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CB2 receptor agonist and L- arginine combination attenuates diabetic cardiomyopathy in rats via NF- κ B inhibition

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Title: CB₂ receptor agonist and L- arginine combination attenuates diabetic cardiomyopathy in rats via NF-κβ inhibition

Running Title: BCP and LA attenuate diabetic cardiomyopathy.

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Abstract:

Beta-caryophyllene (BCP), a cannabinoid 2 receptor (CB₂) agonist has recently been found to have cardioprotective activity as an anti-inflammatory and antioxidant molecule. L-arginine (LA), a nitric oxide (NO) donor is a potential regulator of cardiovascular function. Considering the role of CB₂ receptor activation and NO regulation in cardiovascular diseases, the combination of BCP with LA may be a possible treatment of diabetic cardiomyopathy (DCM). Hence, we investigated the efficacy of the novel combination of BCP with LA on cardiovascular inflammation and oxidative stress in diabetic rats. DCM was induced by Streptozotocin (55 mg/kg) in SD rats intraperitoneally. BCP, LA and BCP with LA were administered to diabetic rats for 4 weeks. After completion of the study, hemodynamic parameters, biochemical parameters, and inflammatory cytokine levels were analyzed. Also, oxidative stress parameters, NF- κ B expression and histopathology in cardiac tissues were estimated. The combination of BCP (200 mg/kg) with LA (200 mg/kg) significantly normalized the hemodynamic parameters and decreased the glucose, cardiac markers, IL-6 and TNF- α levels. Treatment of BCP and LA showed a significant decrease in oxidative stress and down-regulated the cardiac expression of NF- κ B. Thus, the combination of BCP with LA improves cardiac functions by attenuating inflammation through NF- κ B inhibition in DCM.

Keywords: Beta-Caryophyllene, L-arginine, Diabetic cardiomyopathy, NF- κ B inhibition, Streptozotocin

Abbreviations:

- ANOVA: Analysis of variance
- ARRIVE: Animal Research Reporting in In vivo Experiments
- BCP: Beta-caryophyllene
- CAT: Catalase
- CB2: Cannabinoid receptor 2
- CK-MB: Creatine Kinase- Myocardial Band
- COM1: Beta-caryophyllene (100 mg/kg) + L-arginine (100 mg/kg)
- COM2: Beta-caryophyllene (200 mg/kg) + L-arginine (200 mg/kg)
- CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals
- DBP: Diastolic Blood Pressure
- DCM: Diabetic Cardiomyopathy
- DM: Diabetes Mellitus
- ECG: electrocardiogram
- ECL: enhanced chemiluminescence
- GSH: Reduced Glutathione
- HDL: high-density lipoproteins,
- HR: Heart Rate
- HRP: Horseradish Peroxidase
- IAEC: Institutional Animal Ethics Committee
- IgG1k – Immunoglobulin G 1 kappa
- IL-6: Interleukin-6
- LA: L-arginine
- LDH: Lactate dehydrogenase
- MAP: Mean Arterial Pressure
- MDA: Malondialdehyde
- m-IgGκ BP- Mouse IgG kappa binding protein

71 NF- κ B: Nuclear factor-kappa beta
72 NO: Nitric oxide
73 PGL: Plasma Glucose Level
74 PVDF: polyvinylidene fluoride
75 QTc: Corrected QT interval
76 RIPA: Radioimmunoprecipitation assay
77 SBP: Systolic Blood Pressure
78 SD rats: Sprague Dawley rats
79 SDS PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.
80 SOD: Superoxide Dismutase
81 STZ: Streptozotocin
82 TBST: Tris Buffered Saline with Tween 20
83 TC: Total Cholesterol,
84 TG: Triglycerides
85 TGF- β : Transforming growth factor-beta.
86 TNF- α : Tumor Necrosis Factor-alpha

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95 1. Introduction:

