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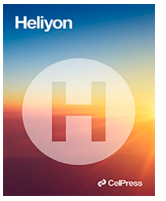

Research article

Cardioprotective effect of CB1 receptor antagonist AM251 against β receptor-stimulated myocardial infarction via modulation of NF-kB signaling pathway in diabetic mice

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ABSTRACT

We substantiated the effect of AM251, a cannabinoid receptor-1 (CB1R) antagonist, against β-receptor stimulated myocardial infarction (MI) in streptozotocin (STZ)-induced diabetic mice via modulation- of the NF-kB signaling pathway. The different parameters were assessed such as ECG, hemodynamic, cardiac injury markers, oxidative stress parameters, pro-inflammatory cytokines, and histopathological abnormalities. Mice were fed a high-fat diet for 30 days. On day 7, to trigger diabetes, 150 mg/kg of STZ was injected intraperitoneally. On day 10, to determine whether diabetes developed, the blood level of glucose was monitored. From days 11–30, diabetic mice were injected with either CB1R agonist oleamide or antagonist AM251 or both, with concurrent administrations of β-agonist isoproterenol on days 28 and 29 to induce MI. In comparison to normal, the myocardial infarcted diabetic animals demonstrated alterations in ECG, hemodynamic profiles, and diminished enzymatic activities (CK-MB, LDH, SOD, GSH, catalase), with concurrently increased MDA levels, which indicated increased oxidative stress in the myocardium. Additionally, higher concentrations of cytokines that signal myocardial inflammation, such as IL-1β, IL-6, and TNF-α, were also noted. Furthermore, elevated myonecrosis, edema, and cell infiltration which is confirmed by histopathology of heart tissue. Treatment with AM251 significantly ameliorated myocardial redox status, reduced cytokines, and repaired enzymatic activities leading to subsequent recovery in cardiac function. AM251 effectively suppressed myonecrosis and edema. This study also showed that AM251 protects against myocardial inflammation and oxidative stress triggered by isoproterenol by blocking NF-kB signalling pathway. However, upregulation of the CB1R through oleamide showed significant cardiac toxicity. Conversely, the concurrent administration of oleamide and AM251 failed to induce cardiotoxic effects in isoproterenol-induced MI in diabetic mice which indicates downregulation of the CB1R might be associated with the cardioprotective effect.

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1. Introduction

The leading cause of mortality among those with type 2 diabetes mellitus (T2DM) is myocardial infarction (MI). The risk of primary MI is more than 20 % within 10 years of developing T2DM, which is comparable to the probability of recurring MI in those without diabetes who experienced MI in previous years. Noteworthily, the risk of reoccurrence of MI in T2DM patients rises above 40 % [1]. The comorbidities associated with T2DM like oxidative stress, hyperglycemia, hyperlipidemia, and inflammatory conditions increase the risk of MI [2–4]. Researchers have been exploring various potential therapeutic targets for the effective management of devastating cardiovascular conditions like MI. The endocannabinoid system plays a crucial role in the regulation of cardiovascular functions.

Recent investigations suggest the involvement of the endocannabinoid system in the pathophysiology of cardiovascular diseases [5–7]. The cannabinoid receptor-1 (CB1R) expression is higher in the cardiomyocytes of infarct hearts, and this is associated with reduced cell survival signaling [8]. Similarly, in the heart of obese mice, increased mRNA expression of CB1R was observed in the myocardium, endothelium, vascular smooth muscles, and fibroblasts. These changes were accompanied by increased endocannabinoids (anandamide and 2-arachidonoylglycerol) contents in the myocardium, indicating augmentation of CB1R-mediated signaling by the endogenous ligands. Further, increased arachidonic acid contents in the cardiac tissue signifies the development of a proinflammatory environment [9]. It is possible that activation of CB1R by endocannabinoids may trigger a proinflammatory response, oxidative stress, and necrosis in the myocardium that results in cardiac dysfunction [10,11]. In obese people, coronary dysfunction is associated with CB1R upregulation, and elevated endocannabinoid plasma levels, This eventually leads cardiomyocytes to undergo cellular dysfunction and cell death [12]. Consequently, remedies that target only CB1Rs have been proposed as potential treatment for cardiovascular disorders like hypertension and heart failure that are related to an augmented sympathetic tone [13]. In this connection, it is worth noting that CB1R antagonist improves hemodynamics and cardiac contractility functions [8].

The primary objective of the present research was to investigate the safeguarded properties of AM251 (a CB1 antagonist) against MI in mice with diabetes by enhancing cardiac functionality tissue architecture, and cardiac injury indicators. Streptozotocin (STZ)-induced mouse model of T2DM was used to trigger MI by administration of nonselective β-adrenergic receptor agonist isoproterenol (ISO). This model shows pathophysiological changes similar to those observed in type 2 diabetic patient with MI. ISO causes necrosis in the myocardium by generating cytotoxic free radicals, pro-inflammatory cytokines, apoptosis, and an excessive load of cytosolic calcium [14,15].

2. Materials and methods

2.1. Animals

We employed adult male Swiss albino mice ranging 20–25 g. Over a natural light-dark cycle, they were maintained at a constant room temperature of 25 ± 2 °C and humidity of 55 ± 5 %. The animals consumed pelleted chow food (Nutrivet Life Sciences Pvt. Ltd., Pune, India) or HFD (Research Food Inc., New Brunswick, NJ, USA. The Institutional Animal Ethics Committee of RCPIPER, Shirpur, Maharashtra, India, has authorised the research protocols (Approval No. IAEC/CPCSEA/RCPIPER/2018-19/11).

2.2. Chemicals

AM251, STZ, and ISO suppliers were Sigma-Aldrich, located in Saint Louis, MO, USA. We bought oleamide (ODA) from Tokyo Chemical Industries in Japan. Kits for measuring serum glutamic pyruvic transaminase (SGPT), serum glutamic-oxalacetic transaminase (SGOT), creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) have been purchased from ERBA Diagnostics, Germany. The nuclear factor-kappa B (NF-kB) ELISA kit was purchased from Elabscience, USA, and the ELISA kits for tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) were bought from eBioscience, San Diego, CA, USA. Additional analytical-grade reagents were acquired from nearby suppliers.

