# ATTENUATION OF METABOLIC DYSFUNCTIONS IN THE SKELETAL MUSCLES OF TYPE 1 DIABETIC RATS BY *STEVIA REBAUDIANA* EXTRACTS, VIA AMPK UPREGULATION AND ANTIOXIDANT ACTIVITIES

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### ABSTRACT

**Objectives.** *Stevia rebaudiana* Bertoni leaves are well-known for their sweetness and have been used as a non-caloric sweetener in several countries. It has numerous therapeutic properties which have been proven safe and effective over hundreds of years. In the present study, we aimed to evaluate the possible antioxidant effects of *stevia* extracts and their role in regulating AMPK in type-1 diabetic rats.

**Methods.** Fifty male Sprague Dawely rats were divided into: (1) normal control (NC) group; normal rats receiving 0.5 ml normal saline, (2) DM group; diabetic rats receiving 0.5 ml normal saline, (3) DM + MSE group; DM rats receiving 200 mg/kg of methanolic extract of stevia, (4) DM + S group; DM rats receiving 2 mg/kg of pure stevioside, and (5) DM + CGA group; DM rats receiving 10 mg/kg of pure chlorogenic acid. Four weeks after treatment, AMPK activity, GLUT4 mRNA and oxidative stress markers were measured in frozen muscles. Also, fasting blood glucose in serum, insulin and HbA1c were measured at the end of experiment.

**Results.** DM caused a significant increase in serum fasting glucose, HbA1c and muscle MDA with significant reduction in serum insulin, muscle SOD, catalase, GPx, AMPK activity and GLUT4 expression ($p < 0.05$). Treatment with stevia extract, pure stevioside and chlorogenic acid caused significant improvements in the studied parameters ($p < 0.05$).

**Conclusions.** We concluded that stevia extracts and derivatives may improve metabolic dysfunction in skeletal muscles via upregulation of AMPK and GLUT4 and suppression of oxidative stress.

**Keywords:** type-1 diabetes, stevia leaf extracts, AMPK, antioxidant

## INTRODUCTION

Diabetes mellitus (DM) is a systemic metabolic disease, the incidence of which is increasing at an exponential rate around the world. Although type 2 DM (T2DM) accounts for around 90% of all diabetes patients, type 1 DM (T1DM) is also on the increase (Laron et al., 2015). The hallmark of DM is

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hyperglycemia, which results from the disruption of glycemic control due to the lack of insulin secretion from pancreatic beta cells, or the impairment of insulin action (Thomas and Philipson, 2015). The generation of reactive oxygen species (ROS) secondary to hyperglycemia is enhanced in diabetes, as evidenced by significant elevation in the products of lipid peroxidation, such as 4-hydroxy-2-nonenal (4-HNE) or malondialdehyde (MDA) (Sahin et al., 2012; Sies, 1997). Oxidative stress plays an important role in the pathogenesis of diabetes complications due to glucose autoxidation and glycation of proteins, which depletes the antioxidant defense system and thus promotes free radical generation (Sahin et al., 2012; Sies, 1997). Recently, much attention has been focused on dietary natural antioxidants capable of inhibiting reactive oxygen radical-mediated oxidative stress (Ghanta et al., 2007). Diabetic complications affecting skeletal muscles include contractile weakness, changes in muscle fibre type, reduction in oxidative activity and peripheral insulin resistance (Petersen and Shulman, 2002). Muscle is the most important organ in which glucose uptake is insulin-dependent (Wasserman, 2009); therefore, impaired hormonal signaling has a deleterious effect on muscle glucose uptake. Also, in skeletal muscles, the activation of both insulin and AMPK stimulates glucose uptake by enhancing GLUT4 translocation to the cell plasma membrane (Do et al., 2012). When activated, AMPK phosphorylates many downstream targets that lead to the inhibition of pathways that consume energy, such as fatty acid synthesis, cholesterol synthesis, and gluconeogenesis (Alcantara-Aragon et al., 2015). This downstream phosphorylation also leads to the stimulation of pathways that generate energy, glucose uptake, fatty acid oxidation and glycolysis (Alcantara-Aragon et al., 2015). Activation of AMPK requires both an increase in the intracellular AMP : ATP ratio and phosphorylation of Thr172 on the "activation loop" of the α-subunit (Birnbaum, 2005). Physiological activators of AMPK are thought to be exercise and calorie restriction, as they help to increase the cellular ratio of AMP : ATP (Chen et al., 2003). Also, a polyphenol derived from plants which are used in traditional Asian medicines are thought to activate AMPK (Hawley et al., 2010). *Stevia rebaudiana* Bertoni is a small perennial shrub that belongs to the aster family. It grows primarily in the Amambay mountain range of Paraguay (Mizutani and Tanaka, 2002). Steviol glycosides (SGs) are found in the leaves of a scrub plant native to the subtropical regions of South America. In South America, *Stevia rebaudiana* Bertoni extract has been used for centuries as a sweetening food additive in South America (Geuns, 2003). Six steviol glycosides (stevioside, steviolbioside, rebaudioside A, B, C and dulcoside) can be detected in stevia leaves (Geuns, 2003). Non-caloric sweeteners are increasingly introduced into common foods as a sugar replacement, and are for this reason recommended for weight loss and for individuals suffering from glucose intolerance and diabetes (DuBois and Prakash, 2012). We hypothesized that stevia could activate AMPK, and thereby improve metabolic dysfunctions and Glut4 upregulation in skeletal muscles in diabetic rats. Therefore, our study was designed to investigate the role of stevia leaf extract and its derivatives (stevioside and chlorogenic acid) as anti-diabetic and antioxidant agents, as well as their effects on the expressions of a AMP-activated protein kinase (AMPK) involved in the translocation of GLUT 4.

