Available online at www.journalijmrr.com

INTERNATIONAL JOURNAL OF MODERN RESEARCH AND REVIEWS

ISSN: 2347-8314

International Journal of Modern Research and Reviews

Volume 2, Issue 1, pp 26-32 January, 2014

ORIGINAL ARTICLE

COMPARATIVE EFFECT OF INDOLE-3-CARBINOL AND ITS METABOLITE 3, 3'- IINDOLYLMETHANE ON GLYCEMIC CONTROL; A DOSE RESPONSE STUDY

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Article History: *Received 20 th Dec,2013, Accepted 30th Dec,2013,Published 31st Jan,2014*

ABSTRACT

The current study was hypothesised to monitor the effect of indole-3-carbinol (I3C) and its metabolite, 3, 3′- diindolylmethane (DIM) in a dose dependant manner thereby these could able to attenuate the type 2 mellitus in C57BL/6J mice. The HFD mice was administered with I3C at the doses of 20, 40, 80 mg/kg. b.wt and DIM at the doses of 5, 10, 20 mg/kg. b.wt. After treatment body weight, glucose, hepatic markers (AST, ALT, ALP and GGT) and nephritic markers (urea, uric acid and creatinine) were measured in control and HFD mice. HFD mice show increased body weight, plasma glucose, hepatic markers and nephritic markers. I3C and DIM at the doses of 40 and 10 mg/kg. b.wt reduce the body weight and glucose levels. The hepatic and nephritic markers where moderately reduced in the HFD mice treated with I3C (20 and 40 mg/kg. b.wt) and DIM (5, 10 and 20 mg/kg. b.wt) in contrast the above markers were elevated in mice administered with I3C at its maximum dose (80 mg/kg. b.wt). Hence the effective dose for the I3C and DIM were found to be 40 and 10 mg/kg. b.wt respectively and also among the two compounds DIM (10 mg/kg. b.wt) exerts accelerated glycemic control.

Keywords: Insulin resistance, Diabetes mellitus, High fat diet, Hepatic marker enzymes.

1.INTRODUCTION

The World is facing diabetic explosion and the cause may be both nature and nurture. According to the World Health Organization (WHO), diabetes mellitus affects approximately 171 million people worldwide and the number is expected to reach to 366 million in 2030 (Amos et al., 2010). Evidence is accumulating that the fundamental defect of type 2 diabetes mellitus (T2DM) is primarily associated with insulin resistance. The consistent data throughout the world also shows that obesity, associated insulin resistance, and T2DM have dramatically increased in locations where lifestyles have recently been westernized (Martin et al., 1992). Even though there were loads of drugs that can be used as treatment modalities for T2DM they can cause undesirable side effects or contraindications consequently there was a necessity to develop the plant based drugs to overcome myriad of problems. In this study the genetically modified mice (C57BL/6J) that mimic human abnormalities of T2DM were used for preclinical studies.

I3C (shown in figure 1) is a dietary component derived from indolyl-methyl glucosinolate, accumulated in cruciferous vegetables such as cabbage, broccoli, Brussels sprouts, cauliflower, napa cabbage, kale and mustard. Upon maceration, endogenous enzyme particularly myrosinase, lead to the breakdown of indolyl-methyl glucosinolate to form a range of indoles, including I3C and its predominant metabolite DIM, shown in figure 2 (Bharat et al., 2005). In this study, we sought to address whether the I3C and its metabolite DIM exerts glycemic control in a dose dependant manner on HFD-induced C57BL/6J mice and also to access its extent of toxicity by monitoring hepatic and nephritic markers.

Figure 1. Structure of indole-3-carbinol (I3C)