2.3. Experimental protocol for induction of diabetes by using STZ

Six experimental groups including a total of six mice were randomly assigned (n = 6). All mice, except for those in the control group, were maintained on an HFD ad libitum from day 1 to day 30. On the seventh day of the protocol, HFD-fed animals received a single 150 mg/kg intraperitoneal (i.p.) dosage of freshly prepared STZ. Animals showing a fasting glucose level of more than 250 mg/dl following three days of STZ injection were classified as diabetic and utilized in the following investigations.

2.4. Experimental protocol for induction of MI using ISO

To trigger experimental MI in diabetic mice, ISO was injected subcutaneously (s.c.) for a couple of days (on days 28 and 29) [16].

2.5. Experimental groups

The following treatments were given to the different animal groups.

2.5.1. Group I: normal

Mice on a normal chow diet were administered with saline from day 11 to day 30 of the study.

2.5.2. Group II: disease control [HFD + STZ (150 mg/kg) + ISO (150 mg/kg)]

On day seven, STZ 150 mg/kg was administered i.p. to HFD-fed mice. After that, on days 28 and 29, 150 mg/kg ISO. was administered s.c. at 24-h intervals.

2.5.3. Group III: AM251 (1 mg/kg)

1 mg/kg/day AM251 was administered i.p. to HFD-fed diabetic mice from day 11 to day 30, and 150 mg/kg ISO s.c. was given concurrently on days 28 and 29 at 24-h intervals.

2.5.4. Group IV: AM251 (3 mg/kg)

3 mg/kg/day AM251 was administered i.p. to HFD-fed diabetic mice from day 11 to day 30, and 150 mg/kg ISO s.c. was given concurrently on days 28 and 29 at 24-h intervals.

2.5.5. Group V: ODA (10 mg/kg)

10 mg/kg/day ODA was administered i.p to HFD-fed diabetic mice from day 11 to day 30, and 150 mg/kg ISO s.c. was given concurrently on days 28 and 29 at 24-h intervals.

2.5.6. Group VI: AM251 (3 mg/kg) + ODA (10 mg/kg)

3 mg/kg/day AM251 was administered i.p. to HFD-fed diabetic mice 30 min before 10 mg/kg/day ODA i.p. from day 11 to day 30, and 150 mg/kg ISO s.c. was given concurrently on days 28 and 29 at 24-h intervals.

Variations in body weight and energy consumption were observed throughout the experiment, and the experimental animals' mortality rate was subsequently reported.

2.6. Assessment of hemodynamic parameters

A hemodynamic evaluation was performed employing the process outlined in our previous investigation [17]. The mice were positioned supine on a wooden board. Skin electrodes (three lead electrodes) were used to record an electrocardiogram (ECG), continuously, the two electrodes were placed toward the heart on the right and left fore limb and the third electrode as neutral was placed in the hind limbs. The electrodes were interfaced with a data acquisition system.

2.7. Biochemical evaluation

A homogenate of heart tissue was prepared in pH 7.4 phosphate buffer, and CK-MB and LDH were estimated using the aliquot amount of homogenate as per the manufacturer's instructions using a microplate. Serum samples were simultaneously used to determine the fasting blood glucose level, liver makers (SGPT, SGOT), and lipid profile (TC, TG, LDL-C, HDL-C).

2.8. Oxidative stress estimation

The levels of glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were analyzed for the assessment of oxidative stress [15,17].

2.9. Estimation of proinflammatory cytokines and NF-kB

A manufacturer's guidelines were implemented in order to quantify the activity of IL-1β, TNF-α, IL-6, and NF-kB using an ELISA kit. An H1M Multimode Microplate Reader, manufactured by BioTek, India, was used to detect the intensity of color.

2.10. Microscopic examination

A microtome was used to cut the cardiac tissue into serial slices. The sections were stained with hematoxylin and eosin and assessed under a microscope. Motic Image 2.0 was used to take digital pictures of the stained tissue.

2.11. Statistical evaluation

Each group's data were shown as mean ± standard error mean (SEM). Analysis of variance (ANOVA) followed by post hoc Bonferroni multiple comparison test was performed for statistical analysis by using Graph Pad Prism software (Version 8.4.2). A statistically significant result was defined as a p-value of less than 0.05 ($p < 0.05$). A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Effect of AM251 on body weight and energy consumption

All groups of mice except the control offered with HFD showed a significant body weight gain till day 7. However, after STZ administration, they started losing body weight till day 10. Various treatments were given to the animals from day 11. Up to day 30 of the experiment, the body weights of disease-control animals were significantly lower than the normal control. Further, as compared to disease control, a significant body weight gain was observed in groups treated with AM251. Interestingly, co-administration of AM251 with ODA showed significant weight gain as compared to ODA per se treated animals (Fig. 1A).

Additionally, thirty days' calories intake was evaluated in all the groups. The energy intake of disease-control animals ($p < 0.01$) was significantly lower than the normal control. Treatment with AM251 showed a significant increase ($p < 0.01$) in energy intake as compared to disease control. However, AM251 with ODA treatment increased total Kcal consumption (Fig. 1B).

3.2. Effect of AM251 on electrocardiogram

ISO-injected diabetic mice showed ST-segment elevation ($p < 0.001$) when compared with the ECG waveforms in normal mice, which indicates the induction of myocardial necrosis. On the other hand, ST-segment elevation was normalized by AM251 ($p < 0.001$) as compared to that in disease-control animals. However, AM251 significantly attenuated ($p < 0.001$) the ST-segment elevating effect of ODA. This might be attributed to the blockade of CB1 receptors, which is responsible for the cardioprotective effects (Fig. 2).

