Original Article

Beta Sitosterol Mitigates Diabetic Nephropathy By Facilitating The Expression Of Insulin Signalling And Modulating The Pro-Inflammatory Molecules In Experimental Diabetic Rats

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Abstract

Background: One of most abundant antioxidative component of vegetable oil and other plants, β-sitosterol (SIT), is a highly effective anti-diabetic drug. Our previous studies have shown that β-sitosterol significantly improved glycaemic control in skeletal muscle and adipose tissue by facilitating insulin metabolic signalling molecules but its effects on the kidney is not known.**Aim:**The current study sought to determine

whether SIT could also exert anti-diabetic effects by regulating insulin signalling and preventing inflammation the kidney, which is a major contributor to insulin resistance and diabetic nephropathy.To achieve this, adult male albino rats weredivided in to four groups. Group-1: control; Group 2; high fat diet-induced type-2 diabetic rats; Group 3: Diabetic rats treated with β-sitosterol (20mg/kg b.wt, orally for 30 days) and group-4: Type-2 diabetic rats treated with metformin (50mg/kg.b.wt, orally for 30 days).**Methods:**Insulin receptor (IR), insulin receptor substrate-2 (IRS-2), Akt and interleukin -6 (IL-6), and kidney injury molecules mRNA (KIM-1) were analysed by Real Time-PCR analysis using gene specific primers. **Results:**mRNA expression of insulin signalling molecules (IR/IRS-1/Akt) were significantly improved in diabetic animals treated with 20mg dose of β-sitosterol ($p<0.05$). Conversely, it reduced the mRNA levels of pro inflammatory signalling (IL-6) and gene involved in tissue remodelling (KIM-1) effectively ($p<0.05$). **Conclusion:**Our present findings for the first time clearly indicate that β-sitosterol attenuates insulin resistance in the kidney by modulating the expression of proinflamamtion and KIM-1. Therefore, β-sitosterol could be considered as a potential therapeutic natural drug candidate for the treatment of diabetic nephropathy and associated complications.

Ke words: β-sitosterol; insulin resistance; diabetic nephropathy; insulin signalling; inflammation.

Introduction

Worldwidethereare463millionpeopleaffectedwithdiabeteswhichcausesmillions of deaths in the present scenario. It is estimated that 578 million people will havediabetesby2030and 700millionbythe yearof2045[1].Diabetes isfastgaining the status of a potential epidemic in India with an estimated more than 77 millionpeople suffering from the condition, the largest in any country in the world and thenumbers are predicted to double in the next 20 years. Diabetes has become a major healthcare problem in India and it became indomitable diabetes capital of the world. It has beenreportedthatChinahas116.4milliondiabetics,whencorrelatedwithpopulationandIndiaistheindomitablediabetescapitaloftheworld.Reportsaffirmedthatanuncommon increase in the prevalence of diabetes in India is the outcome of lifestylemodification in the background of nutrition, genetic predisposition, high-fat diet (Westerndiet)intakeandlessphysicalactivity[1].Moreover,thereisasubstantialregionalvariationin prevalenceofdiabetesandits management.

Natural medication is a profoundly mainstream reciprocal or elective method of treatment with a yearly turnover of US$ 60 billion with a continually developing business sector. In each nation, conventional drugs discover establishment in mystical or strict convictions, or well-known insight and the World Health Organization (WHO) is locked in to build up conclusive rules for the procedure of clinical examination and the evaluation of the adequacy of customary medication. A few factors that contribute towards the spread of this treatment are the common root of the medications, the confidence in their protected use and the supposed absence of side effects. In the present situation, herbal medicines' usage has significantly increased and published studies from developed countries emphasize that a paramount proportion of medicines supplied by them have herbal origin, so growing and producing the herbal medicines could be helpful to both economic development and community's health. The WHO reported that about 80% of inhabitants of planet rely on herbal medicine for their primary health care needs which involves the use of plant materials or their bio- active components[2].

