatogram, at 520 nm, is shown in Figure A1. The chromatogram contained four peaks within the retention time of 31–38 min, indicating the presence of four different ANCs in mulberry fruit (Table B1). Peak 1, with a retention time of 31.3 min, M+ at *m/z* 449.1 (mw of cyanidin 3-glucoside), and a fragment ion at *m/z* 287.0 (mw of cyaniding-3 glucoside - mw of glucose), was identified as cyanidin 3-O-glucoside (51.4%). Peak 2, with a retention time of 33.0 min, M+ at *m/z* 595.2 (mw of cyanidin 3-rutinoside), and fragment ions at *m/z* 449.1 (mw of cyanidin 3-rutinoside - mw of glucose) and 287.0, was identified as cyanidin 3-rutinoside (45.3%). Peak 3, with a retention time of 36.4 min, M+ at *m/z* 433.1, and a fragment ion at *m/z* 271.0, was identified as pelargonidin 3-glucoside (2.1%). Peak 4, with a retention time of 38.0 min, M+ at *m/z* 579.1, and fragment ions at *m/z* 433.1 (pelargonidin 3 -rutinoside) and 271.0 (glucose), was identified as pelargonidin 3-rutinoside (1.2%). Results of UV-Vis quantification of total ANC showed that the phenolic-rich extract contained 28 mg/g of total ANC (calculated as cyanidin-3-O-glucoside equivalents). The total phenolic content of ANC extracts, expressed as mmol of Fe (II) equivalents and gallic acid equivalents, was 67.28 GAEmM/Gfw and 22.67 FeFmM/gFW, respectively.

4.2 Results in ZDF Rats

4.2.1 Hypoglycemic Effects of ANCs and Histology of Pancreatic Islets in ZDF Rats

Table B2 shows the changes in body weight observed in the six groups of rats. Body weight was significantly greater in ZDF rats than in their lean littermates from 8 weeks of age, and progressively increased with age ($P < 0.05$). ANC treatment did not affect body weight in either genotype. In fact, after 4 weeks of treatment, ZDF rats treated with 250 mg/kg ANC tended to gain more weight than those treated with CMC alone or 125 mg/kg ANC, although this was not statistically significant ($P = 0.3$ vs. CMC; $P = 0.11$ vs. 125 mg/kg ANC).

Blood glucose levels were measured in all of the rats for 5 weeks before starting the study and throughout the experimental period (Figure A7 [A]). At 7 weeks of age, the ZDF rats treated with vehicle showed mild hyperglycemia (~159 mg/dL) that rapidly progressed, reaching levels of ~396 mg/dL after 3 weeks. Administration of ANCs did not affect glucose levels in lean ZDF rats. Glucose levels increased significantly from 105.5 ± 8.7 mg/dL at 0 weeks to 396.25 ± 21 mg/dL ($P < 0.0001$) at 5 weeks in ZDF rats treated with CMC, but were significantly lower in rats treated with 125 (228.25 ± 45 mg/dL) and 250 (131.75 ± 10 mg/dL) mg/kg ANC (both, $P < 0.001$; Fig. 12B). Treatment with 250 mg/kg ANC reduced glucose levels in ZDF rats to values similar to those in lean littermates (Figure A7).

At the start of treatment at 5 weeks of age, plasma insulin levels were significantly higher in ZDF rats than in lean rats (11 ± 0.2 vs. 4.2 ± 0.0 pg/mL; $P < 0.001$; Fig. 12). Between 0 and 5 weeks, insulin levels decreased from 10.88 ± 0.0 to 7.9 ± 0.4 ng/mL ($P < 0.001$) in CMC-treated ZDF rats, and from 11.51 ± 0.0 to 8.72 ± 1.4 ng/mL ($P < 0.001$) in ZDF rats treated with 125 mg/kg ANC. Interestingly, plasma insulin levels did not decrease in ZDF rats treated with 250 mg/kg ANC (0 weeks: 10.8 ± 0.6 ng/mL; 5 weeks: 10.93 ± 0.4 ng/mL).

We also conducted histologic evaluation of pancreatic islets of 10-week-old ZDF and lean rats. Hematoxylin/eosin staining revealed no significant pathological abnormalities in islets from lean rats; their islets were round or oval with well-defined

boundaries (Figure A9 [A-C]). However, histological examination of pancreatic islets from CMC-treated ZDF rats revealed substantial changes in islet morphology. In particular, the islets were hypertrophic and compressed adjacent exocrine tissue, and there was marked vascular congestion or hemorrhagic degeneration (Figure A9 [D], upper panel). Furthermore, the islets were disorganized with finger-like projections into surrounding exocrine tissue. The degenerated islets also showed β cell vacuolation and degeneration (Figure A9 [D], lower panel). By contrast, histological assessment of pancreatic sections from ZDF rats treated with 125 mg/kg ANC showed a normal distribution of islet within the exocrine tissue and some β cell vacuolation (Figure A9 [E]). Interestingly, evaluation of pancreatic tissue samples collected from ZDF rats treated with 250 mg/kg ANC suggested that this dose had some protective effects, as there were fewer abnormal morphological features and fewer degenerated islets. Additionally, the islets had a regular shape with well-defined boundaries (Figure A9 [F]). In cell culture studies, we confirmed that ANCs had no cytotoxic effects on murine macrophages or rat kidney cells (Figure A10).

4.3 Result in Pre Diabetes Patients

4.3.1 Hypoglycemic Effects of ANCs in Pre Diabetes Patients

Data from 18 subjects were included in our study. The characteristics of the study population are shown in table B3. Thirteen men and six women took part in the study (Table B3).

FBS were measured in all subjects from both groups before starting the study and throughout the experimental period. Administration of ANCs reduced FBS in the subjects statistical significantly compared with the ones taking gelatin. After 12 weeks of treatment, FBS decreased significantly in pre diabetes patients treated with ANCs from 112.8 $\pm$ 5.5 mg/dl to 93.0 $\pm$ 10.45 mg/dl ($p=0.0001$) (Figure A11), but not in the patients treated with gelatin ($p= 0.1$) (Figure A12). Figure 18 shows the FBS level for both groups. The mean absolute difference calculated from the individual glucose concentrations was 19.89 + 4.94 mg/dl in the ANC group and 6.9 $\pm$1.4 mg/dl in the gelatin group (Table B4).

