Journal of Advanced Zoology

ISSN: 0253-7214
*Volume **44** Issue **04 Year 2023** Page **157:165***

ß- Caryophyllene Ameliorates Insulin Resistance in Liver of High Fat Diet and Fructose-Induced Type-2 Diabetic Rats

Vadivel Mani[1]*, Sangeeta Chandrashekar[2], Jenny Jayapal[3,4], Nithya Rajapandian[5], Nisha K[6]

[1]*Department of Biochemistry, Konaseema Institute of Medical Sciences and Research Foundation, Amalapuram, East Gothwari - 533201, Anadhra Pradesh, India.*
[2]*Department of Medical and Health Sciences (Physiology), Bangor University, UK*
[3]*Research Scholar, Department of Physiology, Chettinad Hospital and Research Institute, Chettinad Academy of Research and Education, Kelambakkam- 603103, Tamil Nadu, India.*
[4]*Department of Physiology, PSP Medical College Hospital and Research Institute, Oragadam, Kanchipuram, Tamil Nadu -631604, India.*
[5]*Research Scholar, Department of Physiology, Mahatma Gandhi Medical College and research institute, Sri Balaji Vidyapeeth, Puducherry-607402, India.*
[6]*Department of Community Health Nursing, KIMS Nursing College, KIMS&RF Amalapuram, East Gothwari - 533201, Andhra Pradesh, India.*

**Corresponding author's E-mail: velvdm.vel5@gmail.com*

Article History

Received: 06 June 2023
Revised: 05 Sept 2023
Accepted: 07 Nov 2023

Abstract

Insulin resistance may impact organs that transmit insulin signals, like skeletal muscle, adipose tissue, and the liver. Hyperglycemia and hypertriglyceridemia in the fasting state of type 2 diabetes mellitus are more significantly impacted by insulin resistance in the liver. Hypertriglyceridemia is the culprit behind beta-cell failure in type 2 diabetes. Treating insulin resistance in liver as a primary mechanism of traditional antidiabetic drugs, but unfortunately traditional drugs has unalterable advisory effect. In the present research, insulin resistance in the liver is addressed with the versatile natural phytonutrient beta-caryophyllene to demonstrate effectiveness in managing diabetes. An effective dose of -Caryophyllene taken by non-parental route (200 mg/kg b.wt) was given for 30 days to experimentally induced (high-fat diet and fructose-fed) type-2 diabetic rats to find out whether β-Caryophyllene regulates the IRS-2/akt pathway of insulin signaling. In the liver of diabetic rats, the data demonstrates that ß-caryophyllene treatment significantly increased the mRNA and protein expression of insulin receptor (IR) and GLUT-2. However, there was a significant variation in the mRNA expression of akt and insulin receptor-substrate-2 (IRS-2) between groups. Taking caryophyllene supplements can be beneficial for the control of type 2 diabetes. Studies conducted with this sesquiterpene could have tremendous potential and benefit for the treatment of type 2 diabetes.

Keywords: *Fructose, Insulin, Protein, Rats*

1. Introduction

Diabetes mellitus (DM) is a common metabolic disease characterized by hyperglycemia brought on by inadequate or absent pancreatic insulin synthesis, with or without concurrent impairment of insulin action [1]. The most prevalent kind of diabetes is type 2. It is a reversible condition characterised by an impaired circulatory level of triglyceride in which the body gradually loses beta-cell function of the pancreas to produce enough insulin, and this loss of function is due to lipotoxicity along with free radical toxicity in diabetes [2]. It is the main cause of many complications related to coronary artery disease, cerebrovascular disease, renal failure, blindness, limb amputation, neurological complications, and pre-mature death. According to Luca Visconti *et al*., 2016 [3], Western-style diets that are high in saturated fatty acids and poor in dietary fibre are linked to an elevated risk of diabetes and obesity. Insulin insensitivity manifests itself in three target organs: the liver, adipose tissue, and skeletal muscle [4]. In addition, the predominance of insulin insensitivity, a more vital pathophysiological parameter that contributes to the development of T2DM and is an independent risk factor for the metabolic syndrome, is much more generalised [5]. When lipid metabolism, a crucial energy function, is organised by insulin, the insulin receptor tyrosine kinase is activated [6]. It