Several anti-inflammatory drugs including metformin, thiazolidinediones, etc., are widely used to

suppress inflammation and thus insulin resistance in type 2 diabetic patients [3]. However, their long term administration is highly limited due to adverse side effects. This pathetic scenario has forced the researchers and clinicians to think of using natural drugs to target the inflammatory milieu and insulin resistance. Natural medicines always garner more attention in diabetic pharmaceuticals due to their long term efficacy and safety. SIT is one such ideal and safe antidiabetic drug with high potential to control hyperglycemia. This natural micronutrient and the primary phytosterol found in nuts, oils and vegetables possess a chemical structure analogous to cholesterol. Owing to its antioxidant, immunomodulatory, antidiabetic and hepatoprotective properties, SIT is used as an effective phytomedicine for treating obesity, diabetes, atherosclerosis and cancer [4]. More importantly, SIT does not exert any genotoxic or cytotoxic effects on models under study and hence is considered to be a safe drug for pharmaceutical applications. Our previous studies show that SIT controls hyperglycemia and insulin resistance by promoting insulin signaling via activation of insulin receptor and glucose transporter 4 (GLUT 4) proteins in adipose tissues of obesity induced type-2 diabetic rats [5]. However, nephroprotective effect. Hence, the present study was designed to assess the possible role of β-sitosterol on insulin signal transduction and glucose oxidation in high fat diet and sucrose-induced type-2 diabetic adult male rats. Herein, we investigated if SIT could reverse obesity induced insulin resistance by ameliorating inflammatory events in adipose tissue of high fat diet (HFD) and sucrose induced type 2 diabetic rats.

Materials and Methods

Chemicals

The entire chemicals and reagents used in this research were of the molecular and analytical grade acquired from Sigma Chemical Company (St. Louis, MO, USA); MP Biomedicals (Santa Ana, CA 92,707 USA) and Sisco Research Laboratories (Mumbai, India).

Animals

The present study was conducted at central animal house facility, MAHER, Kanchipuram-631552, Tamil Nadu, India as per the guidelines of institutional animal ethics committee (IAEC no: 006/2016dt 04.07.2016). Adult albino rats (180–200g) healthy adult male rats were maintanined in hygienic polypropylene cages in specific humidity (65%±5%) and temperature (21±2°C) with stable 12 h light and 12h dark.

Type-2 diabetes induction in animals

Type-2 diabetes in the experimental was induced based on report of [6]. After the treatment period, rats showed the fasting blood glucose above 120 mg/dL, were considered as type-2 diabetic rats.

Experimental Design

Animals were grouped into five and each group consisted of 6 animals. Normal rats (Group 1); Type-2 diabetic rats (Group 2); Type-2 diabetic rat treated with β-sitosterol (20 mg/kg) was served as group 3; Type-2 diabetic rats treated with metformin (50 mg/kg) was served as group 4. After the completion of treatment period, the animals were anaesthetized, serum was collected. Kidneys from both control and treated animals were removed and used for the assessment of various assays.

Geneexpressionanalysis

mRNA expression analysis of Kidney Injury Molecule-1 (KIM-1), insulin receptor(IR), Insulin receptor substrate-2 (IRS-2), Akt and Interneukin-6 (IL-6) by RealTime-PCR

TotalRNAisolation, cDNAconversionandreal-timePCR

Using aTRIRkit(TotalRNA IsolationReagentInvitrogen),totalRNA wasisolated from control and experimental samples. In brief, to 100 mg fresh tissue, 1 ml ofTRIRwasaddedandhomogenized.Thecontentwastransferredtoamicrocentrifugetube instantly and 0.2 ml of chloroform was added,vortexed for 1 min thenkept at 4ºCfor5min.Later,thecontentswerecentrifugedat12,000×g for15minat4ºC.Theaqueous phase (upper layer) was carefully transferred to a fresh microfuge tube and anequal volume of isopropanol was added, vortexed for 15 S and placed on ice for 10 min.After centrifugation of the content at 12000 ×g for 10 min at 4ºC, the supernatant wasdiscarded and RNA pellet was washed with 1 ml of 75% ethanol by the vortex. TheRNAconcentrationwasexpressedinmicrogram(µg).

