Inhibition of Mitochondria Permeability Transition Pore and Antioxidant Effect of Delta-9-Tetrahydrocannabinol Reduces Aluminum Phosphide-Induced Mitochondrial Dysfunction and Cytotoxicity

Ahmad Salimi (salimikd@yahoo.com)
Ardabil University of Medical Sciences https://orcid.org/0000-0003-3026-6398
Elham Mojarad Aylar
Ardabil University of Medical Sciences
Mohammad Shabani
Ardabil University of Medical Sciences

Research Article

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Abstract

Purpose: Previous studies have demonstrated that phosphine gas (PH_3) released from aluminum phosphide (AlP) can inhibit cytochrome oxidase in cardiac mitochondria and induce generation of free radicals, oxidative stress alteration in antioxidant defense system and cardiotoxicity. Available evidence suggests that cannabinoids have protective effects in the reduction of oxidative stress, mitochondrial and cardiovascular damages. The objective of this study was to evaluate the effects of trans-Δ-9-tetrahydrocannabinol (THC) on AlP-induced toxicity in isolated cardiomyocytes and mitochondria.

Methods: Rat heart isolated cardiomyocytes and mitochondria were cotreated with different concentrations of THC (10, 50 and 100 μM) and IC50 of AlP, then cytotoxicity and mitochondrial toxicity parameters were assayed.

Results: Treatment with AlP alone increased the cytotoxicity, depletion of cellular glutathione (GSH), mitochondrial reactive oxygen species (ROS) generation, lipid oxidation, mitochondria membrane potential (ΔΨm) collapse and mitochondrial swelling, when compared to control group. However, incubation with THC (10, 50 and 100 μM) attenuated the AlP-induced changes in all these parameters in a THC concentration-dependent manner. Interestingly, the obtained results showed remarkably significant protective effects of THC by attenuation the different parameters of cytotoxicity, mitochondrial toxicity and oxidative stress induced by ALP in isolated cardiomyocytes and mitochondria.

Conclusion: It is the first report showing the protective effects of THC against AlP-induced toxicity, and these effects are related to antioxidant potential and inhibition of mitochondria permeability transition (MPT) pore by THC. Based on these results, it was hypothesized that THC may be used as a potential therapeutic agent for the treatment of AlP-induced mitochondrial dysfunction and cardiotoxicity.

Introduction

Rice tablet or aluminum phosphide (AlP) is a cheap and potent pesticide, without impact on seed viability and remaining in the food grains (Moghadamnia, 2012). When AlP comes in contact with moisture and acid (gastric acid in stomach), the toxic gas of phosphine (PH3) is released by it (Moghadamnia, 2012). In the acute toxicity induced by AlP, the heart, liver and lungs are the main targets in in exposed individuals (Etemadi-Aleagha et al., 2015). In various studies have been demonstrated that the mortality rates caused by AlP poisoning is high and about 70–100% (Etemadi-Aleagha et al., 2015, Farahani et al., 2016). The main causes of death caused by AlP poisoning are the refractory cardiogenic shock, severe hypotension, various electrocardiographic changes, dysrhythmias and severe and refractory metabolic acidosis (Farahani et al., 2016). It has been reported that most deaths occur due to cardiovascular toxicity after exposure with AlP and the cardiovascular system is the main target of this poison (Aziz and Husain, 2015). The main mechanisms of action of AlP are oxidative stress and inhibition of cytochrome oxidase c (Anand et al., 2013). It has been demonstrated that AlP is a potent mitochondrial respiratory chain enzyme inhibitor and the inhibition of cytochrome c oxidase is the most important target in

mitochondria (Anand et al., 2013). The inhibition of cytochrome c oxidase as a last enzyme in the respiratory electron transport chain of mitochondria and other mitochondrial and cellular enzymes leads to the production of superoxide radicals (O□ □) and cellular hydrogen peroxide (H_2O_2), and subsequent cellular injury through lipid peroxidation and oxidative stress (Anand et al., 2013, Nourbakhsh et al., 2019). This condition stimulates a vicious cycle of the production of reactive oxygen species, hydrogen peroxide, MDA and increase superoxide dismutase and the inhibition of peroxidase, catalase and glutathione (Anand et al., 2013, Nourbakhsh et al., 2019). Therefore, mitochondria as a main target in the toxicity of AlP can play a pivotal role in the cardiotoxicity caused by this pesticide (Sciuto et al., 2016). Moreover, mitochondria are abundant in heart tissue and about constituting 45% of the cardiomyocytes volume (Boengler et al., 2017). Accordingly, it can be anticipated that the development of antioxidant and mitochondrial protective agents is a rational strategy to prevent AlP-induced mitochondrial toxicity, oxidative stress, cellular injury and cardiotoxicity.

Hemp or *cannabis sativa L.* (*C. sativa L.*) is one of the oldest known medicinal plants for recreational and medicinal applications by human for over thousands of years (Raja et al., 2020). This plant contains more than 500 natural compounds including fibers, metabolites, proteins and oils, of which, more than 200 are metabolites including phytocannabinoids, alkaloids and flavonoids and terpenoids (Brenneisen, 2007). The most bioactive metabolites of cannabis are a chemical class of C21 terpenophenolic compounds, which are called phytocannabinoids and produced uniquely by this plant (Bonini et al., 2018). One of the most potent cannabinoids responsible for the pharmacological effect of cannabis is trans-Δ-9-tetrahydrocannabinol (THC) (Cooper and Haney, 2009). THC and other cannabinoids activate cannabinoid receptors (CB1 and CB2), as a group of receptors in the body which are responsible for various pharmacological and physiological processes (Zou and Kumar, 2018). THC is a partial agonist to cannabinoid receptors CB1 and CB2 (Zou and Kumar, 2018). These G-protein–coupled receptors are found in the cardiovascular system (blood vessels and heart) (LIU et al., 2000, Bonz et al., 2003), although cannabinoid receptors are also profusely expressed in the brain and immune cells (Matsuda et al., 1990, Munro et al., 1993). In addition to cannabinoid receptors in the heart, other constituents of the endocannabinoid signaling system are present in the cardiac tissue (Mechoulam et al., 1995, Deutsch et al., 1997). Available evidence suggests that cannabinoids have protective effect in the reduction of cardiovascular damages (Lépicier et al., 2003, Underdown et al., 2005). For example, cardiac myocyte hypertrophy is prevented by via cannabinoid receptor signaling and infarct size is alleviated by synthetic and endogenous cannabinoid agonists (Lagneux and Lamontagne, 2001, Lépicier et al., 2003, Underdown et al., 2005). Moreover, it has been reported that CB1 receptors are predominantly localized in mitochondria in skeletal and myocardial muscles, and the activation of these receptors may participate in the mitochondrial regulation of the oxidative activity (Mendizabal-Zubiaga et al., 2016). Aside from above activities of THC has considerable antioxidant effects (Hacke et al., 2019). Due to the promising effect of THC it deserves to be more studied. Therefore, here we aim to evaluate the effect of THC against AlP-induced toxicity in rat heart isolated cardiomyocytes and mitochondria.

Materials And Methods

Animals

Five male Wistar rats (180-220 g) were procured from the animal house of the Baqiyatallah University of Medical Sciences (Tehran, Iran) and were kept in ordinary cages at room temperature of 25 ± 2 ºC with a 12 h dark/light cycle and humidity 50–60 %. Animals were allowed free access to food and water, and were adapted for 2 weeks before the experiments. The study protocol was approved by Ethical Committee of the Ardabil University of Medical Sciences (Ardabil, Iran) with ethics code IR.ARUMS.REC.1399.484, for the animal care and experimentation.

