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2013

Insulin Secretagogue Activity of Ellagic Acid-Rich Muscadine (Vitis Rotundifolia) and Indian Gooseberry (Emblica Officinalis) Extracts on Pancreatic Beta Cells

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INSULIN SECRETAGOGUE ACTIVITY OF ELLAGIC ACID-RICH
MUSCADINE (*VITIS ROTUNDIFOLIA*) AND INDIAN GOOSEBERRY
(*EMBLICA OFFICINALIS*) EXTRACTS ON PANCREATIC BETA CELLS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Food Science

by
Srikanth Earpina
B.Tech., Acharya N G Ranga Agricultural University, 2009
December 2013

I dedicate this work to my
beloved Parents, Mr. Satyanarayana Earpina and Mrs. Venkata Ramana Earpina,
my Guru Sri Brahmasri Chaganti Koteswararao garu,
and
the Almighty

TABLE OF CONTENTS

ABSTRACT

Diabetes is an inflammatory disease associated with hyperglycemia. Chronic exposure of pancreatic β-cells to glucolipotoxicity stimulates a low-grade inflammation associated with the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β). Increased levels of IL-1β can initially lead to decreased insulin secretion and finally β-cell death.

Ellagic acid and quercetin have been reported to be anti-inflammatory in several studies. *Vitis rotundifolia* (muscadine) or *Emblica officinalis* (amla) are good sources of ellagic acid and quercetin. Ellagic acid or quercetin is bioavailable as is or are metabolized into bioavailable urolithins or isorhamnetin, respectively. The objective of this study was to evaluate the effect of ellagic acid, urolithin A, isorhamnetin, muscadine or amla extracts standardized to their ellagic acid content on glucose-, palmitic acid-, or glucose + palmitic acid-induced IL-1β and insulin secretion.

Acid-hydrolyzed and neutralized extracts of muscadine or amla were prepared and the ellagic acid content in the extracts was measured by HPLC. The ellagic acid content in muscadine and amla extracts was 9.4 ± 2.3 mg/g and 19.4 ± 1.5 mg/g, respectively. NIT-1 pancreatic β-cells were incubated with 33.3 mM glucose, 250 μM palmitic acid or 33.3 mM glucose + 250 μM palmitic acid for 24 h followed by addition of 0.01 − 10 μM each of ellagic acid, urolithin A, isorhamnetin, extracts of muscadine or amla standardized to its ellagic acid content followed by additional incubation for 72 h. All incubations were performed at 37°C in a 5% CO_2 humidified incubator. IL-1β and insulin were analyzed in the supernatants by ELISA. Glucose, palmitic acid or glucose + palmitic acid up-regulated IL-1β and reduced insulin secretion in NIT-1 cells. Glucose induced more IL-1β secretion than palmitate and reduced insulin secretion than palmitic acid. Ellagic acid, muscadine or amla extracts containing ellagic

acid equivalent dose-dependently inhibited IL-1β secretion. Urolithin A or isorhamnetin did not significantly inhibit IL-1β. Ellagic acid, urolithin A, isorhamnetin, or extracts from muscadine or amla dose-dependently increased insulin secretion. Muscadine or amla extracts standardized to their ellagic acid content showed higher stimulation of insulin secretion and IL-1β down-regulation compared to pure ellagic acid. A tentative explanation for this inhibition is the presence of other bioactive compounds in the muscadine or amla extracts. The results of this study show that ellagic acid, muscadine or amla are effective modulators of glucose-, palmitic acid- or glucose+palmitic acid-induced IL-1β secretion and promoters of insulin secretion in β-cells.

Keywords: NIT-1 cells; inflammation; glucose; palmitic acid; muscadine; amla; ellagic acid; urolithin A; isorhamnetin; IL-1β; insulin

CHAPTER 1
INTRODUCTION

Diabetes is a chronic degenerative disease that develops due to defects in insulin secretion function of pancreatic β-cells resulting from excess intake of nutrients such as carbohydrates or fats (*1-3*). The presence of high levels of glucose or free fatty acids increases free radical production and thereby induce oxidative stress (*4, 5*) that can be translated into a chronic inflammatory process by the release of cytokines (*6, 7*). The combination of glucose toxicity and lipid toxicity is referred to as glucolipotoxicity. Interleukin-1β (IL-1β) is the major proinflammatory cytokine released under glucose, lipid or glucolipotoxicity (*8*). Elevated IL-1β levels lead to an initial faulty insulin secretion (*9, 10*) and ultimately β-cell failure (*11*). Synthetic anti-diabetic drugs or plant foods that can modulate inflammation can help in diabetes management.

The demand for functional foods has been increasing due to the growing public awareness on the potential health benefits of dietary bio-actives (*12*). Phenolic compounds in plant foods have been ascribed with anti-oxidant (*13*), anti-inflammatory (*14*) and anti-diabetic (*15*) potentials. Ellagic acid is a phenolic bioactive compound with high anti-oxidant activity present in plant foods as ellagitannins (*16*). Ellagitannins are hydrolyzed by the intestinal microflora into ellagic acid and further metabolized to urolithins (*17, 18*). Ellagic acid showed hypo-glycemic and insulin stimulating effects upon administration to diabetic rats (*19, 20*). On the other hand, isorhamnetin is a metabolite of quercetin with anti-inflammatory (*21*), hypoglycemic, and anti-oxidant activities (*22*). The inhibition of IL-1β as the possible mechanism behind the potential of ellagic acid, urolithins or isorhamnetin on insulin stimulation needs to be explored.

Muscadine (*Vitis rotundifolia*) is a native fruit of the southern part of the United States. It is a good source of ellagic acid and quercetin (*23-25*). The anti-diabetic activity of muscadine has been demonstrated in an in vivo study (*26*). Treatment with muscadine extracts inhibited diabetes associated complications such as advanced glycation end products (*27, 28*) and enzymes related to carbohydrate and lipid metabolism (*29*). Amla (*Emblica officinalis*) is a fruit native to India and has anti-inflammatory and anti-oxidant properties (*30, 31*). Amla is a good source of ellagic acid and quercetin (*32, 33*). In vivo studies in diabetic rats (*34*) or human subjects (*35-38*) showed that amla exhibited a strong anti-diabetic activity by lowering blood glucose levels. The studies, however, did not examine the effect of amla on inflammatory cytokines as a potential mechanism involved in lowering the blood glucose.

The objective of the present study was to investigate the effect of ellagic acid, urolithin A, isorhamnetin, or muscadine or amla standardized to their ellagic acid content on glucose-, palmitic acid- or glucose + palmitic acid-induced IL-1β and insulin secretion. To achieve this objective, the following steps were followed:

1. Determine the ellagic acid content in muscadine and amla extracts

2. Determine the effect of ellagic acid, urolithin A, isorhamnetin, or muscadine or amla extracts on cell viability of glucose-, palmitate- or glucose+palmitic acid-treated NIT-1 cells

3. Investigate the effect of ellagic acid, urolithin A, isorhamnetin, or muscadine or amla extracts on the glucose-, palmitate- or glucose+palmitic acid-induced IL-1β in NIT-1 cells

4. Study the effect of ellagic acid, urolithin A, isorhamnetin, or muscadine or amla extracts on the insulin secretion in NIT-1 cells exposed to glucose, palmitate or glucose + palmitic acid.

CHAPTER 2
LITERATURE REVIEW

2.1. Diabetes

"Diabetes is a group of diseases marked by high levels of blood glucose resulting from defects in insulin secretion, insulin action or both" (*39*). It is anticipated that the number of diabetics in the world will increase 1.5-fold from 371 millions in 2012 to 552 millions by 2030 and the USA has one of the highest death rates (183,633/year) from diabetes in the world (*40*). According to the National Diabetes Fact Sheet from CDC, 8.3% (25.8 millions) of Americans are diabetics. Among them, 10.9 millions are those aged above 65 years, 14.7 millions are those aged between 20 and 65 and 215,000 people aged less than 20 years had diabetes in 2010 (*39*). The estimated costs associated with diabetes in the US have risen to $245 billion in 2012 from $174 billion in 2007 (*41*). The reports stress the necessity to develop a strategic therapeutic plan associated with dietary management to attenuate the disease and restore insulin activity.

2.1.1. Types of Diabetes

There are basically two types of diabetes, Type 1 diabetes (T1D) and Type 2 diabetes (T2D). T1D is an autoimmune mediated process, which results in the destruction of pancreatic β-cells thereby impairing insulin secretion and leading to hyperglycemia. Exogenous insulin injection is required to control the metabolic disorder (*42*). T2D is lifestyle-related, developing as a result of excess pressure on β-cells to maintain glucose homeostasis generated by increased energy consumption. The excess calories from carbohydrates induce a hyperinsulinemic condition which leads to chronic insulin resistance. Dietary balance in combination with physical activity and insulin therapy or insulin secretagogue administration can improve the condition (*43, 44*).

Normal pancreatic tissues contain nearly one million islets (1-2% of total pancreas) and approximately one billion β-cells (50% of islets), of which at least 40% of the β-cell mass is

4

required for the maintenance of glucose homeostasis (*45, 46*). Under normal healthy conditions and prediabetic state, the β-cell mass is maintained through the self-replication of β-cells and regeneration from other pancreatic cells. The increased mass helps the β-cells to maintain stable glucose levels and compensate for insulin resistance (*47*). The stress of continuous hyperglycemic load eventually overwhelms the pancreas, leads to the failure of β-cell functionality and regeneration. The initial hyperinsulinemic conditions ultimately causes other tissues to become insulin resistant which leads to clinical diabetes (*45, 48*).

Diabetes is associated with 40% - 60% decrease in islet volume (*49-51*), reduced β-cell area by 24% (*52*), and 30% lower concentration of pancreatic insulin (*50*) due to loss in β-cells compared to non-diabetic condition. The defective mitochondria in the β-cells in diabetic condition lead to decrease in the cellular ATP/ADP ratio to ≤ 40%. (*53*).

2.2. Risk factors for diabetes
Genetics is a non-modifiable risk factor for diabetes. Identification of candidate genes has been postulated as a reliable method to identify genes susceptible to development of the disease. However, it is challenging to segregate a group of common genes as they vary depending on ethnic groups, exposure to environment and gene-environment interactions (*54*). Life style factors such as excessive consumption of high levels of saturated fats, sugar-sweetened beverages and starchy foods, low fiber diets and sedentary lifestyle contribute to the development of diabetes (*55*).

2.2.1. Hyperglycemia, free fatty acids and type 2 diabetes
The duration of exposure of β-cells to high concentrations of nutrients such as glucose or fatty acids will determine the effect of the later on the β-cells. Acute exposure to high concentration of nutrients stimulates insulin secretion whereas chronic exposure impairs β-cell function and affects β-cell survival as observed in type 2 diabetic patients (*56*). Type 2 diabetes

is characterized by a failure to increase β-cell mass that can compensate for the increased demand for insulin (*51, 57*). The combination of glucose toxicity and lipid toxicity is referred to as glucolipotoxicity.

Pancreatic β-cells are highly responsive to stimuli from glucose in serum. Increase in glucose levels can lead to β-cell hyperactivity to maintain glucose homeostasis. (*58*). Chronic hyperactivity pressurizes β-cells to adapt to provide sufficient insulin. This process increases the demand for production of more amount of adenosine tri phosphate (ATP) through glycolytic and mitochondrial activity. Hyperactivity of mitochondria increases ROS production. Additionally, the inherent low levels of key anti-oxidant proteins in pancreatic islets make β-cells highly susceptible to metabolic stress (*59*).

Obesity and hyperlipidemia are the primary environmental risk factors leading to diabetes. Dietary fat and adipose tissue are the source of free fatty acids in plasma. Increased adipose tissue mass under obese conditions increases the concentration of free fatty acids that serves as the major risk factor associated with hyperlipidemia in diabetes (*1-3, 60*). Chronic exposure of β-cells to free fatty acids has shown impaired glucose-stimulated insulin secretion and insulin synthesis (*61*). Moreover, free fatty acids contribute to insulin resistance and also prevent the β-cell response to overcome insulin resistance by secreting more insulin (*62*). The combination of insulin resistance and β-cell dysfunction leads to inability to control blood glucose levels and as a result diabetes develops (*63*). High levels of free fatty acids (FFA) resulting from hyperlipidemia also cause an influx of immune cells such as macrophages and T-cells in the pancreas. Due to this, a low-grade inflammatory process develops through the production of inflammatory cytokines such as IL-1β which otherwise is usually transient or milder in individuals that are not at risk of developing type 2 diabetes (*64*).

Long-term exposure to saturated free fatty acids has deleterious effects on β-cells. Cultured human islets exposed to 500 µM palmitic acid for 4 days resulted in increased levels of β-cell apoptosis markers, decreased β-cell proliferation and suppression of insulin secretion (*65*). Rat pancreatic islets exposed to 250 µM palmitic acid for 48 h displayed type 2 diabetic characteristics such as decreased insulin synthesis and impaired glucose stimulated insulin secretion in β-cells (*66*).

2.3. Oxidative stress and β-cells

Imbalance between cellular anti-oxidant levels and superfluous generation of reactive oxygen species (ROS) is defined as oxidative stress. The generation of ROS takes place either due to increased demand for ATP leading to incomplete oxygen reduction or by controlled reduction yielding the highly reactive superoxides, hydroxyl radicals and hydrogen peroxide, most of which are degraded by anti-oxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxin (*67, 68*). Free radicals induce chronic degenerative diseases by damaging components such as lipids, proteins and nucleic acids (*69*).

Glucose serves as the source of energy for β-cell, signals the production of insulin within the cell, and provides the signal for insulin secretion (*70*). β-cells maintain glucose homeostasis by the conversion of stimuli from glucose to insulin secretion (*71*). During hyperglycemic conditions, increased glucose influx provides the substrate for the increase of mitochondrial respiratory activity and subsequent reduction of molecular oxygen to free radicals yielding superoxide anions (*72, 73*).

Insulin secretion process is highly susceptible to the ROS. Diets rich in carbohydrates or fatty acids can lead to hyperactivity of respiratory metabolic process and generate ROS (*4*). Even a fleeting exposure to oxidative stress due to glucolipotoxicity can cause inactivation of mitochondria and interception of signals involved in glucose stimulated insulin secretion (*74*).

7

Concurrently, the β-cells are quite frail in their expression of anti-oxidants posing themselves susceptible to fail even to smaller degree of exposure to oxidative stress unlike other organs of the body (*75, 76*).

2.4. Inflammation and β-cells

Inflammation is a response to an injury of a tissue. It is identified by the invasion of immune cells, release of cytokines and damage to tissue structurally and even functionally in a few cases. The positive effects of inflammation include prevention of infection and regeneration of affected tissue. Persistence of inflammation over a long period can induce excess production of cytokines and aggravate the disease (*77*). Exposure of pancreatic β-cells to hyperglycemia or hyperlipidemia and subsequent oxidative stress can initially stimulate an inflammatory process that helps for the β-cell repair and regeneration. Uncontrolled levels of high glucose or free fatty acids can cause chronic inflammation which involves infiltration of immune cells, release of cytokines and eventually result in apoptosis followed by fibrosis (*6*). The slow development of the inflammatory process and the rapid clearance of the dead cell debris by the immune cells offers resistance to rapid detection of inflammation in vivo (*78*). Among the group of inflammatory cytokines that affect β-cell functionality, IL-1β is the most prominent one (*8*).

2.4.1. Interleukin-1β (IL-1β) and β-cells

Cytokines are products and effectors of inflammatory process that cause oxidative stress and cell dysfunction (*79, 80*). IL-1β is the major proinflammatory cytokine produced under stress conditions of high concentrations of glucose or free fatty acids. The cytokine can enhance its production by auto stimulation (*8*). Endogenous IL-1β released by β-cells can attract macrophages that can contribute to the production of additional amount of IL-1β. Increase in IL-1β concentration ultimately results in faulty insulin secretory process and β-cell failure.

Furthermore, it can also induce the expression of other cytotoxic factors that lead to β-cell destruction (*9, 81*).

IL-1β mediated inflammatory process can be examined by an initial oxidative stress followed by down regulation of insulin signal transduction. This effect can be worse in the presence of other cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (*10*). Exposure to the cytokines, TNF-α and IL-6 released by adipocytes due to inflammation can impair β-cell functionality (*82, 83*).

IL-1β and TNF-α released from infiltrating immune cells in diabetic conditions can cause significant destruction of β-cells (*84*). IL-1β toxicity is amplified in the presence of TNF-α and both IL-1β and TNF-α can induce the activation of apoptotic pathways (*85*). Pancreatic β-cells cultured with recombinant TNF-α exhibited impaired insulin action. (*86*). However the negative effect of TNF-α was neutralized when the cells were treated with salicylates.

High glucose or free fatty acids cleave pro IL-1β to generate IL-1β (*73, 87-93*). Caspase-1 activation by saturated free fatty acids and resulting cleaving of pro IL-1β occurs through mitochondrial ROS production in NLRP3 dependent manner (*94*). The generation of IL-1β by high glucose or free fatty acids is illustrated in Figure 1.

The first step of IL-1β activation involves the accumulation of electrons resulting from catabolism of excess glucose or fatty acids on the electron carriers such as nictotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide ($FADH_2$). The electrons are then transported to the oxygen molecules involved in mitochondrial respiratory chain. Free radicals are produced through the single electron reduction of oxygen molecule leading to ROS that cause oxidative stress (*73, 87*).

9

Thioredoxin plays an important role in cell proliferation and prevention of apoptosis. In normal cells, thioredoxin binds the thioredoxin interacting protein (TXNIP) thereby masking its availability for interaction with nucleotide binding domain and leucine rich repeat containing protein 3 (NLRP3), an aggregation of proteins that mediates the autocatalytic activation of cysteine protease, caspase-1 (*88*). However, ROS produced by oxidative stress sets the TXNIP free enabling its binding to NLRP3 (*89*). Moreover, a 10-fold increase in TXNIP protein levels has been related to an equivalent rise in the cleavage of the apoptotic protein, caspase-3. This effect of TXNIP also signifies its involvement in β-cell dysfunction (*90*).

The activation of the NLRP3 inflammasome protein complex requires the efflux of K^+ ions from the cell. During hyperactivity of β-cell, extracellular ATP levels increase and lead to activation of the P2X7 receptor. Activated P2X7 receptor paves the way for efflux of K^+ ions from the cell which can be observed by the drop in K^+ ions in β-cell to 70 mM during stress conditions compared to the concentration around 140 mM under normal conditions (*91*).

Another protein involved in this pathway is the apoptosis-associated speck-like protein (ASC). The shift in cytoplasmic K^+ ions result in the interaction of the NLRP3 and ASC followed by the interaction of ASC and procaspase 1 through their caspase recruiting domains (CARDs). It results in the dimerization of procaspase 1 to active caspase 1 and the cleavage of inactive pro IL-1β is facilitated by active caspase 1. The IL-1β is then released by the β-cell which is mediated by an influx of Ca^{+2} ions (*92, 93*).

