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Research Article

Silver Nanoparticles Of Tetrahydrocurcumin Attenuate The Oxidative Stress And Mitochondrial Mediated Apoptosis In Isoproterenol Induced Toxicity

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ABSTRACT

Cardiovascular diseases (CVDs) is usually accompanying with a build-up of fatty deposits inside the arteries (atherosclerosis) and an enhanced risk of blood clots. It is mainly caused due to unhealthy diet and lack of physical activities. The recent studies showed that intake of healthy and antioxidant rich foods have the high ability to reduce and prevent the cardiovascular diseases (CVDs) now a days. Isoproterenol is broadly used for inducing CVDs in both in vitro and in vivo models. Isoproterenol is a nonselective beta-adrenergic agonist. In this study it is used as the inducer to induce CVDs in H9C2 cell line. It causes elevated level of apoptosis, mitochondrial membrane depolarization and ROS formation. The result of the study indicated that the exposure of silver nanoparticles of Tetrahydrocurcumin (THC-AgNPs) has ameliorate the, isoproterenol induced mitochondrial dysfunction, ROS formation and apoptosis due to its antioxidant properties.

INTRODUCTION

Cardiovascular diseases (CVDs) is the world's leading cause of death and reason for morbidity in middle aged peoples. The most common form of CVD is myocardial infarction (MI), whose pathophysiology is centred on the loss of cardiomyocytes as a result of apoptosis [1]. Research indicates that cardiovascular diseases (CVDs) cause 17.9 million deaths annually, or 31% of all fatalities globally. Apoptosis is preceded by lipid peroxidation, DNA damage, structural protein change, and the depletion of intracellular antioxidants caused by reactive oxygen species (ROS). Consequently, one of the main goals of research to control the apoptotic cascade in cardiomyocytes is the lowering of

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intracellular ROS [2]. Synthetic catecholamine and β-adrenergic agonist isoproterenol (ISO) is well known for producing free radicals that induce lipid peroxidation, which in turn causes severe stress in the myocardium and may be a major role in the irreversible damage to the myocardial membrane [2]. Myocardial hyperactivity, hypoxia and coronary hypotension [3], calcium overload, and infract-like necrosis [4] are the consequences. When ISO is administered, the heart rate increases, which raises the need for oxygen, increases the calcium burden, and causes accumulation. In addition, the myocardium's shape and membrane integrity are altered, and the myocardial cells' cAMP levels are boosted [3]. Isoproterenol-induced myocardial infarction is a simple and non-invasive model that is frequently used in experiments to examine the beneficial effects of various medications and cardiac function [2–5]. It is comparable to the pathophysiological alterations seen in myocardial infarction in humans. The search of innovative strategies for the treatment of cardiovascular disease continues to be a top priority for worldwide research. The need for novel medications that may prevent myocardial damage is critical. There could be a significant therapeutic window for the treatment of heart conditions with medicinal herbs. Combining antioxidants can maximize their effectiveness. They are capable of carrying out a large range of metabolic processes, scavenging free radicals, and prophylactic functions. Numerous studies have demonstrated the synergistic effects of vitamin combinations with other antioxidants [6–9]. More antioxidant and ROS scavenging activity is seen in foods high in polyphenols. Turmeric, a dried, orange-yellow-coloured rhizome derived from Curcuma longa L., belongs to the ginger family (Zingiberoside) and is used as a spice and food additive. The biological and pharmacological characteristics of tetrahydro curcumin are more efficient than those of curcumin. Its excellent antioxidant capacity has been reported as the cause of its effectiveness as a chemo preventive agent against a range of ailments, such as diabetes, hypertension, atherosclerosis, neurotoxicity, cardiovascular disease, hepatotoxicity, and liver fibrosis [10]. Silver nanoparticles possess anti-inflammatory and anti-cancer properties, and conjugated silver nanoparticles are essential for medication transport to many organs. Despite silver's interesting material features and abundance and low cost, the usage of silver-based nanomaterials has been limited due to their instability, e.g., potential for oxidation in an oxygen-containing fluid. The goal of the current investigation was to determine if AgNPs-THC could protect the oxidant-antioxidant imbalance, mitochondrial dysfunction and apoptosis in ISO induced cellular (H9C2) model of MI.

