Modulation of Metabolic and Contractile Dysfunctions of Soleus Muscle in Type 1 Diabetic Rats by Stevia Rebaudiana Bert. Extracts and Some of Its Derivatives: Comparative Experimental Study

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Abstract: This study was carried out to evaluate effect of stevia extracts and some of its derivatives (chlorogenic acid, CGA and stevioside, S) on metabolic dysfunctions and contractile functions of soleus muscle and glucose transporter 4 (GLUT4) expression in STZ-induced type 1 diabetic rats. Forty-eight male Sprague Dawely rats were divided into 6 equal groups 1) normal control (NC) group; normal rats received 0.75 ml normal saline, 2) DM groups; diabetic rats received 0.75 ml normal saline, 3) DM+ I(insulin); as DM group but rats received 1.0 IU mixtard insulin/100 g, 4) DM + MSE group; as DM but rats received 200 mg/ kg of methanolic extract of stevia, 5) DM+ S as DM but rats received 2 mg /kg of pure stevioside and 6) DM+ CGA as DM but rats received 10 mg / kg of pure chlorogenic acid. Four weeks after drug treatment, serum fasting glucose, insulin and lipid parameters (TG, TC, LDL and HDL), the expression of GLUT4 by real time PCR and contractile parameters of soleus muscle of rats were measured. DM showed significant increase in FBS, LDL, TG and TC with significant decrease in fasting insulin, HDL, GLUT4 expression and contractile parameters of soleus muscle ($p< 0.05$). Treatment with MSE, stevioside or CGA caused significant decrease in FBS, LDL, TG and TC and significant increase in fasting insulin, HDL, GLUT4 expression and contractile parameters of soleus muscle ($p< 0.05$). We concluded that STZ- induced type 1 MD is manifested by hyperglycemia and dyslipidemia and impaired contractile functions of slow type skeletal muscle fibres with down regulation GLUT4 expression. These deleterious effects might be corrected by treatment with methanolic stevia extracts, pure stevioside or CGA.

Keywords: *Stevia rebaudiana, DM, chlorogenic acid, soleus muscle, GLUT4*

Background

Diabetes mellitus (DM) is a systemic metabolic syndrome which results from either lack of insulin (type 1 DM) or lack of insulin action (type 2 DM) and characterized by hyperglycemia, insulin resistance, and relative insulin deficiency and characterized by hyperglycemia and dyslipidemia *(Maitra and Abbas ,2005)* . Type 1 DM usually associated with many complications such as cardiovascular diseases, neurological complication, chronic renal failure, and diabetic retinopathy and myopathy *(Nanchen et al.,2012; Kumar et al., 2012 ; Brosius and Alpers , 2013 ; Kan , et al., 2012).* Diabetic myopathy in skeletal muscle is a common clinical condition and much less studied complication of poorly controlled diabetes and characterized by reduction in muscle mass, power, and an overall reduced physical capacity (*Andersen et al. 1997; Andersen, Gjerstad, and Jakobsen. 2004; Andersen et al. 1996; Andersen, Schmitz, and Nielsen , 2005*). Several mechanisms tried to study the mechanisms underlying diabetic myopathy such as oxidative stress caused by hyperglycemia (*Brownlee, 2005; Ghahary et al., 1991*). Glucose transporter -4 (GLUT4), the main facilitative glucose carrier responsible for the insulin-regulated glucose uptake in skeletal muscle *(Scheepers et al., 2004)* showed high expression by exercise training and low expression during states of insulin deficiency *(Host et al., 1998)* .

Nowadays, herbal supplements and other alternative medicine are necessary for handling of diabetic hyperglycemia. Stevia (Stevia rebaudiana Bertoni) is a shrub of the Asteraceae family native to South America, especially to Northeast of Paraguay *(Soejarto, 2002)*and the use of its leaves as a powerful alternative to artificial sweeteners spreads the Stevia crops to other regions of the world such as Canada, Asia and Europe *(Lemus-Mondaca et al., 2012).* In addition to their sweetening

properties, stevia extracts possess other therapeutic effects such anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrhea, diuretic and immunomodulatory effects *(Chatsudthipong and Muanprasat, 2009).* Also, stevia contains phytochemical compounds that help to reduce blood sugar, cholesterol and blood pressure and phenolic compounds such as flavonoids, phenolics are known to possess potential antioxidant properties *(Shukla et al., 2011; Ramya et al., 2014)* . The steviol glycosides which include as rebaudioside A and stevioside are the cause of their sweetness and stevioside differs from rebaudioside A by having one less glucose moiety *(Wheeler et al., 2008)* . Also, chlorogenic acid (CGA) is a type of phenolic acid created by the condensation of caffeic acid and quinic acid, also known as 5-coffee quinic acid (5-CQA, the IUPAC numbering***)*** and extracted from Stevia extract *(van Dam and Hu , 2005).* The plant extract containing CGA helps improve glucose and lipid metabolism, lowers plasma glucose and C-reactive protein levels, and improves liver function in diabetic patients *(Herrera-Arellano et al ., 2004; Abidov et al ., 2006)* . So, in the present study we investigated the medicative effects of methanolic extract of leaves from stevia rebaudiana (MSE) and its derivatives (stevioside and CGA) on body weight, fasting blood glucose, fasting insulin levels, lipid profile, soleus muscle contractility and GLUT4 RNA expression in (STZ) induced type 1 diabetic rats.

Material and Methods

Sample collection and solvent extraction

Stevia plant leaves (S. rebaudiana Bertoni) were obtained from Stevia International Company from Agro-industry Product (SICAP), Cairo, Egypt. Stevia plant leaves were dried out from direct sunlight. Plants were dried under shade condition for one month and cut into small pieces, A Soxhlet extractor apparatus was used for

extraction, with petroleum ether and methanol solvent(HPLC grade) for extraction of non-polar and polar compounds, respectively and cycled 10–15 times. After that, it was decanted into the beaker and was left open, so that the solvents evaporated using rotary vacuum evaporator at 40–45°C. Stevioside and chlorogenic acid separation was performed using different system on a reverse phase C18 column and the compounds were monitored with Diode array detector (JASCO HPLC system**) (Rao et al., 2012; Ross et al., 2009) .**

Experimental animals:

Forty eight male Sprague Dawely rats, weighting 170-200 g were used in this study. The animals were purchased and housed at the Medical Experimental Research Centre (MERC), faculty of medicine, Mansoura University. They were housed in a controlled environment that was maintained under a 12 hour light\dark cycle and a temperature of 25°C (±3ºC).The rats were allowed free access to a standard water and diet. This research was approved by the Medical Research Ethics Committee of Mansoura University.

