doi.org/10.36721/PJPS.2020.33.3.SUP.1261-1270.1

In-vivo and *in-vitro* studies to investigate the anti-diabetic mechanisms underlying *Moringa oleifera* leaf ethanol extracts

Hafiz Muhammad Irfan[1,2]*, Mohd Zaini Asmawi[2], Nurzalina Abdul Karim Khan[2], Alamgeer[3], Sajid Bashir[1], Zahid Rasool[4], Shafiq-ur-Rehman[5] and Humayun Riaz[6]

[1]College of Pharmacy, University of Sargodha, Sargodha, Pakistan
[2]School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia
[3] Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan
[4]Department of Basic Health Sciences, Faculty of Pharmacy, Gomal University, DI Khan, Pakistan
[5]Department of Pharmacy, University of Central Punjab, Lahore, Pakistan
[6]Rashid Latif College of Pharmacy, Lahore, Pakistan

Abstract: Obesity, insulin resistance and cardiovascular complications are endemic global health diseases due to ingestion of high carbohydrate. To prevent such health complications, this study was conducted to report another appealing option that can prevent postprandial hyperglycemia. For *in vivo* studies, 95% ethanol (v/v) extract of *Moringa oleifera* and its butanol fraction were given orally once a day for 14-days to streptozotocin-induced diabetic rats. The blood samples were collected to determine plasma insulin, monocyte chemoattractant protein-1 (MCP-1) and glucose-6-phosphate (G6P) level. α-amylase and α-glucosidase enzyme kinetics against fixed concentrations of samples (100 µg/mL) was conducted. The *M. oleifera* ethanol (v/v) extract at 1000 and 500 mg/kg and butanol fraction at 500 mg/kg dose raised the level of G6P and lowered MCP-1 level significantly but failed to increase the level of insulin significantly. The results also indicated non-competitive inhibition of α-amylase, α-glucosidase and a significant increase in glucose uptake by rectus abdominus muscle.

Keywords: α-Amylase; α-glucosidase; glucose-6-phosphate; monocyte chemoattractant Protein-1; *Moringa oleifera*; skeletal muscle.

INTRODUCTION

Obesity and its related problems like diabetes, cardiovascular diseases and metabolic syndrome have worldwide distribution. Consumption of diet with high carbohydrate contents can raise the index of such medical problems (Barrett and Udani, 2011). Natural products of plant origin (herbs) are being ingested as dietary components due to their edible nature. Many are reported to have potential to treat diabetes mellitus either by numerous anti-diabetic mechanisms (Coman *et al.,* 2012). α-Amylase hydrolyzes soluble starch, amylose, and amylopectin present in food (Dutta *et al;* 2006), while α-glucosidase causes further breakdown of disaccharides (maltose) into monosaccharides (glucose). By inhibiting these two enzymes, the glucose absorption into the blood and postprandial glycemia can be deferred. Consequently, rigorous scientific researches have been conducted to identify naturally occurring α-amylase and α-glucosidase inhibitors present in plants (Seo *et al.,* 2005). *M. oleifera* Lam. is a famous edible plant that has been evaluated worldwide for its anti-diabetic effect (Irfan *et al.,* 2016). Researchers have reported that it contained α-amylase and α-glucosidase inhibitors and caused regeneration of pancreatic β-cells (Adisakwattana and Chanathong, 2011; El-Desouki *et al.,* 2015). Methanol extract has also been reported to enhance glycogen synthesis and uptake of glucose through peripheral tissues (Olayaki *et al.,* 2015). Recently, the bioactive compounds in *M. oleifera* 95% ethanol (v/v) extract responsible for anti-hyperglycemic effect were identified (Irfan *et al.,* 2017). Therefore, here in this study an attempt was made to see the effect of *M. oleifera* 95% ethanol (v/v) extract and its butanol fraction on intestinal glucose absorption inhibition, plasma insulin, MCP-1, glucose-6-phosphate and glucose uptake by skeletal muscle. Moreover, this study also provides enzyme kinetic studies underlying its intestinal enzyme inhibition.

MATERIALS AND METHODS

Chemicals and apparatus

α-Amylase from *Aspergillus oryzae* (Sigma-Aldrich, USA), 3,5-Dinitrosalicylic acid (Sigma Aldrich, USA), acarbose (Glucobay, Bayer, Germany), α-glucosidase *saccharomyces cerevisiae* (Sigma-aldrich, USA), wheat soluble starch, p-nitro-phenyl-α-D-glucopyranoside (Sigma aldrich, USA), anhydrous monobasic sodium phosphate, sodium phosphate monobasic dehydrate (Sigma-aldrich, USA), sodium phosphate dibasic (Sigma-aldrich, USA), quercetin 3-β-Dglucoside (Sigma-Aldrich, USA), kaempferol-3-O-glucoside (Extrasynthese), cryptochlorogenic acid (Wuhan ChemFaces Biochemical Co., Ltd.), 96-well microtitre sterile plates (Nunc,

*Corresponding author: e-mails: muhammad.irfan@uos.edu.pk

Rosklide, Denmark), insulin ELISA kit (Abcam), rat MCP-1 ELISA kit (Elabscience Biotechnology Co., Ltd.) and glucose-6-phosphate assay kit (Sigma, USA).

Plant preparation

Moringa oleifera leaf was purchased from Herbagus Sdn. Bhd. (Pulau Pinang, Malaysia). The plant specimen was authenticated by Dr. Rahmad Zakaria and assigned the herbarium voucher no. 11626. The powdered *Moringa oleifera* leaf was extracted with 95% ethanol (v/v) through maceration process and butanol fraction was prepared through solvent-solvent extraction.

Animal study design

Forty-two healthy male adult Sprague Dawley rats (230 ± 30 g) were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia. Six rats were placed in normal control group (distilled water, 10 ml/kg) and 36 rats received administration of Streptozotocin (45 mg/kg) intraperitoneally to induced diabetes. Three days after Streptozotocin induced diabetes, the rats were then divided into six groups receiving oral gavage once a day for 14-days as follows: 10 ml/kg of distilled water (diabetic control); metformin, 500 mg/kg (postive control); *M. oleifera* leaf 95% ethanol (v/v) extract (group 3, 4 and 5 at doses of 250, 500 and 1000 mg/kg respectively) and butanol fraction of 95% ethanol (v/v) extract at a dose of 500 mg/kg. At the end of 14-days treatment, the animals were anesthetized (Pentobarbitone, 6 mg/100g) and the blood samples were collected to determine plasma insulin, MCP-1 and G6P level using ELISA kits. The animal studies were performed after approval from the Animal Ethics Committee of Universiti Sains Malaysia (approval No. AEA/2015/613).