96 According to the International Diabetes Federation, approximately 12-32 % of patients with
97 diabetes have suffered from diabetic cardiomyopathy (DCM); a major cause of mortality and have
98 a 2-5-fold higher risk than non-diabetic patients suffering from DCM (IDF Diabetes Atlas 9th
99 edition, 2019). The pathophysiology of DCM is well established by various pre-clinical and
100 clinical research. Hyperglycemia is a hallmark of diabetes; which is a very well-known risk factor
101 in the development of DCM, which is attributed to cardiac hypertrophy, vascular endothelial
102 dysfunction, increased oxidative stress, inflammation and mitochondrial dysfunction (Knapp, Tu
103 and Wu, 2019; Paolillo *et al.*, 2019; Kaur *et al.*, 2021). Collectively, all these factors contribute to
104 the accumulation of pro-inflammatory cytokines such as Tumor Necrosis Factor-alpha (TNF- α),
105 Interleukin-6 (IL-6) and transforming growth factor-beta (TGF- β) in the cardiac tissue that can
106 induce the cardiac injury. It was well reported that nuclear factor kappa beta (NF- $\kappa\beta$) is the main
107 inflammatory pathway and its activation contributes to cardiac fibrosis, apoptosis and left
108 ventricular dysfunction (Atta *et al.*, 2018; Tan *et al.*, 2020). Cannabinoid receptor 2 (CB₂) is a
109 novel therapeutic target that gained interest for its advantageous role in treating numerous diseases
110 such as inflammation, diabetes mellitus (DM) and cardiac disorders (Fulmer and Thewke, 2018).
111 Emerging evidence indicates that CB₂ agonist demonstrates a protective effect in ischemia-
112 reperfusion injury, cardio-protection, diabetes mellitus and inflammation (Defer *et al.*, 2009). Wu
113 et al evaluated the effect of activating CB₂ on cardiac function in STZ induced diabetic
114 cardiomyopathy in mice. HU308, a selective CB₂ agonist when administered in cardiomyopathic
115 mice showed improved cardiac function with attenuation of DCM. (Wu *et al.*, 2018). It was also
116 suggested that CB₂ receptor activation ameliorates inflammation through inhibition of NF- $\kappa\beta$,
117 TNF- α and IL-6 (Liu *et al.*, 2014). Expression of CB₂ receptors decreases in the heart and kidney

due to hyperglycemia which promotes the development of diabetic complications through activation of the inflammatory cascade and oxidative stress. (Barutta *et al.*, 2011). Accumulating evidence suggests that, endothelial dysfunction mainly through nitric oxide (NO) deficiency which is observed in diabetes, is responsible for the development of cardiac hypertrophy and increased myocardial fibrosis (Khanna, Singh and Khullar, 2014). Therefore, anti-inflammatory and NO donating therapeutic strategies could provide a new approach for the treatment of DCM.

Beta-caryophyllene (BCP) is a naturally occurring selective CB₂ agonist; which has shown proven cardioprotective activity as well as anti-inflammatory activity. (Gertsch *et al.*, 2008). Chemically BCP is a sesquiterpene and a volatile oil present in many plants, food and spices, including cloves, cinnamon, black pepper and coriander. Due to the selective binding of BCP to CB₂ receptors, it does not produce any psychotropic side effects. Therapeutic effects of BCP have been demonstrated in various pharmacological activities such as anti-diabetic (Basha and Sankaranarayanan, 2014), anti-cancer, antioxidants (Hashiesh *et al.*, 2020) as well increased insulin secretion (Hashiesh *et al.*, 2020). Suijin *et al* demonstrated enhancement of insulin secretion in the MIN6 cells when treated with BCP (0.1-1.0 μ M) in a dose-dependent manner. However MIN6 cells treated with CB₂ receptor inhibitor (SR144528, 1 μ M) interfere with the effect of BCP on glucose-stimulated insulin secretion (Suijun *et al.*, 2014). Basha *et al* evaluated the role of BCP on glucose homeostasis in STZ induced DM rats. BCP (200 mg/kg oral) has been found to significantly decrease plasma glucose levels and increase plasma insulin to near-normal levels (Basha and Sankaranarayanan, 2014). An effect of BCP in STZ induced hyperglycemia, oxidative stress and inflammation in STZ induced DM rats was assessed. BCP oral supplementation significantly improved the anti-oxidant defence mechanism; and inflammatory cytokines such as TNF- α and IL-6 (Basha and Sankaranarayanan, 2016). Younis *et al* demonstrated that pretreatment

of BCP at the dose of (100 mg/kg and 200 mg/kg orally) protected the isoproterenol-induced myocardial infarction in rats (Younis and Mohamed, 2019). Recently, the effects of BCP has been demonstrated to decrease inflammation by NF- κ B transcription pathway and oxidative stress in doxorubicin-induced cardiotoxicity in rats (Meeran *et al.*, 2019).

L-arginine (LA) is an amino acid present in the body and found widely in dairy products, poultry, seafood, peanut, coconut, etc. It is known to play a vital role in cell division, wound healing, immunity and NO production (Herman and Moncada, 2005). An important finding by Claybaugh *et al* suggested that LA (1 gm/kg) supplementation in the type 2 diabetic rats decreased the plasma glucose level, preserved the renal function and improved the insulin sensitivity by altering the NO pathway (Claybaugh *et al.*, 2014). Sherin *et al.* demonstrated supplementation of LA in diabetic rats led to the protection of the heart from Streptozotocin-induced myocardial fibrosis in rats. Also, the authors revealed that LA supplementation decreased the myocardial inflammatory cytokines and NF- κ B expression (Rizk, El-Maraghy and Nassar, 2014). It is well reported that hyperglycemia decreases endogenic levels of LA in the kidney and heart which develops diabetic complications (You *et al.*, 2014). Pharmacologically LA shows anti-diabetic, anti-inflammatory, anti-obesity as well as pancreatic β -cell regenerative properties (Adeghate *et al.*, 2001; You *et al.*, 2014). Preclinical and clinical studies have recommended several beneficial pharmacological activities such as improvement of the immune response, reduction in cardiac risk and maintaining the integrity of pancreatic cells.