Chemicals

Streptomycin and Penicillin Solution, 199 Medium, Fetal Bovine Serum (FBS), Collagenase (Type II), Taurine, Creatine, 2',7'-Dichlor-fluorescein (DCF), Trypan blue, Carnitine, Rhodamine123, Hank's Balanced Salt Solution (HBSS), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS),Bovine Serum Albumin (BSA), D-mannitol, Butylated hydroxytoluene (BHT), Cyclosporine (Cs.A), Dimethyl sulfoxide (DMSO), Sucrose, Rotenone, Monopotassium phosphate, 3-morpholinopropane-1-sulfonic acid (MOPS), 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Magnesium chloride, Sodium succinate, Potassium chloride and Delta-9-tetrahydrocannabinol (Δ9-THC) in methanol were purchased were purchased from Sigma (St. Louis, MO USA). Aluminum Phosphide with a purity of about 99% was gifted from the Samiran company (Tehran, Iran). AlP and THC was freshly prepared before use and dissolved in DMSO (0.05 %).

Solutions and Buffers

Calcium chloride ($CaCl_2$) contained: 100 mM $CaCl_2$. Mitochondrial isolation buffer contained; 75 mM sucrose, 225 mM D-mannitol and 0.2 mM EDTA, pH adjusted to 7.4 with NaOH (2 mM). Powell medium contained: 0.3 g $MgSO_4$ $7H_2O$ (1.2 mM), .43 g NaCl (110 mM), 0.19 g KCl (2.5 mM), 0.16 g KH_2PO_4 (1.2 mM), .98 g D (+)-Glucose monohydrate (10 mM) and 6 5.96 g Hepes (25 mM) 1 in Aqua sterile, pH adjusted to 7.4 with NaOH (2 mM) in a sterile medium. Creatine-carnitine-taurine medium (CCT medium) contained: 655.5 mg creatine (5 mM), 625.5 mg taurine (5 mM), 395.4 mg carnitine (2 mM), 10 µM cytosine β-D-arabinofuranoside, 3.6 g Hepes and pH adjusted to 7.4 with NaOH (2 mM) in a sterile medium. Respiration buffer contained: 0.32 mM sucrose, 10 mM TRIS, 20 mM MOPS, 50 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM KH_2PO_4 and 5 mM sodium succinate pH 7.4). Mitochondrial assay buffer contained: 0.5 mmol/L KH_2PO_4, 10 mmol/L NaCl, 140 mmol/L KCL, 2 mmol/L $MgCl_2$, 0.5 mmol/L EGTA, 20 mmol/L HEPES; supplemented with 10 mmol/L succinate and 1 mg/mL rotenone, pH adjusted to 7.4. Mitochondrial membrane potential buffer contained: 5 mM KH_2PO_4, 50 µM EGTA, 220 mM sucrose, 10 mM HEPES, 5 mM sodium succinate, 68 mM D-mannitol, 2 mM $MgCl_2$, 2 µM rotenone and 10 mM KCl. Mitochondrial swelling buffer contained: 0.5 mmol/L KH_2PO_4, 2 mmol/L $MgCl_2$, 10 mmol/L NaCl, 140 mmol/L KCL, 20 mmol/L HEPES, 0.5 mmol/L EGTA; supplemented 1 mg/mL rotenone, pH adjusted to 7.4.

Isolation Of Adult Ventricular Rat Heart Muscle Cells

Adult ventricular rat heart muscle cells or cardiomyocytes were isolated as previously described by Nippert et al (Nippert et al., 2017) from male Wistar rats. The animals were deeply anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg). The chest was opened by surgical scissors and the heart was quickly removed, and directly perfused with Powell medium for 5 minutes, on a Langendorff perfusion system, then the medium was replaced with enzymatic solution for 25 minutes. The heart was removed from the Langendorff perfusion system and were mechanically cut to small pieces and shaken in the enzymatic mixture for 10 minutes. To remove additional tissues, the digested cell suspensions were filtered through a mesh (180 µm). The obtained cardiomyocytes suspensions were centrifuged at 1000 x g for 10 min. The obtained isolated cardiomyocytes were suspended in CCT medium supplemented with antibiotics (100 µg/ml penicillin and streptomycin) and 10 % FBS at 37°C under a 5% CO_2–95% air atmosphere. One hour after cell isolation and plating, the culture medium was changed to remove dead cells.

Cell Culture and Treatment

Isolated cardiomyocytes obtained from the rat heart has been widely used for pharmacological and toxicological studies. Rat heart isolated cardiomyocytes were incubated in 75 cm^2 flasks (~2.0–2.5 ×10^6 cells/mL) in CCT medium supplemented with antibiotics (100 µg/ml penicillin and streptomycin) and 10 % FBS in a humidified incubator in the presence of 5% CO_2-95% air at 37°C. Cardiomyocytes treated with 20 µg/ml of AlP (IC_{50} 3h) for 3h in the presence or absence of different concentration of THC (10, 50 and 100 µM) simultaneously. Control cells were treated with vehicle (0.05% DMSO) alone.

Cell Viability Assay

For assessment of cell viability, MTT assay was performed as previously described. Isolated cardiomyocytes were seeded at a density of 10^4 cells/well in a 96-well plate. For induction of cytotoxicity, isolated cardiomyocytes was treated for 3 h with 20 µg/ml of AlP according to previous studies (Khezri et al., 2020, Hafez et al., 2021). Simultaneously isolated cardiomyocytes were cotreated with different concentrations of THC (10, 50 and 100 µM) and 20 µg/ml of AlP for 3 h. Control cells were treated with vehicle (0.05% DMSO) alone. After measurement, the produced formazan by viable cells was dissolved in DMSO and the optical density (OD) was measured at 575 nm. The cell viability was shown as a percentage of non-treated cells. Butylated hydroxytoluene (BHT), a well-known antioxidant, was used as a positive control.

Determination of Lipid Peroxidation and Glutathione

Isolated cardiomyocytes reduced glutathione (GSH) and oxidized glutathione (GSSG) content was determined by Hissin and Hilf method (Hissin and Hilf, 1976). Also, lipid peroxidation was measured by detecting the amount of malondialdehyde (MDA) formed during the decomposition of fatty acids within cells (Beach and Giroux, 1992). Briefly Isolated cardiomyocytes were seeded in a 96-well plate, for measurement of MDA and GSH levels, and treated with 20 µg/ml of AlP alone, and simultaneously with different concentrations of THC (10, 50 and 100 µM) and 20 µg/ml of AlP at 37°C for 60 minutes in CCT medium. Butylated hydroxytoluene (BHT), a well-known antioxidant, was used as a positive control.

Control mitochondria were treated with vehicle (0.05% DMSO) alone. The levels of GSH, GSSG and MDA in the cell homogenates were determined using a commercially available assay methods according to the above standard protocols.

Mitochondria Isolation from Rat Heart

Using the standard protocol and technique, mitochondria were isolated from the heart of male Wistar rats as previously described (Atashbar et al., 2021). The animals were deeply anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg). The chest was opened by surgical scissors and the heart was quickly removed, chopped, cleared from blood vessels, and homogenized in the mitochondrial isolation buffer (75 mM sucrose, 225 mM D-mannitol and 0.2 mM EDTA, pH adjusted to 7.4 with NaOH (2 mM) with a glass homogenizer in a 10-fold volume on ice. The homogenized tissue was centrifuged at 1000× g for 10 min, the supernatant was transferred to new tube and the pellet was removed. The supernatant was sedimented at 10000× g for 10 min at 4°C, to obtain of mitochondria in pellet. After isolation of mitochondria, they were kept for 2 h on ice bath. To measure the protein content in mitochondria was used the Bradford assay. The protein content in mitochondria in suspension was 1000 µg/mL.