In-vitro enzyme activity

α-Amylase inhibitory assay

The α-amylase inhibitory activity of 95% ethanol extract, its butanol fraction with querecetin 3β-D-glucoside, kaempferol-3-O-glucoside, cryptochlorogenic acid and acarbose as reference standard was measured at (Saeed and Ahmad, 2015) different concentration (6.25, 12.5, 25, 50 and 100 µg/mL). Briefly, 125µL of all samples and standards were added into their respective labeled 5mL Eppendorf tubes. Then 125µL α-amylase solution was added and incubated for 30 min at room temperature. After that, 250µL starch solution (0.5%) was added. Incubation was done for 3 min and 250µL DNS reagent was added and then heated for 15 min at 85°C in water bath. The reaction mixture was cooled to room temperature and 2.25 mL distilled water was added into each Eppendorf tube. From this, 200µL solution was taken in 96 well microplate and absorbance was measured at 540 nm using microplate reader (Power Wave X 340, Biotek Instruments Inc, US). Similar protocol was prepared for the standards and 100% control activity (DMSO). Blank samples were also prepared for every concentration of extract, fraction and standard. In this assay, the enzyme solution was replaced by equal amount of phosphate buffer solution. For final calculation, the reading of blank was subtracted from sample extract, fraction and standard. The percentage inhibitory activity was determined using following formula. The assay was done in triplicate for every sample to find IC_{50} values.

$$\text{Percentage inhibitory activity} = \frac{\text{(Control absorbance – sample absorbance)}}{\text{Control absorbance}} \times 100$$

Enzyme kinetics/mechanism of α-amylase inhibitors

The enzyme kinetics of α-amylase inhibitory activity was done using the method described by Ali et al. (2006). A fixed concentration of samples (100 µg/mL) and alpha-amylase (0.5 mg/mL) was used in this assay against different substrate concentration (5, 2.5, 1.25, 0.625 and 0.31mg/mL starch solution). Briefly, 250µL of samples (95% ethanol extract, butanol fraction and DMSO for control) were respectively added into 5 mL Eppendorf set of tubes (5 tubes for enzyme activity and 5 for blank). Then, 250µL α-amylase solution (0.5 mg/mL) was added into all set of tubes except blank tubes to which was added equal amount of phosphate buffer without enzyme. The resultant solution was incubated for 10 min at room temperature (25°C). After incubation period, 250µL of five different concentrations of starch were added into their respective set of tubes and incubated again for 10 min at room temperature. Then DNS solution (500µL) was added into all tubes and boiled for 5 min in water bath. From each test tube, 200µL solution was transferred into a 96 well microplate and the absorbance was measured at 540 nm using microplate reader (Power Wave X 340, Biotek Instruments Inc, US). The readings were measured in triplicate. The mechanism of enzyme inhibition was determined through Lineweaver-Burk plot and Michaelis-Menten enzyme kinetics [1/V versus 1/S].

α-Glucosidase inhibitory assay

The α-glucosidase inhibitory activity of 95% ethanol extract, fraction and standards (acarbose, quercetin 3-β-D-glucoside, kaempferol-3-O-glucoside and cryptochlorogenic acid) was measured at different concentration (6.25-100µg/mL) against 100% control activity (Bak *et al.,* 2011) with minor modifications. Briefly, 50µL of extract, fraction, standards or DMSO (control) were added in to 96 well microplate followed by 100µL α-glucosidase enzyme solution and incubated for 10 min. Then 50µL of 5 mM P-nitrophenyl-α-D-glucopyranoside solution was added to each well and the plate was incubated for another 30 min. The absorbance was read at 540 nm using a microplate reader (Power Wave X 340, Biotek Instruments Inc, US). Blank samples were also prepared for every concentration of extract, fraction and each standard. In this assay, the enzyme solution was replaced by equal amount of phosphate buffer solution. For final calculation, the reading of blank

was subtracted from sample extract, fraction and standard. The percentage inhibitory activity was determined using following formula. The assay was done in triplicate for every sample to find IC_{50} values.

$$\text{Percentage inhibitory activity} = \frac{\text{(Control absorbance – sample absorbance)}}{\text{Control absorbance}} \times 100$$

α-Glucosidase enzyme kinetics

The kinetics of α-glucosidase enzyme inhibition was assayed by Michaelis-Menten-Lineweaver Burk plot with minor modifications (Kazeem *et al.,* 2016). The α-glucosidase enzyme activity of active 95% ethanol extract and butanol fraction (100µg/mL) on substrate (P-nitrophenyl-α-Dglucopyranoside) at 2, 1, 0.50, 0.25 and 0.125 mg/mL was measured. The assay for blank and control was the same as in α-amylase kinetics. Briefly, 50µL samples were taken in 96 well microplate and mixed with 100µL α-glucosidase solution (pH 6.8) and incubated for 10 min at room temperature. Then 50µL of different stock solutions (2-0.125mg/mL) of P-nitrophenyl-αD-glucopyranoside were added into their respective tubes. After 30 min incubation at room temperature and absorbance was measured at 540nm using microplate reader (Power Wave X 340, Biotek Instruments Inc, US). All the observations were recorded in triplicate.

Glucose uptake assay

The triceps femoralis and abdominus skeletal muscle glucose uptake was assayed by using the method of Hassan et al, (2010) with minor modifications. The Sprague dawley rats (300-350g) were sacrificed and tricep femoralis and rectus abdominus muscles were exposed and cut into pieces 0.140-0.160g per muscle piece, placed in Kreb's Ringer bicarbonate (KRB) buffer containing samples (1mg/mL) provided with carbogen gas (95% O_2 and 5% CO_2) for 10 min to acclimatize the muscle. Initial base line reading was measured by Stat Fax Analyzer, by adding 30µL of each sample into 3mL of Glucose GOD-PAP reagent (Cobas, Roche) respectively, after incubating the resultant solution for 20 min. Then KRB solution containing samples and muscle was incubated for 30 min on a shaker and final reading was taken in the presence and absence of insulin; Novorapid (1U/mL).

$$\text{Glucose uptake (mg/dL/g)} = \frac{\text{Glucose reading before incubation – glucose reading after incubation}}{\text{Weight of tissue}}$$

STATISTICAL ANALYSIS

The results were analyzed statistically as Mean ± SEM, one way ANOVA followed by Dunnett and independent T-Test. Graph Pad Prism (Version 6) was used to analyze the data graphically and statistically significant.

