RESEARCH ARTICLE

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Punica granatum Seed Essential Oil Suppressed Methadone-Induced Cell Death by Natural Antioxidant Activity

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Abstract

Objectives: Methadone is an opioid used in treating chronic and acute pains as well as opioid dependence. It induces death in neural cells. This study investigates Punica granatum oil's effects as a natural antioxidant on methadone-induced cell death. **Materials and methods:** The cell death index indicating the apoptosis occurrence is calculated using the TUNEL test. Rhodamine123 evaluated mitochondrial membrane permeability. Griess reaction was used to detect nitric oxide production. Furthermore, IL-1β, IL-6, INFγ, and TNFα inflammatory cytokines were measured using the Rat inflammatory cytokine assay kit, Rat Kit V-Plex, and the caspase-3 activity was calculated through the Caspase-3 Colorimetric Assay Kit. **Results:** Different treatment processes of *Punica granatum* oil reduced cell cytotoxicity and cell death index and increased viability and proliferation in methadone-treated PC12 cells. NO production decreased in different treatment processes compared to methadone-induced PC12 cells and decreased IL-1β, IL-6, INFγ, and TNFα inflammatory cytokines. In these treatment processes, mitochondrial membrane potential increased, and caspase-3 activity decreased compared to methadone-induced PC12 cells. **Conclusion:** *Punica granatum* essential oil declined methadone-induced cell death in PC12 cells in a dose-dependent manner through suppressing NO production, IL-1β, IL-6, INF-γ, and TNF-α inflammatory cytokines production, mitochondrial membrane disruption, and caspase-3 activities.

Keywords: Methadone- cell death- apoptosis- *Punica granatum* essential oil

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Introduction

D, L-methadone hydrochloride (Methadone) is an agonist for μ opioid receptors, meaning it activates them. It increases potassium conductance mediated by G-protein-coupled. Methadone is commonly used to alleviate pain and treat some narcotics addictions, such as opium and cocaine (Matsui and Williams, 2010; Quillinan et al., 2011; Singer, 2018; Hanna and Senderovich, 2021). Between opioids used clinically, methadone has the longest elimination half-life, and it seems that it acts as a μ-receptor agonist. After a single intraoperative dose, methadone provides stable blood concentrations (Calcaterra et al., 2019; Murphy and Szokol, 2019). Methadone is used in treating chronic pain due to its activity in N-methyl-D-aspartate (NMDR) and μ opioid receptors and seems to be more effective in neuropathic pains (Leppert, 2009; Neumann et al., 2013). Methadone maintenance treatment has beneficially affected opioid-dependent therapy; however, it can impair brain function and structure. Li et al., (2016) suggest that methadone damages the integrity of the white matter in long-time consumers in a dose-dependent pattern. The radiological studies revealed that methadone in high concentration and long-term consumption affects the structure of the cerebellum, basal ganglia, and deep white matter of cerebral hemispheres (Cerase et al., 2011; Corré et al., 2013). A study on the SH-SY5Y cell line showed that high concentrations of methadone induced cell death in this undifferentiated human neuroblastoma cell line. The cells exposed to methadone exhibited some changes, including the mitochondrial outer membrane's high permeability and BAX's transfer into mitochondria, leading to cytochrome C release (Perez-Alvarez et al., 2010; Lee et al., 2021). Previous studies have demonstrated that chronic opiates use, such as methadone, for medical purposes may cause toxicity in brain cells and repress neurogenesis (Andersen et al., 2011). Two signal paths have been suggested regarding methadone: one for binding to opioid receptors and another for acting as an antagonist in NMDR (Sotgiu et al., 2009; Kazemi Roodsari et al., 2019). It seems that the NMDR pathway would have more evident effects on methadone toxicity in cortical cell cultures (Nylander et al., 2016). A study on mitochondria of methadone-treated

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rat livers showed that methadone causes mitochondrial uncoupling, and since it has ion transferability, it induces cell death due to its ability to significantly decrease cellular ATP levels (Mintzer and Stitzer, 2002; Rass et al., 2014). Methadone alters the mitochondrial outer membrane permeability by translocating Bax into the mitochondria and releasing cytochrome c.

Interestingly, methadone induces a drastic depletion in cellular ATP levels by impairing ATP synthesis. Methadone-induced mitochondrial uncoupling might be due to its ionophoric properties (Perez-Alvarez et al., 2010; Ahmeda et al., 2020). Increasing reactive oxygen species production and calcium are critical for inducing cell death. Methadone does not produce reactive oxygen species directly, and this increase may be due to the toxicity initiated by the primitive increase in intracellular calcium. In this regard, the general deactivation of antioxidants occurs in the presence of glutamate (Vergun et al., 2001).