Succinate Dehydrogenase Activity Assay

For assessment of succinate dehydrogenase (SDH) activity, MTT assay was performed as previously described. Isolated mitochondria were seeded at a density of 100 µg/well in a 96-well plate. Briefly, for measurement of succinate dehydrogenase activity, isolated mitochondria were treated with 20 µg/ml of AIP alone, and simultaneously with different concentrations of THC (10, 50 and 100 µM) and 20 µg/ml of AIP at 37°C for 60 minutes in mitochondrial assay buffer. Control mitochondria were treated with vehicle (0.05% DMSO) alone. After measurement, the produced formazan by intact mitochondria was dissolved in DMSO (100 µL) and the optical density (OD) was measured at 570 nm. Succinate dehydrogenase activity was shown as a percentage of non-treated mitochondria. Butylated hydroxytoluene (BHT), a well-known antioxidant, was used as a positive control.

Mitochondrial Swelling Assay

Mitochondrial permeability transition pore (PTP) opening causes mitochondrial permeability transition (MPT), which leads to uncoupling of oxidative phosphorylation, increase in mitochondrial volume (swelling) and dissipation of mitochondrial membrane potential (MMP). Mitochondrial swelling was monitored spectrophotometrically by the changes of absorbance at 540 nm, which reflects mitochondrial permeabilization to sucrose. Decrease in the absorbance reflects mitochondrial swelling and opening of mitochondrial PTP (Zhao et al., 2010). Briefly, for measurement of mitochondria swelling, isolated mitochondria were treated with 20 µg/ml of AIP alone, and simultaneously with different concentrations of THC (10, 50 and 100 µM) and 20 µg/ml of AIP at 37°C for 60 minutes in swelling buffer. Control mitochondria were treated with vehicle (0.05% DMSO) alone. Mitochondrial swelling was monitored by measurement of light scattering at 540nm, during 60 minutes, with a BioTek microplate reader (US).

Mitochondrial Membrane Lipid Peroxidation Assay

Mitochondrial membrane lipid peroxidation was measured by the reaction of the end product of lipid peroxidation, malondialdehyde (MDA) with thiobarbituric acid (TBA). Briefly, for measurement of mitochondrial membrane lipid peroxidation, isolated mitochondria were treated with 20 µg/ml of AlP alone, and simultaneously with different concentrations of THC (10, 50 and 100 µM) and 20 µg/ml of AlP at 37°C for 60 minutes in mitochondrial assay buffer. Control mitochondria were treated with vehicle (0.05% DMSO) alone. After treatment, in a tube containing 1 ml 0.1% (w/v) TCA, mitochondria were mechanically lysed by homogenizer. The obtained mitochondria homogenate samples were mixed with 0.5% TBA and 20% TCA, then and incubated in boiling water bath for 15 minutes. After cooling on ice, the absorbance was measured at 532nm (Beach and Giroux, 1992).

Mitochondrial ROS level Assay

For mitochondrial ROS detection, mitochondria were stained with 2',7'-Dichlor-fluorescein (DCF). Briefly, for measurement of mitochondrial ROS level, isolated mitochondria were treated with 20 µg/ml of AlP alone, and simultaneously with different concentrations of THC (10, 50 and 100 µM) and 20 µg/ml of AlP at 37°C for 60 minutes in mitochondrial respiration buffer (0.32 mM sucrose, 10 mM TRIS, 20 mM MOPS, 50 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM KH_2PO_4 and 5 mM sodium succinate pH 7.4). Control mitochondria were treated with vehicle (0.05% DMSO) alone. The samples were incubated with DCF (10 µM) for 15 min and then the fluorescence intensity was determined through flow cytometry (Cyflow Space-Partec, Germany). The signals were obtained using a 530 nm bandpass filter (FL-1 channel). Each determination is based on the mean fluorescence intensity of 20000 counts. The results were analyzed by FlowJo software (Ma et al., 2017).

Mitochondrial Membrane Potential Collapse Assay

For mitochondrial membrane potential (MMP) collapse detection, mitochondria were stained with rhodamine 123. Briefly, for measurement of mitochondrial ROS level, isolated mitochondria were treated with 20 µg/ml of AlP alone, and simultaneously with different concentrations of THC (10, 50 and 100 µM) and 20 µg/ml of AlP at 37°C for 60 minutes in mitochondrial membrane potential buffer (5 mM KH_2PO_4, 50 µM EGTA, 220 mM sucrose, 10 mM HEPES, 5 mM sodium succinate, 68 mM D-mannitol, 2 mM $MgCl_2$, 2 µM rotenone and 10 mM KCl). Control mitochondria were treated with vehicle (0.05% DMSO) alone. The samples were incubated with rhodamine 123 (5 µM) for 15 min and then the fluorescence intensity was determined through flow cytometry (Cyflow Space-Partec, Germany). The signals were read on FL-1 channel. Each determination is based on the mean fluorescence intensity of 20000 counts. The data were analyzed by FlowJo software (Atashbar et al., 2021).

Statistical Analysis

Results were analyzed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA). Data were expressed as mean ± standard deviation (SD). Results were analyzed by one- and two-way analysis of variance (ANOVA), Tukey's and Bonferonie's tests were applied for post-hoc analysis. A value of $p < 0.05$ was considered to be statistically significant.

Results

THC exhibited ameliorative effects against AlP-Induced Cytotoxicity in Cardiomyocytes

The cell viability of control group (0.05% DMSO) was accepted as 100% and the viability of the other treated cell groups were expressed as a percentage. In the AlP-treated group (20 µg/ml), cell viability significantly decreased (51.14 ± 0.30%) compared with the control group ($p < 0.001$). While, AlP-plus-THC treatment (10, 50 and 100 µM) increased cell viability compared with the AlP group ($p < 0.001$) in concentration dependent manner. Whereas, THC (100 µM) and BHT (50 µM) treatment did not show significant alterations in the cell viability compared to control group 0.05% DMSO). Cell viabilities of different groups are shown in Figure 1A-B.

THC exhibited ameliorative effects against AlP-Induced Oxidative Stress in Cardiomyocytes

The amount of MDA of the AlP group was found to be at a high level ($p < 0.001$) compared to the control group (0.05% DMSO). Cotreatment application of THC (10, 50 and 100 µM) with AlP dose-dependently reduced the amount of MDA compared with the AlP group ($p < 0.001$). Whereas, THC (100 µM) and BHT (50 µM) treatment did not show significant alterations in the amount of MDA compared to control group (0.05% DMSO). A significant ($p < 0.001$) decrease was seen in the amount of GSH of the AlP-treated cardiomyocytes compared with the control group (0.05% DMSO). THC (10, 50 and 100 µM) coadministration with AlP, increased the amount of GSH compared with the AlP group ($p < 0.001$) in a concentration-dependent manner. Whereas, THC (100 µM) and BHT (50 µM) treatment did not show significant alterations in the amount of GSH compared to control group (0.05% DMSO). The amount of MDA and GSH of all the treated groups are presented in Table 1.

Table 1
Effects of AlP at concentration 20 µg/ml, THC at concentration 100 µM and THC at concentration 10, 50 and 100 µM + AlP on malondialdehyde (MDA), reduced glutathione (GSH) and oxidized glutathione (GSSG) levels of rat heart isolated cardiomyocytes at 3 hours.

