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ORIGINAL ARTICLE

β-Caryophyllene Reduced Oxidative Stress and Expression of Apoptotic Markers in Streptozotocin-Induced Diabetic Rats

Binghong Hua, Yin Xiao[1], Fuling Li[1]

Departments of Endocrinology and [1]Pharmacy, Affiliated Haikou Hospital of Xiangya Medical College, Central South University, Haikou, Hainan, China

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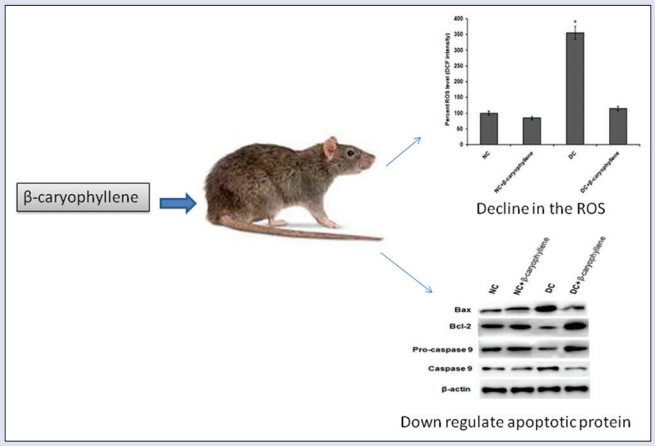
ABSTRACT

Purpose: In this study, we aimed to investigate the effects of β-caryophyllene against oxidative stress-induced apoptosis in the animal model of diabetes. **Materials and Methods:** Experimental diabetes was induced in the rat model through the administration of streptozotocin. Plasma samples were tested for the lipid profile. Plasma insulin levels were measured by performing electrochemical immunoassay. Fasting blood glucose was measured using the glucometer. Intracellular levels of reactive oxygen species (ROS) were measured through dichlorodihydrofluorescein diacetate method. Markers of oxidative stress such as catalase (CAT), reduced glutathione (GSX), malondialdehyde (MDA), and superoxide dismutase (SOD) levels were determined by using the colorimetric kits. Expression of apoptotic proteins such as Bax, Bcl-2, Pro-caspase 9, caspase 9, and β-actin was analyzed using the Western blot technique. **Results:** According to our results, β-caryophyllene normalized the body weight of diabetic rats and improved the lipid profile of the experimental animals. It also normalized the levels of fasting blood glucose. Moreover, the plasma insulin levels significantly increased after the administration of β-caryophyllene. The β-caryophyllene treatment caused a significant decline in the ROS and MDA levels together with an increase in the levels of CAT, SOD, and GSX levels in diabetic rats. Interestingly, the markers of apoptosis such as Bax and caspase 9 decreased after the administration of β-caryophyllene in the neural tissue of diabetic rats. However, the levels of Bcl-2 protein were increased in the β-caryophyllene-treated rats. **Conclusion:** β-Caryophyllene exhibits significant antidiabetic effect, which might be due to its capacity to reduce oxidative stress and inhibit apoptotic markers in the peripheral neural tissue of diabetic rat. These results point toward the potential of β-caryophyllene in the treatment of diabetes.

Key words: Apoptosis, diabetes, malondialdehyde, reactive oxygen species, β-caryophyllene

SUMMARY

- In this study, we aimed to investigate the effects of β-caryophyllene against oxidative stress and apoptosis in the animal models of diabetes. The dichlorodihydrofluorescein diacetate method was used to determine the intracellular reactive oxygen species (ROS) levels. Oxidative stress parameters such as catalase (CAT), reduced glutathione (GSX), malondialdehyde (MDA), and superoxide dismutase (SOD) levels were determined by using the colorimetric kits. Proteins of interest were analyzed for their expression levels using the Western blot technique. The results of this study showed that β-caryophyllene treatment normalized the body weight of diabetic rats. β-caryophyllene significantly increased the levels of plasma insulin in diabetic rats. Furthermore, β-caryophyllene significant decreased the levels of ROS and MDA as well as increased the levels of CAT, SOD, and GSX levels in diabetic rats. Interestingly, β-caryophyllene also reduced the levels of apoptotic markers such as Bax and caspase 9 in diabetic rats. However, the levels of Bcl-2 protein were increased in the β-caryophyllene-treated rats.



