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Ajwain and its phenolic compound, carvacrol improve cadmium-induced
apoptosis in PC12 cells

(Ajwainとそのフェノール化合物であるカルバクロールは、**PC12**細胞に
おけるカドミウム誘導アポトーシスを改善する)

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Abstract

In the modern world, the environment has been contaminated enormously with many types of toxicants. As results, humans are facing the exposure of more than a thousand types of chemicals every day. Among these contaminants, heavy metals such as lead (Pb), cadmium (Cd) and mercury (Hg) are of great concerns worldwide due to their high toxicity against biological functions. Moreover, these metals can lead to a pathophysiological status of diseased conditions at their low concentrations. Among the heavy metals, Cd has picked up more attention for its ubiquitous characters depending on modern anthropogenic activities. Cd is also present in many foods, so Cd is easily taken into the human body. As Cd pollution is a global issue, many researchers have focused on that. Cd has a longer biological half-life and bioaccumulative tendency in biological systems including humans. The acute and chronic exposures with Cd can induce cellular, metabolic and physiological toxicities. In other words, Cd causes diseases involving tissues and organ systems including lungs, liver, gastrointestinal tract, reproductive system and cardiovascular system. Although the pattern of toxicity exerted by Cd is very complex, researchers have already approached to unveil the mechanisms *in vivo* and *in vitro*, and achieved some success. However, there is no therapy by using drug or chelation therapy against the toxicity of Cd. So, it will be required to explore and develop new anti-toxic agents against Cd. On the other hand, dietary interventions, traditional medicinal approaches or natural products for toxicity alleviation can be expected to act as better preventive measures against Cd toxicity without side effects. For the purpose, keeping both preventive measure and development of new therapeutic agent in mind, recently researchers have made efforts to clarify the ameliorating roles of numerous anti-oxidative plant extracts and phytochemicals on heavy metal toxicity and their mechanism. The objective of this research has been set to clarify the effects of ethanolic extract of ajwain (*Trachyspermum ammi* L.) seed and its phenolic compound, carvacrol on Cd-induced cytotoxicity, oxidative stress and apoptosis. Moreover, the molecular mechanisms behind these alleviating roles have also been resolved.

Ajwain is a popular spice, and has historically been used for the remedy of many pathological consequences such as inflammatory, toxic, neurological, genital and respiratory tract disorders. These symptoms are also common in Cd toxicity manifestations. Moreover,

the revalidation of these numerous therapeutic use has been achieved by demonstrating research works about the therapeutic effects of ajwain extracts on disease model animals with molecular clarifications. Additionally, various ajwain extracts have been found as Toxicity ameliorating agents against few organic and inorganic toxicants *in vivo* and *in vitro*. In chapter 2, ethanolic extract of ajwain (AE) has been investigated to clarify the ameliorating effects against Cd-induced cytotoxicity and apoptosis in PC12 cells. First, the non-toxic doses of AE were determined to observe the effects of AE on the toxicity against Cd (5 or 10 μ M) upon simultaneous exposures for 24 h. The safe but most effective dose of AE (240 μ g/mL) considerably improved the viability of PC12 cells treated with both concentrations of Cd. From the results of cell viability and lactate dehydrogenase (LDH) activity in the media, it was indicated that AE reduced cytotoxicity and increased cell membrane integrity. Oxidative stress and apoptosis were observed in PC12 cells treated with Cd. These were proved by decreases in intracellular reduced glutathione (GSH) levels and higher DNA fragmentations. AE significantly improved the cellular anti-oxidative status by increasing the GSH levels, and decreased the Cd-induced DNA fragmentation. In addition, the western blotting results revealed that AE down-regulated the Cd-induced increased expression of apoptotic protein Bax, and up-regulated suppressed expressions of anti-apoptotic proteins Bcl-2, Bcl-xL and NF-KB. Moreover, the Cd-induced release of cytochrome c from mitochondria into the cytosol was reduced by co-treatment with AE. The expressions of caspase-3 increased by Cd were also significantly decreased by AE. From all these findings, it has been suggested that AE reduced Cd-induced cytotoxicity and apoptosis in PC12 cells due to improvement of intrinsic pathway of apoptosis.

Like other ajwain extracts, AE has already been phytochemically characterized in previously reported research. It was found that AE contains carvacrol (iso-thymol) as the major phenolic compound in highest concentration. Moreover, in this research, the highest intensity peak in LC-MS analysis profile of AE was shown as carvacrol. Therefore, the next aim of the research was turned towards to clarify whether the improvement effects of AE on Cd-induced cytotoxicity and apoptosis was due to carvacrol or not.

Carvacrol, is a monoterpenoid phenol found in many *Labiatae* and *apiaceae* family plants. Numerous *in vivo* and *in vitro* studies have conducted on biological activities of carvacrol including anti-inflammatory and anti-carcinogenic effects. Previous studies have

already reported that carvacrol showed high antioxidative potential and also the protective effects against various toxicants *in vivo* and *in vitro*. In chapter 3, the effects of carvacrol on Cd-induced cytotoxicity and apoptosis in PC12 cells has been investigated. First, the non-toxic concentrations of carvacrol were found in between 0 and 200 μ M in PC12 cells. Upon co-exposure with Cd (10 μ M) for 48 h the dramatic improvement of cell viability was found for 100 μ M of carvacrol. The simultaneous exposure of carvacrol with Cd reduced LDH level, and increased the GSH levels and glutathione reductase expression. Carvacrol also reduced Cd-induced DNA fragmentation. The rate of apoptosis in Cd-exposed cells was considerably reduced by the co-exposure of carvacrol. Co-exposure of carvacrol also up-regulated the down-regulated expressions of mTOR, Akt, NF- κ B, ERK-1 and NRF2 by Cd. Additionally, carvacrol reduced the cleavage of caspase 3, the cytosolic level of cytochrome c, and expression of apoptosis inducing factor (AIF). Carvacrol induced the metallothionein expression in PC12 cells upon co-exposure with Cd. Overall, it was proposed that carvacrol reduced Cd-induced cytotoxicity, oxidative stress, and caspase-dependent and -independent apoptosis in PC12 cells.

From the results in chapter 3, it was able to guess that the improvement effects of AE on Cd-induced apoptosis were due to carvacrol. Therefore, it was suggested that both AE and carvacrol were able to act as anti-oxidative and anti-apoptotic agents in bio-organism. Finally, these two agents have been recommended as potential and safe therapeutic agents against Cd toxicity and other toxicants in the biological system.

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1.1 Background

Conventionally, herbal and dietary interventions are considered as key regulatory approaches affecting human health and diseases. A newly introduced field of study unveiling the facts regarding relationship between nutrition and gene expression namely 'nutrigenomics' elucidates that diet influences genetic expressions and disease incidents. Thus, the healing system of nutritional therapy has evidently been paid more attention (Koithan and Devika, 2010). It was considered that plants have potential for preventing and treating many diseases. It was proved that a part of plant-derived natural products serves as a source of chemical diversity and biochemical specificity which make these suitable for the modulation of various signaling pathways in many chronic diseases including cancer and neurodegenerative diseases (Hussain et al., 2018). These provide an infinite number of

opportunities for new drug development because of diversities and convenience of phytochemical compounds (Cos et al., 2006). The protective roles of phytochemicals on chronic diseases are generally defined as bioactivity of plant compound (Wang et al., 2013b). About 10,000 phytochemicals including tannins, flavones, terpenoids, steroids, saponins and alkaloids have already been identified as bioactive compounds (Barbosa et al., 2013). Since overproduction of oxidants in biosystem is involved in the pathogenesis of many chronic diseases, the preventive actions of these phytochemicals either as pure compound or crude compositions (i.e., extracts) may be associated with antioxidant activities (Cos et al., 2006; Zhang et al., 2015).

Environmental pollution leads disease and death to human due to be exposed to hazardous environmental pollutants. It has already been established that even in exposure to trace amount environmental pollutants in early life can result in permanent abnormality of functions for organs because such abnormality may cause acute or chronic disease at any stage throughout the life span and ultimately can lead to death (Landrigan et al., 2016). World Health Organization (WHO) recently reported that environmental pollution is accountable for approximately 7 million deaths per year which is roughly greater than death number collectively caused by HIV, malaria and tuberculosis (WHO, 2014a; WHO, 2014b; WHO, 2014c; WHO, 2014d).

In recent years, especially heavy metals are under a supreme consideration among all of the environmental pollutants due to their high toxicity, persistence in the environment, and bio-accumulative nature (Ali et al., 2019). Additionally, heavy metals have received an increased ecological and public health concern due to dramatic rise of their use in industrial, technological, agricultural, and domestic purpose (Bradl, 2005). Although some heavy metals have essential roles in normal biological functions, few non-essential heavy metals such as cadmium (Cd), mercury (Hg) and lead (Pb) are extremely toxic even at very low concentrations in biosystems (Espín et al., 2014; Cao et al., 2017). Nevertheless, the extent of toxicity and probable health consequences of each non-essential toxic heavy metals depend on the type and form of the element, exposure route and duration, and vulnerability of individual. However, generally in biological system toxic metal-induced pathophysiology and homeostatic disruption include damage to vital biomolecules and generation of oxidative stress (Jan et al., 2015).

Cd ranks the uppermost among all toxic heavy metals in terms of adverse effects on plant growth and human health (John et al., 2008). In recent decades, Cd pollution has turned out as a global concern because of its increasing environmental load from industrial, agricultural, municipal, and energy sources. Moreover, neurotoxic, carcinogenic and other toxicological effects of Cd may be depending on its high solubility and relatively high bioavailability indicating high hazardous environmental pollutant for Cd (Mahmood et al., 2012). Although the cellular and molecular toxicological mechanisms of Cd toxicity including toxicokinetics have been studied extensively, numerous features regarding patterns of exposure, impacts on biological system, and probable ways of prevention or remediation are not yet elucidated (Jacobo-Estrada et al., 2017). It has already revealed that Cd toxicity is associated with the formation of elevated reactive oxygen, reduction of antioxidant molecules and subsequent generation of oxidative stress (Kukongviriyapan et al., 2016).

Since heavy metal chelating agents possess variability in efficacy with intrinsic limitations, new exploration and development for anti-toxic therapeutic agents against Cd toxicity could be urgent matter. For this purpose, phytochemicals should be given the prioritized considerations (Renugadevi and Prabu, 2009). Recent numerous studies unveiled the mechanisms of ameliorating role of several anti-oxidative phytochemicals including plant extracts against Cd-induced oxidative stress and toxicity (Zhai et al., 2013). However, there may be a lot of prospective phyto-medicinal materials having properties for the reduction of Cd-induced toxicity yet to be unveiled. In this purpose, the ethanolic extract of ajwain and its phenolic compound, carvacrol have been chosen for candidature for future consideration. In this study, first the toxicological molecular mechanisms of the toxic heavy metal, Cd, will be clarified. Second, feasibility of the prospective effects of ethanolic extract of ajwain and its phenolic compound, carvacrol on Cd-induced toxicity amelioration have been investigated. In the following, the properties of the materials relevant to this study explained in more detail.

1.2 Cadmium (Cd)

Cd is the most abundant naturally occurring toxic heavy metal in the environment majorly sourced from earth crust (in which concentration is 0.15-0.2 µg/g) through volcanic activity and rock weathering. However, anthropogenic activities by human rather than these natural sources are responsible for the uplift in Cd concentration in Cd cycle in nature

(Charles and Rini, 2014; Hutton, 1983). Anthropogenic sources of Cd contribute about 3-10 times more than the natural contribution of it to the environment; electroplating, fossil fuel combustion, waste incineration, welding, sewage sludge application, production and usage nickel-cadmium batteries, pigments, fluorescent paint semiconductor of solar cells, and phosphate fertilizer (Huang et al., 2017; Ji et al., 2019; SGS, 2019). Both occupational and non-occupational exposure of Cd ultimately affects human health adversely. Occupational exposures mostly happen through mining and smelting of non-ferrous metals, production or processing of compounds containing Cd and recycling of electronic waste (Huang et al., 2017). Usually, emitted Cd frequently translocate between the three major environmental compartments, such as air, water and soils, for probable achievement of steady state flux and can be utilized for non-occupational exposure to populations. Since Cd is highly mobile in soil system, and plants have the high transfer factor properties, the primary non-occupational exposure (among non-smokers) of Cd occurs via food chain upon bio-concentration (Chunhabundit, 2016). Various pathways evidently have been found to increase population health risk upon Cd exposure such as consumption of food grown in contaminated field, direct ingestion of water and accidentally soil, inhalation of dust, and dermal contact of soil and water (Wu et al., 2016). Although the routes of exposure are the main factor determining the amount of absorbed Cd, the absorption following exposure depends on many other factors, such as age, gender, smoking and nutritional status. Additionally, Cd exposure affects human dose-dependently, i.e., it gradually cause cellular injury and organ failure at high doses and disrupts specific mechanisms without marked cytotoxicity at low doses (Branca et al., 2018). Absorbed Cd is primarily but slowly excreted in urine. One of the most probable reasons for this low excretion rate is that, Cd remains tightly bound to metallothionein (MT) even though after degradation of MT, released Cd reabsorbed in the renal tubule and re-incorporated into newly biosynthesized MT. From the reason, Cd has a markedly long biological half-life in human between 6 and 38 years and in liver ranges from 4 to 19 years (ATSDR, 1999). Nonetheless, the liver and the kidney, particularly renal cortex are the main pool sites for Cd (Jadán-Piedra et al., 2018).

1.2.1 Cd: clinical manifestations

Cd is ranked 7th out of 275 most significant risk posing chemicals (ATSDR, 2017) and is classified as group-1 carcinogen (IARC, 2012). Exerting toxicity primarily to the kidneys, Cd

can also cause bone demineralization and may impair lung functions. Prolonged exposure of Cd can give toxic effects in a variety of tissues of organs including lungs, livers, gastrointestinal tract, reproductive system, and cardiovascular system (Bernard, 2008; Godt et al., 2006). It has been shown that occupational and environmental exposure to Cd can cause early signs of renal damage, calcium loss, proteinuria and tubular lesion. Moreover, acute toxicity may induce nephrotoxicity with polyuria, glycosuria, aminoaciduria, hyperphosphaturia, hypercalciuria, and decreased buffering capacity which may lead to renal tubular dysfunction and may cause ultimate fate to death (Inaba et al., 2005; Gonick, 2008; Nishijo et al., 2006). Renal diseases are associated with both acute and chronic Cd intoxication, but in the case of acute toxicity due to accumulation of Cd in liver, hepatocellular damage, hepatic necroinflammation and non-alcoholic fatty liver disease (NAFLD) may be concerned (Arroyo et al., 2012; Hyder et al., 2013). The most severe disease of chronic Cd exposure involving bone lesion is itai-itai disease which was first recognized in Toyama prefecture, Japan. In this case Cd intoxication initially manifest femoral and low back pain, and later pain spreads to whole body with skeletal deformities (Umemura and Wako, 2006). Numerous studies have already explained that Cd exposure can also cause skeletal demineralization, inhibits collagen production, osteoporosis and osteomalacia (Staessen et al., 1999; Nawrot et al., 2010). It has been proposed on the basis of *in vivo* studies that Cd restricts spermatogenesis and secretory functions, decrease in the levels of serum testosterone, reduction of steroidogenesis, and occurrence of ovarian hemorrhage and necrosis (Chandel and Jain, 2014). As Cd was also found to be associated with the loss of endothelial cell structure leading to cell death, Cd was recognized as a causative agent for cardiovascular disease and myocardial infarction (Everett and Frithsen, 2008). Additionally, an *in vivo* study clarifies that Cd can promote the formation of atherosclerotic plaques (Fagerberg et al., 2012).

Recent studies revealed that the normal functioning of the nervous system is adversely affected by cadmium exposure; these include olfactory dysfunction, peripheral neuropathy, neurological disturbances, mental retardation, and learning disabilities. Moreover, Cd-induced neurotoxicity was also found to be associated with neurodegenerative diseases like as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis and multiple sclerosis, and myalgic encephalomyelitis (Branca

et al., 2018; Chin-Chan et al., 2015; Sheykhansari et al., 2018). Cd can alter pH of gastrointestinal tract and thus can induce the inflammation (Waisberg et al., 2005). Cd, besides being a lung carcinogen, can cause pulmonary diseases such as chronic obstructive disease and emphysema (Lampe et al., 2008).

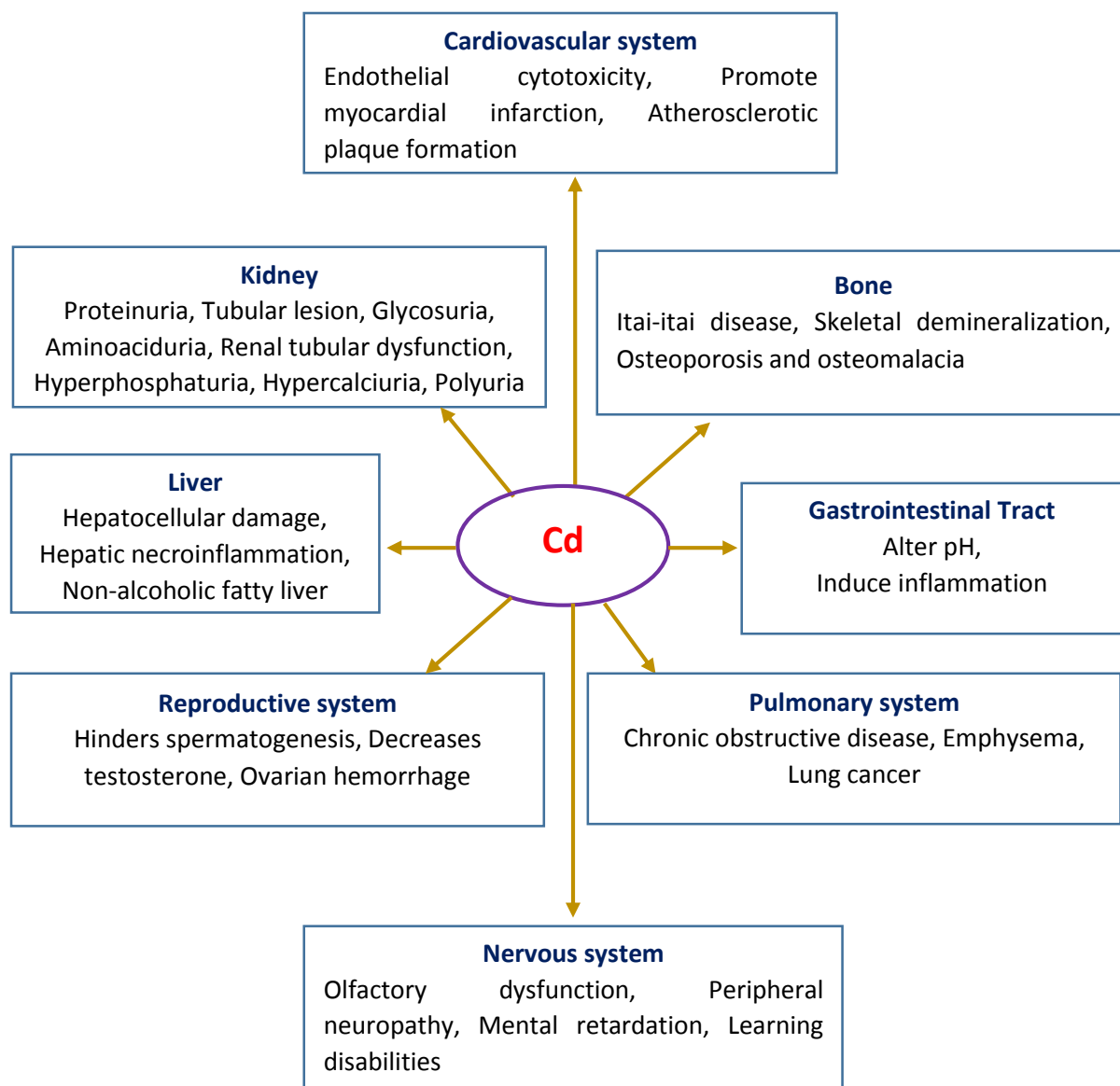


Fig. 1.1 Exposure and toxicity of Cd associated with disease development in human body.

1.2.2 Cd: cellular and bio-molecular toxicology

Studies have been carried out extensively to clarify the cytotoxic effect exerted by Cd *in vivo* and *in vitro*. Before, Cd-induced cell death was considered as a form of necrosis even

after the concept of apoptosis had been familiarized in the cell biology. In 1991, based on ultra-structural observation of proximal renal tubular cells in atrophic kidney, it was firstly described that Cd-induced cell death was apoptosis in nature (Hamada et al., 1991; Hamada et al., 1997). Later, it was also established that Cd also inhibits autophagy-related pro-survival proteins and may modulate apoptosis through an interplay (Messner et al., 2016; So et al., 2018). Although there are huge numbers of studies, the mode of cell death induced by Cd is partially unresolved; however, the characterization of the death mechanism induced by Cd depends on dose, duration, and cell system type. In general, like other heavy metals, upon exposure Cd generates oxidative stress through increase in reactive oxygen species (ROS) leading to an increase in lipid peroxidation, depletion of sulfhydryls, alteration of calcium homeostasis, and DNA damage (Méndez-Armenta and Ríos, 2007).

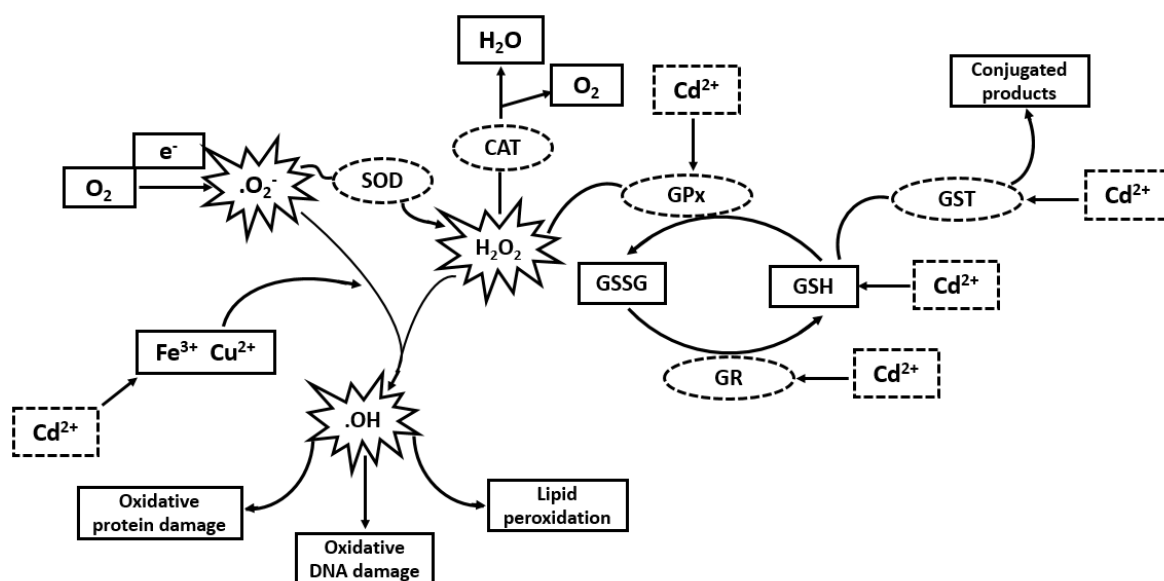


Fig. 1.2 Cd damages antioxidant enzyme activity defense system and of the non-enzymatic component glutathione, GSSG and GSH. Cd also increases the levels of Fenton metals (Fe^{3+} , Cu^{2+}), which can break down hydrogen peroxide, H_2O_2 to a reactive hydroxyl radical, $\cdot OH$ (image modified from Valko et al., 2005). Superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione-S-transferase, GST; glutathione reductase, GR.