A preliminary study by Kaur and coworkers showed the combination of BCP with LA (150 mg/kg) significantly decreased the plasma glucose level and protected the pancreas from oxidative stress damage in diabetic rats. In addition to that effect, the combination of BCP with LA has also explored insulin secretion in rat insulinoma (RIN-5F) cell lines by glucose challenge. A

combination of BCP with LA was found to significantly increase insulin secretion (Kumawat and Kaur, 2020).

At present, there is no effective and specific therapeutic approach in the treatment of DCM and its management is difficult because of numerous factors involved in the pathogenesis of DCM. Therefore, there is a need to venture into a novel, effective and multifaceted treatment for the management of DCM. Hence the present study aims to evaluate the combinational effect of BCP with LA on NF- κ B expression and oxidative stress in the STZ induced DCM. In this study, we also investigated whether the combination of BCP and LA showed a more protective effect when compared with the alone treatment of BCP and LA in DCM.

2. Materials and Methods:

2.1 Drugs, Chemicals and Antibodies:

BCP and STZ were purchased from Sigma-Aldrich (St. Louis, MO, USA). LA was purchased from Arrobiochem (India). NF- κ Bp65 Antibody Mouse monoclonal IgG1k, β -Actin Antibody mouse monoclonal IgG1k mouse, mouse IgG kappa binding protein (m-IgGk BP) conjugated to Horseradish Peroxidase (HRP) (secondary antibody) were procured from Santa Cruz Biotechnology, Inc. USA. All diagnostic biochemical kits were purchased from Transasia Biomedicals Ltd., India.

2.2 Experimental animals:

Male Sprague Dawley (SD) rats (180-200 gm) were obtained from the National Institute of Biosciences, Pune. The experimental animals were housed in controlled temperature $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and humidity ($55 \pm 5\%$) at SVKM's NMIMS, Animal house facility. A normal pellet diet was provided to the animals along with water *ad libitum*. The animals were treated in accordance with

the Guide for the Care and Use of Laboratory Animals (Eight editions, National Academies Press)

This research manuscript reporting adheres to the ARRIVE guidelines. The research protocol was approved by the Institutional Animal Ethics Committee and procedures were performed as per the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines Govt. of India. (Registration no: CPCSEA/ IAEC/ P-38/2019).

2.3 Induction of DCM:

DCM was induced with single-dose administration of STZ (55 mg/kg, i.p.) (Zhang and Xu, 2018). Animals with plasma glucose levels (PGL) higher than 250 mg/dl were included in the study and after six weeks, animals were randomized into different groups.

2.4 Experimental Design:

Animals were divided into seven groups, each group containing eight rats. All treatments were administered orally except Insulin (s.c). BCP and LA were freshly prepared in corn oil and water respectively. The animals in group I received corn oil and water (1:1) and served as normal control. The diabetic animals in group II were considered as a disease control group. The diabetic animals in group III received insulin (2 IU s.c.) (Chen *et al.*, 2018). The diabetic animals in group IV received BCP (200 mg/ kg p.o.) (Basha and Sankaranarayanan, 2016). The diabetic animals in group V received LA (200 mg/kg p.o.) (Gad, 2010). The diabetic animals in group VI received BCP (100 mg/kg p.o.) and LA (100 mg/kg p.o.) and served as COM1. The diabetic animals in group VII received BCP (200 mg/kg p.o.) and LA (200 mg/kg p.o.) and served as COM2. The combination dose was given in 15 mins of interval. The treatment was given to the animals daily for 28 days. At the end of the study, body weight and hemodynamic parameters were recorded.

The blood samples were collected, and heart tissue samples were isolated after the sacrifice of animals.

2.5 Parameters Measured:

2.5.1 Assessment of electrocardiogram (ECG) parameters:

After 4 weeks of treatment, rats were anaesthetized using terminal anaesthesia (Urethane, 1.2 g/kg, i.p.) for ECG parameters. The ECG was recorded using a Lead II configuration. The needle electrodes (29-gauge, 12 mm) were placed beneath the skin at the left inguinal (positive lead), right inguinal (neutral lead), and right axilla (negative lead). Electrodes were connected to the data acquisition system (AD Instrument, Australia) and responses were recorded for 5 minutes after stabilization of animals for 30 min. The heart rate (HR), R-R interval, QRS interval, QT interval, and corrected QT interval (QTc) were calculated from ECG recordings (Younis and Mohamed, 2019).