Groups	MDA (nM)	GSH (µM)	GSSG(µM)
	3h	3h	3h
Control	43 ± 2.3	21 ± 0.6	10 ± 0.6
AlP (20 µg/ml)	89 ± 3.1[a]	8 ± 0.3[a]	19 ± 1.4[a]
AlP + THC (10 µM)	76 ± 2.0[b]	13 ± 0.4[b]	16 ± 0.3
AlP + THC (50 µM)	64 ± 1.9[b]	13 ± 0.4[b]	17 ± 0.4[b]
AlP + THC (100 µM)	52 ± 1.6[b]	16 ± 0.7[b]	18 ± 0.3[b]
AlP + BHT (50 µM)	48 ± 1.8[b]	18 ± 0.5[b]	18 ± 0.8[b]
THC (100 µM)	42 ± 1.0	19 ± 1.2	9 ± 0.5
[a] shows significant difference ($p<0.05$) with control			
[b] shows significant difference ($p<0.05$) with AlP treated group with 20 µg/ml			

Effects of THC on Succinate Dehydrogenase Activity of AlP-Treated Isolated Mitochondria

The succinate dehydrogenase activity of control group was accepted as 100% and the activity of the other treated mitochondria groups were expressed as a percentage. Only AlP treatment on rat heart isolated mitochondria decreased succinate dehydrogenase activity ($p < 0.001$). THC (10, 50 and 100 µM)-plus-AlP significantly enhanced succinate dehydrogenase activity in a concentration-dependent manner. However, only THC treatment (100 µM) did not change the succinate dehydrogenase activity compared with the control group (0.05% DMSO). Succinate dehydrogenase activity of the experimental groups are shown in Figure 2A-B.

Effects of THC on Mitochondrial Swelling of AlP-Treated Isolated Mitochondria

Figure 3A, shows that compared with control group, heart mitochondrial swelling rate, an index of mitochondrial permeability transition (MPT), was significantly ($p < 0.001$) increased in AlP-treated group as compared to the control group (0.05% DMSO). Such high mitochondrial swelling rate was significantly ($p < 0.001$) reduced when THC (10, 50 and 100 µM) was cotreated with AlP as compared to the AlP alone treated mitochondria. However, only THC treatment (100 µM) did not change the heart mitochondrial

swelling rate compared with the control group (0.05% DMSO). $CaCl_2$ (100 μM), as a known inducer of mitochondrial permeability transition (MPT) was used as a positive control. Cyclosporine A (5 μM), a PTP inhibitor, was added to verify PTP dependence of mitochondrial swelling.

Effects of THC on Mitochondrial Lipid Peroxidation of AlP-Treated Isolated Mitochondria

As shown in Figure 3B, AlP treatment on rat heart isolated mitochondria increased significantly ($p < 0.001$) MDA levels, an index mitochondrial lipid peroxidation. THC (10, 50 and 100 μM)-plus-AlP significantly ($p < 0.001$) reduced mitochondrial MDA levels in a concentration-dependent manner. However, only THC treatment (100 μM) did not change the mitochondrial MDA levels compared with the control group (0.05% DMSO). Mitochondrial lipid peroxidation state of the experimental groups is illustrated in Figure 3B.

Effects of THC on Mitochondrial ROS Formation of AlP-Treated Isolated Mitochondria

The fluorescence intensity of 2',7'-dichlor-fluorescein (DCF) was investigated to measure mitochondrial ROS formation in cardiac isolated mitochondria. Figure 4 showed that the fluorescence intensity of DCF or ROS amount in the mitochondria treated with AlP (20 μg/ml) was significantly ($p < 0.001$) increased compared to the control group (0.05% DMSO). On the other hand, the fluorescence intensity of DCF was significantly ($p < 0.001$) decreased in mitochondria co-treated with THC (10, 50 and 100 μM) and AlP (20 μg/ml) in a concentration-dependent manner, when compared with the group treated with only AlP (20 μg/ml). However, only THC treatment (100 μM) did not change the mitochondrial ROS formation compared with the control group (0.05% DMSO). These findings showed that AlP induced mitochondrial toxicity in cardiac mitochondria via ROS formation and THC co-treatment reduced AlP-induced mitochondrial ROS formation through antioxidant potential. Hydrogen peroxide (H_2O_2) was used as a positive control (100 μM). BHT (50 μM), as a known antioxidant, was added to verify antioxidant effect of THC inhibits ROS formation.

Effects of THC on Mitochondrial Membrane Potential Collapse of AlP-Treated Isolated Mitochondria

The fluorescence intensity of rhodamine 123 was investigated to measure mitochondrial membrane potential (MMP) collapse in cardiac isolated mitochondria. Figure 5 showed that the fluorescence intensity of rhodamine 123 in the mitochondria treated with AlP (20 μg/ml) was significantly ($p < 0.001$) increased compared to the control group (0.05% DMSO). While cotreatment of THC (10, 50 and 100 μM) with AlP (20 μg/ml) was found to be significantly ($p < 0.001$) restored the mitochondrial membrane potential collapse in a concentration-dependent manner, as compared to the AlP alone treated group. However, only THC treatment (100 μM) did not change the mitochondrial membrane potential collapse compared with the control group (0.05% DMSO). $CaCl_2$ (100 μM), as a known inducer of mitochondrial

permeability transition (MPT) was used as a positive control. Cyclosporine A (5 μM), a PTP inhibitor, was added to verify PTP dependence of mitochondrial swelling.

Discussion

Poisoning with pesticides is a global health problem and leads to the deaths of about 300,000 people each year worldwide (Hosseini et al., 2020). AlP is one of the most widely used pesticides with a high mortality rate about 40–80% (Mathai and Bhanu, 2010). AlP causes a wide range of effects, including oxidative stress, cell toxicity, metabolic disorders, electrolyte imbalances, circulatory failure and damage to vital organs (Sciuto et al., 2016). AlP produces phosphine gas (PH_3) in presence of the moisture and stomach acid and known as a mitochondrial toxin which leads to severe toxicity in cells (Sciuto et al., 2016). One of the main targets for phosphine gas are cardiomyocytes and AlP-induced cardiac toxicity has been reported as the main cause of death in the poisoning cases (Hosseini et al., 2020). The leading causes of death in AlP poisoning is cardiomyocyte death that leads to circulatory collapse, cardiac dysfunction and cardiac toxicity (Hosseini et al., 2020). Cardiomyocytes construct close to 75% of the heart tissues and have a main role in cardiac function. It has been reported that AlP directly effects on cardiomyocytes poisoning individuals (Hosseini et al., 2020). Due to limited antioxidant system, high metabolic activity and high oxygen intake by cardiomyocytes, the heart is very sensitive to AlP toxicity known as an oxidative stress agent (Baghaei et al., 2016). Our results on isolated cardiomyocytes showed that cardiac myocytes are very sensitive to direct toxicity induced by AlP during 3h, and AlP-induced cytotoxicity is associated with oxidative stress and mitochondrial dysfunction. Mitochondria are abundant organelle in cardiomyocytes, and provide 90% of the energy of these cells by producing ATP through the process of oxidative phosphorylation, which contribute to the contractile function of cardiomyocytes (Baghaei et al., 2016). Phosphine at the mitochondrial level impairs the synthesis of proteins and enzymes (Sciuto et al., 2016). This gas penetrates the intracellular space and disrupts mitochondrial function by reacting with the mitochondrial respiratory chain. The mitochondrial respiratory chain as the primary source of ROS production is disrupted by AlP, which in turn disrupts cardiomyocytes energy demand (Sciuto et al., 2016). Previous studies have been demonstrated that AlP inhibits cytochrome c oxidase and complex IV as main sites in the ETC and in turn decreases ATP generation, and eventually reduces cardiomyocytes energy (Asghari et al., 2017). Our results on isolated mitochondria showed that AlP directly induces mitochondrial toxicity and these observations are consistent with previous studies. Previous studies have been shown that decreased energy production in the cardiomyocytes and mitochondria is associated with the generation of free radicals, especially ROS, and oxidative stress, which lead to lipid peroxidation, AlP-induced cardiac toxicity (Goharbari et al., 2018, Hosseini et al., 2020). Our results on isolated cardiomyocytes and mitochondria showed that exposure with AlP is associated with reducing antioxidant enzymes and producing lipid peroxidation. The exact mechanism of AlP is still unclear but most studies have estimated a decrease in antioxidant capacity, an increase in oxidative stress and mitochondrial dysfunction as the primary mechanism of cardiotoxicity (Valmas et al., 2008). According to above mechanisms, mitochondria in the cardiomyocytes are attractive targets, because of their roles in AlP-induced cardiotoxicity.