HbA1c decreased in pre diabetes patients treated with ANCs from 5.87 $\pm$ 0.13 % to 5.46 $\pm$ 0.37 (p=0.006) (Figure 19) but not in the patients treated with gelatin (p=0.43) (Figure A14). The mean absolute difference calculated from the individual HbA1C level in the ANC group was 0.41 $\pm$ 0.24 % and 0.15 $\pm$ 0.13% in the gelatin group (Table B4).

There were no statistical significantly differences of BMI in ANC and gelatin group before and after treatment (p=0.88 and p=0.92, respectively). At the baseline, BMI in pre diabetes patients treated with ANCs was 22.86 $\pm$ 1.5 kg m^{-2}, whereas in the patients treated with gelatin was 23.01 $\pm$ 2.23 kg m^{-2}. After 12 weeks treatment, BMI in pre diabetes patients treated with ANCs was 22.65 $\pm$ 1.56 kg m^{-2} and in the patients treated with gelatin was 22.92 $\pm$ 2.05 kg m^{-2}. The mean absolute difference calculated from the individual BMI in the ANC group was 0.2 $\pm$ 0.05 kg $m^{-2}$and 0.1 $\pm$ 0.17 kg m^{-2} in the gelatin group (Table B4).

There were no statistical significantly differences of body weight in ANC and gelatin group before and after treatment (p=0.88 and 0.94, respectively). At the baseline, body weight in pre diabetes patients treated with ANCs was 61.08 $\pm$ 8.74 kg, whereas in the patients treated with gelatin was 65.2 $\pm$ 4.3 kg. After 12 weeks treatment, body weight in pre diabetes patients treated with ANCs was 60.47 $\pm$ 9.1 kg and in the patients treated with gelatin was 65.05 $\pm$ 5.04 kg. The mean absolute difference calculated from the individual body weight in the ANC group was 0.61 $\pm$ 0.36 kg and 0.15 $\pm$ 0.74 kg in the gelatin group (Table B4).

There were no adverse effects of the ANCs extract reported by the participants.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

Our results in prediabetes patients and rat model showing that ANCs reduced blood glucose levels are consistent with those of a prior study showing that ANCs extracted from black soybean seed coats had antidiabetic and antioxidative effects in streptozotocin-induced diabetic rats (Nizamutdinova et al., 2009), while administration of ANCs extracted from *C. officinalis* fruits significantly increased insulin release from pancreatic β cells *in vitro* (Jayaprakasam et al., 2005).

In this study, the chromatogram of the purified product after acid hydrolysis of the ethanol extract revealed that cyanidin 3-O-glucoside (51.4%) and cyanidin-3-rutinoside (45.3%) were the major ANCs present in Thai *Morus alba* L.. The minor ANCs, which comprised 3.3% of the ANCs, were pelargonidin 3-O-glucoside and pelargonidin 3-O-rutinoside. Our results are relevant to those reported by Qin et al. (Qin et al., 2010), although the ANC content differs, probably because of differences between mulberry species and cultivars, as well as differences in extraction, separation, purification, and analysis between the two studies.

Lean ZDF rats are less sensitive to the exogenous glucose-induced hyperglycemia. The ZDF rats in our study exhibited marked hyperglycemia at 7 weeks of age and blood glucose levels continued to increase with age. These results are consistent with those of a previous study in which diabetes occurred spontaneously in male rats aged about 6 weeks, and was associated with hyperphagia, polyuria, and polydipsia (Jones, Nugent & Jenkins, 2010). It was also revealed that the β cell mass decreased by 51% from 9 to 12 weeks of age. In rats aged 6-12 weeks, the β cell mass cannot compensate for insulin resistance, resulting in compensatory β cell proliferation (Pick et al., 1998).

In a recent report, treatment with the ANC cyanidin 3-O-glucoside reduced the body weight and fat accumulation in visceral adipose and liver tissues of KK-Ay mice by improving triglyceride metabolism, and regulating lipoprotein lipase activity (Wei et al., 2011). In another study, mulberry water extract had antiobesity effects by upregulating hepatic peroxisome proliferator-activated receptor α and carnitine palmitoyltransferase-1 expression, and reducing fatty acid synthase and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase expression (Peng et al., 2011). However, in our study, the ANC extract from mulberry fruit did not promote reductions in body weight both in the rat model and prediabetes patients. In fact, the dose of 250 mg/kg ANCs actually resulted in some weight gain in ZDF rats from the age of 9 weeks ($P > 0.05$), without changes in food intake. The differences in results may be due to differences in polyphenols contained in the extracts, their free radical scavenging properties and mechanisms of action.

We report for the first time that mulberry fruit extract contains abundant cyanidin 3-O-glucoside (approximately 28 mg/g of crude ANC extract). The highest ANC dose (250 mg/kg body weight) contains approximately 7 mg cyanidin 3-O-glucoside. Blood glucose levels were 66% lower and insulin levels were 27% higher in ZDF rats treated with 250 mg/kg ANC compared with those treated with CMC for 5 weeks to 10 weeks of age. In addition, consumption of ANCs did not affect glycemia in lean rats. The maximum dose of ANC used in this study was derived from the cyanidin 3-O-glucoside concentration (10 mg/kg) used in a prior study (Andallu et al., 2012).

In human study, the ANC dose (750mg) contains approximately 21 mg cyanidin 3-O-glucoside. After 12 weeks of treatment, FBS decreased significantly in pre diabetes patients treated with ANCs from 112.8 $\pm$ 5.5 mg/dl to 93.0 $\pm$ 10.45 mg/dl (p=0.0001). HbA1c decreased in pre diabetes patients treated with ANCs from 5.87 $\pm$ 0.13 % to 5.46 $\pm$ 0.37 (p=0.006) (Figure A12) but not in the patients treated with gelatin (p=0.43). In addition, consumption of ANCs did not affect glycemia and BMI in both groups. So far, there are limited data on the mechanism of ANC on insulin-mediated glucose uptake. Some studies have shown that cyanidin 3-O-glucoside from black beans significantly upregulated glucose transport 4 (GLUT4) expression, induced adipocyte differentiation and glucose uptake *in vitro* (Tetsuya Inaguma & Hiroko, 2011), and prevented insulin resistance and pancreatic apoptosis in streptozotocin-induced diabetic rats (Nizamutdinova et al., 2009).