The IL-1β released into extracellular matrix can re-enter the cell and induce the activation of pro IL-1β by an auto stimulation process. This uncontrolled production of IL-1β leads to a greater degree of inflammation and a vicious cycle of inflammatory process (*95*).

```mermaid
flowchart TB
    A[High glucose / FFA] --> B[Reactive oxygen species]
    C[Extracellular ATP] --> D[Activation of P2X7 receptor]
```

High glucose / FFA

↓

Reactive oxygen species

⊣ **Thioredoxin**

↓

Thioredoxin interacting protein (TXNIP)

↓

Nucleotide binding domain and leucine rich repeat containing protein 3 (NLRP3)

Extracellular ATP

↓

Activation of P2X7 receptor

↓

Efflux of K$^+$ ions

↓

Dimerization of ASC and caspase 1

↓

Active caspase 1

↓

Cleavage of pro IL-1β to active IL-1β

Auto stimulation

Influx of Ca^{+2} ions

↓

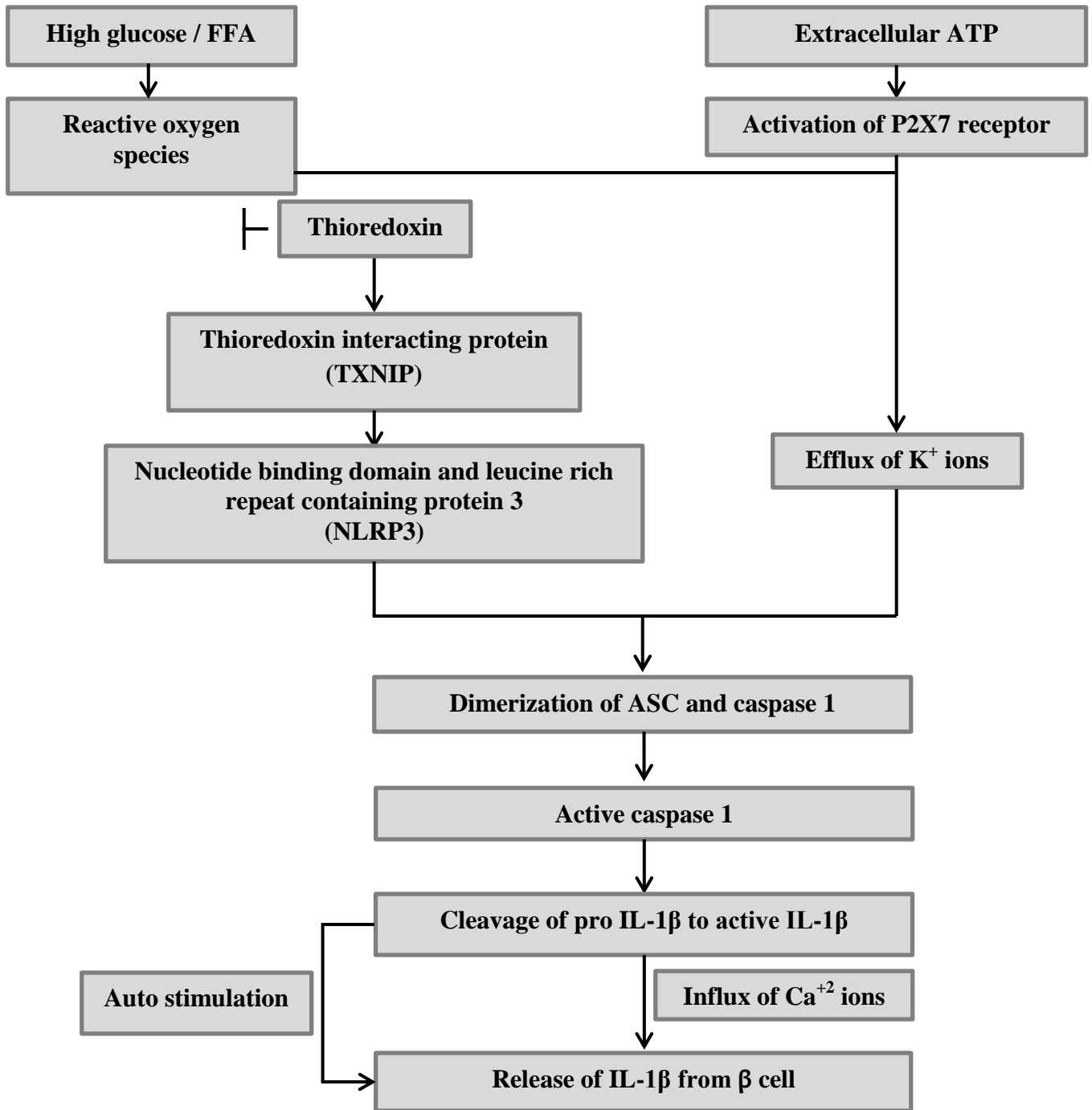**Release of IL-1β from β cell**

Figure 1. Activation and release of IL-1β by glucose or free fatty acids (*73, 87-93*).

Thereafter, the released IL-1β destabilizes the insulin production activity of β-cell and prompts the activation of NF-κB and its nuclear translocation resulting in DNA fragmentation. Such phenomenon was observed in the pancreatic sections of type 2 diabetic patients due to the

IL-1β released by β-cells. Moreover, induction of nuclear translocation of NF-κB by IL-1β on its own or by combining with IFN-γ has also been reported (*11, 96*).

Keeping the above information in view, the deleterious effects of inflammatory cytokines, oxidative stress and IL-1β in particular, therapies aimed at suppression of these cytokines may be beneficial in conserving β-cell functionality.

2.5. Diabetes Management
2.5.1. Therapeutic approach
Diabetes has no cure. Several short and long term therapies aimed at preserving β-cell function and improvement of insulin secretion are currently in use. These include insulin injections and oral anti-diabetic agents such as sulfonylurea, biguanides and alpha-glucosidase inhibitors. Diabetic conditions have been reported to be restored upon usage of treatments over a short term. Besides, such treatments have been observed to be detrimental to the β-cell mass (*97*). Elevated cytoplasmic Ca^{+2} levels can cause excitotoxicity of β-cells and depolarize them by direct closure of K_{ATP} channels upon long term treatment with drugs such as sulphonylurea. This can lead to abnormal activity of β-cells (*98*).

On the other hand, long term treatments proved to be effective by exerting stimulating effects on β-cell proliferation. However, the associated side effects such as hypoglycemia and weight gain due to sulfonylureas, gut problems, liver toxicity due to thiazolidinediones and skin rashes due to insulin injection in some cases and cost are the limiting factors (*99-103*). Immunosuppression therapy by the usage of pancreatic islet cell implantation increases the metabolic stress on β-cells and subsequently drains their ability to produce insulin. These effects are the limiting factors in using islet implantation as an absolute cure for diabetes (*104*). Considering the above factors, dietary approach can be a promising alternative to mitigate the deleterious effects of inflammation in diabetes.

2.5.2. Dietary approach

The role of diet in diabetes management is quite significant. For decades, traditional medicines from plants formed the main sources of dietary management of diabetes before the introduction of therapeutic treatment with insulin (*105*). Consumption of fruits and vegetables has been associated with decreased occurrence of chronic oxidative stress-related diseases such as diabetes, cancer and cardiovascular diseases, besides an enhanced cellular anti-oxidant defense (*106-108*). Plant foods are good alternatives to synthetic anti-diabetic drugs because the side effects associated with plant food use are negligible (*30, 109*). Phenolics and flavonoids are the focus of anti-diabetic dietary compounds. This research is focused on muscadine and amla as sources of dietary bioactives for type 2 diabetes management.

2.5.3. Muscadine or amla bioactives and diabetes

2.5.3.1. Muscadine *Vitis rotundifolia* is a grape that is native to southeastern region of the United States. Muscadine has been cultivated since the 16th century and is considered as a southern specialty product. It grows over a wide range extending from Delaware to the Gulf of Mexico and towards west from Missouri to Texas (*110*). The highly resistant nature of muscadine to Pierce's disease and thriving ability in soil and climatic conditions unfavorable for other grape varieties places it in a unique position in contributing to the economy of southeastern states (*23*).

Muscadine skins are rich in phenolics such as ellagic acid, myricetin, quercetin and kaempferol whereas gallic acid, catechin and epicatechin are the major phenolics in seeds (*23, 111*). Yi et al. identified ellagic acid, quercetin, myricetin and kaempferol in muscadine grapes (*24*) and the presence of those compounds were also reported by Wang et al. in muscadine pomace extracts (*69*). The concentration of ellagic acid in various products of muscadine has been studied to range between 36-91 mg/100 g fresh weight in fruits (*25*), 8-84 mg/L in juices

and 2-65 mg/L in wines (*112*). Muscadine grapes are consumed as juices, jams, jellies, or wines (*69, 113*). They are a good source of dietary fiber (*114*). The rich aroma and unique taste are the positive factors, whereas the tough skins are the limiting factor for muscadine to be accepted by the consumer the way other grapes have been accepted.

Greenspan et al. reported that muscadine skin extracts dose-dependently inhibited the release of TNF-α, IL-1β and IL-6, and superoxides stimulated by LPS (lipopolysaccharide) in blood mononuclear cells (*110*). Muscadine pomace extracts showed dose-dependent DPPH free radical scavenging activity (*69*). Investigation on the anti-diabetic efficacy of muscadine juice, wine or dealcoholized wine in type 2 diabetic subjects showed that consumption of dealcoholized muscadine grape wine for 28 days altered blood insulin levels in type 2 diabetic subjects and the fasting blood glucose: insulin ratio increased from 8.5 to 13.1 (*26*). A low glucose: insulin ratio of less than 7 is predictive of insulin resistance and dealcoholized wine consumption has improved the ratio indicating better response to insulin. Consumption of wine or juice did not show any effect on fasting blood glucose, insulin, or glycated hemoglobin levels, whereas the triacylglycerols and low density lipoprotein levels have been reduced.

Hyperglycemia increases protein glycation and formation of advanced glycation end products (AGEs). Dicarbonyls such as glyoxal and methylglyoxal react with proteins to form AGEs that have been reported to be a causative factor for diabetic complications (*115*). Increased free radicals production, altered gene expression and release of proinflammatory cytokines are the diabetic complications promoted by AGEs. Moreover, the concentration of methyglyoxal in diabetic patients was found to be five times higher than in healthy ones (*116*). Wang et al. investigated AGEs inhibition and methylglyoxal scavenging activities of muscadine extracts (*27*). AGEs generation was inhibited by approximately 53% and methylglyoxal by 46% thereby

suggesting the beneficial effects of muscadine in preventing AGEs associated chronic diseases such as diabetes. The suppressive effect of ethanolic extracts of muscadine skins on AGEs formation has been reported (*28*). Muscadine skin extracts inhibited the AGEs formation by 40%, but did not affect methyglyoxal-mediated glycation of albumin. You et al. reported the potential inhibitory effect of muscadine extracts on carbohydrate hydrolyzing and lipid metabolizing enzymes that are linked to play a role in diabetes. (*29*). The study examined the inhibition of α-glucosidase and pancreatic lipase inhibition by phenolics in muscadine extracts. Among the phenolics of muscadine extracts tested, ellagic acid and quercetin were reported to be the most potent enzyme inhibitors.

2.5.3.2. Amla (Indian gooseberry) *Emblica officinalis*, commonly called amla or Indian gooseberry, belongs to the *Euphorbeaceae* family. The edible fruit is generally consumed in raw, cooked or pickled forms. In the traditional Indian medicine or Ayurveda, asthma, diarrhea, rheumatic pains can be treated with amla (*117, 118*). The anti-inflammatory, anti-oxidant and radical scavenging activity of amla has been observed in studies on animal models (*30, 31*).

Amla is a good source of gallic acid (*32*), ellagic acid (*33*), quercetin (*33*) and quercetin-3-O-β-D-glucoside (*119*). Oral administration of commercial enzymatic or polyphenol rich extracts of amla to diabetic rats dose-dependently reduced the expression of creatinine and 5-hydroxy-methyfurfural that are indicators of oxidative stress (*120*). Diabetic rats fed with amla juice for 8 weeks had higher plasma levels of anti-oxidant enzymes, superoxide dismutase and catalase than rats that were not fed with amla juice. Amla juice administration also attenuated hyperglycemia and hypercholesterolemia in the diabetic rats (*34*).

Amla has been used to treat type 2 diabetes patients. Consumption of amla juice by diabetic patients for 3 months along with other plant extracts showed reduced fasting blood

glucose levels (*35*). Diabetic subjects given with 500 mg tablets consisting of 25% amla for 3 months showed reduced fasting blood sugar levels (*36*). Consumption of medium sized amla fruit (35 g) for 2 months induced hypoglycemia (*37*). Oral consumption of amla powder for 21 days dose-dependently decreased blood glucose and cholesterol levels and improved the levels of high-density lipoprotein cholesterol in type 2 diabetes patients (*38*).

The above reported studies support amla as a promising dietary source for attenuating diabetic condition. The mechanism by which amla improves diabetic condition and the bioactive compounds responsible for such an activity, need to be investigated at the molecular level.

2.5.3.3. Ellagic acid and urolithins Ellagic acid exists in food as hydrolysable tannins of the non-flavonoid polyphenol group known as ellagitannins, which are a combination of hexahydroxydiphenic acid and glucose. Hydrolysis of ellagitannins sets the acid group free, which thereafter undergoes spontaneous cyclation and through the formation of internal lactone bridges, yields ellagic acid. The in vitro high anti-oxidant activity exhibited by ellagitannins is attributed to the phenolic hydroxyls groups in the ortho position of their structure (*16*).
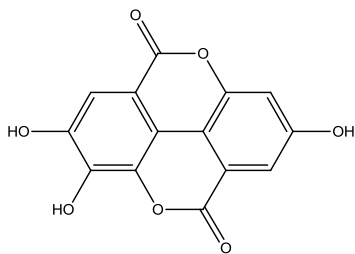

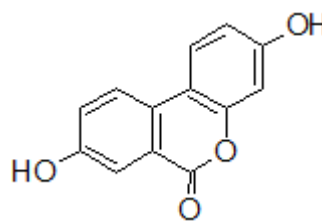Figure 2. Ellagic acid structure.   Figure 3. Urolithin A.

Studies on the physiological changes of ellagic acid from different portions of intestine had confirmed its release from ellagitannins in the small intestine and the microbial transformation to urolithins in jejunum, where they are absorbed. Ellagic acid metabolize into

16

tetra, tri, di and monohydroxy-dibenzopyrazone derivatives through the loss of lactone rings of ellagic acid and successive removal of hydroxyls, forming the di and mono forms usually referred to as urolithin A and B, respectively (*16, 17, 121*).

The rate of conversion of ellagic acid to its metabolites depends on the composition of gut microorganisms and can differ based on an individual's microbial profile (*18*). Cerda et al. reported that concentrations of ellagic acid can reach up to 100 µM in the intestine. Despite such higher concentrations of ellagic acid in the gastrointestinal tract, most of the ellagic acid is converted to metabolites. The plasma concentration of ellagic acid metabolites including urolithin A and B in plasma can reach up to 5 µM (*121*).

Ellagic acid is bioavailable at micromolar concentrations. Consumption of 180 ml pomegranate juice containing 25mg ellagic acid by a human subject showed that ellagic acid concentration in plasma was 31.9 ng/ml (0.1 µM) after 1h of consumption and eliminated after 4 h. Low bioavailabiltiy of ellagic acid was attributed to its poor water solubility and transformation by intestinal bacteria to metabolites (*122-124*). Twenty healthy volunteers were fed with 40 mg ellagic acid capsules. Plasma concentration of ellagic acid was 30-50 ng/ml (0.1-0.2 µM) after 15 min and 200 ng/ml (0.7 µM) after 1h. Mean serum elimination half-life was around 8.4h (*125*).

The maximum plasma level of urolithin A was reported to range between 14 - 25 µM after 6 – 8h and varied among volunteers confirming the metabolism of ellagic acid into urolithin A and B (*126*). The anti-diabetic activity of ellagic acid in vivo has been reported in diabetic rats. Ellagic acid administration for 35 days restored the plasma insulin that was suppressed by streptozotocin and decreased the streptozotocin-induced increase of plasma glucose, blood

glycosylated hemoglobin and hexokinase activity (*19*). Oral administration of ellagic acid to streptozotocin-nicotinamide-induced diabetic rats dose-dependently inhibited plasma glucose levels. Maximum reduction of glucose by ellagic acid was at 2 h. Ellagic acid stimulation of β-cell for increased insulin secretion was proposed to be the possible mechanism behind the hypoglycemic effect of ellagic acid (*127*).

The bioavailability and in vivo anti-diabetic activity of ellagic acid has been reported in earlier studies. However, investigation on the attenuation of diabetes development by ellagic acid in vitro can help to elucidate the mechanism involved in its anti-diabetic activity.

2.5.3.4. Isorhamnetin Isorhamnetin (3, 4', 5, 7-tetrahydroxy-3'-methoxyflavone) is an active flavonol aglycone and metabolite of quercetin. Onions, apples, tea or red wine are among the best sources of quercetin (*128*). Oral administration of the isorhamnetin diglucoside from mustard leaf to diabetic rats lowered blood glucose and 5-hydroxymethylfurfural expressing anti-diabetic and anti-oxidant activities. (*22*). However, intraperitonal administration did not show any such inhibition. It has been hypothesized that the observed inhibitory activity of isorhamnetin diglucoside might have been due to the aglycone form, isorhamnetin formed during metabolic conversion in the gut. Intraperitoneal administration of isorhamnetin exhibited similar effects as observed by oral administration of isorhamnetin diglucoside.
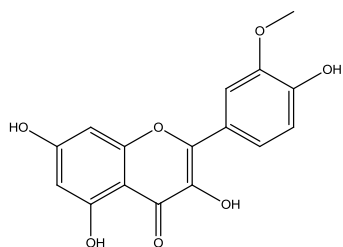

Figure 4. Isorhamnetin structure.

The concentration of quercetin or its metabolites at 10µM tested against IL-1β-induced nitrite production and insulin secretion suppression in rat pancreatic cells showed that quercetin was effective but quercetin metabolites were not (*129*). Interestingly, all the metabolites considered were glucosides including isorhamnetin 3-glucuronide, suggesting that reduced efficacy was associated with glucoronidation (*22*). Aglycone forms of quercetin metabolites have anti-inflammatory and anti-oxidant properties. Lipopolysaccharide-stimulated IL-1β, IL-6 and TNF-α mRNA levels along with nitric oxide synthase in macrophages were suppressed by quercetin and isorhamnetin at 10µM level, but quercetin-3-glucuronide did not (*21*). Under physiological conditions, quercetin glycosides in foods are metabolized to sulfates and glucuronides and aglycones of quercetin and isorhamnetin (*130*). Quercetin is bioavailable in its original form or as a metabolite. The maximum attainable plasma level of pure quercetin has been estimated to be 0.1 to 0.4 µM (*131*). Keeping this in view, studies should examine the efficacy of quercetin or its metabolites such as isorhamnetin as diabetic attenuators at concentrations lower than 10 µM.

CHAPTER 3
MATERIALS AND METHODS

3.1. Reagents

HPLC grade methanol and acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Acetonitrile was purchased from J.T Baker (Phillipsburg, NJ) and hydrochloric acid was obtained from VWR (Bridgeport, NJ). Dimethyl sulfoxide (DMSO) was purchased from RESEARCH ORGANICS (Cleveland, OH). Urolithin A (UR) was a gift from Dr. Tomas Barberan, F.A., (CEBAS, Spain). Glucose (Glu), palmitic acid (PA), ellagic acid (EA), isorhamnetin (IS), and bovine serum albumin (BSA) were obtained from SIGMA-ALDRICH (St. Louis, MO). F12k medium for NIT-1 cells was obtained from ATCC (Manassas, VA) and fetal bovine serum (FBS) was purchased from ATLANTA® Biologicals (Lawrencevillie, GA). Sodium bicarbonate and glutamate used for medium preparation were purchased from Life Technologies (Grand Island, NY). Ison variety of muscadine (MS) pomace was obtained from Feliciana Cellars (Jackson, LA) and frozen fruits of amla (AM) were purchased from Deep Foods Inc. (Union, NJ).

3.2. Fruit extracts preparation

MS pomaces or AM fruits were blended, freeze dried and stored at - 20°C until use. Five grams of freeze dried MS or AM was taken and dissolved in 100 ml of 80% methanol + 20% 6N HCl. Acid hydrolysis was carried out in a water bath (Labline orbit microprocessor shaker bath) at 60°C and 200 rpm for 2 h for the conversion of flavonoid glycosides to aglycones. Samples were then sonicated (Branson 2510, Danbury, CT) for 10 min to maximize the extraction (*111*). The extracts were vacuum filtered through a Whatman No.1 filter paper using a Buchner funnel. The solvents were removed in a rotary evaporator (Buchi Rotavapor, New Castle, DE) and neutralized with 1N NaOH. The resultant extracts were freeze dried (Genesis 35 XL lyophilizer, VirTis Co., NY) and stored at -20°C until analysis.

3.3. Determination of ellagic acid content in muscadine or amla extracts

Agilent 1100 series HPLC (Agilent, Santa Carla, CA) equipped with an Agilent Zorbax SB-C18 column (5 µm, 4.6 x 250 mm) connected to a G1315B Diode Array UV Detector was used to estimate the ellagic acid content. Phenolic acids profiles and contents in MS or AM extracts were analyzed using the method of Pastrana et al. with modifications (*111*). One mg of freeze dried extract MS or AM was dissolved in 1ml of 80% methanol + 20% 6N HCl and filtered through a 0.2 µm nylon syringe filter (Fisher, GA) before injecting into HPLC. Three mobile phases were used namely, solvent A, methanol/acetic acid/water (10:2:88, v/v/v); solvent B, acetonitrile; and, solvent C, water. All the solvents were filtered through 0.2 µm filter paper and sonicated (Branson 2510, Danbury, CT) for 10 min prior to use.

