Ethanolic seeds extract of *Centratherum anthelminticum* reduces oxidative stress in type 2 diabetes

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Abstract: This work was accomplished to assess the *in-vitro* antiglycation and antioxidant activities of ethanolic seeds extract of *Centratherum anthelminticum* (CSEt), followed by its *in-vivo* examination in type 2 diabetes. Overnight fasted rabbits were divided into control and diabetic groups. Rabbits in diabetic group were fed with 35% fructose solution to develop hyperglycemia that was well-monitored by glucometer. These were divided into diabetic control (distilled water 1ml/kg), positive control group (pioglitazone 15mg/kg) and two test groups (CSEt 400 and 600mg treated). All treatments were given orally. After 14 days, rabbits were sacrificed and blood samples were used to estimate glycated haemoglobin (HbA_{1c}) while total bilirubin (direct and indirect), uric acid, alanine aminotransferase (ALT) and creatine kinase (CK) were done in sera. In addition, antioxidant parameters *viz.,* catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and lipid peroxidation (LPO) were in liver tissues. The *in-vitro* studies showed good antiglycation and antioxidant potential of CSEt. Similarly, *in-vivo* investigation showed significant reduction in glycemia and body weights in type 2 diabetic test groups. Plus values of HbA_{1c}, ALT, CK, uric acid and bilirubin were almost back to normal along with improvement found in efficiency of antioxidant parameters.

Keywords: *Centratherum anthelminticum*, fructose, type 2 diabetes, antioxidant parameters.

INTRODUCTION

Insulin resistance, the basic feature of type 2 diabetes induces hyperglycemia that severely affects enzymes, electron transport chain (ETC) and accelerate the production of free radicals which influence all body metabolism including β-cells performance (Savu *et al*., 2012). If this oxidative stress persists for a long time, the beta cells of pancreas easily lost their insulin secretary ability (Poitout and Robertson, 2008). The risk of insulin resistance also enhances globally with the high rate of fructose utility which is related to its higher catabolic activity inside the cells to generate energy deficit environment that equally supports the free radical pool as well as membrane damage (Thomas *et al.,* 2015). This situation lowers the efficiency of oxidative stress fighting enzymes that makes pancreatic β-cells completely defenceless while oxygen containing reactive species like peroxides, superoxide and singlet oxygen become free to initiate micro- and macro-vascular level of cellular failure by oxidizing lipid and proteins (Baynes, 1991; Nishikawa *et al*., 2000; Li *et al*., 2008).

Literature also proved that insulin resistance induce glucose stress via activation of hexosamine pathway that amplifies UDP-N-acetylglucosamine level and stimulates O- & N-linked glycosylation of other proteins by altering the activity of acetylglucosamine transferase enzyme (Vasconcelos-dos-Santos *et al*., 2017; Zhang *et al*., 2013). Additionally lipid peroxidation which occurs either enzymatic (activation of phospholipase A_2 to digest lipids) or non-enzymatic (mitochondrial generation of ROS) also flare up oxidative stress in diabetes (Arora *et al*., 2013) that causes deterioration of membrane lipids and initiates many problems of body tissues (Devi *et al*., 2005; Thomas *et al*., 2015). Therefore, the detoxification of free radicals should be considered as one of the strategies for the treatment of type 2 diabetes to overcome the difficulties of this world health hazard, besides normalizing the blood glucose level by reducing insulin resistance. After observing noteworthy impact of ethanolic seeds extract of *Centratherum anthelminticum* (CSEt) on decreasing insulin resistance in fructose-induced type 2 diabetes (Mudassir and Qureshi, 2015), this study was intended for further *in-vitro* and *in-vivo* antiglycation and antioxidant assessments of same extract. In addition, hepato- and cardio-protective effect of CSEt was evaluated.

MATERIALS AND METHODS

Formation of seeds extract

C. anthelminticum seeds were bought from nearby market; its identification was done by expert in Botany department, University of Karachi and sampled as KU/BCH/SAQ/08. Seeds extract (in ethanol) of *C. anthelminticum* (CSEt) was prepared according to the procedure described in our earlier paper (Mudassir and Qureshi, 2015).