During regular aerobic metabolism ROS is continuously generated, but body has integral bio-molecular antioxidant defense systems which play pivotal roles in averting probable damages driven by overproduction of ROS. Cd increases ROS production through the

suppression of cellular free radical scavengers e.g., glutathione (GSH) and by limiting the actions of some detoxifying enzymes e.g., catalase (CAT), superoxide dismutase (SOD), GSH peroxidase (GSH-Px or GPx) and GSH reductase (GR). The impairing action of Cd against cellular antioxidant defense system is shown in Fig. 1.2 (Valko et al., 2005). Another metal-binding protein molecule namely MT which physiologically maintains the homeostasis of essential metals such as Zn and Cu, but protects cells by scavenging free radicals during Cd-induced cytotoxicity (Sabolic' et al., 2010). Moreover, elevated level of ROS inhibits the activity of cellular survival proteins such as mTOR, Akt, NFkB, Nrf2 and ERK1 which can promote DNA fragmentation (Rahman et al., 2018; Roy et al., 2014; Singh et al., 2012; Xie and Shaikh, 2006; Méndez-García et al., 2019; Hu et al. 2015). However, the ROS-derived oxidative stress can finally induce DNA damage, abolish the functions of proteins and lipids, and can activate signaling pathway(s) ultimately leading to cell death (Chang et al., 2013).

To date, a huge number of reports has demonstrated that Cd can induce apoptosis in numerous cell lines and tissues both *in vivo* and *in vitro* including cells of the kidney (Hamada et al., 1997), liver (Wang et al., 2015), immune system (Azzouzi et al., 1994), and respiratory system (Hart et al., 1999).

Apoptosis is the death mechanism which can be characterized by some morphological and biochemical features that include chromatin condensation, nuclear fragmentation, activation of caspases, and loss of mitochondrial membrane potential (Kroemer et al., 2009). Cd-induced apoptosis may be caspase-dependent which can be accomplished through intrinsic or extrinsic pathways. The intrinsic pathway primarily involves mitochondria and reduces the membrane potential to interrupt the integrity of mitochondrial membrane which triggers the Bcl-2 family protein (e.g., Bax is increased and Bcl2 and BclxL are decreased)-mediated release of cytochrome c into the cytosol. Cytosolic cytochrome c binds with Apaf-1 leading to the cleavage and activation of caspase 9 and subsequent activation of caspase 3 through cleavage, leading to the dismantling of cellular compartments and ultimately to apoptotic cell death (Jiang et al., 2014). Extrinsic pathway involves the binding of cytokine ligands to the death receptors (e.g., Fas ligand and Fas receptor) which activate caspase-8 and subsequently activate caspase 3 or merges to the intrinsic pathway. Thus, both intrinsic and extrinsic pathways converge on activation of caspase 3, and finally apoptosis happens (Fulda et al., 2001). Additionally, Cd can also trigger

the caspase-independent pathway of apoptosis in various cell lines. Caspase-independent pathway involves in the release of apoptosis inducing factor (AIF) and endonuclease G (Endo G) from mitochondria which upon translocation to the nucleus can fragment DNA without the engagement of caspase reactions (Shih et al., 2003). Fig 1.3 shows a convenient scheme of the caspase dependent and the caspase-independent pathway induced by Cd.

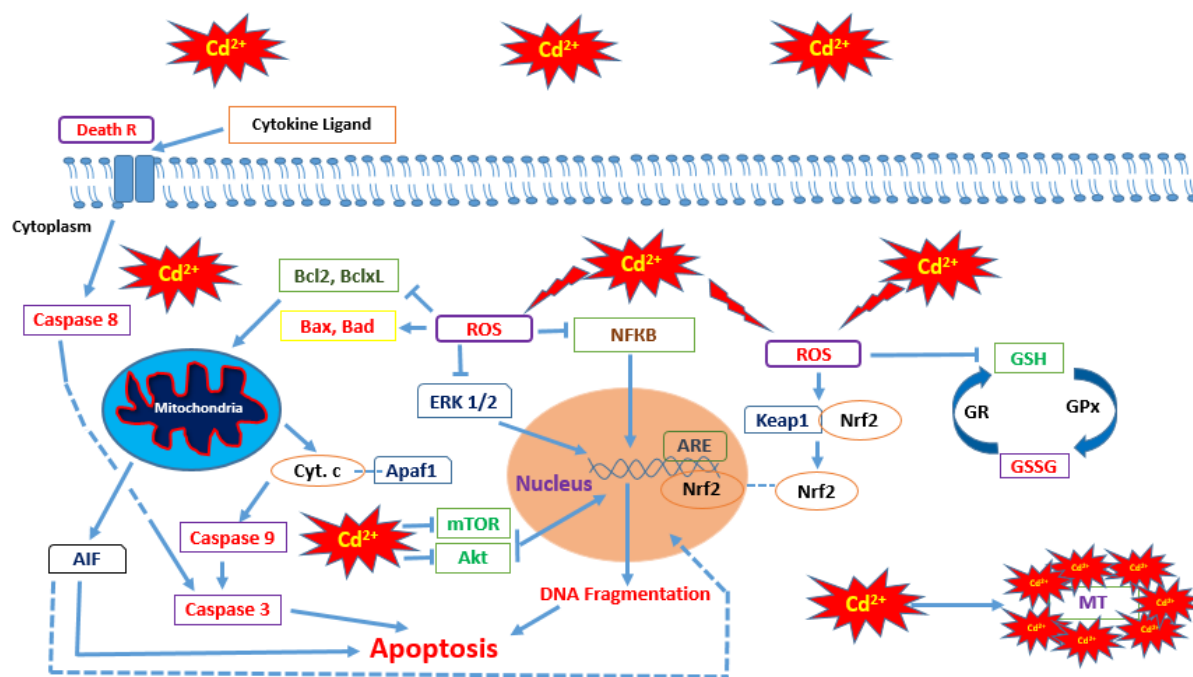


Fig. 1.3 Cd can induce apoptosis through caspase dependent (both intrinsic and extrinsic) and/or caspase independent pathway.

1.2.3 Phyto-extracts and phytochemicals ameliorating Cd toxicity

From many plant sources, numerous phyto-extracts and phytochemicals especially possessing high antioxidant properties have already been found to be protective against Cd-induced toxicity *in vivo* and *in vitro*. Since Cd is a pro-oxidant in biological system, the interest among the scientists has been rising in the possible uses of natural high antioxidant agents to prevent and to treat the unfavorable effects of heavy metals (Brzóška et al., 2016). Based on recent *in vivo* and *in vitro* studies, few examples of phyto-extracts and phytochemicals ameliorating Cd toxicity, model organ tissue or cell type in which toxicity

was improved, toxic doses of Cd exposure, clarified molecular mechanisms and references has been listed in Table 1.1 and Table 1.2.

Table 1.1 Cd toxicity reducing phyto-extracts, target tissue/cells, cadmium doses and molecular mechanisms

Phyto-extract	Tissue/Cell	Cd Dose	Molecular Mechanism	References
Ginger (<i>Zingiber officinale</i>) ethanol extract	Rat kidney tissues	5 mg/kg body weight	Raised cadmium-induced reduced total antioxidant status (TAC), DNA content, and restored histopathological changes	Gabr et al., 2019
Barries (<i>Aronia melanocarpa</i> L.) aqueous extract	Rat liver tissues	1 or 5 mg/kg diet respectively for 3 to 24 months	Increased Cd-induced lowered SOD, CAT, GPx, GR, GST and total thiol groups (TSH), reduced GSH.	Mężyńska et al., 2019
Grape seed extract (GSE)	Rat brain tissue	15 mg CdCl ₂ /kg bodyweight/day for 3 months	Increased Cd-induced decreased acetylcholinesterase, MAO-A, and GR and expressions of GST, GPx, dopamine and 5- hydroxytryptamine transporters.	El-Tarras et al., 2016
<i>Monodora myristica</i> aqueous extracts	Rat liver tissues	200 mg CdCl ₂ /L in drinking water for 21 days	Reduced Cd-induced elevated total cholesterol (TC), triglycerides (TG) and malondialdehyde; Increased GSH, SOD, and CAT levels.	Oyinloye et al., 2016
Aged garlic extract in 0.1% DMSO	1321NI and HEK293 cells	5, 10, 50 µM CdCl ₂ for 24 h	Increased Cd-induced reduced cell viability; Reduced TBARS production and LDH leakage; Increased GSH levels and NAD(P)H:quinone oxidoreductase (NQO1) expression.	Lawal and Ellis, 2011

Table 1.2 Cd toxicity reducing phytochemicals, target tissue/cells, cadmium doses and molecular mechanisms

Phytochemical	Tissue/Cell	Cd Dose	Molecular Mechanism	References
Sulforaphane (compound of broccoli and cabbages)	Mouse Leydigs (TM3) cells	10 $\mu\text{mol/L}$	Increased total-SOD, GSH-Px activity and GSH content; Inhibited the production of MDA or ROS; Reduced apoptotic rate; Up-regulated <i>Nrf2</i> , <i>GSH-Px</i> , <i>HO-1</i> , <i>NQO1</i> , and γ -GCS expressions.	Yang et al., 2019
Quercetin (found in apple, onion, mulberry etc.)	Rat proximal tubular cells	2.5, 5 $\mu\text{mol/L}$	Increased cell viability; Decreased apoptotic rate, Decreased intracellular ROS, MDA, Ca^{2+} ; Increased MMP and GSH; Activated Na^+ , K^+ ATPase, Ca^{2+} ATPase, GSH-Px, CAT and SOD.	Wang et al., 2013a
Taxifolin (found in various conifers)	Human keratinocyte (HaCaT)	30 $\mu\text{mol/L}$	Increased cell viability; Decreased apoptotic rate; Reduced DNA fragmentation; Decreased intracellular ROS; downregulated cathepsin B and D; upregulated hsp27, cyclophilin A and peroxiredoxin-1; Reduced cleaved PARP.	Moon et al., 2019
Rosmarinic acid (found in basil, rosemary, sage etc.)	isolated mouse proximal tubular epithelial cells	4 mg/kg body weight	Reduced ROS, NO level, NADPH oxidase; Deactivated NF- κ B/PKC- δ /TNFR2; Reduced fibrosis by deactivating TGF- β 1/SMAD3/ α -SMA/collagen signaling.	Joardar et al., 2019

Resveratrol (berries, peanut plants, pine trees etc.)	PC12 cells and Primary murine neurons	10, 20 $\mu\text{mol/L}$	Increased cell viability; Reduced nuclear fragmentation; Deactivated caspase 3; prevented Cd-induced activation of Erk1/ 2 and JNK pathways; prevented suppression of protein phosphatases 2A (PP2A) and 5 (PP5) activity	Liu et al., 2015
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1.3 Ajwain

Ajwain (*Trachyspermum ammi* L.) is a herbal and a valued medicinally important plant belonging to the Apiaceae family. It is widely cultivated in its native country, Egypt and also in many other countries around Mediterranean and Southwest Asia (Asif et al., 2014). This is an erect, glabrous or minutely herbaceous plant containing the greyish brown fruits or seeds (Chauhan et al., 2012). The ajwain seed is mostly found in cooking as a spice in which is known as carom seed (Davidson and Jaine, 2014). However, in traditional medicinal treatment system it has been largely used for its numerous medicinal and therapeutic values. In Iranian traditional literature ajwain has been cited as a therapeutic agent used in flatulence, indigestion, colic, dyspepsia and diarrhea (Dashti-Rahmatabadi et al., 2007). Practically, ajwain seed is used as ethnobotanical medicine for anorexia, ascites, asthma, bronchitis, cachexia, cancer of abdomen, cardiopathy, cholera, cold, colic, congestion, cough, cramp, debility, diarrhea, dipsomania, dyspepsia, edema, emphysema, enteritis, epilepsy, fever, flu, hemorrhoid, hepatosis, hiccup, high blood pressure, hysteria, laryngitis, mycosis, nausea, nematode, nephrosis, ophthalmia, pain, paralysis, pneumonia, rheumatism, sinusitis, snakebite, sore throat, syncope, toothache, and wounds (Asif et al., 2014).

1.3.1 Phytochemistry of ajwain

The nutritive value of ajwain seed has already been analyzed by researchers. It also contains bio-active compounds which made the nutritive status more potential for beneficial pharmaceutical uses. It has been revealed that ajwain seed contains fiber (11.9%), carbohydrates (38.6%), tannins, glycosides, moisture (8.9%), protein (15.4%), fat (18.1%),

saponins, flavone and mineral matter (7.1%). It also contains riboflavin, thiamine, nicotinic acid, carotene, calcium, chromium, cobalt, copper, iodine, iron, manganese, phosphorus, and zinc (Duke, 1992). Ajwain has 2-4% yield of essential oil of which contains thymol, p-cymene, γ -terpinene, α - and β -pinenes, dipentene, α -terpinene, and carvacrol (Bairwa et al., 2012). They possess very high anti-oxidative potential. The aqueous, methanolic, acetonetic and ethanolic extracts of ajwain seed have shown high 2,2-diphenyl-1-picryl-hydrazyl hydrate (DPPH) radical scavenging potential and ferric reducing antioxidant potential so as ajwain could be comparable to commercially used antioxidant, butylated hydroxytoluene (BHT) (Goswami and Chatterjee, 2014). Studies have already indicated the phytochemical profiles of various ajwain extracts. Using spectral analyses, 25 compounds were identified in water soluble portion of methanol extract for ajwain; those include 5 monoterpenoid glucosides, 2 aromatic compound glucosides, and 2 glucides (Ishikawa et al., 2001). The acetone extract of ajwain contains 18 identified compounds which include thymol, oleic acid, linoleic acid, γ -terpinene, p-cymene, palmitic acid, and 4-hydroxy-4-methylpenta-2-one (Singh et al., 2004). Ethanolic ajwain extract was phytochemically characterized using GC-MS, and carvacrol (iso-thymol) showed dominant concentration followed by emersol. It also contains phenol, 4-methoxy-2,3,6-trimethyl-, ethyl ester, terpineol, terpinene and cymene as minor compounds (Tariq et al., 2014).

1.3.2 Therapeutic activities of ajwain extracts

Numerous *in vivo* experiments demonstrated the therapeutic effects of various ajwain extracts in many experimental models. These experiments have been conducted aiming the revalidation of the traditional use of ajwain in various diseases and ailments. Moreover, besides deciphering the molecular basis of the traditional use of ajwain, new links among potential therapeutic efficiencies of ajwain in other diseased conditions have been established in some results. Few findings of the therapeutic effects of various ajwain extracts on different *in vivo* diseased conditions implying the pharmaceutical potentiality of ajwain have been represented in this section.

Antidiarrheal and gastro-protective activity:

Balaji et al. (2012) resolved that the ethanolic (95%) and aqueous extracts of ajwain seed inhibited potentially and dose dependently the castor-oil induced diarrhea in experimental rats. In the experiment they found activity of ajwain extracts similar to that of

the standard drug loperamide in intestinal fluids. The ethanolic (70%) extract of ajwain also exhibited potentiality for the treatment of gastrointestinal ailments. It showed antibacterial activity against various strains of *Helicobacter pylori* and thus indicating that it can produce gastro protective activity (Zaidi et al., 2009).

Antihypertensive, antispasmodic, broncho-dilating and hepatoprotective activity:

The traditional use of ajwain as antihypertensive, antispasmodic and broncho-dilating agents was rationalized by Gilani et al. (2005). This study demonstrated that the aqueous-methanolic extract of ajwain lowered dose dependently arterial blood pressure in anaesthetized rats. The extract showed calcium channel blocking effect in isolated rabbit aorta and jejunum preparations which were similar to that of verapamil.

Additionally, in isolated tracheal preparation of guinea-pig, the extract shifted the dose-response curve of carbachol and histamine to the right, suggesting its non-specific broncho-dilating effect. Moreover, pre-treatment of the extract in rat prevented paracetamol-induced rise in serum alkaline phosphatase (ALP) and aminotransferases (AST and ALT), and inhibited CCl₄-induced prolongation in pentobarbital-induced sleeping. Thus it was indicated that the ajwain extract exhibited hepatoprotectivity.

Anti-hyperlipidemic activity:

Cardiovascular diseases are positively related to the hyperlipidemia. The relationship between the amount of circulating lipids in blood and mortality rates from coronary atherosclerosis has been already established. There are many synthetic drugs are available with a lot of side effects to treat such diseases. So, phyto-chemical based natural drugs may be the best choice for the prevention of the cardiovascular diseases. In this purpose, ajwain can be an option. The methanolic extract of ajwain was found to decrease total cholesterol by 71%, LDL-cholesterol by 63%, triglycerides by 53% and total lipids by 49% on 135th post-treatment day in albino rats (Kumari and Prameela, 1992).

Antitussive effect:

Application of two different concentrations of aqueous and macerated extracts for ajwain demonstrated that both extracts significantly reduced the cough number produced by citric acid aerosol in guinea pig tracheal chain. The antitussive effect of ajawin extracts was even greater than that of codeine at concentrations used. However, the major

constituent, carvacrol did not show any antitussive effect, indicating carvacrol is not responsible for the effect (Boskabady et al., 2005).

Anti-inflammatory activity:

In a contemporary study, total aqueous extract and ethanolic extracts of ajwain exhibited significant anti-inflammatory activity in rat animal model. Both extracts inhibited the carrageenan-induced paw oedema. The weight of the adrenal glands was increased by the treatments of the both extracts of ajwain which were better results in comparison with the results shown by the treatments of anti-inflammatory drugs, aspirin and phenyl butazone (Thangam and Dhananjayan, 2003).

Anti-platelet aggregatory effect:

It has been demonstrated that an ethereal ajwain extract hindered platelet aggregation induced by arachidonic acid, epinephrine and collagen. It showed the most effectiveness against arachidonic acid-induced aggregation in the blood from healthy volunteers (Srivastava, 1988). Ethereal ajwain extract reduced thromboxane B₂ formation in intact platelet preparation from added arachidonic acid. It was indicated that lipoxygenase derived products from exogenous arachidonic acid in ajwain extract-treated platelets was evolved because of the redirection of arachidonic acid from the cyclooxygenase to the lipooxygenase pathway.

Antibacterial activity:

Ajwain was found to be a bacteriostatic and bactericidal agents. The ethanolic extract of ajwain showed antibacterial activity against *P. aeruginosa*, and acetone extract possessed the potential activity against *Escherichia coli*. Additionally, acetone and aqueous extracts of ajwain were found active against *P. aeruginosa*, *Salmonella typhi*, *S. typhimurium*, *Shigella flexneri*, *E. coli*, *Enterococcus faecalis* and *S. aureus* (Kaur and Arora, 2008). It has also been proved that ajwain ethanolic extract loaded MnFe₂O₄ NPs coated with PEGylated chitosan was found precisely active against *E. coli*, *klebsiella* and *P. aeruginosa* (Esmaeili and Ghobadianpour, 2016).

1.3.3 Toxicity ameliorating effects of ajwain extracts

Imbalance in redox homeostasis is thought to be one of the major basic reason of disease development. We have experienced exposure to many drugs and toxicants that can imbalance the redox homeostasis in our body. On the other hand, toxicants are thought to be the etiological reasons for many acute and chronic diseases. It can be postulated that natural antioxidant rich foods or phyto-medicines such as ajwain can be remedial agents to prevent toxicant-derived oxidative stress and apoptosis. However, various ajwain extracts have already been found to be active against toxicant-induced oxidative stress, cytotoxicity, genotoxicity and apoptosis. Several *in vivo* and *in vitro* studies have been conducted clarifying the ameliorative effects of numerous ajwain extracts against organic and inorganic toxicants including heavy metal-induced toxicity. In this section examples have been represented depicting the effect of various ajwain extracts against oxidative stress, cytotoxicity and apoptosis *in vivo* and *in vitro*.

Protection in Hepatotoxicity:

Anilkumar et al. (2009) revealed that pre-feeding of ethanolic extract of ajwain resulted in decreased hepatic levels of lipid peroxides and increased GSH, GSH-peroxidase, glucose-6-phosphate dehydrogenase (G-6-PDH), SOD, CAT and GST activities on hexachlorocyclohexane-induced oxidative stress and toxicity in rats. Ajwain extract also altered the hexachlorocyclohexane-induced changes in glutamyl transpeptidase. Moreover, the extract lowered significantly the formation of micronuclei in femur bone marrow.

Amelioration of chromium-induced cytotoxicity, genotoxicity, apoptosis and oxidative stress:

Chromium is a toxic heavy metal which generates oxidative stress, cytotoxicity and genotoxicity in biological system and can lead the cells to apoptotic death. A modern study conducted by Deb et al. (2012) found that the methanolic extract of ajwain can reduce the hexavalent chromium-induced cytotoxicity, genotoxicity, apoptosis and oxidative stress in human bronchial epithelial cells (BEAS-2B) and isolated human peripheral blood lymphocyte (PBL) *in vitro*. The pre-treatment of ajwain extract before potassium dichromate ($K_2Cr_2O_7$) treatment increased cell viability, and reduced the DNA damage. Ajwain extract lowered the level of ROS, raised the mitochondrial membrane potential, and reduced the

caspase 3 activity resulting ameliorated apoptosis induced by oxidative stress. The reversed action of ajwain methanolic extract against chromium-induced reduction of antioxidant enzymes, SOD and GPx was also confirmed.

1.4 Carvacrol

Carvacrol, (5-isopropyl-2-methyl-phenol; chemical formula is $C_6H_3CH_3(OH)(C_3H_7)$) is a monoterpenoid phenol found in many aromatic plants which are generally used as spices in culinary and folk medicinal therapy. These plants are mostly belong to *Labiatae* and *apiaceae* families including oregano, thyme, pepperwort, black cumin, summer and winter savory and ajwain (*T. ammi*) (Bayir et al., 2019; Tariq et al., 2014; Boskabady et al., 2005).

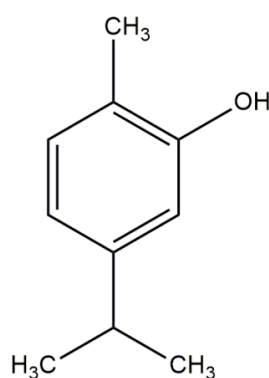


Fig. 1.4 Chemical structure of carvacrol

Carvacrol is generally used as a flavoring agent in beverages, sweets and chewing gum. Recently, the potential use of carvacrol as active packaging material in food industry has become very prospective. Moreover, it is approved as “Generally Recognized as Safe (GRAS)” by the U.S. Food and Drug Administration (Ramos et al., 2016; Nostro et al., 2012). It has been reported that carvacrol is rapidly and almost completely metabolized and excreted by mammals. The excretion rate is very low and the molecule remain unchanged while excreted (Austgulen et al., 1987). The ring hydroxylation in structure and lipophilicity have made carvacrol suitable for the rapid uptake and metabolism (Austgulen et al., 1987; Michiels et al., 2008).

1.4.1 Carvacrol: biological activities

The increasing uses of carvacrol as food additive, flavoring and preservative agent have attracted researchers for searching probable clinical implications. In line with these,

numerous *in vivo* and *in vitro* studies regarding biological activities of carvacrol have already been conducted.

Anti-inflammatory effects:

Carvacrol reduces neurogenic and inflammatory pain in mammals. It attenuates hypernociception and inflammatory responses by reducing TNF- α levels in pleural lavage in mouse, and suppresses the recruitment of leucocytes without morphological alteration (Guimarães et al., 2012). Furthermore, without being cytotoxic to macrophages carvacrol inhibits the lipopolysaccharide-induced nitrite generation *in vitro*. It was shown that the anti-inflammatory activity of carvacrol is mediated through the suppression of COX-2 expression, peroxisome proliferator-activated receptors α and γ and inhibitory effect on the generation of nitric oxide (Hotta et al., 2010).