MATERIALS & METHODS

Cell lines and culture medium

Chemicals.

Isoproterenol, tetrahydrocurcumin, thiobarbituric acid (TBA), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-diacetyl dichlorofluorescein (DCFH-DA), rhodamine 123 (Rh-123), heat-inactivated fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), glutamine, penicillin-streptomycin, EDTA, and trypsin were purchased from Sigma Chemicals Co., St. Louis, USA.

Cell culture

H9C2 cell lines were procured from the National Centre for Cell Science, Pune, India. Cells were grown in (DMEM) 12 (1 : 1), supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO2 at 37oC until confluent. Cells were maintained at 37∘C in

CO2 incubator in a saturated humidity atmosphere containing 5 % CO2.

MTT assay

The viability of cells treated with various concentrations of Isoproterenol, and/or tetrahydro curcumin was determined by the MTT assay based on the detection of mitochondrial dehydrogenase activity in the live cells [11]. MTT was added to each well, and the plates were incubated at 37°C for 4 h. later, the cells were centrifuged for 10 min, and the supernatant was removed, 200 µL of DMSO was added into each well, and absorbance was measured in a microplate reader (Molecular Devices, CA, USA) at 595 nm.

Mitochondrial membrane potential by Rhodamine-123 staining

The control and experimental H9C2 cells (2 × 104 cells/well) washed with PBS and then treated with fresh media mixed with Rh-123 solution (10mg/mL). Then, they were incubated in the dark for 30 mins. After washing the images were taken using a fluorescence microscope [12].

Reactive oxygen species by DCFH-DA staining

The percentage of ROS was estimated in the control, isoproterenol, and tetrahydrocurcumin - treated H9C2 cells. Briefly, an aliquot of the isolated cells 8 × 106 cells/mL was made up to a final volume of 2 mL in normal PBS (pH 7.4). Then, 1 mL aliquot of cells was taken to which 100 µL DCFH-DA (10 µM) was added and incubated at 37°C for 30 min. fluorescent measurements were made with excitation and emission filters were set at 485 ± 10 nm, and 530 ± 12.5 nm respectively (Shimadzu RF-5301 PC spectrofluorometer). All initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were expressed as percentage; increase in fluorescence was calculated using the formula [(F t30 − F t0)/(F t0 × 100)], and the fluorescence intensities at 0 and 30 min were measured [12].

Apoptosis by Acridine orange-ethidium bromide (AO-EB) staining

Dual staining method is used to analyze the apoptotic morphological changes by treating the control and experimental cells with fluorescent probes acridine orange and ethidium bromide (AO and EB) and using fluorescence microscope. After treatment schedule as described in previous experiments, medium was removed from the plates; cells (1 × 105) were washed with PBS twice and then fixed with 4% paraformaldehyde for 20 min and stained with 100 µg/mL AO and EB. These cells were incubated for 20 min at room temperature and washed with warm PBS to remove excess dye. Cellular morphology was examined using fluorescence microscopy and photographed and quantified at 535 nm in spectrofluorometer [12].

TBARS Assay

After being suspended in a solution of 130 mM KCl and 50 mM PBS containing 0.1 mL of 0.1 M dithiothreitol (DTT), H6C9 cells were centrifuged at 20,000 g for 15 minutes at 4∘ C. A sample of the supernatant was obtained for biochemical analysis. TBARS analysis was performed as previously described [13] to ascertain the degree of lipid peroxidation. It was measured how much pink chromogen 2-TBA produced when it reacted with the breakdown products of lipid peroxidation.

Assay for SOD Activity

The approach based on the inhibition of the formation of the (NADH-PMS-NBT) complex, as previously described [14], was used to measure the activity of superoxide dismutase (SOD).

Assay for Catalase Activity

As previously mentioned, the breakdown of hydrogen peroxide was used to measure the activity of catalase [15]. For one minute, a drop in absorbance at 240 nm caused by H2O2 degradation was observed.