phosphorylates and recruits several substrate adapters, including the IRS protein family [7]. The fast action of insulin to accelerate the absorption and metabolism of glucose in tissues is a crucial component of the sustainability of glucose homeostasis [8]. It was suggested that the primary tissues causing postprandial hyperglycemia in an insulin-resistant person were the liver and skeletal muscle because these were the primary sites of glucose clearance in the insulin-stimulated state [9]. The ability of insulin to increase glucose transport in skeletal muscle and adipose tissue was evoked by the translocation of GLUT-4 and in the liver by GLUT-2, so it is also known as a "glucose sensor" of the main glucose transporter regulated by insulin, intracellular vesicles in the plasma membrane, and transverse tubules [10]. Elevated fat levels have caused many problems with the insulin signalling molecule, leading to insulin susceptibility [11]. Currently, more synthetic drugs used for the management of diabetes that are available on the market cause deleterious side effects [12]. The problem is overcome by natural remedies like plant phytochemicals, especially sesquiterpene [13], used to increase insulin sensitivity. In this study, we used natural phytochemicals like β -caryophyllene to treat insulin resistance. A natural sequiterpene called β -caryophyllene is found in large quantities in cannabis as well as numerous herbs and spices used in cooking [14]. This terpene can be found in abundance in hemp, black pepper, cloves, cinnamon, hops, and rosemary. It has a variety of biological actions, including anti-inflammatory, antioxidant, and antilipidemic properties [15]. In streptozotocin (STZ) -induced diabetic mice, chronic oral treatment of β -caryophyllene lowers glycemic index, depressive-like behaviour, and neuropathic pain [16]. Furthermore, it has recently come to light that β -caryophyllene effectively protects β -cells by reducing hyperglycemia by increasing insulin release, as well as by reducing oxidative stress and inflammation in the pancreatic tissue of experimental diabetic rats [17]. However, the effect of β -caryophyllene on the insulin signaling pathway, which is remarkably altered in diabetic conditions, is still unclear. Insulin regulates the activities of these insulin signaling markers, and its deficiency results in a derangement in insulin signaling markers. Hence, in the present study, we focused on exploring the effect of β -caryophyllene on liver-key insulin signaling molecules in HFD-induced type 2 diabetes.

2. Materials And Methods

Chemicals

All of the chemicals, reagents, and metformin utilized in this investigation was of the molecular and analytical grade and were bought from Sisco Research Laboratories in Chennai, India, and Sigma-Aldrich Chemical Company in St. Louis, Missouri, USA. The supplier of β- caryophyllene was Tokyo Chemicals Industry Co., LTD in Tokyo, Japan. Plus, On-Call In San Diego, California, USA, ACON Laboratories, Inc. sold in blood glucose test strips. Santa Cruz Biotechnology in the United States supplied the -actin monoclonal antibody, polyclonal insulin receptor β-subunit (IR-β), and GLUT2 antibodies.

Animals

According to the National Guidelines and Protocols, the Institutional Animal Ethics Committee accepted the experimental study under registration number 765/03/ca/CPCSEA with approval certificate number 007/2019 dated April 11, 2019. At the Meenakshi Medical College and Research Institute (Meenakshi Academy of Higher Education and Research), the Central Animal House Facility collected and cared for healthy adult male Wistar albino rats (150–180 days old weighing 180–200g). They were given a regular rat pelleted meal (provided by Lipton India, Mumbai, India), and free access to clean drinking water was provided.