Study design

1. **Normal Control (NC) group (n=8)**: normal rats received 0.75 ml normal saline subcutaneous (S.C) once daily for 4 weeks.

2. **Diabetes Mellitus (DM) group (n=8)**: type 1 diabetic rats received 0.75 ml normal saline subcutaneous (S.C) once daily for 4 weeks.

3. **Diabetes Mellitus + Insulin (DM+I) group (n=8)**: type 1 diabetic rats received mixtard insulin 30 in at a dose of 1.0 IU/ 100g dissolved in 0.75ml normal saline subcutaneous (S.C) once daily for 4 weeks (**Unlucerci ,et al. 2002)** . Injection was started 48 hours after induction of diabetes.

4. **Diabetes Mellitus + Stevia Extract (DM+MSE) group (n=8)**: as DM+I group with 200 mg / kg of methanolic extract from stevia.

5. **Diabetes Mellitus + Stevioside group (DM+S) group (n=8)**: as DM+I group with 2 mg /kg of pure stevioside extracted from Stevia.

6. **Diabetes Mellitus + chlorogenic acid group (DM+CGA) group (n=8)**: as DM+I group with 10 mg / kg of pure chlorogenic acid extracted from stevia.

Animal model for type 1 diabetes

Type 1 DM was induced by single dose of 50 mg/kg of freshly prepared Streptozocin (STZ) (Sigma chemical Company, Saint Louis, MO, USA) in 0.1 M citrate buffer pH 4.5. Induction of diabetes was confirmed after a week of treatment by estimation of fasting blood glucose level. Only those rats with blood glucose level between >300 mg/dl were included in the study **(Marianna et al., 2006) .**

Collection of blood samples and harvesting soleus muscle specimens

By the end of experiment, rats were weighted, then anaesthetized by high dose of Na thiopental (120 mg/Kg), then blood samples were collected by heart puncture. The blood samples were centrifuged at 1000 rpm and sera were kept at -20°C till the time of biochemical analysis. Also, the soleus muscle (right and left) were rapidly and carefully dissected from the surrounding fasciae and superficial layer of the muscle and the muscle on left side was broken into small cubes (about 30 g) and placed into RNAse free cryotubes and stored in liquid nitrogen (< - 196 ºC) until PCR analysis. The muscle on the right side was preserved in Krebs solution (NaCl 7 g, Kcl 0.37 g, Ca gluconate 0.1 g, MgSO4 0.029 g, Na2HPO4 0.17 g, NaHCO3 2.1 g and glucose 2,07 g, pH 7.4) at 30 ºC with continuous bubbles of 5% CO2 and 95% O2 for isolated contractile study.

Biochemical Tests

Serum fasting blood glucose and insulin levels detected by a competitive assay method with an enzyme-linked immunosorbent assay (ELISA) by commercially available kit ***(Biocheck, USA)*** and serum TGs, HDL, LDL were measured by commercially available kits according to manufacturer instructions (*Human Diagnostics, Germany*).

HOMA index was calculated from the following equation

$$\text{HOMA} = \frac{\text{Fasting insulin (mIU/L) x fasting glucose (mmol/L)}}{22.5}$$

Isolated study of the contractile changes in soleus muscle

This was done by **BIOPAC SL system**. Hardwares (MP30, BSLSTM stimulator, force transducer (SS121LA) were turned on. The BSL PRO software on the computer was started. The muscle template performed by selecting file menu then open then select graph template file. The tension adjuster placed on the ring stand and attach force transducer so that the hook hole is pointing down and should be stetted that its level both horizontally and vertically. The optimal force range for the experiment (force transducer 100g for soleus muscle) was determined and the muscle (soleus) fixed in the organ path, then start the electrical stimulation by putting the electrode on the muscle. Single electrical stimulation applied; the speed of contractile function was measured by time taken to reach peak and time taken to relax 50 %.Then, continuous stimuli were applied till fatigue occurs. Determine time taken by the muscle to produce complete fatigue in seconds. Also, the tension (g) developed in muscle before and after complete fatigue was recorded.

Real time PCR assay:-

Relative quantification of GLUT4 transcripts

The kit used was purchased from Bioline Reagents scientific company *for cDNA Synthesis* and *Relative Quantitation by Real Time PCR* Using SensiFAST SYBR® No-ROX Kit with the amplicons of up to 200 bp. However, the cycling conditions can be varied to suit different machine-specific protocols. It is not recommended to use annealing temperatures below 60 °C or combined annealing/extension times longer than 30 seconds. According to gene bank, primers sequences were :(NCBI Reference Sequence (SLc2a4): NM_012751.1 (Pruitt et al., 2007) Primers used: Forward and reverse primer sequences were Glut 4 (SLc2a4), forward primer 5´CTTGGGTTGTGGCAGTGAGT 3, reverse primer 5´ TTCCCCATCTTCAGAGCCGAT 3´ and β-actin forward primer 5´ CACCATGTACCCAGGCATTG ´3 and reverse primer 5´ CACACAGAGTACTTGCGCTC 3´.

Statistical analysis

The parametric data obtained by the experiments have been analyzed by using SPSS 20 .The quantitative data are expressed as the mean ± SD. Multiple comparisons were performed among experimental groups by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. $P < 0.05$ was considered statistically significant.

Results

Effects of MSE, Stevioside and CGA on body weight

Compared to NC group, the rat body weight was significantly low in DM, DM+MSE and DM+S groups ($p<0.05$), while DM+ I and DM+CGA groups showed no statistical significance with NC group. Also, compared to DM group, DM+MSE and DM+S groups showed no statistical significance in body weight. Moreover, DM+ I and DM+CGA groups showed statistical significant increase in body weight compared to DM group ($p<0.05$) (fig.1).