Assay for Glutathione Peroxidase Activity

Spectrophotometric analysis was used to measure the activity of glutathione peroxidase (GPx), as previously reported [16]. In short, for a with a specific amount of enzyme preparation. After the reaction, the amount of GSH that remained was measured.

Glutathione Estimation

The procedure previously outlined was used to measure the total GSH content [17]. This procedure was based on the observation that adding 5,5-dithiobis (2-nitrobenzoic acid) to a chemical containing sulfhydryl groups caused the substance to turn yellow.

predetermined amount of time, hydrogen peroxides (H2O2) and GSH were allowed to react

Isoproterenol treatment (2.5, 5, 50, 100, and 200 nM for 24 h) of H9C2 cells induced a dose-dependent reduction in cell proliferation, with approximately 50% cell death was observed at 10 µM. THC-AgNPs dose dependently (5, 10, 20, 50, 100 and 200 µg/mL concentration) attenuated the changes in cell proliferation induced by 10 µM Isoproterenol, with approximately 85% protection following treatment with 20 µg THC-AgNPs after 24 h.

RESULT

MTT assay

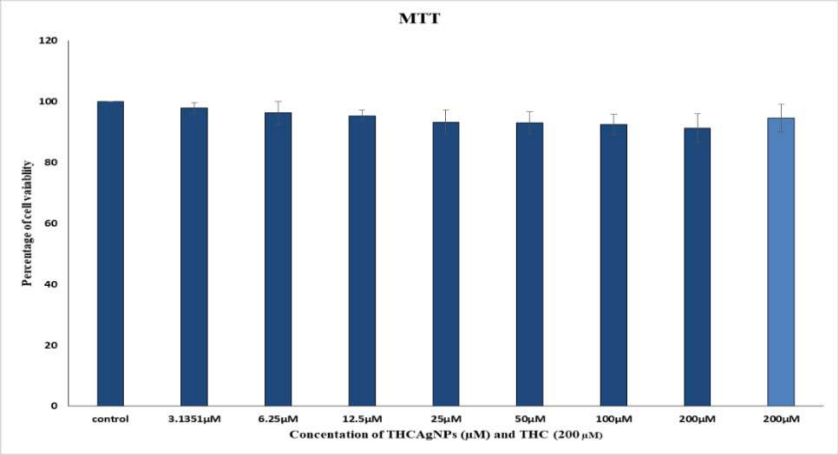


Figure 1: Effect of THCAgNps and THC on cell viability of H9C2 cells

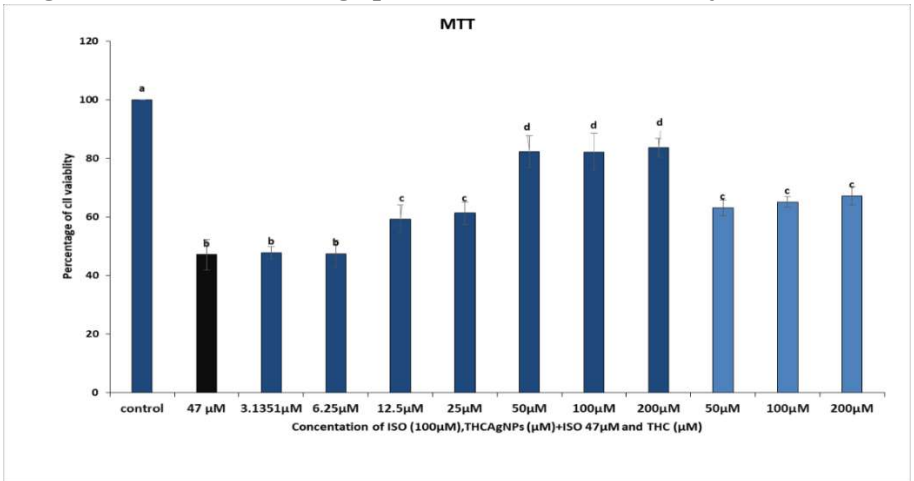
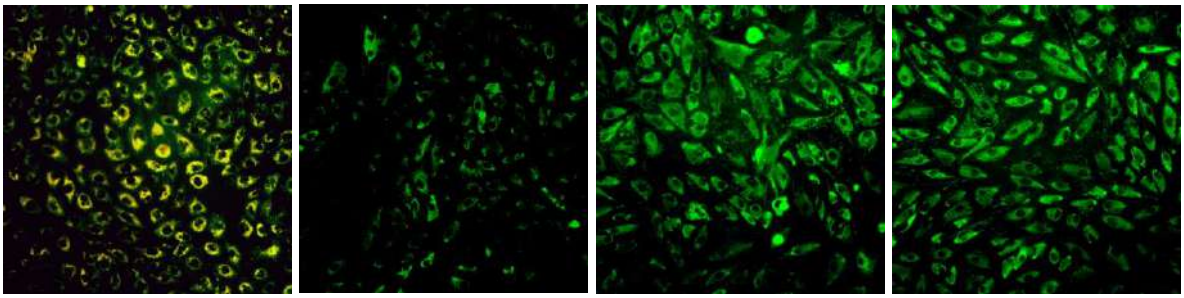