Induction of Type-2 Diabetes

By giving rats a high-fat diet containing 2% cholesterol, 1% cholic acid, 30% coconut oil, 67% traditional rat feed, and 25% fructose through drinking water for 60 days, rats were made diabetic (type-2) [18]. Animals were recruited for the experiment if their fasting blood glucose levels were greater than 120 mg/dl after 60 days. The high-fat diet and sugar feeding continued until the conclusion of the study. The control rats received standard pelleted rat food, and water was provided *ad libitum.*

Experimental design

The following experimental design was framed, and accordingly the rats were subjected to treatment for a period of one month. Healthy adult male Wistar rats were divided into the following groups of 6 rats each.

Group I: Control (Normal rats).

Group II: Rats were made diabetic (type-2) after feeding high fat diet & fructose through drinking water (30%) for 60 days.

Group III: Type-2 diabetic rats treated orally with β-caryophyllene (200 mg/kg b.wt/day) for 30 days

Group IV: Type-2 diabetic rats treated orally with metformin [19] (50 mg/kg, b.wt/ day for 30 days

Group V: Control rats administered orally with β-caryophyllene (200 mg/kg b.wt/day) for 30 days.

After 30 days, the animals were fasted overnight, physiological saline was infused into them while they were under sodium thiopentone anaesthesia (40 mg/kg b.wt.), and the liver was cut out to assess various qualities. Blood was then collected.

mRNA expression analysis

Total RNA Isolation, cDNA conversion and real-time PCR

A TRIR kit (Total RNA Isolation Reagent Invitrogen) was used to extract total RNA from the control and experimental samples. In a nutshell, 100 mg of fresh tissue received 1 ml of TRIR, which was then homogenised. The material was then immediately transferred to a micro centrifuge tube, combined with 0.2 ml of chloroform, vortexed for 1 minute, and stored at 4°C for 5 minutes. Then, the mixture was centrifuged at 12,000 g for 15 minutes at 4 °C. Carefully transferring the top layer of the aqueous phase to a fresh microfuge tube, equal parts of isopropyl alcohol were then added, vortex for 15 seconds, and then placed on ice for 10 minutes. Following centrifugation of the material at 12000g for 10 minutes at 4C, the supernatant was separated. The RNA pellet was washed in 1 ml of 75% ethanol using the vortex. The extracted RNA was calculated using spectrometry according to Fourney et al. Each sample's RNA content was quantified in micrograms.

Using a reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was created from 2 micrograms of total RNA in accordance with the manufacturer's instructions. A 45 µl reaction mixture containing 2x reaction buffer (Takara SyBr green master mix), forward and reverse primers for the target and housekeeping genes, water, and β-actin (primer sequences are supplied in (Table:1) was made in order to perform real-time PCR. About 5 µl of control DNA for the positive control, 5 µl of water for the negative control, and 5 µl of template cDNA for the samples were extracted and added to each individual PCR vial along with the reaction mixture (45 µl). The reaction was set up for 40 cycles (95°C for 5 min, 95°C for 5 s, 60°C for 20 s, and 72°C for 40 s), and the PCR machine (Stratagene MX 3000P, Agilent Technologies, 530l, Stevens Creek Blvd, Santa Clara, CA, 95051) showed the findings on a graph. From the examination of the melt and amplification curves, relative quantification was derived.

Table: 1 Primer sequences of insulin signaling molecules

Name of the gene	Primer Sequence	Reference
Rat IR	Sense primer: 5'- GCC ATC CCG AAA GCG AAG ATC-3' Anti-sense primer: 5'- TCT GGG TCC TGA TTG CAT-3'	Gonzalez et al, 2003[20]
Rat IRS-2	Sense primer: 5'- CCCCAGTGTCCCCATCCT-3' Anti-sense primer: 5'- TTTCCTGA GAGAGACGTTTTCCA-3'	Melissa et al, 2006 [21]
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'	Sharma et al, 2010 [22]
Rat GLUT2	Sense primer: 5'- CTGGGTCTGCAATTTCATCA- 3' Anti-sense primer: 5'- CGTAAGGCCCGAGGAAGT- 3'	Liu et al, 2006 [23]
Rat β-actin	Sense primer: 5'- AAG TCC CTC ACC CTC CCA AAA G-3' Anti-sense primer: 5'- AAG CAA TGC TGT CAC CTT CCC-3'	Peinnequin et al, 2004 [24]