## MATERIALS AND METHODS

### Extraction and purification
Stevia plant leaves (*S. rebaudiana* Bertoni) were obtained from the Stevia International Company for Agro-industry Product (SICAP), Cairo, Egypt. Procedures for extraction and isolation were performed to produce methanolic stevia extract, isolation and purification two natural steviosides from *S. rebaudiana* leaves were done according to El-Mousalamy et al. (2017).

### Experimental animals
A total of adult fifty male Sprague Dawely rats, weighing 180–210 g were obtained and housed in a controlled environment that was maintained under a 12-hour light\dark cycle and a temperature of 25°C (±3°C) at the Medical Experimental Research Centre (MERC), Mansoura university. Rats were housed individually in separate cages and fed on normal standard rat chow, with free access to water. All procedures were approved by our local IRB-committee (# r.17.12.275).

### Type 1 DM animal model

Induction of type 1 DM was achieved by a single intraperitoneal (i.p.) dose of streptozotocin (STZ) (50 mg/kg b.w.) as previously described by our research group (El-Mousalamy et al., 2017). The onset of DM was confirmed by the measurement of fasting blood glucose level one week after STZ administration. Rats with a blood glucose level of more than 300 mg/dl were included in the study (Muranyi et al., 2006).

### Study design

The rats were segregated randomly into 5 groups of 10 rats, as follows:

1. **Normal control (NC) group:** normal rats receiving 0.5 ml normal saline by mouth by gastric gavage once daily for 4 weeks.

2. **Diabetes mellitus (DM) group:** diabetic rats receiving 0.5 ml normal saline by mouth by gastric gavage once daily for 4 weeks.

3. **DM + methanolic stevia extract (MSE) group:** diabetic rats receiving 0.5 ml normal saline containing 200 mg/kg b.w. methanolic extract of stevia leaves, by mouth, by gastric gavage once daily for 4 weeks (El-Mousalamy et al., 2017).

4. **DM + stevioside (S) group:** diabetic rats receiving 0.5 ml normal saline containing 2 mg/kg b.w. of pure stevioside, by mouth, by gastric gavage once daily for 4 weeks (El-Mousalamy et al., 2017).

5. **DM + chlorogenic acid (CGA) group:** diabetic rats receiving 0.5 ml normal saline containing 10 mg/kg b.w. of pure chlorogenic acid, by mouth, by gastric gavage once daily for 4 weeks (El-Mousalamy et al., 2017).