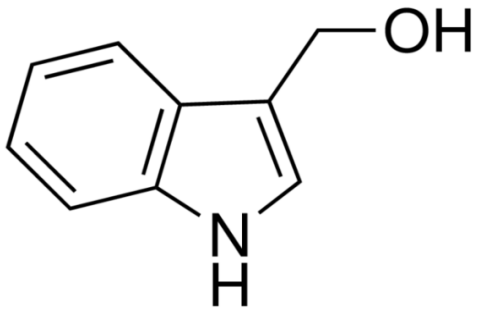

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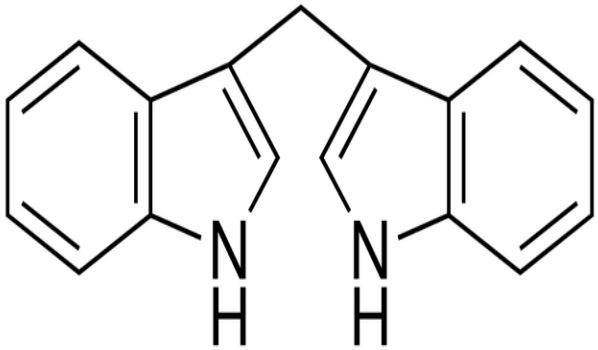
Figure 2. Structure of 3,3´-diindolylmethane (DIM)



2.MATERIALS AND METHODS

Chemicals

Indole-3-carbinol and 3,3'-Diindolylmethane were purchased from Sigma-Aldrich. Unless specifically stated, all other reagents were purchased from Sigma.

Animals

Male C57BL/6J mice with a body weight ranging from 20 to 25 g, were purchased from the National Institute of Nutrition (NIN), Hyderabad. All experimental procedures were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No. 160/1999/CPCSEA, Proposal number: 912), Annamalainagar. Mice were housed in a room maintained at a controlled temperature (23 ± 1°C) and 12 h dark/light cycles. Food and water provided *ad libitum* to all the animals.

Diet

The standard diet consisted of a balanced diet containing protein 21.1%, fat 5.1%, carbohydrate 60.0%, minerals 7.9%, fiber 3.9% and vitamins 2.0%. High fat diet (HFD) of about 40 % was prepared by mixing beef tallow (34.9%) with standard pellet diet every day. All measures were taken to ensure uniform mixing of the additives of the diet before kneading using a little water.

Preliminary study

After adaptive feeding, preliminary study was carried out to assess the effect of I3C and DIM on blood glucose levels in HFD mice. The animals were randomly divided into ten groups of six animals each as given below.

Group I	:	Control
Group II	:	Control + I3C (80 mg/kg. b.wt)
Group III	:	Control + DIM (20 mg/kg. b.wt)
Group IV	:	High fat diet (HFD)
Group V	:	HFD + I3C (20 mg/kg. b.wt)
Group VI	:	HFD + I3C (40 mg/kg. b.wt)
Group VII	:	HFD + I3C (80 mg/kg. b.wt)
Group VIII	:	HFD + DIM (5 mg/kg. b.wt)
Group IX	:	HFD + DIM (10 mg/kg. b.wt)
Group X	:	HFD + DIM (20 mg/kg. b.wt)

The compounds were suspended in 0.5% DMSO vehicle solution and fed by gastric intubation. Blood was collected from the tail after fasting i.e. 0 hour (h), 2 h and 15 days (d) after giving the I3C at a dose of (20, 40 and 80 mg/kg. b.wt) and DIM at a dose of (5, 10 and 20 mg/kg. b.wt) in 0.5% DMSO. Glucose values were measured by enzymatic method using commercial kit (Agappe Pharmaceutical, Kerala, India) with a semi-autoanalyser.

Long term study

In the long term study I3C and DIM were administered orally once in a day in the morning at 10.00 am for 35 d. The initial and final body weight, food and water intake of all the groups were monitored. At the end of 35 d, all animals were anesthetized using ketamine (24 mg/kg b. wt), intramuscular injection), and sacrificed by cervical dislocation between 8:00 am and 9:00 am. Blood samples were collected in tubes containing potassium oxalate and sodium fluoride (3:1) mixture for the estimation of plasma glucose. The serum also collected to analyse various hepatic and nephritic markers. The tissue of kidney was also collected for histopathological studies.

Biochemical parameters

Glucose was estimated by the method of Trinder using a reagent kit (Trinder et al., 1969). The serum was used for the determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT) which were estimated (by using commercially available kits), by the method of Reitman and Frankel (Reitman and Frankel , 1957). The activities of serum alkaline phosphatase (ALP) and γ-glutamyl transferase (γ-GT) were estimated by the methods of Kind & King and Rosalki & Rau respectively (Kind and King, 1954; Rosalki and Rau, 1972). Urea and Uric acid in the plasma was estimated by using the diagnostic kit based on the method of Fawcett and Scott and Caraway (Fawcett and Scott, 1960; Caraway, 1955). Creatinine in the plasma was estimated using the diagnostic kit based on the method of Tietz (Tietz, 1987).