Protein expression analysis

Protein isolation and western blotting

Proteins were isolated from 100 mg of liver tissue from experimental and control animal. 100 mg of liver tissue were combined with 1 ml of buffer A (5 mM NaN3, 0.25 M sucrose, and 10 mM NaHCO3), homogenised, then centrifuged at 1300 x g at 4 °C for 10 minutes. At 4°C, the supernatant was separated and centrifuged for 15 minutes at 12,000 x g. The final supernatant was sampled as a total protein in order to assess the insulin signaling molecules found post-receptor. The Lowry et al., method [25] was used to estimate the protein.

Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel), the lysate proteins (50 g/lane) were separated and electro-blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc). The membranes were marked with primary antibodies at dilutions of 1:1000 and blocked with 5% non-fat dry milk. After three TBS-T washes, the membrane was incubated for 1 hour with a 1:5000 dilution of rabbit-anti-mouse or goat-anti-rabbit secondary antibody coupled with horseradish peroxidase (GeNei, Bangalore, India). The membrane was rinsed three times with TBS and TBS-T after the incubation period. Thermo Fisher Scientific Inc., Waltham, MA, USA, developed a sophisticated Chemiluminescence detection system to visualise the protein bands. Once the specific signals were identified, the protein bands were photographed and quantified using Bio-Rad Laboratories' Chemidoc and Quantity One image analysis systems. After that, the membrane was stripped in a solution of 50 ml, 62.5 mM Tris-HCl (pH 6.7), 1 g SDS, and 0.34 ml -mercaptoethanol for 30 minutes at 50°C. After that, the membranes were re-probed with a 1:5000 anti β-actin antibody. β- actin was under the invariant control that was utilised.

Statistical analysis

Using one-way analysis of variance (ANOVA) and Duncan's multiple range test, computer-based software, the data were analyzed to determine the significance of individual variance within the control and treated groups (Graph Pad Prism version 5). Duncan's test was used to determine significance at the level of $p<0.05$.

3. Results and Discussion

β- Caryophyllene increases insulin receptor (IR) mRNA and protein expression in the lever of experimental rats

The effect of β- Caryophyllene on IR expression in liver was seen in Fig 1 and 2. In type-2 diabetic rats, we found a substantial reduction in insulin receptor mRNA and protein expression. Treatment with β- Caryophyllene increased insulin receptor mRNA and protein levels in diabetic experimental rats, to a level comparable to that of the conventional medication metformin.