Effects of MSE, Stevioside and CGA on fasting blood glucose, insulin, HOMA index

By the end of experiment, DM, DM+ MSE, DM+S and DM+ CGA groups showed significant higher values for FBS than NC group ($p<0.005$). Also, DM+MSE, DM+S and DM+CGA groups showed significantly lower values for FBS than DM group ($p<0.05$). Moreover, DM+CGA group showed significantly lower values for FBS than DM+MSE and DM+S groups ($p<0.05$). There was no statistical significant difference between NC and DM+I groups (fig.2A).

Also, DM group showed significant reduction in serum fasting insulin levels compared to NC group ($p<0.05$), on the other hand, all treated groups (DM+I, DM+I+SE, DM+I+S, DM+I+CGA) showed significant increase fasting insulin level compared to DM group ($p<0.05$) and the highest significant elevation in serum insulin was noticed in DM+I group (fig.2B)

HOMA-IR was significantly elevated in DM and treated groups (DM+I+SE, DM+I+S, DM+I+CGA) compared to NC and DM+I groups ($p<0.05$), although its level still was within normal range in all groups (fig.2C)

Effects of MSE, Stevioside and CGA on Lipid profile (serum TC, TGs, LDL and HDL)

DM group showed significant increase in serum TGs, TC and LDL with significant decrease in serum HDL compared to NC group ($p<0.05$) and this significant elevation was significantly attenuated in treated groups (DM+I, DM+MSE, DM+S and DM+CGA) compared to DM group ($p<0.05$). On the other hand, HDL showed significant decrease in DM group compared to NC group and this attenuation was improved in all treated groups ($p<0.05$). Moreover, DM+CGA showed more significant reduction in serum TG and LDL with more significant elevation HDL compared to DM+MSE and DM+S groups ($p<0.05$) (table 1).

Effects of MSE, Stevioside and CGA on contractile properties of isolated soleus muscle

DM group showed significant reduction in developed muscle tension before and after fatigue compared by normal group ($p<0.05$). Compared to DM group, all treated (DM+I, DM+MSE, DM+S and DM+CGA) groups showed significant increase in muscle tension with the maximum significant increase was noticed in DM+I and DM+ CGA groups ($p<0.05$) (fig.3A-B).

The time taken by the muscle to reach complete fatigue was significantly reduced in DM group when compared to NC group and significantly increased in all treated (DM+I, DM+MSE, DM+S and DM+CGA) groups when compared with DM group ($p<0.05$) with the maximum increase was observed in DM+CGA group (fig.3C). Also, the time to reach the peak of contraction and half relaxation time showed significant increase in DM group compared to NC group and significant reduction in all treated (DM+I, DM+MSE, DM+S and DM+CGA) groups compared to DM group with the maximum reduction was observed in DM+I and DM+CGA groups ($p<0.05$) (fig.3D-E).

Effects of MSE, Stevioside and CGA on expression of mRNA of GLUT4 gene

Quantitative RT-PCR analysis revealed significant reduction in Glut4 mRNA expression in DM group compared to NC group ($p<0.05$). Also, all treated (DM+I, DM+MSE, DM+S and DM+CGA) groups showed significant elevation GLUT4 expression compared DM group with the maximum elevation was noticed in DM+I and DM+CGA groups ($p<0.05$) (fig.4)

Discussion

The main findings of the present study can be summarized as follow a) type 1 DM caused significant impairment in contractile function of soleus muscle which was associated with dyslipidemia and downregulation of skeletal muscle GLUT4, b) pretreatment with MSE or stevioside or CGA caused improvement in fasting blood glucose, insulin, dyslipidemia and contractile dysfunctions in skeletal muscle with upregulation of GLUT4.

Assessment of rat body weight is considered as one of the parameters which monitor the growth and development of the lab rat model (*Sengupta, 2011).* It has been known that insulin is anabolic hormone and loss of its effects is the main cause for loss of body weight in diabetic patients (*King , 2012; Novikova et al., 2013*). Lack of insulin make the muscle and adipose tissue cells unable to take up glucose which results in stimulation of muscle proteolysis and adipose tissue lipolysis *(Finn and Dice, 2006).* Depletion of fat stores in adipose tissues and breakdown of muscles contribute to weight loss in diabetic patients *(Eiselein et al., 2004).* In agreement with these findings we found in the present study significant reduction in body weight of

rats in DM group. Treatment with insulin restored the body weight, while treatment with MSE and stevioside caused non-significant increase in body weight, while CGA (10 mg/kg) caused significant increase in body weight of diabetic rats. Similar findings were reported by previous studies. Misra et al., (2011) and Assaei et al., (2016)demonstrated that *Stevia* extracts caused significant improvement in FBS and body weight in alloxan and STZ-induced diabetic rat model respectively. Also, Karthikesan et al., (2010) demonstrated that CGA (5 mg/kg) caused significant improvement in body weight and FBS in STZ-induced diabetic rats.

The metabolic dysfunctions in type 1 DM is characterized by hyperglycemia and hyperlipidemia with low insulin level (*Guo et al., 2014; Ford et al., 2001*). In agreement with these findings, the present study showed that STZ injection at a dose of 50 mg/Kg caused significant elevation in fasting blood sugar and significant reduction in fasting insulin with significant increase in HOMA index but still in the range of normal levels. Moreover, we demonstrated in the present study that, treatment with MSE, stevioside and CGA caused significant improvement in fasting blood glucose and insulin with the maximum improvement with CGA treatment. These findings suggested antidiabetic effects for CGA, stevioside and stevia extracts and confirmed the findings reported by previous studies. The antihyperglycemic effects for stevia extracts were demonstrated in diabetic animal models by Misra et al., (2011), Assaei et al., (2016) and Das et al., (2017) . While, the antidiabetic effects of CGA was demonstrated by *Karthikesan et al., (2010)* in STZ- induced diabetic rats and that of stevioside and steviol was demonstrated by *Jeppesen et al., (2000)* . The hypoglycemic effects of the stevia extracts and its derivatives might be due to central effects by enhancing insulin secretion (as evidenced by significant elevation in fasting

insulin these animal groups treated with these agents) or due to peripheral action through improvement of insulin sensitivity (as evidenced by significant reduction in HOMA index and elevation GLUT4 expression in muscle).