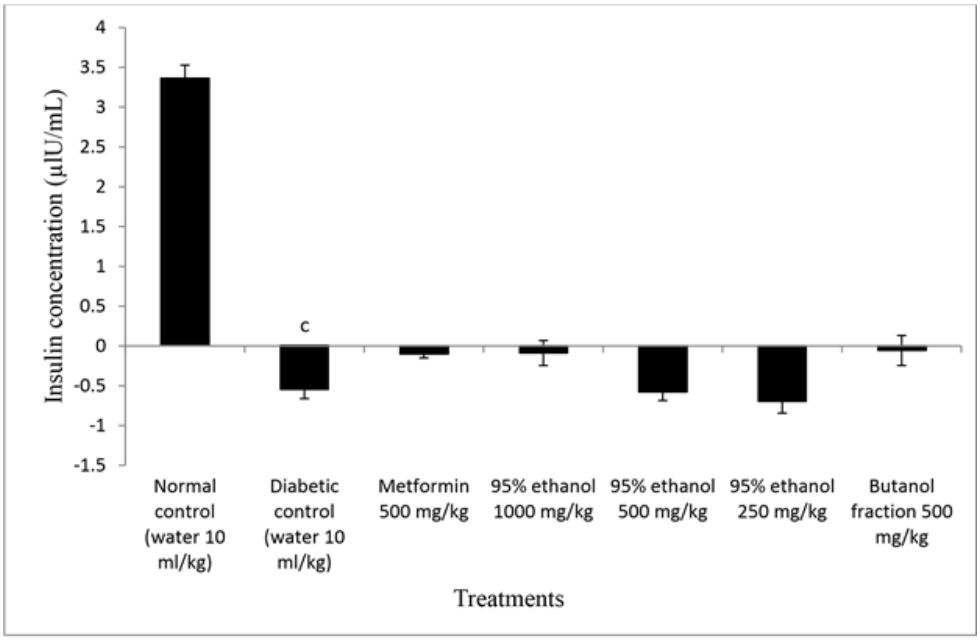

Fig. 1: Effect of daily oral treatment for 14 days of various doses (250, 500 &1000mg/kg) of 95% ethanol extract and its butanol fraction on plasma insulin concentration of streptozotocin induced diabetic rats. Values are expressed as Mean±S.E.M and negative values are due to background noise. $^{c}p<0.001$ vs normal control analyzed by using independent-sample T test.

RESULTS

Effect on insulin release from pancreatic β-cells of pancreas

The insulin level was significantly higher in normal rats compared to diabetic-induced rats as shown in fig. 1. While the insulin level in diabetic control rats was negative, diabetic rats treated with metformin, 1000 mg/kg of 95% ethanol extract and butanol fraction (500 mg/kg) exhibited an improvement in the plasma insulin level; although relatively non-significant when statistically compared to diabetic control group. In contrast to 1000 mg/kg of 95% ethanol extract, lower doses of 250 and 500 mg/kg showed no improvement in insulin plasma level in diabetic rats.

Effect on plasma MCP-1 level

Intraperitoneal administration of STZ resulted in a significant increase (by 51.61%) in the plasma MCP-1 level of diabetic control rats. However, metformin caused significant reduction in plasma MCP-1 level compared to diabetic control. Similarly, the diabetic rats treated with 95% (v/v) ethanol extract (500 and 1,000 mg/kg) and butanol fraction 500 mg/kg significantly lowered plasma MCP-1 level as compared to diabetic control rats. In contrast, *M. oleifera* ethanol extract at 250 mg/kg dose failed to show significant reduction on MCP-1 (fig. 2A).

Effect on glucose-6-phosphate level

The glucose-6-phosphate level was significantly reduced in diabetic control rats as compared to normal rats (fig. 2B). Diabetic rats treated with metformin, 95% ethanol extract and its butanol fraction for 14 days had showed significant dose-dependent increase in glucose-6phosphate level.

Effect on intestinal glucose absorption

The dose dependent inhibition of α-amylase was observed with 95% ethanol extract of *M. oleifera* leaf, its butanol fraction and standard compound (acarbose). The results indicated that 95% ethanol extract showed the highest α-amylase inhibitory activity compared to marketed drug acarbose. Among the standard marker compounds, maximum α-amylase inhibition was observed with kaempferol-3-O-glucoside (66.67%) at 100 µg/mL, suggesting that kaempferol-3O-glucoside contributed the most in alpha amylase inhibition by 95% ethanol extract and butanol fraction (table 1). Among all the samples,