Abbreviations used: ROS: Reactive oxygen species; CAT: Catalase; GSX: Reduced glutathione; MDA: Malondialdehyde; SOD: Superoxide dismutase; NC: Normal control; DCFH-DA: Dichlorodihydrofluorescein diacetate; LDL: Low-density lipoprotein; HDL: High-density lipoprotein.

Correspondence:

Dr. Fuling Li,
Department of Pharmacy, Affiliated Haikou Hospital of Xiangya Medical College, Central South University, Haikou, Hainan 570208, China.
E-mail: 416015110@qq.com

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INTRODUCTION

Diabetes mellitus is a chronic metabolic condition resulting in high blood glucose levels caused due to the low production of insulin or impairment in its secretion from the pancreas.[1] It is currently one of the most prevalent metabolic disorders at the global level.[2] The incidence of diabetes by 2035 has been predicted to increase to 592 million worldwide.[3]

Neuropathy is generally defined as nerve damage in the peripheral nervous system. It is prevalent worldwide which affects nearly 2% of the global population.[4] Diabetic peripheral neuropathy or neuropathy associated with diabetes is quite common. Approximately 50% of the patients with diabetes can develop peripheral neuropathy and among them 30% patients can experience painful diabetic neuropathy.[5] The

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clinical manifestations associated with diabetic peripheral neuropathy include tingling and burning sensation, neuroglia, and numbness.[6] The high levels of blood glucose are known to trigger the generation of reactive oxygen species (ROS) which in turn activate many downstream signals leading to the development of diabetic peripheral neuropathy.[7]

Recent studies on the experimental animal diabetic models have advanced the understanding of the molecular events related to diabetes and diabetic peripheral neuropathy to a great level.[8] This prompted us to explore the chemical agents that can be used to treat diabetic peripheral neuropathy. With this in mind, we aimed to evaluate the effects of β-caryophyllene [Figure 1] against diabetic neuropathy using the experimental rat model.

MATERIALS AND METHODS

Experimental

Animals, induction of diabetes, and β-caryophyllene administration

A total of 50 2-month-old male Wistar rats were obtained from the National Research Center Laboratory, Egypt. The animals were maintained in fully ventilated rooms with free access to pellet diet and fresh water. The animals were kept under 12 h day/night cycle. Forty animals were used in this study. Animal Ethics Committee of the Central South University (Haikou, China) approved all experimental protocols involving animal studies.

To induce experimental diabetes, 12-week-old Wistar rats were fasted for 14 h. Subsequently, the animals were administered with streptozotocin (70 mg/kg body weight) dissolved in citrate buffer through intraperitoneal injections. Four days after the administration, the animals were examined for the induction of diabetes by determining their fasting blood glucose levels. Animals with >250 mg/dL glucose levels were used for further experiments. β-Caryophyllene used in this study was obtained from Sigma-Aldrich, St. Louis, MO, USA (≥80% purity). The animals were divided into four groups (10 animals per group) and were administered with β-caryophyllene for up to 6 weeks. Group 1 normal control (NC) consisted of NC rats; Group II (NC + β-caryophyllene) consisted of normal rats treated with 200 mg β-caryophyllene/kg body weight; Group III (DC) consisted of diabetic rats without β-caryophyllene treatment; and Group IV (DC + β-caryophyllene) consisted of diabetic rats treated with 200 mg β-caryophyllene/kg of body weight.

Biochemical analysis of plasma samples

After 6 weeks of treatment, the animals were anesthetized using ketamine and the blood samples were collected from the from the retro-orbital vein. Then, the plasma was separated and stored at −40°C until further experimentation. The glucose levels in the blood were determined using glucometer. Lipid profile was estimated colorimetrically with the help of commercially available kits (Salaucea Company). To estimate the plasma insulin level, an electrochemical immunoassay was performed using the enzyme-linked immunosorbent assay kits (Thermo Scientific).