3.3. Effect of AM251 on the heart weight-to-body weight ratio, atherogenic index (AI), and cardiac risk ratio (CRR)

Compared to the normal group of animals, disease-control animals showed a considerably higher heart weight to body weight ratio ($p < 0.001$). The cardiac weight to body weight ratio was drastically normalized after treatment with AM251 ($p < 0.001$). However compared to the ODA per se treated groups, AM251 considerably reduced the effect of ODA, as shown by the lowered heart weight to body weight ratio ($p < 0.001$) (Fig. 3A).

Compared to normal groups, disease-control animals exhibited substantially higher levels of AI and CRR ($p < 0.001$). Comparing AM251-treated mice to disease-control mice, the AI and CRR remained substantially decreased. The concomitant pre-treatment of AM251 significantly reduced the AI and CRR ($p < 0.001$) below the level of ODA-treated animals. The attenuation of the effect of ODA by AM251 indicates that CB1R blockade might be associated with cardioprotection (Fig. 3B and C).

3.4. Effect of AM251 on blood glucose levels and lipid profile

Compared to normal mice, the disease-control mice showed a significantly higher glucose level ($p < 0.001$). When compared to the disease control mice, AM251 treatment (3 mg/kg) significantly lowered the blood glucose level ($p < 0.001$). Nonetheless, AM251 drastically decreased blood glucose levels in mice ($p < 0.001$) as compared to ODA per setreated mice, suggesting that AM251

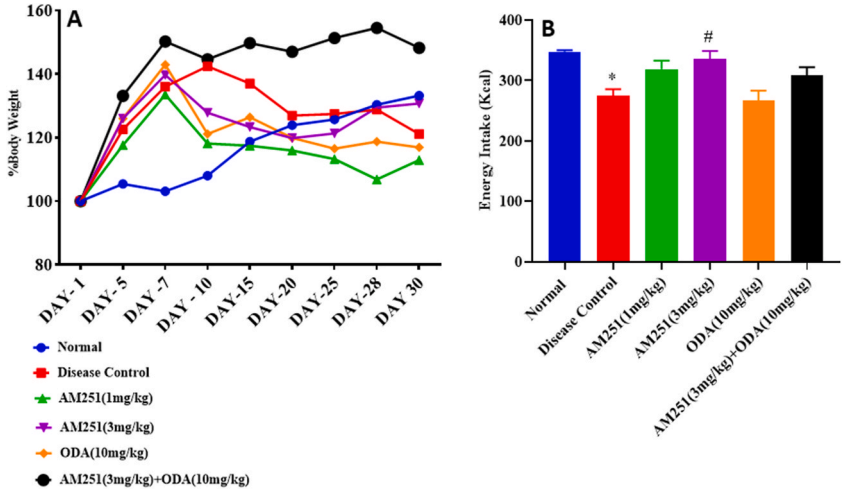

Fig. 1. The effect of CB1 receptor (CB1R) modulators on percentage change in body weight (A) and total energy intake (B) in diabetic mice with myocardial infarction. The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. The data are expressed as mean ± SEM, and analyzed by two-way analysis of variance (ANOVA) and one-way analysis of variance (ANOVA) respectively, followed by post hoc Bonferroni's multiple comparisons test. *$p < 0.001$ vs. normal; #$p < 0.001$ vs. HFD + STZ + ISO. HFD, High-fat diet, ISO, Isoproterenol; ODA, Oleamide; STZ, Streptozotocin.

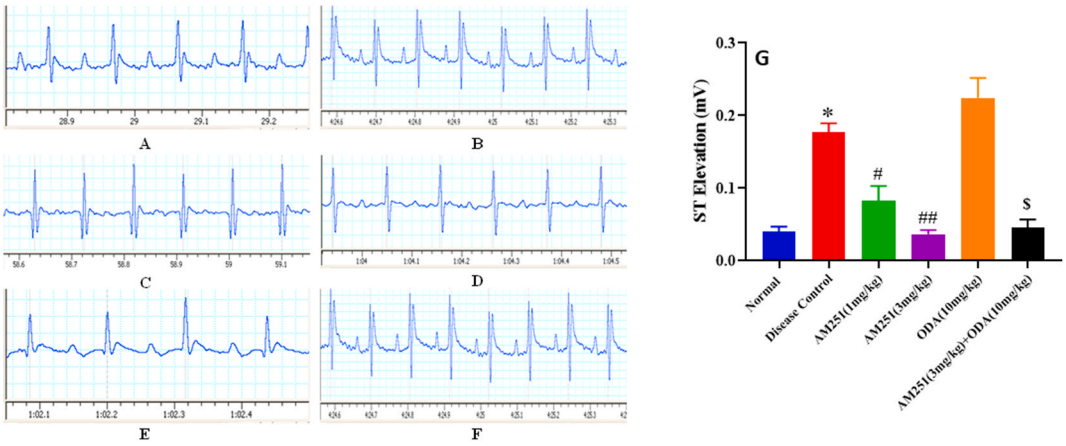

Fig. 2. The effect of CB1 receptor (CB1R) modulators on ECG (ST elevation) in diabetic mice with myocardial infarction. The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. Normal (A), Disease control (B), AM251 (1 mg/kg) (C), AM251 (3 mg/kg) (D), AM251 (3 mg/kg) + ODA (10 mg/kg) (E), ODA (10 mg/kg) (F), groups representing ST-segment elevation (G). The data are expressed as mean ± SEM, and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparisons test. *p < 0.001 vs. normal; #p < 0.01, ##p < 0.001 vs. disease control (HFD + STZ + ISO); $p < 0.001 vs ODA. HFD, High-fat diet; ISO, Isoproterenol; ODA, Oleamide; STZ, Streptozotocin.