Histopathological examination

Kidney was excised, fixed in 10% neutral formalin, dehydrated in graded alcohol (80-100%), cleared in xylene and embedded in paraffin. Then the tissue was sliced into 5 µm pieces using microtome, deparaffinated in xylene, passed through 80 to 100% alcohol and stained with hematoxylin and eosin (HE), for Olympus BX40 photomicroscope assessments.

Statistical analysis

Values are given as means ± S.D. for six mice in each group. Data were analyzed by one-way analysis of variance followed by Duncan's Multiple Range Test (DMRT) using SPSS version 10 (SPSS, Chicago, IL). The limit of statistical significance was set at $p < 0.05$.

3.RESULTS

Table 1 showed the effect of I3C and DIM in preliminary study on plasma glucose levels and body weight changes in high fat diet induced C57BL/6J mice. The plasma glucose was measured at 0 h, 2 h and 15 d. HFD mice administered with I3C at the dose of 40 mg/kg. b.wt reduce the blood glucose levels when compared to the other two doses 20 and 80 mg/kg. b.wt. Also when compared to the other two doses of DIM (5 and 20 mg/kg. b.wt.), 10 mg/kg. b.wt augments the antihyperglycemic effect.

Table 2 showed the effect I3C and DIM on bodyweight and plasma glucose in high fat diet induced C57BL/6J mice on long term study followed by preliminary study. HFD mice showed elevated bodyweight and blood glucose level and oral

treatments of I3C, at its maximum dose of 80 mg/kg. b.wt showed the elevated levels of hepatic markers when compared to the other two doses of I3C (20 and 40 mg/kg. b.wt) and the three doses of DIM (5,10 and 20 mg/kg. b.wt).

Table 1. Effect of I3C and its metabolite DIM in preliminary study on plasma glucose levels in high fat diet induced C57BL/6J mice

Groups	Plasma glucose (mg/dL)				
	0 h	2 h	% change	15 th d	% change
Control	90.95 ± 8.30 [a]	93.96 ±7.75 [a]	+3.20	96.98 ± 6.64 [a]	+6.63
Control + I3C (80 mg/kg b.wt)	89.96 ± 7.75 [a]	87.98 ± 6.64 [a]	-2.20	84.96 ± 7.75 [a]	-5.55
Control + DIM (20 mg/b.wt)	91.96 ± 7.75 [a]	88.96 ±7.75 [a]	-3.26	85.98 ± 6.64 [a]	-6.50
High fat diet (HFD)	285 ± 17.71 [b]	273.96 ± 16.61 [b]	+3.87	282 ± 17.71 [b]	+1.05
HFD + I3C (20 mg/b.wt)	268 ± 17.71 [b c]	262 ± 17.71 [c]	-2.23	255.96 ±16.61 [c]	-4.85
HFD + I3C (40 mg/b.wt)	264.98 ± 19.92 [c]	240.46 ±18.43 [c]	-9.28	225.73 ± 16.12 [d]	-14.81
HFD + I3C (80 mg/b.wt)	261.96 ± 16.61[c]	250.31 ± 17.97 [c]	-4.44	242.96 ± 16.61 [c]	-7.25
HFD + DIM (5 mg/b.wt)	259.98 ±15.49 [c]	253.96 ± 18.73 [c]	-2.32	239.96 ± 18.80 [c]	-7.70
HFD + DIM (10 mg/b.wt)	263.98±19.92 [c]	227.83 ± 15.43 [d]	-13.74	206.96 ± 14.38 [e]	-21.60
HFD + DIM (20 mg/b.wt)	266.86 ±18.80 [b c]	256 ± 17.71 [c]	-4.06	245 ± 17.71 [c]	-8.19

Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$. (DMRT).