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Positive control and vehicle for CSEt

Pioglitazone was used as positive control, whereas 0.05% DMSO (dimethyl sulphooxide) of Fisher Scientific (UK) was used as vehicle for giving CSEt to experimental rabbits.

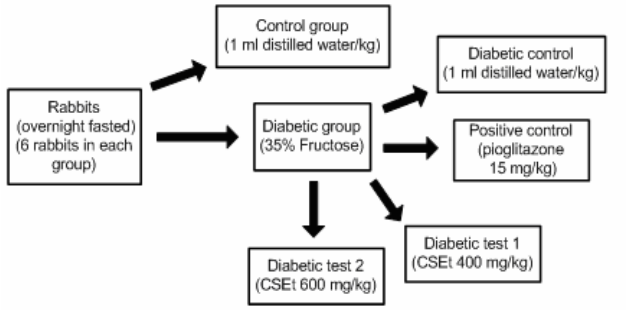
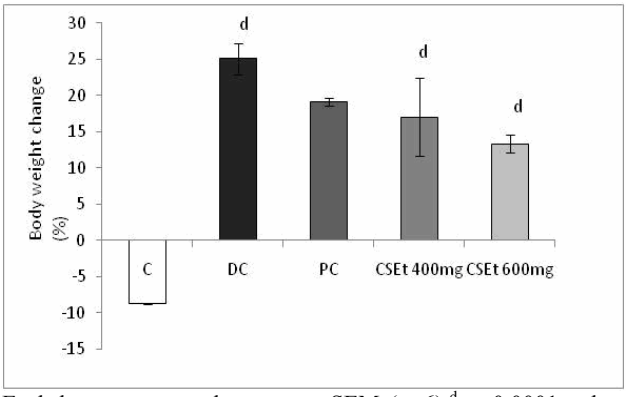
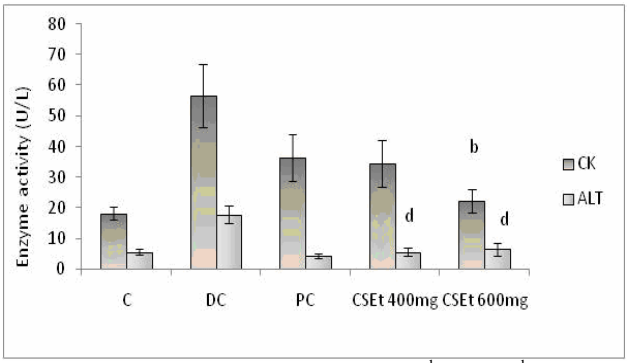


Fig. 1: Animal grouping with their specific treatment



Each bar represents the mean ± SEM (n=6).$^{d}p< 0.0001$, when test groups compared with diabetic control (DC).

Fig. 2: Effect of CSEt on Percent Body Weight Change



Each bar represents the mean ± SEM (n=6). $^{b}p<0.01$, $^{d}p<0.0001$, compared with diabetic control (DC)

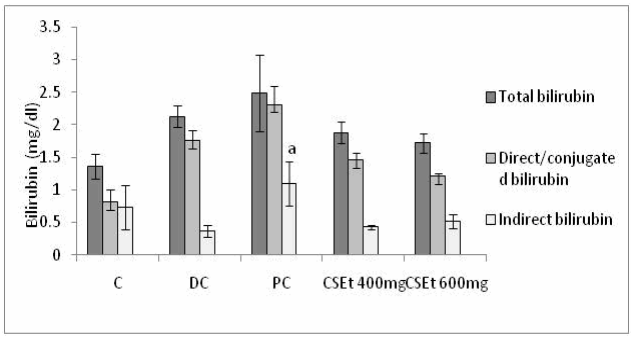
Fig. 3: Effect of CSEt on ALT and CK.