Dyslipidemia in diabetic rats was demonstrated in the present study in the form of significant elevation in LDL, TC and TGs with significant reduction in HDL. Treatment with MSE, stevioside and CGA improved significantly this dyslipidemia. In consistence with these findings, *Aghajanyan et al., (2017)* and *Assaei et al., (2016)* demonstrated that treatment with aqueous extract of stevia significantly reduced TC, TG, and LDL levels and increased HDL level in hyperglycemic rabbits and *Wan et al., (2016)* reported that CGA (10 mg/kg) significantly reduced total and LDL-cholesterol, increased HDL cholesterol, and improved both the atherogenic index and the cardiac risk factor.

Diabetic myopathy in type 1 DM is characterized by reduction in either the isometric or isotonic maximal contractile force (g) in skeletal muscles (Andersen et al. 1996; Andersen et al., 2005; Andersen. 1998). Also, there is loss of muscle fiber size and an increase in the relative percentage of fast-glycolytic muscle fibers in the expense of slow-oxidative muscle fibers in diabetic adult skeletal muscles (Fritzsche et al. 2008). In the present study, we examined the contractile functions in diabetic rats in type 1 slow oxidative muscle (soleus muscle). We found that DM caused significant reduction in isometric contraction (muscle tension) compared to normal group before and after fatigue with significant lengthening of the timing of contraction and relaxation. These findings are in agreement with those reported by *Eshima et al., (2015).* Moreover, treatment with insulin restored contractile functions

to the normal levels and treatment with MSE, stevioside or CGA caused significant improvement in the contractile functions with the maximum improvement was observed with CGA treatment. These effects could be explained by improvement in glucose uptake and enhanced protein synthesis in soleus muscle fibers by these agents. In the present study we examined their effects on GLUT4 expression.

GLUT4, which is 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 enhances glucose uptake and explaining 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 revert the effect of the reduction of glucose uptake caused by methyl glyoxal, an inhibitor of the insulin receptor/PI3K/Akt pathway (*Rizzo et al., 2013; Ong et al., 2012)* demonstrated for the first time that CGA stimulates glucose transport in skeletal muscle via the activation of AMPK.

Conclusions

Methanolic extracts of stevia, stevioside and CGA have hypoglycemic effects and improve the lipid profile and contractile dysfunctions in slow twitch (soleus) muscle in type 1 diabetic rats. These effects were associated with upregulation of GLUT4 in soleus muscle. CGA offered more powerful effects than MSE and stevioside.

Conflict of interest

Authors declared that there is no conflict of interest

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Table (1): Effects of methanolic stevia extracts, stevioside and chlorogenic acid on lipid profile

	NC (n=6)	DM (n=6)	DM+ I (n=6)	DM+MSE (n=6)	DM+S (n=6)	DM+ CGA (n=6)
Serum Cholesterol (mg/dl)	153.71± 9.81	175.21± 13.9*	155.38± 4.89#	150.45± 3.21 #	155.50± 3.83 #	143.73± 2.32 #
Serum TG (mg/dl)	94.80± 7.18	241.93± 3.32 *	98.20± 5.05#	226.36 ± 3.38*#$	237.53 ± 5.74*$	184.48 ± 16.78*#$& @
Serum HDL (mg/dl)	44.45± 1.02	26.13± 3.10 *	41.61± 1.32 #	34.21 ± 1.84 *#$	36.58± 1.77 *#$	39.43± 0.76 *#&
Serum LDL (mg/dl)	90.10± 8.96	175.88± 7.25 *	120.23± 5.77 *#	157.66± 2.22 *#$	138.60 ± 5.36 *#$&	122.13± 2.75 *#&@

All results are expressed as mean ± SD. One way ANOVA to evaluate the difference following Tukey post hoc test. p≤ 0.05 is considered significant.*significant vs NC group , # significant vs DM group ,$significant vs MD+I group ,&significant vs DM+MSE group ,@ significant vs DM+S group**.**