In recent years, many articles indicated that medicinal plants had therapeutic properties. These studies revealed that natural drugs are the only treatment modality in some cases, and their available compounds have been used in pharmaceutical industries (Rawat et al., 2016; Bouyahya et al., 2017; Dar et al., 2017). In this regard, Punica granatum, as a member of the Punicacea family, is an ethnic medicinal plant with therapeutic potential for many diseases (Arun and Singh, 2012; Moga et al., 2021). It possesses antioxidant, antibacterial, and cardioprotective properties and is beneficial for many diseases, such as bronchitis, diarrhea, digestive problems, and wounds, which might be due to its chemical constituents and components (Jasuja et al., 2012). Anthocyanins, phenolic acids, flavones, and alpha estradiol are the most important components of *P. granatum* peels and seeds essential oil, responsible for its therapeutic properties (Kim and Kim, 2002; Lansky and Newman, 2007). The *P. granatum* seems to be a good choice for assessing its protective potential against cellular damage because of its antioxidant and therapeutic properties. So far, no study has been conducted on the protective properties of Punica granatum essential oil against the opioid substances' destructive effects on nerve cells. Therefore, in this study, we aimed to measure the effect of different concentrations of Punica granatum essential oil on methadone-induced cell death in nerve-like PC12 cells.

Materials and Methods

Plant sample collection and essential oil extraction

This empirical study collected 1500gr of Punica granatum seeds in Kermanshah, Iran (geographical coordinates: 34.3277°N and 47.0778°E). The plant was identified, and a voucher specimen (no. 2886 RUH) was deposited at the herbarium of the Research Center of the Faculty of Agriculture, Razi University, Kermanshah, Iran. The leaves were dried in shadow, and after grinding, each time 200gr of the obtained powder was dissolved in 2000mL ethanol and put in Clevenger apparatus for eighth. Subsequently, the hydrolate was collected and centrifuged at 10,000rpm for 10min. The organic phase was removed with a Pasteur pipette and subsequently transferred to an Eppendorf tube, intricate in parafilm and aluminum foil, and stored in a freezer at -20ºC. The oil's yields were assessed based on the dry mass. The experiment was performed in triplicate.

Gas chromatography/Mass spectrometry (GC/MS)

P. granatum essential oil was analyzed by GC-MS (Thermo Quest Finnigan, UK). The GC-MS device was equipped with HP-5MS 5% phenyl methyl silicone capillary column (30.00m length × 0.25mm ID and 0.25µm film thickness). Helium flow rate with the split ratio Mohammad Mahdi Zangeneh et al., / TEOP 21 (5) 2018 1349 - 1358 1350 of 1:3 was kept at 2mL/min. The GC analysis was performed three times. The components were quantified using a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector (GC-FID). A Supelco SPB-5 (5% diphenyl/95 % dimethylpolysiloxane) capillary column (30m × 0.25mm with a 0.25µm film) was utilized while using nitrogen as the carrier gas. Each component's percentage, retention index, and time were computed from the relative peak regions. The analyses were carried out twice.

Cell culture

A rat neuron-like cell line, PC12 (ATCC® CRL-1721TM) cells were cultured using Gibco RPMI1640 cell culture medium. The cell culture was supplemented with 10% fetal bovine serum (FBS, Gibco), 100IU/ml penicillin (Sigma), and 100µg/ml streptomycin (Sigma). The cells were incubated at 37ºC and 5% CO2 and kept in T-25cm2 tissue culture. DMSO solved the essential oil and essential oil was added to the culture medium with a final volume of 0.1%. The cells were plated and after 12h PBS, at 37°C, washed, and the cells were treated in different treatments for 48h. the nine groups are listed below: methadone cell culture containing 100mM methadone, Control: cell culture medium without methadone and *P. granatum* essential oil; Treatment-1: cell culture contains 100mM methadone and 1µg of *P. granatum* essential oil; Treatment-2: cell culture contains 100mM methadone and 2µg of *P. granatum* essential oil; Treatment-3: cell culture contains 100mM methadone and 5µg of *P. granatum* essential oil; Treatment-4: cell culture contains 100mM methadone and 10µg of *P. granatum* essential oil; Treatment-5: cell culture contains 100mM methadone and 20µg of *P. granatum* essential oil; Treatment-6: cell culture contains 100mM methadone and 40µg of *P. granatum* essential oil; and Treatment-7: cell culture contains 100mM methadone and 80µg of *P. granatum* essential oil.

Cell viability

In this study, Trypan blue 0.4% solution was used to measure cell viability. The cells were plated in 96-wells cell culture plates for 12h at a 5×10^4 cells/mL density. After 48h incubation at 37ºC in 5% CO_2, the cells were trypsinized, and 100µL of the cell suspension was combined with 20µL of Trypan blue 0.4%. After 1-2 minutes, the cells were suspended by the Neubauer Lamel, and the cell viability was measured by dividing the uncolored cell number into the total number (Strober,

2015).

Cell proliferation

5mg/mL of MTT powder (sigma) was added to different treatments and incubated for 3h. The supernatant from each well was removed, and 100µL of DMSO was added to dissolve the formazan crystals at room temperature for 30min. Then 200µL of DMSO was added to each well, and an ELIZA Reader reported different treatments' absorption rates at 570nm by a reference of 630nm (Loveland et al., 1992).