In our study, the islets of lean rats showed normal histological features. By contrast, there were marked morphological changes, including islet hypertrophy and cellular degeneration, in CMC-treated ZDF rats. These pathological findings support those of earlier studies showing pancreatic islet hypertrophy in ZDF rats (Janssen et al., 2001). By the time diabetes is diagnosed, β cells try to secrete sufficient insulin to overcome the insulin resistance in a process that involves islet hyperplasia. Degenerating islet cells show cytoplasmic vacuolation, possibly caused by autodigestion following cell death (Pick et al., 1998). In the present study, histological assessment of pancreatic islet from ZDF rats showed that 250 mg/kg ANC attenuated the degenerative changes in most of the rats. Furthermore, ANC prevents marked reductions of plasma insulin levels in these rats. These effects may be coupled with enhanced hepatic/peripheral tissue glucose uptake. As the current results, we cannot clarify the mechanism. Further experimental studies are clearly needed to identify the mechanisms of action of ANCs using isolated islets or β cells to examine whether ANC has direct effects on insulin secretion.

5.2 Conclusion

This study provides evidence that ANCs extracted from mulberry fruit have significant antidiabetic properties by improving blood glucose levels in ZDF rats as an animal model of type 2 diabetes and in pre diabetes patients. Here, we show for the first time that consumption of ANC of Thai *Morus alba* L. fruits, may have glucose-lowering effects in both type 2 diabetes rats and pre diabetes patients. ANCs protect insulin reduction and attenuate islet degeneration in ZDF rats.

In conclusion, our results suggest that the ANC extract of mulberry fruit is an effective antidiabetic agent, with marked glucose-lowering effects and prevents the progressive decline in insulin secretion. Although ANCs may protect against β cell damage, further experimental studies are needed to examine the pharmacokinetics and the molecular basis for the pharmacological activity of ANCs on insulin resistance and glucose handling in the management of diabetes mellitus. An increasing number of human subject and long-term studies are needed to confirm the present results and establish the durability of the improvements in glucose levels.