Strong antioxidant effects of THC have demonstrated in previous study (Hampson et al., 2000, Raja et al., 2020). Hampson et al demonstrated that this cannabinoid can be considered more potent protective compounds than tocopherol and ascorbate (Hampson et al., 2000). As demonstrated by Raja et al, THC is able to prevent hydroperoxide-induced oxidative stress in neurons obtained from rats (Raja et al., 2020). In another study has been that THC can prevent oxidative cell death and act as antioxidant at sub-micromolar concentrations (Chen and Buck, 2000). Further studies conducted by Comelli et al demonstrated that sativa extract containing 4% of THC improved the oxidative stress protection by reducing diabetic neuropathic pain due to its antioxidant activity (Comelli et al., 2009). Another study conducted by Hacke et al, demonstrated by several biochemical analyzes the antioxidant effects of THC (Hacke et al., 2019). Oxidative stress and mitochondrial dysfunction have been implicated in AlP-induced cardiotoxicity (Hosseini et al., 2020). In the present study, the coadministration of THC with AlP for 3 hours was associated with significant and dose-dependent decrease in cell death. In the current study we showed that, THC is a potent antioxidant and plays an important role in the maintenance of the antioxidant capacity of the cell and in protecting against oxidative stress by ALP, compared to BHT as positive antioxidant. MDA and GSSG, as main markers of lipid peroxidation and oxidative showed a significant decrease in rat heart isolated cardiomyocytes at sub-micromolar concentrations, suggesting a potent antioxidant effect for the THC. The antioxidant activity of THC has been demonstrated in the other animal and cellular studies, and this is consistent with our results in the current study (Hampson et al., 1998, Abdel-Salam et al., 2015, Atalay et al., 2020).

The effects of THC on mitochondrial function vary in different organs (Sarafian et al., 2003, Wolff et al., 2015, Lu et al., 2020). Our studies showed that concentrations of 10 to 100 μM of THC did not cause any serious damage to cardiac mitochondria and can even have beneficial effects in reducing mitochondrial damage and oxidative stress induced by AlP. This difference in effect may be related to the number of mitochondrial CB1 receptors in different organs. Interestingly, these mitochondrial CB1 receptors has been shown in the hippocamp for first time and then in the peripheral tissues, such as sperm cell, cardiomyocytes and muscles. Mitochondrial CB1 receptors are localized at mitochondrial membranes with a small but significant proportion, where they control mitochondrial respiration (Mendizabal-Zubiaga et al., 2016). As demonstrated by Lu et al, activation of CB receptors exerted early protective effects on mitochondrial function in cardiac myocytes exposed to a prohypertrophic agonist (Lu et al., 2020). They reported that dual agonism of CB1 and CB2 receptors restored the mitochondrial membrane potential ($\Delta\Psi m$) and prevented depression of fatty acid oxidation-related mitochondrial bioenergetics (Lu et al., 2020). Given that fatty acids are the primary energy source in the heart, the ability of CB to restore fatty acid oxidation strengthens its cardioprotective potential (Lu et al., 2020). Our results on isolated mitochondria showed that THC exerts protective effects on mitochondrial function, restores mitochondrial membrane potential collapse and prevents mitochondrial swelling and ROS formation in cardiac mitochondria exposed to AlP. It has been reported that activation of CB2 receptor by JWH133 as a CB2 receptor agonist protects the isolated heart of the rat from ischemia-reperfusion injury (Li et al., 2014). This report has been shown that inhibition of MPTP opening in mitochondria may be its possible mechanism (Li et al., 2014). On the other hand, it has been reported that these inhibitors of the

mitochondrial permeability transition pore can play an effective role in reducing myocardial damage (Hausenloy et al., 2002). Therefore, presumably inhibition of MPT pore and antioxidant activity of THC may be involved in mitochondrial protection effect in the presence of AlP. However, the underlying mechanisms need further investigation.

We previously reported that AlP can directly cause toxicity in cardiac mitochondria and cardiomyocytes, which is associated with cytotoxicity, mitochondrial toxicity, reduction of antioxidant molecules, ROS formation and oxidative stress. Antioxidants and mitochondrial protective agents represent a viable strategy to ameliorate cardiac toxicity induced by AlP. Here, antioxidant activity and inhibition of MPT pore by THC exerted protective effects on mitochondrial function, restored mitochondrial membrane potential collapse and prevented mitochondrial swelling and ROS formation in cardiac mitochondria exposed to AlP. At the end, it is suggested that more studies be performed on animal models and clinical trials.

Declarations

Ethical Approval

This research was approved by the Ethics Committee at the Ardabil University of Medical Sciences, Ardabil, Iran. The approval code is IR.ARUMS.REC.1399.484.

Consent to Participate

All authors have given consent to their contribution.

Consent to Publish

All authors have agreed with the content and all have given explicit consent to publish.

Author Contributions

A.S. conceived and designed research. EMA and MS conducted experiments. A.S. contributed new reagents or analytical tools. A.S. analyzed data. A.S. wrote the manuscript. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Competing Interests

The authors declare no competing interests.

Data Availability

Not applicable.