Cell cytotoxicity

LDH cytotoxicity kit was used for measuring the cell cytotoxicity percentage. At first, the cells were plated in 24-well culture plates at a 104cells/mL density for 12h; then, they were exposed to different treatments for 48h. The cytotoxicity percentage was measured through the company's protocol. ELISA Reader (EL800; USA) measured the samples' absorbance at 490nm, indicating the LDH activity. The reference wavelength should be more than 600nm (Fotakis and Timbrell, 2006).

Cell death index

According to the company's protocol, the TUNEL staining test was performed by an in-situ cell death detection kit (Roche) to measure the cell death index and apoptosis in different treatments. In this regard, PC12 cells were fixed, and an Olympus AX-70 fluorescent microscope was used to count the TUNEL-positive cells in eight treatments randomly. The cell death index was achieved by dividing the apoptotic cell number into the total (Kyrylkova et al., 2012).

Nitric oxide test

After 48h exposing the cells to different treatments, the produced NO in the cells was measured using the Griess reaction (Sun et al., 2003).

Anti-inflammatory and anti-inflammatory cytokines Secretion

After 48h of exposing the cells to different treatments, the pre-inflammatory cytokines IL-1β, IL-6, INFγ, and TNFα were measured using the rat inflammatory cytokine assay kit, Rat Kit V-Plex, based on the company's protocol.

Mitochondrial membrane potential (MMP)

This study used rhodamine-123 (sigma), as lipophilic cationic fluorescents penetrating cells were used to detect the potential changes in the mitochondrial membrane. In this regard, 10mg/mL rhodamine-123 was used for each treatment for half-hour. Then, PBS was used for cell washing, and 900µL triton-x100 (sigma) was added to each treatment for 2h at 4◦C. After centrifuging the cells at 130000rpm for 15min, the cells went through a fluorescence microplate reader to measure fluorescence at 488nm excitation and 520nm emission wavelength (Baracca et al., 2003).

Caspase-3 activity

PC12 cells in a 5×10^5cell/mL density were cultured in a 24-well cell plate using PRMI 1640 medium culture and 0.2% BSA. After 12h, the plates were washed with PBS; then, they were exposed to different treatments for 48 h. Trypsin separated the cells from the wells. The cells were centrifuged for 10min, and their supernatant was removed. Afterward, the cells were centrifuged for 1min at 10000rpm after adding 25mL of lysate buffer. Supernatants containing proteins were transferred to a 96-well plate, and 5µl N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline DEVD-pNA was added to the plate and incubated at 37◦C for 2h. The release of pNA indicates the caspase-3 activity, calculated using the Caspase-3 Colorimetric Assay Kit and Biotek (USA) Spectrophotometer (Gurtu et al., 1997).

Statistical analysis

In this study, statistical analyses were performed using ANOVA and T-test. In all cases, the P value of less than 0.05 was considered statistically significant.

Results

Chemical composition

P. granatum essential oil yield was 7.13 (19.21mL), calculated on a plant's fresh seeds. Overall, eight compounds, including Octanoate ethyl ester, Doconoate ethyl ester, Farnesene, Palmitate ethyl ester, Linoleic acid ethyl ester, Docosane, Tetracosane, and Trans-Squalene were identified in *P. granatum* essential oil using GC-MS (Table 1). Farnesene (44.2.0%) and Trans-Squalene (32.9%) were the most frequently detected compounds.

Cell viability

The cells were exposed to different treatments, and after 48h, trypan blue was used to measure the cell viability in these treatments. In the methadone group, all the cells died, and the cell viability percentage was 0%. The cell viability of treatments 1-7 was higher than the methadone group ($p<0.05$). The cell viability of treatments 1-6 was significantly lower than the control treatment, but the difference between treatment 7 and the control group was insignificant. A significant intra-group difference was observed between treatments 1-7 in terms of cell viability ($p<0.05$) (Figure 1).

Table1. Main Composition of the Essential Oil of *Punica granatum* Seeds Using Gas Chromatography Mass Spectrometry Method

No.	Compound	RI	RT	Percent of total
1	Octanoate ethyl ester	1186	21.234	1.9
2	Doconoate ethyl ester	1307	27.842	2.4
3	Farnesene	1413	30.693	44.2
4	Palmiate ethyl ester	1932	32.171	1.7
5	Linoleic acid ethyl ester	2099	32.985	1.6
6	Docosane	2153	33.694	7.9
7	Tetracosane	2373	34.573	7.3
8	Trans-Squalene	2700	36.41	32.9
Total				99.9