2.5.2 Assessment of hemodynamic parameters:

After recording of ECG parameters, hemodynamic parameters such as mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were evaluated. The right carotid artery was cannulated with a polyethene catheter and connected with the physiological pressure transducer system. After stabilization of the animal, MAP, SBP and DBP were recorded with a data acquisition system (AD Instrument, Australia) (Roslan *et al.*, 2017; Younis and Mohamed, 2019).

2.5.3 Bodyweight and relative heart weight:

227 After the hemodynamic assessment, blood samples were collected, and rats were humanely
228 euthanized, hearts were quickly isolated and weighed. Bodyweight was recorded prior to
229 hemodynamic assessments after four weeks of dose administration. The relative heart weight was
230 calculated by the following formula: (Heart weight / Bodyweight) * 100.

231 2.5.4 Biochemical estimations:

232 Serum and plasma samples were separated from the blood samples by centrifugation (3000 rpm,
233 10 min, 4° C). Plasma glucose, total cholesterol (TC), triglycerides (TG), high-density lipoproteins
234 (HDL), serum creatine kinase-MB (CK-MB), and Lactate dehydrogenase – P (LDH-P) were
235 estimated as per the manufacturer's protocol using Erba chemical kits (Transasia Biomedicals,
236 India) by biochemistry analyzer (Erba Chem-7, Germany).

237 2.5.5 Assessment of TNF- α and IL-6 levels:

238 The TNF- α and IL-6 levels in the plasma were estimated with a multi-mode microplate reader
239 (Epoch 2, BioTek Instruments, Inc., USA) by commercially available ELISA kits (BT Biosciences,
240 China) according to manufactures protocol (Roslan *et al.*, 2017).

241 2.4.6 Assessment of antioxidant parameters:

242 The heart homogenate was used to estimate Malondialdehyde (MDA) (Ohkawa, Ohishi and Yagi,
243 1979) and reduced glutathione (GSH) (Ellman, 1959). Post-mitochondrial supernatant was
244 obtained by centrifugation at 10000 rpm for 20 min at 4°C. This preparation was used further to
245 estimate superoxide dismutase (SOD) and catalase. The SOD (Paoletti *et al.*, 1986) and catalase
246 (Lück, 1965) activity were measured.

247 2.4.7 Assessment of NF- κ B expression by western blotting:

Total proteins from heart tissues were extracted by using Radioimmunoprecipitation assay (RIPA) buffer. Protein samples (40- μ g) with Laemmli sample buffer and 2-mercaptoethanol were separated via 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 μ M, Merck Millipore, Germany). The membrane was blocked with 5% skimmed milk for 1 hr. at room temperature. Primary antibody treatment of NF- κ B and β -actin (loading control) was given at dilution 1:1000 for 2 hours. Then samples were incubated with secondary antibody (m-IgGk BP-HRP) at dilution 1:1250 for 1 hour. Then washed three times for 10 min with TBST. Imaging was performed using enhanced chemiluminescence (ECL) on X-ray film in a dark room. The signal intensity of bands was measured by Image Studio Lite version 5.2 (LI-COR Biosciences, USA) software (Ma *et al.*, 2018).

2.4.8 Histopathological study and determination of cardiac fibrosis by collagen deposition:

Heart tissue fixed in 10% v/v neutral buffered formalin (NBF) were further embedded in paraffin. Thin (3-4 μ m) transverse sections were taken with the Rotary Microtome (Leica, Germany). Slides of heart sections were stained with Hematoxylin & Eosin (H & E) stain Masson's trichrome stained. H & E-stained slides were examined under the microscope to observe histopathological lesions if any. Masson's trichrome stained slides were examined under the microscope to measure cardiac fibrosis. The extend of collagen deposition (blue colour) was analyzed using Image J software (NIH, USA) and results were expressed as % collagen deposition (Othman *et al.*, 2017).

2.4.9 Statistical Analysis:

Values were expressed as mean. GraphPad Prism ver. 5.00 for Windows was used to analyze the data. Analysis of variance (ANOVA) followed by Dunnett's test was used to determine the

statistical significance of the difference between the groups. The values were considered to be significantly different when the P -value was less than 0.05.

3. Results:

3.1 Bodyweight, and relative heart weight:

As depicted in figure 1, diabetic animals showed significantly decreased body weight and increased relative heart weight when compared with the normal group. The combination of BCP with LA significantly improved body weight and reduction in relative heart weight ($P < 0.001$). It was also observed that a high dose of combinational treatment additionally improved the metabolic perturbations when compared with monotherapy. Insulin treatment also significantly increased weight and reduced relative heart weight ($P < 0.001$).