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REFERENCES

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APPENDICES

APPENDIX A

FIGURES

name	GAEmM/Gfw
mulberry	67.28
vitamin C	67.97

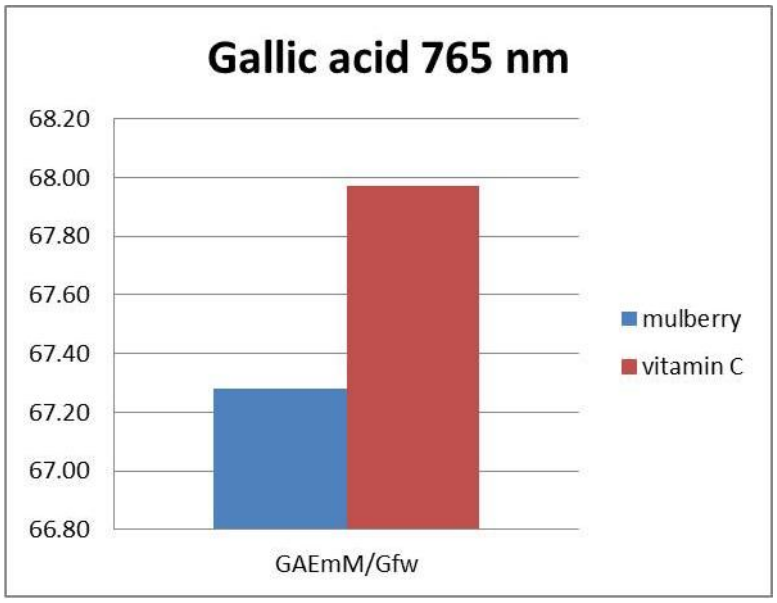

Figure A1 Total phenolic Content in Mulbeery

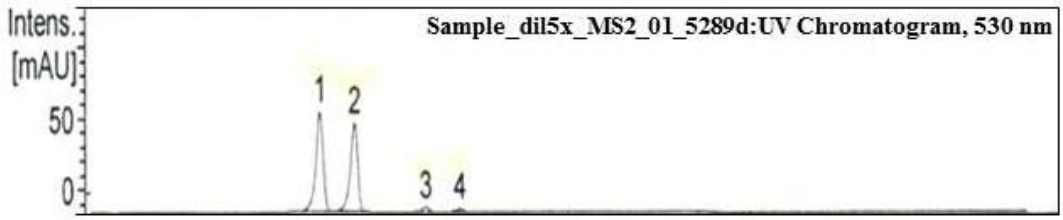

Figure A2 Sample UV Chomatogram