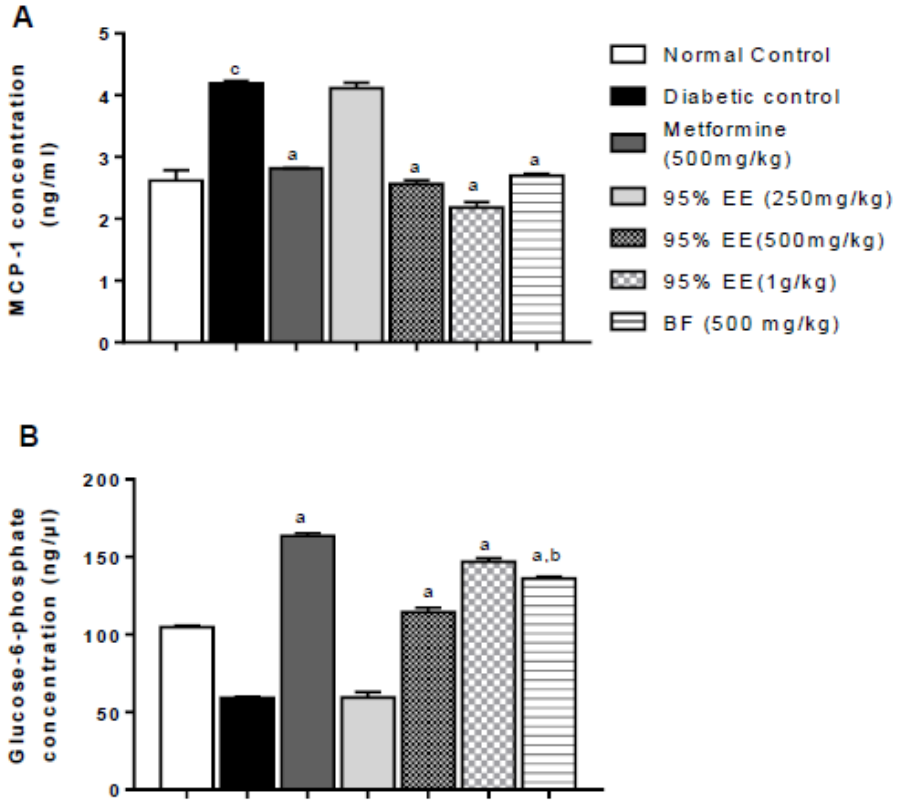

Fig. 2 Effect of daily oral treatment for 14 days of various doses (250, 500 & 1000 mg/kg) of *M. oleifera* 95% ethanol extract and its butanol fraction (500 mg/kg) on plasma MCP-1 level (**A**) and plasma glucose-6-phosphate level (**B**) of streptozotocin-induced diabetic rats. Values are expressed as Mean±S.E.M and statistically analysed by One way ANOVA followed by Dunnett's and independent T-Test was also used. [a]$p< 0.001$ vs diabetic control. [b]$p< 0.05$ vs 95% ethanol 500 mg/kg and [c]$p< 0.001$ vs normal control analyzed by using independent-sample T test.

butanol fraction was found to have the lowest IC_{50} (table 3), indicating it has the highest potency in inhibiting α-amylase enzyme (table 1). In the kinetic study, the slopes of α-amylase inhibition had different y-intercepts (1/Vmax) but the same x-intercepts (Km is unaffected), thus the mode of inhibition with such characteristics was non-competitive inhibition (fig. 3).

The results of α-glucosidase inhibitory activity study indicate that all the samples (acarbose, 95% ethanol extract, butanol fraction and the three standard marker compounds) showed αglucosidase inhibition (table 2). Of all, acarbose showed the highest α-glucosidase inhibition activity. Among the standard marker compounds, quercetin 3-β-Dglucoside exhibited the highest α-glucosidase inhibition activity at 100 µg/ml. Among all the samples, the cryptochlorogenic acid showed the lowest IC_{50} value (table 3). The findings of lineweaver-Burk plot indicates that 95% ethanol extract and its butanol fraction affected both the K_m and the V_{max} of the enzyme when compared to the control, thus exhibiting mixed mode of inhibition (fig. 4).

Effect on glucose uptake by skeletal muscle

The significant glucose uptake by tricep femoralis muscle was not observed with metformin, 95% ethanol extract and its butanol fraction as compared with control in the absence of insulin. Even the addition of 1IU/ml insulin

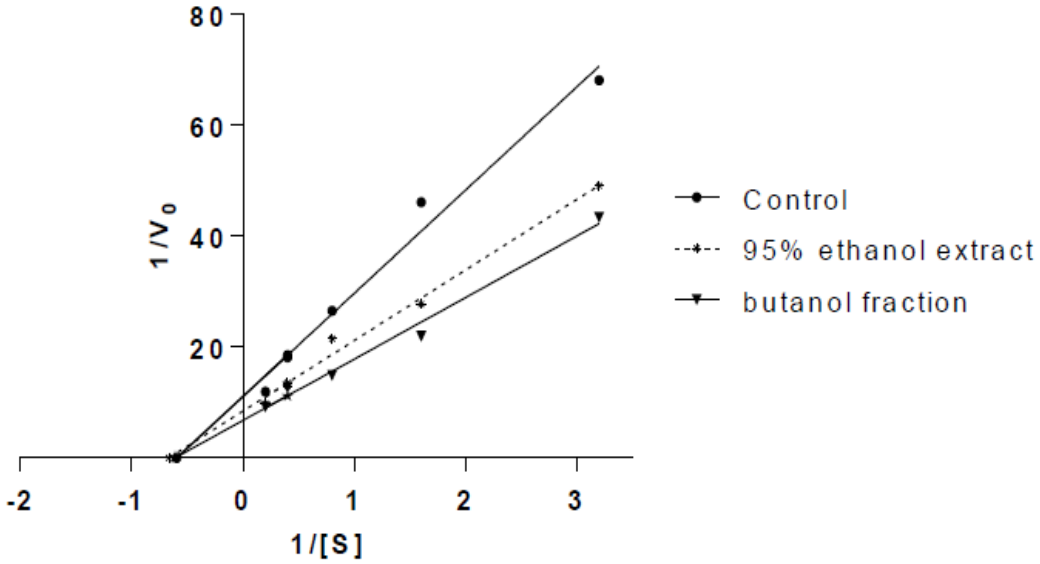

Fig. 3: Lineweaver-Burk plot of α-amylase enzyme kinetics, 95% ethanol extract and butanol fraction of *M. oleifera*. Vo= reaction velocity, S = substrate concentration.

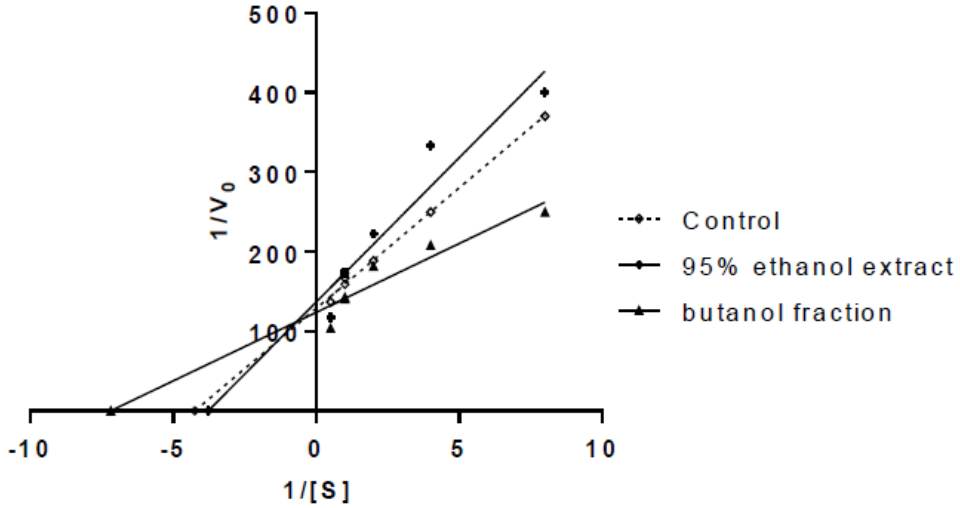

Fig. 4 Lineweaver-Burk plot of α-glucosidase enzyme kinetics, control, 95% ethanol extract and butanol fraction of *M. oleifera*. Vo = reaction velocity, S = substrate concentration.

was unable to promote glucose uptake significantly in all the samples (fig 5A). However, the glucose uptake in the rectus abdominus skeletal muscle was significantly higher compared to control with all samples (metformin, 95% ethanol extract and butanol fraction) as shown in fig. 5B.