ByusingthereversetranscriptasekitfromEurogentec(Seraing,Belgium),complementary DNA (cDNA) was synthesized from 2 µg of total RNA as stated in themanufacturer's protocol. To perform real-time PCR, the reaction mixture containing 2xreaction buffer (Takara SyBr green master mix), Forward and reverseprimers of thetarget gene and house-keeping gene, water and β-actin in total volume of 45 µl expectthe cDNA was made, mixed intensively and spun down. In individual PCR vials, about 5µlofcontrolDNAforpositivecontrol,5µlofwaterfornegativecontroland5µlof template cDNA for samples were taken and reaction mixture (45 µl) were added. 40cycles (95ºC for 5 min, 95ºC for 5 s, 60ºC for 20 s and 72ºC for 40 s) was set up for thereaction and obtained results were plotted by the PCR machine (Stratagene MX 3000 P,Agilent Technologies, 530 l, Stevens Creek Blvd, Santa Clara CA, 95051) on a graph.Relativequantification was calculatedfromthe meltand amplificationcurves analysis.

Table 1: List of primers used in this study.

Statisticalanalysis

S.No	Gene name	Primer sequence	Reference
1	Rat βactin	Sense primer: 5'- AAG TCC CTC ACC CTC CCA AAA G-3' Anti-sense primer: 5'- AAG CAA TGC TGT CAC CTT CCC-3'	[7]
2	Rat-IR	Sense primer: 5'- GCC ATC CCG AAA GCG AAG ATC-3' Anti-sense primer: 5'- TCT GGG TCC TGA TTG CAT-3'	[8]
3	Rat-IRS-1	Sense primer: 5'-GCC AAT CTT CAT CCA GTT GCT-3' Anti-sense primer: 5'-CAT CGT GAA GAA GGC ATA GGG-3'	[9]
4	Rat-Akt	Sense primer: 5'- GGA AGC CTT CAG TTT GGA TCC CAA-3' Anti-sense primer: 5'- AGT GGA AAT CCA GTT CCG AGC TTG-3'	[10]
5	Rat-IL-6	Sense primer: 5' -GTGAGAAGTATGAGAAGTGTGA-3' Anti-sense primer: 5' -GCAGGATGAGAATGATCTTTG-3'	[11]
6	Rat-KIM-1	Sense primer: 5'-GGT CAC CCT GTC ACA ATT CC-3' Anti-sense primer:5'-CTC GGC AAC AAT ACA GAC CA-3'	[12]

The data were subjected to statistical analysis using one-way analysis of variance andDuncan's multiple comparison test to assess the significance of individual variationsbetween the control and treatment groups using a computer-based software (SPSS 7.5using Windows student version). The significance was considered at the level of $p< 0.05$.

Results

β-sitosterolonKIM-1mRNAexpression

In diabetes-induced nephrotoxic rats, mRNA level of KIM-1 was significantly increased.Oral effective dose of β-sitosterol significantly restored the altered levels of the mRNA tothat ofthenormalrange (Fig.1).