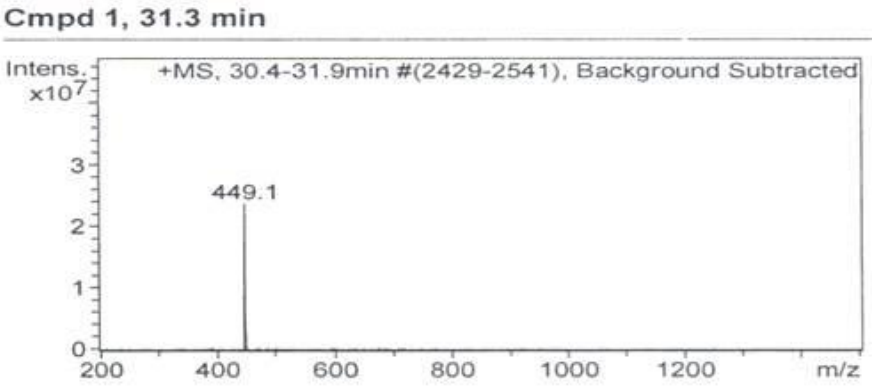

Figure A3 Cyanidin 3-glucoside

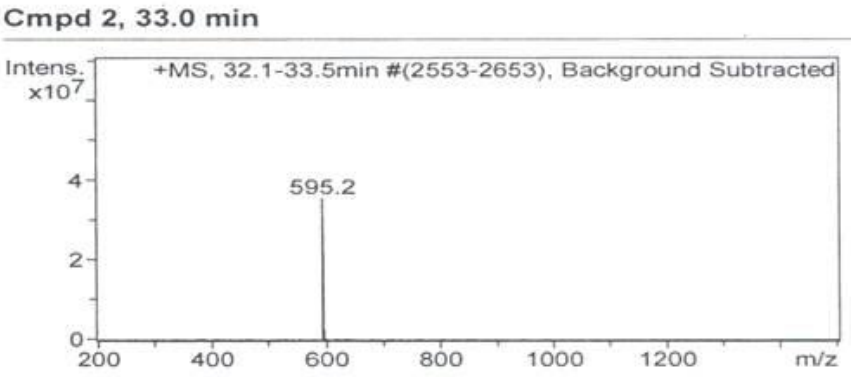

Figure A4 Cyanidin 3-rutinoside

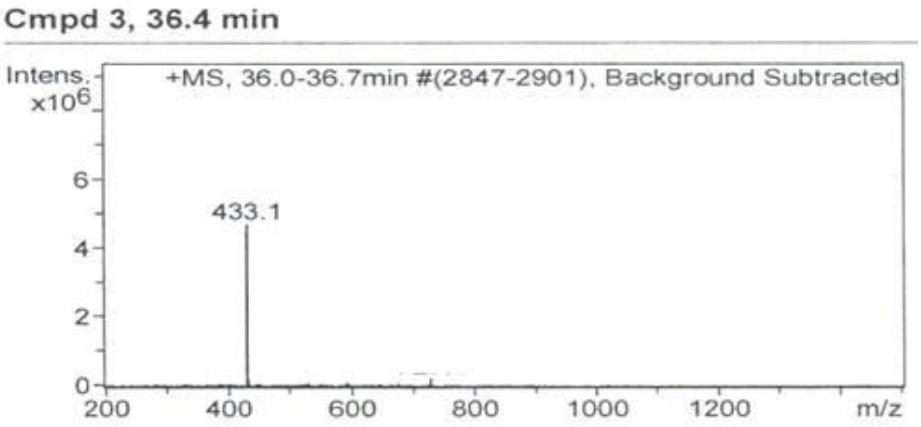

Figure A5 Pelargonidin 3-glucoside

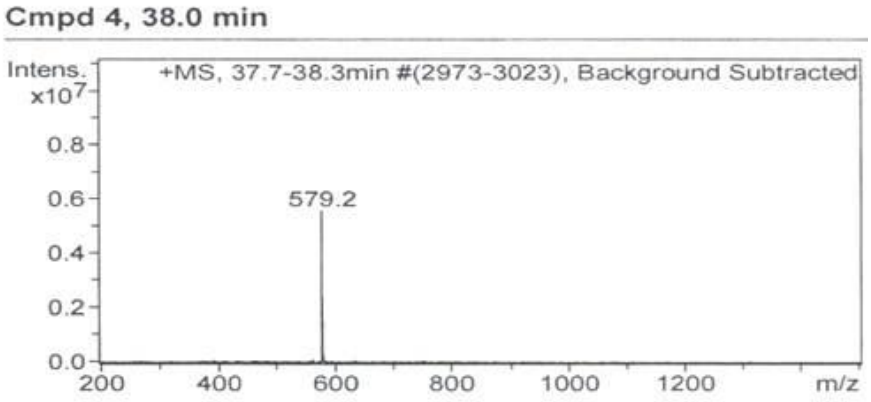

Figure A6 Pelargonidin 3-rutinoside * Results refers to table B1

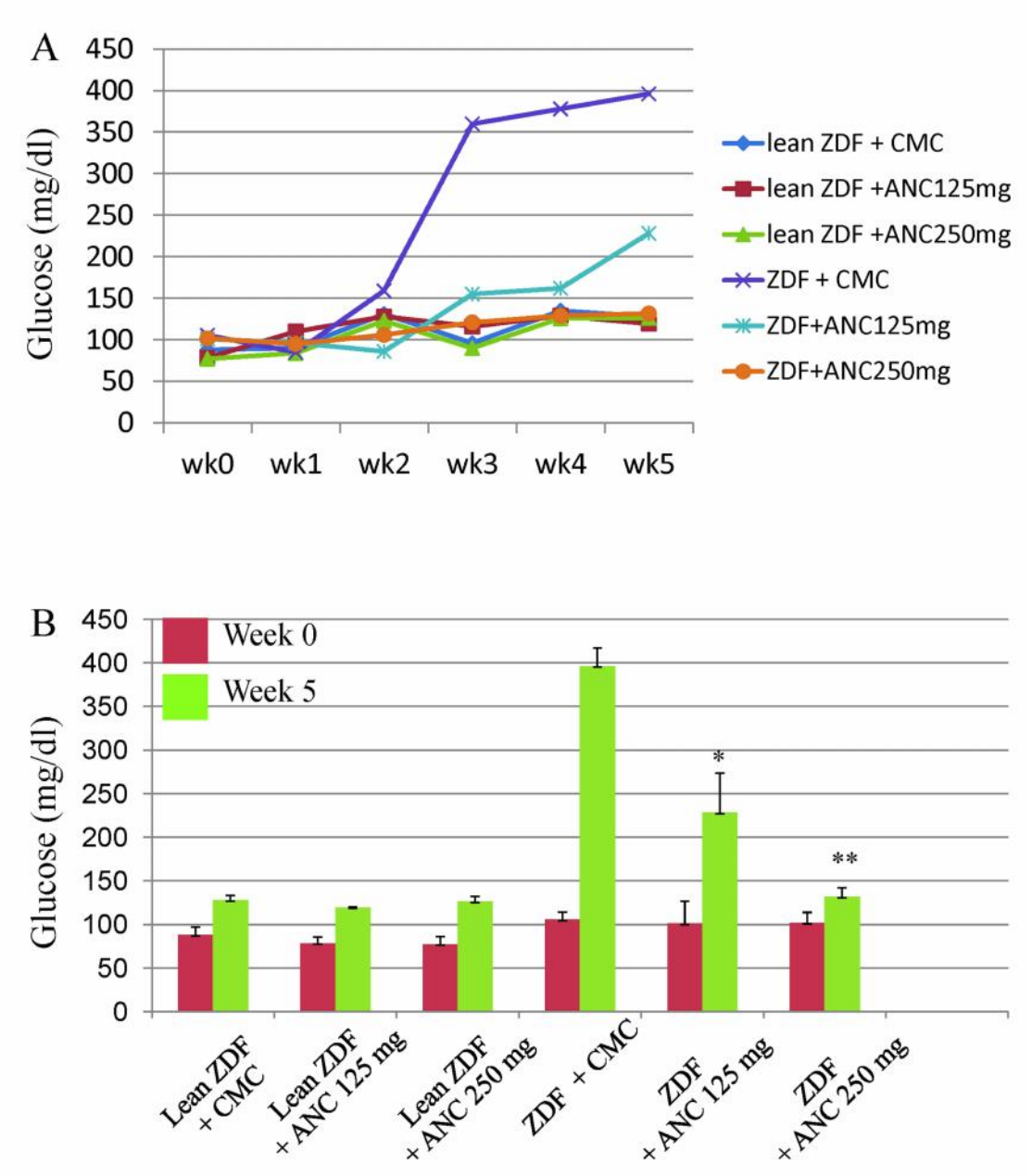