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Figures

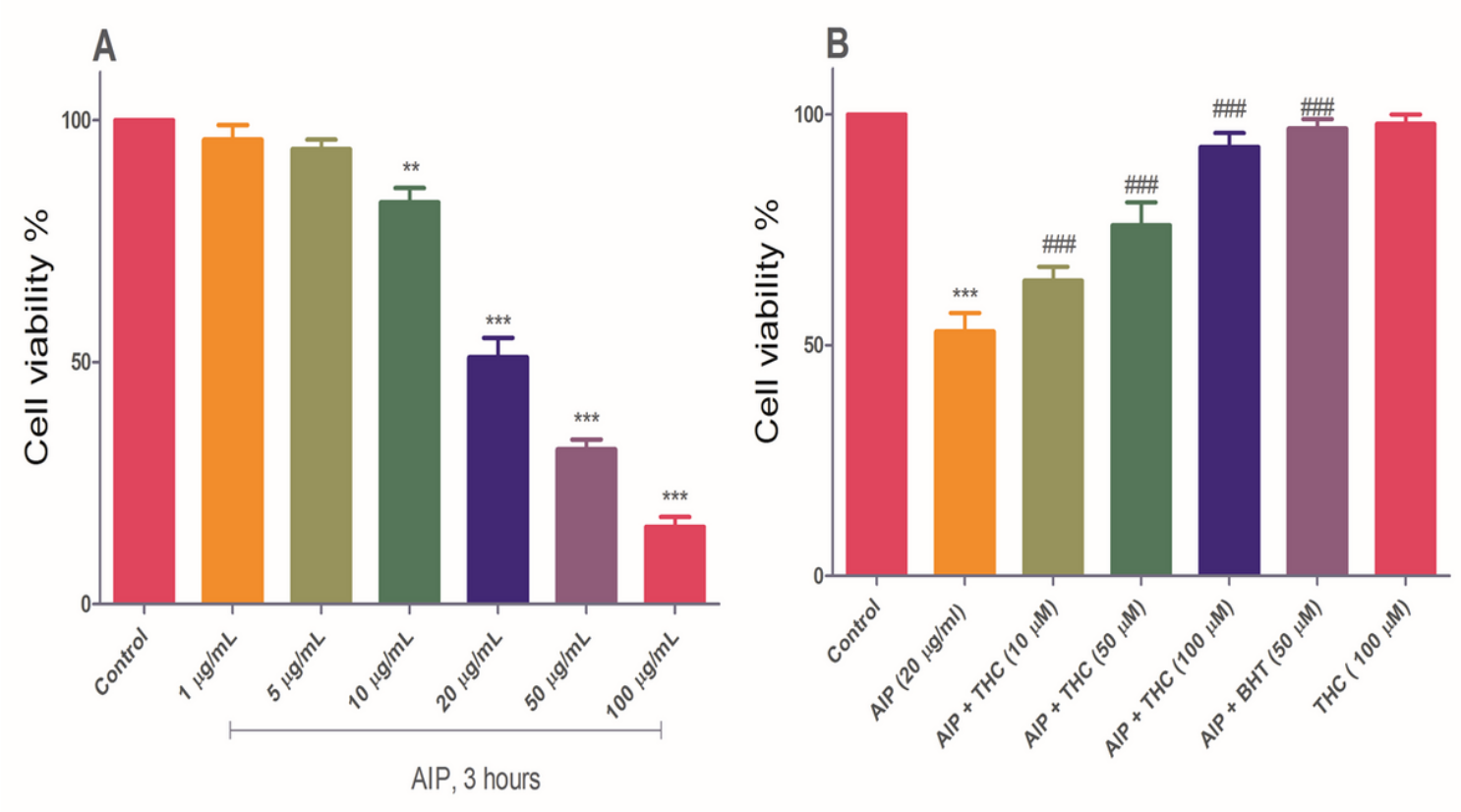

Figure 1

Effects of AlP treatment (20 µg/ml), THC treatment (100 µM) and concomitant treatment of AlP (20 µg/ml) with 10, 50 and 100 µM THC on MTT cell viability assay in rat heart isolated cardiomyocytes at 3 hours. Isolated cardiomyocytes were treated or cotreated with the indicated concentrations (20 µg/ml) of AlP and THC (10, 50 and 100 µM) for 3 hours. Cell viability was evaluated by MTT assay. Data are presented as mean ± SD, n= 3, ***$p < 0.001$: control versus AlP; ###$p < 0.001$: AlP + THC versus AlP-treated cardiomyocytes. THC, trans-Δ-9-tetrahydrocannabinol; AlP, Aluminium Phosphide

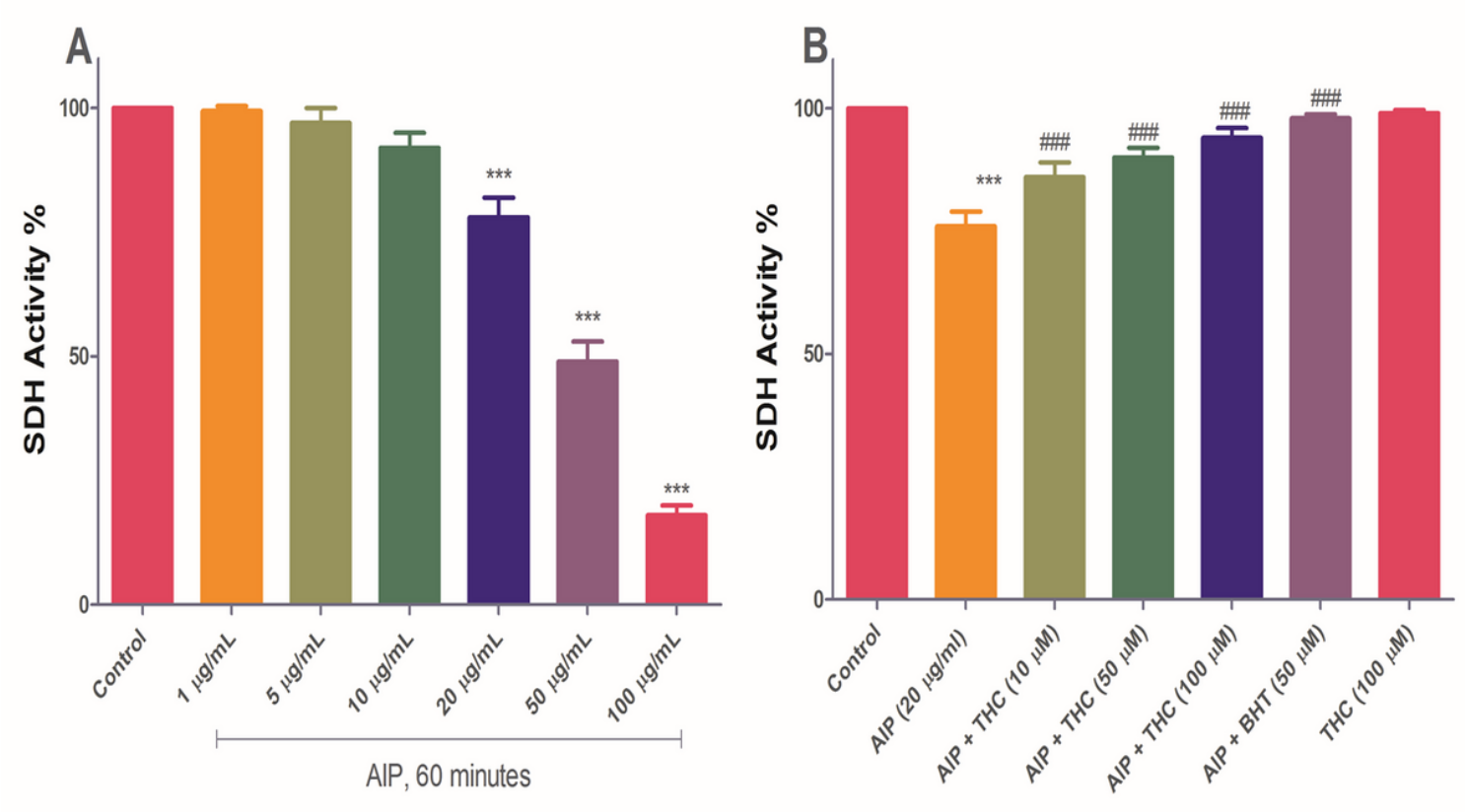

Figure 2

Effects of AlP treatment, THC treatment (100 µM) and concomitant treatment of AlP (20 µg/ml) with 10, 50 and 100 µM THC on succinate dehydrogenase (SDH) activity in rat heart isolated mitochondria at 60 minutes. Isolated mitochondria were treated or cotreated with the indicated concentrations (20 µg/ml) of AlP and THC (10, 50 and 100 µM) for 60 minutes. Succinate dehydrogenase activity was evaluated by MTT assay. Data showed AlP (20 µg/ml) significantly decreased SDH activity compared to control while THC (10, 50 and 100 µM) significantly increased SDH activity compared to AlP treated mitochondria. Data are mean ± SD of three independent experiments. ***p < 0.001 significantly different from control, ###p < 0.001 significantly different from AlP treated mitochondria. THC, trans-Δ-9-tetrahydrocannabinol; AlP, Aluminium Phosphide