Gradient elution was performed as follows: at 0 min, 100% solvent A; at 5 min, 90% solvent A and 10% solvent B; at 25 min, 30% solvent A and 70% solvent B; at 30 min, 100% solvent C; at 35 min, 100% solvent C; at 36 min, 100% solvent A and 4 min postrun with 100% solvent A. Twenty µL of sample was injected, the column temperature was maintained at 40°C and the flow rate was 1mL/min. The detection was done at 280 nm using a diode array detector (DAD) (*113*). The samples were run in triplicates and peaks and concentration of ellagic acid in fruit extracts were confirmed by matching with the retention times obtained by running the calibration curve of ellagic acid standard.

3.4. NIT-1 cell line

This study was conducted using NIT-1, a pancreatic β-cell line developed from non-obese diabetic (NOD) mice, *Mus musculus* that is transgenic for the SV40 T antigen (a proto-oncogen capable of transforming a lot of cell types) under control of the insulin promoter and spontaneously develops beta cell adenomas. These cells showed a low percent (5%) of glucagon, no somatostatin and positive for insulin when stained at passage 18 indicating that they are a

good strain to be employed for in vitro studies (*132*). The cell line was obtained from Dr. Sita Aggarwal (William Hansel Cancer Prevention Laboratory, Pennington Biomedical Research Center, Baton Rouge, LA). The cells were cultured and maintained in F12k medium (ATCC, Manassas, VA) containing 10% FBS, 2% sodium bicarbonate and 1% glutamate.

3.5. Conjugation of palmitic acid with bovine serum albumin (PA-BSA)

The conjugate of PA with BSA was prepared as described previously (*133, 134*). Thirty percent BSA was prepared in DPBS (Dulbecco's Phosphate Buffered Saline) that contained 2.5% HEPES. The pH of the solution was adjusted to 7.2 with 1M KOH and then sterile-filtered through a 0.2 µm syringe filter.

A stock solution of 12.5 mM PA was prepared before conjugating with BSA. Ten mg of cell culture grade PA was aseptically weighed and dissolved in hexane at 9% of final volume of stock. The mixture was vortexed for few seconds and then dried under nitrogen to obtain a white, chalky powder. The salt thus obtained was immediately re-dissolved in warm sterile water at 9% of final volume of stock and combined with 30% BSA at 91% of final stock volume. The resultant conjugate of PA was flushed with argon, aliquoted into sterile vials and stored at -20°C.

3.6. Cell viability assay

NIT-1 cells were seeded at 10,000 cells per well in 96-well plate and incubated for 24 h before treatment. Stocks solutions of EA and IS were prepared in methanol and stock solutions of UR, MS, and AM were prepared in DMSO. Stocks were diluted with medium to obtain concentrations of 100 µM and 1 µM before cell treatment. Glu stock was prepared in the medium and sterile-filtered with 0.2 µm nylon syringe filter (Fisher, GA). Cells were treated with 33.3 mM Glu, 250 µM PA, or a combination of 33.3 mM Glu and 250 µM PA and incubated for 24 h at 37°C with 5% CO_2. Thereafter, 0.01, 0.05, 1 and 10 µM of EA, UR, IS, or MS or AM extracts containing 0.01, 0.05, 1 and 10 µM equivalent of EA were added to the cells

followed by incubation for 72 h. Cell viability was then determined after a total incubation of 96

h using the MTS assay with CellTiter 96 A$_{queous}$ One solution (Promega, Madison, WI) according

to the manufacturer's protocol. Absorbance value of the MTS assays were read in a Bio-Rad

Model 680 micro plate reader (Hercules, CA). All the treatments were performed in triplicates

and the results are presented as percentage of control.

3.7. Interaction between inducers and inhibitors of inflammation in NIT-1 cells

To study the effect of the inducer (Glu, PA or Glu+PA) and inhibitor (EA, UR, IS, MS or

AM) on inflammatory markers, 3 x 10^5 NIT-1 cells were seeded in 6-well plates and incubated

for 24 h in a humidified atmosphere at 37°C with 5% CO$_2$. The cells were treated with 33.3 mM

Glu or 250 μM PA or combination of 33.3 mM Glu and 250 μM PA for 24 h. Thereafter, 0.01,

0.05, 1 or 10 μM of EA, UR, IS, or MS or AM containing 0.01, 0.05, 1 or 10 μM equivalent of

EA were added to the wells followed by incubation for 72 h. The supernatants were removed and

stored at -80°C until further analysis. All the treatments were performed in triplicates.

3.8. Analysis of IL-1β

IL-1β levels in the supernatants of control and treated cells were analyzed by sandwich

ELISA using commercially available kits from Peprotech (Rock Hill, NJ) according to the

manufacturer's instructions. All of the samples were tested in duplicates.

3.9. Analysis of insulin concentration

The supernatants from control or treated cells were analyzed for insulin secretion using

the commercially available ultra-sensitive mouse insulin ELISA kit from Crystalchem (Downers

Grove, IL). The assay was performed according to manufacturer's protocol and all the samples

were analyzed in duplicates.

3.10. Statistical analysis

Results are expressed as means ± standard deviation. Statistical analysis was performed using Analysis of variance (ANOVA). Separation of means and difference between control and treatments was determined by Tukey's analysis (SAS, version 9.3). Statistical significance was set at P-value < 0.05.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Ellagic acid content in muscadine and amla extracts

To quantify the EA content in MS and AM, HPLC analysis was performed. The results are presented in Fig. 4.1. EA eluted at 12.6 min. HPLC analysis showed that the EA content in MS and AM was 9.4 ± 2.3 mg/g and 19.4 ± 1.5 mg/g, respectively. On a fresh weight (F.W.) basis, the estimated EA was 88.23 mg/100g in MS and 219 mg/100g in AM. Pastrana-Bonilla identified ellagic acid, myricetin, quercetin and kaempferol as major phenolics in MS skins (*111*). Gallic acid and ellagic acid are the major phenolics in AM (*32, 33*). Quercetin is a minor phenolic in AM (*33*). The range of EA concentration in MS found in this study was 36-91 mg/100 g on F.W. basis and fit within the range reported by Torronen et al (*25*). The average EA content in different varieties of MS was 16.5 mg/100g of F.W. EA content in Ison variety of MS was 8.7 mg/100g F.W in whole fruit and 22 mg/100g of F.W in skins alone (*111*). Paltanov et al. reported that HPLC analysis of commercially available extract of AM showed EA content between 2.42 and 1.29 mg/g (*135*). Thin layer chromatographic (TLC) analysis of water extract of AM showed that the ellagic acid content in AM was 6.45 g/100 g F.W (*32*).
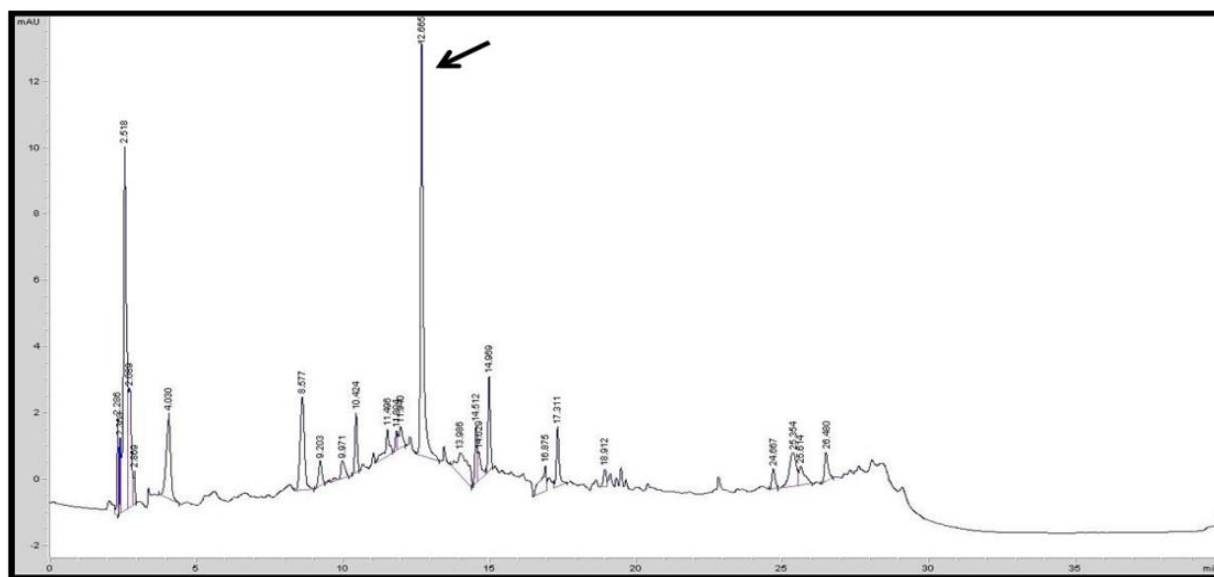

Figure 4.1.A. HPLC profile of muscadine fruit extract. EA eluted at 12.6 min.
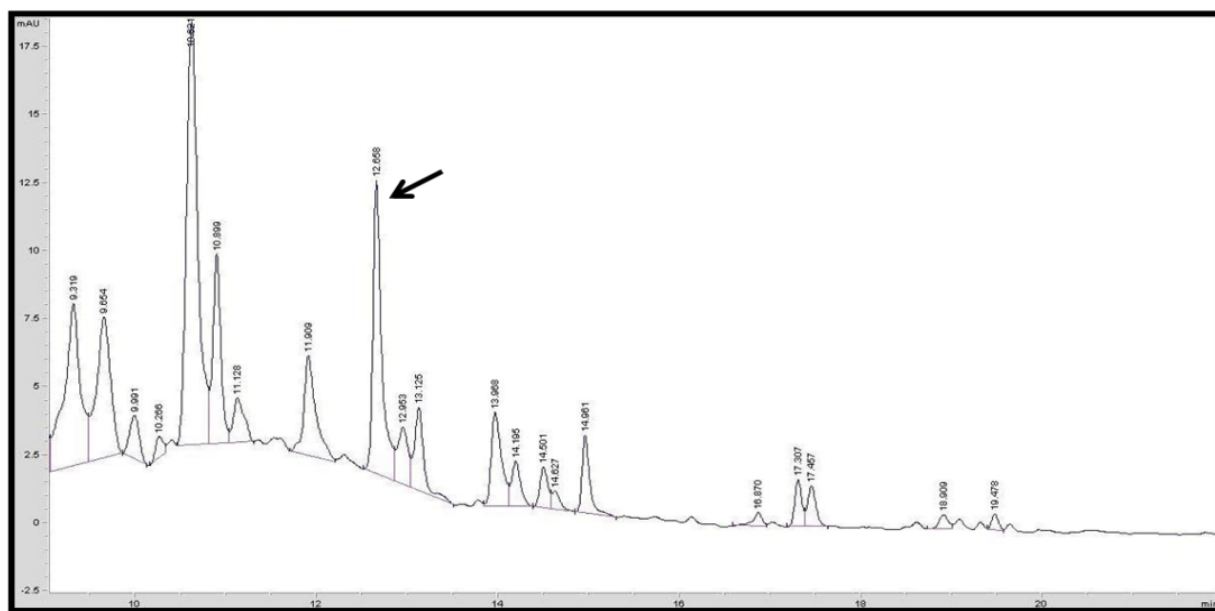
Figure 4.1.B. HPLC profile of amla fruit extract. EA eluted at 12.6 min.

The variation in the amount of ellagic acid among different studies may be associated to the difference in cultivars, growing conditions and harvest time (*136*).

4.2. Effect of EA, UR, IS, MS or AM on the viability of NIT-1 cells treated with Glu, PA or Glu+PA

To evaluate the effects of Glu or PA on cell viability, NIT-1 cells were treated with Glu, PA or Glu+PA for 96 h. Cell viability was analyzed by MTS assay and expressed as percentage of viable cells in the control. The results are presented in Fig 4.2.1.

The solubility of free fatty acids is high in aqueous solutions when conjugated to BSA. Moreover, BSA contains two high affinity binding sites for long-chain free fatty acids (*137*). Therefore, PA conjugated to BSA was used in the experiments. To confirm if BSA by itself did not affect cell viability, cells were treated with BSA alone and compared with control. The results showed that BSA did not affect NIT-1 cell viability and Glu, PA or Glu+PA treatment exhibited a proliferative effect, which however was not significantly different from control. Nevertheless, neither Glu nor PA was toxic to NIT-1 cells. The results in this study with PA were in contrast to the reported decrease in cell viability and increased apoptosis in the

pancreatic β-cells treated with PA for 24 h (*138*). The present study was conducted for 96 h and inhibition of cell viability in PA treated cells was not observed.

The effect of EA, UR, IS or extracts of MS or AM along with Glu, PA or Glu+PA on cell viability was also tested. NIT-1 cells were exposed to Glu, PA or Glu+PA for 24 h and treated with EA, UR, IS or extracts of MS or AM followed by 72 h incubation. The concentrations of the EA, UR or IS tested were 0.01, 0.05, 1 and 10 µM, respectively. For the treatments with extracts of MS or AM, equivalent concentrations of ellagic acid quantified by HPLC were used. Cell viability was analyzed using the MTS assay and expressed as percentage of control viable cells. The results are presented in Fig 4.2.2. to 4.2.6.

The bioavailability of phenolics dictates their efficacy as against chronic diseases (*16*). Therefore, the concentrations tested in the study were in the range of bioavailable levels of EA (0.1 µM - 0.7 µM) as reported in two studies (*122, 125*). The bioavailability of UR differed among studies. One study reported the maximum attainable level as 5 µM (*121*), whereas another study reported a range between 14 - 25 µM (*126*). The concentrations tested in the present study were in the range of those reports.

The viability of NIT-1 cells incubated with Glu and treated with EA, UR, IS, MS or AM was not significantly different from the viability of control cells. A dose-dependent decrease in viability was observed with increase in concentration of EA, UR, IS, MS or AM. However, the viability of treated cells was not significantly different from control cells except for cells treated with 10 µM IS (Fig.4.2.4), suggesting that the compounds were not cytotoxic to NIT-1 cells. The observed dose-dependent decrease in cell viability in the present study is similar to the reported increase in cell death from 3.8% at higher dilution of muscadine skin extract of 1:400 to 8.7% at a lower dilution of 1:100 (*110*). The difference in response of the bioactive compounds at

different concentrations may be associated to the hormetic effect of these antioxidants that show a stimulatory effect at low concentration and toxic effect at high concentration (*139, 140*).

The viability of cells exposed to PA or Glu+PA and treated with EA, UR, IS, MS or AM showed that there was no significant difference in cell viability compared to control cells. The concentrations (0.1-10µM) tested either showed proliferative effect or did not significantly inhibit NIT-1 cell viability. The dose-dependent decrease in cell viability was similar to the response observed with Glu and EA, UR, IS, MS or AM-treated NIT-1 cells.
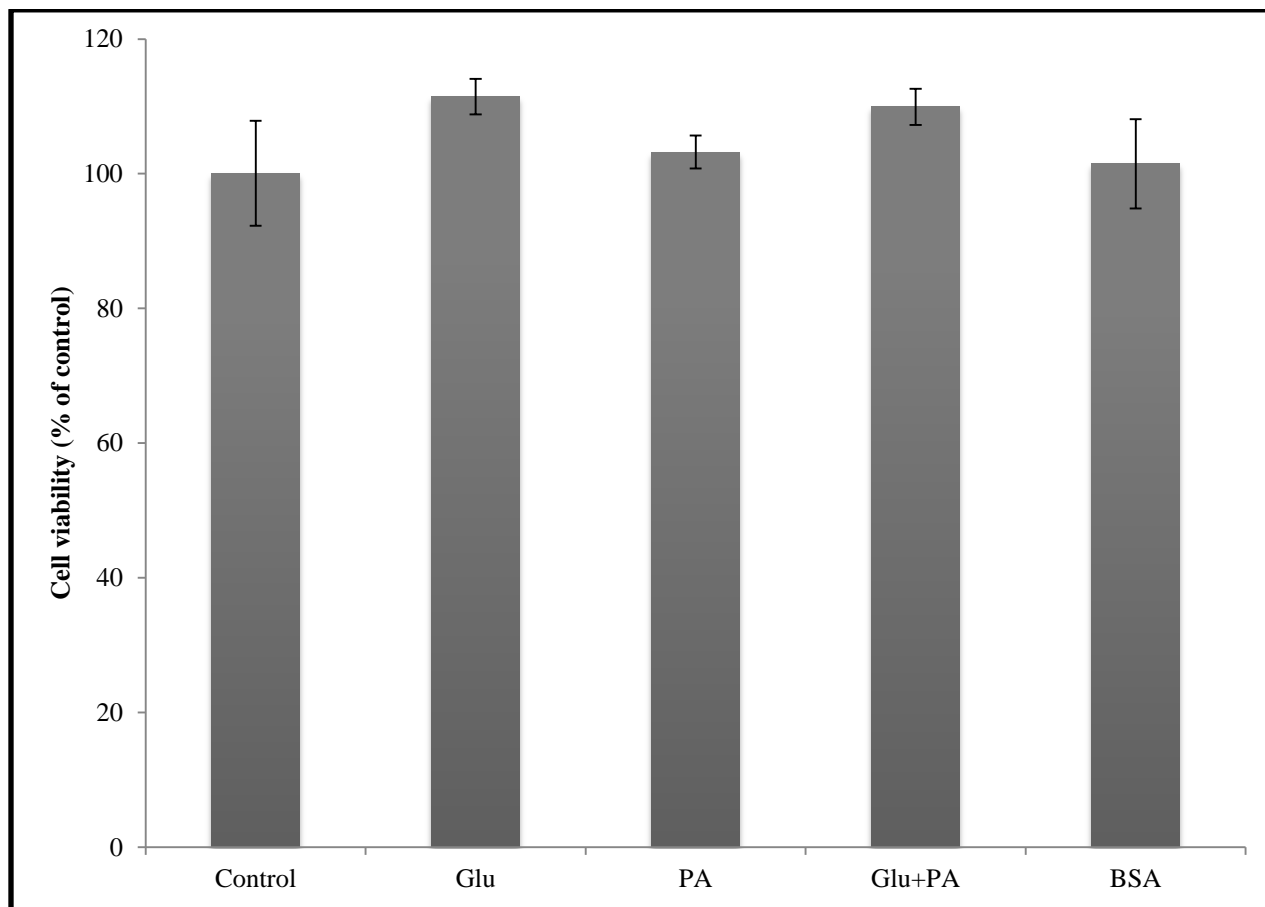