Table 2. Effect of I3C and its metabolite DIM on body weight and plasma glucose levels in high fat diet induced C57BL/6J mice

Name of the group	Body weight (g)			Plasma glucose (mg/dL)		
	0 d	35th d	Change (%)	0 d	35th d	Change (%)
Control	21. 13±1.97	26. 43±2.16 [a]	+20.05	95.00±8.30 [a]	97.32±9.31 [a]	+2.38
Control + I3C (80 mg/kg. b.wt)	22. 03±2.04	29. 89±2.38 [a]	+26.29	89.96 ±7.75 [a]	82.45±7.92 [a]	-9.10
Control + DIM (20 mg/kg. b.wt)	20. 47±1.84	28.11 ±2.56 [a]	+27.17	91.96 ±7.75 [a]	80.21±7.64 [a]	-14.64
High fat diet (HFD)	20. 66±1.63	41.39± 3.90 [b]	+50.08	285 ±17.71 [b]	287.88±21.30 [b]	+1.00
HFD + I3C (20 mg/kg. b.wt)	21. 89±1.92	38.68±3.26 [c]	+43.39	268 ±17.71 [b c]	214.56±18.45 [c]	-24.90
HFD + I3C (40 mg/kg. b.wt)	20. 81±1.78	36.14±3.13 [c]	+42.41	264.98±19.92 [c]	153.98±11.34 [d]	-72.08
HFD + I3C (80 mg/kg. b.wt)	22. 32±2.13	35.70±3.30 [d]	+37.47	261.96±16.61[c]	178.32±13.78 [d,e]	-46.90
HFD + DIM (5 mg/kg. b.wt)	20.50±1.81	36.33±2.94 [c]	+43.57	259.98±15.49 [c]	168.34±12.95 [d,e]	-54.43
HFD + DIM (10 mg/kg. b.wt)	20.25±1.97	28.41±2.51[d,e]	+28.72	263.98±19.92 [c]	142.47±10.12 [f]	-85.28
HFD + DIM (20 mg/kg. b.wt)	21.92±1.68	29.57±2.42 [d,e]	+26.87	266.86 ±18.80 [b c]	155.56±11.31 [d]	-71.54

Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$.(DMRT).

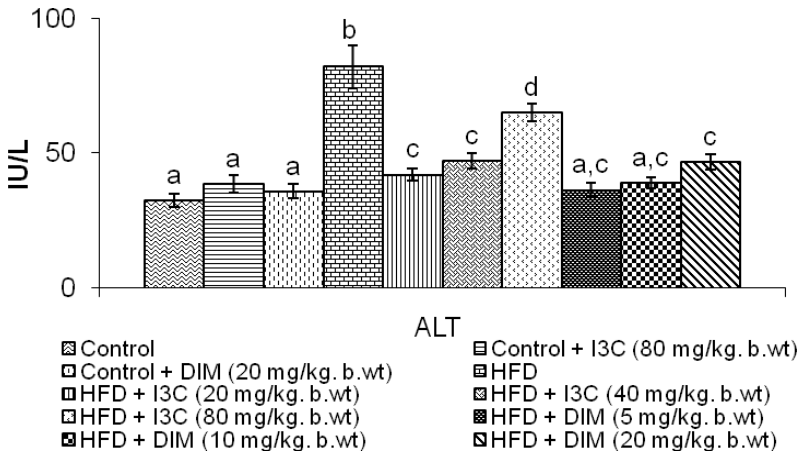
administration of 13C and DIM for 35 d reduced body weight and plasma glucose levels significantly Treatment with 13C and DIM at the dose of 40 mg/kg.b.wt. and 10 mg/kg.b.wt. lowered the plasma glucose and body weight significantly. Moreover when compared to I3C, DIM at the dose of 10 mg/kg. b.wt was found to be more effective by regaining the glucose level to near normalcy.

The effect I3C and DIM on hepatic markers (AST, ALT, ALP and GGT) in high fat diet induced C57BL/6J mice were showed in figures 3, 4, 5 and 6. Incredibly, the hepatic markers were significantly increased in HFD mice. The observation after the

Figures 7, 8 and 9 illustrated the effect I3C and DIM on nephritic markers (urea, uric acid and creatinine) in HFD induced C57BL/6J mice. The HFD mice showed elevated levels of nephritic markers. Similar to that of the above results I3C at its maximum dose of 80 mg/kg. b.wt shows increased levels of nephritic markers when compared to the other two doses of I3C and three doses of DIM.