Anti-carcinogenic effects:

The anti-carcinogenic effects of carvacrol have been confirmed by several studies. Carvacrol can hinder dose dependently the proliferation of human gastric adenocarcinoma cells via intrinsic mitochondrial apoptosis (Günes-Bayir et al., 2017). It has been demonstrated that carvacrol can reduce tumor incidence significantly with survival time prolongation in 3,4-benzo[a]pyrene treated rats (Karkabounas et al., 2006). Carvacrol was found to suppress the diethylnitrosamine (DEN)-induced hepatocellular carcinogenesis by lowering serum tumor marker enzymes, carcinoembryonic antigen and α -fetoprotein (Subramaniyan et al., 2014).

Antimicrobial activity:

Carvacrol shows antimicrobial activity against numerous pathogenic bacteria including *Campylobacter*, *Pseudomonas*, *E. coli*, *Salmonella*, *S. aureus*, and *Listeria* (Friedman et al., 2002). It has been shown that carvacrol has synergistic effect on the growth inhibition of bacteria such as *Staphylococcus aureus* and *Enterococcus faecium* by tetracycline resistance and biofilm formation (Miladi et al., 2017). One of the important reasons behind the antibacterial activity of carvacrol is the presence of a free hydroxyl group in its structure which effects on the delocalized electron system to reduce the gradient across the cytoplasmic membrane (Ultee et al., 2002).

Anti-obesity effects:

Obesity increases the risk of several metabolic diseases such as diabetes mellitus and cardiovascular disease. Anti-obesity effects of carvacrol have been demonstrated in several studies. For instance, carvacrol was found to reduce body and visceral fat pad weight and plasma lipid levels in high-fat diet fed mice. This obesity prohibiting effect is supposed to be due to the carvacrol-induced suppressions of bone morphogenic protein, fibroblast growth factor-1 and galanin-mediated signaling. In addition, the attenuating effect of carvacrol on the synthesis of proinflammatory cytokines in visceral tissues was also found (Cho et al., 2012).

1.4.2 Carvacrol: an emerging anti-toxic agent

Carvacrol possesses a strong antioxidant potential *in vivo* and *in vitro*. It inhibits the production of food-product deterioration compounds such as peroxides and has shown high scavenging activity against nitric oxide and lipid peroxidation (Quiroga et al., 2015; Guimarães et al 2010). Carvacrol was found to protect human lymphocyte from mitomycin C-induced DNA damage, and to increase antioxidant capacity in cultured primary rat neuron cells (Aydin et al., 2005; Aydin et al., 2014). The antioxidant effects of carvacrol is contributed by the aromatic ring attached –OH group which donate hydrogen atom to an unpaired electron and leads to the production of another radical at a molecular resonance structure (Aeschbach et al., 1994).

Since oxidative stress due to the over-generation of ROS is one of the major reasons for toxicity development by various toxicants, the reduction of oxidative stress or enhancement of anti-oxidative protection might be effective strategies for reduction of toxicity. In this regard, carvacrol shown high anti-oxidant properties has been established as a toxicity remediating agent against numerous toxicants in biological system. Some examples and mechanisms of protective effects of carvacrol against toxicants have been discussed briefly in this section.

Neurotoxicity protection:

Carvacrol has been shown as an agent which potentially alleviates various toxicant-induced neuronal toxicity *in vitro* and *in vivo*. It was demonstrated that carvacrol dose dependently protected PC12 cells from 6-hydroxydopamine (6-OHDA)-induced cytotoxicity by increasing cell viability, reducing ROS and lipid peroxidation, and lowering

the apoptosis rate. In the same study it was also found that carvacrol upgraded locomotion activity, catalepsy, akinesia, bradykinesia and motor coordination in 6-OHDA-intoxicated rats. It also increased the GSH level, and reduced melondialdehyde (MDA) level (Manouchehrabadi et al., 2017). In another research it was established that carvacrol reduced ethanol-induced oxidative stress and apoptosis in mice hippocampal neurons by modulating expressions of Bcl-2, Bax, caspase-3, and p-ERK (Wang et al., 2017).

Hepatotoxicity alleviation:

Khan et al. (2019) found that carvacrol pre-treatment in mice reversed the ethanol-induced toxic effects on liver function, antioxidant markers, matrix metalloproteinases activities and histological changes. Additionally it was shown that carvacrol inhibits cytochrome P450 by binding in its active pocket. Aristatile et al. (2009) have shown that carvacrol reduced aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyl transpeptidase in D-galactosamine (D-GaIN) - induced hepatotoxic rats. It also reduced SOD, CAT and GSH. An *in vitro* study has shown that carvacrol can reduce inflammation and oxidative stress in acetaminophen (APAP)-exposed HepG2 cells (Palabiyik et al., 2016).

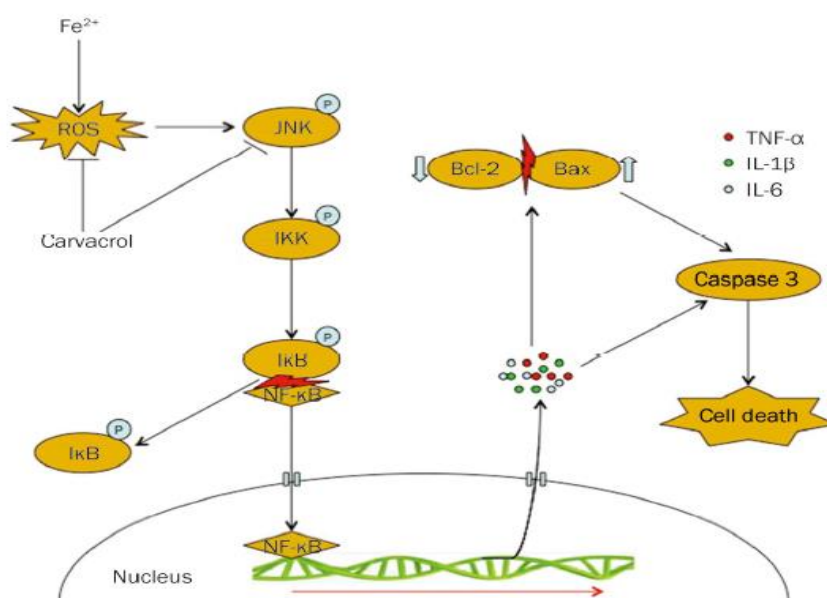


Fig. 1.5 Schematic representation of the mechanisms of carvacrol cytoprotection against Fe²⁺-induced apoptosis in neuroblastoma SH-SY5Y cells (Cui et al., 2015).

Protection against Fe²⁺ induced cytotoxicity and apoptosis:

The effect of carvacrol on Fe²⁺-induced apoptosis and its mechanism in neuroblastoma SH-SY5Y cells were clarified by Cui et al. (2015). The cell viability reduced by Fe²⁺ treatment was increased by carvacrol pretreatment. Carvacrol reduced Fe²⁺-induced elevated NF-κB, expression of the pro-inflammatory cytokines, and apoptosis in the cell. It also inhibited Fe²⁺-induced phosphorylation of JNK and IKK, but not p38 and ERK in the cells. Thus, carvacrol protected neuroblastoma SH-SY5Y cells against Fe²⁺-induced apoptosis, which may result from suppressing the MAPK/JNK-NF-κB signaling pathways as shown in Fig1.5.

1.5 Research motivation

Environmental contamination and exposure to human due to the toxic heavy metal such as Cd is now obvious all over the world. This phenomenon is more exacerbate in some countries. For instance, the soil and water systems in Bangladesh are extremely contaminated with Cd. It has already contaminated the food chain considerably in Bangladesh. Despite the maximum permitted Cd level in rice samples is 0.4 mg/kg (Codex Alimentarius, 1995), researchers found that Cd in many samples of rice grain from Bangladesh was higher than 1.0 mg/kg Cd (Meharg et al., 2013). It has been also reported that this enormously high concentration of Cd in Bangladeshi rice grain is the highest among Cd concentrations found in rice samples collected from 12 rice producing countries of 4 continents. A comparative descriptive statistics for Cd (mg/kg) in rice grains reported by Meharg et al. (2013) has shown in Table 1.3.

Table 1.3 a descriptive statistics for Cd (mg/kg) in rice grain by countries surveyed

Country	N	Mean	Median	Minimum	Maximum
Bangladesh	260	0.099	0.057	<0.0005	1.31
Cambodia	14	0.006	0.001	0.0010	0.03
France	37	0.010	0.006	0.0030	0.10
Ghana	428	0.020	0.013	<0.005	0.27
India	58	0.078	0.028	0.0020	1.00
Italy	114	0.038	0.027	0.0030	0.16
Japan	18	0.059	0.050	0.0101	0.14
Nepal	12	0.050	0.048	0.0139	0.08
Spain	92	0.024	0.016	0.0008	0.14
Sri-Lanka	75	0.081	0.024	<0.0005	0.80

Thailand	18	0.027	0.020	0.0057	0.07
USA	21	0.018	0.017	0.0095	0.04

Moreover, it was already found that the rice-based diet of Bangladeshi populations are excessively contaminated with Cd. Research findings on the dietary intake of Cd from Bangladeshi foods have shown that the total daily intake (TDI) of Cd for Bangladeshi is about 35 µg/day which is much higher than that of the other populations (Al-Rmalli et al., 2012). The chronic exposure thus has been ascertained by the food intake. This chronic exposure of Cd may lead to the ultimate lethal fate through bioaccumulation and bio-magnification. For instance, many Japanese people suffered from itai-itai disease originated from the chronic exposure through the intake of Cd contaminated rice (Kaji, 2012). Considering the consequences, preventive methods should be taken to bypass this situation. Of course, preventing the flow of contamination in the environment is the best policy; however, once the Cd contamination spreads to the food cycle then measures are necessary to prevent its toxic effects in biological system. There is no drug available directly for the chronic toxic exposure of Cd. Even, yet, no chelation therapy has been approved for the clinical use against Cd-induced toxicity (Zhai et al., 2015). However, dietary interventions especially phyto-medicines having high antioxidant properties are exceedingly effective to neutralize the daily xenobiotic effect of Cd. To date, there are many plant-based remedies for Cd toxicity using complex compositions (extracts) and their active compounds have been available. Moreover, some of their modes and mechanisms of them against Cd toxicity have been clarified by researcher that was briefly introduced in the previous sections of this chapter.

Like in many other countries, ajwain (*T. ammi* L.) seed has been used as a spice in culinary items or as a phyto-therapeutic agent for various diseases in Bangladesh. First, the traditional uses of ajwain (for thousands years) as a phyto-medicine motivated me to conduct the research against Cd-induced cytotoxicity and apoptosis *in vitro*. Second, Cd has been found worldwide as the etiological reason for many diseases (e.g., inflammatory disorders and hepatocellular damage) which have been traditionally treated by herbal medicines containing ajwain in many countries including Bangladesh. Finally, from the results already reported ajwain extracts and its phenolic active compound, carvacrol can be

expected to ameliorate the oxidative stress (from previous studies) which can be one of the probable strategies to alleviate Cd-induced toxicity *in vivo* and *in vitro*. Hence, considering and connecting all of the accumulated information, ethanolic extract of ajwain and its active compound monoterpenic phenol, carvacrol have been hypothesized to be potential toxicity-remediating agents against Cd in a cellular system.

1.6 Aims and objectives

The broader aim of this research is to expand the spectrum of diet-based therapy of cheap plant sourced complex, the ethanolic extract of ajwain and its active phenolic compound, carvacrol for amelioration of Cd toxicity. To achieve these targets, the objectives have been set as outlined as follows:

- i) To examine the effects of ethanolic extract of ajwain under the safe dosage on Cd-induced cytotoxicity and apoptosis and to clarify the underlying molecular pathway of the extract.
- ii) To study the effects of the phenolic compound in ethanolic extract of ajwain, carvacrol on the amelioration of Cd-induced cytotoxicity and apoptosis with molecular toxicological understandings.

1.7 Thesis outline

This thesis is consisting of four consecutive chapters. The Chapter-1 explains the background of the emergence of the phyto-extracts and phyto-chemical based therapeutic practices as the preventive measures for diseases caused by various modern-world toxicants especially Cd. Additionally, information on the pollution, exposure and disease manifestations of the toxic heavy metal, Cd has been included in this chapter. Chapter-1 also include the examples of the modes and mechanisms of the effectiveness of some phyto-extracts and phytochemicals against the Cd-induced toxicity *in vivo* and *in vitro*. It includes the background knowledges of ajwain, the phytochemistry of ajwain and its various extracts, examples of therapeutic activities of various ajwain extracts in various diseases and intoxicated conditions in various cellular and animal models. In addition, the background knowledge of carvacrol, various biological and anti-toxic activities with future prospects of carvacrol, have also been discussed in Chapter-1. The protective effects of ethanolic extract of ajwain on Cd-induced cytotoxicity, oxidative stress and apoptosis are shown in Chapter-2.

This chapter also clarify the role of various related molecular components in toxicity/apoptosis, and its amelioration of bio-molecular toxicological pathway in PC12 cells. The combating effects of the phenolic compound of the ethanolic extract of ajwain, carvacrol on Cd-induced cytotoxicity, oxidative stress and apoptosis in PC12 cells with molecular mechanisms has been indicated in Chapter-3. The Chapter-4 is the general conclusion through all of the thesis. The chapter enhances all of the findings focusing on the potential uses of ajwain and carvacrol in Cd toxicity, and proposes uses of these agents in various toxicological and biological system.

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Chapter 2: Protective effects of ajwain (*Trachyspermum ammi* L.) extract against cadmium-induced cytotoxicity and apoptosis in PC12 cells

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Abstract

Cadmium (Cd^{2+}) is a heavy metal that can induce cytotoxicity leading to many chronic diseases. Ajwain (*Trachyspermum ammi* L.) is a popular spice with diverse pharmacological properties. It is used for the remedy of many pathological consequences those could be manifested by Cd^{2+} exposure e.g. inflammatory, toxic, neurological, genital or respiratory tract disorders. To reduce the Cd^{2+} -induced cytotoxicity and apoptosis, PC12 cells were exposed/co-exposed to Cd^{2+} (5 or 10 μM) with/without non-toxic dose (240 $\mu\text{g}/\text{mL}$) of ethanolic extract of ajwain (AE) for 24 h. The cytotoxicity and apoptosis were evaluated by cell viability, lactate dehydrogenase (LDH) activity, glutathione levels, genomic DNA fragmentation and expressions of Bax, Bcl-2, Bcl-xL, NF-KB, cytosolic cytochrome c and caspase-3. Cd^{2+} reduced the cell viability significantly 24 h after exposure; however, the co-exposure of AE with Cd^{2+} reduced the cell death. The co-exposure also raised up glutathione levels, and decreased LDH activity. AE lessened the DNA fragmentation caused by Cd^{2+} . AE suppressed the Cd^{2+} -induced increased expression of apoptotic protein Bax, and promoted suppressed expressions of anti-apoptotic proteins Bcl-2, Bcl-xL and NF-KB. Moreover, it reduced the cytosolic cytochrome c levels and the caspase-3 expressions increased by Cd^{2+} . Thus, it was suggested that AE reduced cytotoxicity and apoptosis caused by Cd^{2+} in PC12 cells. It inhibited Cd^{2+} -induced apoptosis through intrinsic pathway possibly boosting up the antioxidant defense. Therefore, ethanolic ajwain extract (AE) can be a candidate for being a potential agent against metal and/or other toxicants. Additionally, this study validated the ethno-pharmacological therapeutic uses of ajwain in various pathological conditions.

2.1 Introduction

Cadmium (Cd^{2+}), a toxic heavy metal, is one of the lethal environmental and occupational pollutants. It is ranked 7th on the priority list of Hazardous Substances by the Agency for Toxic Substances and Disease Registry (ATSDR) (Rennolds et al., 2012). Globally, population of health risk due to Cd^{2+} exposure has risen steadily through various pathways, e.g. direct ingestion of water and accidentally soil, and consumption of food grown in contaminated fields, inhalation of dust and dermal contact of soil and water (Wu et al., 2016). Notwithstanding that the factors like route, quantity and rate of exposure determine the clinical fate of Cd^{2+} toxicity, toxic exposure may lead to renal tubular dysfunction, osteoporosis, endocrine disruption, respiratory tract irritation, hypertension, anemia,

hepatotoxicity and even pancreatic or lung cancer (Adikwu et al., 2013; Bernhoft, 2013; Hossain et al., 2018). Moreover, Cd^{2+} also exerts severe adverse effects on the normal functioning of the nervous system because of its high blood-brain barrier permeability (Wang and Du, 2013). Although toxicity mechanism of Cd^{2+} is very complex and still debated, it is evident that Cd^{2+} can potentially induce oxidative stress, epigenetic changes in DNA expression, inhibition or up-regulation of transport pathways, competitive interference with essential metals, inhibition in heme synthesis and impairment of mitochondrial function by inducing apoptosis (Bernhoft, 2013).

Recently, attention has been turned towards the therapeutic use of many traditional chemo-preventive agents against the toxic effects of many toxicants including heavy metals. In this regard, plant derived extracts and essential oils owning complex composition and high antioxidant properties are very potent in averting toxicological cellular damage (Anilkumar et al., 2009). A range of extracts from numerous plant sources those possess high antioxidant properties has been experimented to assess their beneficial activity against various toxicant derived oxidative stress-induced cytotoxicity *in vivo* and *in vitro*. For instance, treatment of aged garlic extract on 1321N1 and HEK293 cells showed protective effects against Cd^{2+} -induced cytotoxicity (Lawal and Ellis, 2011).

Trachyspermum ammi L. known as ajwain is an aromatic annual herb, native of Egypt and widely grows around Mediterranean and in Southwest Asia. Although ajwain seed is a popular spice, it also has been used in traditional herbal treatments for its diverse pharmacological properties. It is well known for its carminative properties from very ancient period of time and are also regarded as antispasmodic. In Traditional Persian Medicine (TPM) ajwain has been used factually as a therapeutic agent for gastrointestinal disorders, neurological disorders, respiratory tract disorders and genital disorders; and also has been used as analgesic, aphrodisiac, galactagogue, diuretic, anthelmintic, anti-inflammatory agent and antidote for various natural toxic agents (Zarshenas et al., 2013). Ajwain seeds are commonly used as agent for the household remedy of cholera, colic, diarrhea, dyspepsia, hypertension, asthma and hepato-biliary complications (Gilani et al., 2005). However, a number of *in vivo* experiments proved antibacterial (Esmaeili and Ghobadianpour, 2016), antihypertensive, antispasmodic, bronchodilator and hepatoprotective (Gilani et al., 2005),

anti-inflammatory (Thangam and Dhananjayan, 2003), antiepileptic (Rajput et al., 2013), analgesic (Dashti-Rahmatabadi et al., 2007) activities of various extracts of ajwain.

The constituents found by ajwain seed analysis includes fiber (11.9%), carbohydrates (38.6%), tannins, glycosides, moisture (8.9%), protein (15.4%), fat (15.4%), saponins, flavone and mineral matter (7.1%) containing calcium, phosphorus, iron and nicotinic acid (Bairwa et al., 2012). All the aqueous, methanolic, acetonic and ethanolic ajwain seed extracts and essential oil have shown high antioxidant activity and free radical scavenging potential (Goswami and Chatterjee, 2014). Additionally, many researchers have already characterized various ajwain extracts and essential oil phytochemically. Thymol and p-cymene were found in ajwain essential oil as major phenolic and non-phenolic component respectively (Singh et al., 2004). Twenty five compounds including new aromatic glucosides and glucides were obtained in water soluble portion of the methanol extract of ajwain (Ishikawa et al., 2001). The major compound found in ethanolic extract of ajwain is carvacrol (iso-thymol) which is followed by emersol and minor compounds- phenol, 4-methoxy-2,3,6-trimethyl-, ethyl ester, terpineol, terpinene and cymene (Tariq et al., 2014). A recent study showed that the crude ethanol extract of ajwain showed antioxidant activity more effectively than its purified thymol rich fraction (Sameera and Shamim, 2017).

PC12 is a model cell line which has ease of culture with ample background knowledge on their proliferation, has versatility for pharmacological manipulations and has been extensively using for toxicity studies (Rahman et al., 2017; Westerink and Ewing, 2008). Recently, numerous studies have come up to unveil the cytotoxicity mechanisms posed by Cd^{2+} on many cell lines including PC12 cells (Hossain et al., 2018; Rahman et al., 2017). Historically, ajwain has already been used for the remedy of many pathological consequences (such as inflammatory, toxic, neurological, genital or respiratory tract disorders) those could be manifested by Cd^{2+} exposure. Cd^{2+} poses oxidative damage to biomolecules and induce cytotoxicity leading to many chronic diseases; hence the traditional medicinal antioxidant herb, ajwain could be effectively manipulated as influential therapeutic against it. That's why it was thought that the cytotoxicity induced by Cd^{2+} could be relieved by the antioxidant ability depending on of ajwain extracts. Therefore, the present study was designed with novelty to elucidate the effects of ethanolic ajwain extract (AE) on Cd^{2+} induced cytotoxicity in PC12 cells.

Moreover, it was also aimed to examine the underlying molecular mechanism of cytotoxicity following separate and/or combined exposure of Cd²⁺ and AE.

2.2 Materials and methods

2.2.1 Materials

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's (DMEM), ribonuclease A (RNase) and ethyidium bromide were procured from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biosera (Kansas City, MO, USA). Trypan blue solution (0.4%) was purchased from Bio-Rad (Hercules, CA, USA). Polyclonal antibodies against β -actin (#4967, Cell Signaling Technology), Bax (sc-7480, Santa Cruz Biotechnology), Bcl-2 (# PC68, OncogeneTM Research Products), Bcl-xL (#610211, BD Biosciences), NF κ B p65 (sc-109, Santa Cruz Biotechnology), caspase-3 (#611428, BD Biosciences) were purchased. The Cytochrome c Release Apoptosis Kit (Q1A87-1KIT) was procured from Calbiochem^R. Anti-rabbit IgG (H+L) HRP conjugate (W4011) and anti-mouse IgG (H+L) HRP conjugate (W4021) were purchased from Promega Corporation (Madison, WI USA). ECL western blotting reagent was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Proteinase K was purchased from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

2.2.2 Preparation of AE

Sun dried seeds of *Trachyspermum ammi* (ajwain) were procured from local market, Dhaka, Bangladesh and authenticated by Dr. M. Sayedur Rahman, Senior Scientific Officer of Bangladesh National Herbarium. The seed sample along with plant specimen was deposited with voucher specimen number DCAB-46748 at Bangladesh National Herbarium, Mirpur, Dhaka-1216, Bangladesh.

The whole dried and cleaned ajwain seeds were crushed into powder in a mortar pestle to prepare the ajwain seed powder. One gram of ajwain seed powder was soaked and mixed well with 25 ml of 80% ethanol. The mixture was shaken vigorously and kept in water bath at 60°C with shaking in air-tight condition for 48h. The AE was then filtered twice by using filter paper and was stored in -20°C in air-tight condition for the use in the present experiment. The steps for AE preparation has been represented in Fig. 2.1.