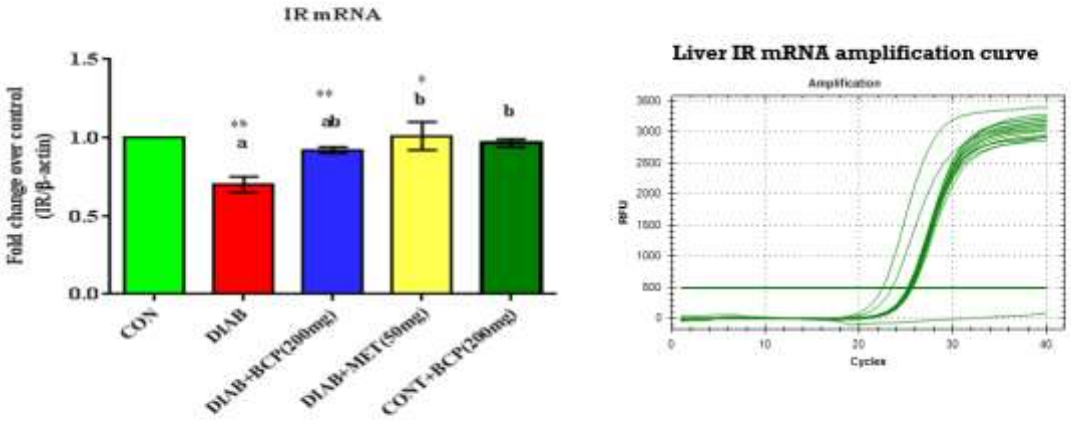

Figure.1: Effect of β- caryophyllene on IR mRNA expression in liver of experimental rats. Each bar represents Mean ± S.E.M of 6 animals. a -compared with control; b –compared with diabetic control rats. Significance was considered at the levels of p<*0.05*

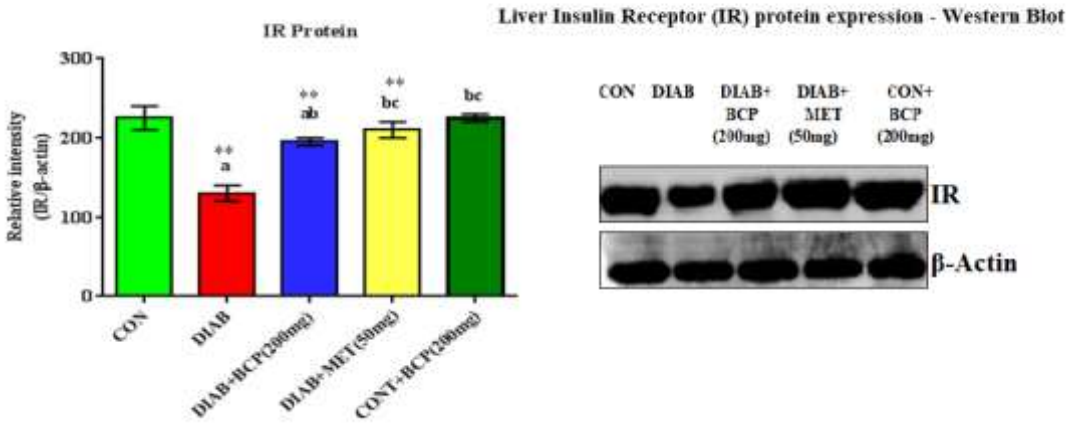

Figure.2: Effect of β- caryophyllene on IR protein expression in liver of experimental rats. Each bar represents Mean ± S.E.M of 6 animals. a- compared with control; b – compared with diabetic control rats; compared with 200mg/b.wt β-Caryophyllene. Significance was considered at the levels of p<*0.05.*

Effect of β - Caryophyllene on mRNA expression of insulin receptor substrate-2 (IRS-2) in the liver of type-2 diabetic rats.

Insulin receptor substrate-2 (IRS-2), a key component, was involved in the metabolic consequences of insulin signaling pathways, primarily in the liver. There was a significant difference in mRNA expression of IRS-2 between the experimental groups was observed (**Fig: 3**).