3.2 Plasma glucose level and cardiac markers:

The plasma glucose level was significantly increased in diabetic animals ($P < 0.001$) when compared with the normal control group. The elevated plasma glucose level was significantly reduced in all treatment groups; especially the insulin ($P < 0.001$) and COM2 ($P < 0.001$) group showed a reduction in plasma glucose level. Individual treatment of BCP and LA reduced the plasma glucose levels to some extent, but the combinational treatment additionally improved the glycemic status. The diabetic animals showed a significant ($P < 0.001$) increase in the levels of serum CK-MB and LDH compared to normal control rats. However, treatment with COM1 and COM2 resulted in a significant decrease in the levels of CK-MB ($p < 0.05$) and LDH ($p < 0.001$) when compared with diabetic animals. There was no significant difference was observed in the monotherapies of BCP and LA on the CK-MB and LDH levels (Figure 2).

3. 3 Lipid Profile:

Figure 3 showed the effect of the combination of BCP with LA on the lipid profile. In the disease control rats, the plasma TC and TG levels were significantly raised, and HDL level was significantly reduced when compared to the normal control group. Rats receiving combination treatment at both dose levels showed a significant reduction in TC and TG levels ($P<0.001$) and showed a significant increment in the HDL level ($P<0.001$) when compared to the disease control group. Moreover, COM2 treatment showed a significant ($P<0.001$) reduction in the TG and TC levels when compared to monotherapies of BCP and LA.

3. 4 Hemodynamic parameters:

Blood pressure parameters such as SBP, DBP and MAP were found to be reduced in the diabetic rats when compared to normal rats. Monotherapy of BCP treatment significantly ($P<0.05$) increased SBP and DBP when compared to the diabetic control group. Interestingly, a combination of both such as COM1($P<0.01$) and COM2 ($P<0.001$) markedly normalize the SBP, DBP and MAP when compared to the disease control rats. However, COM2 showed significantly ($P<0.001$) normalized the blood pressure parameters when compared to the LA monotherapy (Figure 4).

3.5 Electrocardiogram (ECG):

The effect of treatment on the ECG pattern of animals shown in figure 5. ECG parameters such as QT interval, QTc and RR interval were found to be increased and heart rate and QRS complex were decreased in the diabetic rats when compared to normal control animals. Treatment of combination BCP with LA significantly ($p<0.001$) increased heart rate and QRS complex when compared with the disease control group. Moreover, a combination of BCP and LA showed

significantly ($p < 0.001$) normalized the QT, QTc and RR interval when compared to the disease control group.

3.6 Inflammatory markers:

ELISA results illustrated that STZ induced diabetic cardiomyopathy in rats significantly increased inflammatory markers such as TNF- α and IL-6 when compared with the control group as shown in figure 6. Oral administration of BCP ($P < 0.01$), COM1 ($P < 0.001$) and COM2 ($P < 0.001$) showed significantly decreased levels of TNF- α and IL-6 when compared with the disease control group. Additionally, treatment with insulin also decreased TNF- α ($P < 0.01$) and IL-6 ($P < 0.05$) levels in diabetic rats when compared with the disease control group. Treatment with LA alone decrease the TNF- α and IL-6 levels but the results were not significant, while COM2 treatment significantly ($P < 0.001$) lowered the TNF- α and IL-6 levels when compared with LA treated group.

3.7 Oxidative stress parameters:

Figure 7 shows the effect of treatment on antioxidant enzymes such as GSH, MDA, CAT and SOD in the heart tissue samples. Disease control animals showed a significant decrease in GSH, CAT, SOD levels and a significant increase of MDA levels in the heart tissues when compared with the normal control group ($P < 0.001$). After 28 days of treatment, COM1 and COM2 showed a significant increase in GSH ($P < 0.01$), CAT ($P < 0.01$) and SOD ($P < 0.01$) enzymes when compared with the disease control group. MDA levels were significantly ($P < 0.01$) decreased in the COM1 and COM2 treated animals when compared with the STZ treated group.

3.8 Effect of treatment on NF- κ B expression by western blotting:

Figure 8 represents the effect of treatment on NF- κ B in heart tissues. Expression of NF- κ B protein in heart tissue significantly increased in the disease control group when compared with the normal

control group ($P < 0.001$). Oral administration of BCP ($P < 0.05$), LA ($P < 0.05$), COM1 ($P < 0.01$) and COM2 ($P < 0.001$) to DCM rats for 28 days showed significantly decreased expression of NF- κ B in heart tissue. Overall results of COM2 showed better anti-inflammatory activity and decreased NF- κ B expression.