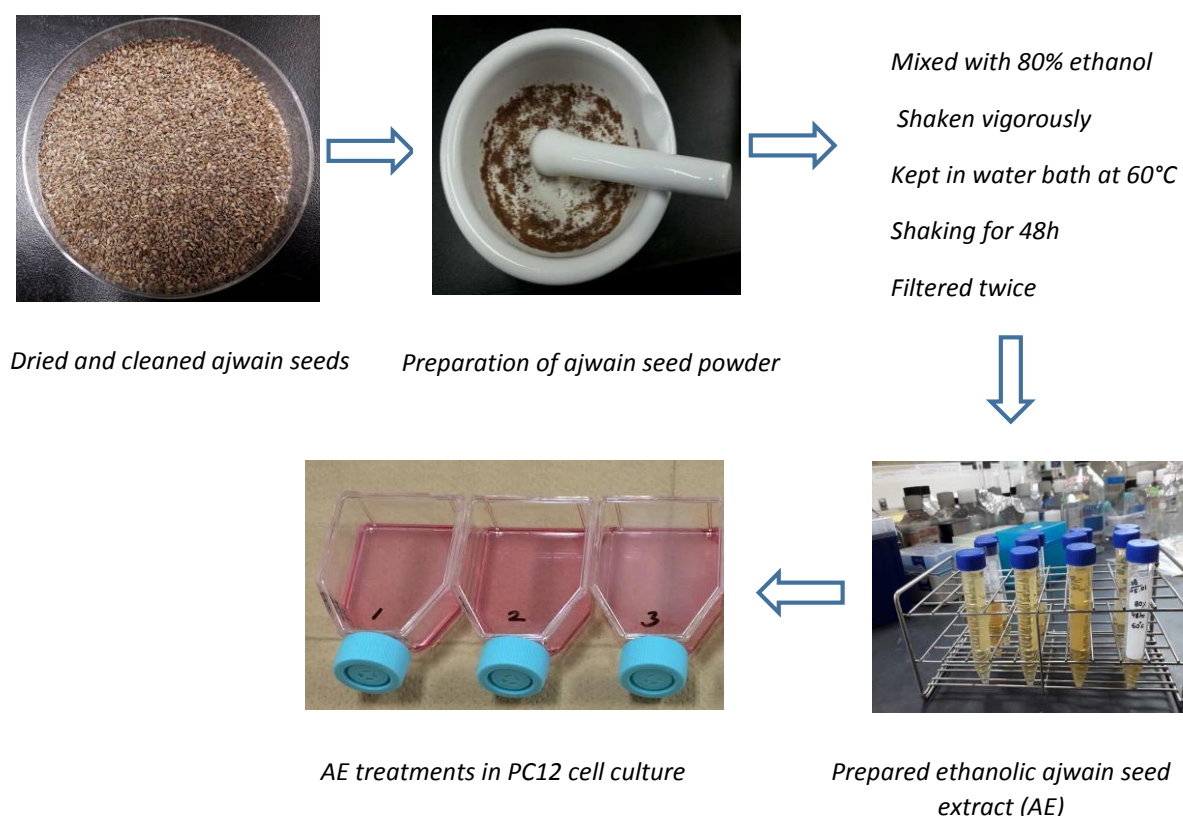


Fig. 2.1 Preparation of ethanolic ajwain (*Trachyspermum ammi* L.) seed extract (AE)

The recovered volume of the filtrate was measured 16.60 mL. So, 1 μ L of AE contained extract of 60 μ g of dried ajwain seed. In this study, AE was added into cell culture media in various concentrations presented in Table 2.1.

Table 2.1 Concentrations of AE treated/co-treated in PC12 cells

Volume of AE	Volume of media	Concentration of AE in media
5 μ L	5 mL	Extract of 60 μ g of dried ajwain seed/mL media (60 μ g/mL)
10 μ L	5 mL	Extract of 120 μ g of dried ajwain seed/mL media (120 μ g/mL)
20 μ L	5 mL	Extract of 240 μ g of dried ajwain seed/mL media (240 μ g/mL)
40 μ L	5 mL	Extract of 480 μ g of dried ajwain seed/mL media (480 μ g/mL)
80 μ L	5 mL	Extract of 960 μ g of dried ajwain seed/mL media (960 μ g/mL)
160 μ L	5 mL	Extract of 1920 μ g of dried ajwain seed/mL media (1920 μ g/mL)

2.2.3 Cell culture

PC12 cells were cultured in DMEM supplemented with 10% FBS, and the culture conditions were maintained in a humidified incubator at 37°C with 5% CO₂. The cells were

seeded and pre-incubated in 25 cm² flasks for 48 h, and then the medium was replaced with FBS supplemented DMEM with or without treatments/co-treatments of various concentrations of Cd²⁺ as CdCl₂ and AE. The cells were incubated at 37°C for 24 h following treatments/co-treatments. The safe doses of AE were primarily selected from cell viability results of PC12 cells treated with various concentrations (60, 120, 240, 480, 960 and 1,920 µg/mL) of AE for 24 h.

2.2.4 Cell viability

Determination of cell viability was accomplished by using trypan blue exclusion assay. PC12 cells were seeded in supplemented DMEM and pre-incubated for 48 h. After 48 h, the cells were treated with AE (60, 120, 240, 480, 960 and 1,920 µg/mL) for 24 h. The safe but active toxicity ameliorating dose of AE against Cd²⁺ was selected as 240 µg/mL (Fig. 2.2A). Afterward, the PC12 cells were treated with Cd²⁺ (5 or 10 µM) and AE (240 µg/mL), separately or in combination of both, and the cells were further incubated for 24 h. After incubation the cells were collected and stained with 0.2% trypan blue in phosphate-buffered saline (PBS) solution. Total cells and trypan blue-stained cells were counted using a cell counter (TC10™ Automated Cell Counter, Bio-Rad). Cell viability was expressed as percentage of the alive cells. Cell viability experiments were carried out at least in triplicate to ensure biological reproducibility and statistical validity.

2.2.5 Lactate dehydrogenase (LDH) activity assay

Damaged or compromised cell membrane integrity due to cytotoxicity was assessed by LDH activity assay of the treated medium using a nonradioactive cytotoxicity assay kit as described by Kihara et al. (2012). In brief; 48 h after pre-incubation, PC12 cells were cultured in the medium containing Cd²⁺ (5 or 10 µM) with/without AE (240 µg/mL) for 24 h. After incubation, 50 µL of the cultured medium was taken to a 1.5 mL tube, and then, 50 µL of a substrate mixture containing terazolium salts was added to the tube. The tube was then incubated at room temperature for 30 min, and then 50 µL of the stop solution was added. The amount of formazan dye formed was determined by measuring the absorbance at 490 nm using an iMark™ microplate reader (BioRad; Hercules, CA, USA). LDH activity was calculated as LDH activity/1 × 10⁶ cells. These experiments were accomplished at least three times to ensure reproducibility.

2.2.6 Measurement of intracellular free sulfhydryl (-SH) levels

Intracellular free -SH levels were measured by following the method previously described by Kihara et al., (2012) and Rahman et al., (2018). PC12 cells were treated Cd^{2+} (5 or 10 μM) with and without AE (240 $\mu\text{g}/\text{mL}$) for 24 h. The cells were subsequently harvested, washed with $1 \times \text{PBS}$, added to 150 μL of a lysis buffer, and then incubated for 10 min at 4°C . Then, two freeze-thaw cycles were to break the cell membranes. After centrifugation, the supernatant was collected and the total protein content were measured spectrophotometrically using protein assay dye reagent (Bio-Rad; Hercules, CA, USA). Intracellular free -SH levels were assessed using 2.5 μM of (DTNB) at 412 nm wavelength. The concentrations of free -SH in the PC12 cells were finally measured by a molecular coefficient factor of 13,600 per 1×10^6 cells. Experiments were repeated at least in triplicate to achieve reproducibility.

2.2.7 Extraction of genomic DNA from PC12 cells

PC12 cells were harvested using a scraper 24 h after the treatment with Cd^{2+} (5 or 10 μM) and/or AE (240 $\mu\text{g}/\text{mL}$). Cells were washed with $1 \times \text{PBS}$ and the genomic DNA was isolated using High Pure PCR Template Kit (Roche Life Science; Penzberg, Germany) following the manufacturer's instructions as described by Hossain et al. (2018) and Kawakami et al. (2008). The extracted DNA solution was allowed to stand overnight at -20°C . Precipitated DNA was suspended in $1 \times \text{TBE}$ solution and DNA concentration was measured using a Personal Spectrum Monitor (Gene Quant Pro, GE, USA). Then, DNA concentrations were made unified for all samples by adding $1 \times \text{TBE}$ solution.

2.2.8 Agarose gel electrophoresis of genomic DNA

Genomic DNA was extracted from PC12 cells cultured in the medium containing Cd^{2+} (5 or 10 μM) with and without AE for 24 h, and was subjected to agarose gel electrophoresis to determine the fragmentation level. Electrophoresis of DNA in an amount of about 3 -5 μg along with loading dye was conducted on 1.5% agarose gel using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). After electrophoresis, DNA in gel was visualized followed by staining with ethidium bromide for 10 min. A ChemiDoc XRS system (Bio-Rad; Hercules, CA, USA) was used to take digital images of the agarose gel under UV illumination. The fluorescence intensity of DNA in the gel was analyzed to evaluate the

amount of fragmentation by using 'Quantity One' software. Every experiment was conducted minimum in triplicate to ascertain biological reproducibility.

2.2.9 Western blot analysis for the determination of protein expression

PC12 cells were harvested using a scraper 24 h after the treatment with Cd²⁺ (5 or 10 µM) and/or AE (240 µg/mL). Western blot analysis was accomplished by following the procedure described by Rahman et al., (2018). Briefly, PC12 cells were suspended in 150 µL ice-cold lysis buffer (2 mM HEPES, 100mM NaCl, 10mM EGTA, 0.1mM PMSF, 1 mM Na₃VO₄, 0.1 mM Na₂MoO₄, 5mM 2-glycerophosphorrate, 10mM MgCl₂, 2mM DTT, 50mM NAF and 1%triton X-100) for the total protein extraction. The cells suspended in lysis buffer was allowed to undergo two cycles of sonication followed by centrifugation at 1500 rpm for 10 min. Additionally, cytosolic proteins were extracted using the cytosolic lysis buffer provided in the 'Cytochrome c Release Apoptosis Kit (Q1A87-1KIT)' for the western blot of cytochrome c. Then the protein concentration in the supernatant of the lysate was measured using a protein assay dye reagent (Bio-Rad, Hercules, CA, USA). Thereafter the extracted protein from each sample (20 µg) was separated by 12.5-15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis and subsequently transferred on nitrocellulose membrane using a semi-dry blotting system (type- AE6678; ATTO, Tokyo, Japan). The protein transferred nitrocellulose membrane was then blocked overnight by 5% skim milk at 4°C. After blocking, each membrane was washed three times with 0.1% Tween buffer and was sequentially incubated with desired primary antibodies at 37°C for 1 h. Then each membrane was again washed three times with 0.1% Tween buffer and incubated with specific secondary antibody for 60 min at 37°C. The nitrocellulose membrane was washed again and the protein bands on the membrane were visualized by chemiluminescence. Lastly, the band images were analyzed using a ChemiDoc XRS (Bio-Rad, USA). The intensity of a specific protein band was expressed as the ratio to that of the intensity of β-actin. All the experiments were done at least in triplicate to confirm the reproducibility.

2.2.10 LC-MS analysis of AE

Liquid chromatography-mass spectrometry (LC-MS) analysis of ethanolic extract of ajwain (AE) was conducted to be confirmed about the presence of carvacrol which was detected as the major compound by Tariq et al., 2014. LC-MS was conducted on an Agilent 1100 Series HPLC system coupled with a Bruker Daltonics micrOTOF-HS mass spectrometer

(ESI). The HPLC system was equipped with a Cadenza CD-C18 column (2 x 150 mm, 3 μ m) under the following conditions: 0-15 min, gradient elution of 20-80% MeCN with 0.1% (v/v) formic acid in H₂O. 15-25 min., isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in H₂O; at 25° C and a flow rate of 0.2 mL/min.

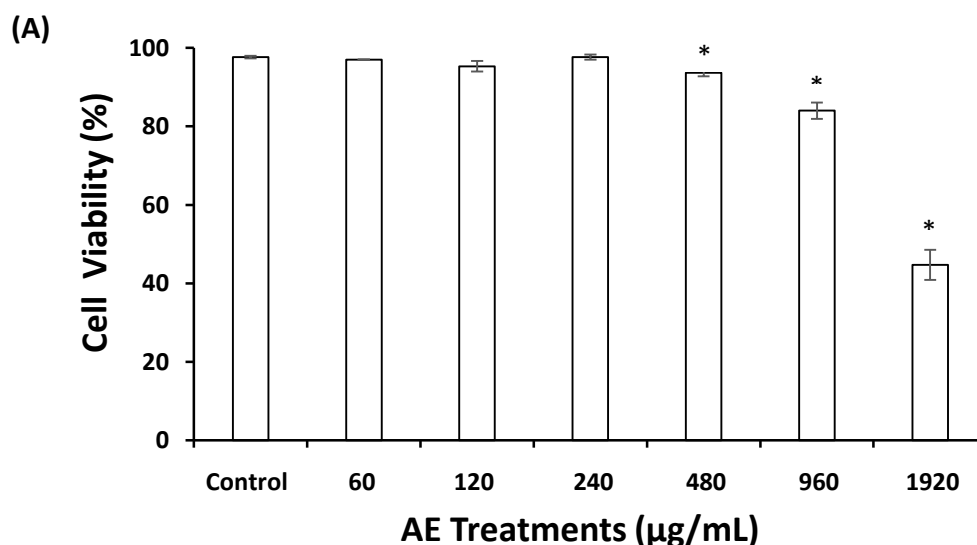
2.2.11 Statistical analysis

All data were statistically represented as means $\pm$ standard error of mean (SEM). Statistical significance was measured by unpaired Student's t-test following transformation to normalized data; $P < 0.05$ was considered to indicate significance. Statistical analysis was performed using MS excel 2007 program.

2.3 Results

2.3.1 Cell Viability

The effects of AE on PC12 cells were investigated by treating cells with various concentrations of AE (60, 120, 240, 480, 960 and 1920 μ g/mL) for 24 h. Based on the cell viability results *via* trypan blue staining, 60, 120 and 240 μ g/mL of AE showed no toxicity in PC12 cells (Fig. 2.2A). Then, non-toxic doses of AE and Cd²⁺ (5 or 10 μ M) were co-treated to choose the appropriate dose of AE for co-exposure with the Cd²⁺ (5 or 10 μ M) for the later experiments. Although the cell viability results showed that treatments of both 5 and 10 μ M of Cd²⁺ reduced the cell viability, only co-exposure of 240 μ g/mL of AE recovered the cell viability of the cell treated with Cd²⁺ significantly (data not shown).



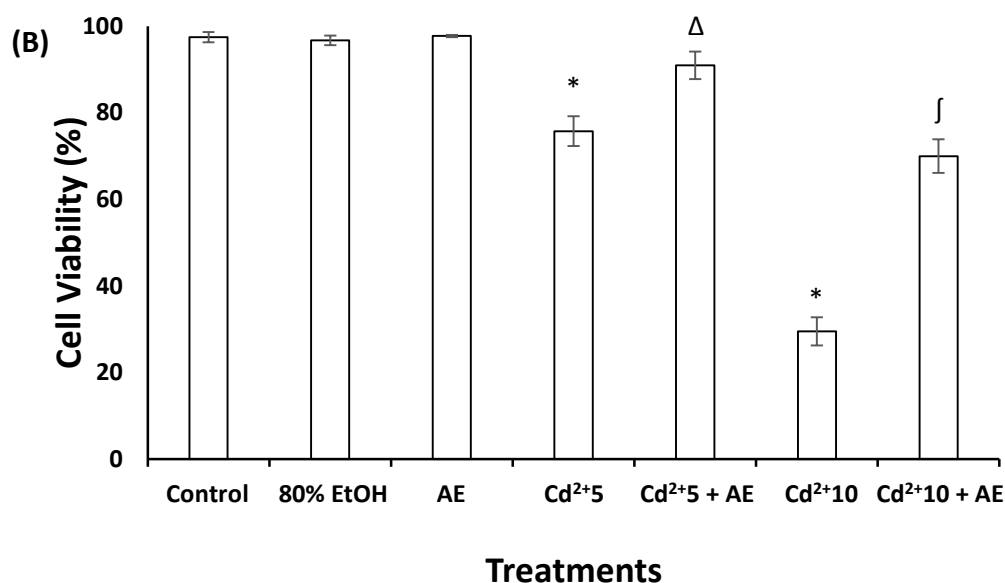


Fig. 2.2 (A) Viability of PC12 cells treated with different concentrations of AE for 24 h. (B) Viability of PC12 cells treated/co-treated with Cd²⁺ (5 or 10 μ M) and with or without AE (240 μ g/mL) for 24 h. Error bars indicate mean $\pm$ SEM (n = 4). * denotes significant difference at $p < 0.05$ compared to control group, Δ denotes significant difference at $p < 0.05$ compared to only 5 μ M Cd²⁺ treated group and J denotes significant difference at $p < 0.05$ compared to only 10 μ M Cd²⁺ treated group.

Thus, the dose of AE in the entire experiments was determined as 240 μ g/mL from the cell viability result. In summary, as shown in Fig. 2.2B, the cell viability was significantly reduced ($P < 0.05$) after the exposure of both 5 and 10 μ M of Cd²⁺ for 24 h. The reduced cell viability was significantly increased ($P < 0.05$) when AE (240 μ g/mL) was co-exposed with either 5 or 10 μ M of Cd²⁺ in PC12 cells for 24 h.

2.3.2 Effects of Cd²⁺ and AE co-exposure on LDH activity as an indicator of cytotoxicity

To confirm the effects of AE on cell toxicity induced by Cd²⁺ in PC12 cells, LDH assay was performed. The relative levels of LDH activity was remarkably increased ($P < 0.05$) in the medium for the cells treated with 5 or 10 μ M of Cd²⁺. However, co-treatments of AE with 5 or 10 μ M of Cd²⁺ in the medium for PC12 cells showed significantly decrease ($P < 0.05$) of the LDH activity (Fig. 2.3).

In addition, treatment of only AE has no effect. The obtained results were in good agreement with the results of cell viability study (Fig. 2.2B). These results of LDH activity suggest that Cd²⁺ (5 or 10 μ M) posed cytotoxic effects on PC12 cells leading to the cell membrane damage and AE (240 μ g/mL) recovered it.

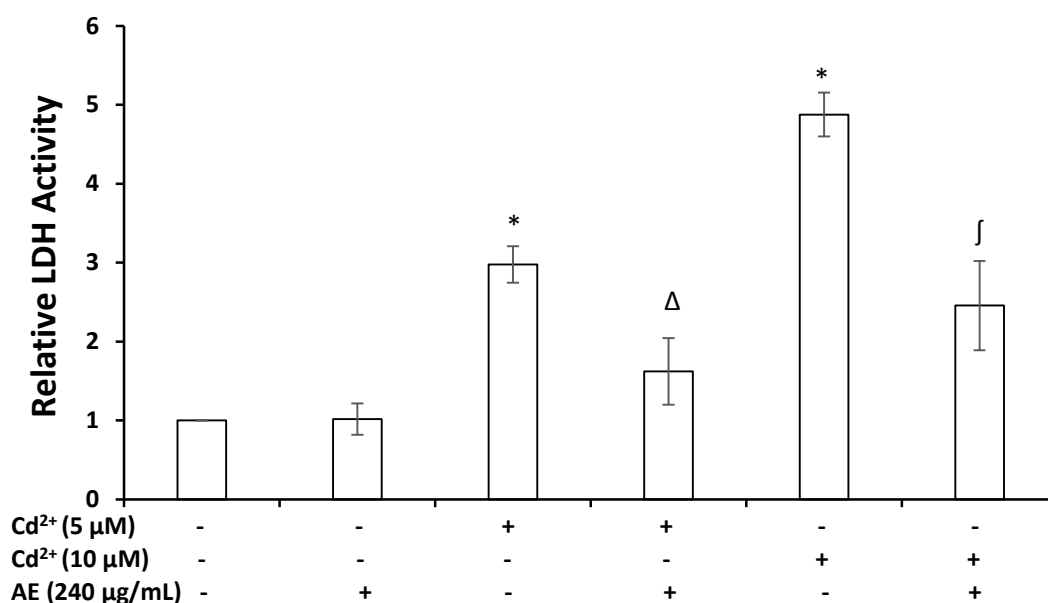


Fig. 2.3 Relative LDH activity levels in the PC12 cells culture medium treated/co-treated with Cd²⁺ (5 or 10 μM) and with or without AE (240 μg/mL) for 24 h. Error bars indicate mean ± SEM (n = 5). * denotes significant difference at p < 0.05 compared to control group, Δ denotes significant difference at p < 0.05 compared to only 5 μM Cd²⁺ treated group and J denotes significant difference at p < 0.05 compared to only 10 μM Cd²⁺ treated group.

2.3.3 Intracellular free sulfhydryl (-SH) levels after Cd²⁺ and AE co-exposure

Glutathione is the most abundant low-molecular weight thiol compound synthesized in cells. Reduced glutathione (GSH) acts critically as an important component of cellular antioxidant system in guarding cells from oxidative damage and maintains redox homeostasis (Forman et al., 2009; Rahman et al., 2017). Intracellular free SH levels were measured in lysates of PC12 cells after treatment of Cd²⁺ (5 or 10 μM) and/or AE for 24 h.

Treatments of both of Cd²⁺ significantly decreased the intracellular free SH levels in comparison to that of the control group. The reduction of intracellular free SH level due to Cd²⁺ treatment was improved significantly (P < 0.05) when AE (240 μg/mL) were co-exposed (Fig. 2.4).

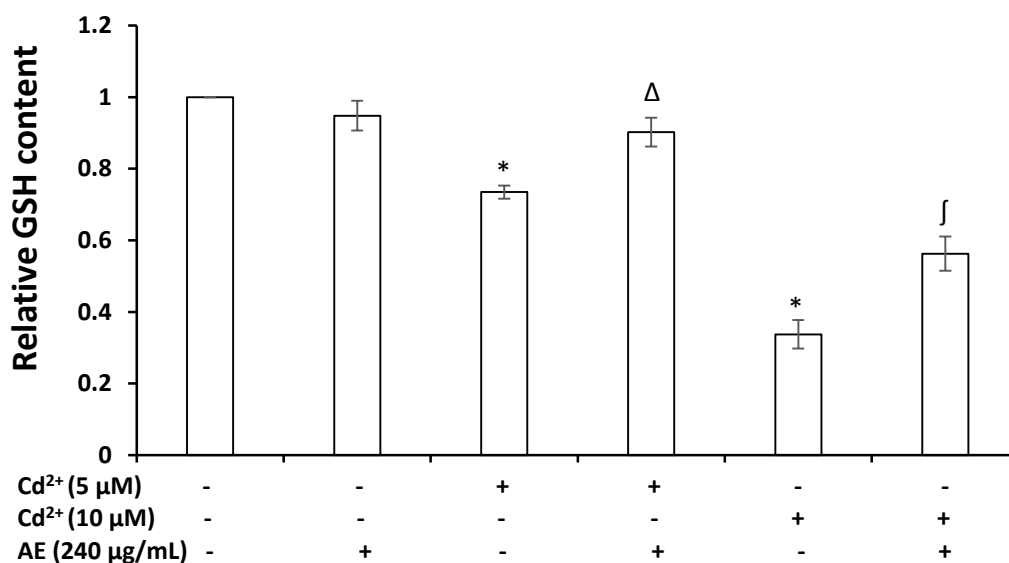


Fig. 2.4 Relative intracellular GSH contents in the PC12 cells treated with Cd²⁺ (5 or 10 μM) and with or without AE (240 μg/mL) for 24 h. Error bars indicate mean ± SEM (n = 3). * denotes significant difference at p < 0.05 compared to control group, Δ denotes significant difference at p < 0.05 compared to only 5 μM Cd²⁺ treated group and J denotes significant difference at p < 0.05 compared to only 10 μM Cd²⁺ treated group.

Here, the findings of intracellular free SH levels together with cell viability (Fig. 2.2B) and LDH activity (Fig. 2.3) indicated that AE dose could lessen oxidative stress posed by Cd²⁺ alone.

2.3.4 Analysis for genomic DNA fragmentation

Ladder pattern of genomic DNA due to the cleavage of chromatin into small fragments becomes visible when apoptosis is induced in the cells (Matassov et al., 2004).