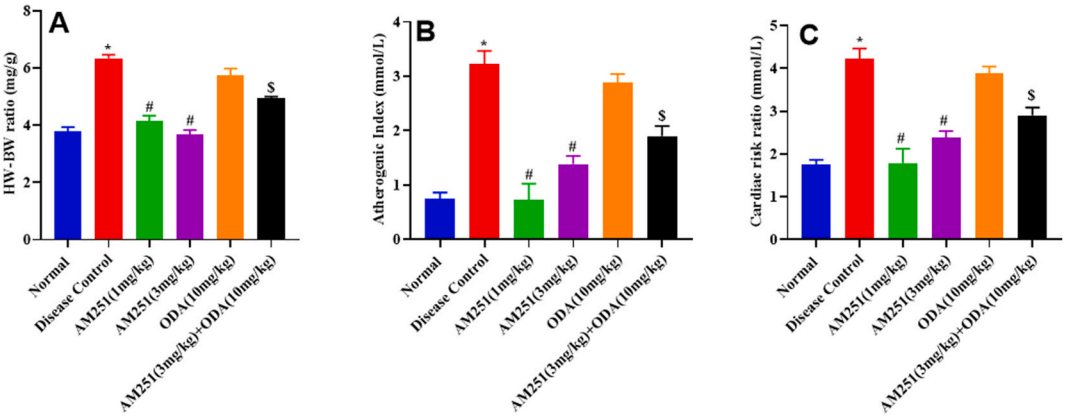

Fig. 3. The effect of CB1 receptor (CB1R) modulators on the heart weight-to-body weight ratio (A), AI (B), and CRR (C) in diabetic mice with myocardial infarction. The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. The data are expressed as mean ± SEM, and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparisons test. *p < 0.001 vs. normal; #p < 0.001 vs. HFD + STZ + ISO; $p < 0.001 vs. ODA. AI, Atherogenic index; CRR, cardiac risk ratio; HFD, High-Fat Diet; ISO, Isoproterenol; ODA, oleamide; STZ, Streptozotocin.

possesses anti-diabetic properties (Fig. 4A).

A high-calorie diet was consumed, which altered the lipid profile and caused the disease-control mice's lipid level to be higher than that of the normal group animals ($p < 0.001$). Conversely, AM251 (1 or 3 mg/kg) markedly restored the lipid profile, including TC, TG, and LDL-C, along with increasing HDL-C. Nevertheless, the level of TC, TG, and LDL-C was substantially decreased ($p > 0.05$) by prior AM251 administration in combination with ODA (Fig. 4B, C, and D) and raised the HDL-C level significantly ($p < 0.001$) in comparison to the mice treated with ODA per se (Fig. 4E).

3.5. Effect of AM251 on cardiac injury markers

As expected, the level of myocardial injury markers like CK-MB and LDH significantly loweres in the cardiac tissue in the disease control group mice ($p < 0.001$) indicating severe damage to the myocardium. Likewise, treatment with AM251 substantially raised ($p < 0.001$) the amount of CK-MB and LDH ($p < 0.01$), suggesting that the integrity of the cardiac membrane was maintained. In addition, pre-treatment of AM251 significantly increased the contents of CK-MB ($p < 0.001$) and slightly increased the levels of LDH ($p > 0.05$) in

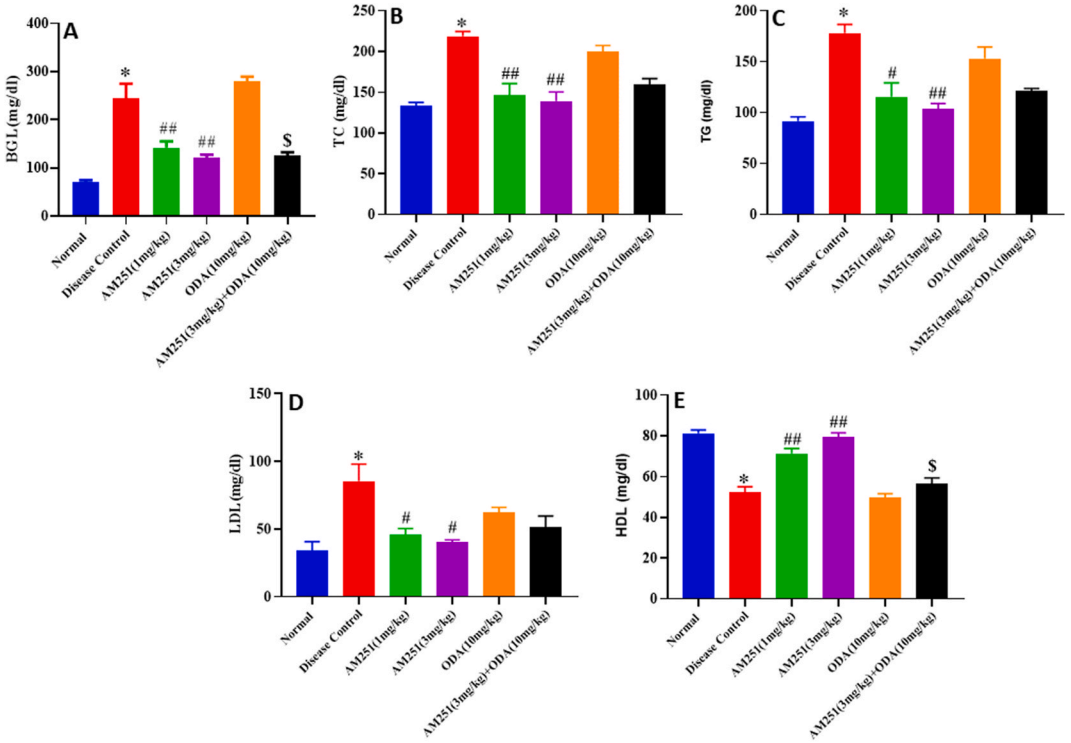

Fig. 4. The effect of CB1 receptor (CB1R) modulators on BGL and lipid profile in diabetic mice with myocardial infarction. The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. The data are expressed as mean ± SEM, and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparisons test. *p < 0.001 vs. normal; #p < 0.01, ##p < 0.001 vs. HFD + STZ + ISO; $p < 0.001 vs. ODA.; BGL, Blood glucose level; HDL-C, High-density Lipoprotein-Cholesterol, HFD, High-fat diet; ISO, Isoproterenol; LDL-C, Low-density Lipoprotein-Cholesterol; ODA, Oleamide; STZ, Streptozotocin; TC, Total cholesterol; TG, Triglycerides.