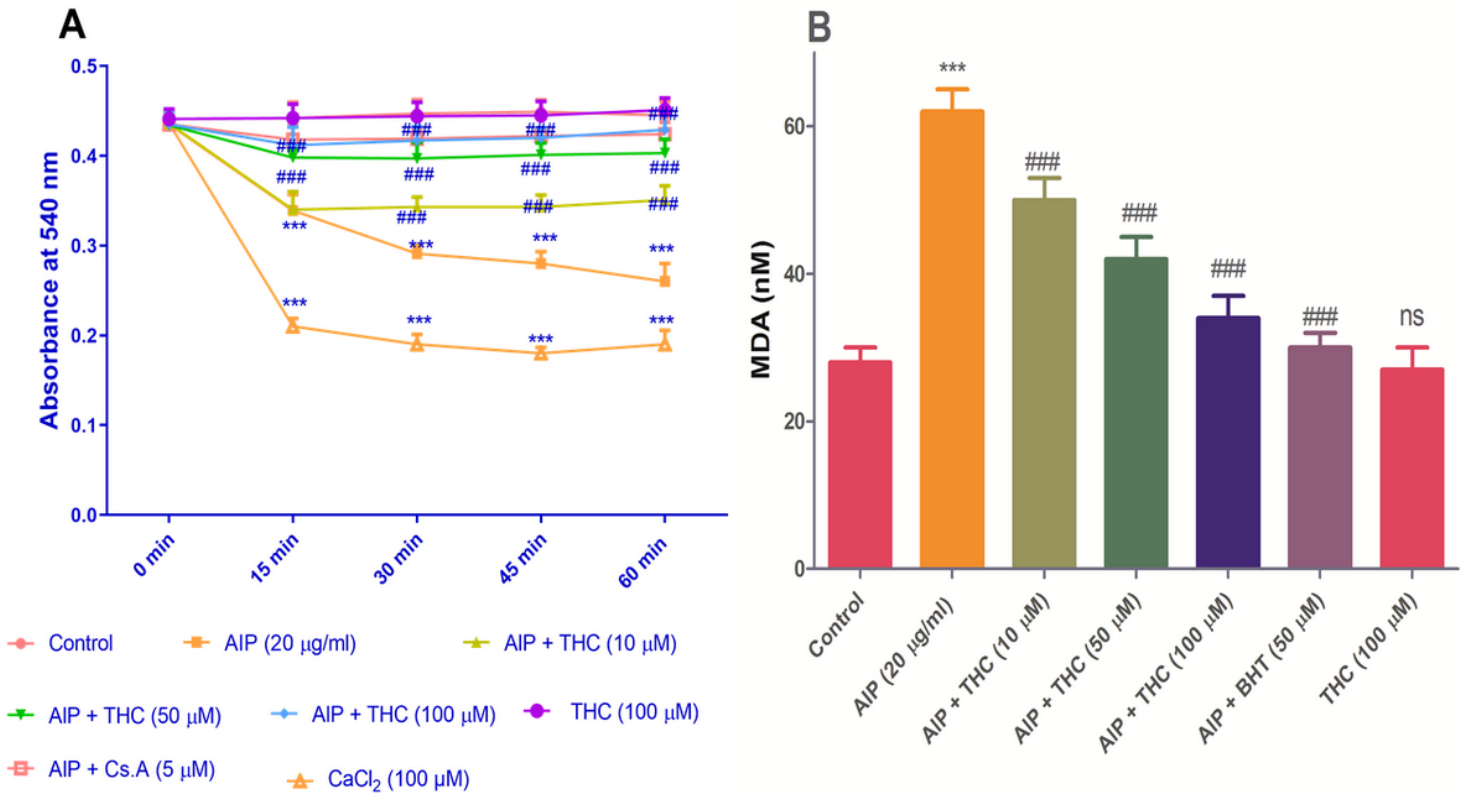

Figure 3

Effects of AlP treatment, THC treatment (100 µM) and concomitant treatment of AlP (20 µg/ml) with 10, 50 and 100 µM THC on mitochondrial swelling (A) and lipid peroxidation (B) in rat heart isolated mitochondria at 60 minutes. Isolated mitochondria were treated or cotreated with the indicated concentrations (20 µg/ml) of AlP and THC (10, 50, and 100 µM) for 60 minutes. (A) Mitochondrial swelling was evaluated by monitoring absorbance at 540 nm. Data showed AlP (20 µg/ml) significantly induced mitochondrial swelling compared to control, while THC (10, 50 and 100 µM) significantly inhibits mitochondrial swelling compared to AlP treated mitochondria. (B) Lipid peroxidation (MDA) levels were measured by production of thiobarbituric acid (TBA) reactive substances (TBARS). Data showed AlP (20 µg/ml) significantly increased lipid peroxidation compared to control, while THC (10, 50 and 100 µM) significantly reduced mitochondrial lipid peroxidation compared to AlP treated mitochondria. Data are mean ± SD of three independent experiments. ***p < 0.001 significantly different from control, ###p < 0.001 significantly different from AlP treated mitochondria. THC, trans-Δ-9-tetrahydrocannabinol; AlP, Aluminium Phosphide; Cs.A, Cyclosporine; CaCl, Calcium chloride