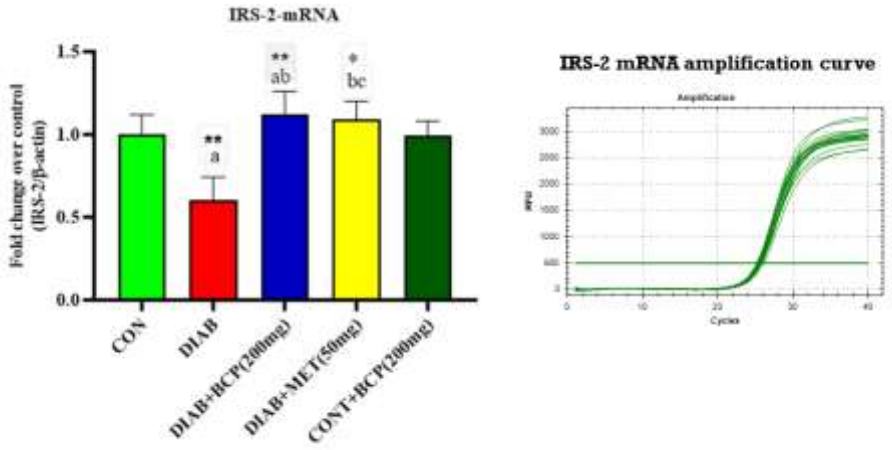

Figure.3: Effect of β- caryophyllene on IRS-2 mRNA expression in liver of experimental rats. Each bar represents Mean ± S.E.M of 6 animals. a- compared with control; b – compared with diabetic control rats. Significance was considered at the levels of p<*0.05**.

β- Caryophyllene improves mRNA expression of Akt in the liver of type-2 diabetic rats.

Akt, a serine-threonine kinase, operates as a master switch for cellular signaling when it gets activated. The Akt mRNA (Fig. 4) was decreased substantially in diabetic groups, whereas the β-Caryophyllene administered group showed an improved level of expression.

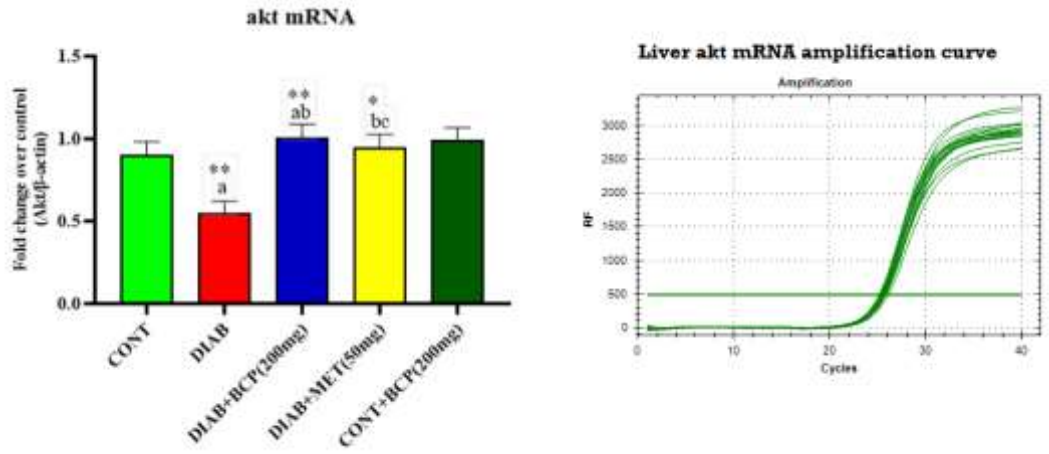

Figure.4: Effect of β- caryophyllene on akt mRNA expression in liver of experimental rats. Each bar represents Mean ± S.E.M of 6 animals. a- compared with control; b – compared with diabetic control rats. Significance was considered at the levels of p<*0.05**.

β- Caryophyllene enhances the GLUT-2 mRNA and protein expression in the liver of type-2 diabetic rats.

The mRNA expression of GLUT-2, an essential transporter protein for glucose transfer from extracellular to intracellular in the liver, was shown in diabetic rats, mRNA (Fig.5) and protein levels (Fig:6) of GLUT-2 were shown to be lower, but β-Caryophyllene therapy dramatically raised the same as the conventional medication metformin.