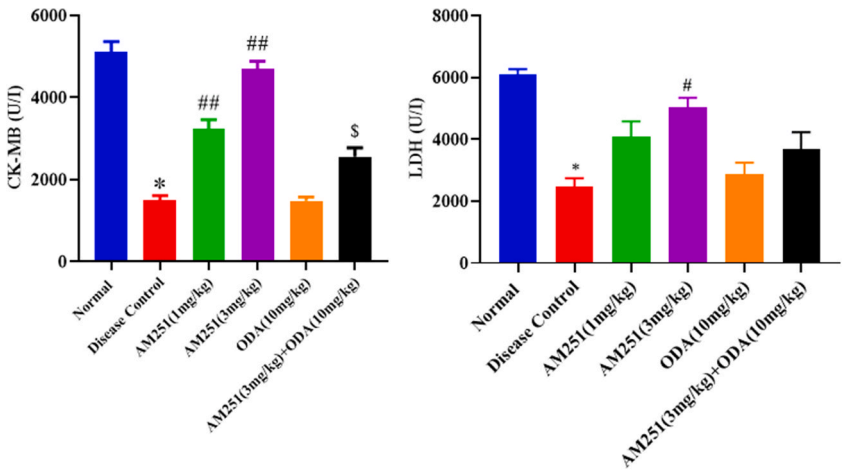

Fig. 5. The effect of CB1 receptor (CB1R) modulators on cardiac injury markers in diabetic mice with myocardial infarction. CK-MB (A), and LDH (B). The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. The data are expressed as mean ± SEM, and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparisons test. *p < 0.001 vs. normal; #p < 0.01, ##p < 0.001 vs. HFD + STZ + ISO; $p < 0.001 vs. ODA.; CK-MB, Creatine kinase-MB; HFD, High-fat diet; ISO, Isoproterenol; LDH, Lactate dehydrogenase; ODA, Oleamide; STZ, Streptozotocin.

the cardiac tissue of the ODA-administered animals (Fig. 5).

3.6. Effect of AM251 on liver injury markers

In disease-control mice, significantly increased ($p < 0.001$) levels of serum SGOT and SGPT were observed as compared to normal. Interestingly, treatment with AM251 significantly reduced the level of serum transaminase as compared to the disease-control mice ($p < 0.001$). Moreover, the prior administration of AM251 significantly increased ($p < 0.001$) the enzyme level as compared to the ODA per setreated group, which indicates AM251 has hepatoprotective effects (Fig. 6A and B).

3.7. Effect of AM251 on oxidative stress

The disease control group of mice showed indications of myocardial injury, including increased LPO (elevated levels of MDA) and a significant ($p < 0.001$) reduction in the contents of GSH, CAT, and SOD. Compared to the disease-control animals, treatment with AM251 significantly prevented the exhaustion of GSH, SOD, and CAT and decreased MDA production in the myocardium ($p < 0.001$). It is interesting to note that in ODA-treated mice, prior AM251 administration raised the contents of GSH ($p < 0.01$), CAT ($p > 0.05$), and SOD ($p < 0.001$) and marginally decreased the levels of MDA ($p > 0.05$). It further suggests that there is a cardioprotective influence of the blocked CB1R (Fig. 7A, B, C, and D).

3.8. Effect of AM251 on NF-kB signaling pathway and pro-inflammatory cytokines

When NF-kB-p65 was detected in mice cardiac tissue, the findings indicated that the disease-control mice showed significantly higher levels of NF-kB-p65. When compared to disease-control animals, treatment with AM251 drastically lowered the expression of NF-kB-p65 (Fig. 8D). Furthermore, the disease-control animals had considerably higher levels ($p < 0.001$) of pro-inflammatory cytokines such TNF-α, IL-1β, and IL-6. AM251 treatment resulted in a substantial decline in TNF-α ($p < 0.01$), IL-1β, and IL-6 ($p < 0.001$) expression. Interestingly, in ODA-treated mice, AM251 prior to treatment lowered the levels of TNF-α ($p > 0.05$), IL-1β, and IL-6 ($p < 0.001$) (Fig. 8A, B, and C).

3.9. Effect of AM251 on the histopathology of heart tissue

Histopathological modifications in the heart tissue were caught in light microscopy (Fig. 9). The control group (A) showed normal myocardial structure. On another hand, the disease control mice (B) showed myocardial necrosis with neutrophilic infiltration as well as extravasation of red blood cells. AM251 (1 mg/kg) group (C) showed mild myocardial injury and edema. AM251 (3 mg/kg) group (D) showed a reduction in necrosis and toxicity. Simultaneously, the concomitant prior administration of AM251 (3 mg/kg) (E) significantly reduced the myocardial necrosis with mild edema, evidently indicating inhibition of CB1R showed cardioprotective activity. However, ODA-administered group (F) mice showed cardiac damage as well as moderate edema, neutrophilic infiltration, and extravasation of red blood cells.

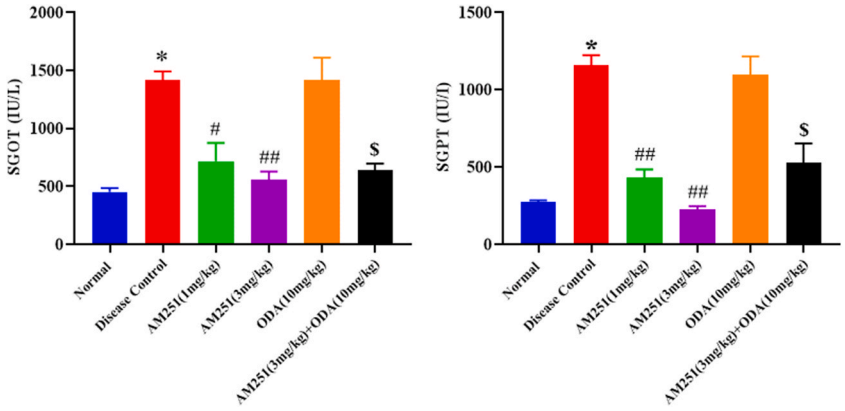

Fig. 6. The effect of CB1 receptor (CB1R) modulators on liver injury markers such as SGOT (A); and SGPT (B) in diabetic mice with myocardial infarction. The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. The data are expressed as mean ± SEM, and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparisons test. *p < 0.001 vs. normal; #p < 0.01, ##p < 0.001 vs. HFD + STZ + ISO; $p < 0.001 vs. ODA. HFD, High-fat diet; ISO, Isoproterenol; ODA, oleamide; SGOT, Serum glutamic-oxalacetic transaminase; SGPT, Serum glutamic pyruvic transaminase; STZ, Streptozotocin.