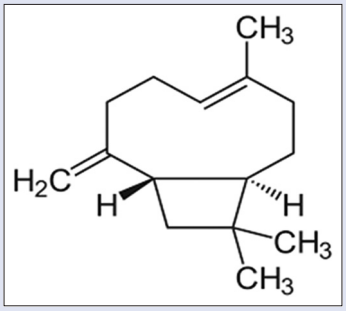

Figure 1: Molecular structure of β-caryophyllene

Evaluation of reactive oxygen species levels and oxidative stress parameters

The ROS levels from the peripheral neural tissue were determined with the help of a fluorescent probe (dichlorodihydrofluorescein diacetate) combined with flow cytometer.[9] To estimate the oxidative parameters such as catalase (CAT), reduced glutathione (GSX), malondialdehyde (MDA), and superoxide dismutase (SOD), peripheral neural tissues were isolated from the experimental animals and homogenized under aseptic conditions, with the help of trypsin (Thermo Scientific). The isolated cellular masses were washed with phosphate-buffered saline and the intracellular levels of oxidative parameters were determined with the help of respective kits.

Western blot analysis

The animals were sacrificed after anesthesia, and the level of protein expression was measured in the sciatic nerve and spinal cord. The homogenized mass of peripheral nerve cells (100 mg) was treated with RIPA lysis buffer for the isolation of total cellular proteins. Then, the concentration of protein was determined with the Bradford method. From each sample, 45 μg of proteins were loaded and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The separated proteins were transferred onto Poly vinylidene fluoride (PVDF) membranes followed by overnight exposure to primary antibodies against Bax (CST: 2772, 1:1000), Bcl-2 (Santa Cruz: sc-7382, 1:1000), Pro-caspase 9 (Thermo scientific: JJ08-05, 1:1000) caspase 9 (Santa Cruz: sc-73548, 1:1000), and β-actin (CST: 4967, 1:1000) at 4°C, followed by incubation with secondary antibody conjugated to horseradish peroxidase (Santa Cruz: sc-2357, 1:5,000) for 2 h at the room temperature. Finally, the bands were visualized with the help of an efficient chemiluminescence reagent.

Statistical analysis

Data are presented as mean of three biological replicates ± standard deviation calculated from at least three experimental replicas. Analysis of variance and Duncan's test were used to estimate the significance of the statistical difference between two/multiple data points. $P < 0.05$ was considered statistically significant.

RESULTS

β-Caryophyllene improved lipid profile in diabetic rats

The effects of β-caryophyllene treatment on the lipid profile of the normal or diabetic rats were determined by analyzing the level of triglycerides, low-density lipoprotein-cholesterol, high-density lipoprotein-cholesterol, and total cholesterol. Body weight measurements (in grams) were taken with/without β-caryophyllene administration in normal and diabetic rats. According to the results, β-caryophyllene increased the body weight of diabetic animals as well as increased their plasma lipid profile [Table 1].

In each row, a statistically significant difference is subscripted with The symbol is (*) (differing from other row values at $P < 0.05$).

β-Caryophyllene decreased glucose levels and increased the plasma insulin in diabetic rats

The effects of the administration of β-caryophyllene in diabetic rats were also examined in terms of the fasting blood levels and the levels of

plasma insulin. According to the results, β-caryophyllene significantly reduced the level of blood glucose in diabetic rats [Figure 2a]. Diabetic rats exhibited significantly higher levels of plasma insulin which were as good as the normal Wistar rats [Figure 2b]. However, the effects were not significant when the normal male rats were administered using β-caryophyllene. The results further highlight the potency of β-caryophyllene to act as an antidiabetic agent.

β-Caryophyllene inhibited the oxidative stress in rat models of diabetes

β-Caryophyllene significantly decreased the levels of ROS in the peripheral neural tissue of diabetic rats. The percentage formation of ROS was as low as that of the normal rat models [Figure 3]. The level of CAT, GSX, and SOD was also increased significantly in the peripheral neural tissues of the diabetic animals administered with β-caryophyllene [Figures 4a, b, and 5b]. The MDA levels in the neural tissues were significantly decreased after the administration of β-caryophyllene [Figure 5a].