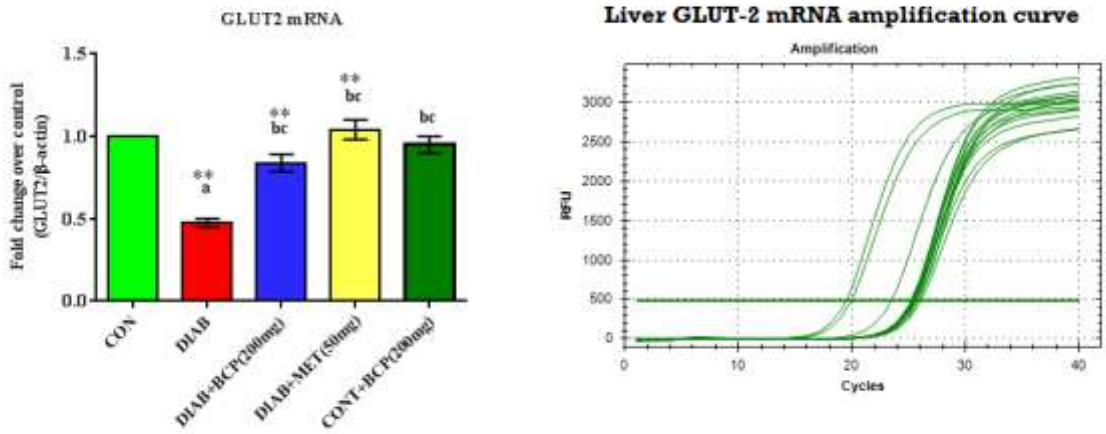

Figure.5: Effect of β- caryophyllene on GLUT-2 mRNA expression in liver of experimental rats. Each bar represents Mean ± S.E.M of 6 animals. a- compared with control; b – compared with diabetic control rats; c- compared with 200mg/b.wt β-Caryophyllene. Significance was considered at the levels of p<*0.05*.

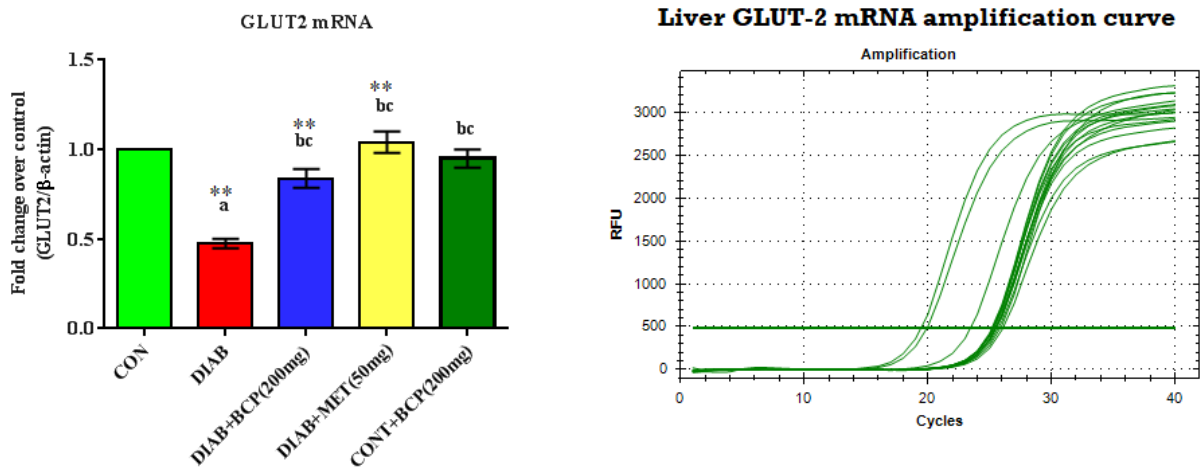

Figure.6: Effect of β- caryophyllene on GLUT-2 Protein expression in liver of experimental rats. Each bar represents Mean ± S.E.M of 6 animals. a -compared with control; b -compared with diabetic control rats; c -compared with 200mg/b.wt β-Caryophyllene. Significance was considered at the levels of $p<0.05$.