RI, Retention index; RT, Retention time

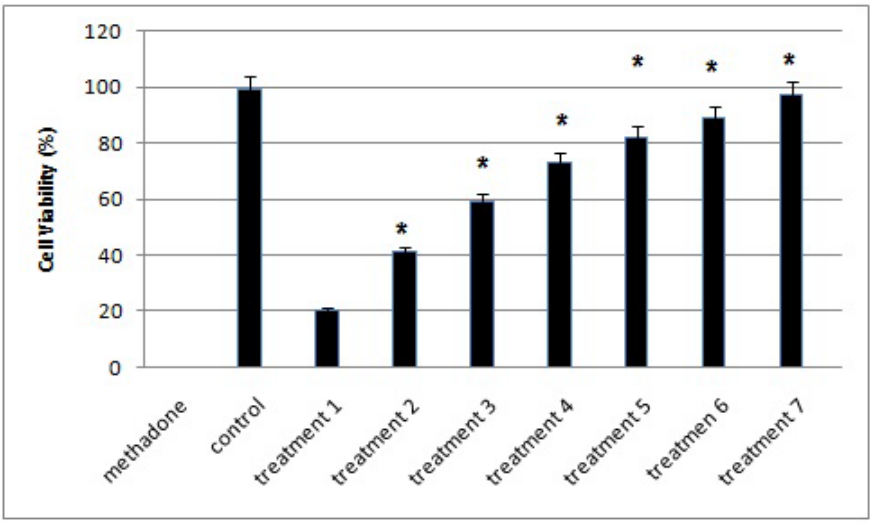

Figure 1. The Cell Viability of Different Treatments after 48h. Methadone cell culture contain 100mM methadone, Control: cell culture medium without methadone and P. granatum essential oil; treatment 1 through 7 correspond to cell culture containg 100mM and P. granatum essential of 2, 5. 10, 20, 40, 80, and 160 ug. All data represented by mean ± S.E.M ($p<0.05$).

Cell proliferation

MTT test was performed to assess cell proliferation in different treatments after 48h. The killer dose of 100μM of methadone suppressed cell proliferation in PC12 cells. The cell proliferation percentages in treatments 1-7 were higher than the methadone group by significant intragroup difference. The cell proliferation percentage of treatments 1-7 was lower than the control group, but there was no significant difference between treatment 7 and the control group ($p<0.05$) (Figure 2).

Cell cytotoxicity

The result of the LDH kit after 48h showed that PC12's cell toxicity in the apoptotic dose of methadone was 0%. The cell cytotoxicity percentage of treatments 1-7 was lower than the methadone group by significant intra-group differences. Cell cytotoxicity of treatments 1-6 was significantly higher than the control group, but the difference between the cell cytotoxicity of treatment 7 and the control group was insignificant ($p<0.05$) (Figure 3).

DNA fragmentation and cell death index

After 48h, different treatment images were taken in the TUNEL test. The TUNEL test describes DNA fragmentation condition and apoptosis occurrence. The TUNEL test's results showed that 100% of PC12 cells in the methadone group were exposed to DNA fragmentation and apoptosis. The cell index in treatments 1-7 was significantly lower than in the methadone group. The cell death index in treatments 1-6 was higher than that of the control cell culture by an intra-group difference, but the

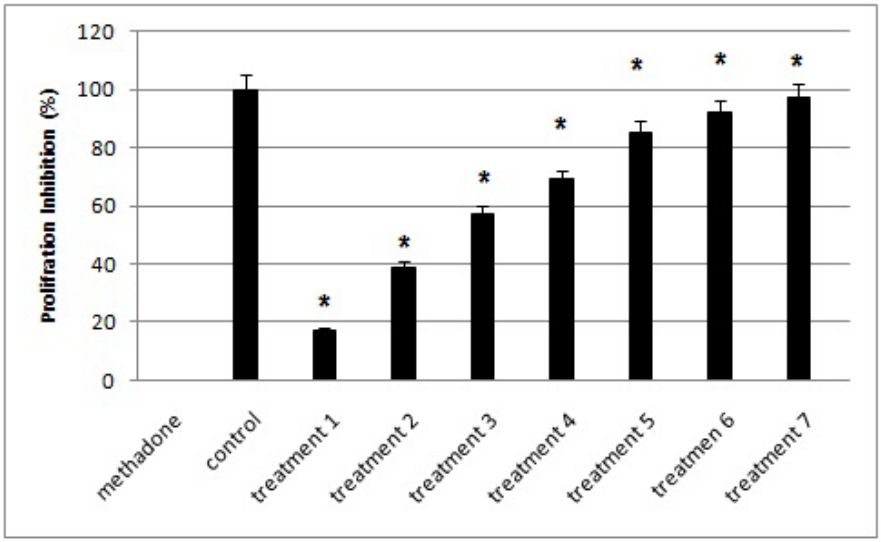

Figure 2. The Cell Proliferation of Different Treatments after 48h. Methadone cell culture contain 100mM methadone, Control: cell culture medium without methadone and P. granatum essential oil; treatment 1 through 7 correspond to cell culture containg 100mM and P. granatum essential of 2, 5. 10, 20, 40, 80, and 160 ug. All data represented by mean ± S.E.M ($p<0.05$).