Determination of in-vitro antiglycation and antioxidant activities of CSEt

Antiglycation activity of CSEt was done by advanced glycated end products (AGEs) on human serum albumin (HSA) method (Singh *et al*, 2001) whereas antioxidant activity of same extract was done by diphenyl-l-picrylhydrazyl radical scavenging assay (DPPH) (Thadhani *et al*, 2011).

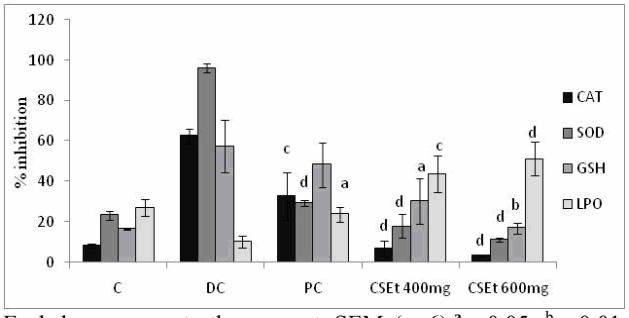
Study protocol of type 2 animal trial

Albino rabbits (male, 1.0 to 1.2kg in weight) were bought from Dow University of Health Sciences (DUHS), Karachi. They were placed in animal house of Biochemistry department, University of Karachi. Following international guidelines, standard laboratory diet and water were provided. 35% fructose in drinking water were given to rabbits after overnight fasting (12-14 hrs) to develop insulin resistance for 14 days without break (Neeharika *et al.*, 2012) whereas animal grouping were done according to fig. 1.



Each bar represents the mean ± SEM (n=6).$^{a}p<0.05$ compared with diabetic control (DC).

Fig. 4: Effect of CSEt on Serum Bilirubin



Each bar represents the mean ± SEM (n=6).$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, & $^{d}p<0.0001$, compared with diabetic (DC) group

Fig. 5: Effect of CSEt on antioxidant parameters.

Determination of percent body weight change

Percentage of body weight change of rabbits of all groups was calculated by the authentic formula (Azmi and Qureshi, 2016).

$$\text{Percent body weight change} = \left(\frac{\text{Final day weight}}{\text{Initial day weight}}\right)\times 100 \quad \text{(Initial day weight)}$$

In-vivo biochemical analysis

Percent glycemic change was also calculated with the help of same above mentioned formula by putting the values of fasting blood glucose of initial and final days of animal trial.

HbA_{1c} (glycated haemoglobin) was assessed in whole blood through Turbidimetric Inhibition Immunoassay

Table 1: *In vitro* antiglycation and antioxidant activities of CSEt

Samples	Antiglycation Activity		Antioxidant Activity	
	Percent Inhibition	IC_{50}±SEM [ug/ml]	Percent Radical Scavening Activity	IC_{50}±SEM [ug/ml]
CSEt	80.5	217.6±5.2	67.36	321.31±0.66
Rutin (0.5mg/ml)	94.5	27.0±0.15	-	-
Gallic Acid (0.094ng/ml)	-	-	93.13	4.3±0.43

IC_{50} ± SEM = 50 % inhibitory concentration ± Standard Error Mean (n=3)

Table 2: Effect of CSEt on percent glycemia, HbA_{1c} and serum uric acid

Groups	FBG (mg/dl)		Percent Glycemic Change	HbA_{1c} (mg/dl)	Uric Acid (mg/dl)
	Initial Day	Final Day			
Control	112.5±11.79	100.75±6.49	-8.51±7.25	4.45±0.14	10.39±0.29
Diabetic Control	102.75±3.59	125.25±1.18	21.59±4.39	6.67±0.13	14.3±1.89
Positive Control	100±16.41	119.25±1.7	26.28±14.7	5.4±0.20^{d}	11.61±1.57
CSEt 400mg	114±4.14	103.25±3.32^{bc}	-9.26±2.55^{c}	5.97±0.09^{c}	9.86^{b}±0.13
CSEt 600mg	115.5±2.02	102.5±3.8 ^{bc}	-11.18±3.49	5.62±0.11^{d}	9.43^{b}±0.29

Values are mentioned as mean ±SEM (n=6). $^{b}p<0.01$, $^{c}p<0.001$, & $^{d}p<0.0001$, test groups compared with diabetic control (DC)

(TINIA) on Roche/Hithachi 902 analyzer. Whereas total bilirubin (direct and indirect), uric acid, alanine aminotransferase (ALT) and creatine kinase (CK) were estimated in serum by Randox kits (UK).