Figure 4.2.1. Effects of 33.3mM Glu, 250 µM PA, 33.3mM Glu + 250 µM PA, or BSA on the viability of NIT-1 cells. Cells were incubated with Glu, PA, Glu+PA or BSA for 96 h and the viability was determined by the MTS assay. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=3).
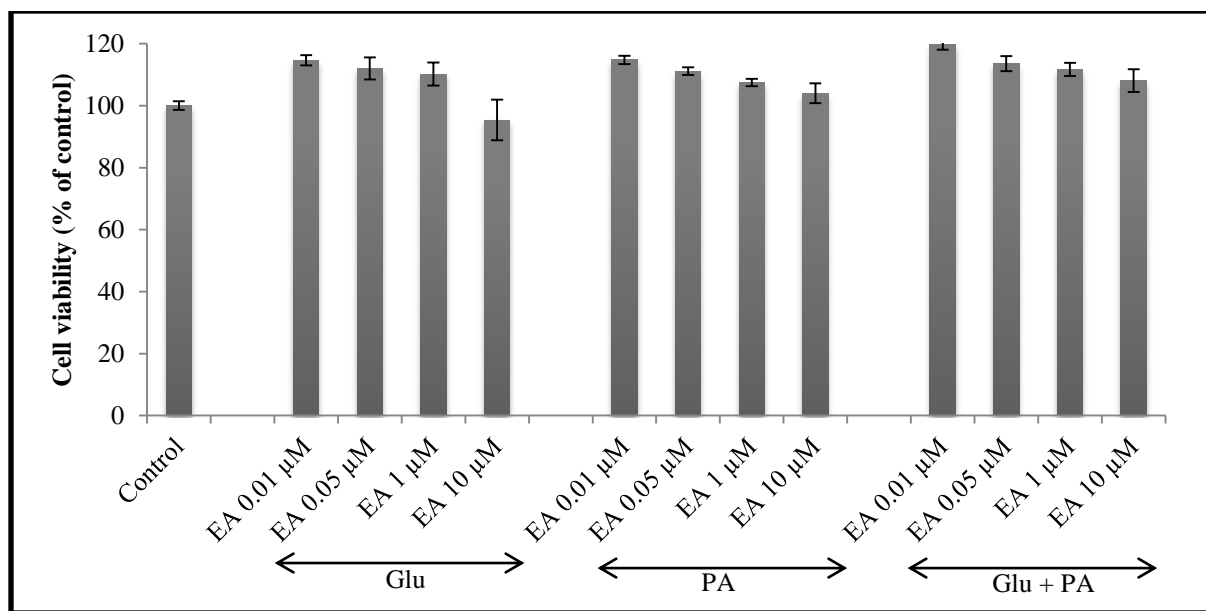
Figure 4.2.2. Effects of 33.3mM Glu, 250 μM PA or 33.3mM Glu + 250 μM PA and different concentrations of EA on the viability of NIT-1 cells. Cells were incubated with Glu, PA, Glu+PA or BSA for 24 h and EA (0.01-10μM) was added followed by additional incubation for 72 h. The viability was determined by the MTS assay. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=3).
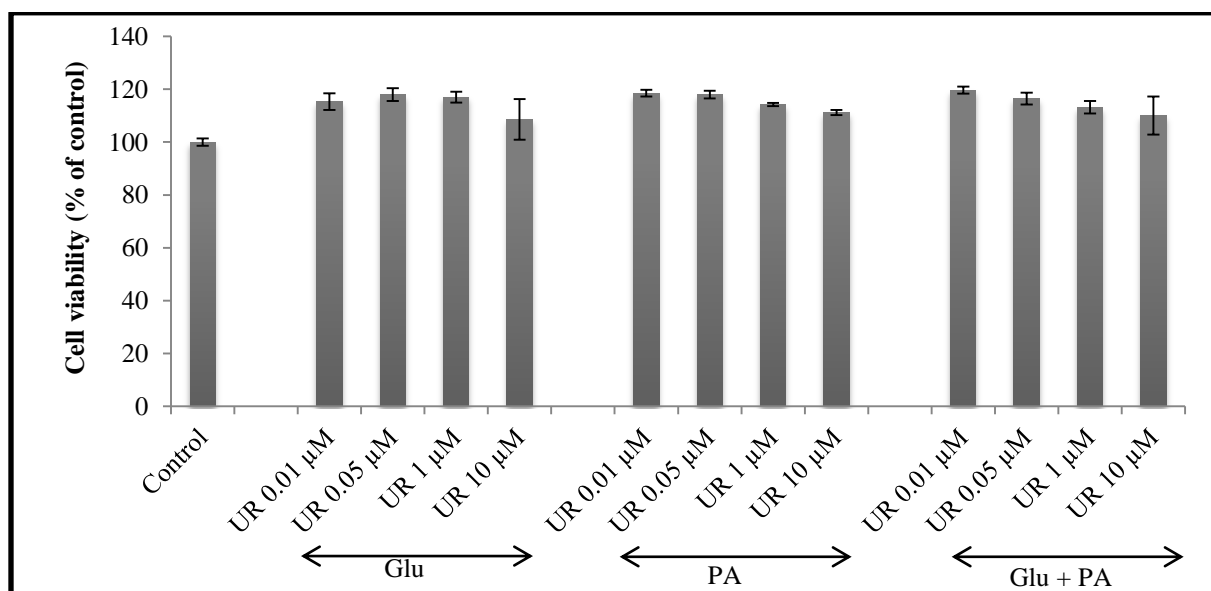

Figure 4.2.3. Effects of 33.3mM Glu, 250 μM PA or 33.3mM Glu + 250 μM PA and different concentrations of UR on the viability of NIT-1 cells. Cells were incubated with Glu, PA, Glu+PA or BSA for 24 h and UR (0.01-10μM) was added followed by additional incubation for 72 h. Cell viability was determined by the MTS assay. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=3).

Figure 4.2.4. Effects of 33.3mM Glu, 250 µM PA or 33.3mM Glu + 250 µM PA and different concentrations of IS on the viability of NIT-1 cells. Cells were incubated with Glu, PA, Glu+PA or BSA for 24 h and IS (0.01-10µM) was added followed by additional incubation for 72 h. Cell viability was determined by the MTS assay The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=3).
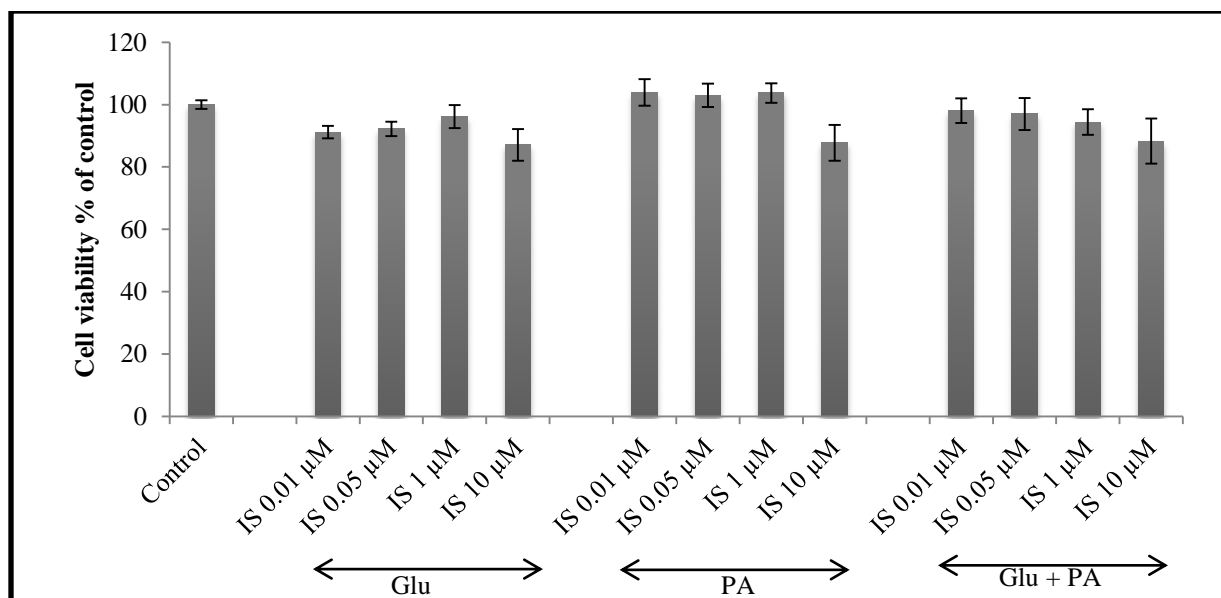

Figure 4.2.5. Effects of 33.3mM Glu, 250 µM PA or 33.3mM Glu + 250 µM PA and different concentrations of MS on the viability of NIT-1 cells. Cells were incubated with Glu, PA, Glu+PA or BSA for 24 h and MS (0.01-10µM) was added followed by additional incubation for 72 h. Cell viability was determined by the MTS assay. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=3).
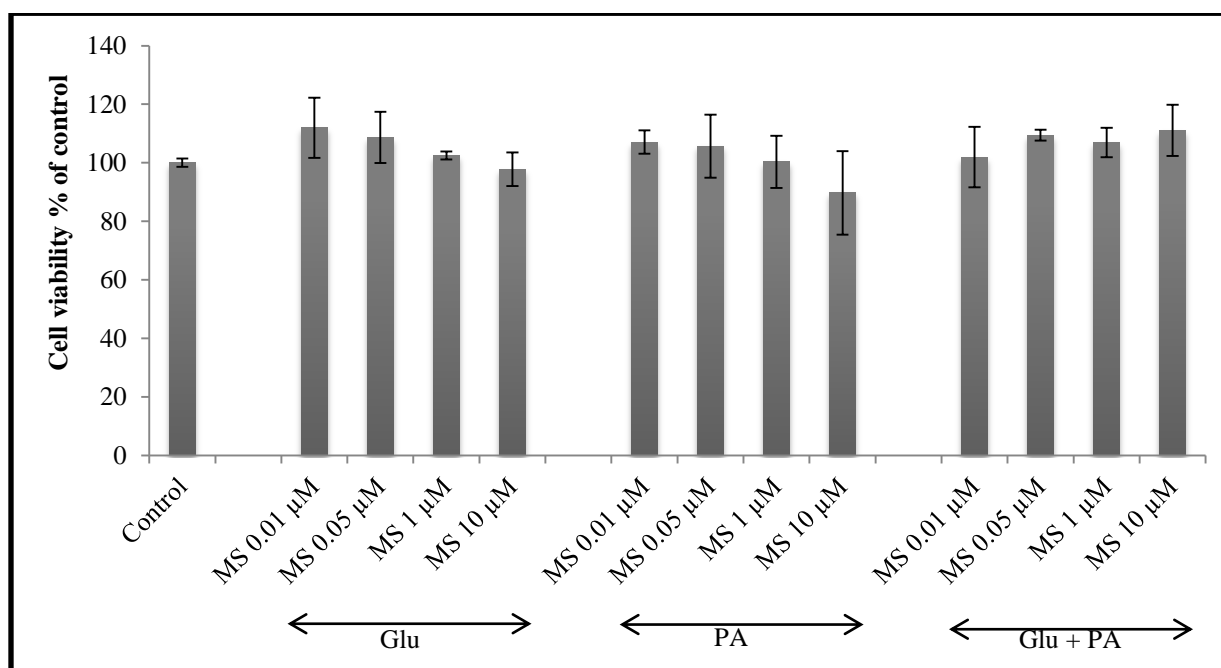
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Figure 4.2.6. Effects of 33.3mM Glu, 250 µM PA or 33.3mM Glu + 250 µM PA and different concentrations of AM on the viability of NIT-1 cells. Cells were incubated with Glu, PA, Glu+PA or BSA for 24 h and AM (0.01-10µM) was added followed by additional incubation for 72 h. Cell viability was determined by the MTS assay. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=3).
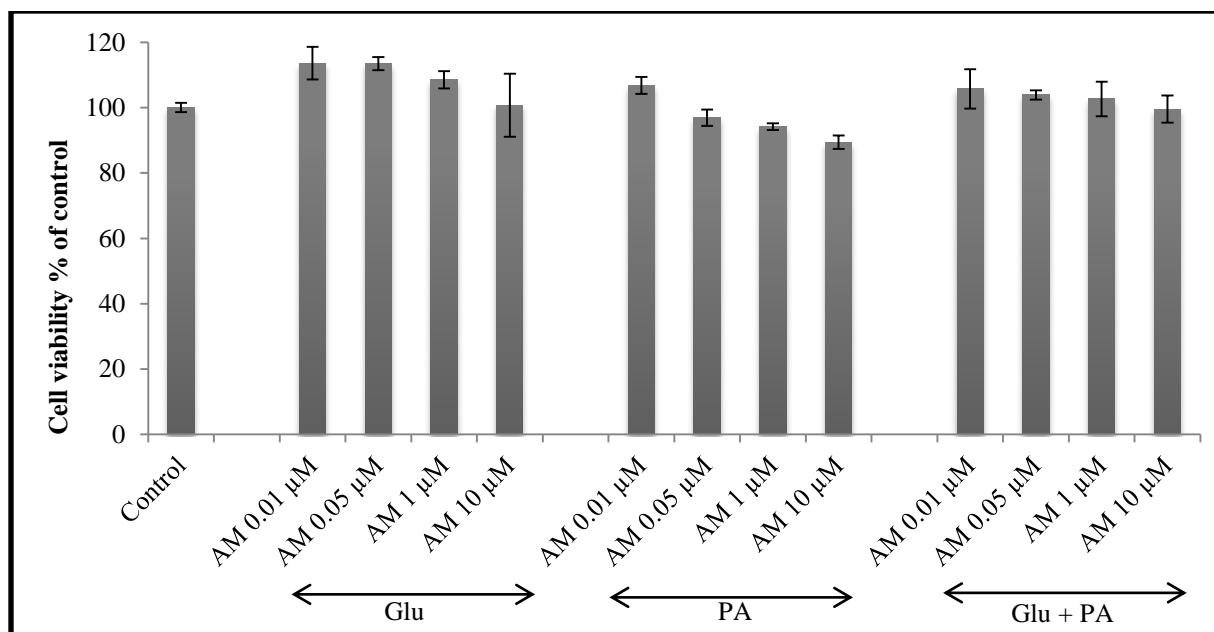
4.3. Effect of EA, UR, IS, MS or AM on Glu-induced IL-1β

To observe the effect of EA, UR, IS, MS or AM on Glu-induced IL-1β, ELISA was performed to determine the IL-1β concentration in the supernatants. The results are presented in Fig. 4.3 and reported as IL-1β (% of control). Exposure to 33.3 mM Glu significantly increased ($P < 0.05$) the levels of IL-1β by 38.29 ± 1.85 % compared to control. The observed results in the present study are in agreement with the reported significant increase of IL-1β levels at glucose concentrations higher than 11mM (*11*). Donath et al. reported that IL-1β has been identified as the major proinflammatory cytokine produced in response to high concentrations of glucose or free fatty acids (*8*). Treatment with EA, MS or AM showed significant (p < 0.05) dose-dependent inhibition of IL-1β, whereas none of the concentrations (0.01-10 µM) of UR or IS tested showed any significant inhibition.

31

EA at concentrations between 0.01 and 10µM significantly inhibited (P < 0.05) IL-1β secretion (Fig 4.3.1). At nanomolar concentrations of EA, IL-1β levels were equal to that of control and the maximum inhibition was observed at 1µM of EA with IL-1β reduced by 80 ± 3.4 % of control. Plant phenolics quench free radicals. The mechanism by which EA suppressed IL-1β may be associated with the inhibition of oxidative stress and subsequent attenuation of inflammatory cytokines (*68*).

The results in Fig 4.3.2 and 4.3.3. show that none of the concentrations of UR or IS tested had any significant inhibitory effect on IL-1β induced by Glu. The low antioxidant potential of UR compared to its parent compound EA may have been associated with the inability of UR to inhibit IL-1β (*141*).

Fig. 4.3.4 and 4.3.5 show that EA-rich MS or AM dose-dependently inhibited IL-1β similar to EA. IL-1β was significantly inhibited (P < 0.05) in cells treated with MS, whereas AM showed significant inhibition only at 1 and 10 µM. MS or AM containing 10 µM equivalent EA inhibited IL-1β by 89.6 ± 1.6% or 68.3 ± 1.7%, respectively compared to control. MS skin extract standardized to its ellagic acid dose-dependently inhibited lipopolysaccharide (LPS)-induced IL-1β in blood mononuclear cells (*110*).

A low-grade inflammatory process precedes the development of diabetes and can be observed before the onset of the disease (*10, 142*). The time of exposure, concentration and composition of the nutrients available in humans are the factors that influence the increase of IL-1β concentration (*143*). The results in the present study show that NIT-1 cells exposed to 33.3 mM Glu induced IL-1β secretion. Treatment with EA, MS or AM dose-dependently inhibited IL-1β, whereas the treatment with metabolites, UR or IS was not effective.
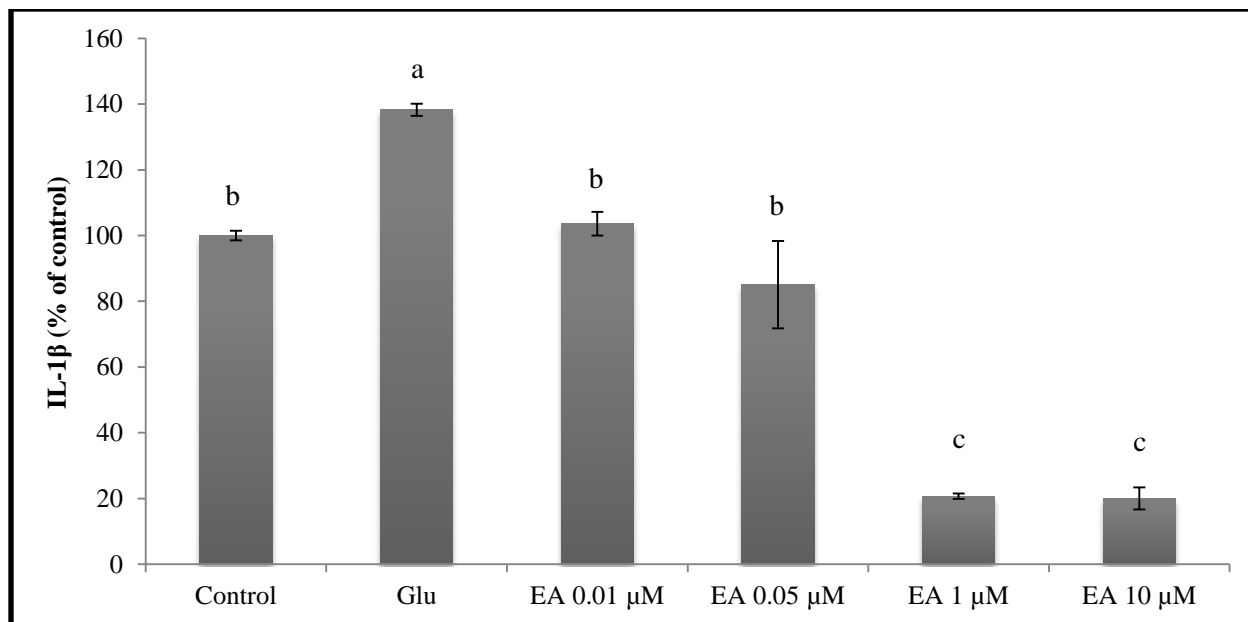
Figure 4.3.1. Effect of ellagic acid (EA) at different concentrations on glucose (Glu)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu for 24 h and EA (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

Figure 4.3.2. Effect of urolithin A (UR) at different concentrations on glucose (Glu)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu for 24 h and UR (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