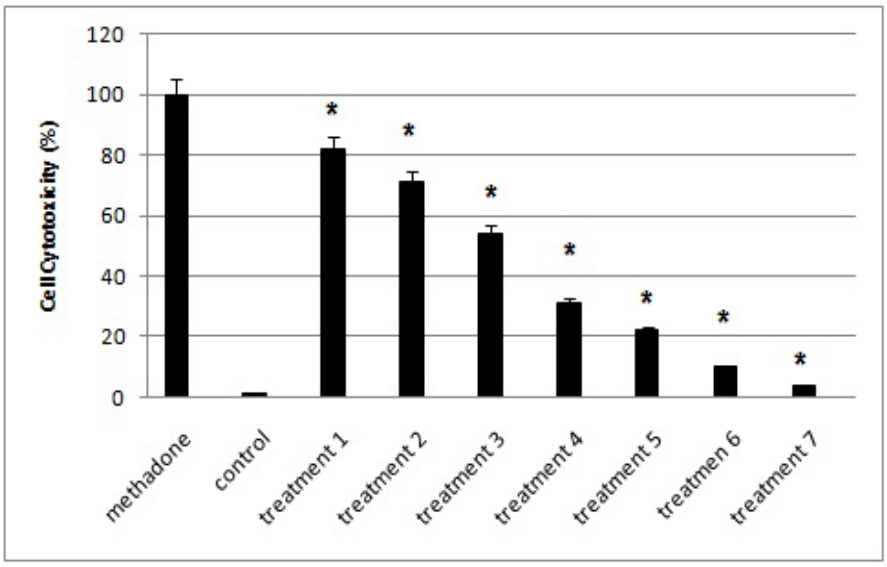

Figure 3. The Cell Cytotoxicity of Different Treatments after 48h. Methadone cell culture contain 100mM methadone, Control: cell culture medium without methadone and *P. granatum* essential oil; treatment 1 through 7 correspond to cell culture containg 100mM and P. granatum essential of 2, 5. 10, 20, 40, 80, and 160 ug. All data represented by mean ± S.E.M ($p<0.05$).

cell death index of treatment 7 and the control group were similar ($p<0.05$) (Figure 4).

Production of nitric oxide in different treatments

After 48h, the production of nitric oxide (NO) was measured using the Griess reaction assay. The concentration of NO in Treatments 1-7 was significantly lower than the methadone group, with an intra-group significant difference. The NO levels of treatments 1-6 were lower than the control group but the NO level of treatment 7 has no difference by the control group ($p<0.05$) (Figure 5).

The Concentration of inflammatory cytokines in treated cells

The results showed that the concentration of IL-1β, IL-6, INF-γ, and TNF-α inflammatory cytokines in treatments 1-7 were lower than the methadone group by significant intra-group differences ($p<0.05$). The concentration of IL-1β, IL-6, INF-γ, and TNF-α inflammatory cytokines in treatments 1-6 was higher than in the control group ($p<0.05$), but there was no significant difference between treatment 7 and the control group (Figure 6).

Mitochondrial membrane potential

Rhodamine-123 absorbance results after 48h showed that 100mM methadone caused fatal mitochondrial membrane potential disruption in PC12 cells. The

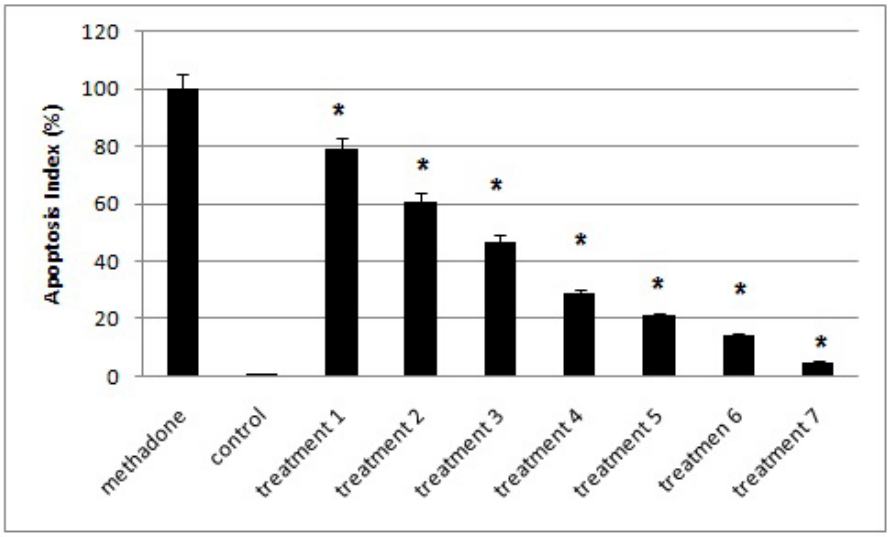

Figure 4. The Cell Death Index of Different Treatments after 48h. Methadone cell culture contain 100mM methadone, Control: cell culture medium without methadone and P. granatum essential oil; treatment 1 through 7 correspond to cell culture containg 100mM and P. granatum essential of 2, 5. 10, 20, 40, 80, and 160 ug. All data represented by mean ± S.E.M ($p<0.05$).