DISCUSSION

The presence of very little or low level of insulin is the primary characteristic of diabetes mellitus (Hou *et al.,* 2009). In type I diabetes mellitus, the body is totally dependent on exogenous insulin due to little insulin production (Kaneto *et al.,* 2007) while in type II, the body depends on exogenous treatment (oral hypoglycemic drugs) due to the presence of low level of insulin or insulin resistance (Jain and Saraf, 2010). Therefore, the insulin levels were measured in normal rats treated with water and diabetic rats treated with water, metformin, 95% ethanol extract and butanol fraction of 95% ethanol extract respectively for 14 days (fig. 1). Streptozotocin being selectively toxic to β-cells causes damage or necrosis to pancreatic β-cells that lead to the decrease in insulin secretion (Eleazu *et al.,* 2013). Metformin has no direct stimulating effect on β-cells to release insulin as glibenclamide does. Therefore, it is assumed that metformin and 95% ethanol extract of *M. oleifera* leaf were unable to raise insulin levels in diabetic rats, suggesting that they have no effect on β-cells. A parallel can be drawn with the plant *Ficus lutea,* which also exhibited substantial *in vitro* activity but was also reportedly unable to induce increase in plasma insulin level (Olaokun *et al.,* 2014; Vargas-Sánchez *et al.,* 2019). MCP-1 and TNFα are the inflammatory mediators released during inflammation that contribute to oxidative stress and insulin resistance. The herbal constituents (polyphenols) at the level that does not produce any cytotoxic effects could lower the levels of these mediators (Marimoutou *et al.,* 2015). It was reported that inflammatory process (including damage caused by STZ), provokes the increase in the level of MCP-1 (Kouyama *et al.,* 2008; Bettcher *et al.,* 2019). After 14 days treatment diabetic rats with metformin, 95% ethanol extract and its butanol fraction, the plasma MCP-1 level decreased, indicating a repair of β-cells, thus, mitigating the inflammatory processes. It can be further deduced that, while metformin and the extracts do not have a positive insulin stimulating effect on the β-cells, they confer a protective effect against STZ induced damage. The dynamics of this protection is however unclear from our

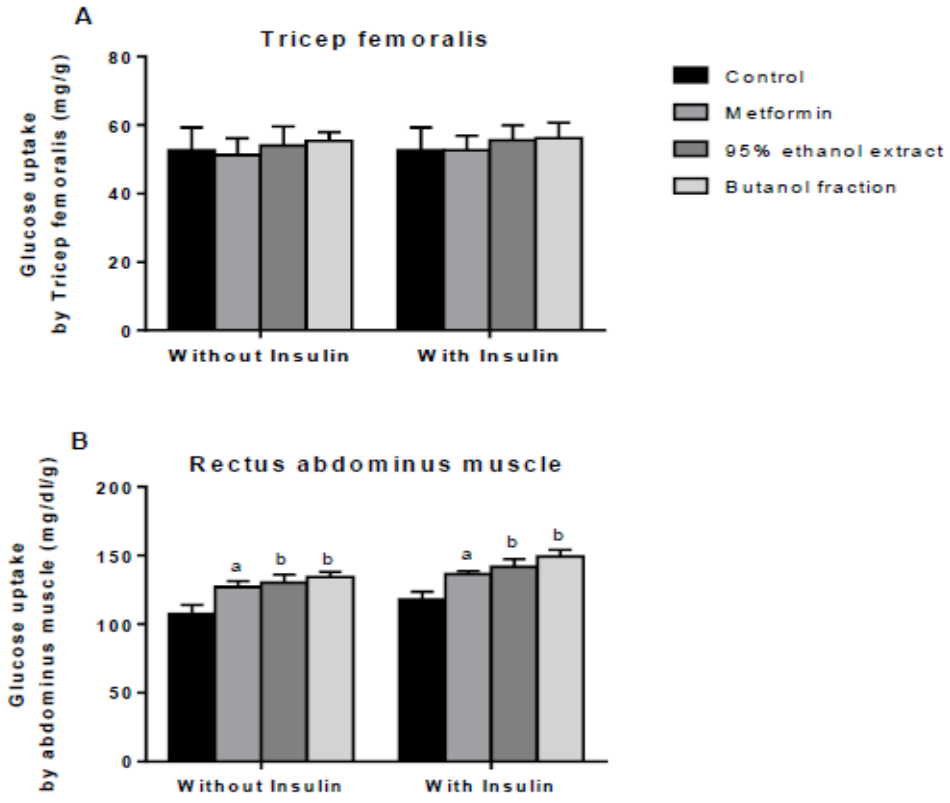

Fig. 5: Effect of *M. oleifera* 95% ethanol extract and butanol fraction on glucose uptake by tricep femoralis skeletal muscle (A) and Rectus abdominus muscle. Results were statistically significant ($p< 0.05$) with control. The results are presented as Mean±SEM and analysed using One Way ANOVA followed by Dunnett's test. $^{a}P < 0.05$, $^{b}P < 0.01$; vs control.

findings. The liver is the primary site for carbohydrate metabolism. Glucose is transported to liver after absorption from intestine. Here, it is phosphorylated by hepatic metabolic enzymes like hexokinase, phosphoenol pyruvate carboxykinase and glucose-6-phosphatase (Watford, 2005) and converted into glucose-6-phosphate. Glucose-6-phosphate acts as the primary intermediate for glycolysis metabolic pathway. In diabetes mellitus, the level of glucose-6-phosphate reduce due to reduction in the activity of hexokinase (Gandgi and Sasikumar, 2012; Villar-Palasi and Guinovart, 1997; Ciftci *et al.,* 2000). Both metformin, high dose of 95% ethanol extract (500 and 1000 mg/kg) and the butanol fraction (500 mg/kg) enhanced the plasma level of glucose-6-phosphate. It was estimated that 61% of diabetic and 17% of cardiac patients are obese (Patel *et al.,* 2016; Fukushima and Lopaschuk, 2016). Obesity usually is a result of excessive intake of dietary carbohydrates that is then stored as fat in adipose tissues of the body. Starch, being the main carbohydrate in human diet, is degraded into oligosaccharides in the presence of α-amylase found in saliva and pancreatic secretion in the intestine. Oligosaccharides are further broken down into monosaccharide (glucose) by αglucosidases enzymes found in intestine. The presence of glucose in the intestine causes the expression of sodium glucose co-transporter-1 in the intestine. The glucose entered into the epithelial cells and then into blood circulation and raises the level of blood glucose concentration (Antipatis and Gill, 2001; Dewi *et al.,* 2007; Hakkim *et al.,* 2007). Due to the low level of insulin or insulin resistance of the diabetic patients, the patients are unable to lower the excess glucose concentration. Therefore, the inhibition of intestinal enzymes (α-amylase and α-glucosidase) is an important strategy to control hyperglycemia associated with diabetes mellitus (Mogale *et al.,* 2011; Fontana *et al.,* 2011).