### Collection of blood samples and harvesting soleus muscle specimens

At the end of experiment, the rats were euthanized by $Na^+$ thiopental (at high dose; 120 mg/kg i.p.) and blood samples were withdrawn by heart puncture. After centrifugation of the blood samples, the sera were stored at –20°C until the time of biochemical analysis. Also, the soleus muscles on both sides were rapidly and carefully dissected from the surrounding fasciae and broken into small cubes (about 30 g), placed in RNAse free cryotubes and stored in liquid nitrogen for PCR and biochemical analysis.

### Measurement of serum parameters

Serum fasting blood glucose and HbA1c were measured by commercially available kits, according to manufacturer instructions (**Cat** #,10121 & **Cat** #,10657 Human Diagnostics, Germany). Also, fasting serum insulin levels were determined by a competitive assay method with an enzyme-linked immunosorbent assay (**ELISA**) using a commercially available kit (**Cat** # K4271-100, **BioVision's**, **USA**). HOMA-insulin resistance index was calculated from the formula of Matthews et al. (1985) as follows:

$$\text{HOMA-IR} = \frac{\text{fasting insulin level, µU/ml} \times}{22.5} = \text{mmol·µU/L}^2$$

### Measurement of oxidative stress biomarkers in soleus muscle

The tissue was homogenized with ice-cold phosphate-buffered saline (PBS; 0.01M, pH = 7.4) to remove excess hemolysis blood, then ground into small pieces to be homogenized in PBS (9 mL PBS would be appropriate for 1 g of tissue). It is recommended to add some protease inhibitor into the PBS with a glass homogenizer on ice. The homogenates are then centrifuged for 5 minutes at 5000×g to retrieve the supernatant. Muscle MDA concentration was measured spectrophotometrically at 532 nm by the method of Ohakawa et al. (1979), while the activity of glutathione peroxidase (GSH-Px) was measured spectrophotometrically at 412 nm, according to Lawrence and Burk (1976). SOD activity in soleus muscle was also assessed by spectrophotometer at 505 nm according to McCord and Fridovich (1969), while catalase (CAT) activity was measured using hydrogen peroxide ($H_2O_2$) as a substrate (Aebi, 1974). The disappearance of $H_2O_2$ was observed at 240 nm.

### Measurement of AMPK activity in tissue homogenates

The activity of AMPK was measured in soleus muscle homogenates using BioVision's AMPK ELISA kit (Cat # E4280-100). All steps of the assay were done according to manufacturer instructions at a wave length of 450 nm. The detection range for this kit is 0.313–20 ng/ml.

## Measurement of mRNA of GLUT4 by real time PCR

Details of the procedures, kits and the sequences of primers of the studied gene (GLUT4) and control gene (β actin) are described in detail in our previous work (El-Mousalamy et al., 2017).

## Statistical analysis

All statistical analyses were done using SPSS (version 16.0). Data is presented as mean ±standard deviation (SD). One-way ANOVA with Scheffe's post-hoc test was carried out. Statistical significance was considered to be $p \leq 0.05$.

## RESULTS

### Effects of methanolic stevia extracts, stevioside and CGA on body weight, fasting blood glucose, fasting serum insulin, HOMA-IR and HbA1c

By the end of experiment, body weight was significantly higher in the NC group than the DM, DM + MSE and DM + S groups ($p < 0.05$). Also, the DM + CGA group showed significant increase in body weight than DM and DM + MSE groups did ($p < 0.05$; Table 1).

Regarding fasting blood glucose level, it was significantly higher in the DM group than the NC group ($p < 0.005$), while its level was significantly lower in all treated groups (DM + MSE, DM + S and DM + CGA) than the DM group ($p < 0.05$). Also, the DM + MSE and DM + S groups showed a more significant reduction in fasting blood glucose than did the DM + CGA group ($p < 0.05$; Table 1).