Estimation of antioxidant parameters

The percent inhibition of catalase (CAT), super oxide dismutase (SOD), reduced glutathione (GSH) and lipid per oxidation (LPO) in liver homogenate were evaluated according to the methods described by Lateef and Qureshi, 2014.

STATISTICAL ANALYSIS

Results are presented as means ± SEM (Standard Error Mean) and measured as significant at $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$ when analysed by ANOVA (SPSS version 18).

RESULTS

In-vitro antiglycation and antioxidant activities of CSEt

CSEt showed good (80-82%) *in vitro* antiglycation activity which seems to be closer to standard rutin (table 1). Similarly CSEt demonstrated moderate *in vitro* antioxidant activity in terms of DPPH- radical scavenging activity than standard gallic acid (table 1).

Effect of CSEt on percent body weight change

The rabbits in diabetic control group showed 25 % gain in body weight after administering 35% fructose solution for 14 days. Whereas positive control (15mg pioglitazone) and two test groups CSEt (400 and 600mg/kg) showed a significant decrease ($p<0.0001$) in the values of the percent body weight gain as 19.15, 17.04 and 13.32% respectively (fig. 2).

Effect of CSEt on percent glycemic change and biochemical parameters

A significant ($p<0.001$) reduction in glycemic percentage was found from -9.26 to -11.18% in test groups treated with CSEt 400 and 600mg/kg than diabetic and positive control groups that showed 21 to 26 % increase in glucose level (table 2). The HbA_{1c} values in both test groups (400 and 600mg/kg) were also reduced significantly ($p<0.001$) and ($p<0.0001$) respectively as compared to diabetic control group (table 2).

Both doses of CSEt (400 & 600mg/kg) in test groups along with positive control (15mg/kg pioglitazone) showed a significant reduction in serum ALT and uric acid concentrations ($p<0.01$) in comparison with diabetic control group (fig. 3, table 2) whereas concentration of serum CK was found decreased ($p<0.05$) only in CSEt (600mg/kg) test group (fig. 3). CSEt (400 & 600mg/kg) also lowered the total bilirubin concentration by improving direct and indirect bilirubin levels in test groups (fig. 4).

Effect of CSEt on percent inhibition of antioxidant parameters

Both test groups treated with CSEt (400 & 600mg/kg) and positive control (15mg/kg pioglitazone) showed a significant decrease ($p<0.0001$ and $p<0.001$) in percent inhibition of CAT, SOD and GSH as compared to diabetic control group (fig. 5). Similarly, LPO was significantly inhibited in all these groups as compared to diabetic control group (fig. 5).

DISCUSSION

In diabetes, the non-enzymatic glycation of proteins gives rise the generation of HbA_{1c} primarily which is almost directly proportional to the blood glucose level (Farhan *et al*., 2012). *In-vitro* antiglycation activity of CSEt revealed 80% inhibition of protein glycation which is nearer to the glycation protective activity showed by standard rutin (flavonoid).This was also confirmed by observing in vivo effect of CSEt in fructose-induced diabetic rabbits *via* monitoring a gradual decrease in HbA_{1c} levels with respect to doses in both test groups than diabetic control group.