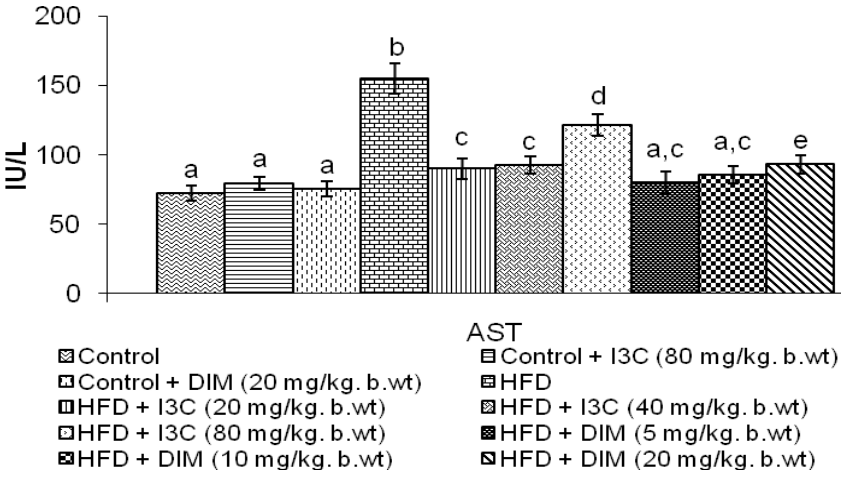
Figure 10 elucidate histopathological changes in kidney (haematoxylin and eosin staining, 20X). The diabetic control (HFD) shows the irregular glomerular architecture. HFD + I3C (80 mg/kg. b.wt) shows glomerular sclerosis and mesangial expansion. Other treated groups show normal structure of glomerulus.

Figure 3. Effect of I3C and its metabolite DIM on ALT in HFD induced C57BL/6J mice



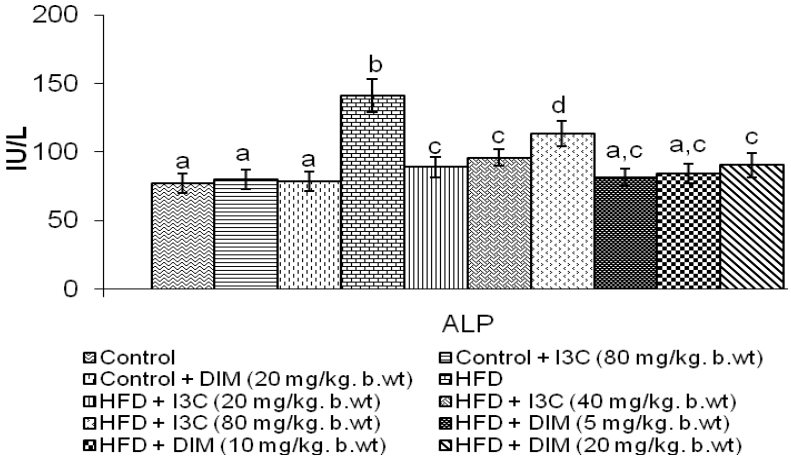
Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$. (DMRT).

Figure 4. Effect of I3C and its metabolite DIM on AST in HFD induced C57BL/6J mice



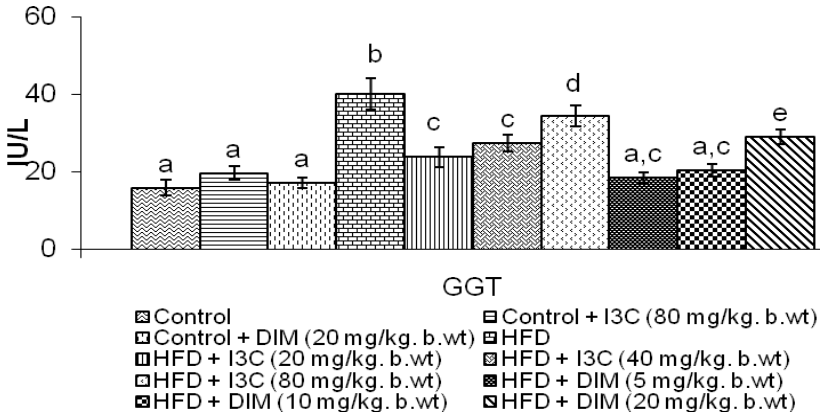
Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$. (DMRT).