Fasting insulin in the serum was also significantly lower in the DM group than the NC group ($p < 0.05$). On the other hand, it was significantly higher in the DM + SE, DM + S, DM + CGA groups than the DM group ($p < 0.05$), with the highest significant elevation noticed in the DM + CGA group (Table 1). HOMA-IR index showed a significant increase in the DM, DM + MSE, DM + S and DM + CGA groups compared to the NC group ($p < 0.05$). Also, HOMA-IR was significantly higher in the DM + MSE group than other studied groups (DM, DM + S and DM + CGA) ($p < 0.05$; Table 1). Moreover, HbA1c levels were significantly higher in the DM group than the NC group ($p < 0.005$), but were significantly lower in all treated groups (DM + MSE, DM + S and DM + CGA) than the DM group ($p < 0.05$). Also, the DM + MSE and DM + S groups showed a more significant reduction in HbA1c than did the DM + S group ($p < 0.05$; Table 1).

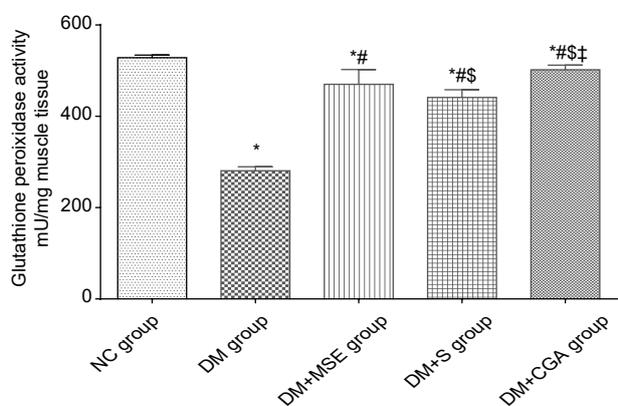### Effects of methanolic stevia extracts, stevioside and CGA on markers of oxidative stress

The activities of GPx, SOD and catalase in soleus muscle were significantly lower in the DM group than the NC group ($P < 0.05$), and their activities significantly increased in the treated groups (DM + MSE, DM + S, DM + CGA) compared to the DM group ($P < 0.05$).

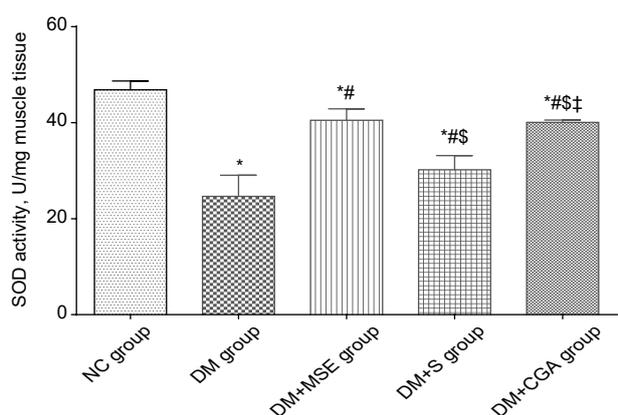**Table 1.** Body weight, FBS, insulin, HOMA-IR and HBA1c in different groups

| Groups | NC (*n* = 6) | DM (*n* = 6) | DM + MSE (*n* = 6) | DM + S (*n* = 6) | DM + CGA (*n* = 6) |
|---|---|---|---|---|---|
| Weight, g | 191.67 ±5.854 | 170.83 ±4.491* | 165.66 ±4.45* | 177.00 ±5.32* | 182.16 ±3.97# |
| FBS, mg/dl | 85.83 ±8.89 | 370.50 ±19.96* | 238.77 ±19.38*# | 285.43 ±16.79*#$ | 188.67 ±10.65*#$‡ |
| Insulin, mIU/L | 15.10 ±0.77 | 5.78 ±0.78* | 11.65 ±1.90*# | 8.32 ±0.33*#$ | 12.58 ±1.69*#‡ |
| HOMA-IR | 3.20 ±0.40 | 5.27 ±0.58* | 6.93 ±1.47* | 5.38 ±0.76* | 5.51 ±1.65* |
| HBA1c, % | 4.68 ±0.23 | 16.95 ±3.40* | 9.35 ±1.51*# | 9.65 ±0.46*# | 7.07 ±0.43*‡ |