3.9 Histopathological study and determination of cardiac fibrosis by collagen deposition:

The histopathological observations of cardiac tissues of the control group showed normal cardiac structure and normal histology of myocyte. The heart sections of disease control animals showed extensive damage in the form of lymphocytic and inflammatory infiltration, degeneration, angiogenesis at the myocardium. Treatment of insulin, BCP and LA alone reduce the severity and distribution of diabetic anomalies. On other hand, diabetic animals treated with a low and high dose of the combination of BCP with LA significantly ameliorated diabetic induced lymphocytic and inflammatory infiltration, degeneration, angiogenesis and prevent the progression of cellular damage in the myocardium. The results also have higher efficacy when compared to the administration of drugs alone.

Masson's trichrome stained sections of the control-treated rat heart indicated normal histology of myocardium and normal deposition of collagen. The heart of diabetic animals displayed immense deposition of collagen in the myocardium. The combination of BCP with LA markedly reduced the collagen deposition at both dose levels (100 and 200 mg/kg) (Figure no. 9-11).

4. Discussion:

The pathophysiology of DCM is multifaceted and multifactorial also it is associated with inflammation, endothelial dysfunction, and oxidative stress. Many evidence suggests that

inflammation is a key factor in the development of DCM (Jia, Hill and Sowers, 2018). Key inflammatory factors such as NF- κ B, TNF- α and protein kinases can directly induce cardiac hypertrophy and leads to the development of DCM. Many previous studies indicate that NF- κ B inhibitor prevents the activation of NF- κ B signalling and protects the heart from cardiac dysfunction and oxidative stress damage (Tan *et al.*, 2020). In recent years, dysregulation of inflammatory cascades has gained more attention in the management of DCM. Also, researchers are targeting the endocannabinoid system in inflammatory diseases such as diabetic complications, ischemic reperfusion injury and cardiotoxicity (Pacher, Bátkai and Kunos, 2006). It was also observed that increased oxidative stress is associated with endothelial dysfunction by NO dysregulation which plays an important role in the development of DCM (Knapp, Tu and Wu, 2019; Murtaza *et al.*, 2019). Recently, oxidative stress and inflammation coupled with the endocannabinoid system contribute to the progression of diabetic complications (Kumawat and Kaur, 2019). Schmidt et al. reported that there is intercalation between CB₂ activation and NO production in the cardiac cells. In a similar study, Delta-9-tetrahydrocannabinol protects cardiac cells from hypoxia via CB₂ receptor activation and nitric oxide production (Shmist *et al.*, 2006). BCP is a phytocannabinoid having an affinity towards non-psychotropic CB₂ receptor which shows numerous pharmacological activities such as anti-diabetic, anti-inflammatory, anti-cancer, and insulin-secreting activity. Whereas LA is a non-essential amino acid involved in many metabolic processes including regulation of inflammation, immune response and maintaining pancreatic β -cells integrity.

To the best of our knowledge, this is the first study to report the cardioprotective effects of the combination of BCP with LA in a rat model of type 1 diabetic mellitus induced cardiomyopathy. The streptozotocin-induced DCM model is a well-established and widely accepted rodent model

for induction of DCM because it provides typical disorder features that are relevant to DCM (Goyal *et al.*, 2016). Increased levels of cardiac markers such as CK-MB and LDH with oxidative stress-induced damage of cardiac tissues are the gold standard parameters to confirm the induction of DCM in rats (Bugger and Abel, 2009). In the present study, cardiac markers, inflammatory cytokines and lipid parameters are elevated in the diabetic rats which resemble previously reported results (Huang *et al.*, 2017).

The important approach to the treatment of diabetes is to control hyperglycemia and this approach is useful in the prevention of development of its complications. The present study indicated that all treatment groups significantly decreased ($P < 0.001$) the levels of plasma glucose. Specifically, the combination of BCP with LA at a high dose showed more glycemic control than the individual treatment of BCP and LA. The present findings report the strong anti-hyperglycemic property of the combination of BCP with LA. In hyperglycemic conditions, glucose consumption as an energy source in the cells is decreased therefore protein and fats are utilized as an energy source instead of glucose which indicates the loss of body weight in rats (Wilcox, 2005). In the present study combination of BCP and LA at high doses showed a significant increase in the body weight in the diabetic rats when compared with the disease control group and monotherapies. These results reflect the positive effects of treatment on glucose homeostasis. The treatment of COM1 and COM2 showed decreased cardiac hypertrophy indicating a possible role in the regulation of metabolic pathways and oxidation of fatty acids (Muoio and Newgard, 2008).

Evaluation of biochemical markers of cardiac injury is an important parameter to detect DCM. An increased level of serum CK-MB and LDH reflects the development of DCM. In this study, treatment of COM1 and COM2 significantly ($P < 0.001$) decreased the levels of cardiac enzymes such as CK-MB and LDH when compared with the disease control group. Increased glucose levels

are directly linked with increased lipids, which leads to the development of DCM and coronary heart disease (Dokken, 2008). TG and TC levels were significantly increased ($P<0.001$), and HDL ($P<0.01$) was decreased in diabetic rats. COM1 and COM2 treatment significantly decreased TG and TC and increment of HDL levels when compared with the disease control group.