β-Caryophyllene downregulates the expression of apoptotic markers

The western blotting study of different apoptosis-related proteins from the peripheral nerve cells indicated that the protein levels of positive regulators of cellular apoptosis i.e., Bax, cleaved caspase 9 were significantly lower in the diabetic rats treated with β-caryophyllene relative to the diabetic control rats [Figure 6]. However, the protein levels of Bcl-2 and pro-caspase 9 were increased upon β-caryophyllene treatment of the rat models. The effects of β-caryophyllene treatment on the protein concentrations of apoptotic marker proteins were less prominent in the normal male Wistar rats. Taken together, the results show that β-caryophyllene has an anti-apoptotic role in the animal models of diabetes.

DISCUSSION

Diabetes mellitus is a chronic metabolic disease with impaired biosynthesis/secretion of insulin from the beta-cells of the pancreas.[1] Lower insulin production significantly increases the level of blood glucose leading to hyperglycemia. Hyperglycemia in turn speeds up the glycolytic pathway which leads to an overload on the mitochondrial electron transport chain and thus the production of ROS.[10] Increased levels of ROS are highly detrimental to normal cellular physiology as they damage the cellular membrane leading to cell death.[7]

Diabetes mellitus-associated nerve cell damage of the peripheral nervous system is known as diabetic peripheral neuropathy.[11] It is the dominant micro-vesicular disorder, and about half of the patients with diabetes are at the risk of peripheral neuropathy during their lifetime.[12] Furthermore, 30% of the patients with diabetes experience painful peripheral neuropathy.[5] Recent studies have revealed the positive effect of chemical agents against diabetic peripheral neuropathy;[13] however, the reports suggest that little success has been achieved at the global level. Therefore, in this study, we evaluated the effects of β-caryophyllene against diabetes in the experimental rat model of diabetes. β-Caryophyllene is a sesquiterpene prevalently found in plant essential oils such as clove oil and is an FDA-approved food additive.[14] It exhibits anti-inflammatory activity, analgesic activity, and antidiabetic activity.[15-17] The results of this study showed that β-caryophyllene significantly increased the levels of insulin in diabetic rats. Furthermore, the level of blood glucose was reduced significantly, which can be attributed to increased insulin levels. β-Caryophyllene normalized the lipid profile as good as the NC rats. Such antidiabetic effects have also been reported for other plant products.[18]

Interestingly, the investigation of ROS levels from the peripheral neural tissues showed that the ROS production in diabetic rats declined markedly after the administration of β-caryophyllene. This decline

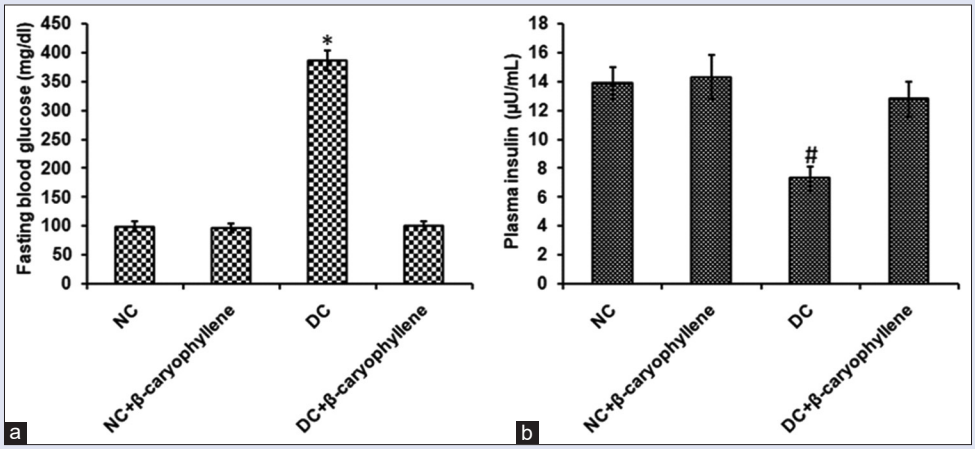

Figure 2: Effect of β-caryophyllene (200 mg/kg) on (a) blood glucose and (b) plasma insulin level. Values presented are the mean of three biological replicates ± standard deviation ($P < 0.05$), where normal control represents normal control male rats and diabetic represents diabetic control rats