Figure 4.3.3. Effect of isorhamnetin (IS) at different concentrations on glucose (Glu)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu for 24 h and IS (0.01-10µM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
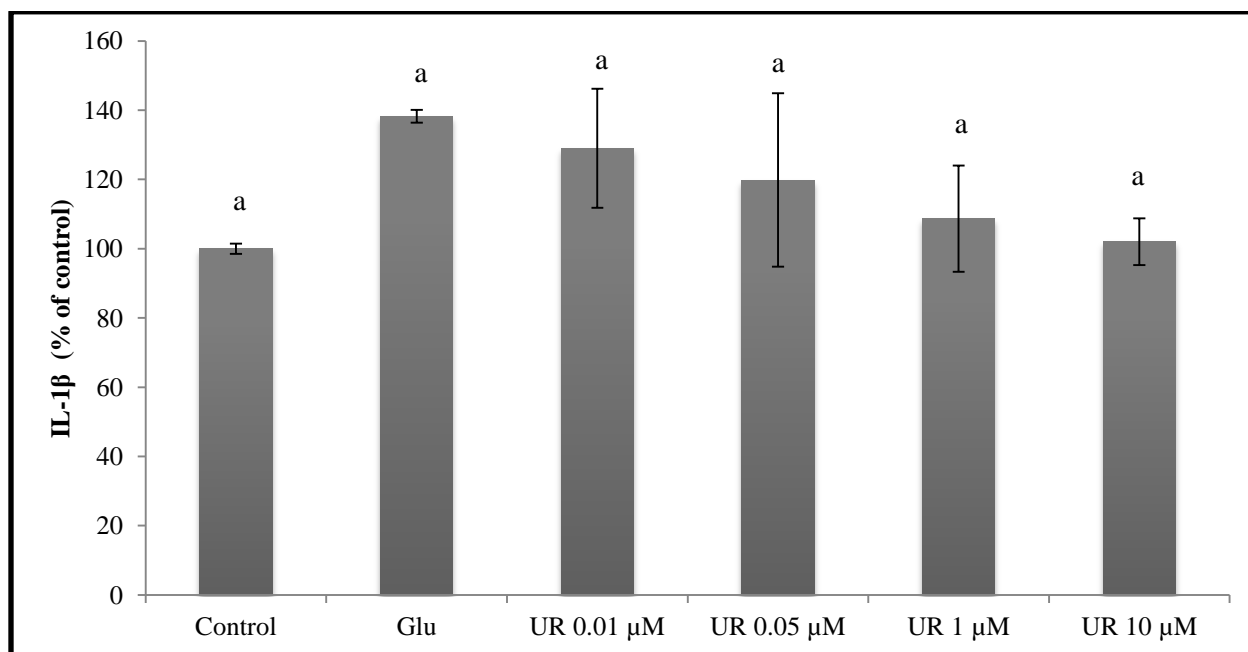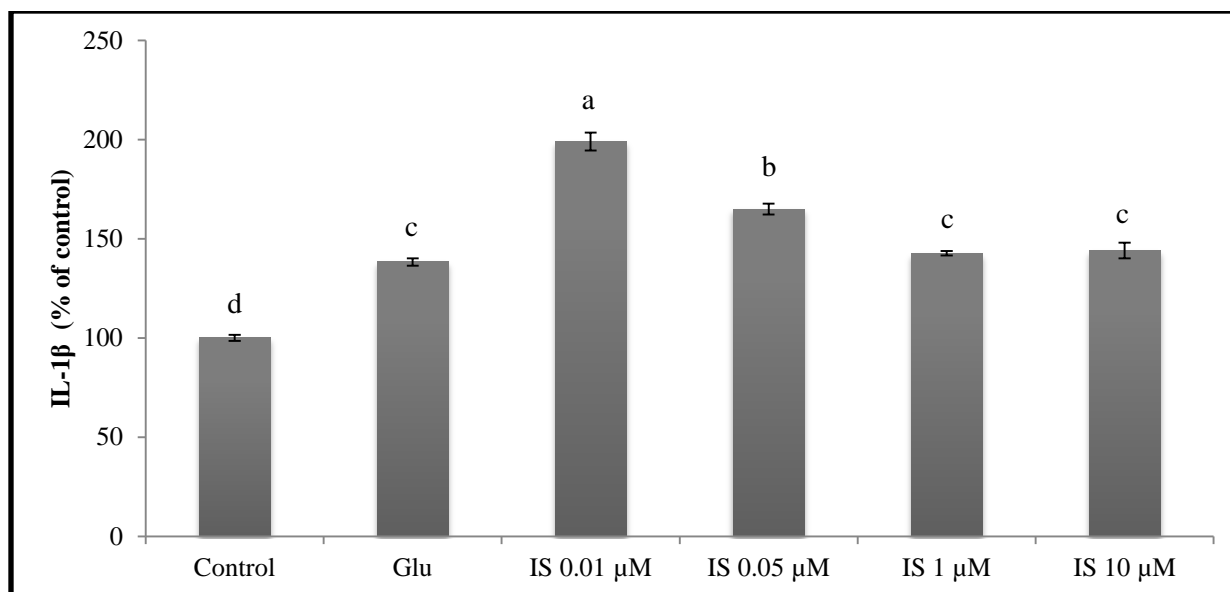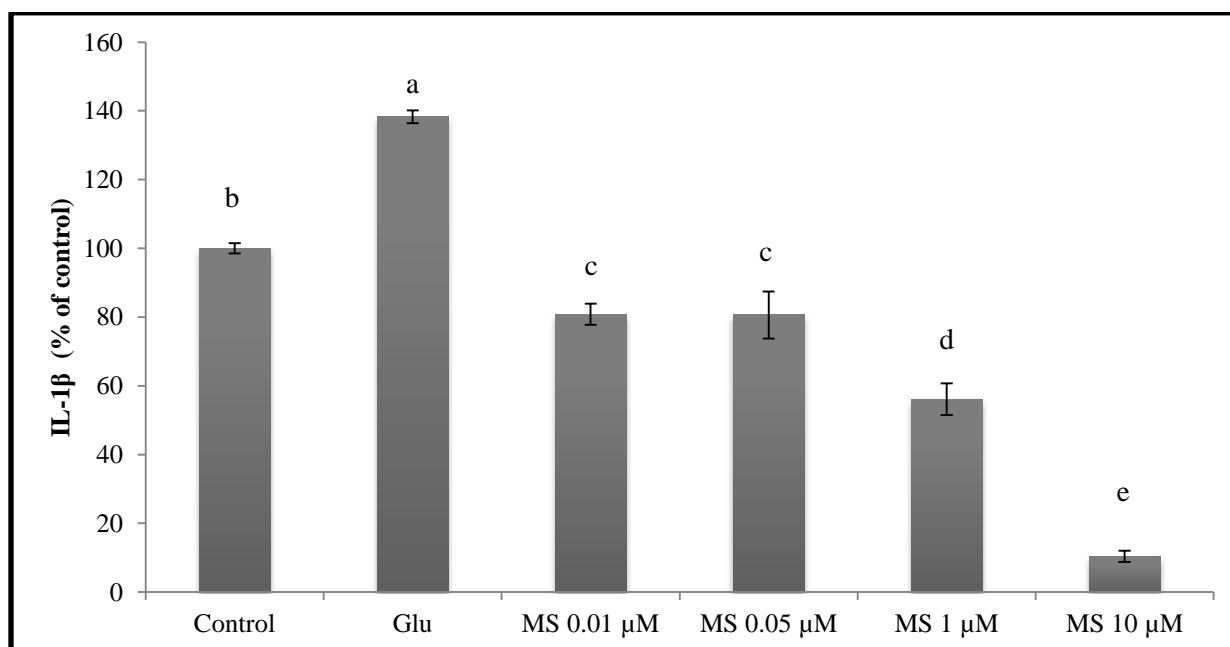

Figure 4.3.4. Effect of muscadine extract (MS) at different concentrations on glucose (Glu)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu for 24 h and MS (0.01-10µM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

Figure 4.3.5. Effect of amla extract (AM) at different concentrations on glucose (Glu)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu for 24 h and AM (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
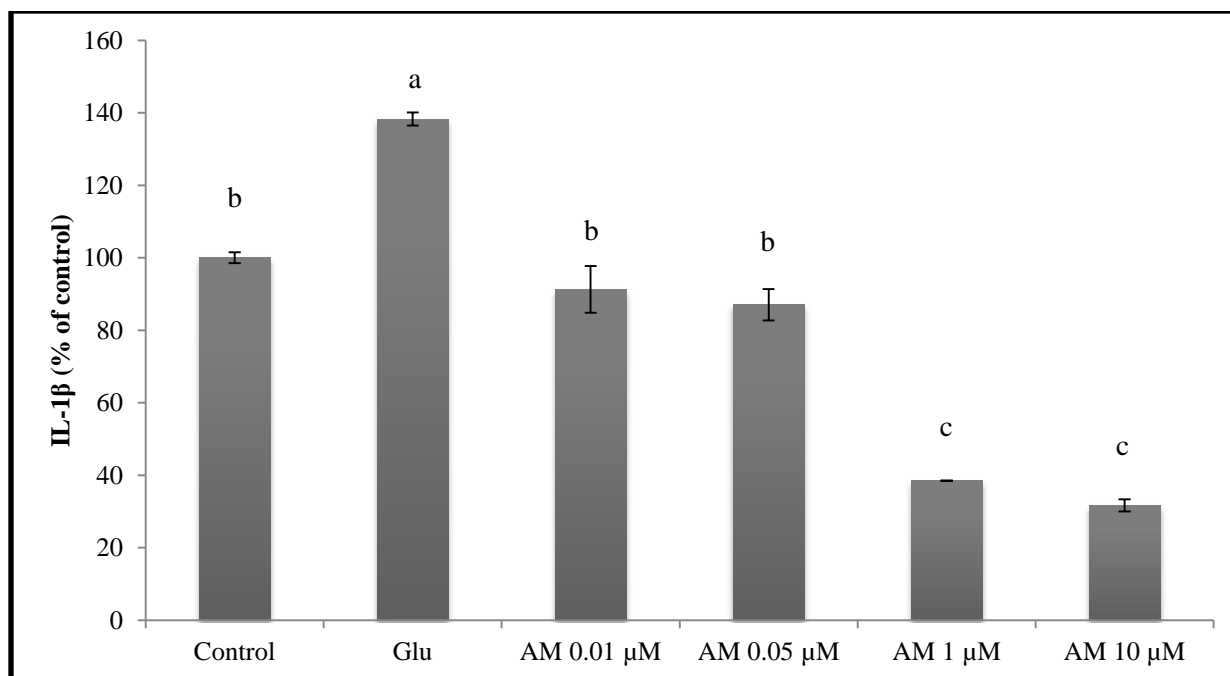
4.4. Effect of EA, UR, IS, MS or AM on PA-induced IL-1β

To study the effect of EA, UR, IS, MS or AM on PA-induced IL-1β, ELISA was performed. The IL-1β levels in the supernatants were determined by ELISA. The results are presented in Fig. 4.4.1 to 4.4.5 and reported as IL-1β (% of control). PA treatment increased IL-1β secretion by 6.84 ± 0.88% compared to control. However, the increase was not significant. The PA-induced IL-1β level was less than that induced by Glu (38.29 ± 1.85 %). BSA treatment did not increase IL-1β level compared to control thereby confirming that the increased IL-1β levels were due to PA.

The response of pancreatic β-cells to saturated fatty acids is dependent on fatty acid chain length (*144*). β-cells have high tolerance for short chain fatty acids such as laurate or octanoate compared to long chain fatty acids such as palmitate. In vitro study for 24 h showed high

amounts of oxygen radicals and inflammatory cytokines expression upon exposure to 200-500

µM of PA in skeletal muscle cells (*5*). Moreover, palmitate, oleate or stearate have been

reported to constitute 80% of the circulating free fatty acids in humans and IL-1β expression was

observed upon exposure to these free fatty acids (*7, 145*).

The results in Fig 4.4.1 show that EA dose-dependently inhibited IL-1β and the inhibitory

effect was significant ($P < 0.05$) only at 1 µM and 10 µM. Although, EA at 0.01 µM and 0.05

µM suppressed IL-1β, the difference was not significant from control. EA at 10 µM reduced the

IL-1β level to 26.96 ± 4.95% of the control. It can be observed from Fig 4.4.2 and 4.4.3 that none

of the concentrations of UR or IS tested could significantly inhibit IL-1β compared to control.

The results were similar to the data obtained from UR or IS in Glu-treated cells (Fig 4.3.2 and

4.3.3).

Figure 4.4.1. Effect of ellagic acid (EA) at different concentrations on palmitic acid (PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with PA for 24 h and EA (0.01-10µM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different ($P < 0.05$) among groups.

Figure 4.4.2. Effect of urolithin A (UR) at different concentrations on palmitic acid (PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with PA for 24 h and UR (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
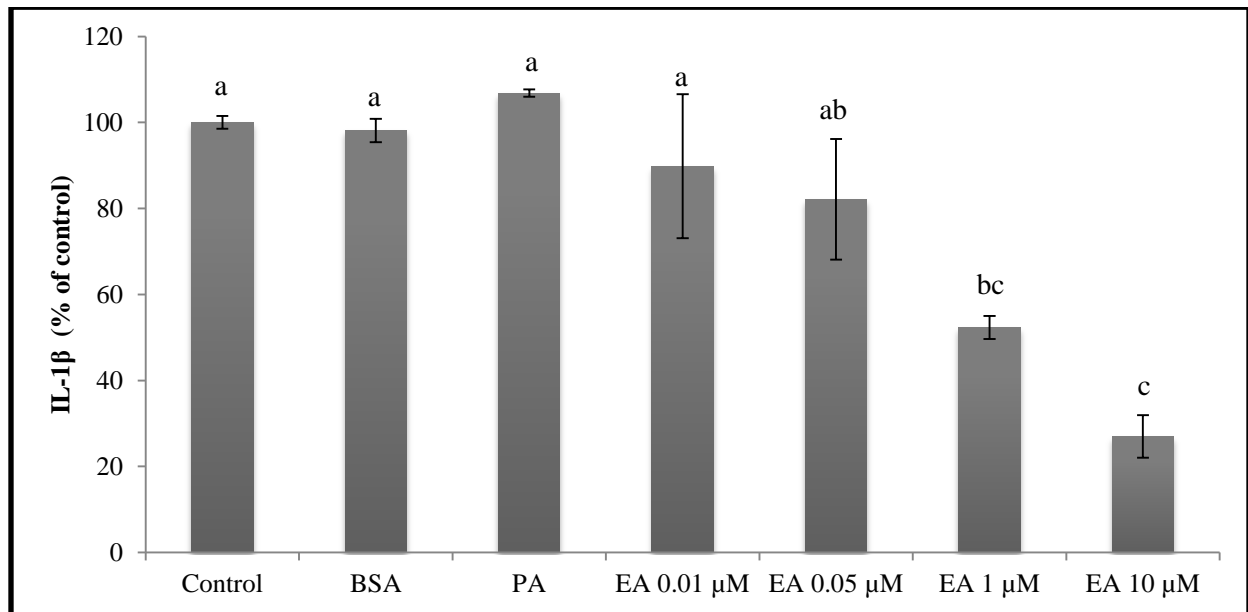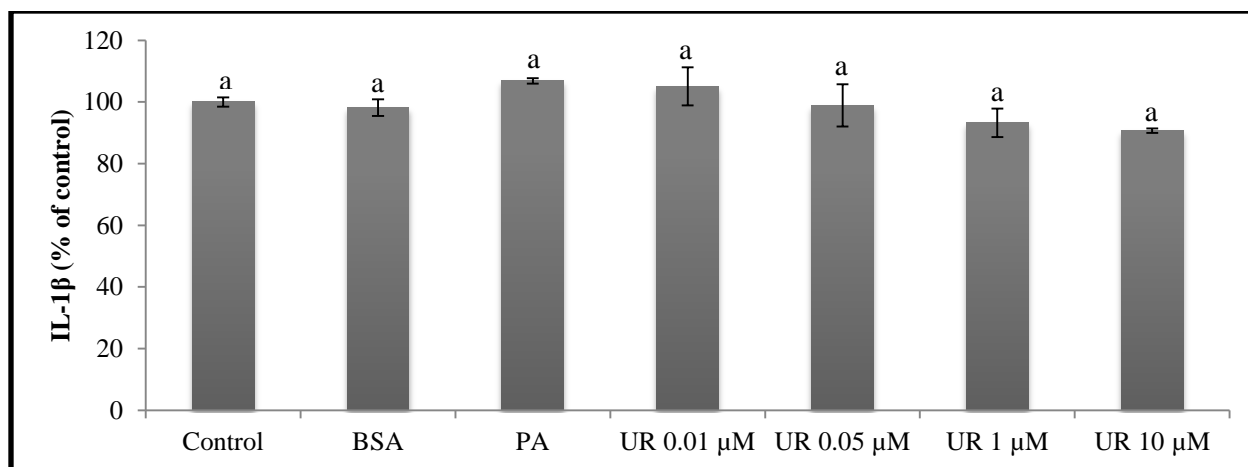

Figure 4.4.3. Effect of isorhamnetin (IS) at different concentrations on palmitic acid (PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with PA for 24 h and IS (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

The results from Fig 4.4.4 and 4.4.5 show that all the concentrations of MS or AM dose-dependently caused significant inhibition (P < 0.05) of IL-1β. MS or AM containing 10 μM EA equivalent inhibited IL-1β by 95% and 99% of control, respectively. The higher inhibition of IL-1β by MS or AM containing EA equivalent compared to pure EA (Fig 4.1.1) may be due to

37

synergistic effect of unidentified compounds along with EA in MS or AM. Free radicals produced by Glu or PA induce IL-1β. IL-1β inhibition by AM may be due to the removal of free radicals by anti-oxidants in AM (*120*).
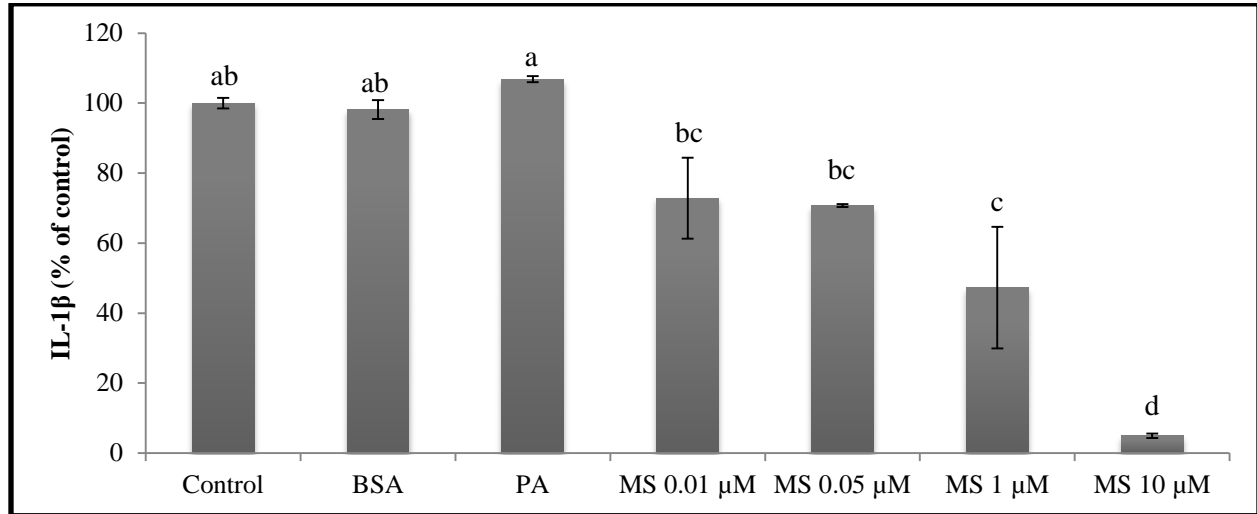
Figure 4.4.4. Effect of muscadine extract (MS) at different concentrations on palmitic acid (PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with PA for 24 h and MS (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
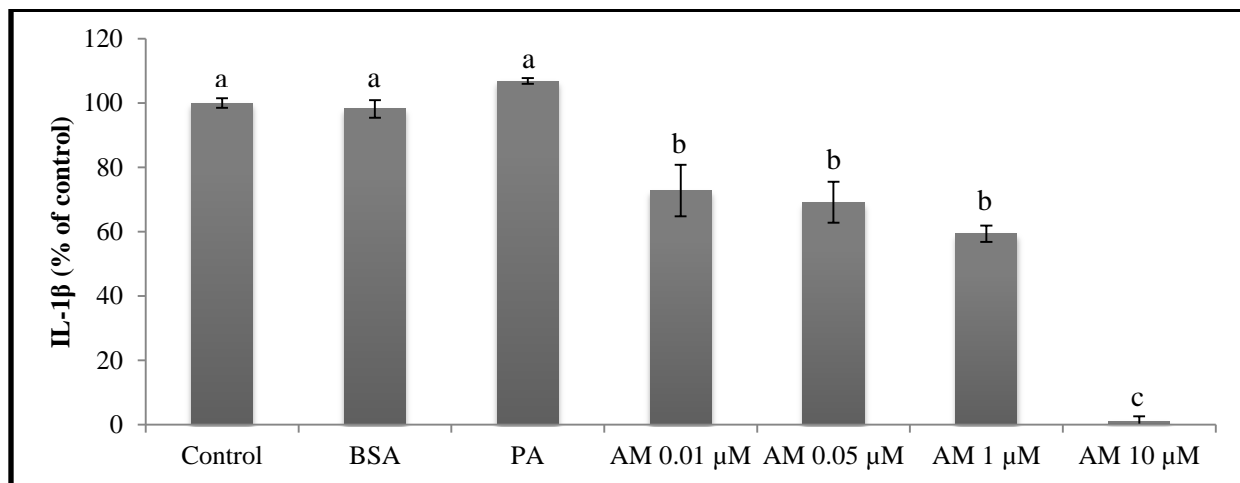
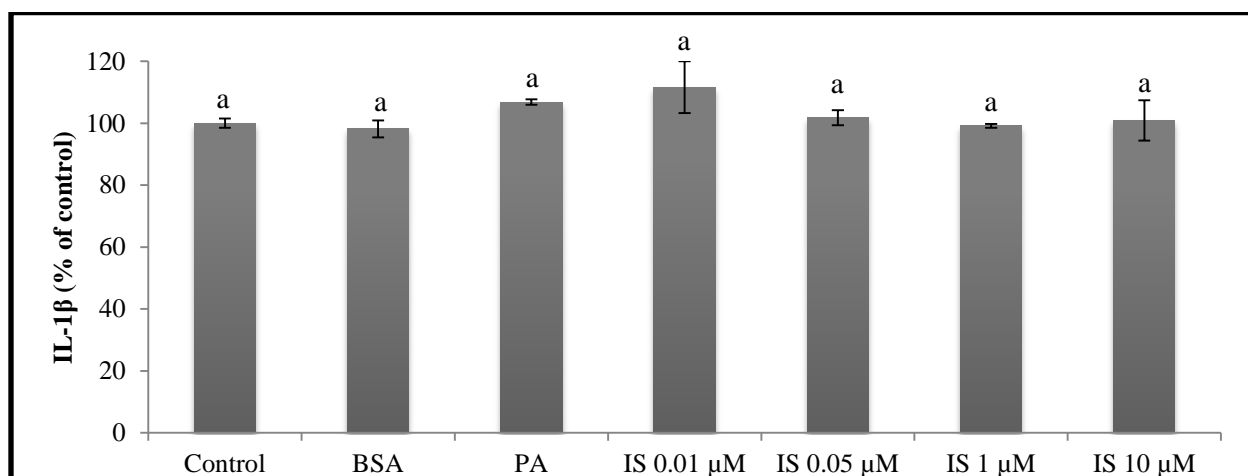Figure 4.4.5. Effect of amla extract (AM) at different concentrations on palmitic acid (PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with PA for 24 h and AM (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

4.5. Effect of EA, UR, IS, MS or AM on Glu+PA-induced IL-1β

To observe the effect of EA, UR, IS, MS or AM on Glu+PA-induced IL-1β, supernatants from the treated NIT-1 cells were measured by ELISA. The results are presented in Fig. 4.5 and reported as IL-1β (% of control). Treatment with a combination of Glu and PA showed a significant increase ($P < 0.05$) of IL-1β levels by $19.91 \pm 1.25\%$ compared to control.