Figures

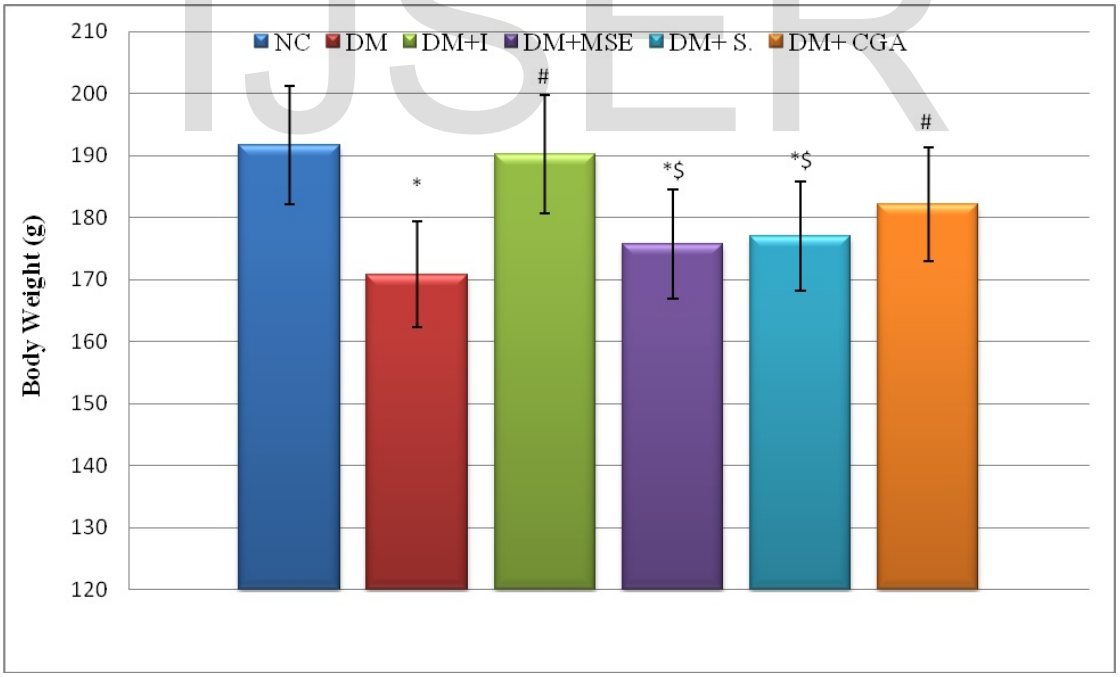
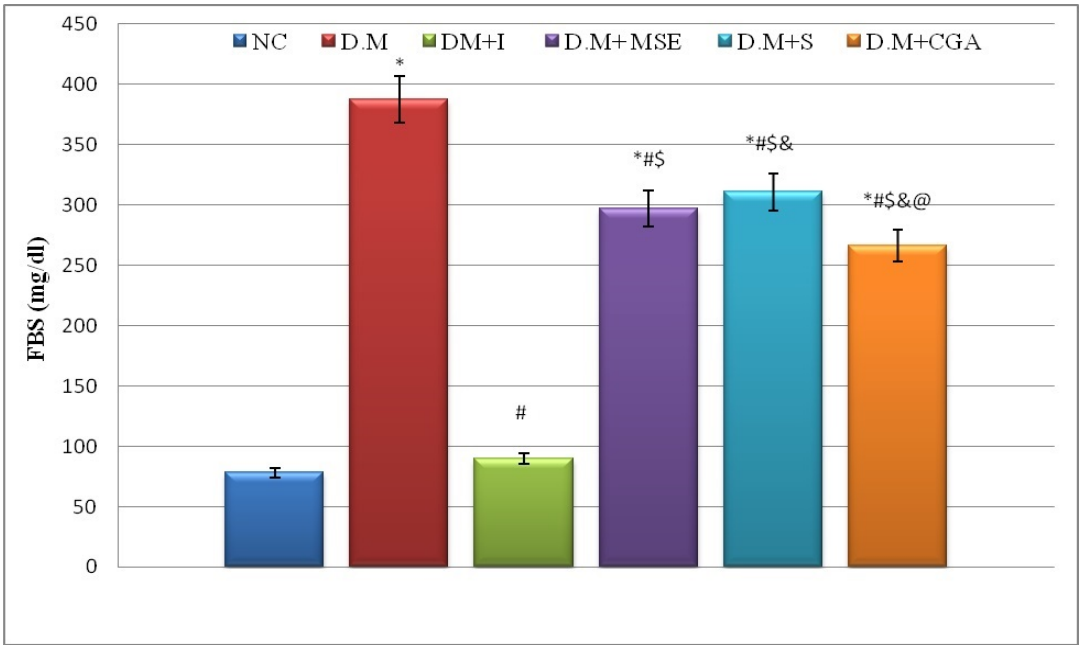
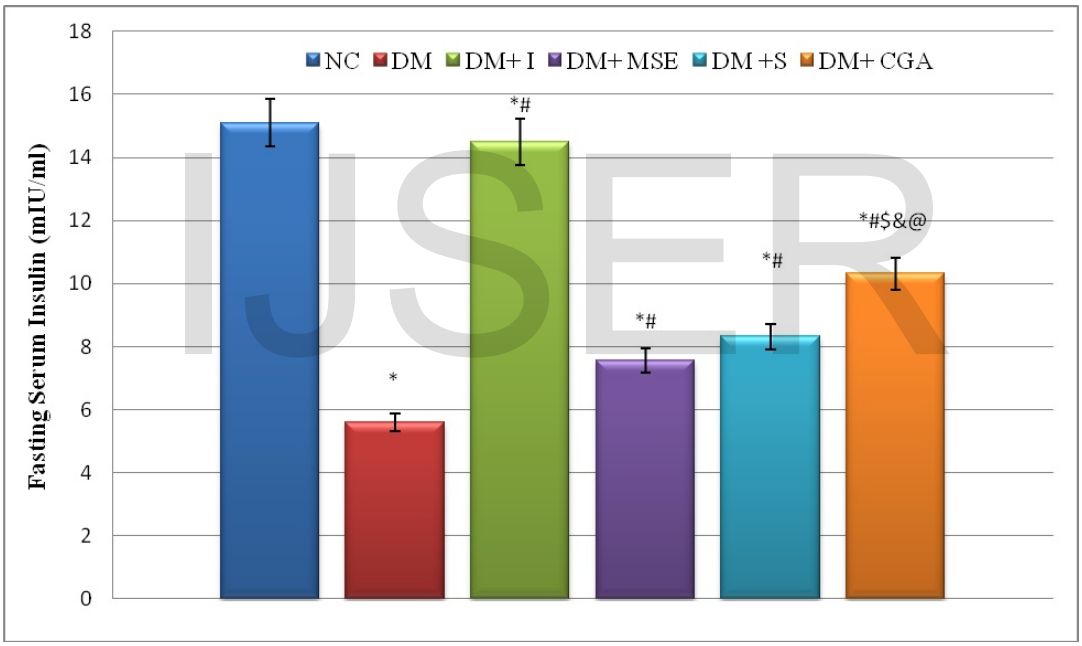


Fig. (1): Effects of insulin, MSE, Stevioside and CGA on body weight (g) in type 1 diabetic rats.*significant vs NC group , # significant vs DM group ,$significant vs MD+I group ,&significant vs DM+MSE group ,@ significant vs DM+S group**.**


NC
D.M
DM+I
D.M+MSE
D.M+S
D.M+CGA
FBS (mg/dl)
450
400
350
300
250
200
150
100
50
0
*
#
*#$
*#$&
*#$&@


NC
DM
DM+ I
DM+ MSE
DM +S
DM+ CGA
Fasting Serum Insulin (mIU/ml)
18
16
14
12
10
8
6
4
2
0
*
*#
*#
*#
*#$&@