Figure 2: Effect of Isoproterenol, THCAgNps with Isoproterenol and THC with Isoproterenol on cell viability of H9C2 neuroblastoma cells

Tetrahydro curcumin inhibits MMP generation in H9C62 cells

ISO exposed cells showed a significant MMP loss as shown in Figure. Significant reduction in MMP

of ISO exposed cells were showed by the diminution of Rh-123 fluorescence. Although no alterations were found in THC-AgNPs alone exposed cells, the pre-exposure of THC-AgNPs attenuated ISO induced reduction in MMP



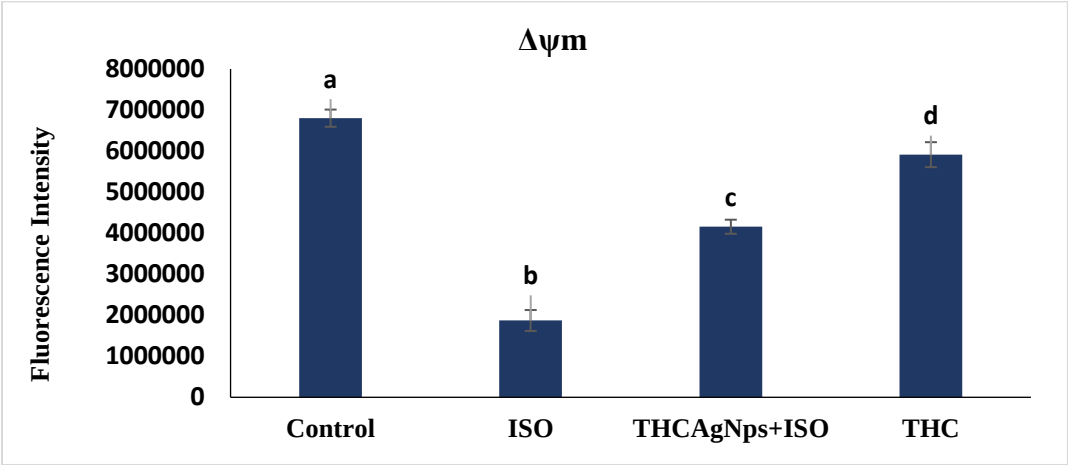
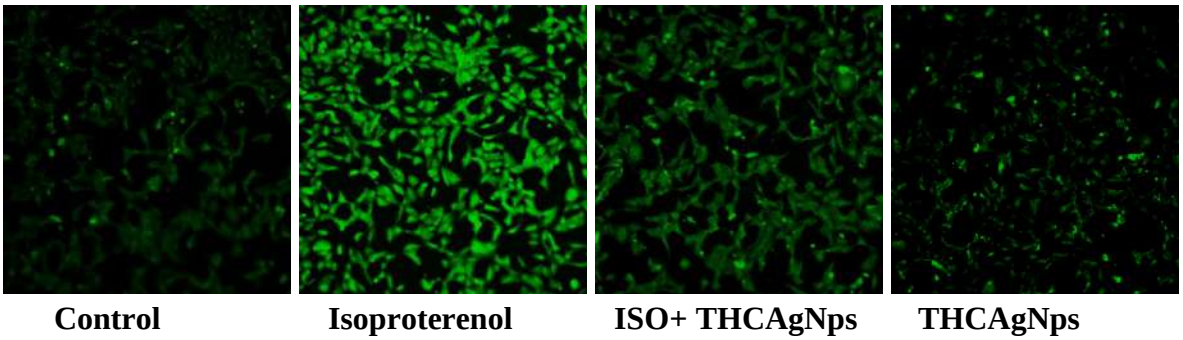