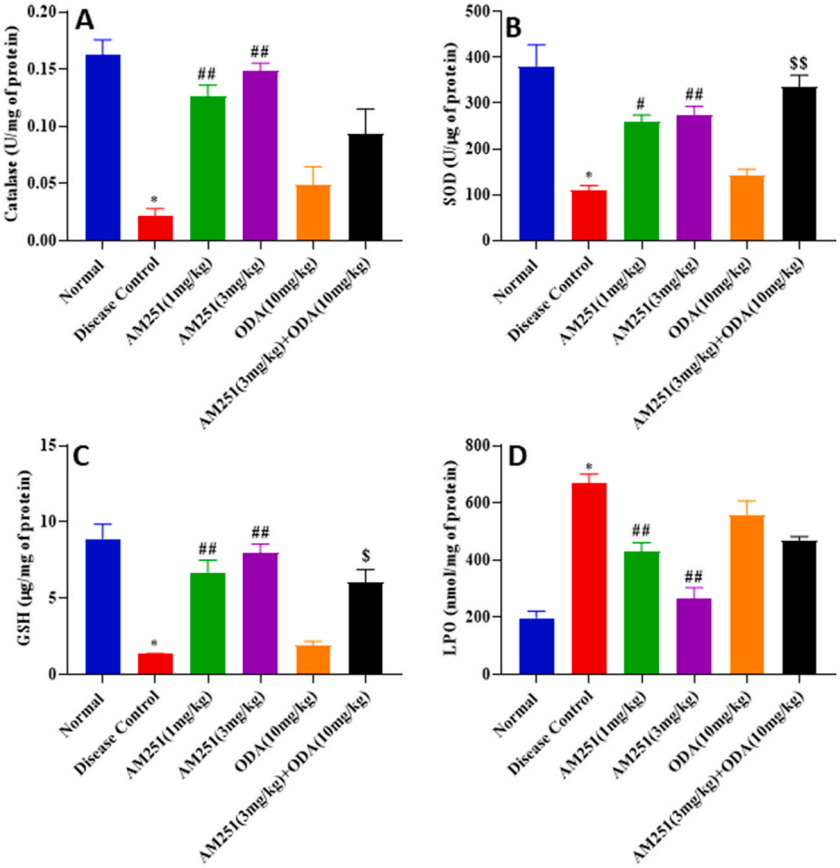

Fig. 7. The effect of CB1 receptor (CB1R) modulators on antioxidants and oxidative stress markers in diabetic mice with myocardial infarction. The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. The data are expressed as mean ± SEM, and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparisons test. *$p < 0.001$ vs. normal; #$p < 0.01$, ##$p < 0.001$ vs. HFD + STZ + ISO; \$$p < 0.01$, \$\$$p < 0.001$, vs. ODA. GSH, Reduced glutathione; HFD, High-fat diet; ISO, Isoproterenol; LPO, Lipid peroxidation; ODA; oleamide; SOD, Superoxide dismutase; STZ, Streptozotocin.

4. Discussion

The result of the present study provides evidence of the cardioprotective potential of AM251 in diabetic conditions in experimental mice. AM251 refilled the endogenous antioxidants mechanism, improved cardiac functionality as well as showed an anti-inflammatory mechanism in myocardial cells. Furthermore, AM251 exhibited multiple activities such as control of blood sugar level, lipids levels (LDL, HDL, TG, and TC), changes in hemodynamic pattern, and preserving myofibril and morphology. The accumulation of excess body fats leads to metabolic disorders such as obesity, T2DM, atherosclerosis, etc. [18]. We utilized HFD for abdominal obesity, and STZ treatment to stimulate diabetic conditions in animals, similar to in humans [19–21]. Recently reports indicate that the blockade of the CB1R improves insulin resistance and shows an anti-hyperlipidemic effect [8,22]. Significant abdominal obesity was observed in HFD-fed mice as compared to chow diet animals, although AM251 significantly maintained the body weight of mice in the presence of HFD (Fig. 1A). Polyuria, polydipsia, and polyphagia are the common symptoms of diabetic patients. STZ-treated mice showed these symptoms by boosting the intake of a high-calorie diet throughout the study protocol (Fig. 1B) [23,24].

In the myocardium of the ischemic heart, CB1R expression is up-regulated, while down-regulation of CB2R expression occurs, which represents the inverse functional association between two receptor systems in the ischemic condition [12]. In obese mice, the mRNA expression of CB1R was increased in the myocardium, endothelial cells, vascular smooth muscles, and fibroblasts. This change was attributed to the increased contents of 2-arachidonoylglycerol and anandamide (myocardial endocannabinoids), suggesting ligand-dependent stimulation of CB1R signaling. The subsequent increase in arachidonic acid concentration in cardiac tissue indicates that elevated metabolic turnover of endocannabinoids might trigger a proinflammatory environment [25]. Endocannabinoids may promote apoptosis, proinflammatory activity, oxidative stress, and cardiac dysfunction via myocardial CB1R [11,12]. Based on this evidence it is possible that the up-regulation of myocardial CB1R might be causally linked to obesity-associated cardiomyopathy. These data are further supported by clinical pieces of evidence. In overweight people, myocardial dysfunctions are associated with the elevated plasma level of endocannabinoids and up-regulation of CB1R, which may cause cellular dysfunctions and myocardial necrosis