The results from Fig 4.5.1 showed that the effect of EA on IL-1β induced by Glu+PA was similar to the effect observed when treated with either Glu or PA (Fig 4.3.1 and 4.4.1). All the concentrations of EA tested showed a statistically significant inhibition of IL-1β except for 0.01 μM EA. EA at 10 μM inhibited IL-1β by $70.2 \pm 5.3\%$ of the control. As shown in Fig 4.5.2, UR treatment dose-dependently inhibited IL-1β, but the inhibition was not significantly different from the control. UR at 10 μM showed maximum inhibition by reducing IL-1β to 80 ± 13.2 % of control.

The results from Fig 4.5.3. show that IS dose-dependently inhibited IL-1β in Glu+PA-treated NIT-1 cells. Interestingly, IS did not have any significant inhibitory effect on the IL-1β produced by either Glu or PA (Fig 4.3.3 and 4.4.3) compared to control. IS at 10 μM inhibited IL-1β by $55.2 \pm 4.7\%$ of the control. IS inhibited LPS-induced IL-1β in murine macrophages (*21*).

The inhibitory effects of MS or AM on IL-1β induced by exposure to Glu+PA are presented in Fig 4.5.4 and 4.5.5. MS or AM showed significant dose-dependent inhibition of IL-1β. Maximum inhibition of 94% and 75% compared to the control was observed with MS or AM containing 10μM EA equivalent, respectively. Higher inhibition of IL-1β in cells treated with MS or AM compared to EA (Fig 4.1.1) may be associated to synergistic effect from other unidentified compounds in MS or AM along with EA.

Figure 4.5.1. Effect of ellagic acid (EA) at different concentrations on glucose + palmitic acid (Glu+PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and EA (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

Figure 4.5.2. Effect of urolithin A (UR) at different concentrations on glucose + palmitic acid (Glu+PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and UR (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
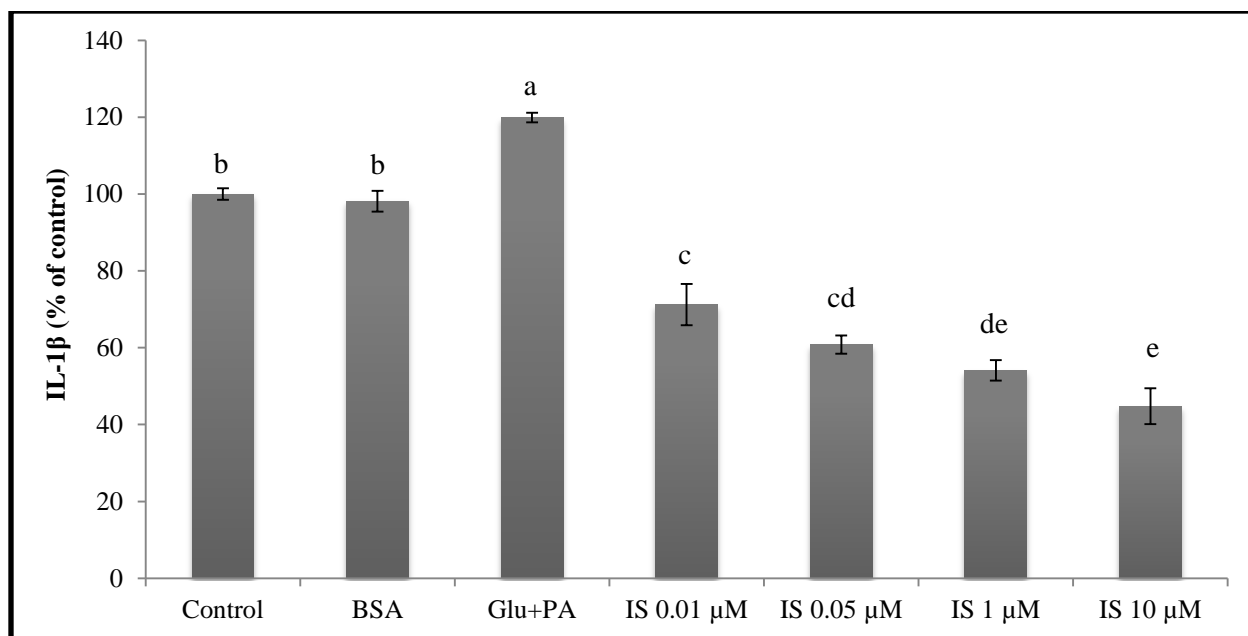
Figure 4.5.3. Effect of isorhamnetin (IS) at different concentrations on glucose + palmitic acid (Glu+PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and IS (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
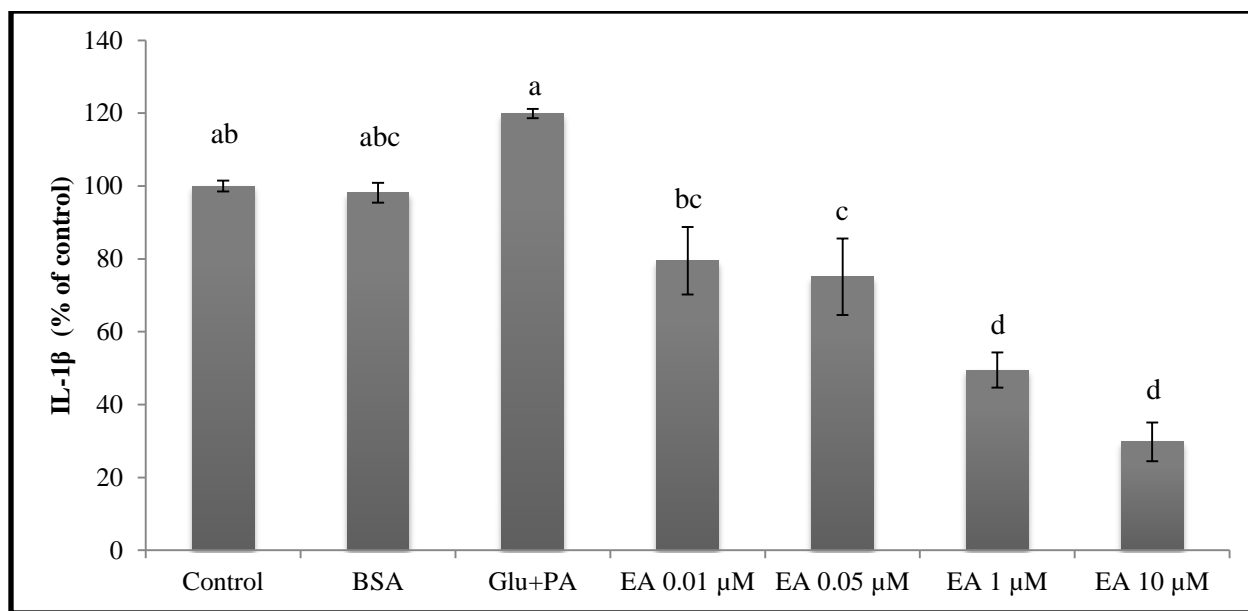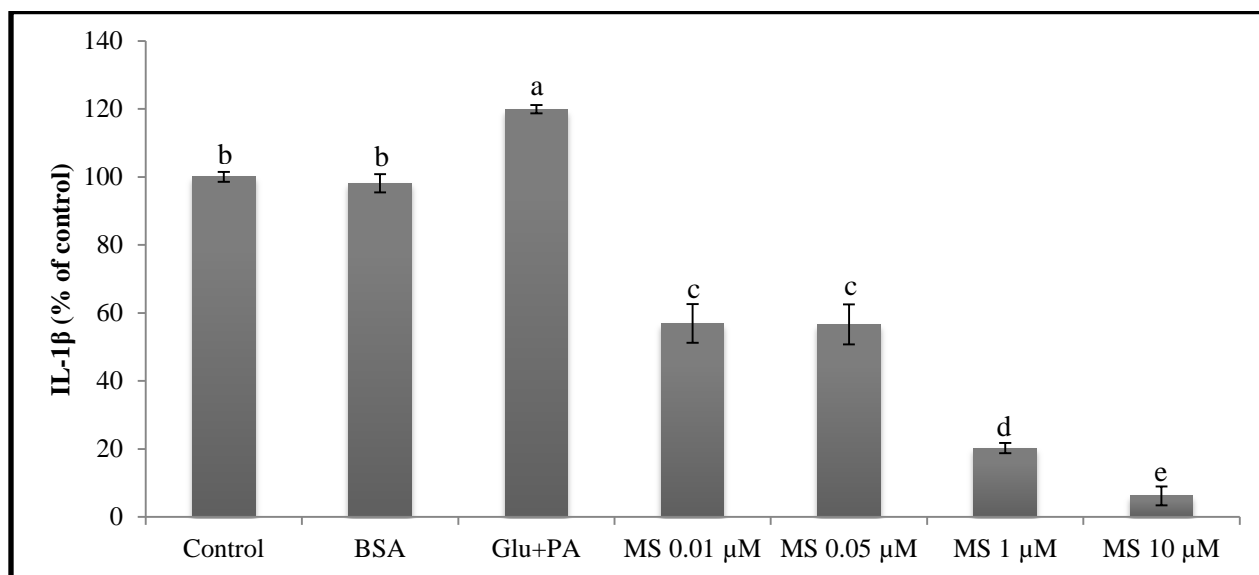

Figure 4.5.4. Effect of muscadine extract (MS) at different concentrations on glucose + palmitic acid (Glu+PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and MS (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
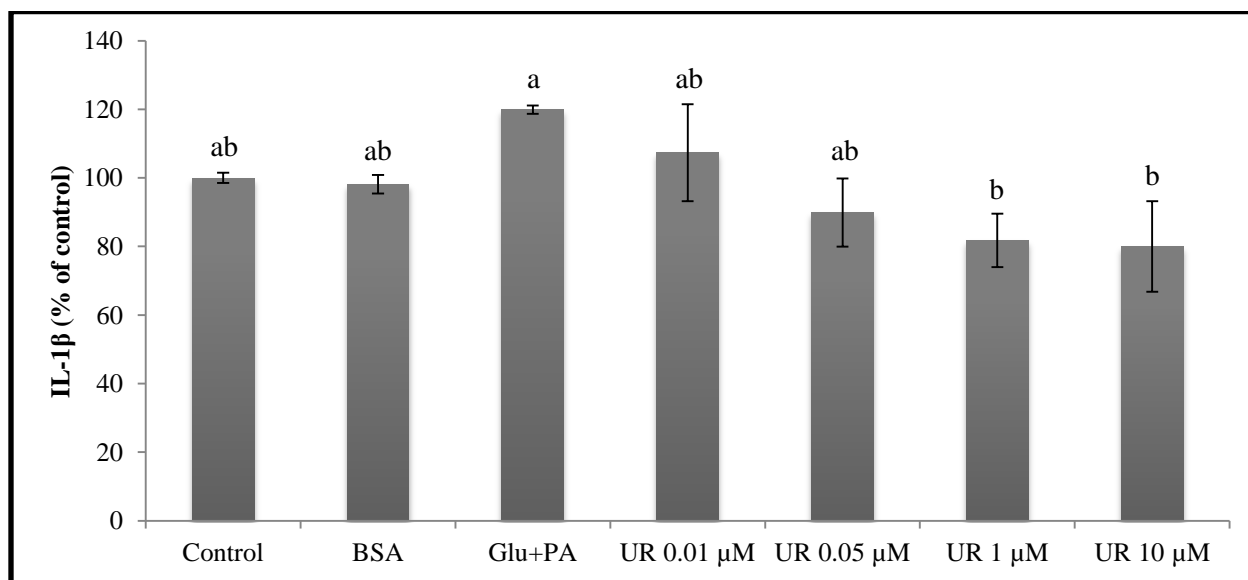
Figure 4.5.5. Effect of amla extract (AM) at different concentrations on glucose + palmitic acid (Glu+PA)-induced IL-1β levels in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and AM (0.01-10μM) was added followed by additional incubation for 72 h. IL-1β concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

## 4.6. Effect of EA,UR, IS, MS or AM on insulin secretion in Glu-treated NIT-1 cells

To observe the effect EA, UR, IS, MS or AM on Glu-reduced insulin secretion, the supernatants from the treated NIT-1 cells were measured by ELISA. The results are presented in Fig. 4.6 and expressed as insulin (% of control). Glu treatment significantly decreased (P < 0.05) insulin secretion by 11.8 ± 0.14 % of control. The decrease in insulin secretion was associated with the increase in IL-1β secretion by Glu treatment (Fig 4.3). Maedler et al. studied the effect of exposure of human islets to 33.3 mM Glu for 4 days and observed that exposure to Glu induced significant increase in IL-1β secretion (*11*). The study also reported nearly complete failure of insulin secretory process after 4 days despite the almost absence of apoptotic cells. However, in our study, we observed a significant increase in IL-1β upon exposure to 33.3 mM Glu, whereas insulin secretion was suppressed by only 11.8 ± 0.14 % of control.

The results in Fig 4.6.1 show that EA at 10μM significantly (P < 0.05) stimulated insulin compared to the control. The insulin concentration in cells treated with 10μM EA is 3.8 ± 0.5 % higher than that of control. EA at 0.01, 0.05 and 1μM dose-dependently stimulated insulin, but

the difference was not significant compared to control. Treatment with UR or IS (Fig. 4.6.2) did not show any significant stimulation on insulin secretion. Although UR or IS dose-dependently increased insulin secretion, they are not statistically similar to that of control. UR or IS did not inhibit IL-1β (Fig 4.3.2 and 4.3.3). A potential explanation is the low stimulatory effect on insulin in UR-or IS-treated cells.

Fig 4.6.4 show that MS dose-dependently enhanced insulin secretion, but the increase was not significantly different from that in Glu-treated or control cells. Fig. 4.6.5. showed that AM dose-dependently increased the insulin secretion only at 1 and 10µM. In vivo studies in diabetic subjects showed that administration of AM juice, tablet or fruits decreased blood glucose levels (*35-37*) and it has been suggested that the reduced blood glucose levels may have been due to insulin stimulation by AM. Moreover, the inhibitory effect of AM on Glu-induced IL-1β (Fig 4.3.5) may be a contributing factor for insulin stimulation.

Figure 4.6.1. Effect of ellagic acid (EA) at different concentrations on glucose (Glu)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu for 24 h and EA (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
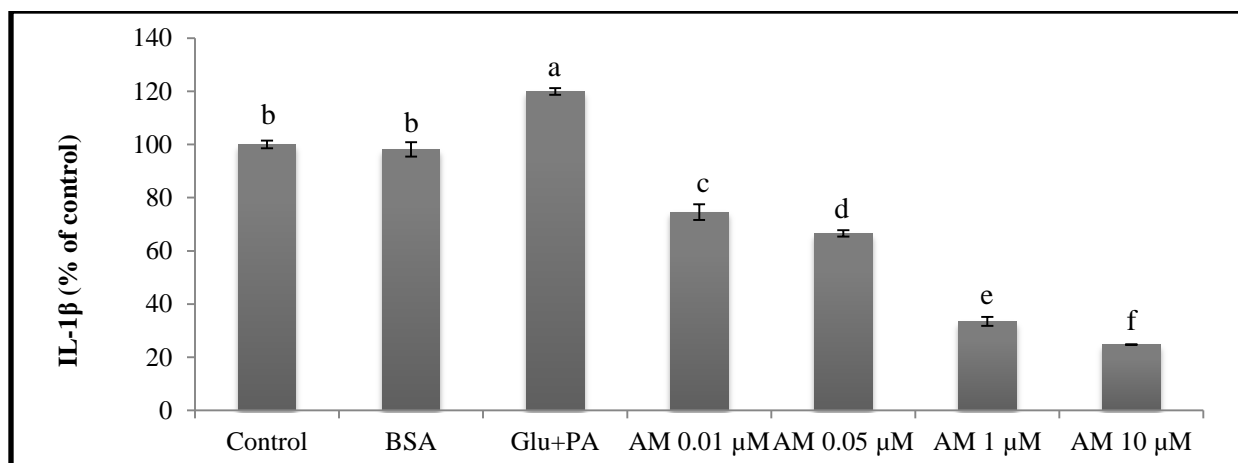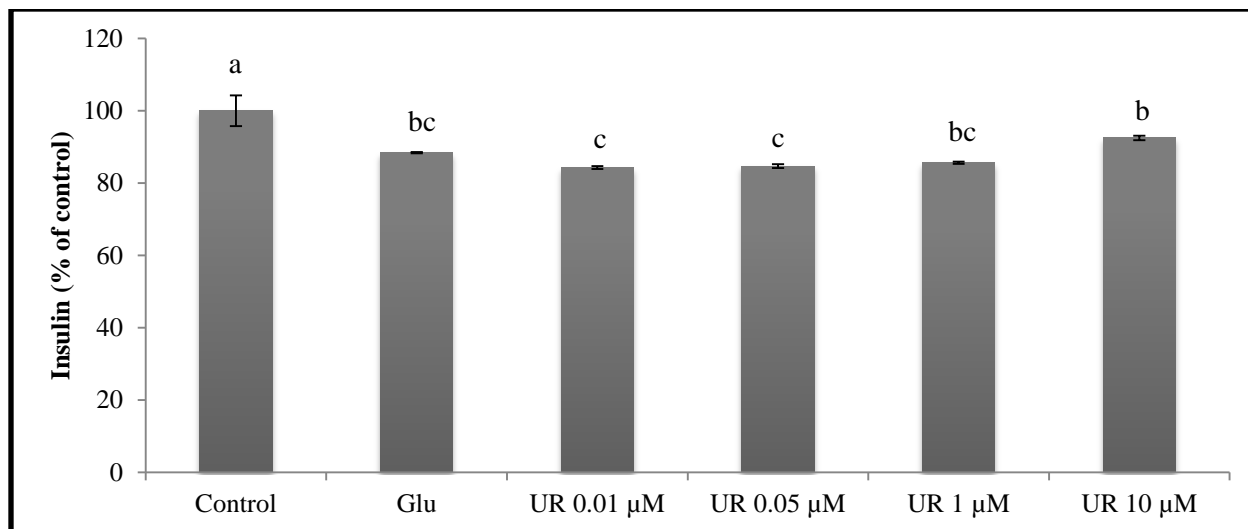
Figure 4.6.2. Effect of urolithin A (UR) at different concentrations on glucose (Glu)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu for 24 h and UR (0.01-10μM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
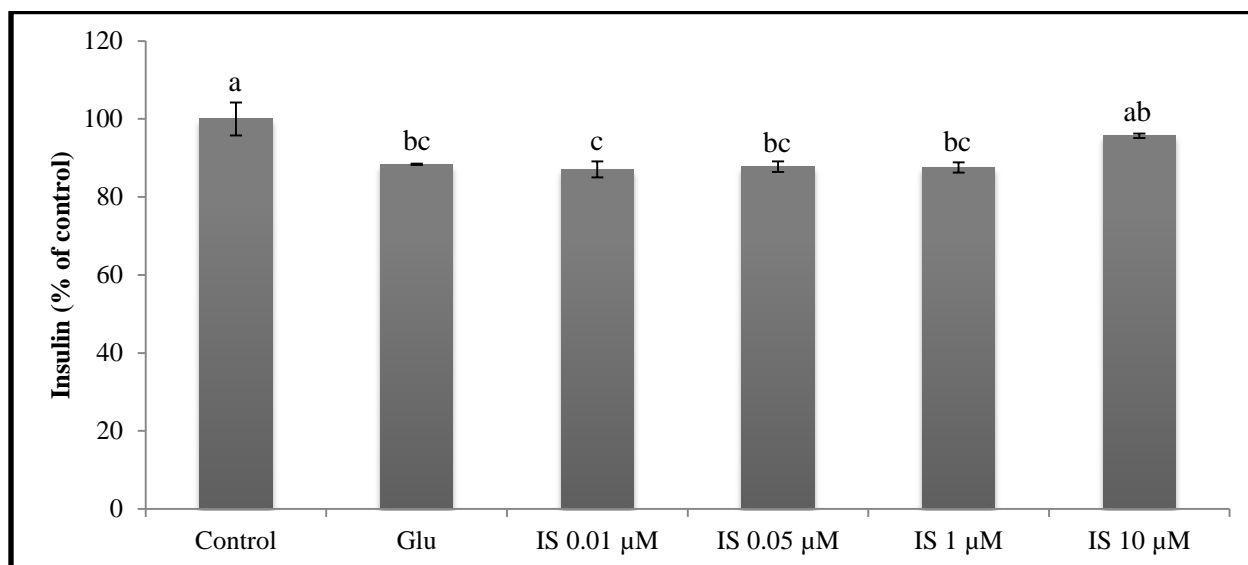
Figure 4.6.3. Effect of isorhamnetin (IS) at different concentrations on glucose (Glu)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu for 24 h and IS (0.01-10μM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
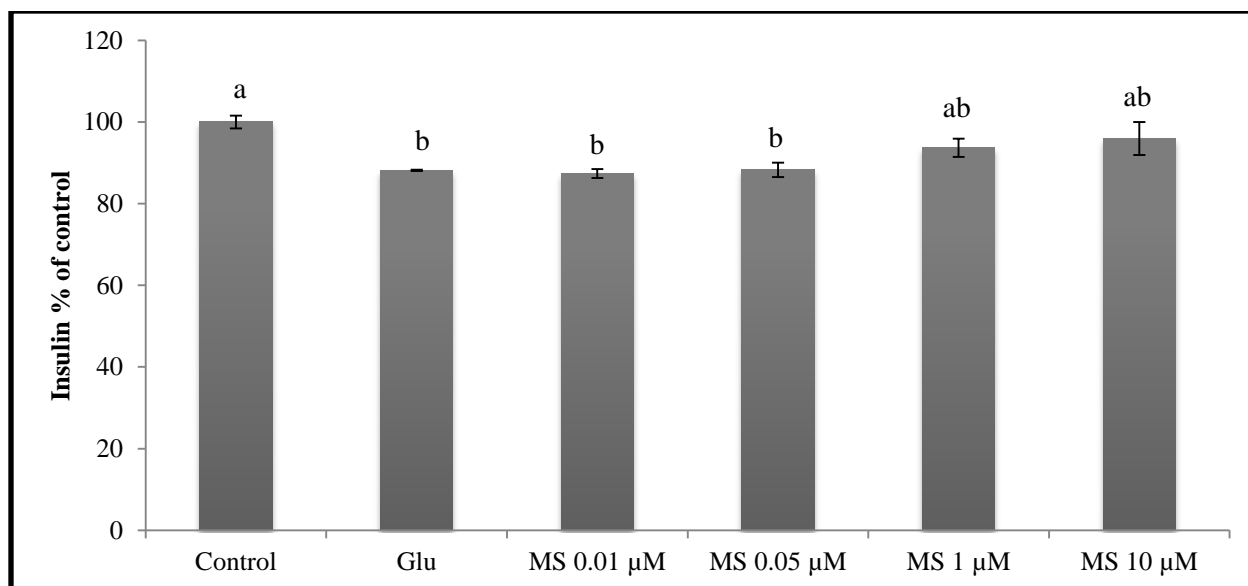
Figure 4.6.4. Effect of muscadine extract (MS) at different concentrations on glucose (Glu)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu for 24 h and MS (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
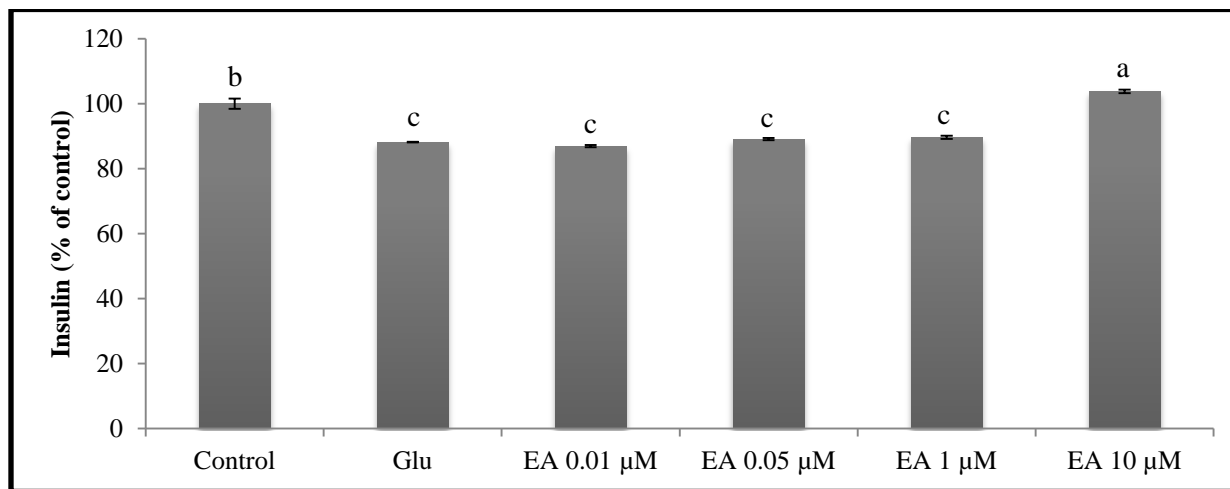