Figure A7 Hypoglycemic Effests of ANCs

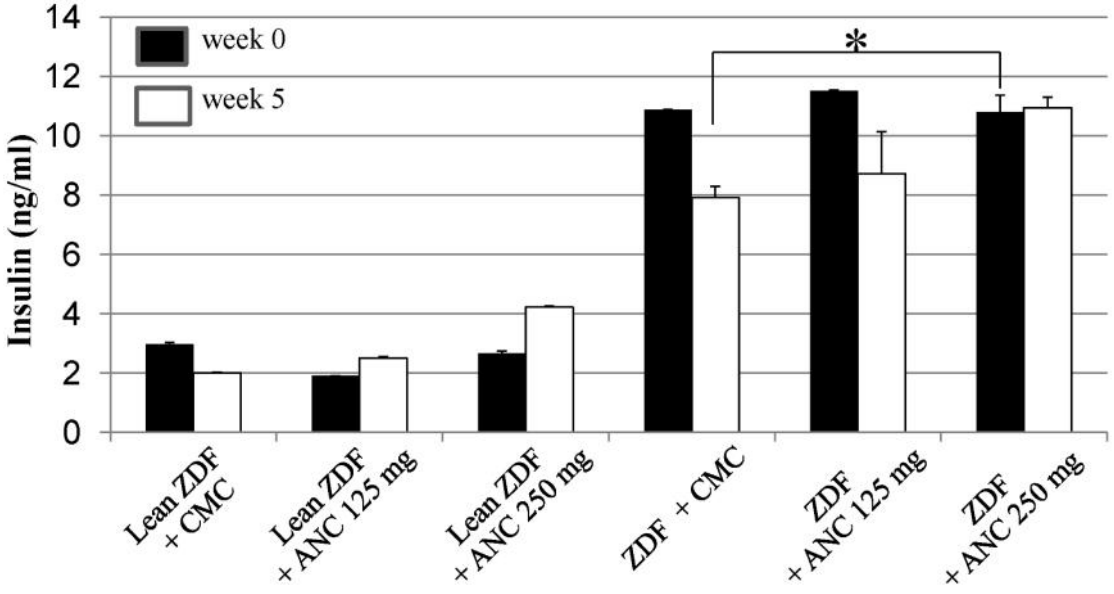

Figure A8 Plasma Insulin Effests of ANCs

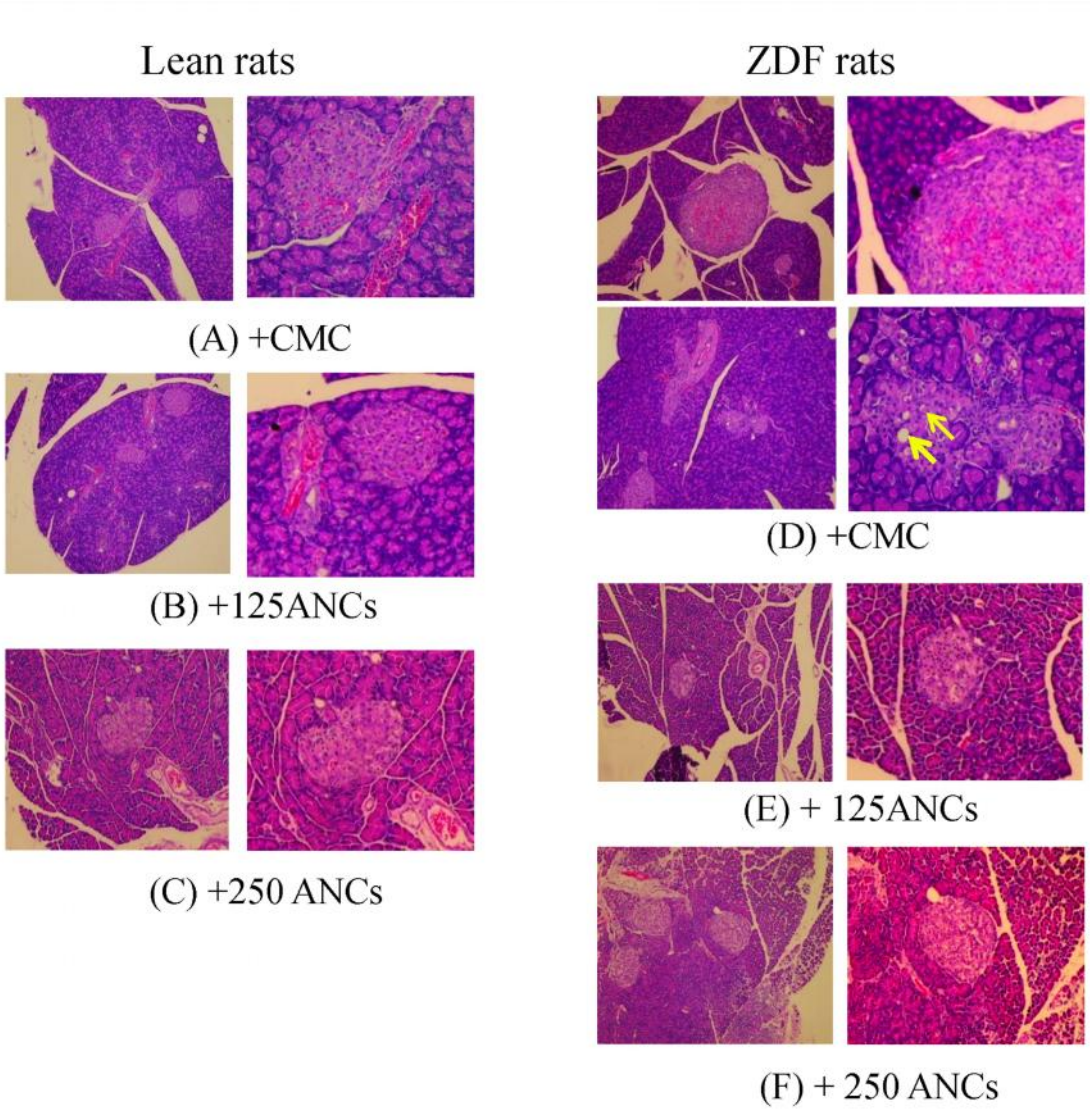

Figure A9 Pancreatic Islet Assessment

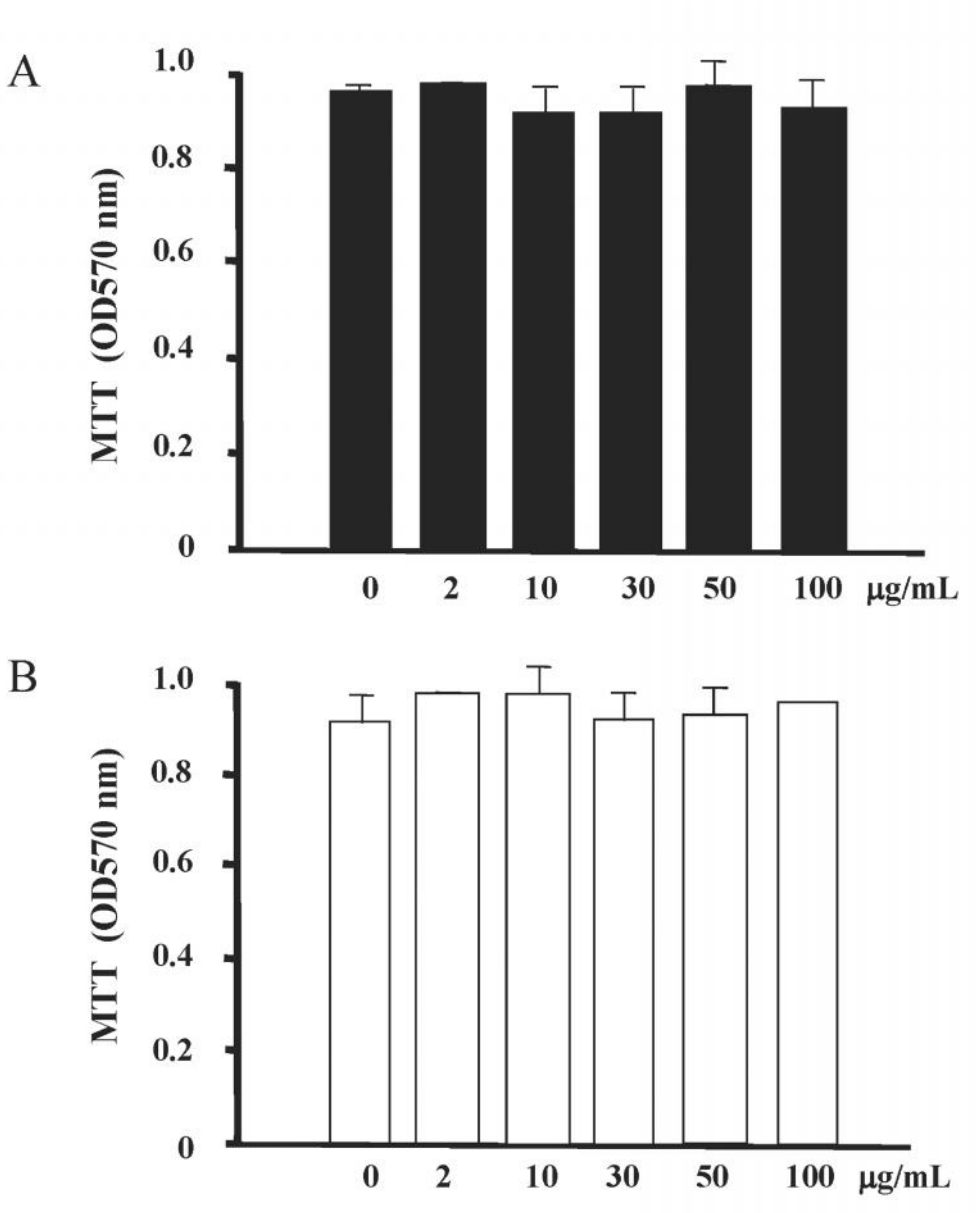

Figure A10 Cytotoxic effects of ANCs on Murine Macrophages and Rat Kidney Cells