In type-2 diabetic rats given a diet high in fat and fructose, we found that administering β -Caryophyllene reduced muscle insulin resistance [26]. After 10 weeks of high-fat and fructose feeding, the rodents' insulin sensitivity decreased as evidenced by their impaired insulin and glucose tolerance [26]. Elevated fasting insulin, fasting glucose, HOMA-IR, and reduced QUICKI values all indicated that the condition was one of insulin resistance [27]. In this research, we improved the gene and protein expression of insulin signaling molecules in the liver of a high-fat and fructose induced type-2 diabetic rat to further investigate the potential mechanisms of β -caryophyllene in improving insulin sensitivity.

Diabetic rats had a significant decline in both mRNA and protein expression of IR, whereas treatment with β -caryophyllene increased IR gene and protein expression. This may provide a molecular explanation for the decreased insulin sensitivity observed in the present experimental research. Since the liver's metabolic process is closely correlated with that of other organs, insulin action on the hepatocyte suggests that indirect processes involving adipocyte and brain signals may be involved [28, 29]. Recent research suggests that direct insulin action on the hepatocyte, rather, exerts a significant influence on the regulation of glucose metabolism [30, 31]. According to animal research [32] a high-fat diet during pregnancy causes hepatic insulin resistance in newborn puppies because it raises the level of free fatty acids in the blood. This is also related to a marked decrease in insulin receptor mRNA and protein expression in the liver [33] reported to be substantially reduced in high-fat-fed animals, along with decreased b-subunit auto -phosphorylation and a lower percentage of tyrosine phosphorylated receptors [34]. Increased lipid peroxidation and free radical production found in our earlier research [35] may have severely disrupted the plasma membrane, resulting in a drop in the concentration of insulin receptors. The production of the gene encoding the insulin receptor (IR) is inhibited by free fatty acids, and this result in less insulin receptor protein being present in insulin target cells [36].

Starting β -caryophyllene treatment remarkably alleviated high fat and fructose-induced insulin resistance in the liver and restored the altered gene and protein expression of insulin signaling molecules- IR. β- Caryophyllene, a potent antioxidant, antilipidemic, and hypoglycemic drug, is thought to be the potential mechanism that increased IR levels in the liver of type-2 diabetic rats [17].

As essential mediators of insulin signaling networks, IRS molecules interact with the transmission of signals from several receptors [37] and are crucial for regulating cell growth, division, and metabolism. The loss of IRS-2 expression and/or activity may be a key factor in the development of insulin resistance, obesity, cell failure, and type-2 diabetes [38]. IRS-2 is essential for maintaining the insulin action in a variety of cell types. Insulin resistance in the liver and skeletal muscles caused the glucose homeostasis of IRS-2-deficient animals to gradually deteriorate [39], these mice also revealed decreased peripheral insulin signaling and pancreatic cell function. Our findings demonstrated that the expression of IRS-2 was down -regulated as a result of a high-fat and high-fructose diet. This is the first proof that a high-fat, high-fructose diet is linked to the expression of the IRS-2 mRNA. In the liver of type -2 diabetes adult male rats, treatment with β -caryophyllene therapy significantly

increased the expression of mRNA of insulin receptor substrate-2 levels, similar to that treated with metformin.

Due to the antioxidant, antilipidemic, and anti-inflammatory properties of β -caryophyllene, which can reduce insulin cascade cross-talk molecules like inflammatory markers, accumulation of ceramides, and ROS formation [40], the IRS-2 mRNA expression in the liver of experimental rats treated with β -caryophyllene increased significantly.

4. Conclusion

Our current results unequivocally demonstrate that β -caryophyllene improves glycemic control in hepatic tissue of high-fat diet and fructose fed type-2 diabetic rats by attenuating insulin resistance through activation of insulin downstream signalling molecules by reducing the high fat diet-induced alteration in gene expression of insulin receptor protein. Additionally, by evaluating the expression pattern of insulin signalling molecules, the compound's potential therapeutic efficacies were determined. Consequently, it can be inferred from the current data that supplementing with β -caryophyllene can be a useful strategy for the control of type-2 diabetes. Clinical investigations using this sequiterpene may be highly interesting and advantageous for the treatment of type 2 diabetes.

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