The extracted genomic DNA from PC12 cells treated with Cd²⁺ (5 or 10 μM) and/or AE (240 μg/mL) was electrophoresed using 1.5% agarose gel to inspect whether the DNA fragmentation took place. Electrophoresis of genomic DNA of cells treated with Cd²⁺ (5 or 10 μM) showed ladder patterns. Thus, it was indicated that Cd²⁺ treatments exerted toxic effects causing apoptotic cell death; however, the intensity of ladder pattern caused by Cd²⁺ treatment was reduced by co-exposure of AE with Cd²⁺ (Fig. 2.5A). This result was also confirmed by measurement of fluorescence intensity for ladder in each lane (Fig. 2.5B). The results also showed that DNA fragmentation level in PC12 cells treated with only AE was not

changed in comparison to the control. Thus, it was indicated that AE can reduce apoptosis induced by Cd^{2+} .

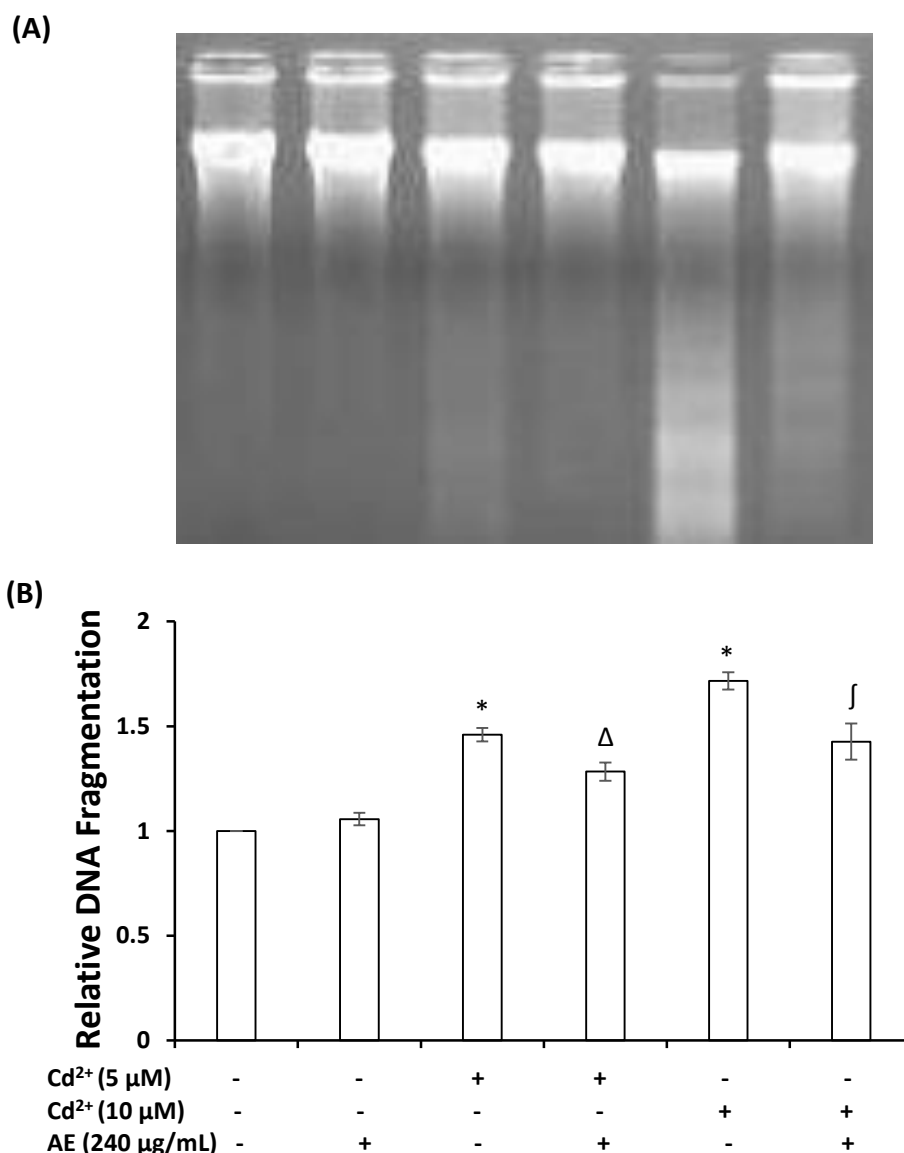
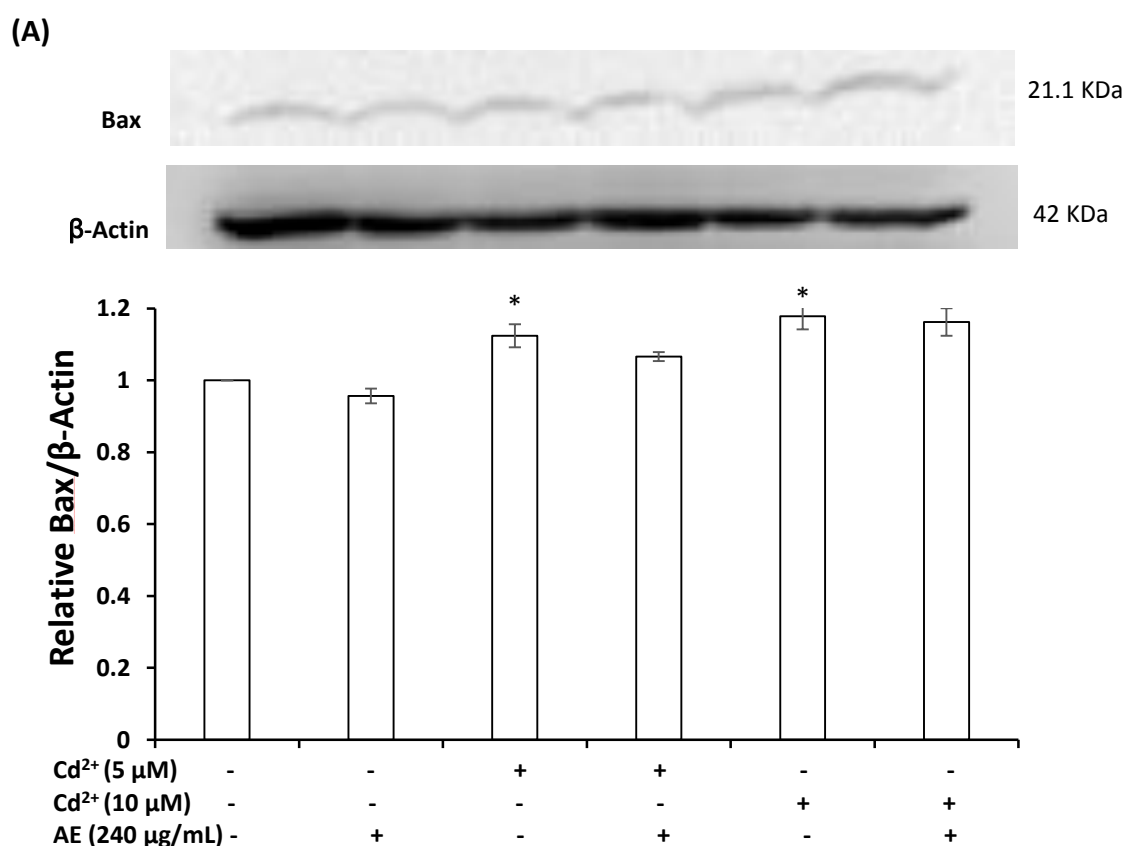


Fig. 2.5 Agarose gel electrophoresis of extracted genomic DNA from PC12 cells treated with Cd^{2+} (5 or 10 μM) and with or without AE (240 $\mu\text{g/mL}$) for 24 h. (A) Representative image of DNA electrophoresis, (B) the fluorescence intensity measured for relative DNA fragmentation. Error bars indicate mean $\pm$ SEM ($n = 3$). * denotes significant difference at $p < 0.05$ compared to control group, Δ denotes significant difference at $p < 0.05$ compared to only 5 μM Cd^{2+} treated group and $\int$ denotes significant difference at $p < 0.05$ compared to only 10 μM Cd^{2+} treated group.

2.3.5 Expressions of Bax and Bcl-2

Active pro-apoptotic protein, Bax ensures cell death via pore formation at the mitochondrial outer membrane, and the anti-apoptotic protein Bcl-2 prevents most forms of

apoptotic cell death (Tsujimoto and Shimizu, 2000; Zhang et al., 2017). However, cell susceptibility to apoptosis is determined by the Bax/Bcl-2 ratio which can be a rheostat (Raisova et al., 2001). In this study, there were significant ($P < 0.05$) increases in relative levels of Bax protein contents 24 h after exposure with 5 or 10 μM Cd^{2+} (Fig. 2.6A). However, these significant increases of Bax were lowered by the co-exposure of AE with Cd^{2+} . In addition, as shown in Fig. 2.6B, the levels of anti-apoptotic protein Bcl-2 contents were significantly decreased ($P < 0.05$) by the treatments of Cd^{2+} ; however, the contents of Bcl-2 were significantly increased ($P < 0.05$) after the co-treatment of AE and Cd^{2+} for 24 h.



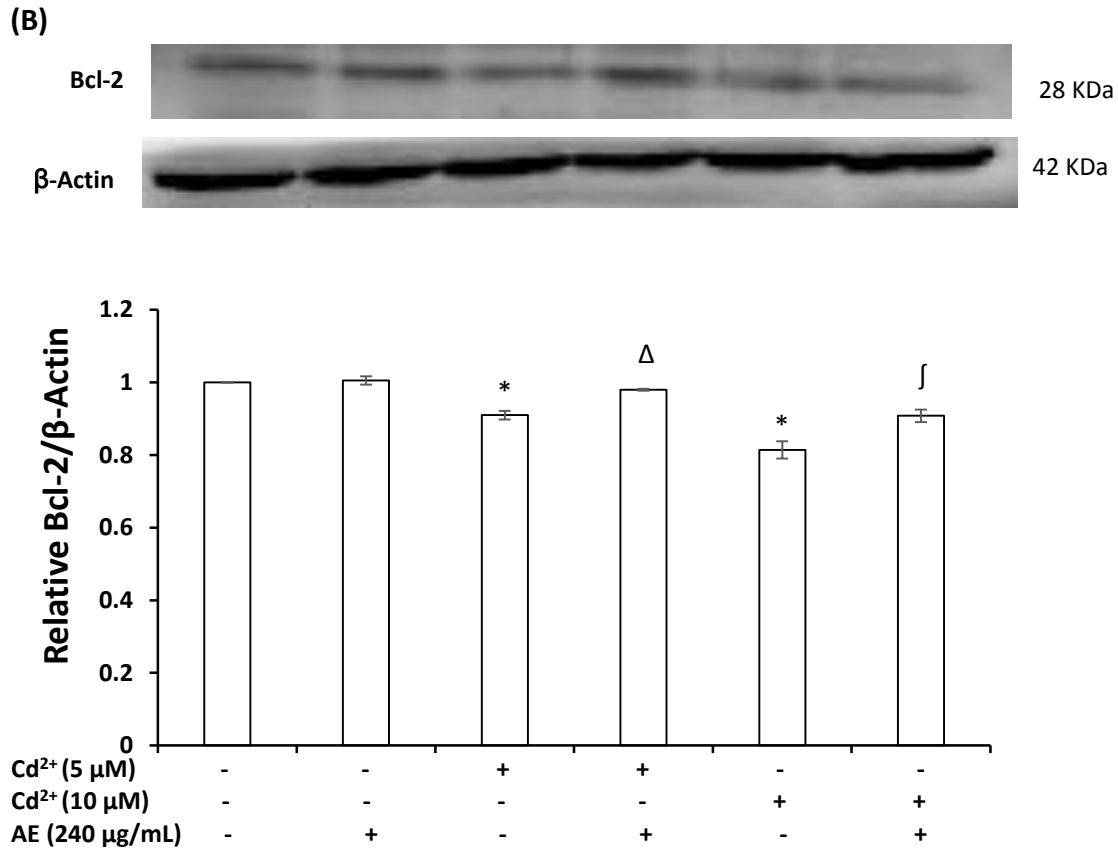


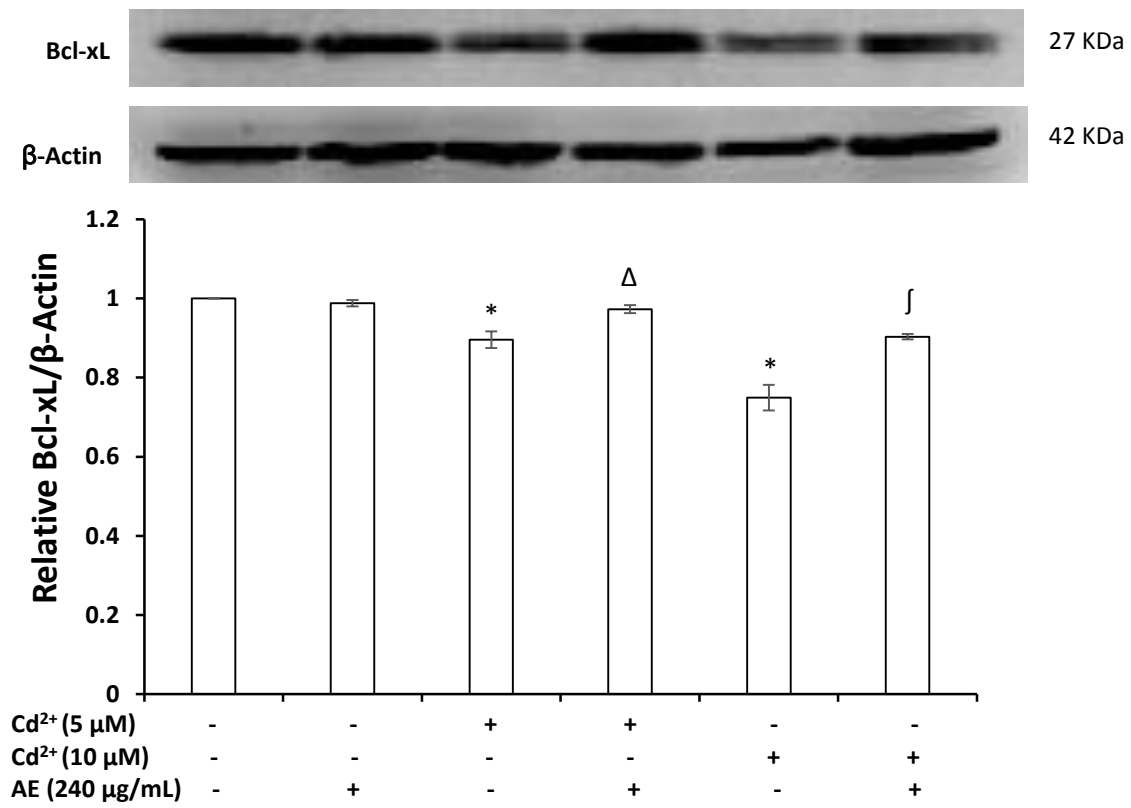
Fig. 2.6 Relative contents of (A) Bax and (B) Bcl-2 in PC12 cells treated with Cd²⁺ (5 or 10 μM) and with or without AE (240 μg/mL) for 24 h. Error bars indicate mean ± SEM (n = 3). * denotes significant difference at p < 0.05 compared to control group, Δ denotes significant difference at p < 0.05 compared to only 5 μM Cd²⁺ treated group and ∫ denotes significant difference at p < 0.05 compared to only 10 μM Cd²⁺ treated group.

Here, there was a net increase in Bax/Bcl-2 ratio. It was supposed that treatment of Cd²⁺ induced apoptosis in PC12 cells. However, to protect the cells against apoptosis, co-treatments of AE and Cd²⁺ could decrease the ratio of Bax/Bcl-2 contents in comparison with the treatments of Cd²⁺ only.

2.3.6 Expressions of Bcl-xL and NF-κB

The control of apoptosis by NF-κB through genomic surveillance on Bcl-xL has already been elucidated earlier (Antwerp et al., 1996; Karin, 2006). Moreover, through neutralization of reactive oxygen species (ROS), NF-κB indirectly prevents mitochondria-mediated apoptosis (Naugler and Karin, 2008).

(A)



(B)

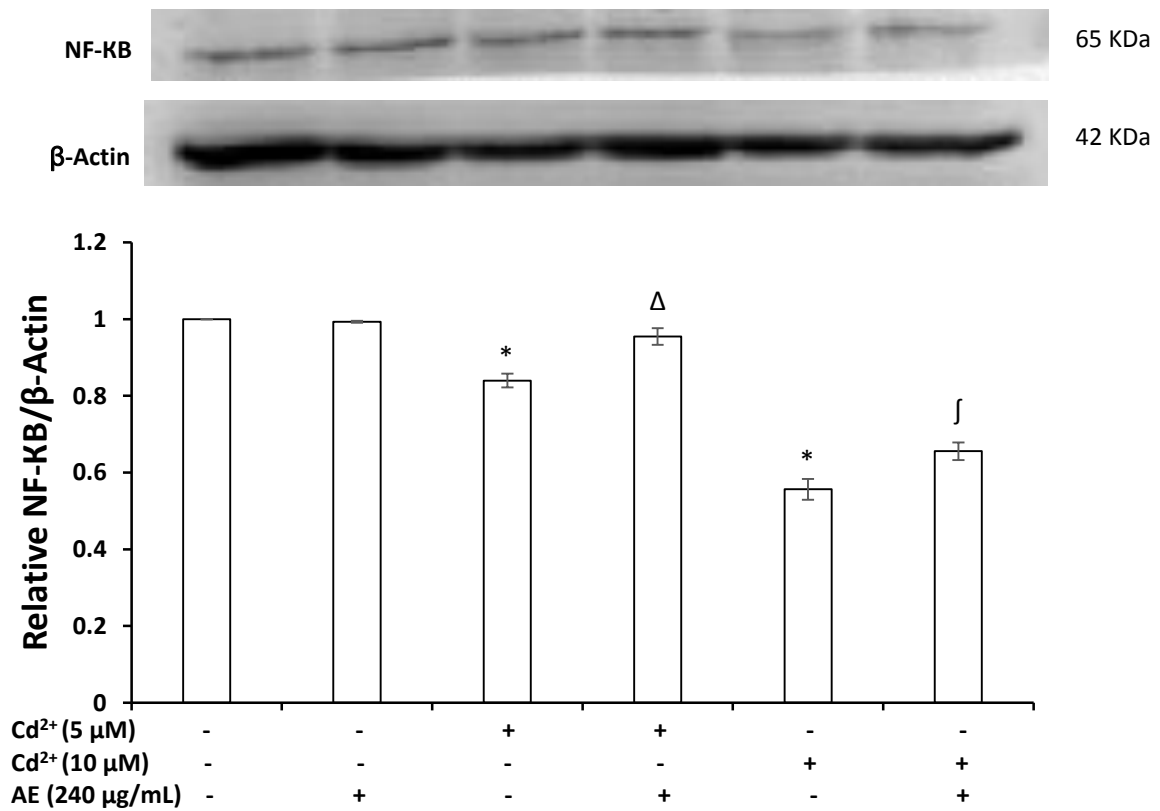


Fig. 2.7 Relative content of (A) Bcl-xL and (B) NF- κ B expressed in PC12 cells treated with Cd²⁺ (5 or 10 μ M) and with or without AE (240 μ g/mL) for 24 h. Error bars indicate mean $\pm$ SEM (n = 3). * denotes significant difference at p < 0.05 compared to control group, Δ denotes significant difference at p < 0.05 compared to only 5 μ M Cd²⁺ treated group and J denotes significant difference at p < 0.05 compared to only 10 μ M Cd²⁺ treated group.

Here, the treatment of 5 or 10 μ M of Cd²⁺ considerably reduced the relative contents of Bcl-xL and NF- κ B which were significantly increased by the co-treatments of AE and Cd²⁺ (Figs. 2.7A and B). The results for both the pro-apoptotic protein Bcl-xL and the cell survival proteins NF- κ B contents in the PC12 cells are in agreement with the results found for relative DNA fragmentation (Fig. 2.5) and Bax and Bcl-2 contents (Fig. 2.6). Thus, the decline in relative contents of both NF- κ B and Bcl-xL proteins after the treatment of Cd²⁺ (5 or 10 μ M) suggested that apoptosis was induced in PC12 cells; however, this decline was subsequently improved by the co-treatments of AE (240 μ g/mL) and Cd²⁺ (5 or 10 μ M).

2.3.7 Cytochrome c released from mitochondria into the cytosol

Cytochrome c is the principal molecule among the molecules released from the mitochondrial compartment which associates with Apaf-1 to initiate the formation of apoptosome during mitochondrial apoptotic pathway (Choi et al., 2006).

Western blot results exhibited that the treatment of Cd²⁺ at both 5 and 10 μ M concentrations significantly (P < 0.05) increased the relative cytochrome c level in cytosol of PC12 cells. It was indicated that Cd²⁺ could induce apoptosis in the cells. However, these higher levels of cytosolic cytochrome c were significantly suppressed by the co-treatments of AE with Cd²⁺ (5 or 10 μ M) (Fig. 2.8). These results of cytosolic cytochrome c levels along with results represented in Figs. 2.5, 2.6, and 2.7 suggested that both the doses of 5 and 10 μ M of Cd²⁺ induced mitochondrial pathway of apoptosis in PC12 cells. It was also suggested that AE could potentially inhibit the apoptosis when co-exposed with Cd²⁺. Thus, mitochondrial pathway of apoptosis induced by Cd²⁺ and ameliorated by AE had been established.

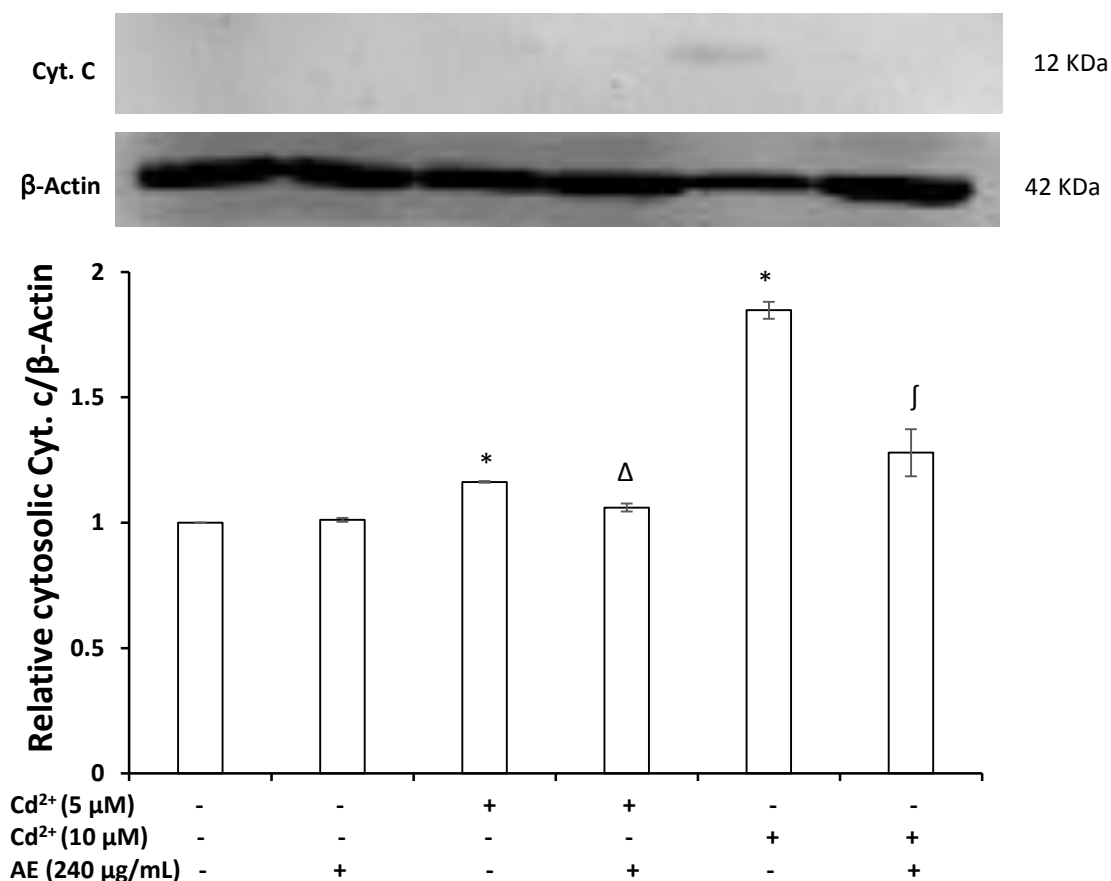


Fig. 2.8 Relative content of cytosolic cytochrome c in PC12 cells treated with Cd²⁺ (5 or 10 μM) and with or without AE (240 μg/mL) for 24 h. Error bars indicate mean ± SEM (n = 3). * denotes significant difference at p < 0.05 compared to control group, Δ denotes significant difference at p < 0.05 compared to only 5 μM Cd²⁺ treated group and f denotes significant difference at p < 0.05 compared to only 10 μM Cd²⁺ treated group.