DCM is a common complication of diabetes mellitus characterized by diastolic and systolic dysfunction. Similarly, the present study showed a significant decrease in heart rate, SBP and DBP and altered the ECG pattern in the diabetic rats when compared with the normal control group. Necrosis, loss of normal cardiac muscle architecture and cardiac contractibility in the diabetic rat heart lead to a decrease in the SBP and DBP (Liu *et al.*, 2008). Treatment of BCP and both combinations significantly ($P<0.001$) increased heart rate, SBP, DBP and normalize the ECG pattern when compared with the disease group.

Chronic hyperglycemia demonstrated in oxidative stress through direct generation of ROS and reduction in the ability of endogenous anti-oxidants and also decreased in the NO levels (Knapp, Tu and Wu, 2019). The presented data simulates previous studies, in which disease control rats show a significant reduction in GSH, CAT and SOD and increment of MDA activity in heart tissues (Huang *et al.*, 2017). COM1 and COM2 treatments show increased SOD, CAT and GSH levels and decreased oxidative stress markers such as MDA. This oxidative stress defence by combination treatment of BCP with LA showed cardio-protection from hyperglycemia.

Results of various animal studies suggested that pro-inflammatory cytokines such as TNF- α and IL-6 were involved directly in the progression of DCM (Filardi *et al.*, 2019). Hence, we investigated the effect of the combination of BCP with LA in the inflammatory cytokines such as TNF- α and IL-6. The present data showed elevated levels of plasma TNF- α and IL-6 in diabetic rats and the treatment with alone BCP and LA, COM1 and COM2 ($P<0.001$) showed a significant

reduction of TNF- α and IL-6 levels. The COM1 and COM2 showed a better reduction in TNF- α and IL-6 levels because individual BCP and LA showed anti-inflammatory activity and these results resemble previous studies which suggested BCP and LA could reduce the inflammatory response in the heart (Basha and Sankaranarayanan, 2016).

NF- κ B is a transcription factor that is activated by various stimuli such as oxidative stress and inflammatory cytokines. The inhibition of NF- κ B expression is a crucial step for the prevention of inflammatory responses in the progression of DCM (Suryavanshi and Kulkarni, 2017). Various animal studies support that, in the hyperglycemic condition the NF- κ B expression is elevated in heart tissues. Our results suggest that the diabetic rats showed increased expression of NF- κ B in heart tissue. After treatment of BCP, LA and a combination of BCP with LA showed a significant reduction in NF- κ B expression when compared with the disease control group. COM2 ($P < 0.001$) showed a better reduction in NF- κ B expression in heart tissues as compared to alone treatment of BCP and LA treatment.

Histopathological examinations of heart tissues strengthen the observed biochemical and enzyme activity findings. The heart of diabetic animals showed massive cell damage in the form of lymphocytic infiltration, degeneration, and angiogenesis at the myocardium. The necrosis of heart tissues is mainly credited to the increased activities of oxidative stress enzymes. The presentation demonstrated that a combination of BCP and LA supplementation protects the heart structure from oxidative stress-induced damage. In addition, our histopathological findings displayed that deposition of collagen in the myocardium and resulted in fibrosis in cardiac muscle fibres of diabetic animals. Collagen deposition and cardiac fibrosis are mainly attributed to the increased TNF- α levels and up-regulation of NF- κ B expression in diabetic conditions (Rivera-Serrano and Sherry, 2017). After four weeks of treatment of BCP with LA combination decreased the TNF- α

levels and Nf- κ B expression which was associated with a reduction in collagen deposition and cardiac fibrosis.

5. Conclusion:

We found that a combination of BCP (200 mg/kg) with LA (200 mg/kg) resulted in the lowering of plasma glucose levels, cardiac enzyme markers and normalizing systolic and diastolic functions as well as decreasing in the inflammatory signalling cascades in diabetic cardiomyopathy when compared with the individual therapies. In view of these results, the combination of BCP with LA could be potentially used as a promising therapeutic drug combination to ameliorate diabetic cardiomyopathy.

6 Conflict of interest:

The authors declare no conflict of interest.

7 Ethics statement:

The animal study was reviewed and approved by the Institutional Animal Ethical Committee of Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management, SVKM's NMIMS.

8 Acknowledgement:

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9 Author Contribution:

GK -Designed the research protocol, read, and approved the manuscript.

VSK - Performed research, analyzed the data and wrote the manuscript.

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631 Figure Legends

632 Figure 1 The effect of the combination of BCP with LA compared with disease control and BCP
633 or LA alone on A) Body weight and B) Relative heart weight.