In this study, substantial inhibition of α-amylase was displayed by 95% ethanol extract and its butanol fraction that exceed the standard compounds (Acarbose, Quercetin-3-O-glucoside and Kaempferol-3-β-D-

Table 1: Samples showing α-amylase enzyme inhibition at different concentrations (6.25-100µg/ml)

α-amylase enzyme inhibition (%) at different Concentrations (µg/mL)					
Samples	6.25	12.5	25	50	100
Acarbose	16.05±0.41	16.42±0.82	23.46±1.09	34.57±0.71	57.9±0.64
95% Ethanolic extract	37.04±0.71	45.68±1.42	51.85±1.89	67.9±0.41	80.25±0.41
Butanol fraction	45.68±0.413	46.91±0.41	56.79±1.09	65.43±0.41	74.07±1.48
Quercetin 3-β-D-Glucoside	33.33±1.09	44.44±0.41	48.15±2.29	53.09±0.82	60.49±1.087
Kaempferol-3-O-Glucoside	34.57±3.93	39.88±0.41	43.21±0.67	56.79±0.41	66.67±0.71
Cryptochlorogenic acid	40.74±0.71	45.68±0.41	49.38±0.41	54.32±1.09	64.2±0.71

Table 2: Samples showing α-glucosidase enzyme inhibition at different concentrations (6.25-100µg/ml)

α-Glucosidase enzyme inhibition (%) at different Concentrations (µg/mL)					
Samples	6.25	12.5	25	50	100
Acarbose	21.62±0.9	40.54±1.8	51.35±1.6	70.27±1.6	94.59±0.90
95% Ethanolic extract	10.81±0.90	18.09±1.80	33.33±1.56	55.86±1.56	72.97±2.38
Butanol fraction	5.41±1.80	33.33±0.90	46.85±0.00	63.06±0.90	88.29±0.90
Quercetin 3-β-D-Glucoside	39.64±0.90	37.84±0.90	51.35±1.80	60.36±0.00	76.58±0.90
Kaempferol-3-O-Glucoside	13.51±0.90	21.62±0.00	27.03±0.00	37.84±0.90	53.15±0.90
Cryptochlorogenic acid	32.43±1.56	45.94±1.56	54.95±0.90	58.56±0.90	67.57±0.90

Table 3: IC_{50} values of 95% ethanolic extract, butanol fraction and standards for alpha amylase

Samples	α-amylase IC_{50} value (µg/mL)	α-glucosidase IC_{50} value (µg/mL)
Acarbose	83.24 ± 0.70	31.47 ± 1.25
95% ethanol extract	24.84 ± 0.98	56.18 ± 1.80
Butanol fraction	14.65 ± 0.94	34.52 ± 0.68
Quercetin 3-β-D-Glucoside	37.63 ± 1.17	29.62 ± 0.90
Kaempferol-3-O-Glucoside	46.87 ± 1.21	85.95 ± 1.48
Cryptochlorogenic acid	35.69 ± 0.70	14.76 ± 0.81

The values were presented as Mean±SEM.

glucoside). The mechanism underlying α-amylase inhibition of 95% ethanol extract, butanol fraction and the standard compounds is deduced to involve inactivation of αamylase by binding on an active site of α-amylase, thus preventing the breakdown of polysaccharides to oligosaccharide. α-Amylase inhibitors, by blocking the degradation of starch, prevent the intestinal absorption of carbohydrates and thus effectively control postprandial hyperglycemia (Tundis *et al.,* 2010; Sidorova *et al.,* 2017). The variation in α-amylase inhibition activity among the samples might be due to the difference in the affinity and intrinsic activity of compounds. The α-amylase enzyme kinetic experiment was carried out to see the mode of α-amylase inhibition by 95% ethanol extract and butanol fraction using Lineweaver-Burk plot of enzyme kinetics. Enzyme inhibitors are classified into competitive, non-competitive, uncompetitive and/or mixed mode of inhibition base on their effect on the affinity (K_m) of the enzyme [E] to the substrate [S] and the relative maximum reaction velocity (V_{max}) in the formation of the enzyme substrate complex [ES]. The competitive inhibitors have the same y-intercepts (Vo) and different x-intercepts (Km). The non-competitive inhibitors show the same x-intercepts and different y-intercepts while both x and y-intercepts are affected in mixed mode of inhibition (Copeland, 2013). Evidently, the extract and fraction exhibits non-competitive inhibition on α-amylase. Similarly, α-Glucosidase inhibitory activity of 95% ethanol extract and butanol fraction was less effective compared to acarbose (fig. 4). This might be due to slow binding of samples, or slow onset of inhibition that is a quite common characteristic among potent glycosidase inhibitors (Shen *et al.,* 2010). The inhibitor binds at a separate site from the substrate active site, to either the free enzyme or the enzyme-substrate complex. A mixed inhibitor displays the characteristic of both noncompetitive and uncompetitive inhibition. Due to a larger difference in the K_m compare to the V_{max}, it can be deduced that the extract and its fraction exhibited a mixed inhibition against αglucosidase that leans more towards competitive inhibition (Zhang *et al.,* 2015). From the results of intestinal enzyme inhibition and IC_{50} values, it is concluded that 95% ethanol extract of *M. oleifera* leaf and butanol fraction are more potent inhibitors of α-amylase enzyme than α-glucosidase enzyme. Skeletal muscle cells depend on the availability of glucose for their normal functioning. GLUT-4 transporter proteins transport glucose via translocating themselves from intracellular membrane to plasma membrane. Insulin further potentiates this uptake process (Vishnu Prasad *et al.,* 2010). Insulin binds to its receptors and causes the phosphorylation of insulin receptor substrates, thus facilitates glucose uptake through GLUT 4 in muscles and phosphorylated by hexokinase into G6P. The antidiabetic drugs provide remedy for diabetes mellitus through enhancing glucose uptake by the skeletal muscle cells, hence reducing hyperglycemia in diabetes. It can be inferred that 95% ethanol extract and its butanol fraction promotes glucose uptake by skeletal muscle. The findings of Olayaki, Irekpita (Olayaki *et al.,* 2015) also demonstrated increased glucose uptake with methanol extract of *M. oleifera* leaf.