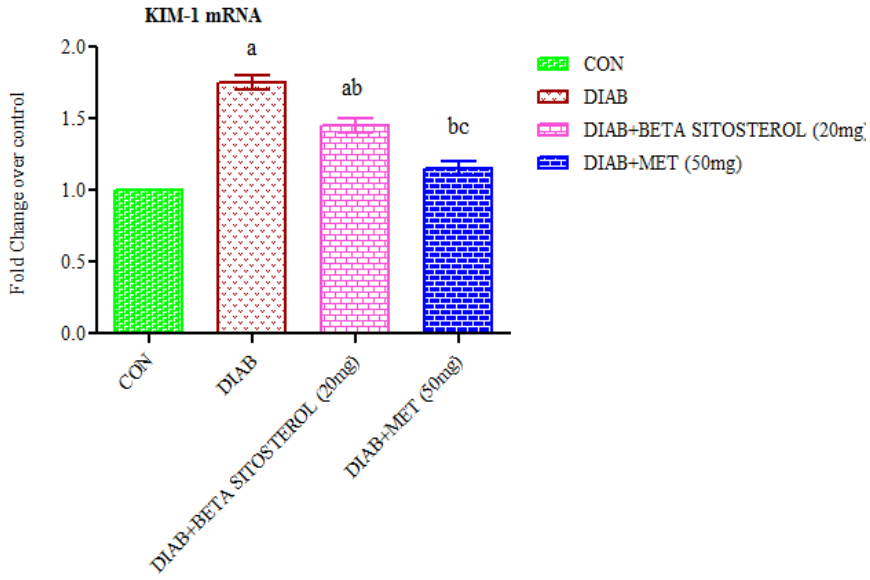

Fig.1: Role of β-sitosterol on KIM-1 mRNA expression in the kidney. Each column indicates mean ±SEM of six rats. $p<0.05$ was considered as significant change, a- comparison to control; b-comparison to diabetes induced; c-comparison with diabetes and β-sitosterol.

β-sitosterol up-regulate mRNA expression of Insulin Receptor (IR) in the kidney

T2DM rats showed a significant decrease in the mRNA expression levels of IR comparedto control rats. However, β-sitosterol treatment enhanced the gene expression of IR in the kidney of diabetic rats thereby suggesting its potential to enhance the insulin signaling in thekidney (Fig.2).

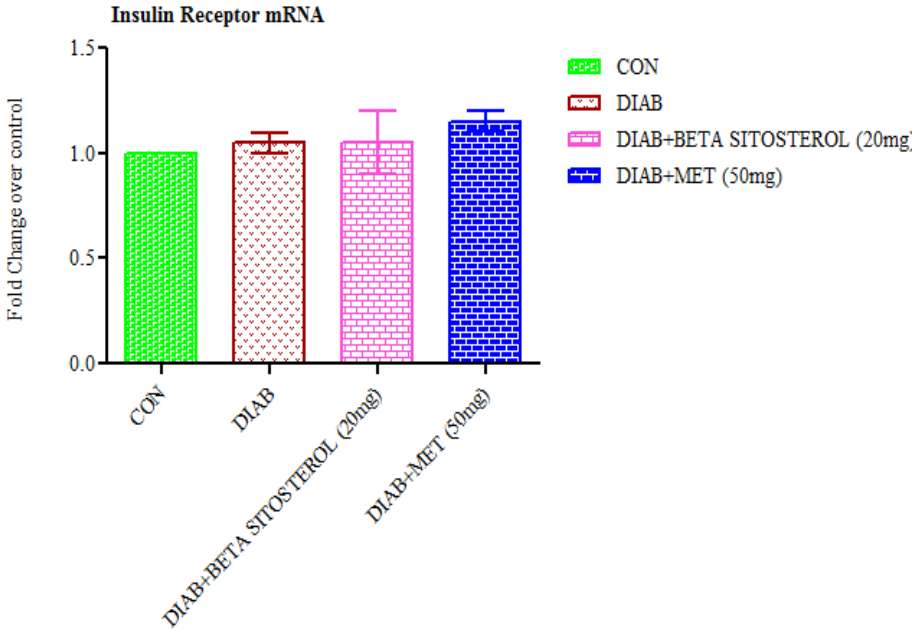

Fig.2: Role of β-sitosterol on IR mRNA expression in the kidney. Each column indicates mean ±SEM of six rats. $p<0.05$ was considered as significant change, a- comparison to control; b-comparison to diabetes induced; c-comparison with diabetes and β-sitosterol.

β-sitosterol on IRS-1 mRNA expression in the kidney

T2DM rats showed a significant decrease in the mRNA expression levels of IRS-2comparedtocontrolrats.However,β-sitosteroltreatmentenhancedthegeneexpressionof IRS-1 in the kidney of diabetic rats thereby suggesting its potential to enhance the insulinsignalinginthekidney (Fig.3).