Table 1: Effects of β-caryophyllene (200 mg/kg) administration on the body weight and lipid profile of experimental animals

	Group I	Group II	Group III	Group IV
Mean body weight (g)	239±8.76	244±8.76	207±10.05*	232±8.87
Triglycerides (mg/dL)	63.21±2.56	61.17±2.12	173.57±5.53*	78.3±3.01
HDL-cholesterol (mg/100 mL)	45.33±1.13	48.16±1.18	27.04±0.72*	43.73±1.54
LDL-cholesterol (mg/100 mL)	26.15±0.64	25.65±0.56	110.45±2.21*	37.53±64
Total cholesterol (mg/dl)	74.21±2.66	71.43±2.09	178.76±4.12*	77.23±2.48

*Symbol (differing from other row values at $P<0.05$). In each row, a statistically significant difference is subscripted with. HDL: High-density lipoprotein; LDL: Low-density lipoprotein

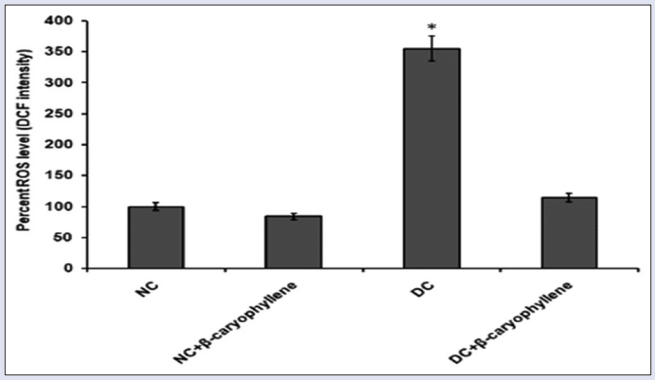

Figure 3: Effect of β-caryophyllene (200 mg/kg) on intercellular levels of reactive oxygen species in the peripheral neural cells. The experiments were performed in triplicate and the values are the mean of three biological replicates ± standard deviation (*P < 0.05), where normal control represents normal control male rats and diabetic represents diabetic control rats

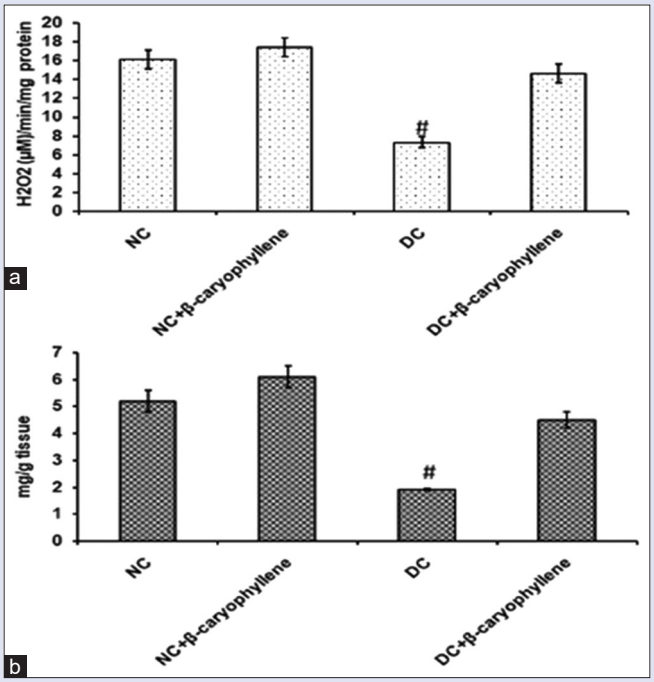

Figure 4: Effect of β-caryophyllene (200 mg/kg) on peripheral neural cell (a) catalase activity (b) glutathione level. Values presented are the mean of three biological replicates ± standard deviation (#P < 0.05), where normal control represents normal control male rats and diabetic represents diabetic control rats