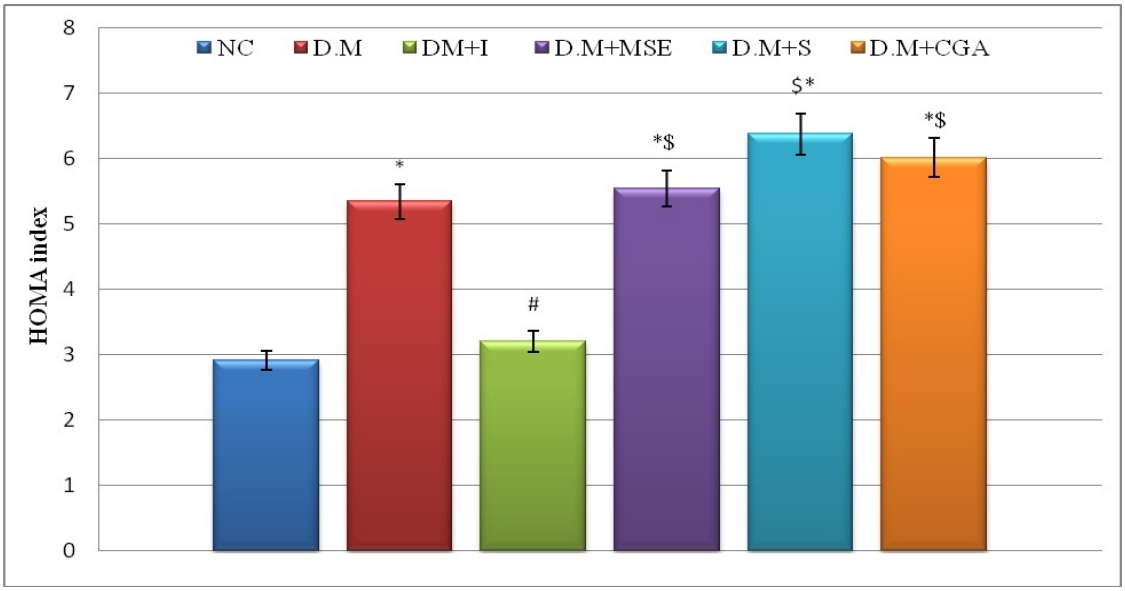
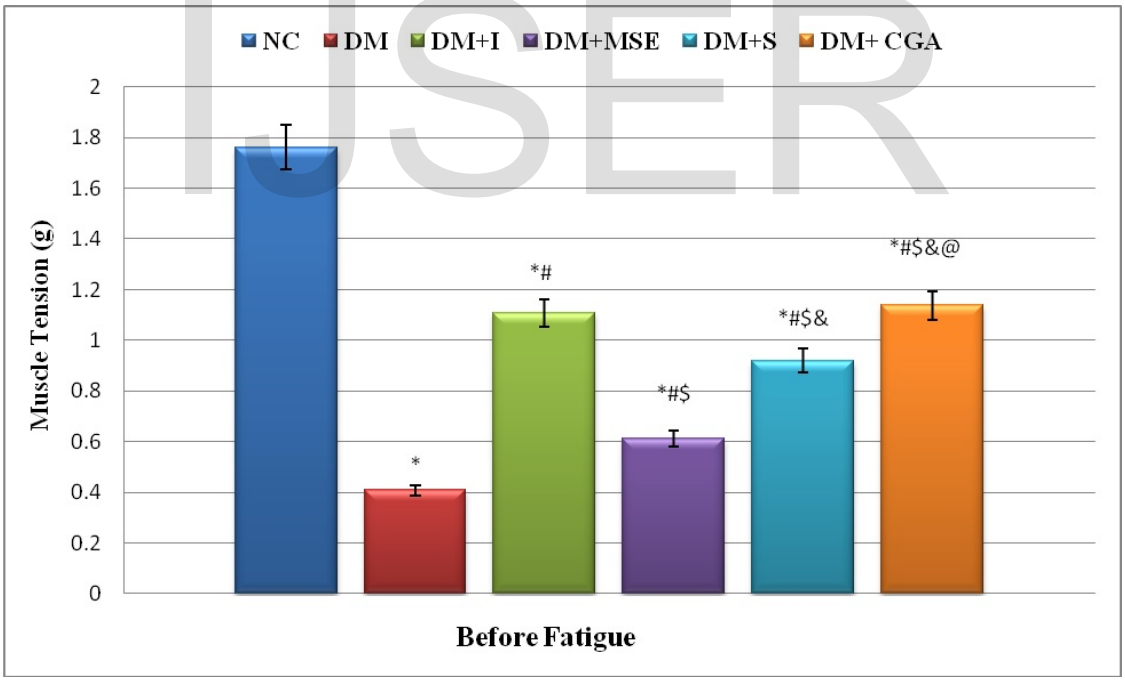
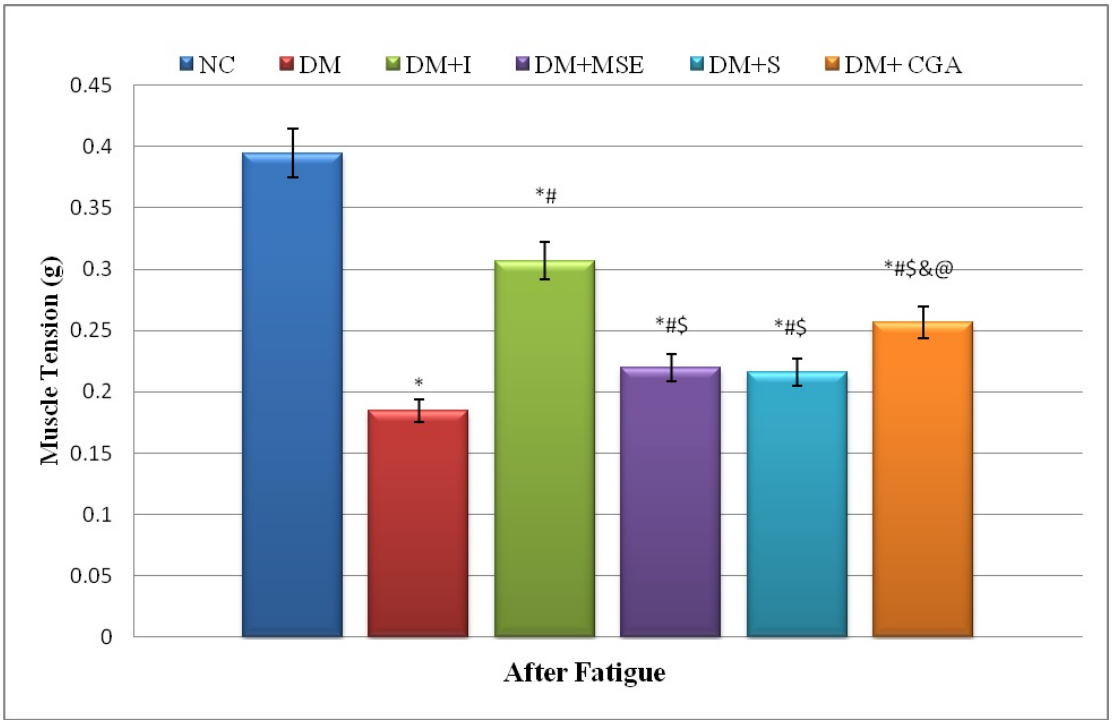