Figure 3: Effect of Tetrahydro curcumin loaded sliver nanoparticles on mitochondrial membrane potential in H9C2 cells

Tetrahydro curcumin inhibits ROS generation in H9C62 cells

The formation of intracellular ROS was measured in terms of fluorescence by DCF. Addition of isoproterenol (10 µM) to cells caused a significant increase in DCF fluorescence. Pretreatment of the cells with THC-AgNPs (20 µg) lowered isoproterenol -induced free radical release as compared to isoproterenol -treated cells alone. No significant changes in ROS formation were detected in H9C2 cells treated with THC-AgNPs alone.



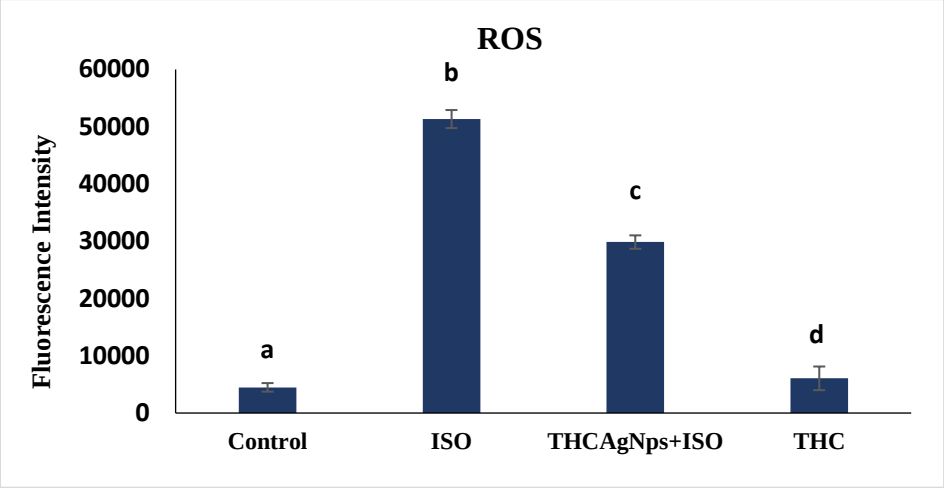
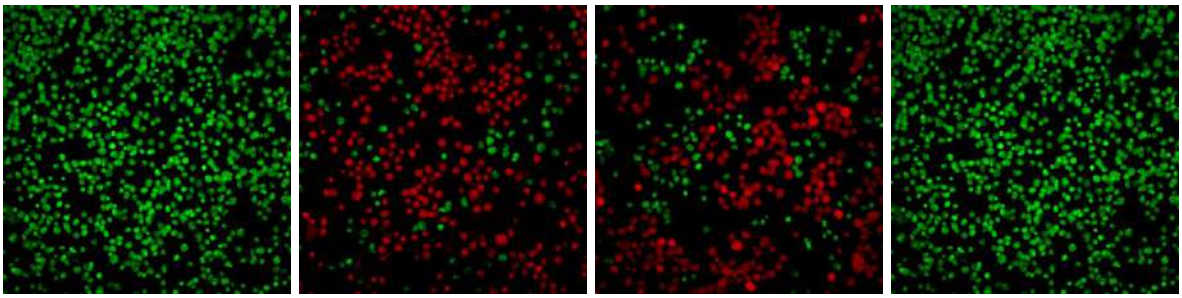