2.3.8 Expression of caspase-3

The release of mitochondrial cytochrome c into cytosol induces activation of caspase-9, and subsequent activation of caspase-3 during intrinsic pathway of apoptosis (Palchaudhuri et al., 2015). In the present study, 24 h after the treatment of 5 or 10 μM of Cd²⁺ in PC12 cells, the relative levels of caspase-3 expression were found significantly (p < 0.05) higher than that in control cells. The elevated levels of caspase-3 was considerably (P < 0.05) reduced by the co-treatments of AE and Cd²⁺ (Fig. 2.9). Thus, AE could reduce intrinsic apoptosis reaction activated by Cd²⁺.

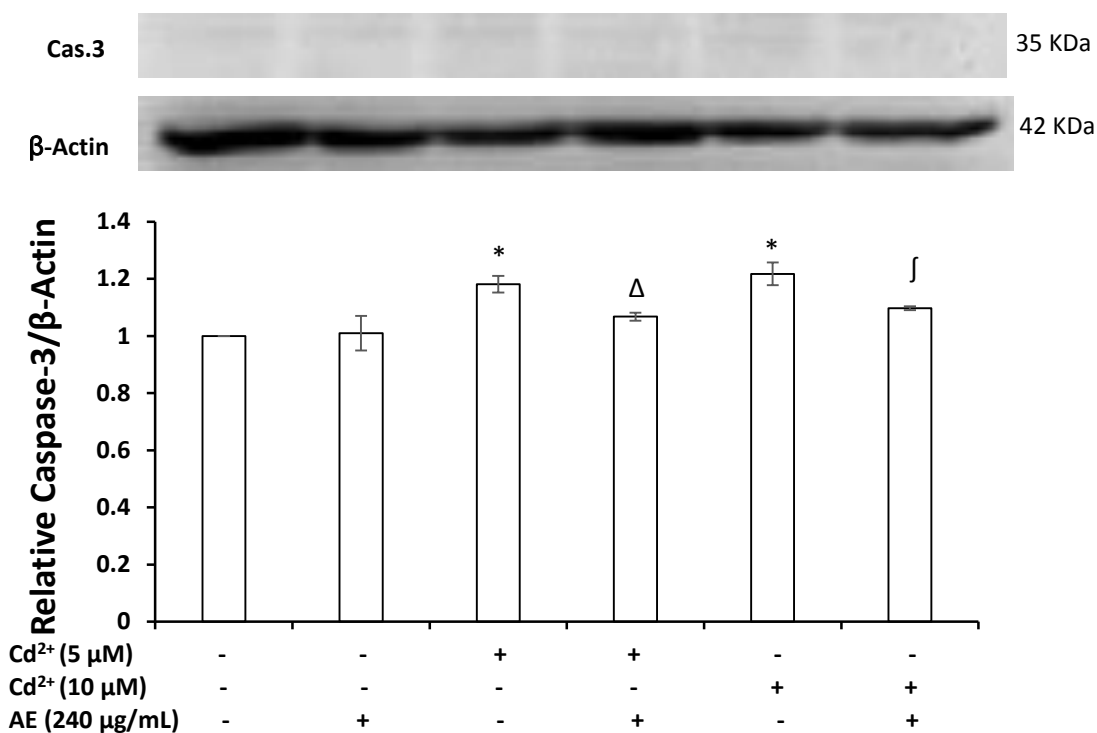
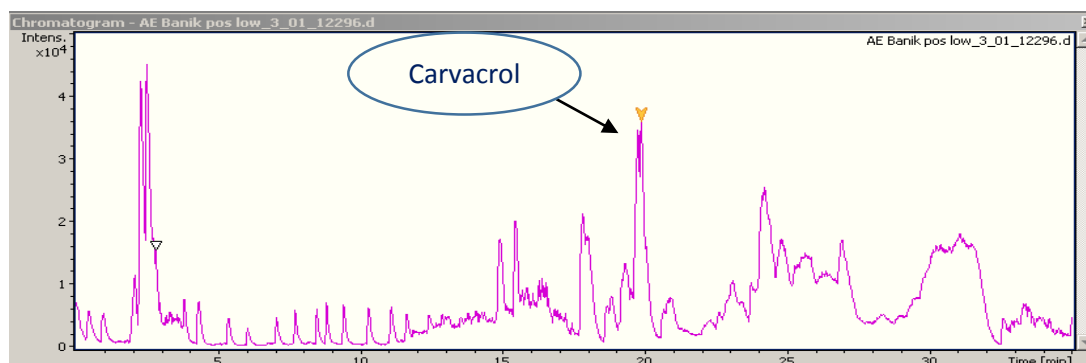


Fig. 2.9 Relative content of caspase-3 expressed in PC12 cells treated with Cd²⁺ (5 or 10 μM) and with or without AE (240 μg/mL) for 24 h. Error bars indicate mean ± SEM (n = 3). * denotes significant difference at p < 0.05 compared to control group, Δ denotes significant difference at p < 0.05 compared to only 5 μM Cd²⁺ treated group and ∫ denotes significant difference at p < 0.05 compared to only 10 μM Cd²⁺ treated group.

2.3.9 LC-MS analysis profile of carvacrol in AE

To reconfirm the major phenolic compound in AE, LC-MS analysis was conducted. It was found that the highest phenolic compound intensity peak (M + H⁺ = 151.1127 g/mole) was calculated for the molecule [C₁₀H₁₄O + H⁺ = 151.1122 g/mole] and was identified as carvacrol.

(A)



(B)

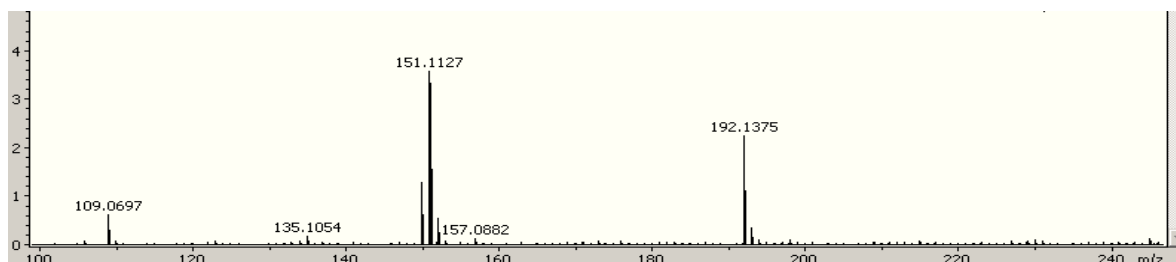


Fig. 2.10 (A) LC-MS chromatogram showing the highest phenolic compound peak for carvacrol in AE. (B) Mass spectrum for carvacrol in ethanolic extract of ajwain (AE).

2.4 Discussion

Ajwain seed is well known for its pharmacological and therapeutic use for a long period of time. Moreover, various ajwain extracts have high antioxidant activity and pose potential ameliorative effects against oxidative stress *in vivo* and *in vitro* (Anilkumar et al., 2009; Goswami and Chatterjee, 2014). Thus, ethanolic ajwain extract (AE) was chosen to investigate the role of AE on the Cd^{2+} induced oxidative stress, cytotoxicity and apoptosis in PC12 cells. Moreover, the molecular mechanism of the improvement of cytotoxicity and apoptosis was also intended to be elucidated using AE. The toxic metal, Cd^{2+} was selected for the study for its ubiquitous presence in the environment as contaminant and also for its harmful impacts on human health. Additionally, ajwain is traditionally used for the remedy of many pathological consequences those could be manifested by Cd^{2+} exposure e.g. inflammatory, toxic, neurological, genital or respiratory tract disorders. Probably, this study is the first one which clarifies the protective effects of AE at most effective dose against heavy metal-induced cytotoxicity and apoptosis with molecular mechanisms. Primarily, based on the cell viability results, the safe and non-toxic doses of AE (i.e., 60, 120 and 240 $\mu\text{g}/\text{mL}$) were nominated and then the dose of 240 $\mu\text{g}/\text{mL}$ was finally selected to be co-exposed with Cd^{2+} (5 or 10 μM) (Fig. 2.2A and B).

The results in the present study revealed that 24 h after treatment with 5 or 10 μM Cd^{2+} induced cytotoxicity in PC12 cells which was considerably reduced by the co-exposure of AE (240 $\mu\text{g}/\text{mL}$) and Cd^{2+} (5 or 10 μM). One of the major reasons of cytotoxicity posed by Cd^{2+} on cell lines supposed to be due to the induction of oxidative stress which refers to elevated

levels of ROS. A plentiful studies showed that Cd^{2+} induces ROS increase exerting oxidative stress in various cell lines such as MCF-7 cells (Khojastehfar et al., 2015), MG63 cells (Hu et al., 2015) and SH-SY5Y cells (Kim et al., 2013). If Cd^{2+} effects cell through oxidative stress, the ROS elevation must be caused indirectly, e.g., by inhibiting antioxidant enzymes or by depleting antioxidant molecules (Stohs et al., 2000). Although oxidative stress has been linked to multitudes of pathologies, it means that high levels of ROS impose the damage to DNA, proteins and lipids (Schieber and Chandel, 2014). However, increasing studies have suggested that oxidative stress, cytotoxicity and cell death of many cell lines proceed via the depletion of the most abundant intracellular non-protein thiol, GSH and other antioxidant markers (Aziz et al., 2014; Im et al., 2006). Since GSH keeps the cellular environment in a reduced state and removes potentially toxic electrophiles and metals, it protects cells from toxic ROS (Armstrong et al., 2002). However, Cd^{2+} with high affinity for thiol group targets the highly abundant cellular GSH (Cuypers et al., 2010). Findings in this study showed that the relative intracellular levels of free -SH group were significantly decreased by the treatment of Cd^{2+} indicating oxidative stress might be induced in the cells. This oxidative stress stimulated due to the exposure of Cd^{2+} was recovered significantly by the co-exposure of AE with both Cd^{2+} (Fig. 2.4). Here, the antioxidant effect of AE was confirmed against the cytotoxicity of Cd^{2+} on PC12 cells. Moreover, a significant increase in the LDH activity in the cells culture medium was found after the treatment of Cd^{2+} . However, it was observed that the co-exposure of AE with Cd^{2+} noticeably repressed the release of LDH into the culture medium (Fig. 2.3). The decrease in LDH release into the culture medium by AE represented the protection of cell membrane integrity relaxed by the toxic Cd^{2+} exposure. From the results, it has been proven that AE can reduce PC12 cell toxicity induced by Cd^{2+} . In a contemporary study, it has been reported that ajwain oil extends lifespan of oxidative stress sensitive *mev-1* mutant *C. elegans* and elevated expression of SOD-3 and GST-4 (Rathor et al., 2017). Furthermore, carvacrol, the major compound found in ethanolic ajwain extract, showed potential protective effects against UVB irradiation-induced oxidative damage in human peripheral lymphocytes (Aristatile et al., 2015). It has also been found that carvacrol prevented t-BHP-induced oxidative stress in Chang cell via suppression of ROS and MDA levels and increases of GSH levels (Kim et al., 2015). Thus, it was established that the compound(s) present in AE can reduce Cd^{2+} -induced oxidative stress and cytotoxicity, and might scavenge ROS in PC12 cells.

There is no uniform mechanism of Cd^{2+} toxicity has so far been proposed. However, many *in vivo* and *in vitro* studies have been approached to unveil the toxicity mechanisms of Cd^{2+} in organisms and cell lines, because previous findings suggested that Cd^{2+} at various concentrations ranging from 1 to 300 μM induced apoptosis *in vivo* and *in vitro* (Yan et al., 2012). Moreover, recent studies on numerous cell lines also revealed that Cd^{2+} exerts toxicity by inducing oxidative damage through the generation of ROS, reacting readily with thiol groups of proteins, producing various genotoxic effects (e.g. DNA damage) and inducing apoptosis (Hossain et al., 2018; Kim et al., 2005; Skipper et al., 2016; Yan et al., 2012). This study also showed that co-exposure of AE and Cd^{2+} significantly increased the viability of PC12 cells reduced by Cd^{2+} only (Fig. 2.2B). In the present study, an increase of DNA fragmentation was also observed after the treatments of Cd^{2+} for 24 h. The relative DNA fragmentation induced by Cd^{2+} was markedly reduced by co-exposure of AE with Cd^{2+} (Fig. 2.5). These results reveal that the occurrence of apoptosis caused by Cd^{2+} was reduced by addition of AE. Therefore, these findings are in agreement with other similar results found by many investigators while studying the effects of Cd^{2+} on various cell lines such as C6 glioma cells (Wätjen and Beyersmann, 2004), human osteoblast-like cell line Saos-2 (Coonse et al., 2007) and HTC cells (Fotakis et al., 2005).

In this study, western blot analyses indicated that apoptosis induced by Cd^{2+} was reduced by co-exposure of AE with Cd^{2+} (Figs. 2.6 to 2.9). There are two well-demarcated regulatory mechanisms of apoptosis namely intrinsic and extrinsic pathways. Starting with the binding of cytokine ligands to the death receptors the extrinsic pathway activates caspase-8 which in turn activates caspase-3 or merges with the mitochondrial pathway via cleavage of Bcl-2 family member, p22 BID (Yuan et al., 2018). On the other hand, B-cell lymphoma 2 (Bcl-2) family proteins mediates the intrinsic pathway of apoptosis (mitochondrial apoptotic pathway). Decrease in the Bcl-2/Bax ratio leads to the release of cytochrome c from mitochondria to the cytosol, and subsequently activates a caspase cascade which ends in apoptosome formation and cellular fragmentation (Kuwana and Newmeyer, 2003). It has been suggested that the release of cytochrome c from the mitochondria into cytosol is a critical step during mitochondrial apoptotic pathway (Chen et al., 2000). The loss of mitochondrial membrane potential has already been reported as the cause to precede cytochrome c in cytosol in numerous cell lines (Wigdal et al., 2002). Although Cd^{2+} activates

various possible signaling pathway, mitochondria are the most relatable in mediating apoptosis via ROS generation (Murugavel et al., 2007). Numerous studies reported that Cd^{2+} exposure activates the caspase-dependent pathway causing increase of cytosolic cytochrome c, activation of mitochondrial Bax protein and executioner caspase-3 in various cell lines including HepG2 cells (Lawal et al., 2015), rat oligodendrocytes (Hossain et al., 2009) and PC12 cells (Rahman et al., 2017). It was observed that there is a decrease in Bcl-2/Bax ratio (Fig. 2.6), significant reduction in anti-apoptotic protein, Bcl-xL (Fig. 2.7A) and also the noticeable decline in survival protein, NF- κ B (Fig. 2.7A) after the exposure of Cd^{2+} in PC12 cells for 24h. These observations are in good agreement with many of studies abovementioned. Moreover, cytosolic cytochrome c was found to be high 24 h after Cd^{2+} treatment (Fig. 2.8). The rise in cytosolic cytochrome c ultimately culminated to initiate the caspase cascade because a significant increase of caspase 3 contents was observed (Fig. 2.9).

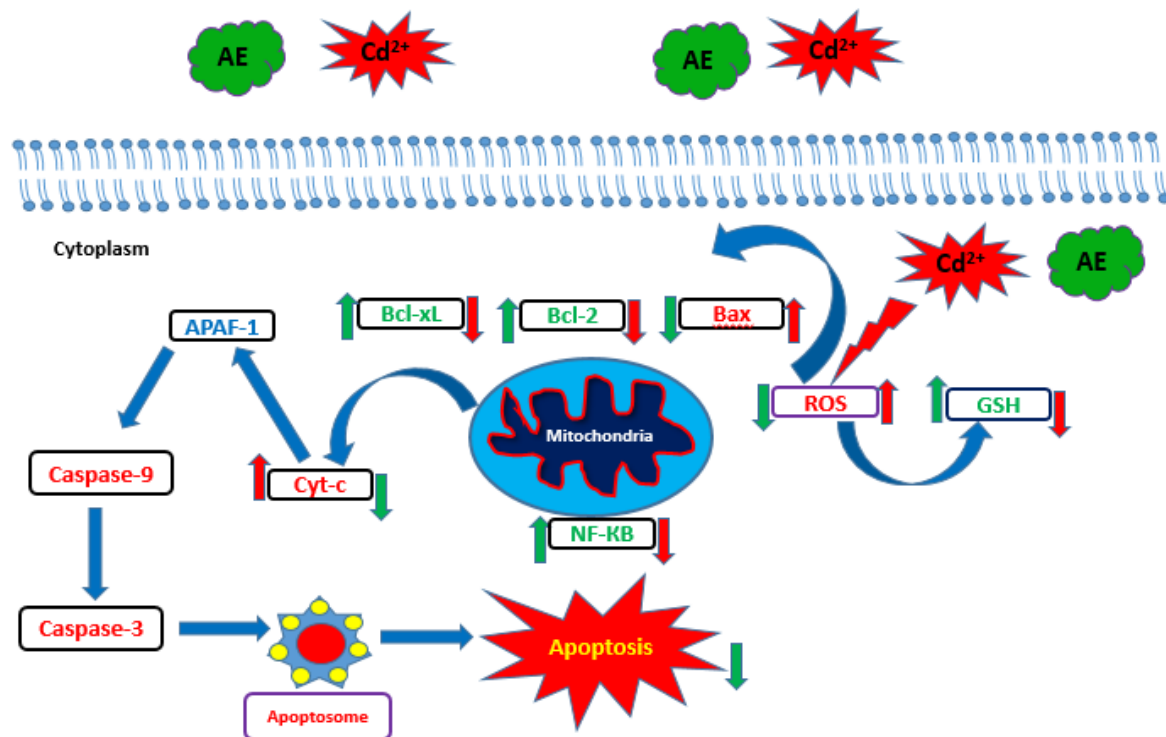


Fig. 2.11 Schematic diagram of protection of ethanolic ajwain extract (AE) against cadmium-induced cytotoxicity and apoptosis in PC12 cells. The red arrows indicate the toxic effects posed by Cd; the green arrows indicate the protective effects posed by AE.

So, in this study, considering the all results; increase of DNA fragmentation, Bcl family protein expressions, decrease of survival protein NF- κ B expressions, increase of cytosolic cytochrome c levels and increase of caspase-3 expressions, the cell death in PC12 after Cd²⁺ treatment could be explained as apoptosis via mitochondrial apoptotic pathway.

As shown in the Fig. 2.11, this study revealed that the co-exposure of AE has a protective effect on the Cd²⁺-induced apoptosis in PC12 cells. AE markedly reduced Cd²⁺-induced DNA fragmentation (Fig. 2.5) and acted as a potent agent for reducing Bax expressions (Fig. 2.6), cytosolic cytochrome c levels (Fig. 2.8) and caspase-3 expressions (Fig. 2.9) increased by Cd²⁺ exposure alone. AE could also increase the levels of Bcl-2 (Fig. 2.6), Bcl-xL and NF- κ B (Fig. 2.7) expressions lowered by Cd²⁺ alone. Many experiments confirmed the antioxidant potential of ajwain *in vivo* and *in vitro*. *In vivo* experiments also suggest that ajwain extracts and ajwain oils considerably reduced the oxidative stress induced cytotoxicity. For instance, ethanolic ajwain extract increased GSH, GSH-peroxidase, G-6-PDH, SOD, catalase and GST activities in hexachlorocyclohexane induced toxicity in rat liver (Anilkumar et al., 2009). Protective effects by ajwain extracts against heavy metal induced cytotoxicity, genotoxicity, apoptosis and oxidative stress were also confirmed by a contemporary *in vitro* research. Deb et al. (2012) reported that methanolic ajwain extract decreased ROS level, increased the mitochondrial membrane potential, and reduced apoptosis and caspase 3 activity on chromium treated BEAS-2B and PBL cell lines. Thus, our results are in good agreement with previous findings, and it can be suggested that AE could probably mollify the oxidative stress exerted by ROS and other redox molecules generated by the Cd²⁺ treatment. It also acted as a protective agent for apoptosis reduction in PC12 cells. Although it is indicated from this study that AE might have high antioxidant potential and free-radical scavenging capacity, it did not specify compound(s) responsible for such potency. But, in Fig. 2.10 it was found from LC-MS result that the presence of the monoterpenic phenol, carvacrol was found in a large quantity. However, future research is necessary to identify the responsible active compound(s) which act against the Cd²⁺-induced apoptosis. Additionally, research works on the effect of various ajwain extracts and/or of their compounds against other metals and non-metal-induced cytotoxicity and in other cell lines are required.

2.5 Conclusion

In conclusion, AE protects cell from oxidative stress and acts as an apoptosis remediating agent. It works against Cd-induced cytotoxicity and apoptosis at specific dose. Thus, ethanolic ajwain extract (AE) can be a candidate for being a potential agent against metal and/or other toxicant. Moreover, the traditional therapeutic uses of ajwain in various pathological conditions such as toxic, neurological, genital or respiratory tract disorders became validated from the findings.

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Chapter 3: Carvacrol inhibits cadmium toxicity through combating against caspase dependent/independent apoptosis in PC12 cells

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Abstract

Carvacrol, a monoterpenic phenol found in essential oils, is considered as a safe food additive, and possesses various therapeutic properties. Numerous studies have also deciphered the protective role of carvacrol on various cytotoxicities. Herein, the effects of carvacrol on cadmium-induced apoptosis in PC12 cells was clarified. Carvacrol while co-exposed with cadmium for 48 h raised PC12 cell viability in comparison to only cadmium exposed group. The co-exposure increased the cellular glutathione levels and promoted the expression of glutathione reductase. The magnitude of DNA fragmentation caused by cadmium was also ameliorated by carvacrol. Flow cytometry exhibited the apoptosis rate augmented by cadmium was reduced by co-exposure of cadmium and carvacrol. Western blotting revealed that cadmium and carvacrol co-exposure alleviated the cadmium-induced down-regulations of mammalian target of rapamycin (mTOR), protein kinase B (Akt), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), extracellular signal-regulated kinase-1 (ERK1) and nuclear factor erythroid 2-related factor 2 (Nrf2) expressions. The co-exposure also reversed action of cadmium by suppressing the cleavage of caspase 3 and reducing the cytosolic levels of cytochrome c and apoptosis inducing factor (AIF). Moreover, carvacrol upon co-exposure significantly increased the intracellular metallothionein content. In conclusion, carvacrol strongly reduced cadmium-triggered oxidative stress, and caspase-dependent and caspase-independent apoptosis in PC12 cells.