The addition of insulin 1IU/ml further potentiated the glucose uptake significantly by abdominus muscle with 95% ethanol extract of *M. oleifera* and its butanol fraction. Insulin further promotes glucose uptake in the rectus abdominus muscle (Hassan *et al.,* 2010). The results indicate that abdominus skeletal muscle showed good glucose uptake ability with and without insulin as compared to tricep femoralis skeletal muscle. Tricep femoralis is more active skeletal muscle compared to rectus abdominus but it has never been reported to be used to study glucose uptake. It was anticipated that glucose uptake induced by metformin would be much more active in this muscle. However, it did not show significant glucose uptake which might be due to the following reasons: (**1**) tricep femoralis used was thick and had to be chopped into thinner pieces- not a single complete muscle. Whereas rectus abdominus was thin single complete muscle and (**2**) when exposed to glucose, rectus abdominus can take the glucose and use it for energy without a trigger from an external stimuli. Tricep femoralis may require not only glucose but nerve stimulation also to actively contract and use the glucose. Therefore, it is concluded that tricep femoralis is not suitable to be used to study glucose uptake by skeletal muscle. This data further supports Hou et al. 2009 findings.

CONCLUSION

The 95% ethanol extract of *Moringa oleifera* leaf produced anti-hyperglycemic effect through αamylase and α-glucosidase inhibition, phosphorylation of glucose, reduction of MCP-1 level and glucose absorption by skeletal muscle. This effect might be possible due to the presence of identified compounds: cryptochlorogenic acid, quercetin 3-β-D-glucoside and kaempferol -3-Oglucoside. It was also concluded that *Moringa oleifera* leaf 95% ethanol extract was more potent inhibitor of α-amylase than α-glucosidase. Furthermore, it was also observed that acarbose and Cryptochlorogenic acid were more potent inhibitor of α-glucosidase than α-amylase.

REFERENCES

Adisakwattana S and Chanathong B (2011). Alpha-glucosidase inhibitory activity and lipid lowering mechanisms of *Moringa oleifera* leaf extract. *Eur. Rev. Med. Pharmacol. Sci.*, **15**(7): 803-808.

Ali H, Houghton P and Soumyanath A (2006). α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus. J. Ethnopharmacol.*, **107**(3): 449-455.

Antipatis VJ and Gill TP (2001). Obesity as a global problem. International Textbook of Obesity, Wiley, Chichester, Australia, pp.3-22.

Bak EJ, Park HG, Lee C, Lee T-I, Woo G-H, Na Y, Yoo Y-J and Cha J-H (2011). Effects of novel chalcone derivatives on α-glucosidase, dipeptidyl peptidase-4, and adipocyte differentiation in vitro. *BMB Rep*, **44**: 410-414.

Barrett ML and Udani JK (2011). A proprietary alpha-amylase inhibitor from white bean (*Phaseolus vulgaris*): A review of clinical studies on weight loss and glycemic control. *Nutr. J.,* **10**(1): 24.

Bettcher BM, Neuhaus J, Wynn MJ, Elahi F, Casaletto KB, Saloner R, Fitch R, Karydas AM and Kramer JH (2019). Increases in a pro-inflammatory chemokine, MCP-1, are related to decreases in memory over time. *Front Aging Neurosci.,* **11**: 25.

Ciftci M, Küfrevioglu ÖI, Gündogdu M and Özmen I (2000). Effects of some antibiotics on enzyme activity of glucose-6-phosphate dehydrogenase from human erythrocytes. *Pharmacol. Res*, **41**(1): 107-111.

Coman C, Rugina OD and Socaciu C (2012). Plants and natural compounds with anti-diabetic action. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* **40**(1): 314.

Copeland RA (2013). Reversible modes of inhibitor interactions with enzymes. Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists, 2nd Edition, John Wiley and Sons, Inc., Hoboken, New Jersey, Canada, pp.57-121.

Dewi RT, Iskandar YM, Hanafi M, Kardono L, Angelina M, Dewijanti ID and Banjarnahor SD (2007). Inhibitory effect of Koji Aspergillus terreus on a-glucosidase activity and postprandial hyperglycemia. *Pak. J. Biol. Sci.,* **10**(18): 3131-3135.

Dutta TK, Jana M, Pahari PR and Bhattacharya T (2006). The effect of temperature, pH, and salt on amylase in *Heliodiaptomus viduus* (Gurney) (Crustacea: Copepoda: Calanoida). *Turk. J. Zool.*, **30**(2): 187-195.

El-Desouki N, Ibrahim B, Hegazi MA and El-Aama M (2015). *Moringa oleifera* leaf extract ameliorates glucose, insulin and pancreatic beta cells disorder in alloxan-induced diabetic rats. *Res. J. Pharm. Biol. Chem. Sci.,* **6**(3): 642-654.

Eleazu CO, Eleazu KC, Chukwuma S and Essien UN (2013). Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans. *JDMD*, **12**(1): 60.

Fontana Pereira D, Cazarolli LH, Lavado C, Mengatto V, Figueiredo MSRB, Guedes A, Pizzolatti MG and Silva FRMB (2011). Effects of flavonoids on α-glucosidase activity: Potential targets for glucose homeostasis. *Nutr*, **27**(11-12): 1161-1167.

Fukushima A and Lopaschuk GD (2016). Cardiac fatty acid oxidation in heart failure associated with obesity and diabetes. *Biochim. Biophys. Acta,* **1861**(10): 1525-1534.

Gandhi GR and Sasikumar P (2012). Antidiabetic effect of *Merremia emarginata* Burm. F. in streptozotocin induced diabetic rats. *APJTB*, **2(4)**: 281-286.

Hakkim FL, Girija S, Kumar RS and Jalaludeen M (2007). Effect of aqueous and ethanol extracts of *Cassia auriculata* L. flowers on diabetes using alloxan induced diabetic rats. *Int. J. Diabetes Metab.,* **15**: 100-106.