All data were expressed as mean ±SD. One-way ANOVA with Scheffe's posthoc test. NC − normal control, DM − diabetes mellitus, MSE − methanolic stevia extract, S − stevioside, CGA − chlorogenic acid, FBS − fasting blood sugar. *Significant vs NC group, #significant vs DM group, $significant vs DM + MSE group, ‡significant vs DM + S group.
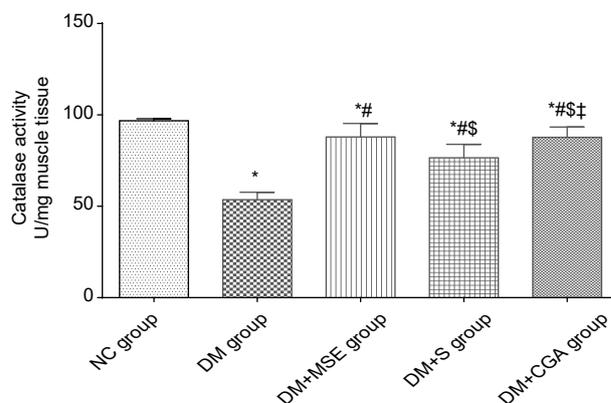
No significant difference was observed in their activity between the DM + CGA and DM + MSE groups ($P > 0.05$; Fig. 1–3). Compared to the NC group, the DM, DM + MSE, DM + S and DM + CGA groups showed significantly higher levels of lipid peroxidation products (MDA) in the soleus muscle ($P < 0.05$). Also, the treated (DM + MSE, DM + S and DM + CGA) groups showed significant reductions in MDA levels compared to the DM group ($P < 0.05$; Fig. 4).
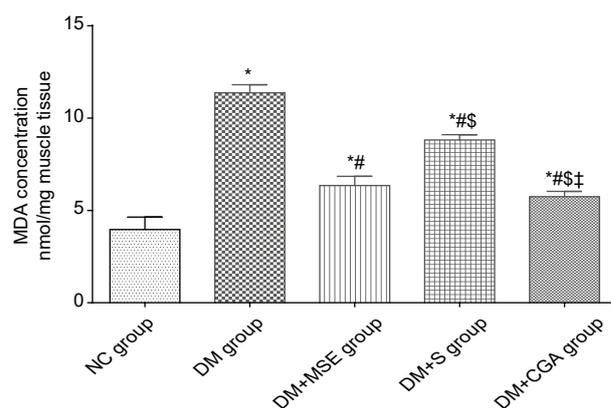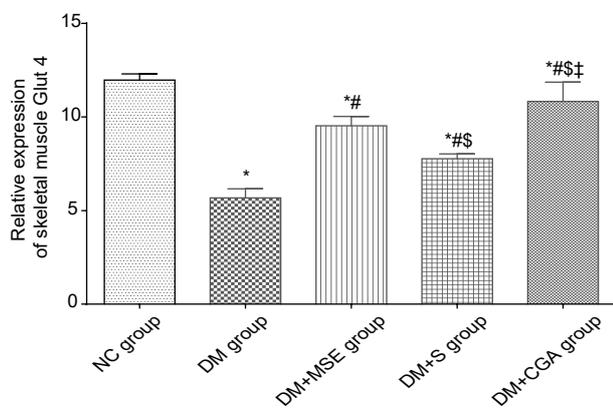


**Fig. 3.** Soleus muscle catalase activity in different groups, U/g muscle tissue: ∗ – significant vs NC group, # – significant vs DM group, $ – significant vs DM + MSE group, ‡ – significant vs DM + S group



**Fig. 1.** Soleus muscle glutathione peroxidase activity in different groups, mU/mg muscle tissue: ∗ – significant vs NC group, # – significant vs DM group, $ – significant vs DM + MSE group, ‡ – significant vs DM + S group



**Fig. 4.** Soleus muscle MDA in different groups, nmol/g muscle tissue: ∗ – significant vs NC group, # – significant vs DM group, $ – significant vs DM + MSE group, ‡ – significant vs DM + S group

### Effects of methanolic stevia extracts, stevioside and CGA on expression of GLUT4 gene

The DM group showed a significant reduction in GLUT4 mRNA expression compared to the NC group ($p < 0.05$), while the treated groups (DM + MSE, DM + S and DM + CGA) showed a significant increase in GLUT4 expression compared to the DM group ($p < 0.05$). Moreover, the DM + CGA group showed significantly higher levels of GLUT 4 mRNA than did the DM + MSE and DM + S groups ($p < 0.05$; Fig. 5).