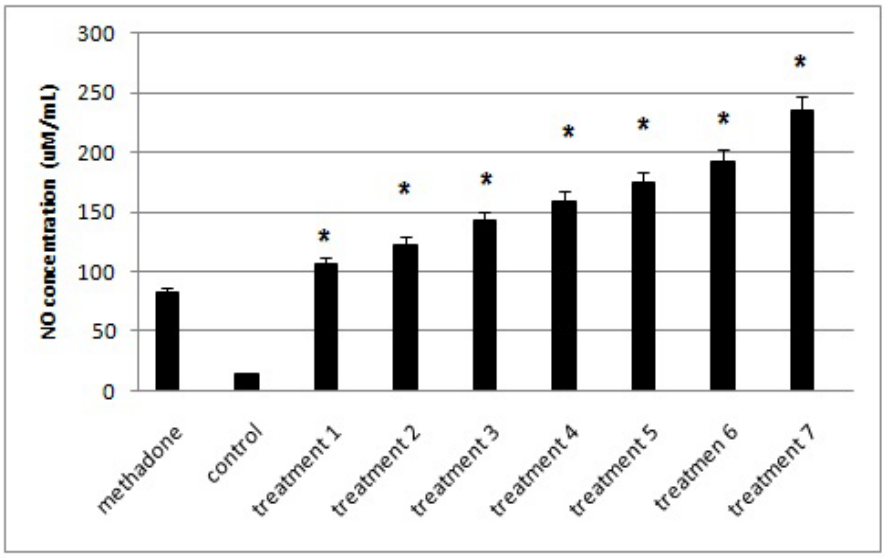

Figure 5. The Production of NO in Different Treatments after 48h. Methadone cell culture contain 100mM methadone, Control: cell culture medium without methadone and P. granatum essential oil; treatment 1 through 7 correspond to cell culture containg 100mM and P. granatum essential of 2, 5. 10, 20, 40, 80, and 160 ug. All data represented by mean ± S.E.M ($p<0.05$).

mitochondrial membrane potentials in treatments 1-7 were higher than in the methadone group by significant intra-group differences. The mitochondrial membrane potentials in treatments 1-6 were lower than the control group, but there was no significant difference between treatment 7 and the control group (Figure 7). The mitochondrial membrane potential in treatments 1-7 was higher than in the methamphetamine group ($p<0.05$).

Caspase-3 activity

Caspase-3 activities in treatments 1-7 were lower than the methadone group by significant intra-group differences. Caspase-3 activity in treatments 1-6 was higher than in the control group, but no significant difference was observed between treatment 7 and the control group ($p<0.05$) (Figure 8).

Discussion

Jin-Soun Jung analyzed the components in the root, stem, and fruit peels of *P. granatum* by thermal desorption gas chromatography/mass spectrometry (TDGC/MS) and reported twenty-four, thirty, and sixty-two compounds in the root, stem, and fruit peels, respectively. Monoterpene hydrocarbons, such as α-Pinene (13.49%), 2-β-Pinene (16.31%), δ-3-Carene (22.94%), and dl-Limonene

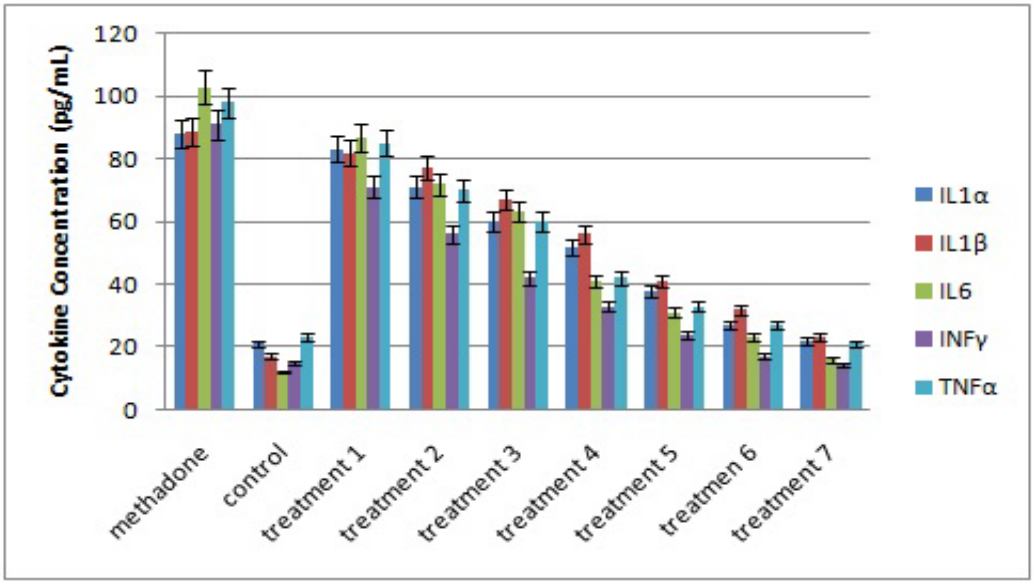

Figure 6. The Production of IL-1β, IL-6, INF-γ, and TNF-α Inflammatory Cytokines in Different Treatments after 48h. Methadone cell culture contain 100mM methadone, Control: cell culture medium without methadone and P. granatum essential oil; treatment 1 through 7 correspond to cell culture containg 100mM and P. granatum essential of 2, 5. 10, 20, 40, 80, and 160 ug. All data represented by mean ± S.E.M ($p<0.05$).