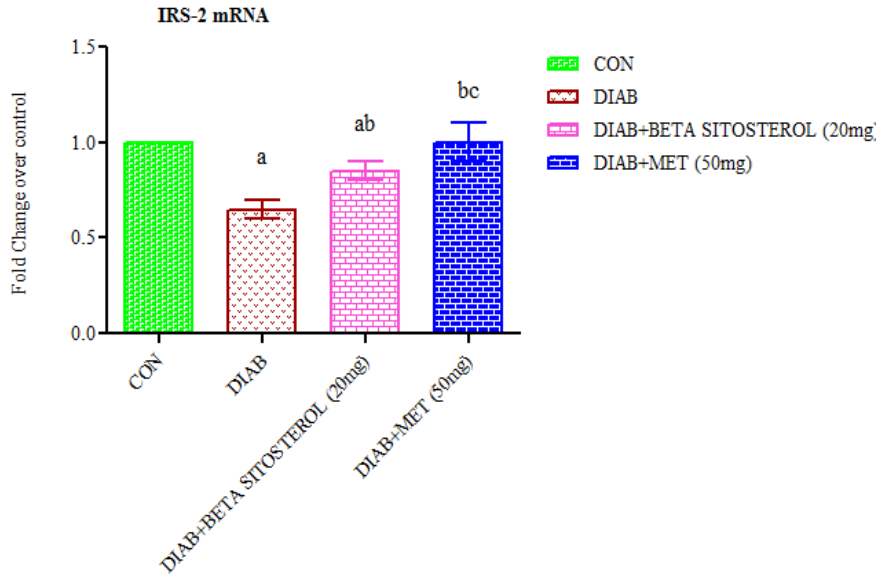

Fig.3: Role of β-sitosterol on IRS-1 mRNA expression in the kidney. Each column indicates mean ±SEM of six rats. p<0.05 was considered as significant change, a- comparison to control; b-comparison to diabetes induced; c-comparison with diabetes and β-sitosterol.

β-sitosterolupregulatedmRNAexpressionofAktinthekidneyoftype2diabeticrats

T2DM rats showed a significant decrease in the mRNA expression levels of Aktcomparedtocontrolrats.However,β-sitosteroltreatmentenhancedthegeneexpressionof Akt in the kidney of diabetic rats thereby suggesting its potential to enhance the insulinsignalinginthekidney (Fig.4).

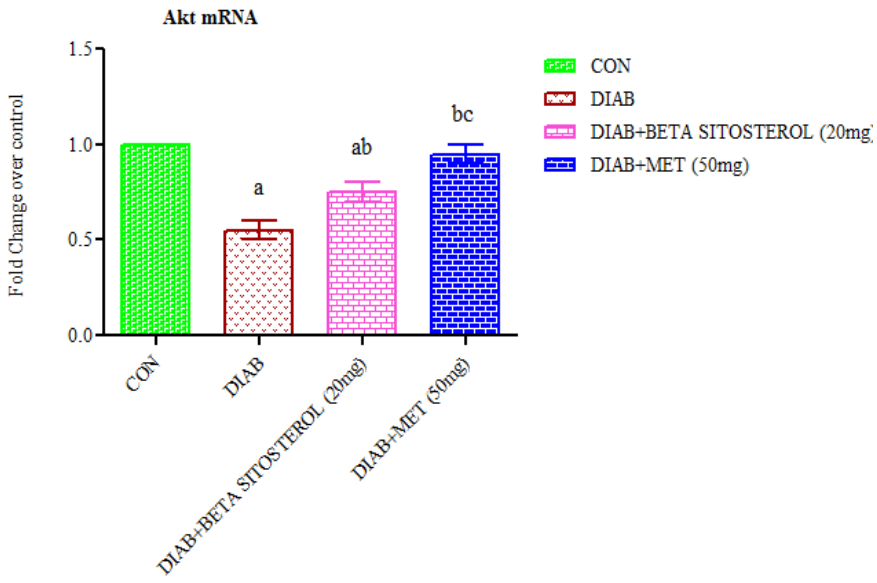

Fig.4: Role of β-sitosterol on Akt mRNA expression in the kidney. Each column indicates mean ±SEM of six rats. p<0.05 was considered as significant change, a- comparison to control; b-comparison to diabetes induced; c-comparison with diabetes and β-sitosterol.