3.1 Introduction

Cadmium (Cd^{2+}), a heavy metal used in many industrial and household products, has become prioritized due to its emergence as a widespread environmental pollutant and a biological toxicant. Concern over Cd^{2+} is due to its extremely high toxicity, a long half-life in humans and being a causative agent for many diseases and disorders upon acute or chronic exposure (Järup and Åkesson, 2009; Jiang et al., 2014). Evidences support that Cd^{2+} exposure is associated with a variety of ailments including renal tubular dysfunction (Nishijo et al., 2006), osteoporosis (James and Meliker, 2013), hepatotoxicity (Dudley et al., 1982),

cardiovascular diseases (Tellez-Plaza et al., 2013) and neurotoxicity (Wang and Du, 2013). Following absorption and distribution, Cd^{2+} affects cellular metabolic processes resulting to alterations in proliferation and differentiation. Cd^{2+} causes cellular injury through exerting oxidative stress (Patra et al., 2011), promoting DNA damage (Badisa et al., 2007), altering transport pathways (Kerkhove et al., 2010), and impairing mitochondrial membrane potential leading to apoptosis (Mao et al., 2011; Jiang et al., 2014). The landscape for Cd^{2+} -triggered death mechanisms have been studied extensively in various cell lines. However, the death mechanisms differ depending on cell type, Cd^{2+} concentration, and duration of exposure. Despite many debates and complexities of mechanisms, previous reports detailed that *in vitro* cells frequently go through an apoptotic death at low to moderate (e.g., 0.1-10 μM) Cd^{2+} concentrations, and undergo necrosis at high (>50 μM) concentrations (Templeton and Liu, 2010). Numerous cell systems showed that diverse signaling pathways have been involved in Cd^{2+} -induced apoptosis, including mitochondrial (Rahman et al., 2017), extrinsic (Liu et al., 2016a) and caspase-independent (Liu and Templeton, 2008) pathways. But, a rise in reactive oxygen species (ROS) levels, increased lipid peroxidation, alterations in antioxidant defense system, and stimulation of metallothionein formation are the common phenomena cells follow upon Cd^{2+} -induced cytotoxicity, irrespective of apoptotic pathway(s) (Stohs et al., 2000; Sarkar et al., 1998).

Carvacrol (5-isopropyl-2-methyl-phenol) is a monoterpenoid phenol predominantly found as a natural constituent of various essential oils of *Labiatae* family plant species such as oregano, thyme and pepperwort (Noshay et al., 2018; Kirimer et al., 1995). It is generally considered as a food additive and flavoring agent (Zotti et al., 2013). Recently edible and non-edible carvacrol-based films have become very useful components in antimicrobial food packaging (Ramos et al., 2016). Numerous studies have reported the diverse biological and therapeutic properties of carvacrol including antimicrobial (Xu et al., 2008), antioxidant (Beena et al., 2013; Aristatile et al., 2009), anti-inflammatory (Landa et al., 2009), anti-carcinogenic (Karkabounas et al., 2006) and neuromodulatory (Zotti et al., 2013) activities. Cumulative evidences from *in vivo* and *in vitro* studies have already confirmed the protective role of carvacrol against various toxicant-induced oxidative stress and apoptosis in various organs and cell lines (Noshay et al., 2018; Aristatile et al., 2009; Wang et al., 2017; Samarghandian et al., 2016; Palabiyik et al., 2016). Another study also demonstrated that

carvacrol can also alleviate Fe²⁺-induced oxidative stress and apoptosis in SH-SY5Y cells (Cui et al., 2015). However, no report becomes available with regards to the potential protective effects of carvacrol on Cd²⁺-induced toxicity in cell system with bio-molecular mechanistic clarifications.

Therefore, it has been hypothesized that carvacrol can induce protective effects against Cd²⁺-induced cytotoxicity in cultured cells. Experiments were conducted to evaluate whether carvacrol has ameliorative effects against the cytotoxicity, oxidative stress and apoptosis caused by Cd²⁺ on PC12 cells. This study is assumed to be the first approach which would elucidate the abovementioned properties of carvacrol in a model cell line.

3.2 Materials and methods

3.2.1 Materials

PC12 cells were obtained from American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM), ribonuclease A (RNase), ethidium bromide, and peroxidase-conjugated avidin were bought from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biosera (Kansas City, MO, USA). High pure polymerase chain reaction (PCR) product purification kit and proteinase K were acquired from Roche Diagnostics (Mannheim, Germany). Polyclonal antibodies against β -actin (cat# 4967), mammalian target of rapamycin (mTOR) (cat# 2972), cleaved caspase 3 (cat# 9661), and protein kinase B (Akt) (cat# 4691) were purchased from Cell Signaling Technology. Polyclonal antibodies against glutathione reductase (GR) (ab16801, Abcam), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (sc-109, Santa Cruz Biotechnology), extracellular signal-regulated kinase 1 (ERK1) (61003c, BD Transduction Laboratories), nuclear factor erythroid 2-related factor 2 (Nrf2) (PM069, MBL; Japan), caspase 3 (GTX110543, GeneTEX), and apoptosis inducing factor (AIF) (cat# 551429, BD Biosciences) were also procured. The Cytochrome c Release Apoptosis Kit (Q1A87-1KIT) was purchased from Calbiochem^R. Anti-mouse IgG (H+L) horseradish peroxidase (HRP) conjugate (W4021) and anti-rabbit IgG (H+L) horseradish peroxidase (HRP) conjugate (W4011) were bought from Promega Corporation (Madison, WI, USA). Enhanced chemiluminescence (ECL) western blotting detection reagent (Amersham Pharmacia Biotech., Buckinghamshire, England) and trypan blue (0.4%) solution (Bio-Rad, Hercules, CA, USA) were purchased. The AnnexinA5-FITC flowcytometry kit (cat#

IM2375) was procured from Beckman Coulter (Marseille Cedex9, France) and Metallothionein (MT) enzyme-linked immunosorbant assay (ELISA) kit (Lot# 160361) was collected from Frontier Institute Co., Ltd. (Hokkaido, Japan). Carvacrol (Lot# SAM5447, Purity: minimum 95.0%) was bought from Wako Pure Chemical Industries Ltd., Japan. All other chemicals used were of analytical grade.

3.2.2 Cell culture and treatments

PC12 cells were cultured on 25cm² cell culture flasks in DMEM supplemented with 10% fetal bovine serum (FBS) in a dehumidified incubator at 37°C with 5% CO₂. After a 24 h pre-incubation, the cells were exposed to CdCl₂ (Cd²⁺), or to carvacrol, or to both for 48 h. The exposure concentrations of carvacrol was first chosen as 0, 50, 100, 200 and 400 µM. But for further experimentation, the concentrations of Cd²⁺ and carvacrol used were selected as 10 µM and 100 µM, respectively.

3.2.3 Cell viability

Trypan blue exclusion test was used for the assessment of cell viability. PC12 cells were seeded at a density of about 1×10⁵/ flask and pre-incubated for 24 h until logarithmic growth phase was reached. The cells were then exposed with different concentrations of carvacrol (0, 50, 100, 200 and 400 µM) and Cd²⁺ (10 µM), separately; Co-exposure was done to cells using different non-toxic concentrations of carvacrol (0, 50, 100 and 200 µM) and Cd²⁺ (10 µM). After treatments and co-treatments, the cells were incubated for 48 h. Then, cells were subsequently collected and stained with 0.2% trypan blue in 1 × phosphate-buffered solution (PBS). The number of trypan blue-stained cells and total cells were counted using a cell counter (TC10™ Automated Cell Counter, Bio-Rad). Cell viability was expressed as percentage (%) of the trypan blue-stained cells.

3.2.4 Lactate dehydrogenase (LDH) activity assay

Cytotoxicity-derived cell membrane disintegration levels were assessed by measuring LDH activity in the cell treatment medium using a nonradioactive cytotoxicity assay kit as described by Rahman et al. (2018). PC12 cells were exposed to Cd²⁺ (10 µM), or carvacrol (100 µM), or Cd²⁺ (10 µM) + carvacrol (100 µM) for 48 h. After exposure period, 50 µL of the culture medium was contained in a 1.5 mL tube and subsequently, 50 µL of substrate mixture (containing tetrazolium salts) was added to the tube. After incubating at room temperature

for 30-min, 50 μ L of stop solution was added. The absorbance at 490 nm was measured using an iMark™ microplate reader (BioRad; Hercules, CA, USA) to determine the amount of formazan dye produced. LDH activity was expressed as “LDH activity/ 1×10^6 cells”. This experiment was repeated at least 3 times for ensuring biological and statistical reproducibility.

3.2.5 Measurement of intracellular glutathione (GSH) levels

Intracellular free-SH levels were determined following the methods previously described by Kihara et al. (2012) and Rahman et al. (2018). PC12 cells were harvested after 48 h exposure to Cd^{2+} (10 μ M), carvacrol (100 μ M), or Cd^{2+} (10 μ M) + carvacrol (100 μ M). The cells were then washed with $1 \times$ PBS. A lysis buffer at an amount of 150 μ L was added to the cells and incubated at room temperature for 10 min. Two freeze-thaw sonication cycles were executed in order to rupture the cell membranes. The resulting solution was centrifuged at 1500 rpm for 10 min to collect the supernatant. Then, the protein contents were measured spectrophotometrically using a protein assay dye reagent (Bio-Rad; Hercules, CA, USA). GSH levels were measured using 2.5 mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB, pH 7). DTNB was added to the cell lysate at a concentration of 200 μ M, and the absorbance at 412 nm was measured using a DU-65 spectrophotometer (Beckman, CA, USA). The free -SH concentration was determined by using a molecular coefficient factor of 13,600 per cell number (1×10^5). The experiments were conducted at least triplicate to achieve reproducibility.

3.2.6 Isolation of the genomic DNA

The genomic DNA of PC12 cells was isolated using high PCR template preparation kit following the manufacturer's instructions. After treatment with Cd^{2+} (10 μ M), carvacrol (100 μ M) or Cd^{2+} (10 μ M) + carvacrol (100 μ M) cells were incubated for 48 h. Then, the cells were harvested using a scraper, and centrifuged at 1500 rpm for 5 min to remove the supernatant. Afterwards, the cells were washed with $1 \times$ PBS and centrifuged at 1500 rpm for 5 min. The procedure for isolation of the genomic DNA was then performed. The isolated DNA was incubated with RNase (10 μ g/mL) for 15 min at 37°C, followed by addition of 100% ethanol and 3M NaOAc buffer (pH 4.5) were added. The solution was allowed to stand overnight at -20°C for DNA precipitation. The following day, precipitated DNA was centrifuged at 15,000 rpm for 7 min, and for another 3 min after being washed with 70% ethanol. The obtained DNA was dried and solved with 50 μ L 1 $\times$ Tris/Borate/Ethylenediaminetetraacetic acid (TBE),

followed by the measurement of DNA concentration using a GeneQuant (GE Heath Care; South East England, UK). Finally, DNA concentrations were equalized for all samples by adding 1×TBE solution.

3.2.7 Agarose gel electrophoresis of genomic DNA

The extracted genomic DNA in PC12 cells was subjected to agarose gel electrophoresis to assess the fragmentation levels. DNA (5 µg) was mixed with loading dye and electrophoresed on a 1.5% agarose gel for 40 min at 100 V, using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). Following electrophoresis, the gel was soaked in ethidium bromide solution for 5-10 min. DNA fragmentation was visualized under UV illumination using a ChemiDoc XRS (Bio-Rad; Hercules, CA, USA). The fluorescence intensity of fragmented DNA was analyzed using a software named “Quantity One”. The amount of intact DNA was expressed as the intensity ratio of the total DNA density to the fragmented DNA density. This experiment was repeated for at least three times to ensure reproducibility.

3.2.8 Detection of apoptosis rate by flow cytometry

The apoptosis rate of PC12 cells was detected by flow cytometric analysis. PC12 cells were harvested after 48 h exposure to 10 µM Cd²⁺, or 100 µM carvacrol or to combined 10 µM Cd²⁺ and 100 µM carvacrol. Then cells were washed with 1 × PBS, and 400 µL ice-cold 1 × binding buffer was subsequently added to the cells. Afterwards, 5 µL of Annexin A5-FITC solution and 2.5 µL of propidium iodide (PI) were added to the cells and the resulting solution was kept for 10 min in the dark. Finally, samples were analyzed using a BD FACSVerse™ Flow cytometer. The experiment was conducted at least in triplicates for biological and statistical reproducibility.

3.2.9 Western blot analysis for the determination of protein expressions

Western blot analysis was accomplished for the determination of protein expressions in PC12 cells 48 h after exposure to the aforementioned Cd²⁺ and/or carvacrol concentrations. Cells were harvested and washed by suspending in ice-cold 1 × PBS, then centrifuged at 1500 rpm for 10 min. After removing the supernatant, the cells were re-suspended in 150 µL of lysis buffer (consisting of 2mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100mM NaCl, 10mM ethylene glycol tetraacetic acid (EGTA), 0.1mM phenylmethylsulfonyl

fluoride (PMSF), 1mM Na₃VO₄, 0.1mM Na₂MoO₄, 5mM 2-glycerophosphate, 10mM MgCl₂, 2mM dithiothreitol (DTT), 50mM sodium fluoride (NaF), and 1% triton X-100) for total protein extraction. The mixture was allowed to stand on ice for 10 min and then cells were disrupted by two cycles of sonication followed by centrifugation at 1500 rpm for 10 min to collect the lysate containing the total cellular protein. On the other hand, the cytosolic protein fractions were extracted using the cytosolic lysis buffer provided in the 'cytochrome c release apoptosis kit (Q1A87-1KIT)'. The concentration of extracted protein in the lysates was determined spectrophotometrically using a protein assay dye reagent (BioRad, Hercules, CA, USA). Thereafter, an equal amount (20 µg) of each protein sample was separated by 12.5-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresed proteins were then transferred into a nitrocellulose membrane using a semi-dry blotting system (type- AE6678; ATTO, Tokyo, Japan). The membranes were incubated overnight at 4°C in 5% skimmed milk as a blocking agent. After blocking, each membrane was washed three times with 0.1% Tween buffer and incubated with the desired primary and secondary antibodies. After washing with 0.1% Tween buffer, the protein band on the nitrocellulose membrane was visualized using enhanced chemiluminescence, and analyzed using a ChemiDoc XRS (Bio-Rad, USA). Finally, the band intensities of the targeted proteins were expressed as the ratio to the intensity of β-actin. Each experiment was conducted at least in triplicates to ensure reproducibility.

3.2.10 Intracellular Cd²⁺ accumulation in PC12 cells

The PC12 cells were incubated for 48 h after being exposed to 10 µM Cd²⁺ and/or 100 µM carvacrol. Following harvesting and washing with 1 × PBS, cells were digested with 1 M nitric acid at 70° C for 1 h. Then, the samples were filtered through a 0.22 µm pore membrane and diluted using deionized water to prepare for measurement. . Intracellular cadmium contents were measured by using an ICPE-9000 inductively coupled plasma mass spectrometry (ICP-MS) (Shimadzu; Kyoto, Japan). To confirm the reproducibility, this experiment was carried out in triplicate.

3.2.11 Determination of metallothionein (MT) content

PC12 cells were exposed to 10 µM Cd²⁺ and/or 100 µM carvacrol for 48 h, harvested, and washed twice with 1 × PBS. The protein from the cells was extracted and the total protein content was measured spectrophotometrically using a protein assay dye reagent (Bio-Rad,

Hercules, CA USA). The MT content in the cell lysate was measured by ELISA kit following the manufacturer's protocol. The provided 96 well microtiter plate was first washed with PBS solution followed by the addition of 50 μ L of standard or sample solution in each well. After adding 50 μ L first antibody in each well, the microtiter plate was incubated at room temperature for 1 h. Then, each of the wells was washed thrice with 350 μ L washing buffer and afterwards, 100 μ L of second antibody was added. Following 1 h incubation at room temperature wells were washed and 100 μ L of substrate mixture was subsequently added to each well while keeping the set-up in the dark. The reaction was stopped by adding 50 μ L of stop solution. Finally, absorbance of the solution was measured at 450 nm in an ELISA reader (ng/mg of protein) and the concentration was calculated from a standard curve of MT protein.

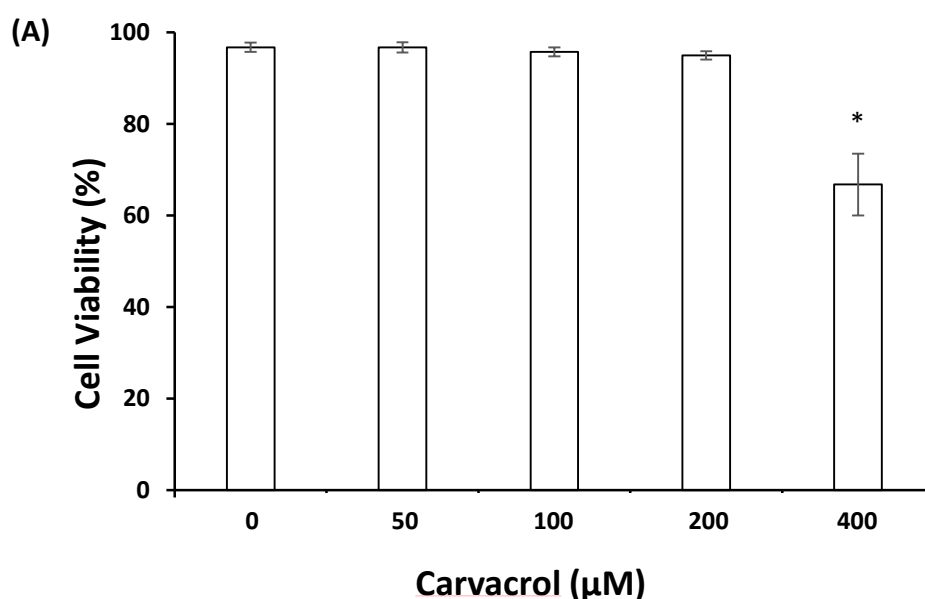
3.2.12 Statistical analysis

Data were represented as the mean $\pm$ standard error of mean (SEM); $p < 0.05$ was considered to indicate significance level. Analysis of statistical significance was achieved by using single-factor analysis of variance (ANOVA) followed by unpaired Student's t-test in MS Excel 2013 program.

3.3. Results

3.3.1 Effects of Cd^{2+} and carvacrol on the viability of PC12 cells

To investigate the toxicity of carvacrol, PC12 cells were exposed to 50, 100, 200, and 400 μ M of carvacrol for 48 h.



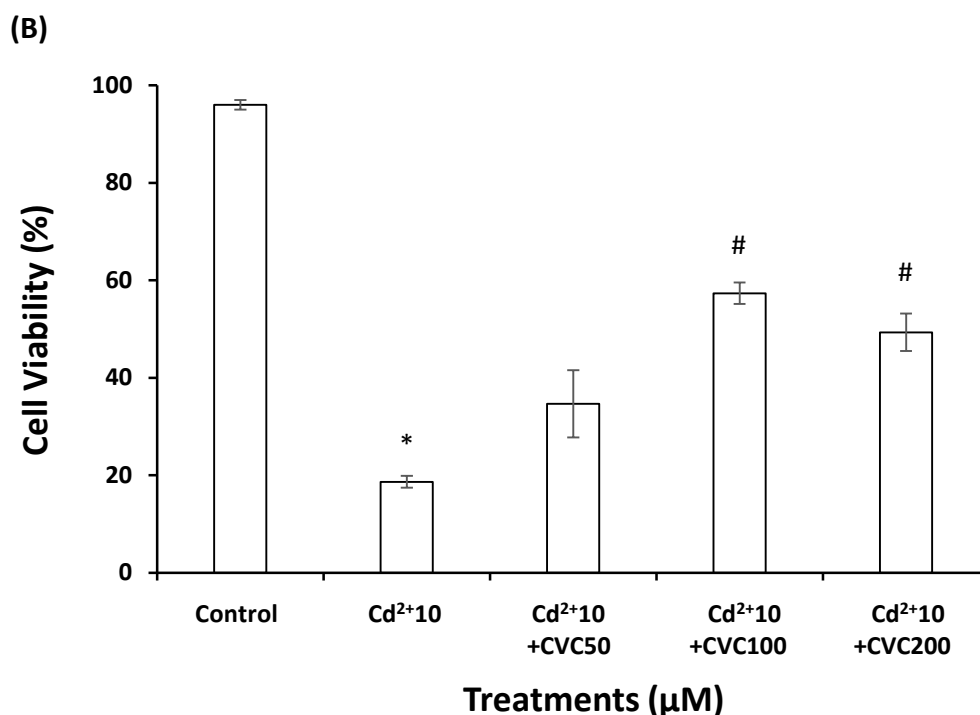


Fig. 3.1 Viability (%) of PC12 cells measured by using trypan blue exclusion method. (A) PC12 cells exposed with 50, 100, 200 and 400 μM carvacrol (CVC) for 48 h. (B) PC12 cells exposed/ co-exposed with 10 μM cadmium (Cd^{2+}) and 50, 100, and 200 μM CVC for 48 h. Error bars indicate mean $\pm$ S.E.M. ($n=4$ for (A) and 3 for (B)), * and # indicate significant differences ($P < 0.05$) from the control group and the Cd^{2+} -exposed group respectively.

Cells were also exposed to 10 μM of Cd^{2+} , and combined Cd^{2+} and carvacrol. Trypan blue staining method was performed to assess the viability of PC12 cells. The results showed that the treatments of carvacrol up to 200 μM did not affect the cell viability (Fig. 3.1A), indicating that the carvacrol concentrations used in this study were not toxic.

On the other hand, the decrease in the PC12 cell viability caused by 10 μM Cd^{2+} was significantly ($p < 0.05$) prevented by carvacrol (100 or 200 μM) (Fig. 3.1B). Therefore, it was proposed from the cell viability results that carvacrol (100 or 200 μM) exerted cytoprotective effects against Cd^{2+} (10 μM)-induced cell death. The results were used as basis of choosing 10 μM Cd^{2+} and 100 μM carvacrol for further experimentation.

3.3.2 Carvacrol reduces Cd^{2+} -induced-LDH leakage

The soluble cytosolic enzyme LDH released in the cell culture media upon plasma membrane disintegration is one of the important indicators of toxicant-induced cell death.

The LDH activity was measured in PC12 cell culture media after 48 h exposure to Cd^{2+} (10 μM) and/or carvacrol (100 μM).

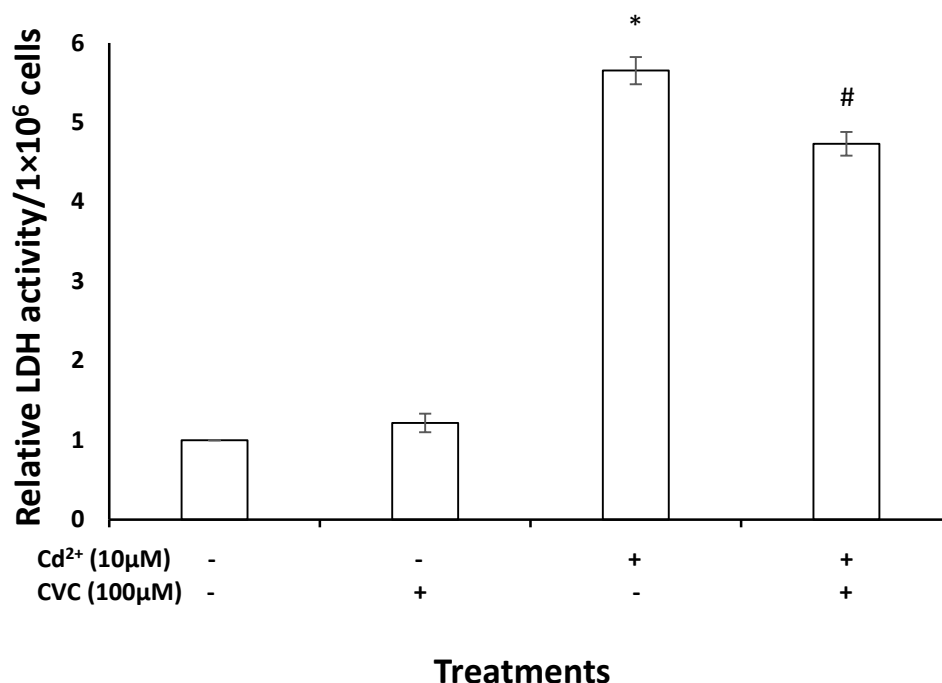


Fig. 3.2 Lactate dehydrogenase (LDH) activity in the culture medium of PC12 cells exposed/ co-exposed with 10 μM Cadmium (Cd^{2+}) and 100 μM carvacrol (CVC) for 48 h measured by a non-radioactive cytotoxicity assay kit. Error bars indicate mean $\pm$ S.E.M. ($n = 3$), * and # indicate significant differences ($P < 0.05$) from the control group and the Cd^{2+} -exposed group respectively.