Hassan Z, Yam MF, Ahmad M and Yusof APM (2010). Antidiabetic properties and mechanism of action of *Gynura procumbens* water extract in streptozotocin-induced diabetic rats. *Molecules,* **15**(12): 9008-9023.

Hou JC, Min L and Pessin JE (2009). Insulin granule biogenesis, trafficking and exocytosis. *Vitam Horm*, **80**: 473-506.

Irfan HM, Asmawi MZ, Khan NaK, Sadikun A and Mordi MN (2017). Anti-diabetic activity guided screening of aqueous-ethanol Moringa oleifera extracts and fractions: Identification of marker compounds. *Trop. J. Pharm. Res.,* **16**(3): 543-552.

Jain S and Saraf S (2010). Type 2 diabetes mellitus – Its global prevalence and therapeutic strategies. *Diabetes Metab. Syndr.,* **4**(1): 48-56.

Kaneto H, Katakami N, Kawamori D, Miyatsuka T, Sakamoto KY, Matsuoka T-A, Matsuhisa M and Yamasaki Y (2007). Involvement of oxidative stress in the pathogenesis of diabetes. *Antioxid. Redox Sign.,* **9**(3): 355-366.

Kazeem MI, Mayaki AM, Ogungbe BF and Ojekale AB (2016). *In-vitro* Studies on *Calotropis procera* leaf extracts as inhibitors of key enzymes linked to diabetes mellitus. *IJPR,* **15**(Suppl): 37.

Kouyama K, Miyake K, Zenibayashi M, Hirota Y, Teranishi T, Tamori Y, Kanda H, Sakaguchi K, Ohara T and Kasuga M (2008). Association of serum MCP-1 concentration and MCP-1 polymorphism with insulin resistance in Japanese individuals with obese type 2 diabetes. *Kobe J. Med. Sci.,* **53**(6): 345-354.

Marimoutou M, Le Sage F, Smadja J, D'hellencourt CL, Gonthier M-P and Robert-Da Silva C (2015). Antioxidant polyphenol-rich extracts from the medicinal plants *Antirhea borbonica*, *Doratoxylon apetalum* and *Gouania mauritiana* protect 3T3-L1 preadipocytes against H_2O_2, TNFα and LPS inflammatory mediators by regulating the expression of superoxide dismutase and NF-κB genes. *J. Inflamm.,* **12**(1): 10.

Mogale M, Lebelo SL, Thovhogi N, De Freitas A and Shai L (2011). α-Amylase and α-glucosidase inhibitory effects of *Sclerocarya birrea* [(A. Rich.) Hochst.] subspecies caffra (Sond) Kokwaro (Anacardiaceae) stem-bark extracts. *Afr. J. Biotechnol.,* **10**(66): 15033-15039.

Muhammad HI, Asmawi MZ and Khan NaK (2016). A review on promising phytochemical, nutritional and

glycemic control studies on Moringa oleifera Lam. in tropical and subtropical regions. *APJTB*, **6**(10): 896-902.

Olaokun OO, Mcgaw LJ, Awouafack MD, Eloff JN and Naidoo V (2014). The potential role of GLUT4 transporters and insulin receptors in the hypoglycaemic activity of *Ficus lutea* acetone leaf extract. *BMC Complement. Altern. Med.,* **14**(1): 269.

Olayaki LA, Irekpita JE, Yakubu MT and Ojo OO (2015). Methanolic extract of *Moringa oleifera* leaves improves glucose tolerance, glycogen synthesis and lipid metabolism in alloxaninduced diabetic rats. *J. Basic Clin. Physiol. Pharmacol.,* **26**(6): 585-593.

Patel SA, Ali MK, Alam D, Yan LL, Levitt NS, Bernabe-Ortiz A, Checkley W, Wu Y, Irazola V, Gutierrez L, Rubinstein A, Shivashankar R, Li X, Miranda JJ, Chowdhury MA, Siddiquee AT, Gaziano TA, Kadir MM and Prabhakaran D (2016). Obesity and its relation with diabetes and hypertension: A cross-sectional study across 4 geographical regions. *Glob. Heart,* **11**(1): 71-79 e4.

Saeed R and Ahmed D (2015). Bioactive compounds from *Carissa opaca* roots and xanthine oxidase and alpha-amylase inhibitory activities of their methanolic extract and its fractions in different solvents. *Pharmacog. Res.,* **7**(4): 295.

Seo WD, Kim JH, Kang JE, Ryu HW, Curtis-Long MJ, Lee HS, Yang MS and Park KH (2005). Sulfonamide chalcone as a new class of α-glucosidase inhibitors. *Bioorg. Med. Chem. Lett,* **15**(24): 5514-5516.

Shen Q, Shao J, Peng Q, Zhang W, Ma L, Chan AS and Gu L (2010). Hydroxycoumarin derivatives: Novel and potent α-glucosidase inhibitors. *J. Med. Chem.,* **53**(23): 8252-8259.

Sidorova Y, Shipelin V, Mazo V, Zorin S, Petrov N and Kochetkova A (2017). Hypoglycemic and hypo-lipidemic effect of *Vaccinium myrtillus* L. leaf and *Phaseolus vulgaris* L. seed coat extracts in diabetic rats. *Nutr,* **41**: 107-112.

Tundis R, Loizzo M and Menichini F (2010). Natural products as α-amylase and α-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: An update. *Mini-Rev. Med. Chem.,* **10**(4): 315-331.

Vargas-Sánchez K, Garay-Jaramillo E and González-Reyes RE (2019). Effects of *Moringa oleifera* on glycaemia and insulin levels: A review of animal and human studies. *Nutrients,* **11**(12): 2907.

Villar-Palasi C and Guinovart J (1997). The role of glucose 6-phosphate in the control of glycogen synthase. *The FASEB Journal,* **11**(7): 544-558.

Vishnu Prasad C, Anjana T, Banerji A and Gopalakrishnapillai A (2010). Gallic acid induces GLUT4 translocation and glucose uptake activity in 3T3-L1 cells. *FEBS Letters,* **584**(3): 531-536.

Watford M (2005). Is the small intestine a gluconeogenic organ? *Nutr. Rev.*, **63**(10): 356-360.

Zhang H, Wang G and Dong J (2015). Inhibitory properties of aqueous ethanol extracts of propolis on alpha-glucosidase. *Evid-Based Complement. Altern. Med*, **2015**: Article ID 587383, 7 pages.