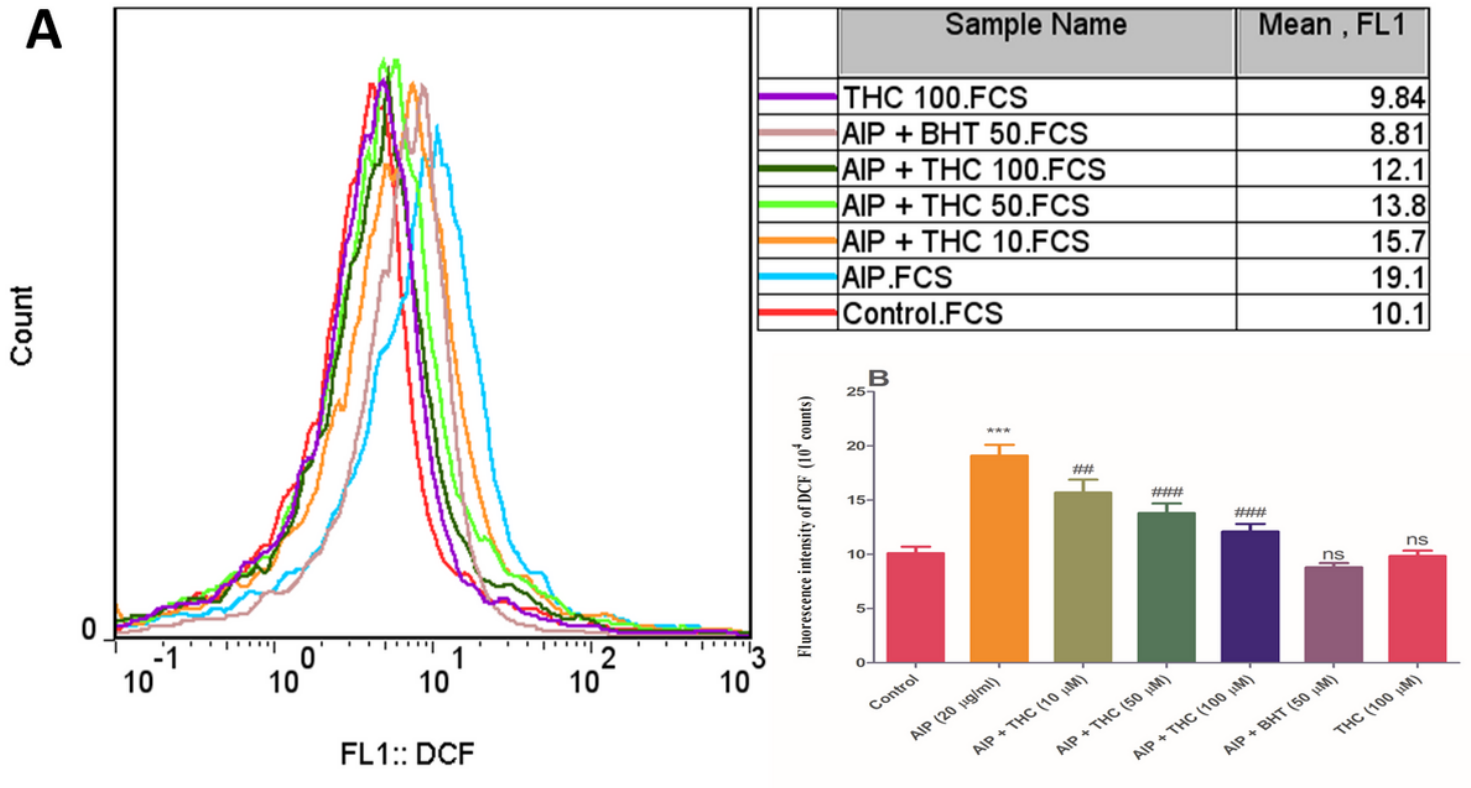

Figure 4

Effects of AlP treatment, THC treatment (100 µM) and concomitant treatment of AlP (20 µg/ml) with 10, 50 and 100 µM THC on mitochondrial ROS formation in rat heart isolated mitochondria at 60 minutes. (A) Mitochondrial ROS formation was analyzed by means of fluorescence intensity of 2',7'-dichlor-fluorescein (DCF) following AlP exposure (60 minutes) in the presence or absence of THC (10, 50 and 100 µM) in rat isolated mitochondria. (B) Bar graphs with statistics. Data are presented as mean ± SD, n = 3, independent experiments repeated at least 3 times, ***p < 0.001: control versus AlP; ###p < 0.001: AlP + THC versus AlP-treated rat isolated mitochondria. THC, trans-Δ-9-tetrahydrocannabinol; AlP, Aluminium Phosphide; BHT, Butylated Hydroxytoluene.

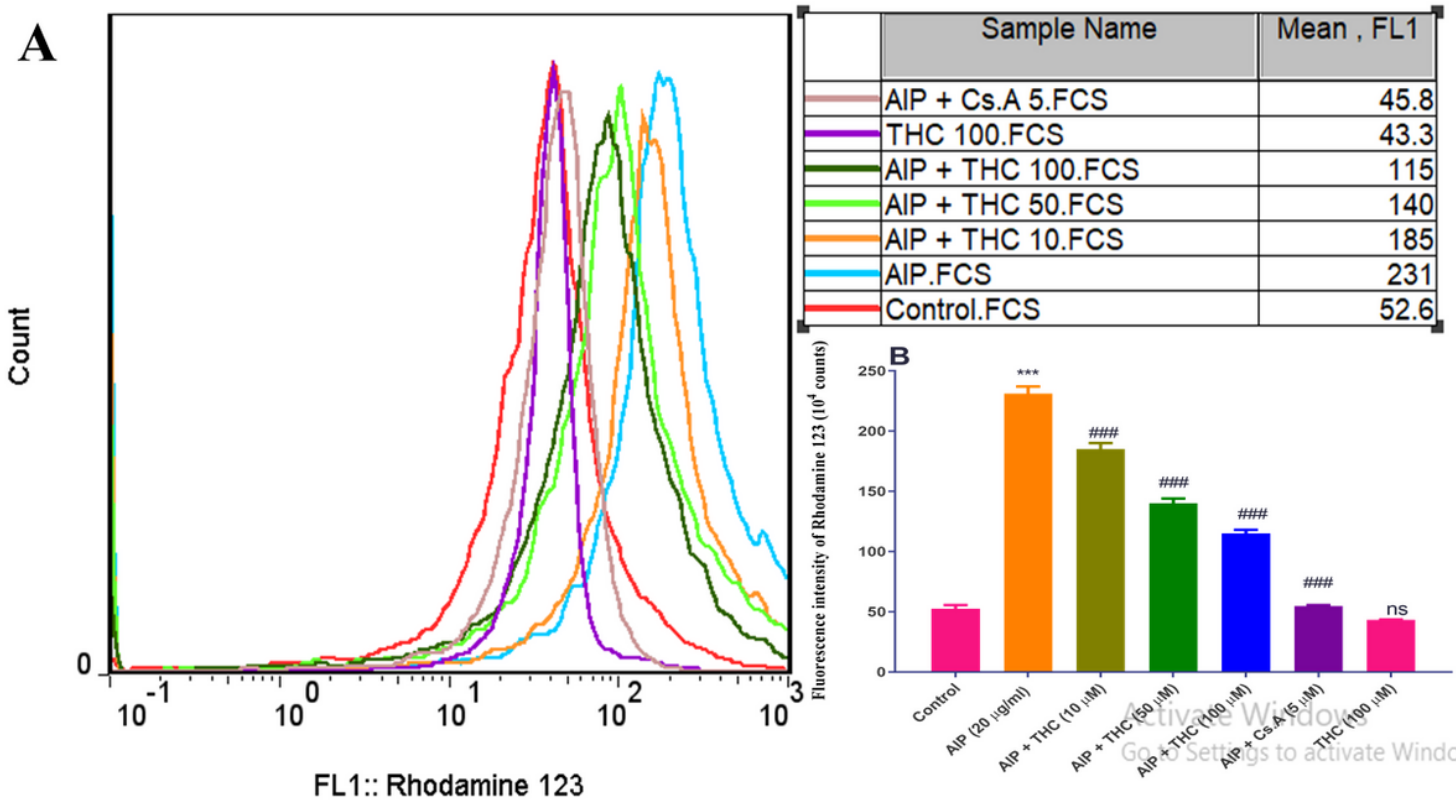

Figure 5

Effects of AlP treatment, THC treatment (100 µM) and concomitant treatment of AlP (20 µg/ml) with 10, 50 and 100 µM THC on mitochondrial membrane potential collapse in rat heart isolated mitochondria at 60 minutes. (A) Mitochondrial membrane potential collapse was analyzed by means of fluorescence intensity of rhodamine 123 following AlP exposure (60 minutes) in the presence or absence of THC (10, 50 and 100 µM) in rat isolated mitochondria. (B) Bar graphs with statistics. Data are presented as mean ± SD, n = 3, independent experiments repeated at least 3 times, ***p < 0.001: control versus AlP; ###p < 0.001: AlP + THC versus AlP-treated rat isolated mitochondria. THC, trans-Δ-9-tetrahydrocannabinol; AlP, Aluminium Phosphide; Cs.A, Cyclosporine; CaCl, Calcium chloride