β-sitosterol upregulated mRNA expression of IL-6 in the kidney

T2DM rats showed a significant increase in the mRNA expression levels of IL-6compared to control rats. However, β-sitosterol treatment controlled and down regulatedthe gene expression of IL-6 in the kidney of diabetic rats thereby suggesting its potential toenhance theinsulinsignalingin thekidney (Fig.5).

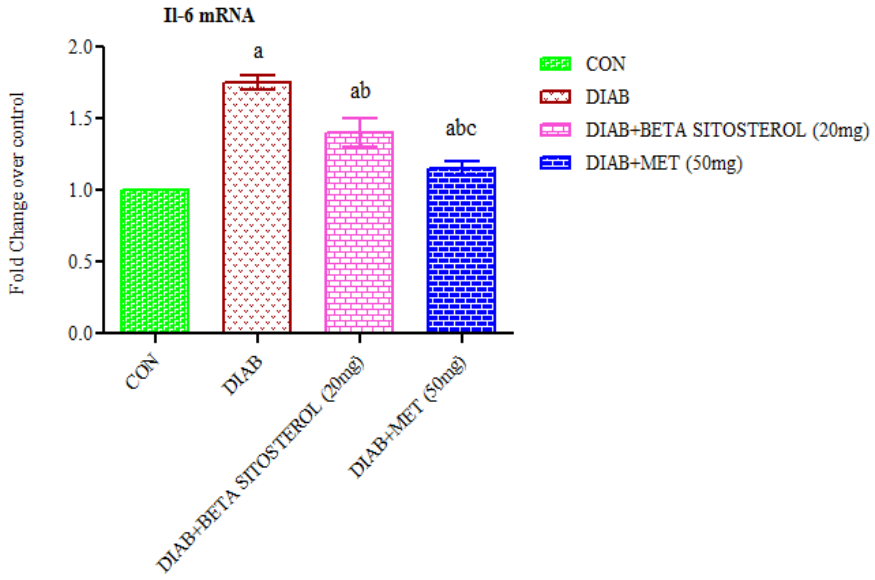

Fig.5: Role of β-sitosterol on IL-6 mRNA expression in the kidney. Each column indicates mean ±SEM of six rats. $p<0.05$ was considered as significant change, a- comparison to control; b-comparison to diabetes induced; c-comparison with diabetes and β-sitosterol.

DISCUSSION

The kidney is usually vulnerable to injury due to its numerous important tasks and a large amount of blood flow. According to the report, 850 million individuals were impacted, which is 20 times more than the prevalence of cancer (42) million and AIDS (36.7 million) and roughly double the number of diabetics (422 million). Globally, CKD is the sixth leading cause of death and the seventeenth leading cause of disability [13]. CKD is a non-communicable disease typically brought on by diabetes and high blood pressure. 10.4% of men and 11.8 % of women in the world have chronic renal disease, respectively.

Numerous scientific studies contend that changes in biology and metabolism serve as fundamental regulators of the prevalence and pathogenesis of obesity and type-2 diabetes. They argue that even a moderate (5 percent) weight loss achieved by low-calorie dieting greatly improves metabolic capacities in adipose tissue, liver, kidney, and muscle insulin sensitivity, as well as pancreatic beta-cell function[14]. In order to find new and anticipated possible treatments for the prevention and treatment of metabolic issues, it is crucial to understand the biology and pathology of adipose tissue. Particularly, a large body of scientific information demonstrating that adipose tissue has recently been discovered thermogenic and endocrine properties strongly suggests that focusing on adipose tissue as a treatment approach is both conceivable and doable[15].

The renal tissues in high fat diet-induced nephrotoxic rats showed dilated congested vascular space and aggregation of inflammatory cells between the renal tubules. Evident histological alterations in β-sitosterol treated rats showed mild condensed glomerulus and mild aggregates of inflammatory cells. This study clearly showed the nephroprotective potential of β-sitosterol.In diabetes-induced nephrotoxic rats, mRNA level of KIM-1 was

significantly increased. Oral effective dose of β-sitosterol significantly restored the altered levels of the mRNA to that of the normal range.