Figure 5. Effect of I3C and its metabolite DIM on ALP in HFD induced C57BL/6J mice



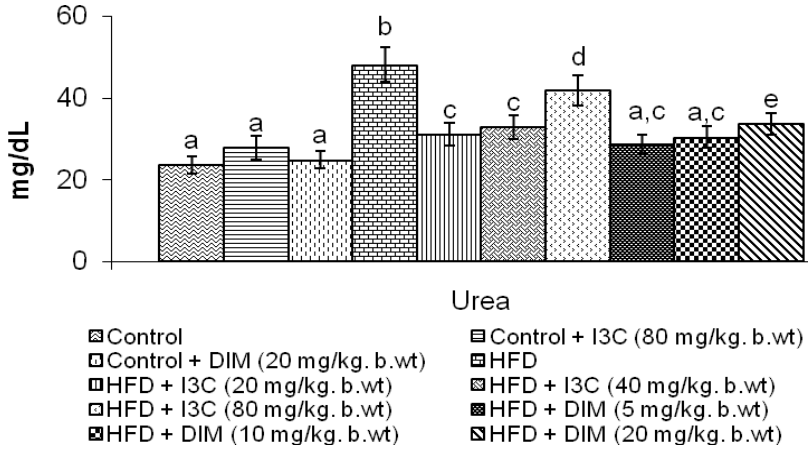
Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$. (DMRT).

Figure 6. Effect of I3C and its metabolite DIM on GGT in HFD induced C57BL/6J mice



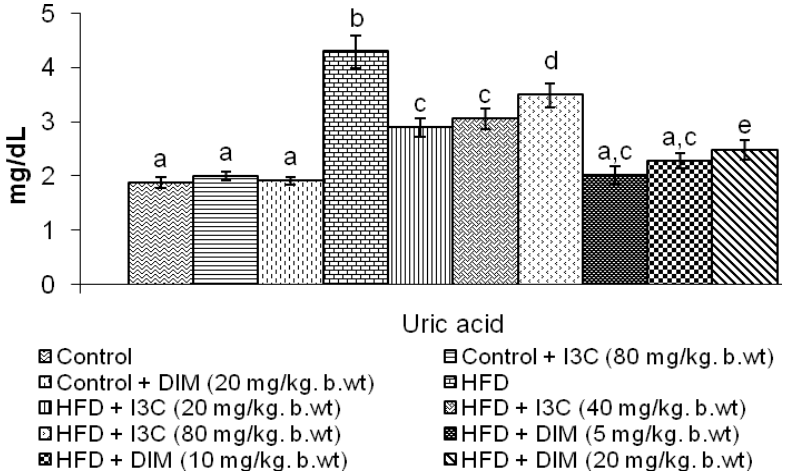
Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$. (DMRT).

Figure 7. Effect of I3C and its metabolite DIM on urea in HFD induced C57BL/6J mice



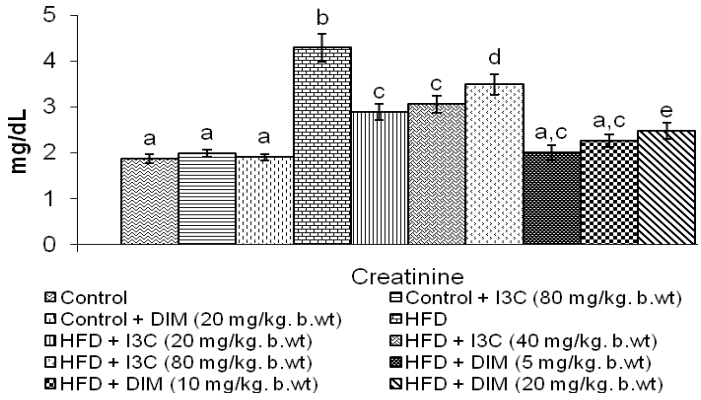
Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$. (DMRT).

Figure 8. Effect of I3C and its metabolite DIM on uric acid in HFD induced C57BL/6J mice



Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$. (DMRT).

Figure 9. Effect of I3C and its metabolite DIM on creatinine in HFD induced C57BL/6J mice



Values are given as mean ± S.D from 6 mice in each group
Values not sharing a common superscript differ significantly at $p < 0.05$. (DMRT).