Figure 4.6.5. Effect of amla extract (AM) at different concentrations on glucose (Glu)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu for 24 h and AM (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
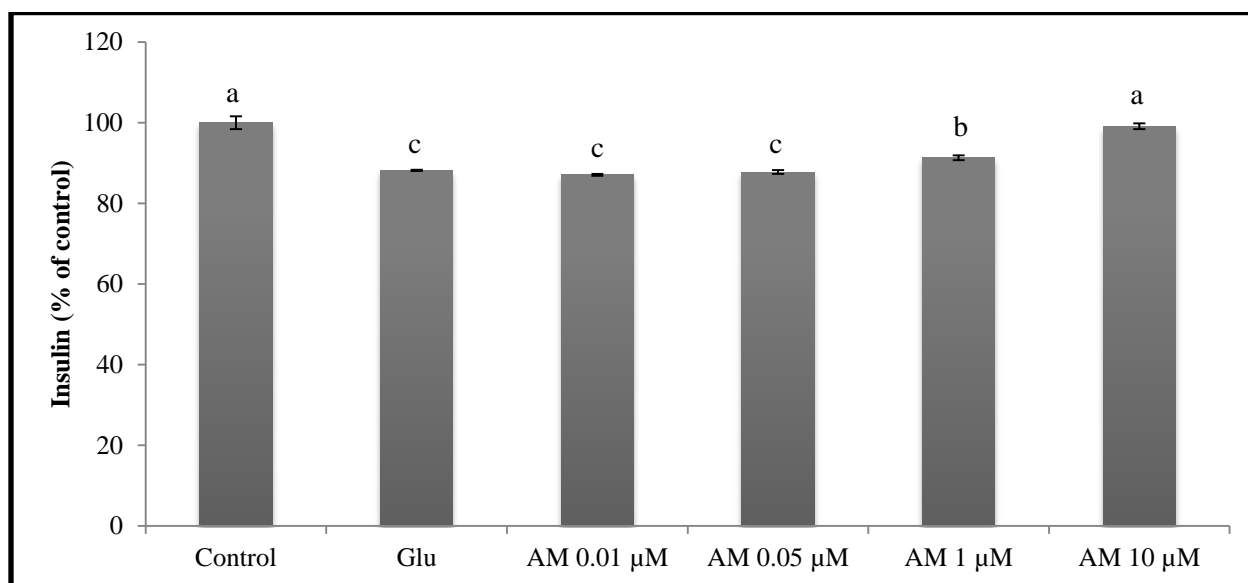
4.7. Effect of EA, UR, IS, MS or AM on insulin secretion in PA-treated NIT-1 cells

To study the effect of EA, UR, IS, MS or AM on PA-reduced insulin secretion, the supernatants from the NIT-1 cells were analyzed for insulin by ELISA. The results are presented in Fig. 4.7. and expressed as insulin (% of control). The results show that PA treatment decreased insulin secretion by $4.8 \pm 0.3\%$ of control. Exposure of NIT-1 cells to Glu reduced insulin secretion to a much lower concentration (11.8 ± 0.14 %) than in the cells that were exposed to PA, compared to control. It may have been due to higher induction of IL-1β (38.29 ± 1.85 %, Fig. 4.3) by Glu than PA ($6.84 \pm 0.88\%$, Fig. 4.4) compared to control.

Treatment with BSA alone did not have any effect on insulin compared to control suggesting that the decreased insulin secretion was due to PA alone. It has been shown that incubation of rat islets with PA for 48 h decreased insulin secretion by 30 - 50% and proinsulin biosynthesis by 30 - 40% (*66*). Rat pancreatic islets exposed to 250 µM PA for 48 h showed inhibition of insulin synthesis as well as glucose stimulated insulin secretion. It was reported by Maedler et al. that incubation of human pancreatic islets with 500 µM PA for 4 days decreased the insulin content as well as chronic insulin secretion (*65*).

The results in Fig 4.7.1 show that EA dose-dependently up-regulated the insulin secretion that was reduced by PA. Among the concentrations of EA (0.01-10 µM) tested, EA at 10µM significantly increased insulin equivalent to control. Malini et al. reported that oral administration of EA at 50mg/kg and 100 mg/kg of body weight for 35 days dose-dependently reduced glucose and increased insulin secretion in streptozotocin-induced diabetic rats (*19*).

Fig 4.7.2 show that treatment of NIT-1 cells with UR at different concentrations (0.01-10 µM) did not have any influence on the stimulation of insulin secretion that was reduced by PA. It may be due to the inability of UR to inhibit IL-1β (Fig 4.4) and also the low antioxidant potential of UR compared to EA (*141*). Among the IS-treated cells (Fig 4.7.3), only IS at 10 µM

46

significantly increased (P < 0.05) insulin secretion by 13.08 ± 0.35 % compared to control. The results of this study show that IS can be effective in up-regulating insulin at concentrations higher than 10 µM.

The results in Fig 4.7.4 and 4.7.5 show that MS or AM containing 0.01-10 µM equivalent EA dose-dependently increased insulin secretion. In the cells treated with 1µM of MS or AM, the insulin concentration was significantly higher (P < 0.05) than PA and similar to the control. MS and AM at 10µM significantly stimulated insulin by 10.4 ± 0.32 and 10.6 ± 0.25%, respectively compared to control. MS or AM contained several bioactives which synergistically may have stimulated insulin secretion. Vattem et al. suggested that the synergistic effect of whole foods is due to the enhanced activity of one bio-active compound in presence of another compound resulting in greater functionality and reduced need of the concentration of each bio-active compound (*68*).

Figure 4.7.1. Effect of ellagic acid (EA) at different concentrations on palmitic acid (PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with PA for 24 h and EA (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
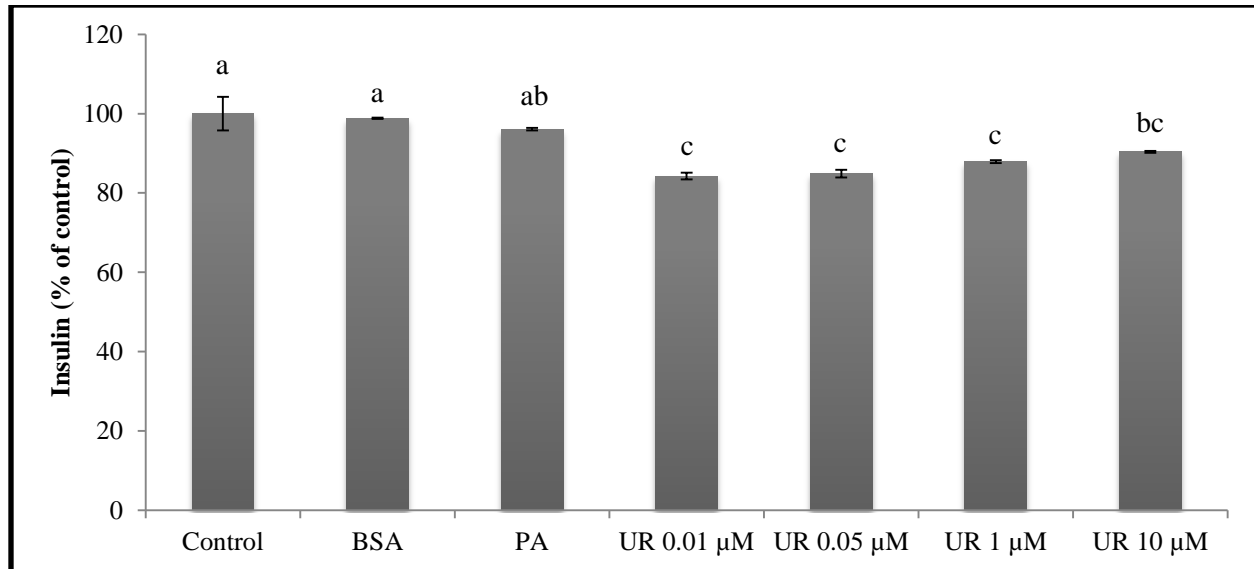
Figure 4.7.2. Effect of urolithin A (UR) at different concentrations on palmitic acid (PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with PA for 24 h and UR (0.01-10μM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
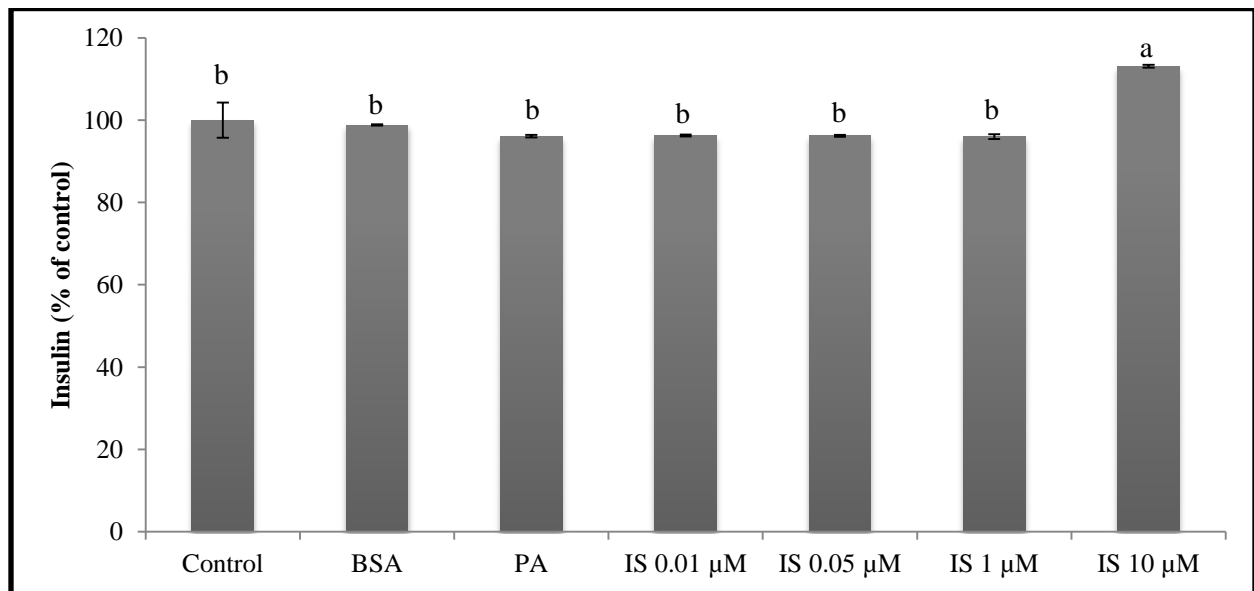
Figure 4.7.3. Effect of isorhamnetin (IS) at different concentrations on palmitic acid (PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with PA for 24 h and IS (0.01-10μM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
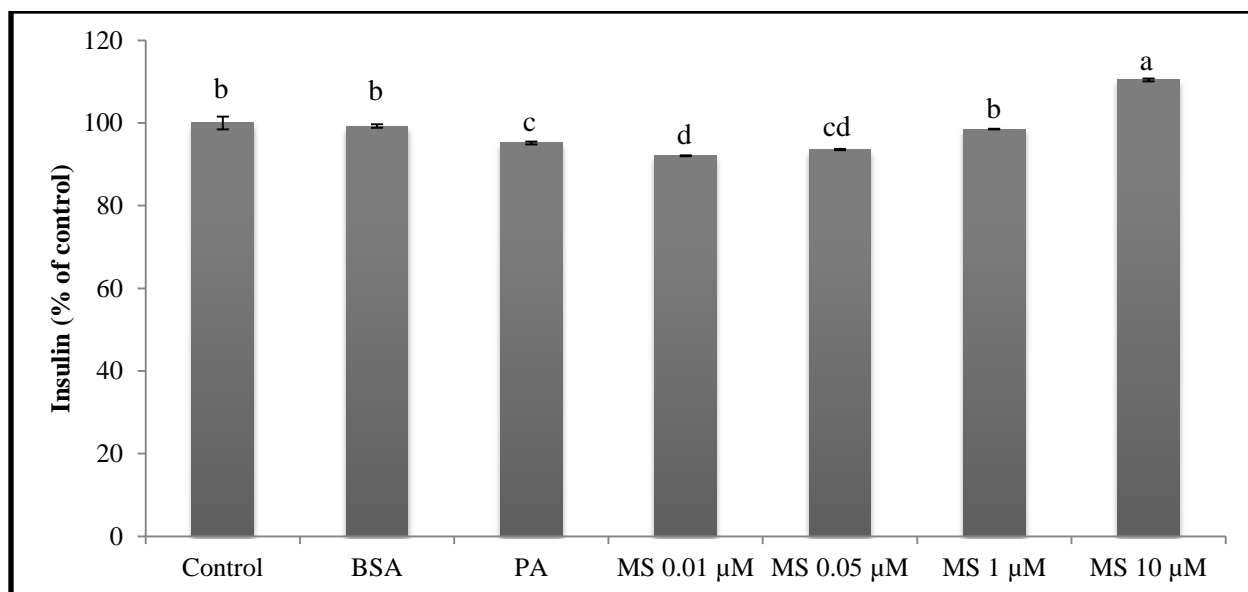
Figure 4.7.4. Effect of muscadine extract (MS) at different concentrations on palmitic acid (PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with PA for 24 h and MS (0.01-10μM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
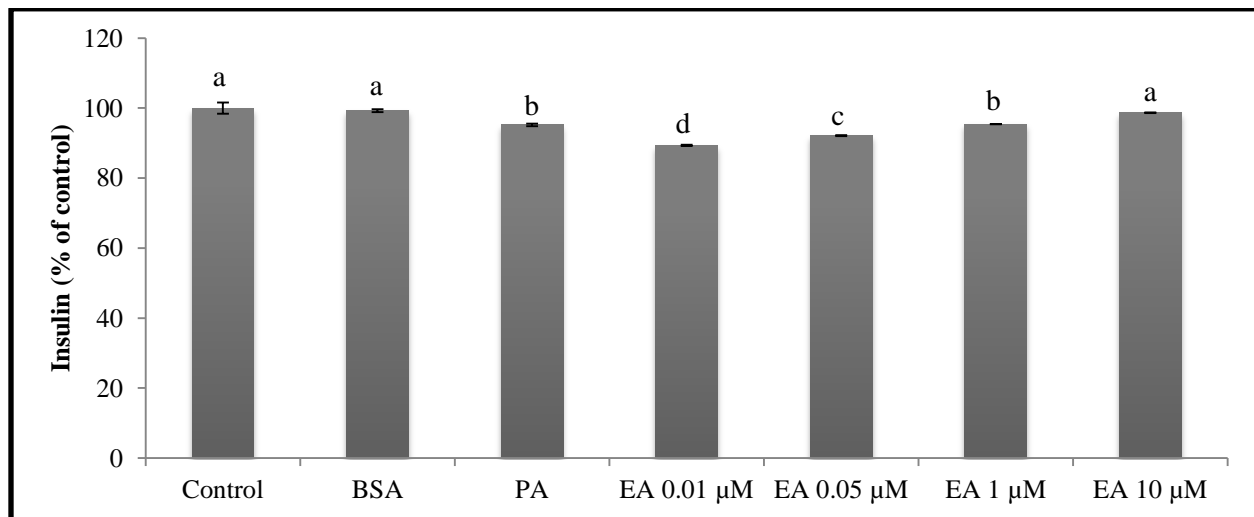

Figure 4.7.5. Effect of amla extract (AM) at different concentrations on palmitic acid (PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with PA for 24 h and AM (0.01-10μM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

4.8. Effect of EA, UR, IS, MS or AM on insulin secretion in Glu+PA-treated NIT-1 cells

To study the effect of EA, UR, IS or MS or AM on Glu+PA-reduced insulin secretion, the supernatants from NIT-1 cells were analyzed by ELISA. The results are presented in Fig 4.8 and reported as insulin (% of control). Exposure to Glu+PA inhibited insulin secretion by $10.4 \pm 0.55\%$ compared to the control.