**Fig. 2.** Soleus muscle superoxide dismutase activity in different groups, U/mg muscle tissue: ∗ – significant vs NC group, # – significant vs DM group, $ – significant vs DM + MSE group, ‡ – significant vs DM + S group

**Fig. 5.** Relative expression of mRNA of GLUT4 in soleus muscle in different groups: * – significant vs NC group, # – significant vs DM group, $ – significant vs DM + MSE group, ‡ – significant vs DM + S group

### Effects of methanolic stevia extracts, stevioside and CGA on activity of AMPK

The DM group showed a significant reduction in AMPK activity compared to the NC group ($p < 0.05$), while the treated groups (DM + MSE, DM + S and DM + CGA) showed a significant increase in AMPK activity compared to the DM group ($p < 0.05$). Moreover, the CGA group showed a significant increase in AMPK activity compared to the DM + MSE and DM + S groups ($p < 0.05$; Fig. 6).
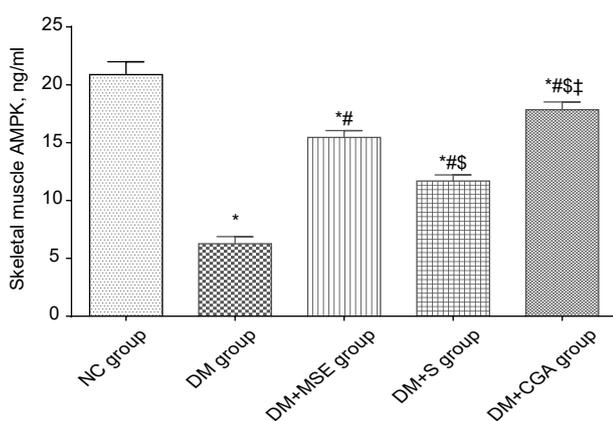


**Fig. 6.** AMPK activity in soleus muscle in different groups, ng/mg muscle tissue: * – significant vs NC group, # – significant vs DM group, $ – significant vs DM + MSE group, ‡ – significant vs DM + S group

## DISCUSSION

Type 1 DM is characterized by hyperglycemia and hyperlipidemia, with low insulin levels (Guo, 2014; Ford and Liu, 2001). As demonstrated by previous studies, the present study showed that streptozotocin (STZ) injection, at a dose of 50 mg/kg b.w., caused a significant elevation in fasting blood sugar and a significant reduction in fasting insulin. Moreover, treatment with stevia extracts, stevioside and CGA caused significant improvements in fasting blood glucose and insulin, with the most significant improvement found after CGA treatment. These findings suggest that CGA, stevioside and stevia extracts have anti-diabetic effects, in line with previous studies that have reported hypoglycemic effects for stevia extracts and derivatives. Previous studies have reported that stevia extracts have hypoglycemic effects in experimental animal models of DM (Assaei et al., 2016; Das et al., 2000; Misra et al., 2011). Also, Karthikesan et al. (2010) demonstrated that CGA at a dose of 5 mg/kg b.w. exerts anti-diabetic potential in STZ (45 mg/kg b.w.) nicotinamide-induced diabetic rats, while Jeppesen et al. (2000) documented stevioside and steviol as potent antihyperglycemic agents. These hypoglycemic effects of the stevia extract and its derivatives may be due to its central effects by enhancing insulin secretion (as evidenced by significant elevation in fasting insulin in the animal groups treated with these agents), or due to its peripheral action, improving insulin sensitivity (as evidenced by the significant elevation in GLUT4 expression in the muscle).