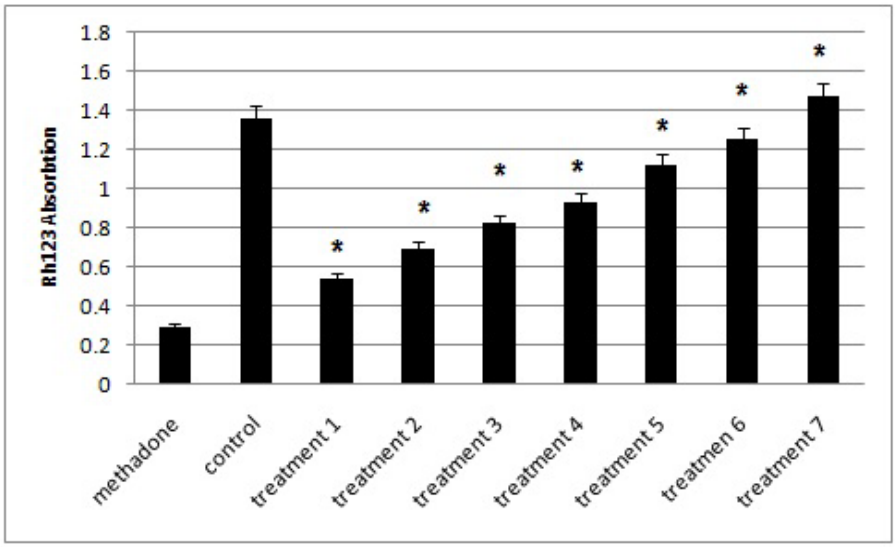

Figure 7. The Mitochondrial Membrane Potential of Different Treatments after 48h. Methadone cell culture contain 100mM methadone, Control: cell culture medium without methadone and P. granatum essential oil; treatment 1 through 7 correspond to cell culture containg 100mM and P. granatum essential of 2, 5. 10, 20, 40, 80, and 160 ug. All data represented by mean ± S.E.M ($p<0.05$).

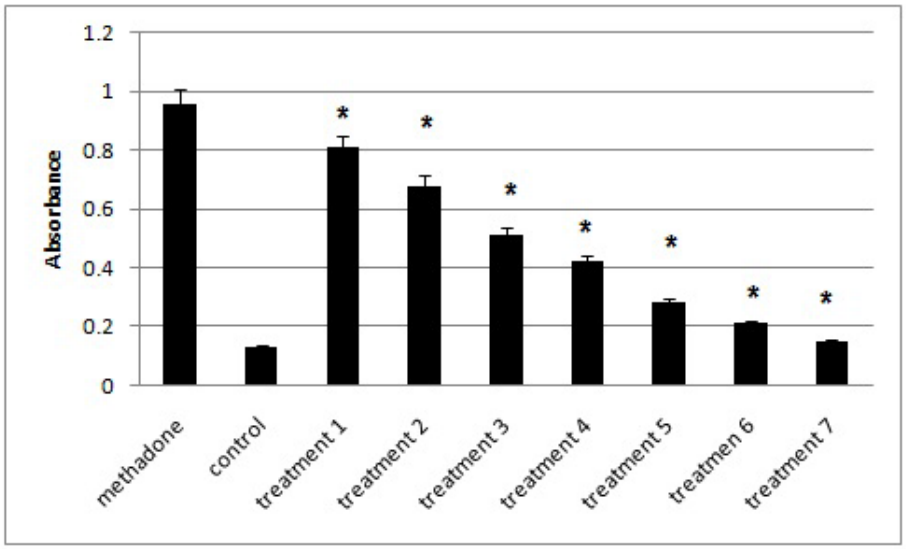

Figure 8. The Activation of Caspase 3 in Different Treatments after 48h. Methadone cell culture contain 100mM methadone, Control: cell culture medium without methadone and P. granatum essential oil; treatment 1 through 7 correspond to cell culture containg 100mM and P. granatum essential of 2, 5. 10, 20, 40, 80, and 160 ug. All data represented by mean ± S.E.M ($p<0.05$).