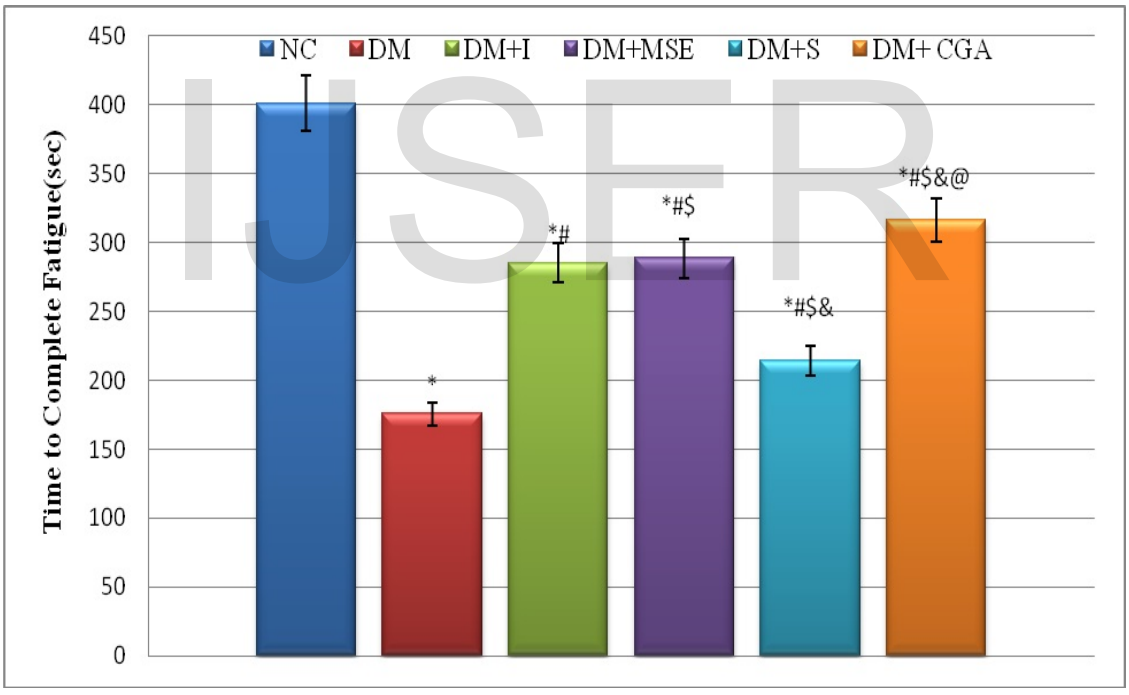
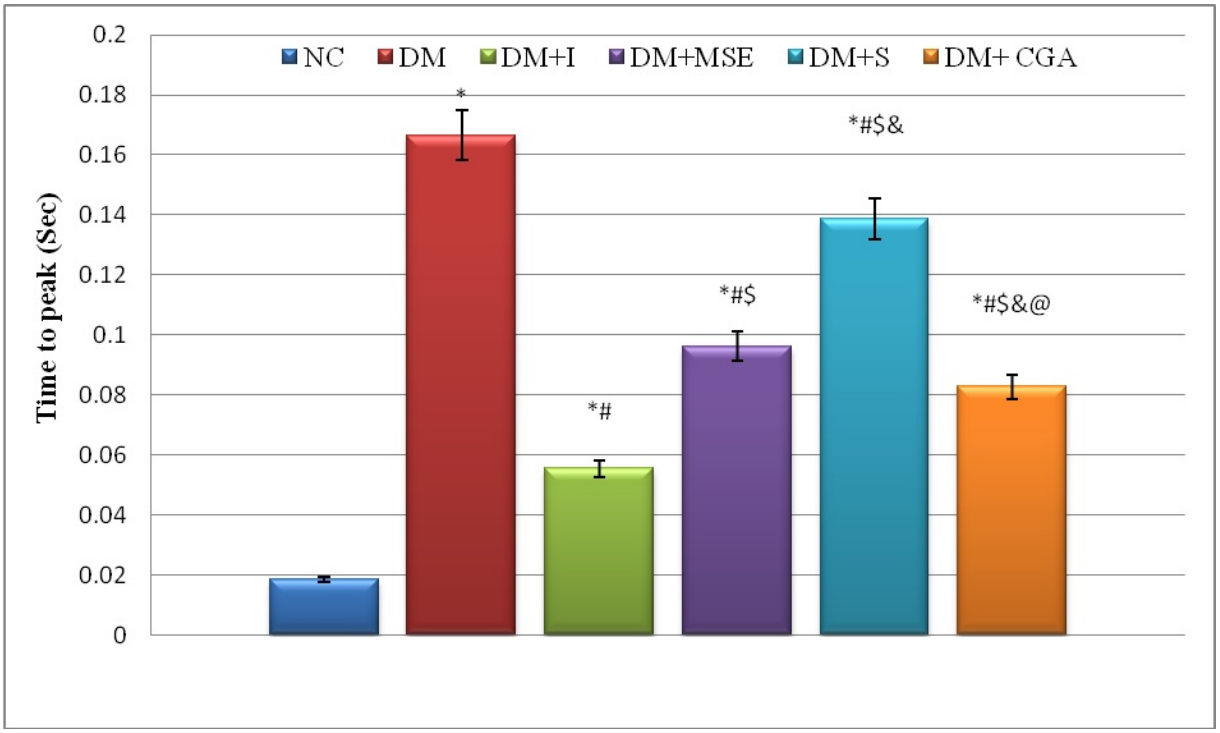