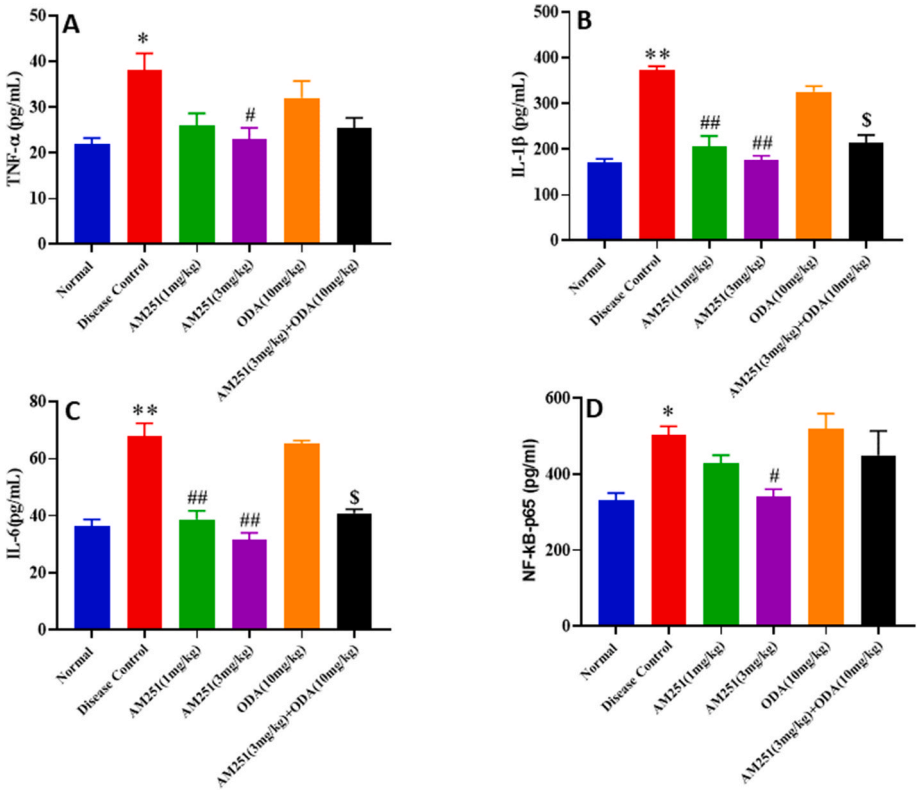

Fig. 8. The effect of CB1 receptor (CB1R) modulators on the level of proinflammatory cytokines and NF-kB-p65 in diabetic mice with myocardial infarction. The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. The data are expressed as mean ± SEM, and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparisons test. *p < 0.01, **p < 0.001 vs. normal; #p < 0.01, ##p < 0.001 vs. HFD + STZ + ISO; $p < 0.001 vs ODA. HFD, High-fat diet; IL-1β, Interleukin-1 beta; IL-6, Interleukin-6; ISO, Isoproterenol; NF-kB-p65, Nuclear factor-kappa B-p65; ODA, oleamide; STZ, Streptozotocin; TNF-α, Tumor necrosis factor-alpha.

[12]. It is interesting to note that the blockade of CB1R leads to antifibrotic and antiatherogenic activity which provides a beneficial effect on cardiomyocytes [13,25]. This suggests that the inhibition of the CB1R-mediated signaling might serve as a protective approach for the cardiomyocytes in ischemic conditions. In the present study, ISO disturbed ECG waveform by elevating the ST height, which indicates damage to the myocardium. AM251 successfully normalized the ST height leads proper waveforms of ECG by blockade of CB1R (Fig. 2). Since CK-MB and LDH are both expressed in cardiomyocytes, injury to the cardiac muscles results in their release into the bloodstream, raising their plasma levels. This eventually caused declined contents of CK-MB and LDH in the myocardial tissue suggesting a myocardial injury. Treatment with AM251 showed significantly higher contents of LDH and CK-MB in the myocardium as compared to the disease control mice (Fig. 5). Lower cardiac output during heart diseases reduces the hepatic blood flow and can results in the hepatic central necrosis, which raises the plasma levels of liver transaminases [26]. In the present study, raised level of SGPT and SGOT was observed indicating myocardial inefficiency. Similar abnormalities were also observed in our previous studies [2]. Treatment with AM251 showed significantly lower contents of these liver enzymes as compared to the disease control animals (Fig. 6A and B). Viewed collectively, the above data suggest that metabolic abnormalities caused increased CB1R expression in the myocardium, and contributes to the myocardial damage. These CB1R-mediated pathological changes can be effectively attenuated by AM251 to exhibit cardioprotective action.

While CB1R is present in pancreatic β cells of humans and rodents, its blockade improves the secretion of insulin and the metabolism of glucose [27]. AM251 profoundly reduced circulating glucose levels by increasing pancreatic β cell function and systemic insulin secretion, which is accountable for a significant reduction in the levels of glucose (Fig. 4A). However, the levels of TG, LDL-C, and TC are significantly higher and drastically reduced the level of HDL-C after feeding an HFD [28], and AM251 significantly restored the level of different lipids in the present study (Fig. 4B, C, D, and E). Additionally, AI and CRR striking in HFD-fed myocardial infarcted diabetic mice indicate abnormal traveling of lipids into the bloodstream. We observed that AM251 administration significantly suppressed the level of LDL-C and induced the level of HDL-C by maintaining the AI and CRR (Fig. 3B and C). These data suggest that AM251 has the potential to rectify the metabolic abnormalities that may lead to coronary artery diseases.