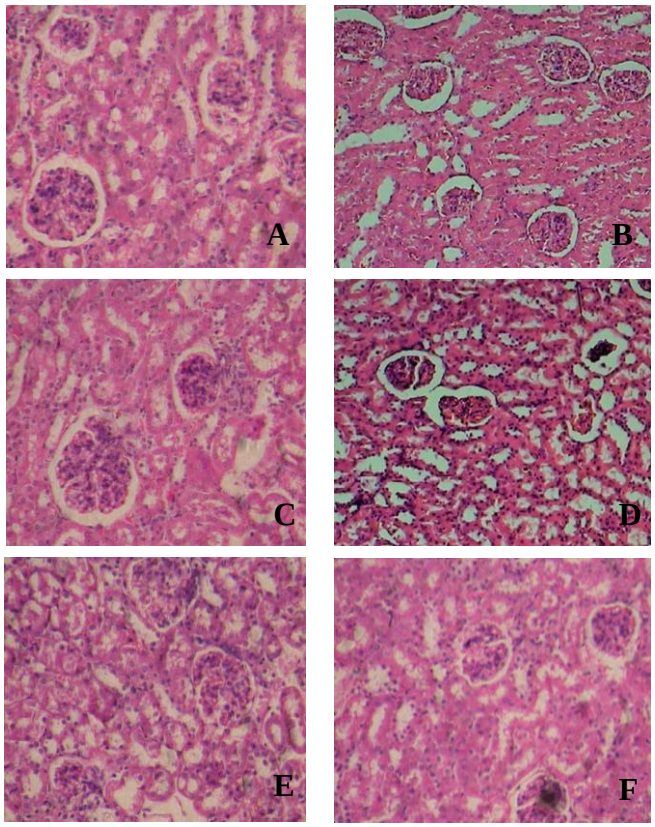


Figure 10. Histopathological changes in kidney (haematoxylin and eosin staining, 20X) (A) Control: Normal control showing typical glomerular structure. (B) Control + I3C (80 mg/kg. b.wt): Showed some mild disformation of glomerular structure (C) Control + DIM (20 mg/kg. b.wt): Showed normal glomerular structure. (D) HFD: Glomerulosclerosis with some area of fibrosis and tubular interstitial injury were observed (E) HFD + I3C (20 mg/kg. b.wt) and (F) HFD + I3C (40 mg/kg. b.wt): Showed near normalization of glomerular structure

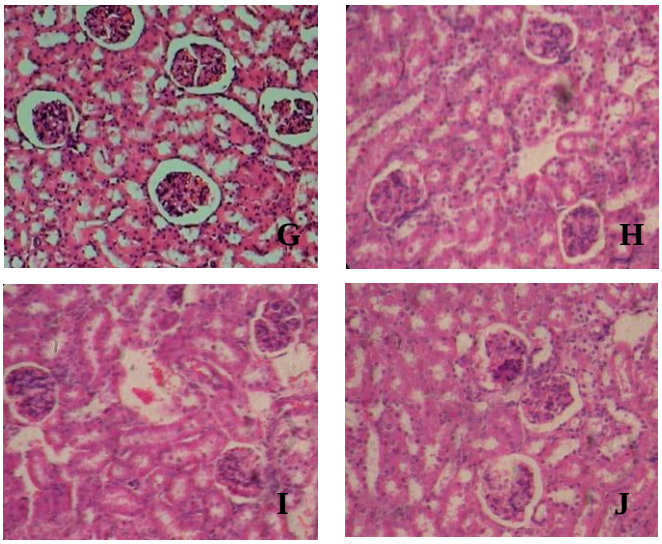


Figure 10. Histopathological changes in kidney (haematoxylin and eosin staining, 20X) (G) HFD + I3C (80 mg/kg. b.wt): Showed abnormal architecture with glomerular sclerosis and mesangial expansion (H) HFD + DIM (5 mg/kg. b.wt), (I) HFD + DIM (10 mg/kg. b.wt) and (J) HFD + DIM (20 mg/kg. b.wt): Regained normal glomerular architecture

4.DISCUSSION

The first part of the discussion focus on the model used in our study. It was well known that in the modern lifestyle the high fat diet was the major diabetogen that was responsible for the development of obesity which leads to T2DM. Also the numerous studies supports the notion that fat, and not carbohydrate, was the critical factor in the development and maintenance of obesity and T2DM. Hence the animal model(C57BL/6J mice) that mimicking human T2DM was chosen for this study. The mice were fed with high fat diet for 10 weeks thereby it induces diabetes and the control mice render to be non- diabetic as it receives standard pellet. To the best of our knowledge emerging data suggest that phytochemicals such as I3C and DIM can act on a number of cellular signalling pathways leading to some biological effects. This part of research was provoked by the therapeutic usefulness of I3C and its metabolite DIM in T2DM, as it remains to be unclear.