Figure 4: Effect of Tetrahydro curcumin loaded silver nanoparticles on intracellular ROS generation in H9C2 cells

Tetrahydro curcumin inhibits apoptosis generation in H9C62 cells

Dual staining of isoproterenol and THC-AgNPs treated H9C2 cells, with AO and EB, was used to determine the rate of apoptosis. Control cells which fluoresced brightly with green nuclei and normal morphology were showed in Figure. In contrast, at 10 µM ISO exposed cells revealed orange luminescent apoptotic body formation, when compared to control ($p < 0.05$), and pre-treatment with THC-AgNPs increased cell viability and decreased apoptotic cell death when compared to cells exposed merely to isoproterenol.



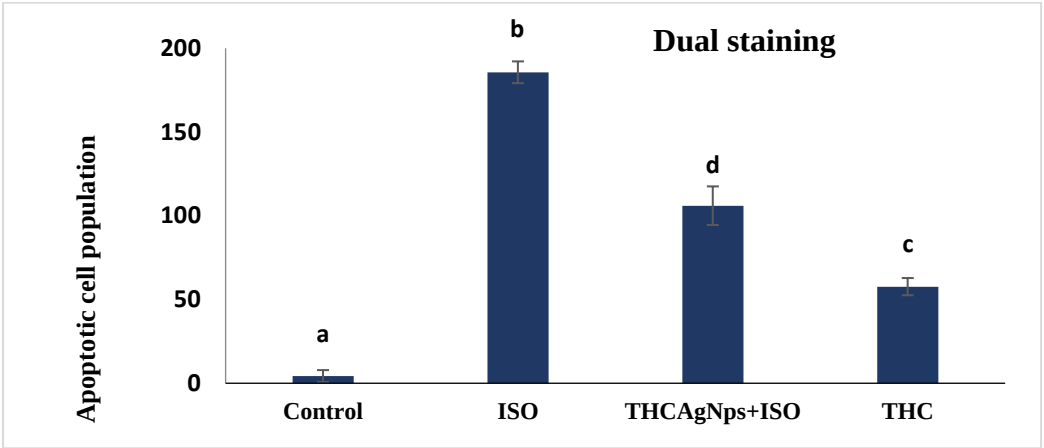
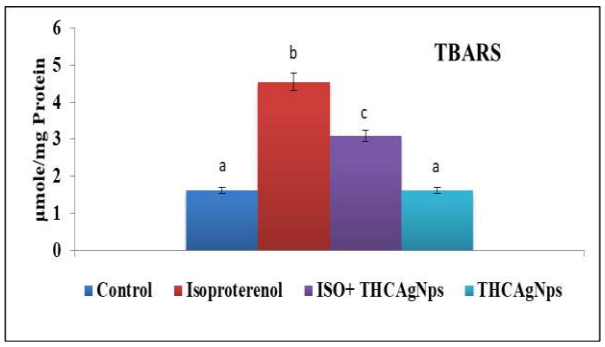


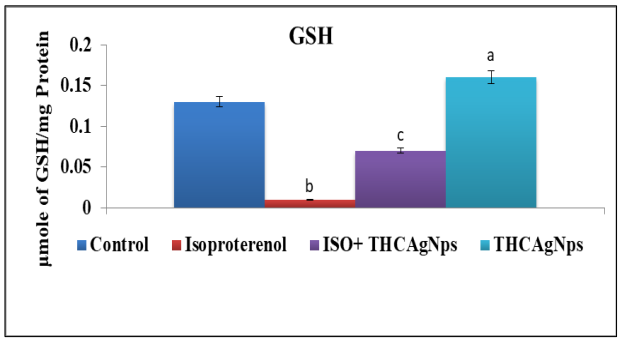
Figure 5: Effect of Tetrahydro curcumin loaded silver nanoparticles on AO/EtBr dual staining assay generation in H9C2 cells