Fructose-induced obesity is the major warning of hyperinsulinema in type 2 diabetes (Wilson and Islam, 2012) that can be corrected by improving the sensitization of insulin receptors (Monnier *et al*., 2009) and this can be achieved by controlling the body weight gain via reducing triglycerides (TG) level. In the present study, the same objective was accomplished in the form of reduced percent body weight gain of test groups (CSEt 400 & 600 mg/kg). This finding is also compatible with our previously reported paper in which same extract effectively decreased lipid profile in type 2 diabetic rabbits (Mudassir and Qureshi, 2015). Therefore, the body weight controlling effect of CSEt is actually the reflection of its hypolipidemic effect that possibly retrieves the unmasking of receptors and accelerates the insulin binding.

The serum concentration of total protein and enzymes that synthesized by liver revealed the healthy or unhealthy behaviour of hepatocytes (Schultz *et al*., 2013). Increased fructose intake is directly associated with hepatic lipogenesis in which lipoprotein and hormone sensitive lipases inhibition produces the TG accumulation in liver tissues that can weakened cell membrane fragility which in turn increased liver-specific enzyme ALT concentration in serum (Azmi and Qureshi., 2016). Any kind of liver dysfunction can be monitored in terms of ALT activity because it is the mirror image of normal hepatocytes' function and often higher ALT levels has been reported in type 2 diabetes (Ballestri *et al*., 2016; Wang *et al*., 2016). Interestingly, CSEt was found to normalize ALT level in test groups. Another important tool for assessing liver physiological turn out is total bilirubin (direct and indirect bilirubins) (Han *et al*., 2010). High fructose consumption enhances the biosynthesis of direct bilirubin that easily diffuses in circulation to raise total bilirubin level. This same was observed in fructose-induced type 2 diabetic control rabbits whereas test groups treated with CSEt showed decreased levels of total and direct bilirubin concentrations. Therefore, normalizing the ALT and total bilirubin values in test groups constitute the defensive role of CSEt towards hepatocytes.

Similar cell protective effect of CSEt was observed as uric acid was decreased in CSEt treated test groups *via* possibly improving the cellular strength of hepatocytes. This could be affected in terms of impaired protein synthesis and decrease availability of adenosine triphosphate (ATP) that is largely consumed in fructose metabolism in liver which severely affects the half life of cells (Mahmood, 2007; Murray *et al*., 2000). The same was clearly observed by elevated levels of uric acid up to 14 mg/dl in diabetic control group. Creatine kinase is a heart-specific and any kind of cardiac muscle injury (heart attack) increases its release in blood (Frank and Finsterer, 2012). CSEt was also found as cardio-protective agent as it maintained the normal CK level in both test groups whereas elevated level of same enzyme found in diabetic control group.

In insulin resistance type 2 diabetes prolonged hyperglycemia and hyperlipidemia are the major reason of liver inflammation and oxidative stress which induce mitochondrial dysfunction and generate high amount of oxygen reactive species (hydrogen peroxide, superoxide, hydroxyl radicals etc) that consequently suppress antioxidant enzymes status (Waggiallah and Alzohairy, 2011; Kumawat *et al*., 2013). These free radicals induce oxidation and damage the membrane lipids of macromolecules (Sheweita *et al.,* 2016). Likewise, diabetic control rabbits showed high percent inhibition of antioxidant enzymes and protein including CAT, SOD and GSH while minimum percent inhibition of lipid per oxidation whereas CSEt significantly reduced percent inhibitions of CAT, SOD, GSH and increased percent inhibition of lipid per oxidation in hepatocytes of both test groups. Even CSEt found more remarkable than pioglitazone (positive control) regarding uplifting the antioxidant enzymes status. Confirmatory evidence for antioxidant potential of CSEt was also obtained by observing 67% i*n vitro* radical scavenging activity in the present study. The previously reported presence of phenols and flavonoids in this extract might be responsible for better activity of antioxidant enzymes and recovering of GSH level in liver tissues of test groups (Mudassir and Qureshi, 2015)

CONCLUSION

The present study proves that *C.anthelminticum* seeds extract has great potential in reducing the oxidative stress and glycation by uplifting the antioxidant enzymes and decreasing the HbA_{1c} levels in fructose-induced diabetic rabbits besides showing hypoglycemic, hepato- & cardiac protective and weight controlling effects in these rabbits.

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