Fig. (2): Effects of insulin, MSE, Stevioside and CGA on fasting blood sugar FBS (A), fasting serum insulin (B) and HOMA index (C) in type 1 diabetic rats.*significant vs NC group, # significant vs DM group, $significant vs MD+I group ,&significant vs DM+MSE group ,@ significant vs DM+S group.




NC
DM
DM+I
DM+MSE
DM+S
DM+ CGA
Muscle Tension (g)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*#
*#$
*#$
*#$&@
After Fatigue


NC
DM
DM+I
DM+MSE
DM+S
DM+ CGA
Time to Complete Fatigue(sec)
450
400
350
300
250
200
150
100
50
0
*
*#
*#$
*#$&
*#$&@



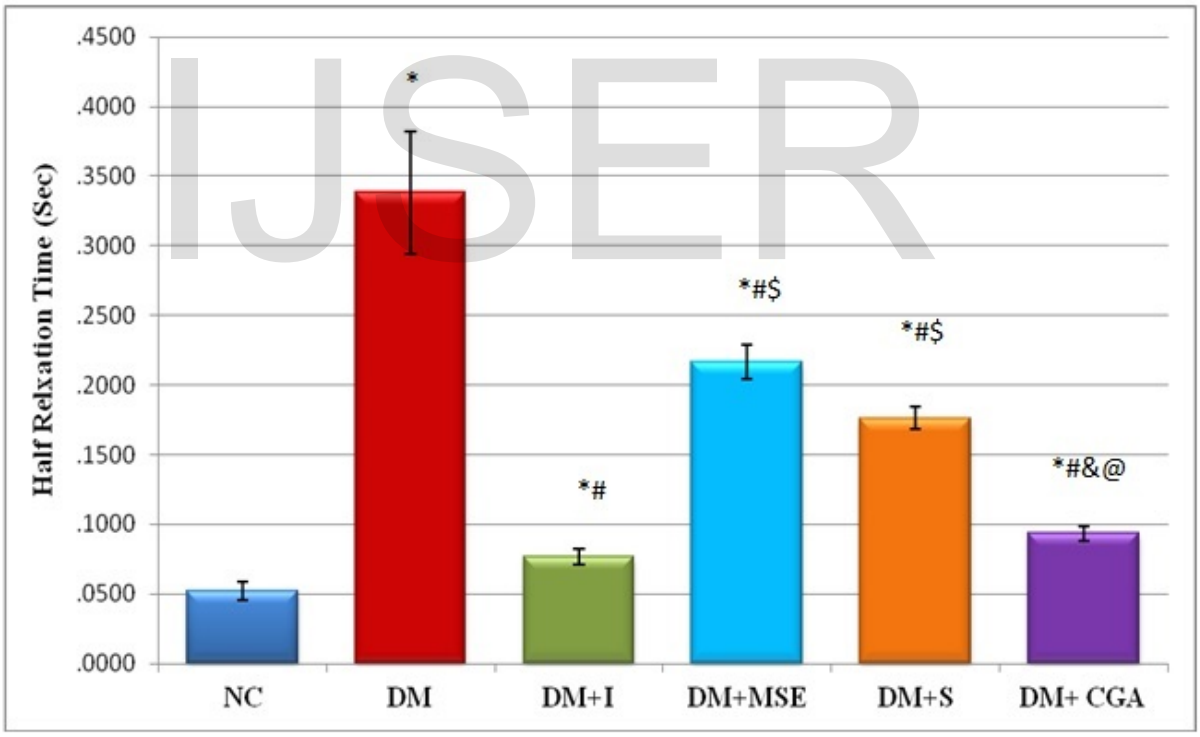


Fig. (3): Effects of insulin, MSE, Stevioside and CGA on muscle tension before fatigue (g) (A), muscle tension after fatigue (g) (B), time to complete fatigue (sec) (C), time to peak (sec) (D), and half relaxation time (sec) (E).*significant vs NC group, # significant vs DM group, $significant vs MD+I group ,&significant vs DM+MSE group ,@ significant vs DM+S group**.**

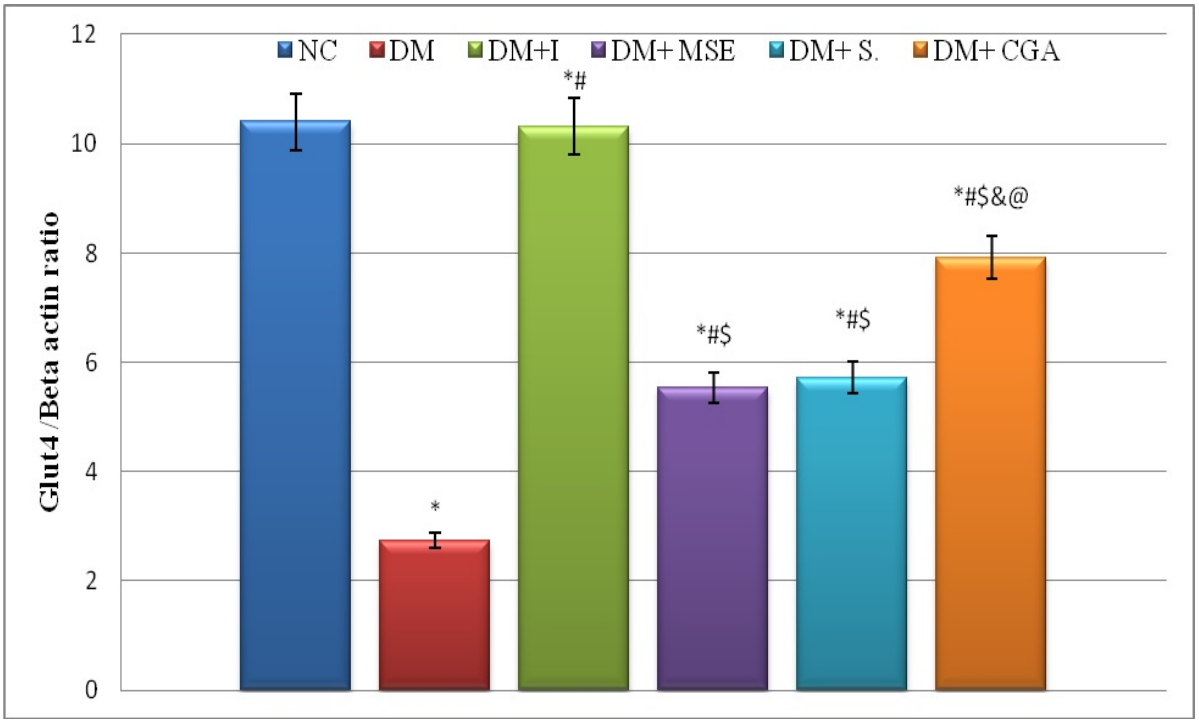


Fig. (4): Effects of insulin, MSE, Stevioside and CGA on expression of mRNA of GLUT4 gene in soleus muscle by real time PCR. $significant vs MD+I group ,&significant vs DM+MSE group ,@ significant vs DM+S group.