Target cells like the liver, fat, and muscle have the insulin receptor, a transmembrane signalling protein that is a member of the receptor tyrosine kinase (RTK) family. These play a significant role in controlling cell metabolism, differentiation, and growth. The -subunit tyrosine autophosphorylates, phosphorylates other substrates, and initiates a signalling cascade in response to insulin binding to the insulin receptor [16]. In the current investigation, type-2 diabetic rats had been given high fat and sucrose levels had reduced amounts of IR protein in their gastrocnemius muscles, whereas diabetic rats that had received -sitoserol treatment had significantly higher levels of IR protein. Increased quantities of free fatty acids have been found to lower the expression of the IR gene, which results in a reduction in the amount of insulin receptor protein in tissues that are targets for insulin [17]. By lowering the lipid levels in type-2 diabetic rats, β -sitosterol, a strong antioxidant and antihyperlipidemic drug, significantly raises the IR levels in gastrocnemius muscle.

It has been thoroughly investigated that altered post receptor insulin signalling is the primary factor causing type 2 diabetes-related insulin resistance and modifying the metabolic effects of insulin [18]. The metabolic master switch of the insulin signalling system is the insulin receptor, a transmembrane tyrosine kinase class of receptor [19]. The cell surface receptor (insulin receptor) undergoes autophosphorylation in the subunit at the time of insulin binding to the subunits, which causes the receptor substrates to be recruited and other insulin signalling pathway proteins to be activated [20]. As a result, the effect of -sitosterol was examined at the protein and IR mRNA levels. While -sitosterol therapy improved both gene and protein expression of IR, diabetic rats showed a significant drop in both mRNA and protein expression of IR, which could be a physiological explanation for the reduced insulin sensitivity in our study. Protein kinase C activation has been found to decrease IR gene expression and lower the quantity of insulin receptor protein in insulin target tissues by enhancing the internalisation of the insulin receptor and/or decreasing the rate of receptor recycling [17]. According to studies, an excess of lipids and a buildup of diacylglycerols are related (DAGs). PKC is activated by an imbalance in the synthesis and oxidation of lipids, and PKC is then translocated to the nuclear area where it inhibits the activation of the IR gene promoter, decreasing the amount of IR on the cell surface and decreasing insulin sensitivity [21]. By partially lowering the levels of lipid in diabetic rats, treatment with -sitosterol restored the expression of IR mRNA, which may be due to the β -sitosterol's potent antioxidant and hypolipidemic properties[22].

Insulin receptor substrates (IRS) are cytosolic adaptor molecules that operate as multifunctional docking proteins in insulin signalling. They are activated by phosphorylation at tyrosine residues, which causes a number of downstream signalling pathways to be triggered. It features multiple domains, including the carboxy-terminal pleckstrin homology (PH), the phosphotyrosine binding (PTB), and the amino-terminal pleckstrin homology (PH), which mediates the interactions of IRS proteins with insulin receptor and IRS effectors [Hançer et al, 2014). The four IRS isoforms IRS-1, IRS-2, IRS-3, and IRS-4 are necessary for insulin responses, including control of the metabolism of carbohydrates, proteins, and lipids. According to reports, IRS-1 affects metabolism by controlling the insulin signalling cascade in muscle and adipose tissue, but IRS-2 has a significant impact on the hepatic