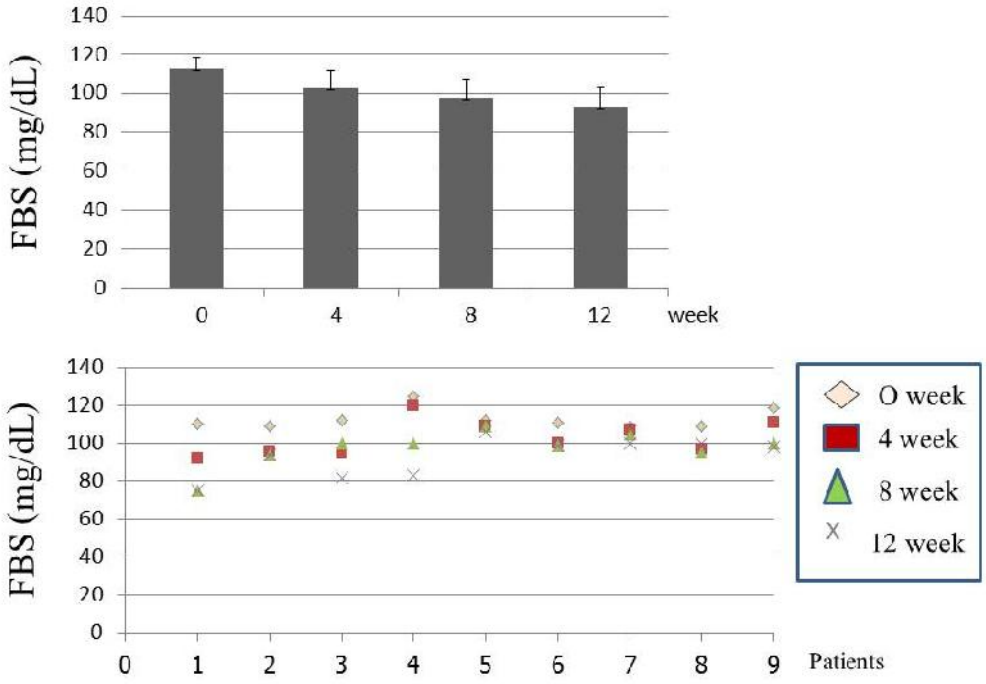

Figure A11 Effects of Freeze Dried Mulberry on FBS in Pre Diabetes Patients

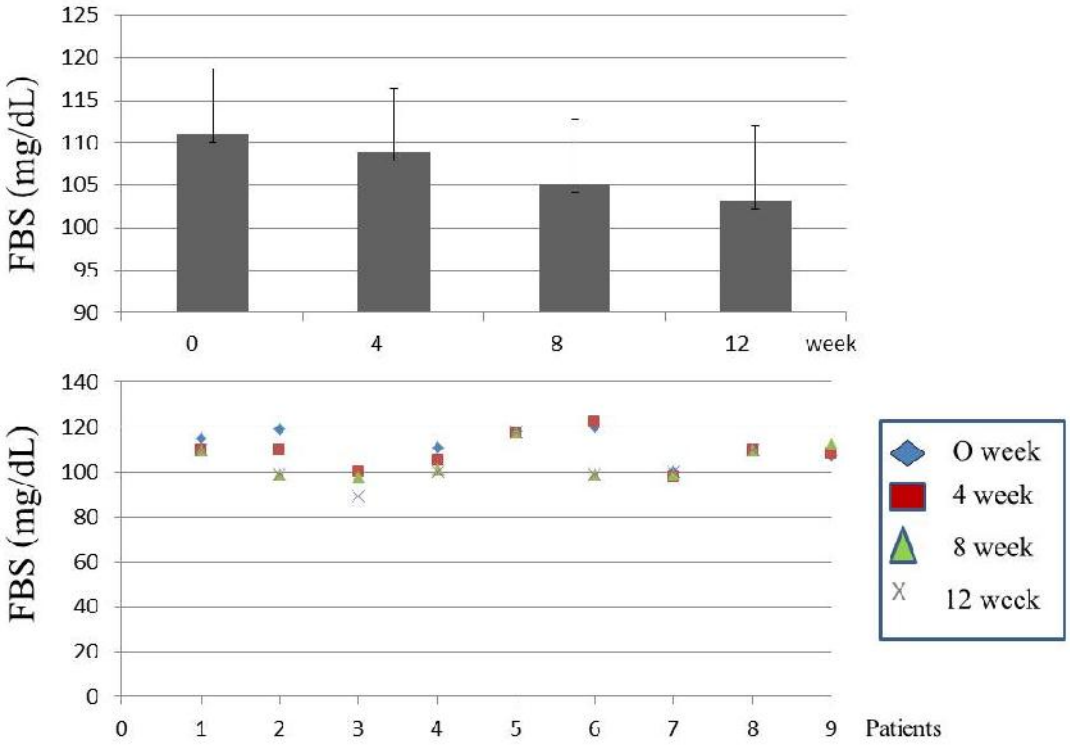

Figure A12 Effects of Gelatin on FBS in Pre Diabetes Patients

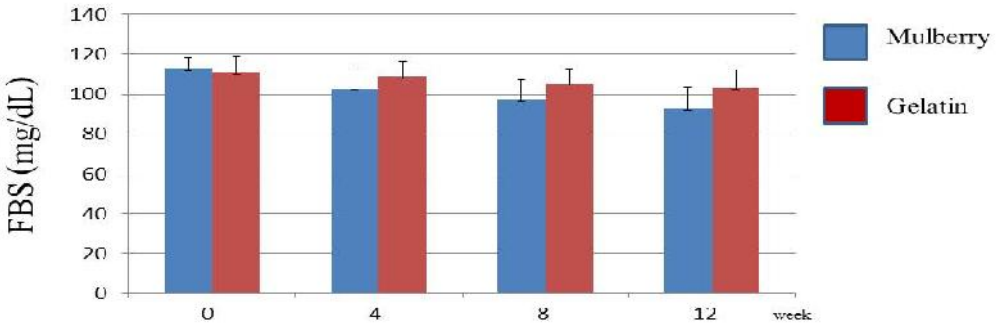

Figure A13 Compared Effects Between Freeze Dried Mulberry and Gelatin on FBS in Pre Diabetes Patients