Antioxidant assay

Figures 6(a) and 6(b) indicate the levels of TBARS and GSH in isoproterenol -treated H9C2 cells incubated with and without THC-AgNPs. Isoproterenol treatment (10 µM) significantly increased the levels of TBARS and decreased levels of GSH in H9C2 cells compared with nontreated cells. Pretreatment with THC-AgNPs (20 µg) to isoproterenol -treated cells significantly decreased the levels of TBARS and increased GSH levels significantly, compared to cells treated with isoproterenol alone. Figures 6(c), 6(d), and 6(e) elucidate the activities of SOD, catalase and GPx in isoproterenol -treated H9C2 cells incubated with and without THC-AgNPs. As compared with untreated cells, isoproterenol (10 µM) treatment increased SOD, catalase and GPx activities in H9C2 cells (Figures 6(c), 6(d), and 6(e)). Pretreatment with THC-AgNPs significantly decreased the activities of SOD, catalase, and GPx, compared to cells treated with isoproterenol alone.



(a)



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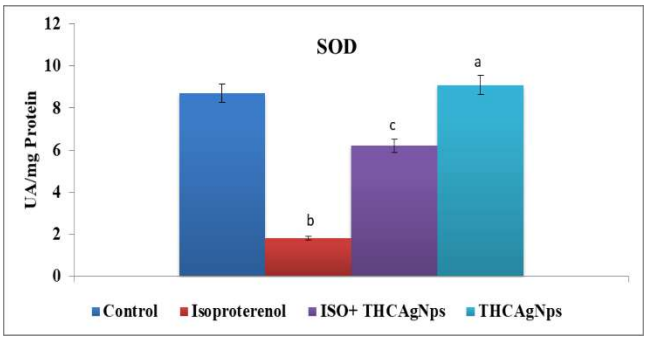
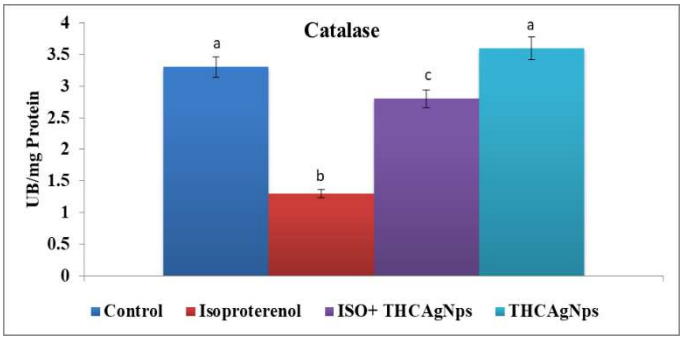
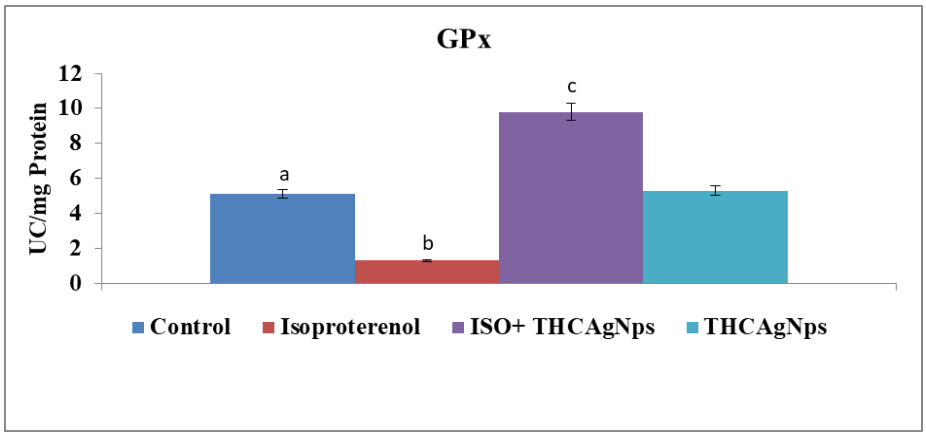


(c)





(e)