GLUT4, which is an insulin-dependent glucose transporter muscle, exerts its function by translocating to the plasma membrane from intracellular stores in response to insulin (Watson and Pessin, 2006) and also in response to muscle contraction (Lauritzen and Schertzer, 2010), allowing the entry of glucose into muscle cells. Changes in GLUT4 expression are observed in physiological states of altered glucose homeostasis. In the present study, we found that the expression of mRNA of GLUT4 was down regulated in type 1 DM. Moreover, the present study demonstrated that treatment with stevia extract and stevioside and CGA upregulated the expression of GLUT4 in skeletal muscle, which enhanced glucose uptake and explains its hypoglycemic effects in DM. Previous studies

suggested that steviol glycosides could act by modulating GLUT translocation through the PI3K/Akt pathway, since treatments with both insulin and Stevia extracts increased the phosphorylation of PI3K and Akt (Rizzo et al., 2013). Furthermore, Stevia extracts were able to reverse the effects of the reduction of glucose uptake caused by methyl glyoxal, an inhibitor of the insulin receptor/PI3K/Akt pathway (Ong et al., 2012; Rizzo et al., 2013), which demonstrated for the first time that CGA stimulates glucose transport in skeletal muscle via the activation of AMPK.

In this study, we demonstrated that stevia extract treatment showed significant induction of Glut4 and its translocation to the plasma membrane via activating AMPK. Furthermore, this data demonstrates that stevia extract, stevioside and CGA act as insulin action-stimulated glucose transport in skeletal muscle via the GLUT 4 translocation, mediated by the activation of AMPK, compared to non-treated diabetic or insulin-treated groups. These findings are in agreement with Prata et al. (2017), who suggested that steviol glycosides caused an increase in glucose uptake into rat fibroblasts by activating the PI3K/Akt pathway, thus inducing Glut4 translocation to the plasma membrane, and also with Ong et al. (2012), who found that CGA stimulated and enhanced both basal and insulin-mediated 2DG transports in soleus muscle. In L6 myotubes, CGA caused a dose- and time-dependent increase in glucose transport. Compound c and AMPKα1/2 siRNA abrogated the CGA-stimulated glucose transport. Consistently with these results, CGA was found to phosphorylate AMPK and ACC, which is compatible with increased AMPK activities.

It is well known that the permanent hyperglycemia characterizing diabetes causes glucose autoxidation and glycation of proteins (Wolff and Dean, 1987), which thereby depletes the antioxidant defense system, thus promoting free radical generation. In addition, the activity of SOD and CAT antioxidant enzymes and GSH levels are significantly reduced in diabetic animals (Shivanna et al., 2013). In agreement with these results, we demonstrated a significant reduction in the activities of SID, CAT and GPx in the present study, with a significant increase in lipid peroxidation marker (MDA). Also, many authors have reported the direct antioxidant activity of extracts from the leaves of *Stevia rebaudiana* Bertoni, owing to the presence

of alkaloids, flavonoids, and polyphenols (Ruiz-Ruiz et al., 2017). In the present study, we demonstrated that Stevia extract, stevioside and chlorogenic acid caused a significant increase in the activities of SOD, CAT and Gpx, together with a significant reduction in lipid peroxidation (MDA) in soleus muscle. Also, Shivanna et al. (2013), showed that feeding of rats with the whole stevia leaf powder and extracted polyphenols reduced the MDA concentration in the liver and improved its antioxidant status through antioxidant enzymes. Also, previous studies have demonstrated the free radical scavenging and antioxidant activity of chlorogenic acid *in vitro* (Xiang and Ning, 2008). Kim et al. (2012) suggested that chlorogenic acid prevented chemically-induced damage in the liver and primary cortical neurons by reducing oxidative damage and apoptosis.

Our data findings, combined with evidence from other studies regarding the insulin-mimetic effects and antioxidant properties exerted by steviol glycosides, suggest their potential beneficial role in the co-treatment of diabetes and health maintenance (Prata et al., 2017).

## CONCLUSIONS

We concluded that stevia extracts, stevioside and chlorogenic acid might improve the metabolic dysfunctions of skeletal muscles in type 1 diabetes via upregulation of GLUT4 and AMPK activity, as well as via suppression of oxidative stress and enhancing the endogenous antioxidants in muscles such as SOD, CAT and GPx enzymes.

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