634 Values are expressed as Mean $\pm$ S.E.M. *** $p < 0.001$ when compared with diabetic control. ###
635 $p < 0.001$ when compared to normal control (n=6).

636

637 Figure 2 The effect of the combination of BCP with LA compared with disease control and BCP
638 or LA alone on A) Plasma glucose, B) CK-MB & C) LDH-P levels.

639 Values are expressed as Mean $\pm$ S.E.M. *** $p < 0.001$ when compared with diabetic control. ###
640 $p < 0.001$ when compared to normal control (n=6).

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642 Figure 3 The effect of the combination of BCP with LA compared with disease control and BCP
643 or LA alone on A) Triglycerides B) Total Cholesterol and C) HDL levels.

644 Values are expressed as Mean $\pm$ S.E.M. *** $p < 0.001$ when compared with diabetic control. ###
645 $p < 0.001$ when compared to normal control (n=6).

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647 Figure 4 The effect of the combination of BCP with LA compared with disease control and BCP
648 or LA alone on A) Systolic blood pressure, B) Diastolic blood pressure and C) Mean atrial
649 pressure.

650 Values are expressed as Mean $\pm$ S.E.M. *** $p < 0.001$ when compared with diabetic control. ###
651 $p < 0.001$ when compared to normal control (n=6).

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Figure 5 The effect of the combination of BCP with LA compared with disease control and BCP or LA alone on A) Heart rate, B) QT interval C) QTc interval, D) QRS Complex and E) RR interval.

Values are expressed as Mean $\pm$ S.E.M. *** $p < 0.001$ when compared with diabetic control. ### $p < 0.001$ when compared to normal control (n=6).

Figure 6 The effect of the combination of BCP with LA compared with disease control and BCP or LA alone on A) TNF- α and B) IL-6 levels.

Values are expressed as Mean $\pm$ S.E.M. *** $p < 0.001$ when compared with diabetic control. ### $p < 0.001$ when compared to normal control (n=6).

Figure 7 The effect of the combination of BCP with LA compared with disease control and BCP or LA alone on A) GSH, B) MDA, C) Catalase and D) SOD activity.

Values are expressed as Mean $\pm$ S.E.M. *** $p < 0.001$ when compared with diabetic control. ### $p < 0.001$ when compared to normal control (n=6).

Figure 8 The effect of the combination of BCP with LA compared with disease control and BCP or LA alone on NF- $\kappa\beta$ expression by western blotting.

Values are expressed as Mean $\pm$ S.E.M. *** $p < 0.001$ when compared with diabetic control. ### $p < 0.001$ when compared to normal control (n=3).

673 Figure 9 The effect of the combination of BCP with LA compared with disease control and BCP
674 or LA alone on cardiac tissues- H.E. staining (400X).

675 1) Normal control: Showing normal histology of myocyte (M); 2) Diabetic control: showing
676 lymphocytic Infiltration (L), degeneration (D), angiogenesis (A) at myocardium; 3) Diabetic +
677 Insulin (2 IU) showing lymphocytic Infiltration (L) in myocardium, 4) Diabetic + BCP (200
678 mg/kg): showing lymphocytic Infiltration (L), degeneration (D) in myocardium; 5) Diabetic + LA
679 (200 mg/kg): showing lymphocytic Infiltration (L) in myocardium, 6) Diabetic + BCP (100 mg/kg)
680 and LA (100 mg/kg): showing lymphocytic Infiltration (L) in myocardium, 7) Diabetic + BCP
681 (200 mg/kg) and LA (200 mg/kg): showing lymphocytic Infiltration (L) in myocardium

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683 Figure 10 The effect of the combination of BCP with LA compared with disease control and BCP
684 or LA alone on cardiac tissues- Trichrome staining (400X).

685 1) Normal control: Showing normal histology of myocardium (M) and normal deposition of
686 collagen (C); 2) Diabetic control: showing increased deposition of collagen (C) in myocardium;
687 3) Diabetic + Insulin (2 IU): showing increased deposition of collagen (C) in myocardium; 4)
688 Diabetic + BCP (200 mg/kg): showing increased deposition of collagen (C) in myocardium; 5)
689 Diabetic + LA (200 mg/kg): showing increased deposition of collagen (C) in myocardium; 6)
690 Diabetic + BCP (100 mg/kg) and LA (100 mg/kg): showing increased deposition of collagen (C)
691 in myocardium; 7) Diabetic + BCP (200 mg/kg) and LA (200 mg/kg): showing decreased
692 deposition of collagen (C) in myocardium

693 Figure 11 The effect of the combination of BCP with LA compared with disease control and BCP
694 or LA alone on % collagen deposition.

695 Values are expressed as Mean $\pm$ S.E.M. ***p < 0.001 when compared with diabetic control. ###
696 p < 0.001 when compared to normal control (n=3).

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Figure 7

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Figure 9 **Figure 10**

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