Figure 6: Effect of THC-AgNPs (20 μg) on isoproterenol (100 nM)-induced oxidative and antioxidative indices. isoproterenol treatment significantly increased and decreased the levels of TBARS and GSH, respectively, as compared to control cells, while THC-AgNPs pretreatment significantly decreased and enhanced the levels of TBARS and GSH as compared to isoproterenol alone treated cells (Figures 6(a) and 6(b)). Values are presented as mean ± SD of four experiments in each group. Isoproterenol treatment enhanced the activities of SOD, CAT, and GPx as compared to untreated cells, while THC-AgNPs pretreatment significantly downregulated the activities of enzymatic antioxidants as compared to isoproterenol alone treated cells ((c), (d), and (e)). Values are given as mean ± SD of four experiments in each group. 1Enzyme concentration required for 50% inhibition of nitroblue tetrazolium reduction in 1 min. 2Micromoles of hydrogen peroxide consumed per minute. 3Micrograms of glutathione consumed per minute.

DISCUSSION

The results of our study, showed that the exposure of ISO is cytotoxic to H9C2 cells and the co-administration of THC-AgNPs can attenuated the toxic cascade in ISO-treated cells. The tetrazolium salt MTT is reduced to form a blue formazan product and is generally used for evaluating cellular viability. This reduction reaction is normally carried out by the dehydrogenases enzymes found in the mitochondria of viable cells [18]. The mitochondrial function is a marker for cell viability and play key role in regulation of apoptotic cell death pathways by controlling the ATP production, levels of ROS formation, and release of apoptotic factors into the cytosol [19]. The results indicated the cytoprotective effect of THC-AgNPs against ISO-mediated mitochondrial dysfunction. As like other cells, mitochondrion is a key cellular organelle in cardiomyocytes also [20]. As MMP indicates the mitochondrial functional status, their estimation is necessary for knowing the mechanisms regulating cardiomyocyte function [18]. Alterations in MMP were found during the cardiac hypertrophy [19]. In the present study, H9c2 cells exposed with ISO showed a reduction in the MMP levels, which is corroborated with previous study [21]. But the pre-exposure of THC-AgNPs reduced the effects of Iso on MMP. As the depolarization of mitochondrial membrane regulates respiration function and trigger apoptosis [22], the THC-AgNPs may attenuate cardiac hypertrophy by preserving mitochondrial integrity and function. Our data showed that the ISO treatment can induce the intracellular ROS in H9C2 cells, as corroborated with previous experiment [22]. Mitochondrial dysfunction may lead to accumulation of electrons within the respiratory chain components. Mitochondria is considered as the key source of ROS and the main target of oxidant-antioxidant imbalance [23]. THC-AgNPs exposure significantly diminished ROS formation in ISO-treated cells, which might be due to ROS scavenging property of THC [24,25]. ISO administration induces the formation of free radicals directly [26] and also by inhibition of β-adrenoceptor mechanism, leading to myocardial cell necrosis [27]. The cytotoxic free radicals induce damage of membrane integrity with disintegration of polyunsaturated fatty acids in the membrane bilayer and exert unfavourable effects on the heart structure and function. SOD is the main enzymatic antioxidant, considering as the first line of defence against oxidative imbalance, as it converts more toxic superoxide anion to H2O2 and O2. catalase and GPx converts H2O2 into H2O by utilising GSH. Therefore, the increased TBARS levels in ISO exposed cells may be due to the inhibition of SOD, GPx and catalase activities. SOD and catalase are protective enzymes and both function in very close

association for the detoxification of highly reactive free radicals. Glutathione in its reduced form is the most abundant intracellular antioxidant, which directly scavenges free radicals or serving as a substrate for the glutathione peroxidase enzyme. In our study, treatment of THC-AgNPs to ISO exposed cells decreased the levels of TBARS and increased the levels of GSH and activities of enzymatic antioxidants. The current study demonstrated the protective effect of THC-AgNPs against cellular model of MI, could be through its mitochondrial protective, antioxidant and anti-apoptotic mechanisms.

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