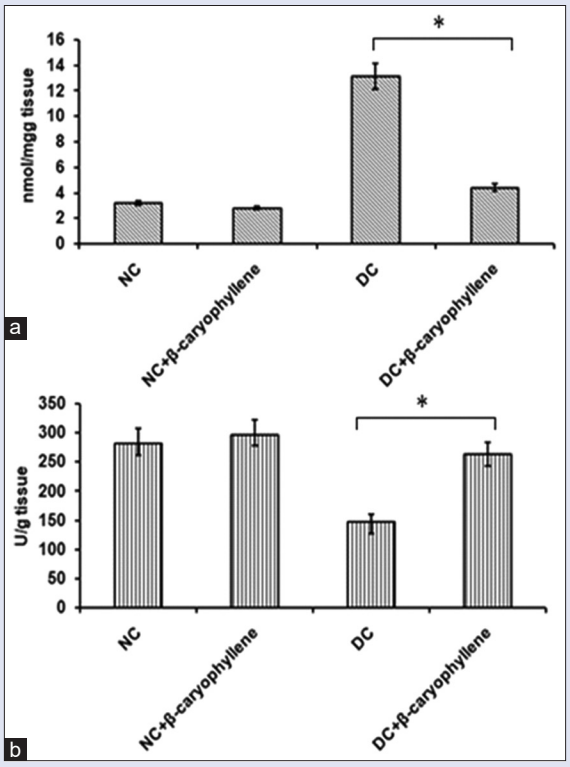

Figure 5: Effect of β-caryophyllene (200 mg/kg) on peripheral neural cell (a) malondialdehyde content (b) superoxide dismutase activity. Values presented are the mean of three biological replicates ± standard deviation (*P < 0.05), where normal control represents normal control male rats and diabetic represents diabetic control rats

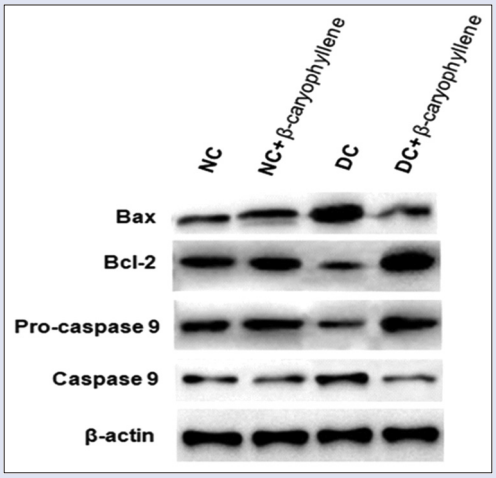

Figure 6: Effect of β-caryophyllene (200 mg/kg) administration on the expression levels of apoptosis marker proteins like Bax, Bcl-2, Pro-caspase 9, caspase 9 and β-actin was performed by Western blot analysis. The experiments were performed in triplicate, where normal control represents normal control male rats and diabetic represents diabetic control rats

might be due to the reduced levels of blood glucose in addition to the increase in the activities of CAT, GSX, and SOD in diabetic rats after the administration of β-caryophyllene. The level of MDA is a representative of cellular damage.[19] The neural tissues exhibited significantly lower MDA levels after treatment with β-caryophyllene, which clearly shows that β-caryophyllene prevents cellular damage. The inhibition of cellular damage by β-caryophyllene was evident as the level of positive regulators of apoptotic cell death proteins such as Bax and caspase-9 was downregulated. Taken together, β-caryophyllene reduced neural ROS levels and inhibited the apoptosis of neural cells in diabetic rats.

CONCLUSION

β-caryophyllene showed potent antidiabetic activity. The aggravation of oxidative stress and inhibition of apoptosis markers in peripheral neural tissues of diabetic rat models by β-caryophyllene administration infers

the potential utility of β-caryophyllene as a vital lead molecule against diabetic peripheral neuropathy.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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