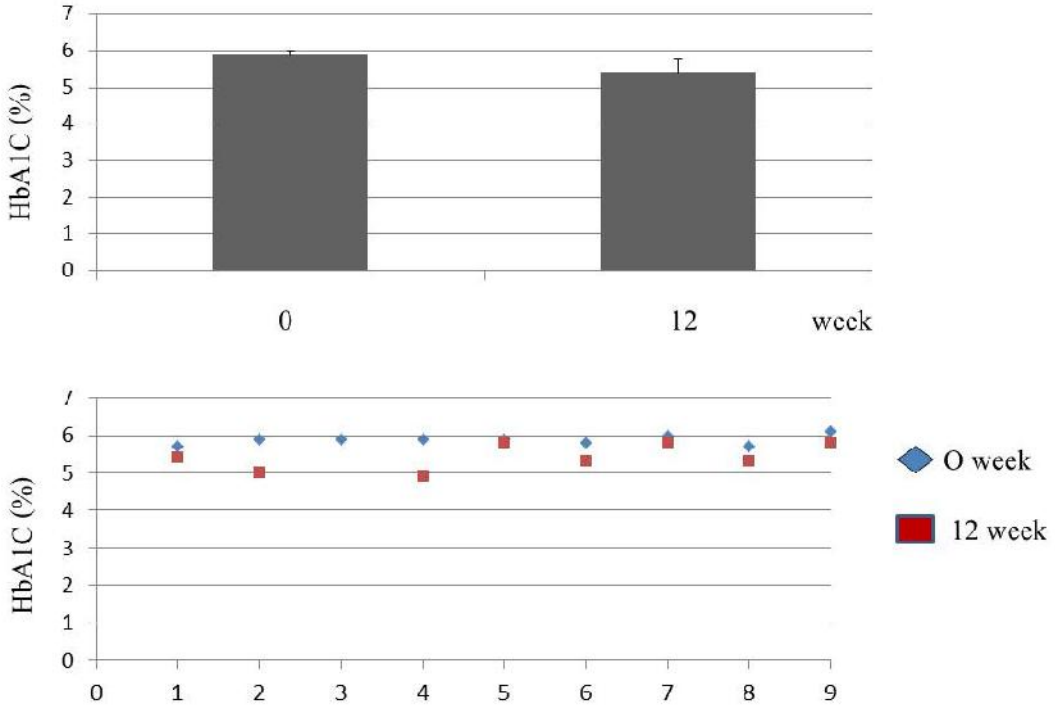

Figure A14 Effects of Freeze Dried Mulberry on HbA1C in Pre Diabetes Patients

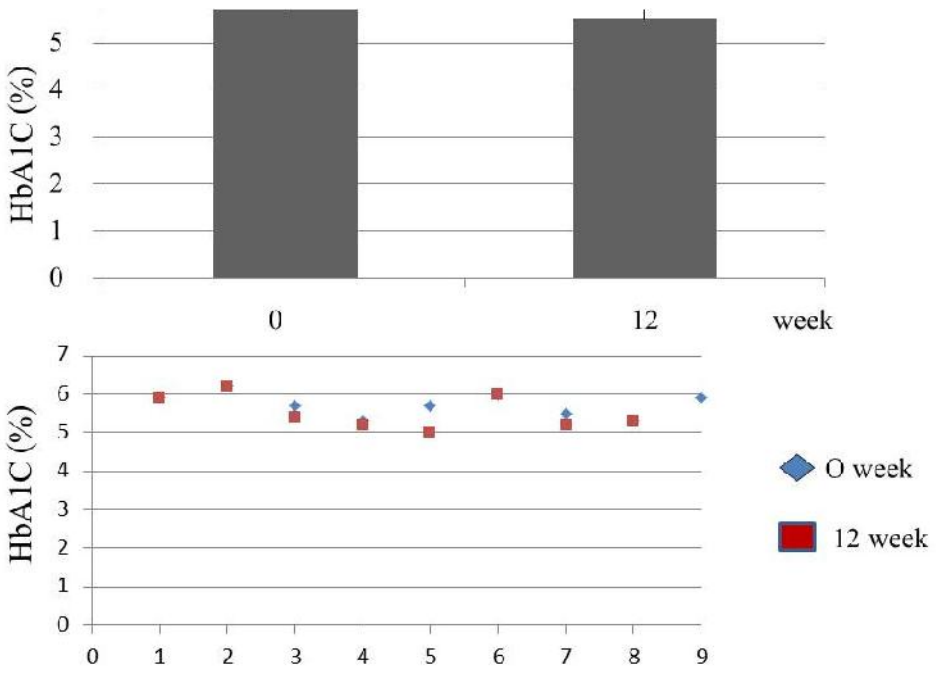

Figure A15 Effects of Gelatin on HbA1C in Pre Diabetes Patients

APPENDIX B

TABLES

Table B1 Identification of ANCs in Mulberry Fruit

Compound number.*	Retention time (min)	MS, M+ (m/z)	MS/MS (m/z)	Assignment**
1	31.3	449.1	287.0	Cyanidin 3-glucoside
2	33.0	595.2	449.1	Cyanidin 3-rutinoside
			287.0	
3	36.4	433.1	271.0	Pelargonidin 3-glucoside
4	38.0	579.1	433.1	Pelargonidin 3-rutinoside
			271.0	

Note. * Diode array detection at 350 nm;

** Based on the fragmentation pattern and its aglycone. The assay was performed in triplicate.

Table B2 Change in body Weight in Each Experimental

	0 weeks	2 weeks	4 weeks	5 weeks
Body weight (g)				
Lean rats				
+1% CMC	124 ± 6	152 ± 2	226 ± 6	277 ± 10
+125 ANCs	121 ± 13	153 ± 6	226 ± 4	288 ± 15
+250 ANCs	118 ± 5	149 ± 7	211 ± 11	270 ± 13
ZDF rats				
+1% CMC	143 ± 2	182 ± 4	256 ± 30	324 ± 24
+125 ANCs	139 ± 3	187 ± 6	257 ± 9	317 ± 34
+250 ANCs	140 ± 6	188 ± 6	273 ± 15	331 ± 7

Note. ZDF, Zucker diabetic fatty rats; +1%CMC, rats treated with 1% carboxymethylcellulose; +125 ANCs, rats treated with 125 mg anthocyanin/kg body weight; +250 ANCs, rats treated with 250 mg anthocyanin/kg body weight.

Table B3 Characteristics of the Study Population

Variable	Mulberry group (n=9)	Gelatin group (n=9)
Gender		
Men (%)	66.67 (n=6)	77.78 (n=7)
Women (%)	33.33 (n=3)	22.22 (n=2)
Age (year)	40.11 $\pm$ 10.3(n=9)	42.0 $\pm$ 13.2 (n=9)
Height (m)	1.62 $\pm$ 0.1 (n=9)	1.69 $\pm$ 0.1 (n=9)
Weight (kg)	59.2 $\pm$ 8.67	65.1 $\pm$ 4.5
BMI (kg m^{-2})	22.4 + 1.57	22.9 + 2.08

Note. Data are means $\pm$ SD.

Table B4 Variable of Glucose, HbA1C, and BMI at Baseline and After the Intervention Period

Variable	Mulberry group (n=9)	Gelatin group (n=9)
Fasting blood glucose at baseline (mg/dl)	112.8 ± 5.5	111.1 ± 7.6
Fasting blood glucose postintervention (mg/dl)	93.0 ± 10.45 ✞	104.2 ± 9.1
Differences ✞ of fasting blood glucose (mg/dl)	19.89 ± 4.94	-6.9 ± 1.4
HbA1C at baseline (%)	5.88 ± 0.13	5.72 ± 0.31
HbA1C postintervention (%)	5.5 ± 0.37 ✞	5.57 ± 0.44
Differences of HbA1C (%)	0.41 ± 0.24	0.15 ± 0.13
BMI at baseline (kg m^{-2})	22.86 ± 1.5	23.01 ± 2.23
BMI postintervention (kg m^{-2})	22.65 ± 1.56	22.92 ± 2.05
Differences of BMI (kg m^{-2})	0.2 ± 0.05	0.1 ± 0.17
Body weight at baseline (kg)	61.08 ± 8.74	65.2 ±4.3
Body weight postintervention	60.47 ± 9.1	65.05 ± 5.04
(kg) Differences of body weight (kg)	0.61 ± 0.36	0.15 ± 0.74

Note. Data are means ± SD.

✞ Significantly different from baseline ($P < 0.005$).

✞ Differences between pre- and postintervention values.

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