In the metabolic disorders pathogenesis, lipid peroxidation and oxidative stress are the common factors [29]. ISO administration causes imbalanced redox status and leads to the formation of highly toxic free radicals. Reactive oxygen species react with

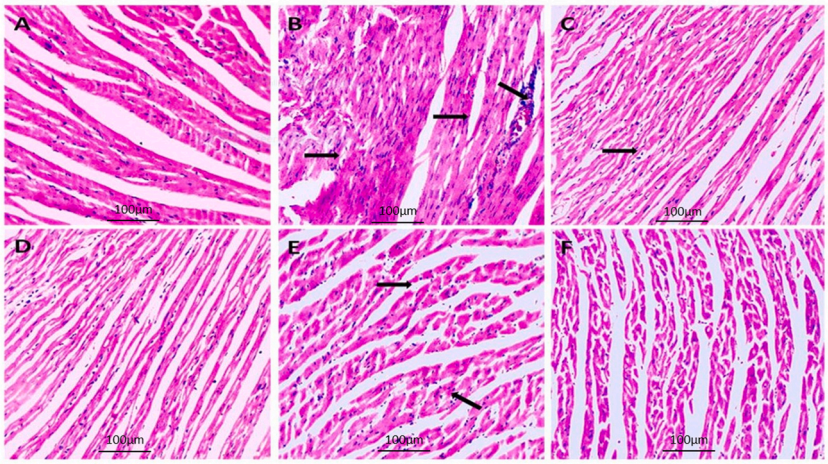

Fig. 9. The effect of CB1 receptor (CB1R) modulators on the heart tissue in diabetic mice with myocardial infarction. The disease condition in different groups of animals was induced by HFD + STZ + ISO. These animals were administered with AM251 (CB1R antagonist) and ODA (CB1R agonist) alone or in combination. A group of healthy chow-fed mice was kept as a normal control. Light micrograph of the normal group showing normal myocardial architecture (A). Disease control mice hearts showing myocardial necrosis, neutrophilic infiltration, and extravasation of red blood cells (B). AM251 (1 mg/kg) shows less myocardial injury, less edema, and inflammatory cells (C). AM251 (3 mg/kg) mice heart tissue showing mild edema without any signs of infarction. The architecture of myocardial fibers is normal (D). ODA (10 mg/kg) showing myocardial injury, edema, neutrophilic infiltration, and extravasation of red blood cells (E). AM251 (3 mg) + ODA (10 mg/kg) showing no signs of myocardial damage with mild edema (F). HFD, High-fat diet; ISO, Isoproterenol; ODA, oleamide; STZ, Streptozotocin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

polyunsaturated fatty acid (PUFA) forming MDA by lipid peroxidation [14,30]. We found that ISO treatment significantly induced lipid peroxidation and downgrade the levels of antioxidant defenses like SOD, CAT, and GSH. Elevated levels of MDA indicate oxidative injury to the cell membrane lipids. Herein, we observed that AM251 significantly protects the myocardium by depleting the level of MDA by managing free radicals. AM251 also refilled antioxidant defense mechanisms such as SOD, CAT, and GSH indicating cardioprotection (Fig. 7A, B, C, and D).

Additionally, several studies have indicated that the CB1R contributes to raised oxidative stress and inflammation, which are related to endothelial dysfunction, diabetic retinopathy, and cardiomyopathy [31,32]. Some findings reported that the Mitogen-activated protein kinases (MAPKs) pathway plays an important role in the activation of CB1R, which causes myocardial stress, cell death, and inflammation in the coronary artery [33,34]. Interestingly, Wei et al., 2022 found that THC activation of CB1 causes oxidative stress and inflammation via NF-kB, TNF-α, and MAP kinase pathways which is significantly attenuated by genistein, a neutral CB1R antagonist [35]. Furthermore, preclinical findings have confirmed that DAMPs produced because of cardiac damage stimulate the TLR4 signaling pathway. Also, some research has proved that TLR4 initiates inflammatory responses through the activation of MAPKs and NF-kB signaling cascades [36,37]. In an unstimulated stimulus, in the cytoplasm the NF-kB is under the custody of IkB, nevertheless, in stressed conditions, IkB degrades by inhibitor-kB (IkB) kinase (IKKB) and thus NF-kB translocated to the nucleus to trigger IL-6, TNF-α, and IL-1β (inflammatory mediators) [38]. Here, we found that the upregulated expression of NF-kB and higher levels of inflammatory mediators due to the activation of CB1R, AM251 significantly attenuate the oxidative stress as well as inflammation in the myocardium by suppressing NF-kB and cytokines level in ISO-induced MI in diabetic mice (Fig. 8A, B, C, and D).

The depletion in heart-to-body weight ratio is one more indication of myocardial injury induced by ISO, we noticed an equivalent loss in the weight ratio [39]. Taken together, the data suggest that AM251 protects the myocardium by alleviating oxidative stress and suppressing the production of pro-inflammatory cytokines (Fig. 3A).

The cardioprotective action of AM251 against ISO-induced MI in diabetic mice was further investigated by the histopathological examination. Histopathological findings of the normal heart indicate normal morphology of cardiac tissue with no sign of infarction. On the other hand, myocardial necrosis with neutrophilic infiltration and extravasation of red blood cells were observed in ISO-treated diabetic mice. This indicates ISO treatment induced myocardial injury in the heart. AM251 treatment preserved myofibril and reduced signs of necrosis with mild edema (Fig. 9). Therefore, the antagonistic activity of AM251 on CB1R resulted in the cardioprotective action against ISO-induced MI in diabetic mice.

5. Conclusion

The present investigation, for the first time, reported the cardio-protective activity of AM251 against β-receptor-stimulated MI in diabetic mice via inhibition of the NF-kB signaling. The data confirmed that CB1R blockade suppressed oxidative stress and inflammatory changes, and regulates cardiac and other metabolic markers in MI.

Data availability

Upon reasonable request, the corresponding author will provide the data supporting the findings of this study.

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Institutional review board statement

Institutional Animal Ethics Committee approved this research protocol (Approval No. IAEC/CPCSEA/RCPIPER/2018-19/11) at R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, Maharashtra, India.

CRediT authorship contribution statement

Harshal D. Pawar: Methodology, Investigation, Conceptualization. **Yugandhara Patil:** Writing – original draft, Methodology, Investigation, Conceptualization. **Ashwani Patil:** Writing – original draft, Methodology, Investigation, Conceptualization. **Kartik T. Nakhate:** Writing – review & editing. **Yogeeta O. Agrawal:** Writing – review & editing. **Kapil Suchal:** Writing – review & editing. **Shreesh Ojha:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Sameer N. Goyal:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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