insulin response [23]. The regulation of the metabolism of carbohydrates, proteins, and lipids is one of the functions of the four IRS isoforms IRS-1, IRS-2, IRS-3, and IRS-4. According to reports, IRS-1 regulates the insulin signalling cascade in muscle and adipose tissue, which has an impact on metabolism, but IRS-2 significantly affects the hepatic insulin response [24]. These mechanisms might investigate the relationship between IRS-1's increased serine phosphorylation and decreased tyrosine phosphorylation. As a result of diminished interaction between IRS-1 and IR caused by phosphorylation of the serine residue in IRS-1, IRS-1 may become a poor substrate for IR. Dephosphorylation of IR and IRS-1 or phosphorylation of IRS-1 at serine both contribute to the suppression of the insulin signalling pathway [25]. After receiving treatment with -sitosterol, the IRS-1tyrosine phosphorylation in adipose tissue was returned to normal levels. In diabetic rats, the hypolipidemic and anti-inflammatory properties of -sitosterol[22] seem to help reduce IRS-1 serine phosphorylation and restore normal insulin signalling. By assembling a complex involving c-Src, IR, and Akt/PKB, -arrestin modifies the function of insulin by acting as a molecular scaffold for several signalling proteins. This β -arrestin and c-Src complex induces the phosphorylation of Akt at Tyr315 and 326 residues. Subsequent therionine and serine phosphorylation (Thr308 and Ser473) has been carried out by PDK1 and PDK2 separately, which are crucial for the full activation of Akt and exerts its action on downstream targets [26]. Researchers have discovered that insulin resistance and type-2 diabetic rat models (db/db and high fat fed diet-induced obesity) and clinical samples of type-2 diabetic humans had lower levels of -arrestin-2 in their skeletal muscle and liver. According to prior research studies stated above, the present findings revealed a significant decrease in the expression of c-Src and -arrestin-2, which may be the cause of the decreased serine phosphorylation of Akt (AktSer 473) in type-2 diabetes experimental rats. The current study examines how -sitosterol regulates -arrestin-2 complex in adipose tissue of type-2 diabetic induced rats. It found that it did so by increasing the expression of -arrestin-2/cSrc at the gene and protein level. Protein kinases must phosphorylate certain residues like Thr308, Ser473, and Tyr315 in order to activate Akt. Akt is phosphorylated by C-Src at Tyr 315, promoting Akt serine/threonine phosphorylation, which is necessary for Akt full activation. Numerous in-vivo and in-vitro studies connect insulin resistance to impairments in both upstream and downstream Akt/PKB targets, including dephosphorylation of insulin receptor substrates, loss of insulin receptor substrates from cell membrane surfaces, decreased PI3K activity, and impaired Akt/PKB substrate phosphorylation [27]. One of the possible causes of decreased Akt phosphorylation in diabetic rats is the decreased -arrestins-2 and c-src signal complex. Diabetes caused by a high-fat diet and sugar decrease IRS-1 tyrosine phosphorylation (IRS-1Tyr632), which impairs PI3K membrane recruitment and reduces Akt phosphorylation. It's interesting to note that treatment with β-sitosterol increased Akt expression at the mRNA and protein levels as well as its phosphorylation at the threonine and serine residues (AktThr308 and AktSer473), which activates downstream molecules of insulin signalling and may result in increased levels of IR and tyrosine phosphorylation of IRS-1 as a result of improved insulin sensitivity. According to Greene et al. (2003)[25], the serine phosphorylation of IRS1/2 causes the IRS-1 protein to be degraded, which reduces glucose absorption and causes hyperglycemia. These events are what cause insulin resistance to develop in target tissues. According to Kuo et al. (2013)[28], type-2 diabetes, insulin resistance, and obesity are all associated with activation of the JNK signalling pathway. It has been discovered that its activity is elevated in obese people, type 2 diabetics, and

diabetic animal models, which is consistent with the findings of the JNK protein expression study, which showed that it was elevated in diabetic rats and was linked to insulin resistance. As a powerful antioxidant and anti-hyperlipidemic drug, β-sitosterol therapy significantly reduced JNK1 expression in diabetic rats. It also repaired dyslipidemia and reduced oxidative stress and pro-inflammatory cytokines.

CONCLUSION

The present study for the first time concludes that β-sitosterolhas significant role on high fat diet and sucrose-induced diabetic nephropathy by modulating the expression of proinflammtory signalling and facilitating the expression of insulin signalling molecules thereby in controls blood glucose levels. Hence, β-sitosterol can be considered as a therapeutic natural drug for the treatment of diabetic nephropathy. Further studies on the role of β-sitosterol on human cell line model are warranted in order to ascertain the potential mechanisms of action prior to clinical trials.

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Declaration of conflicts of interest

The authors report no conflicts of interest.

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