In the present study after fed with high fat diet to mice, there was an increase in body weight when compared to the mice fed with standard diet. There was a significant relation observed between the body weight gain and hyperglycaemia in HFD mice. From the earlier studies it was clear that the body weight gain in HFD mice was not only due to increasing feeding efficiency but also due to lower metabolic efficiency index when compared to normal diet fed mice (Parekh et al., 1998). Increase in feeding efficiency driven towards greater adipose mass which in turn associated with the drastic conversion from impaired glucose tolerance to T2DM or in the other word leads to insulin resistance. The data in this study regarding the body weight change after administration of I3C and DIM provide an important consideration that I3C at the three doses (20, 40, 80 mg/kg. b.wt) and DIM at the different doses (5, 10 and 20 mg/kg. b.wt) have the potency to reduce the body weight in HFD mice

which positively correlates to our study. Further more our data strongly recommends that the body weight gain was reduced more in mice administered with DIM when compared to I3C.

Our result correlates with the earlier studies that the hyperglycaemia in HFD mice was primarily associated with impaired glucose and lipid homeostasis (Marshall et al., 1991). As discussed earlier in our study the high fat diet intake had of paramount importance when it comes to this mice model for the diabetic research. As high fat intake raised the level of free fatty acid, the availability of insulin receptor for maintaining glucose homeostasis was reduced. Treatment with I3C and DIM at the doses of (40 and 10 mg/kg. b.wt) show appreciable level of decrease in the blood glucose when compared to other doses which suggest that the I3C and DIM with the respective doses have some antihyperglycemic effect by regulating the free fatty acid which aid to glucose homeostasis.

There were several reports suggest that the hepatic markers such as AST, ALT, ALP and GGT were drastically increased in HFD mice indicates that there was some hepatic toxicity which leads to leakage of the hepatic enzymes into blood stream (Nilanjan Das et al., 2012). Result from this portion of this study revealed that the HFD mice treated with I3C at the concentration of 20 and 40 mg/kg. b.wt and DIM at all the three doses shows lower serum activity of liver function marker enzymes AST, ALT, ALP and GGT along with improved hepatocellular structure except the I3C at the dose of 80 mg/kg. b.wt indicates some toxicity when compared to other treated groups.

The metabolic waste such as urea, uric acid and creatinine were removed from the kidney thereby the body fluid chemicals homeostasis was maintained in normal condition. In our study, similar to that of the earlier reports in HFD condition renal toxicicity occurs due to the alteration of renal lipid metabolism by an imbalance between lipogenesis and lipolysis, as well as systemic metabolic abnormalities and subsequent renal lipid accumulation leading to the elevation of the above metabolites in the blood (Amin et al., 2009). The HFD mice treated with I3C at the doses of 20 and 40 mg/kg. b.wt and DIM at the doses significantly reduced the elevation of the above marker in contrast I3C at its maximum concentration showed some renal dysfunction by spilling the above marker from the kidney.

In nutshell our result implies that the ability of DIM to promote antihyperglycemic effect was high as discussed earlier by positively regulating glucose homeostasis with less toxicity when compared to I3C. The reason could be that even though the I3C have some antihyperglycemic effect, after ingestion in the acid environment of the stomach, I3C undergoes hydrolysis to a number of products, including a dimeric product, DIM (Rogan et al., 2006). DIM was acid stable and was detected in the bloodstream after oral intake of I3C or DIM (Anderton et al., 2004; Reed et al., 2006). Also in this study its clear I3C exert some toxicity which may be due to its other metabolites and it was not clear and further detailed study implies the priority to choose the compounds for the diabetic medicine.

5.CONCLUSION

From our findings we demonstrated that the I3C at the dose of (40 mg/kg. b.wt) and DIM at the dose of (10 mg/kg. b.wt) administration contributes to the reduced weight gain and blood glucose in addition it reduces the liver and renal toxicity in HFD mice hence these doses can be chosen for the long term study to investigate its potent antihyperglycemic effect. As the mechanistic link between the drugs and the phytochemicals has only begun to be addressed and much still remains to be elucidated about its mechanism to overcome the genesis of diabetes mellitus in further research.

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