(17.33%), were found to be the major volatile compounds in the root peels (Jung, 2014). Ángel Calín-Sánchez et al. revealed that trans-2-Hexenal, 3-Carene, α-Terpinene, and α-Terpineol are the most abundant among the 18 compounds found in *P. granatum* essential oil (Calín-Sánchez et al., 2011). Methadone's adverse effects on the central nervous system seem more critical since it directly affects its receptors (Inturrisi, 2005; Zanin et al., 2010). Methadone's high concentrations toxicity causes cell death and reduces the potential for cell proliferation among nerve cells (Perez-Alvarez et al., 2010; Flanagan and Shepherd, 2022). The results of the present study agreed with these findings and showed that methadone at high concentrations caused cytotoxicity and reduced the cell viability and productive potential in a nerve-like cell line, PC12 cells. Treating these cells with high doses of *P. granatum* essential oil enhanced cell viability and proliferation potential by reducing cell cytotoxicity. Due to its antioxidant potential, *P. granatum* essential oil apparently reduces cell cytotoxicity in neuron-like cells. Today, antioxidants are introduced as cell cytotoxicity reducers since they inhibit ROS production and oxidative stresses in the cells.

Yuan-YuChan et al. revealed that methadone maintenance treatment of opioid-dependent individuals affects the immune system activities; hence, it may also begin long-term inflammations in the nervous system. Furthermore, they exhibited that longer methadone

maintenance treatment increases cytokine IL-1β levels and high-dose methadone increases cytokine IL-6 and TNF-α levels (Chan et al., 2015). Our results showed that high-dose methadone increased NO production leading to an increase in many inflammatory cytokine concentrations, such as IL-1β, IL-6, INF-γ, and TNF-α. These findings indicate that nerve cells are involved in systemic inflammations at high concentrations of methadone. Moreover, our findings showed that *P. granatum* essential oil, in a dose-dependent manner, reduced inflammatory cytokines production by reducing NO production. It may be due to its antioxidant potential.

Claudia Friesen et al. revealed that methadone induces cell death, in leukemia cells, through the induction of apoptosis and inhibition of cell proliferation. Many processes were involved in this pathway, for example, caspase-9 and -3. In addition, Bcl-xl and X chromosome-linked apoptosis inhibitors down-regulated the poly (ADP-ribose) polymerase cleavage in this pathway (Friesen et al., 2008). Caspases are vital for inducing apoptosis. Methadone activates the mitochondrial apoptosis pathway through the ligand-receptor system and can stimulate it directly. The Bcl2 family members are the mitochondrial apoptosis pathway regulators (Kroemer, 1997; Hengartner, 2000; Kaufmann and Earnshaw, 2000). This type of apoptosis causes many changes in the mitochondria. For example, free oxygen radicals are produced, leading to pore formation in the mitochondrial membrane. In the following, some factors, such as cytochrome C, caspase-2 or -9, and the apoptosis-inducing factor, are released from the mitochondrial membrane into the cytoplasm and activate Caspase-3. For caspase-3 activation and apoptosis induction, apoptosis complexes consisting of Caspase-9, Cytochrome C, and Apaf-1 are formed (Kaufmann and Earnshaw, 2000; Elena-Real et al., 2018). In this research, studying apoptosis using the TUNEL test showed that high-dose methadone causes DNA fragmentation and induces apoptosis in nerve-like PC12 cells. Further experiments showed that methadone causes apoptosis in these cells by decreasing the mitochondrial membrane potential and increasing caspase-3 activity. Our findings also indicate that *P. granatum* essential oil increased the mitochondrial membrane potential in PC12 cells treated with methadone. The increased rhodamine123 absorption shows the enhanced mitochondrial membrane potential indicating increased proton pump activity (Zorova et al., 2022). Activated proton pumps regulate mitochondrial membrane activity and suppress apoptosis induction. Reducing the mitochondrial membrane potential leads to cytochrome C release and activates the caspase cascade. Based on this study, *P. granatum* essential oil decrease caspase-3 activity, another sign of this substance's suppressive effect on methadone's apoptosis induction in PC12 cells. *P. granatum* essential oil, with its antioxidant powers, seems to reduce NO production in methadone-treated PC12 cells, ultimately leading to reduced inflammation and apoptosis.

In conclusion, this study concluded that high-dose methadone causes cell death in nerve-like PC12 cells by inducing cell cytotoxicity, inflammation, and apoptosis. *P. granatum* essential oil reduced cell cytotoxicity and increased cell viability and proliferation in high-dose methadone-treated PC12 cells. It suppressed NO production, IL-1β, IL-6, INF-γ, and TNF-α inflammatory cytokines production, mitochondrial membrane disruption, and caspase-3 activity. These events show that *P. granatum* essential oil suppressed methadone-induced cell death in PC12 cells in a dose-dependent manner.

Author Contribution Statement

All authors contributed equally in this study.

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Funding and Ethics approval and consent to participate

This study is approved by the scientific committee of the Kermanshah ACECR Institute of Higher Education and is part of Mr. Mahdizadeh's thesis in collaboration with Dr. Hossein Zhaleh at the Dept. of Medicinal Plants of Kermanshah ACECR Institute of Higher Education, Iran.

Human and animal rights

No Animals/Humans were used for studies that are base of this research.

Availability of data and materials

All data generated during this study are included in this published article. Further data supporting the findings of the current article are available from the corresponding author (Dr. Hossein Zhaleh, Email: hossain_jale@yahoo.com) on reasonable request.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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