Glu+PA reduced insulin secretion more than PA ($4.8 \pm 0.3\%$) and less than Glu (11.8 ± 0.14 %) (Fig 4.8). It may have been due to higher induction of IL-1β by Glu+PA (Fig 4.5) than PA alone (Fig 4.4) and lower than that induced by Glu (Fig 4.3). Exposure to Glu and free fatty acids can induce oxidative stress that can increase IL-1β levels. The increased concentrations of IL-1β may interfere with the insulin signal transduction and lead to faulty insulin secretion process (*9, 10, 81*). In the present study, inhibitory effect of Glu+PA on insulin was observed in agreement with the previous studies.

Fig 4.8.1 shows the effect of different concentrations of EA on Glu+PA treated NIT-1 cells. The results show that EA dose-dependently increased the insulin secretion. Nevertheless, the increase was not significantly different from Glu+PA treated cells and lower than that of control. The results from UR treated cells are presented in Fig 4.8.2. Similar to the results with either Glu (Fig 4.6.2) or PA (Fig 4.7.2) treatment, UR did not show any significant influence on insulin secretion that were reduced by Glu+PA. The secretion of insulin were similar to that of Glu+PA and less than the control.

The results from Fig 4.8.3 show that treatment with IS significantly increased ($P < 0.05$) the insulin secretion compared to treatment with Glu+PA. The insulin secretion in NIT-1 cells treated with IS at 0.01-1µM was similar to that of control. Moreover, IS at 10µM increased insulin secretion by 13.06 ± 0.17 % of control. Yokozawa et al. reported that administration of IS

to diabetic rats decreased the blood glucose levels which may have been due to stimulation of insulin by IS (*22*).

Results in Fig 4.8.4 show that MS containing 0.01-10µM EA equivalent dose-dependently increased insulin secretion. However, the increase was not significantly different from the insulin secretion by the control NIT-1 cells. Supplementation of MS juice to diabetic subjects was associated with decrease in blood glucose (*26*). Results in Fig 4.8.5 show that AM dose-dependently increased insulin secretion that was reduced by Glu+PA. However, only AM at 10 µM up-regulated insulin similar to that of control.
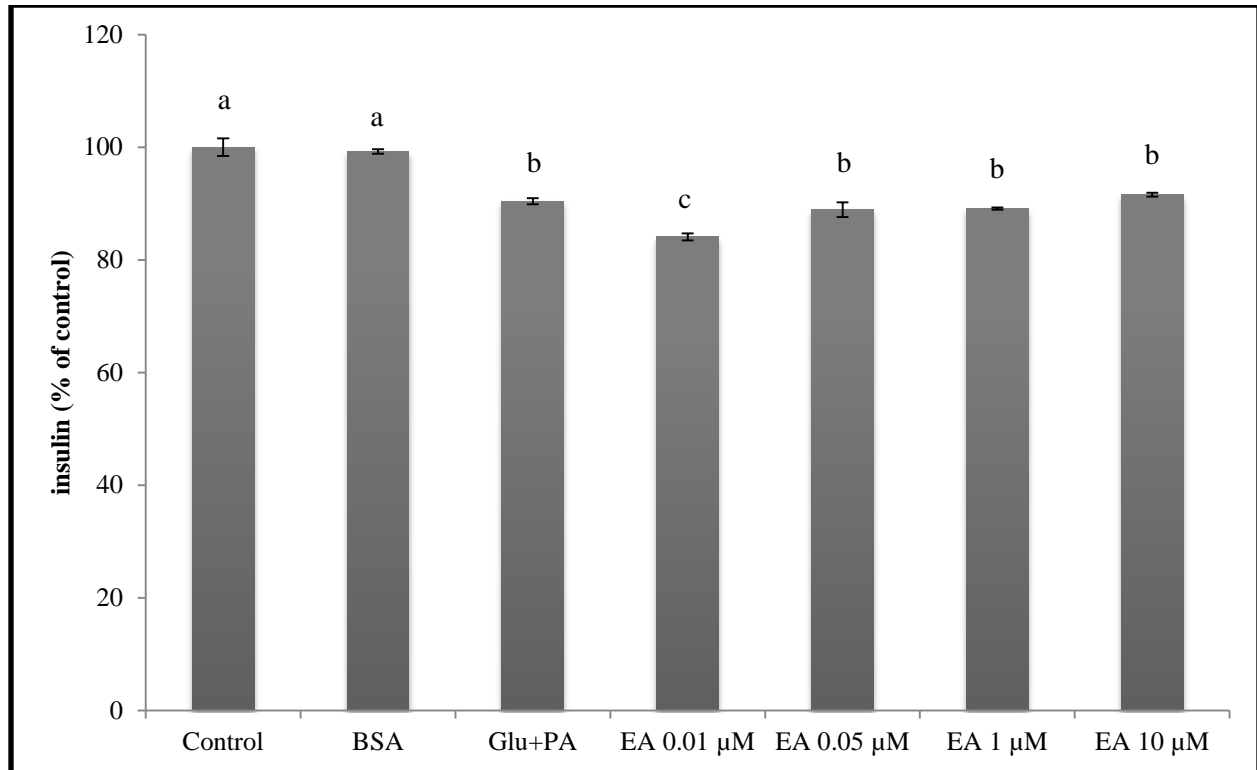
Figure 4.8.1. Effect of ellagic acid (EA) at different concentrations on glucose+palmitic acid (Glu+PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and EA (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
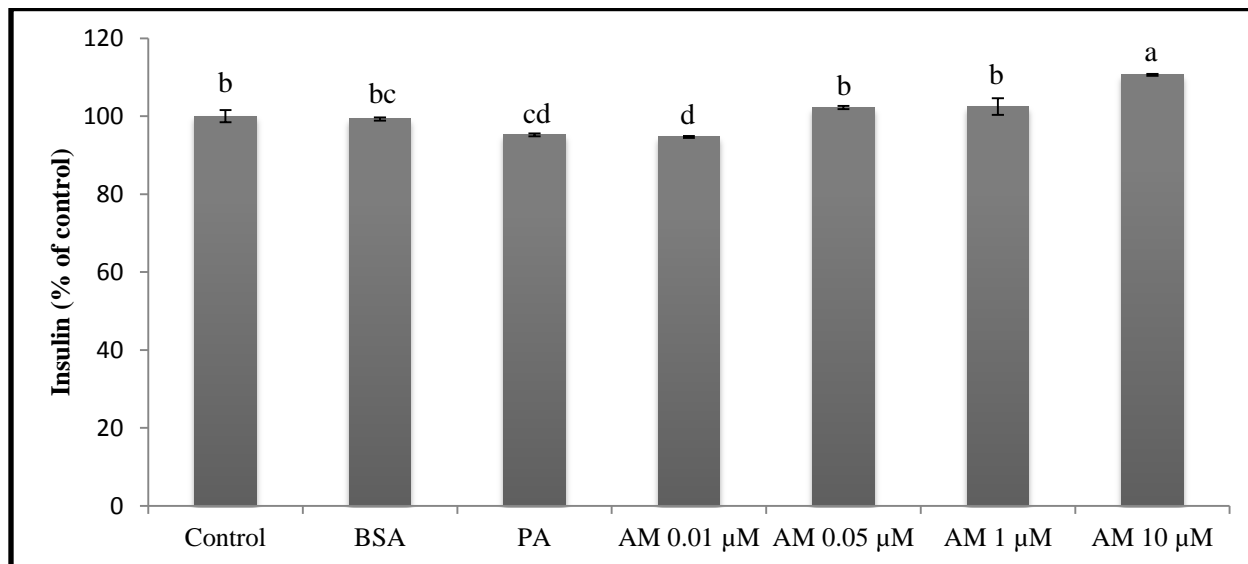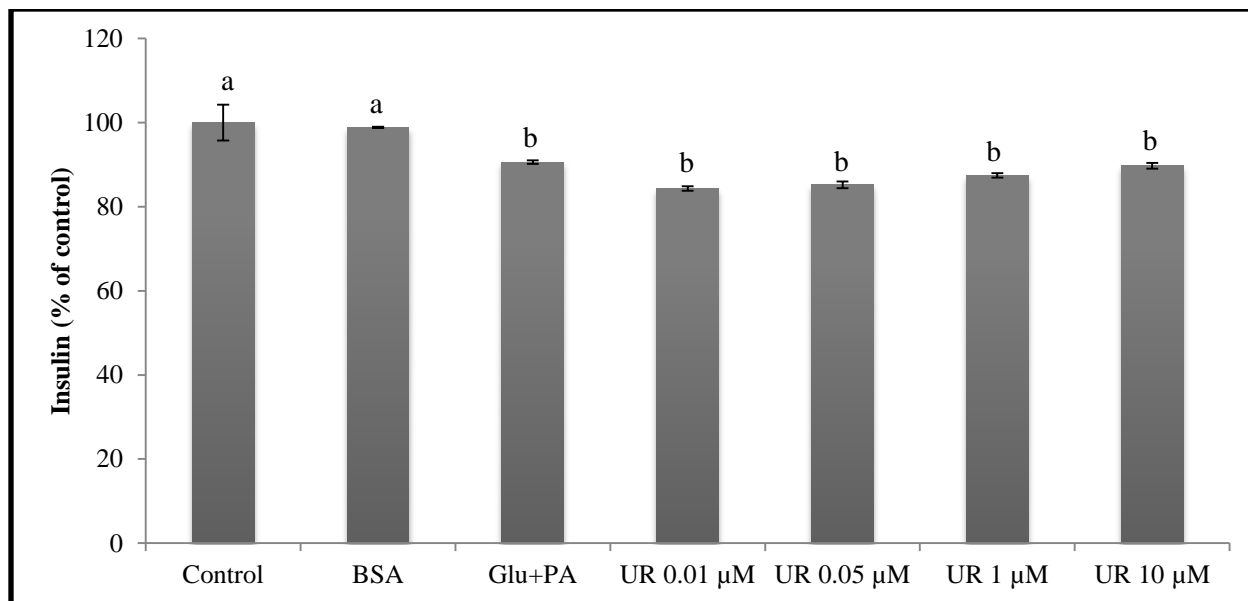
Figure 4.8.2. Effect of urolithin A (UR) at different concentrations on glucose+palmitic acid (Glu+PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and UR (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
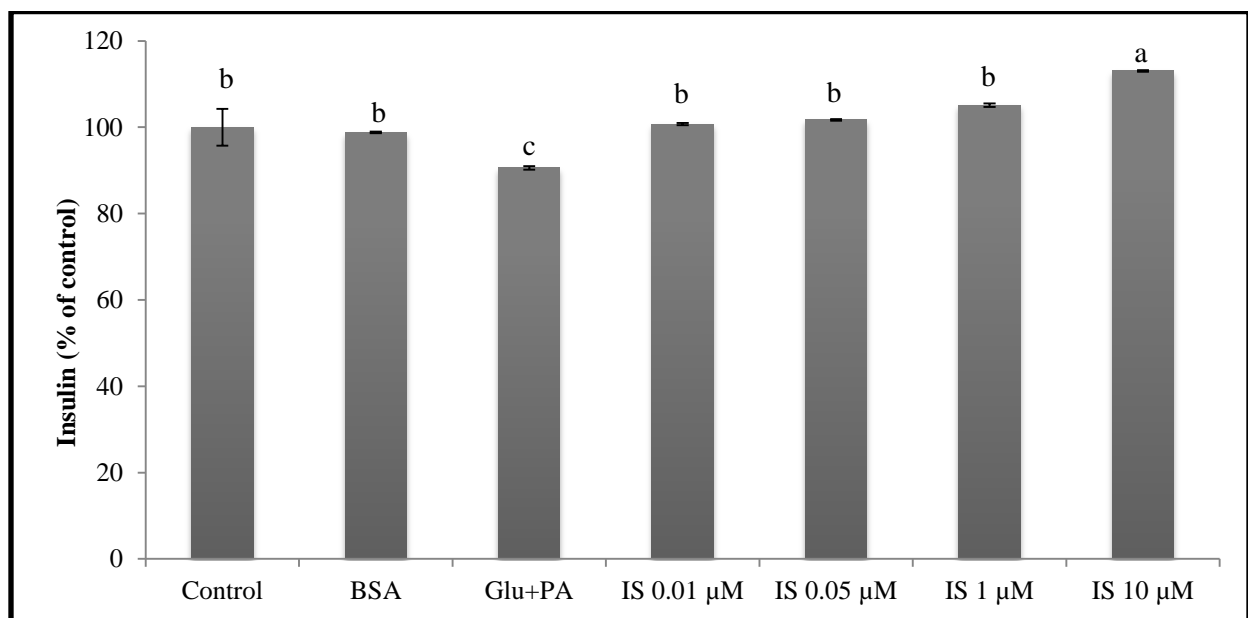
Figure 4.8.3. Effect of isorhamnetin (IS) at different concentrations on glucose+palmitic acid (Glu+PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and IS (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
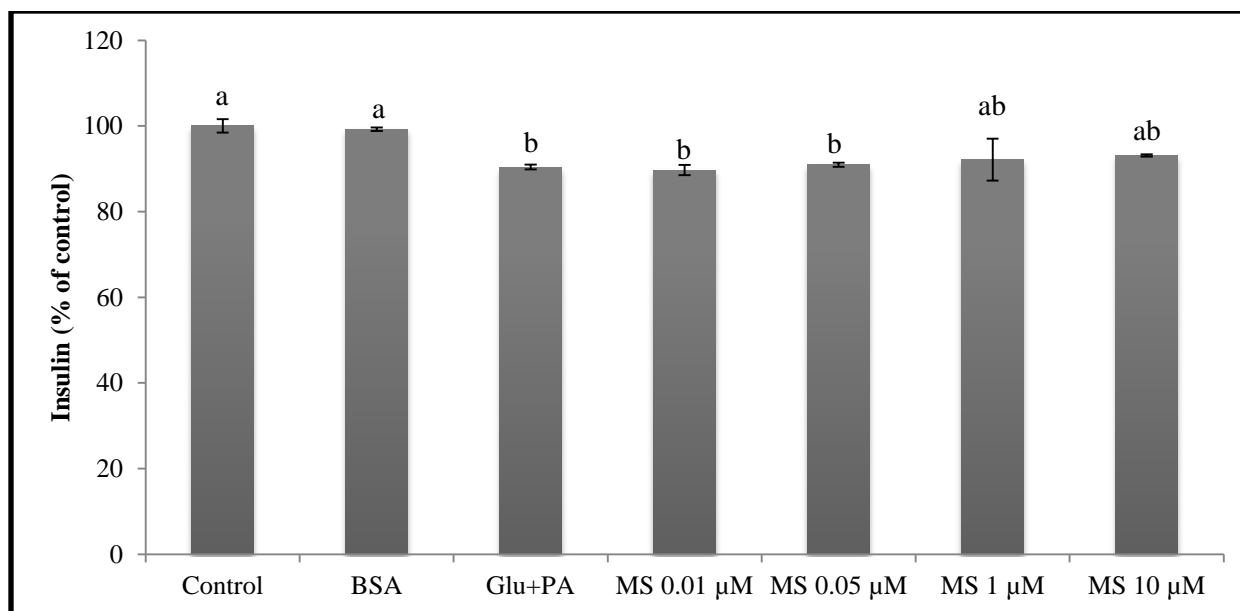
Figure 4.8.4. Effect of muscadine extract (MS) at different concentrations on glucose+palmitic acid (Glu+PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and MS (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.
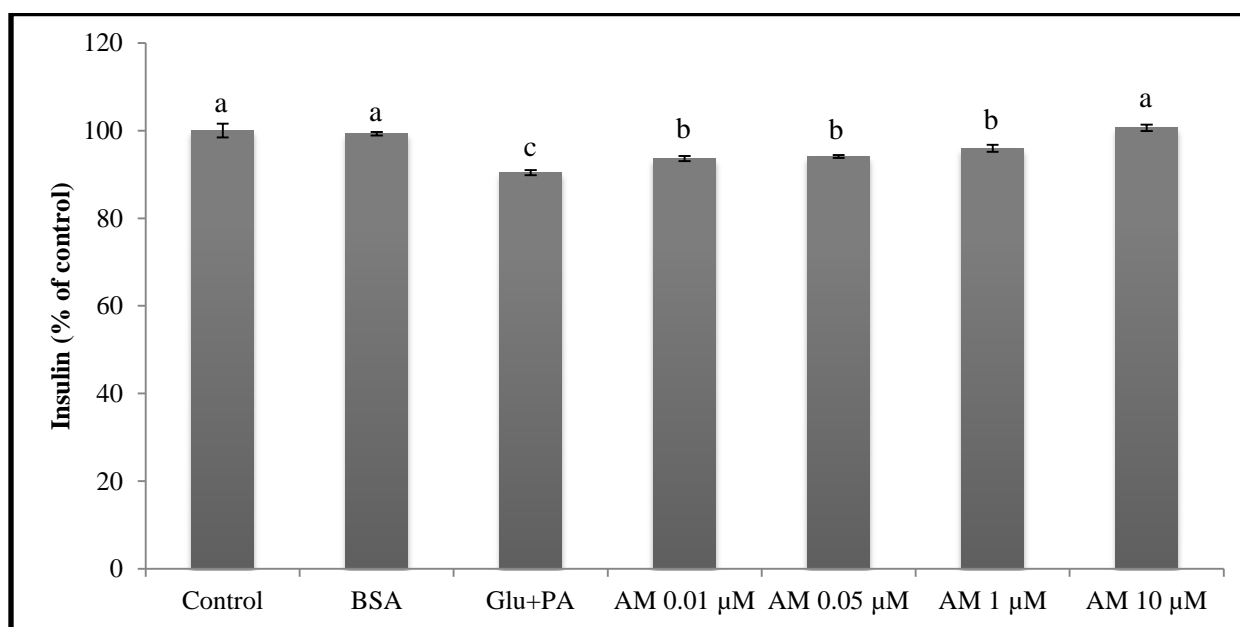
Figure 4.8.5. Effect of amla extract (AM) at different concentrations on glucose+palmitic acid (Glu+PA)-reduced insulin secretion in NIT-1 cells. Cells were incubated with Glu+PA for 24 h and AM (0.01-10µM) was added followed by additional incubation for 72 h. Insulin concentration was measured by ELISA. The values are expressed as percentage of untreated control. Results are presented as mean ± S.D, (n=2). Letters with different superscripts are significantly different (P < 0.05) among groups.

CHAPTER 5
CONCLUSIONS

Exposure of NIT-1 cells to 33.3mM glucose or 250 μM palmitic acid for 96 h significantly increased IL-1β and reduced insulin secretion. Exposure of NIT-1 cells to glucose was associated with more IL-1β secretion and insulin secretion reduction than exposure to palmitic acid. Glucose or palmitic acid-induced IL-1β secretion was dose-dependently inhibited by ellagic acid, muscadine or amla extract standardized to its ellagic acid content, whereas ellagic acid metabolite urolithin A or isorhamnetin treatment did not inhibit the glucose or palmitic acid-induced IL-1β secretion. Glucose + palmitic acid-induced IL-1β secretion was dose-dependently down-regulated by ellagic acid, muscadine or amla extract, or isorhamnetin whereas urolithin A showed significant inhibition only at 1 μM or 10 μM.

Insulin secretion in glucose or palmitic acid treated NIT-1 cells was dose-dependently stimulated by ellagic acid, muscadine or amla extracts. Urolithin A or isorhamnetin did not stimulate insulin secretion in glucose-treated NIT-1 cells. However, palmitic acid-reduced insulin secretion was stimulated by isorhamnetin at 10 μM only, whereas urolithin A did not stimulate insulin in palmitic acid treated cells. Glucose + palmitic acid-reduced insulin secretion was dose-dependently stimulated by muscadine or amla extract, or isorhamnetin. Ellagic acid or its metabolite urolithin did not stimulate insulin secretion in glucose + palmitic acid treated NIT-1 cells.

IL-1β inhibition and stimulation of insulin by ellagic acid, muscadine or amla extracts containing equivalent amount of ellagic acid suggests that ellagic acid or ellagic acid-rich foods can be effective modulators of inflammation in diabetes. The metabolite of ellagic acid, urolithin A did not inhibit IL-1β or stimulate insulin secretion. Isorhamnetin at concentration higher than 10 μM may be an effective modulator of palmitic acid-induced inflammation in NIT-1 cells.

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VITA

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