As shown in Fig. 3.2, compared to the control, Cd^{2+} (10 μM) exposure noticeably ($p < 0.05$) increased the LDH activity in the culture medium. On the other hand, no significant change in LDH activity was observed 48 h after carvacrol (100 μM) exposure. The combined exposure of Cd^{2+} (10 μM) and carvacrol (100 μM) significantly ($p < 0.05$) reduced the LDH activity in the medium compared to that only Cd^{2+} (10 μM) exposed group.

3.3.3 Effects of Cd^{2+} and carvacrol on the intracellular GSH levels and GR expression

GSH is the most abundant intracellular non-protein thiol which acts as an antioxidative defense system, and removes oxidative stress generating electrophiles to maintain the redox homeostasis (Du et al., 2009). PC12 cells were exposed to Cd^{2+} (10 μM) and/or carvacrol (100 μM) for 48 h, and then measured intracellular GSH levels. A significant ($p < 0.05$) decrease in intracellular GSH content was found in the cells exposed to Cd^{2+} (10 μM), which was markedly

($p < 0.05$) increased in the cells with combined Cd^{2+} (10 μM) and carvacrol (100 μM) (Fig. 3.3A). However, no change in GSH level was found in cells exposed to carvacrol (100 μM) only. Thus, it can be suggested that carvacrol ameliorated oxidative stress posed by Cd^{2+} in PC12 cells.

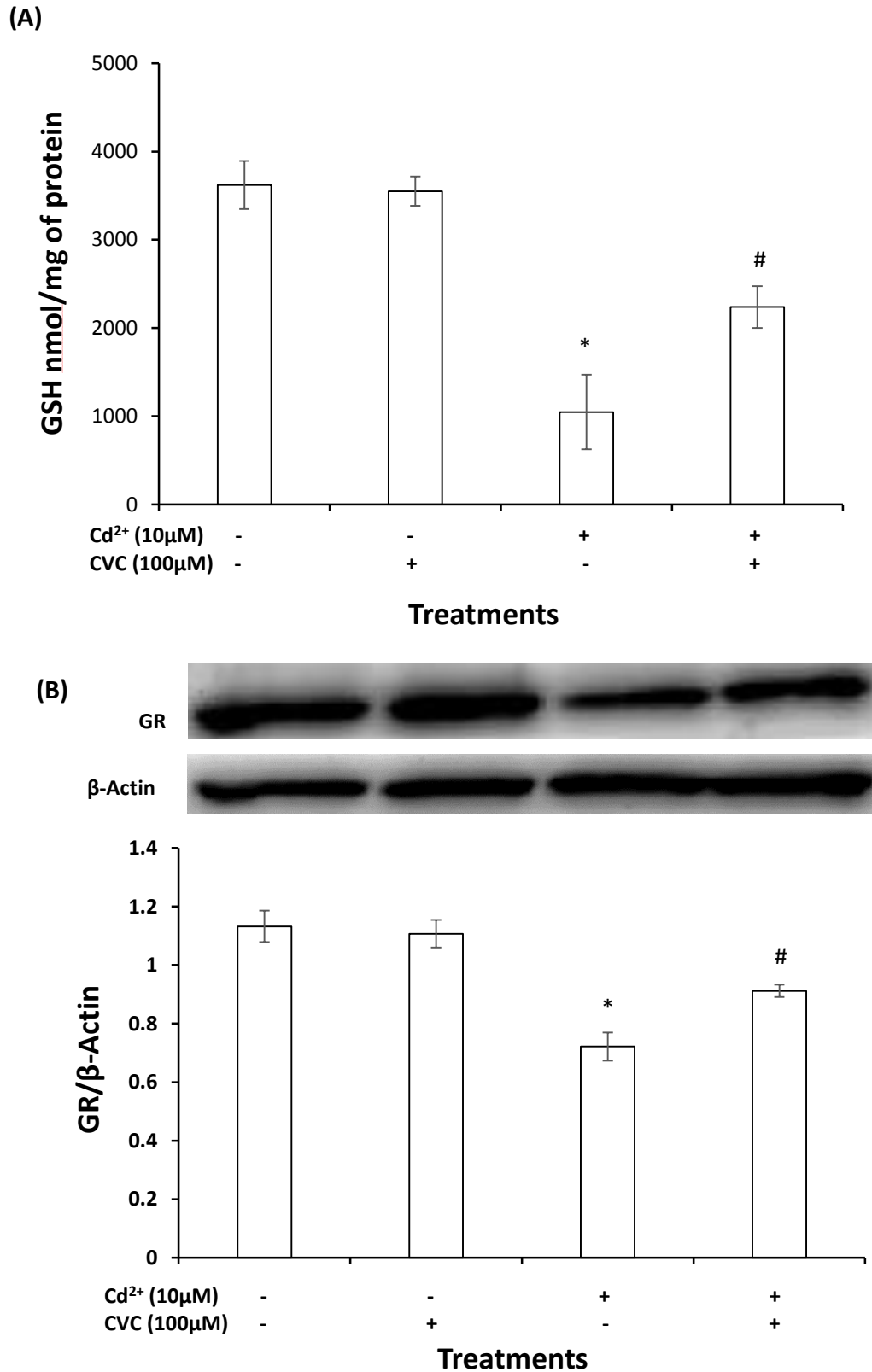


Fig. 3.3 Oxidative stress markers in PC12 cells exposed/co-exposed with Cadmium (Cd^{2+}) and carvacrol (CVC). (A) Intracellular glutathione (GSH) level in PC12 cells exposed/ co-exposed with 10 μM Cd^{2+} and 100 μM CVC for 48 h measured by 2.5 mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) assay kit. (B) Glutathione reductase (GR) expressed in PC12 cells exposed/ co-exposed with 10 μM Cd^{2+} and 100 μM CVC for 48 h analyzed by western blotting. Error bars indicate mean $\pm$ S.E.M. (n= 4), * and # indicate significant differences ($P < 0.05$) from the control group and the Cd^{2+} -exposed group respectively.

Additionally, western blot analysis showed that the expression of GR, the enzyme converting GSSG to GSH, supported the results found for GSH levels (Fig. 3.3B). Therefore, carvacrol showed antagonizing role on Cd^{2+} -induced oxidative damage.

3.3.4 Carvacrol alleviates Cd^{2+} -induced genomic DNA damage

Genomic DNA damage is one of the major biological outcome of cadmium toxicity and is also a prominent route of cell inactivation in apoptosis (Roos and Kaina, 2006). In this study, agarose gel electrophoresis results exhibited that the amount of intact genomic DNA in PC12 cells was severely diminished after 48 h of Cd^{2+} (10 μM) exposure (Fig. 3.4A).

On the other hand, it became visible that the exposure to carvacrol (100 μM) had no effect on the amount of intact genomic DNA compared to that of the untreated control cells. Cd^{2+} (10 μM)-induced DNA fragmentation was lessened in the case of cells exposed to combined Cd^{2+} (10 μM) and carvacrol (100 μM). In line with this, a significant decrease in DNA band density was found in Cd^{2+} (10 μM) exposed cells, while the DNA band density for cells co-exposed with Cd^{2+} (10 μM) and carvacrol (100 μM) was markedly ($p < 0.05$) higher in comparison to only Cd^{2+} (10 μM) treated group (Fig. 3.4B). Thus, the co-exposed cell group possessed a comparatively reduced DNA damage.

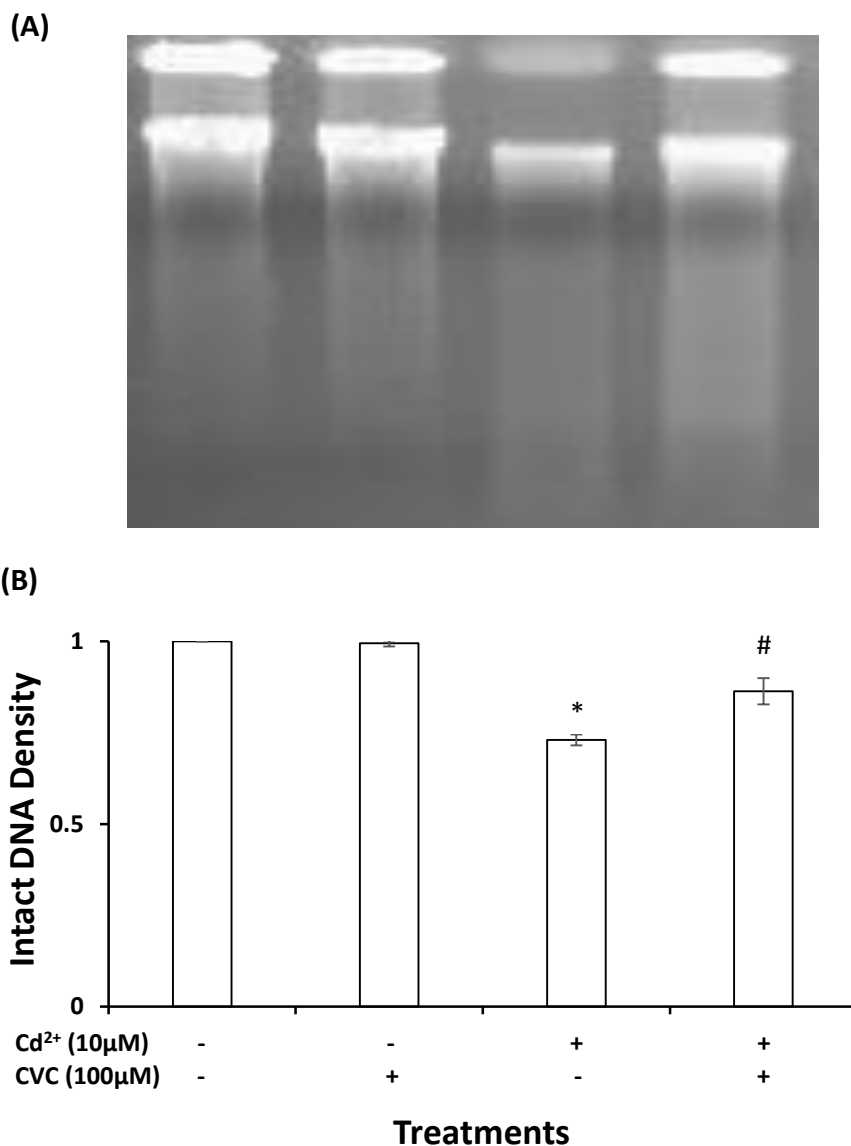


Fig. 3.4 Agarose gel electrophoresis of genomic DNA extracted from PC12 cells exposed/ co-exposed with 10 μ M Cadmium (Cd^{2+}) and 100 μ M carvacrol (CVC) for 48 h. (A) A sample electrophoresis image showing the content of intact and fragmented genomic DNA of PC12 cells exposed/ co-exposed with Cd^{2+} and CVC. (B) Relative band intensity of intact DNA for fragmentation detection. Error bars indicate mean $\pm$ S.E.M. ($n=3$), * and # indicate significant differences ($P < 0.05$) from the control group and the Cd^{2+} -exposed group respectively.

3.3.5 Carvacrol decreases the rate of Cd^{2+} -induced apoptosis in PC12 cells

To determine the rate of apoptosis, PC12 cells were exposed to Cd^{2+} (10 μ M) and/or carvacrol (100 μ M) for 48 h. Then, the apoptotic rate was determined by annexin-V-FITC/PI assay using flow cytometry analysis (Fig. 3.5A).

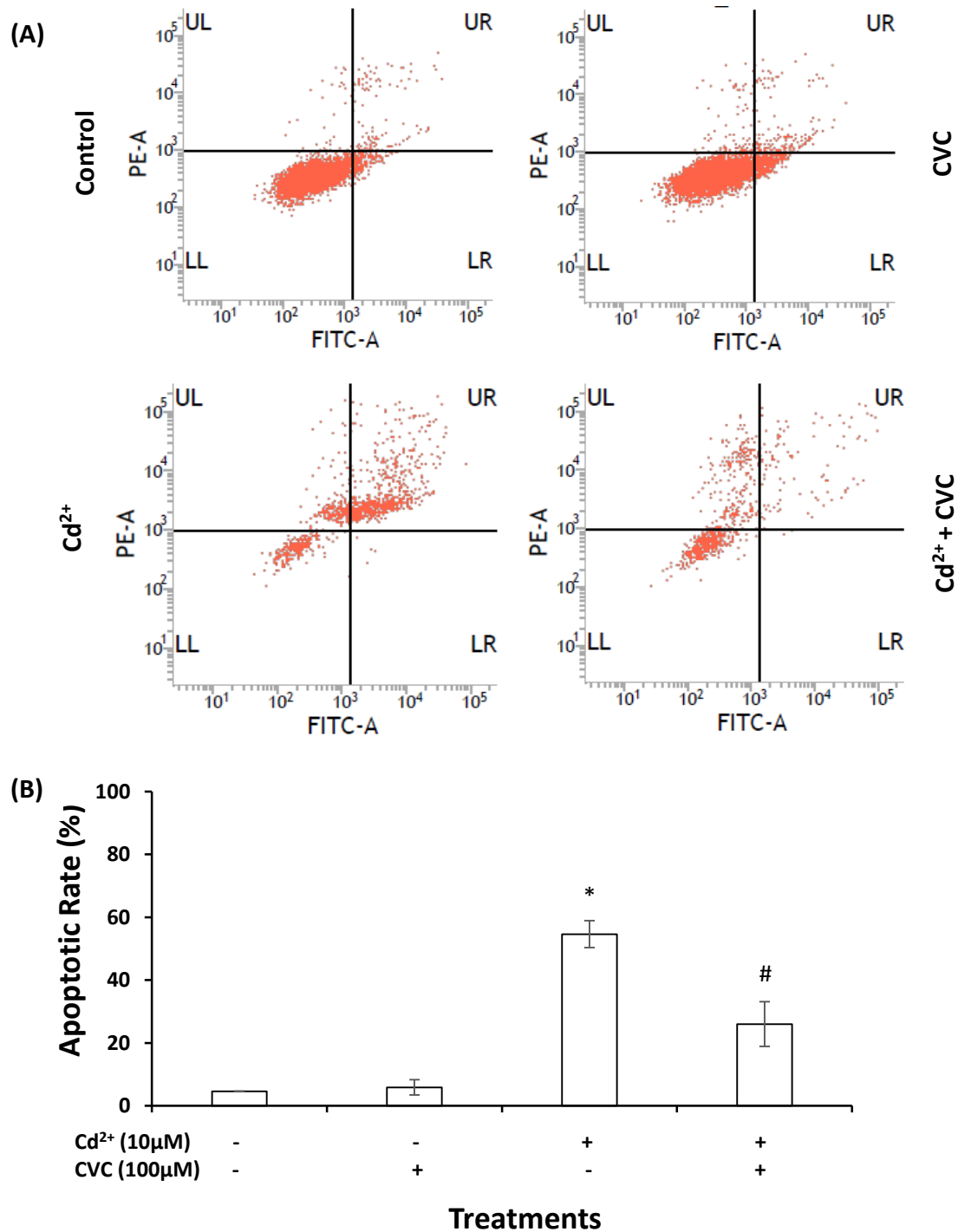


Fig. 3.5 The apoptotic rate of PC12 cells exposed/ co-exposed with 10 μM Cadmium (Cd²⁺) and 100 μM carvacrol (CVC) for 48 h analyzed by flow cytometry. (A) A representative experimental result of flow cytometry followed by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining of PC12 cells exposed/ co-exposed with Cd²⁺ and CVC for 48 h. (B) The apoptotic rate of PC12 cells exposed/ co-exposed with Cd²⁺ and CVC for 48 h, calculated and analyzed using early apoptosis (LR) and late apoptosis (UR). Error bars indicate mean ± S.E.M. (n= 3), * and # indicate significant differences (P< 0.05) from the control group and the Cd²⁺-exposed group, respectively.

As shown in Fig. 3.5B, more than 54% of the Cd^{2+} (10 μM) exposed cells were in either early (in lower right (LR) quadrant) or late (in upper right (UR) quadrant) apoptotic condition. Although only carvacrol (100 μM) exposure did not change the apoptosis rate compared to that of the control group (~5%), Cd^{2+} (10 μM) and carvacrol (100 μM) co-exposure markedly ($p < 0.05$) diminished the apoptosis rate to about 26%. These results suggest that carvacrol strongly reduced Cd^{2+} -provoked apoptosis in PC12 cells.

3.3.6 Effects of Cd^{2+} and carvacrol on the expressions of pro-survival proteins

The expression of protein factors crucial for cell metabolism, proliferation, and survival in PC12 cells was analyzed after exposure to Cd^{2+} (10 μM), or carvacrol (100 μM), or co-exposing with both Cd^{2+} (10 μM) and carvacrol (100 μM).

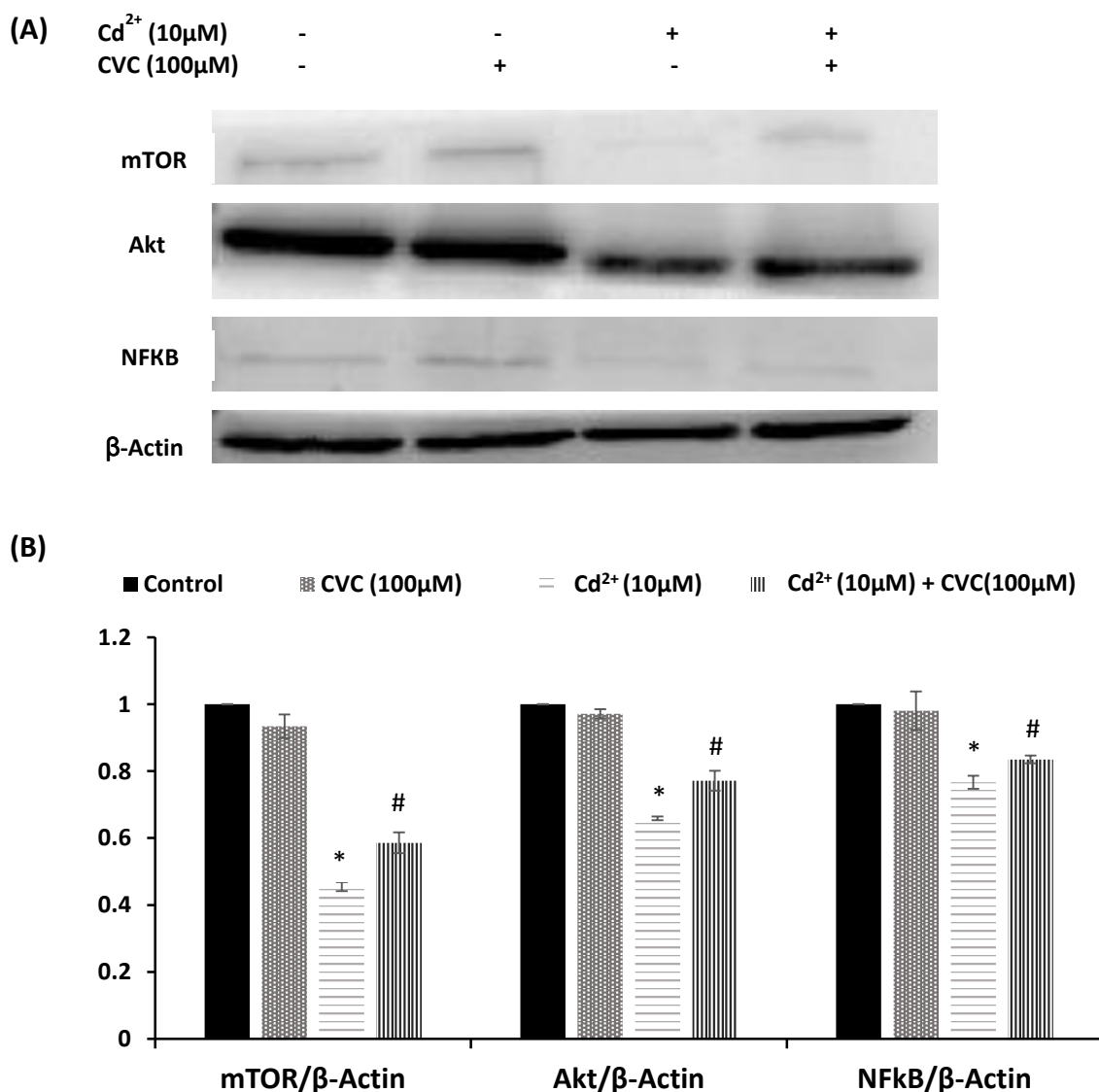


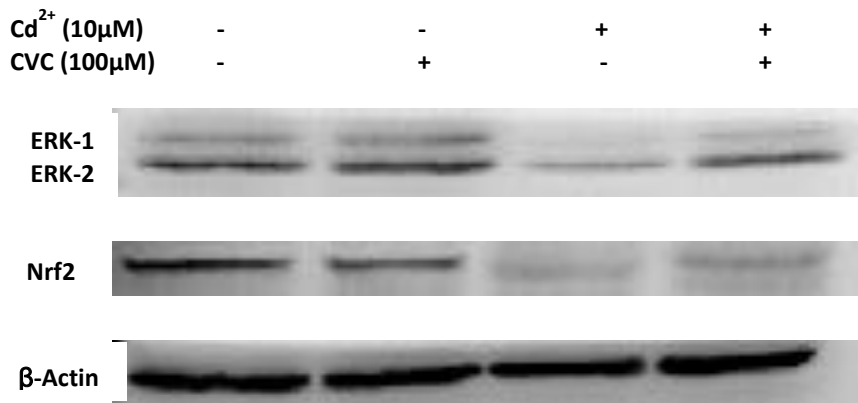
Fig. 3.6 Western blot analysis for the expression of pro-survival proteins in PC12 cells exposed/ co-exposed with 10 μM Cadmium (Cd^{2+}) and 100 μM carvacrol (CVC) for 48 h. (A) The representative images (cropped) of immunoblotting for the expressions of mammalian target of rapamycin (mTOR), protein kinase B (Akt) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) in PC12 cells exposed/ co-exposed with Cd^{2+} and CVC for 48 h. (B) Relative density for the expression bands of mTOR, Akt and NF κ B to β -actin in PC12 cells exposed/ co-exposed with Cd^{2+} and CVC for 48 h. Error bars indicate mean $\pm$ S.E.M. (n= 3), * and # indicate significant differences ($P < 0.05$) from the control group and the Cd^{2+} -exposed group, respectively.

The western blot analyses images for the expressions of mTOR, Akt and NF- κ B were shown in Fig. 3.6A. Results exhibited that only Cd^{2+} (10 μM) exposure significantly ($p < 0.05$) down-regulated the expressions of mTOR, Akt and NF- κ B in comparison to the control. Compared to Cd^{2+} (10 μM) exposed group, all of these pro-survival proteins were evidently ($p < 0.05$) up-regulated in PC12 cells co-exposed to Cd^{2+} (10 μM) and carvacrol (100 μM) (Fig. 3.6B). Thus, the above results support that carvacrol (100 μM) could enhance the survival rate and protect PC12 cells from Cd^{2+} (10 μM)-induced oxidative stress and apoptosis.

3.3.7 Carvacrol enhances the expressions of ERK1 and Nrf2 suppressed by Cd^{2+}

ERK1, a member of mitogen-activated protein kinase (MAPK) protein family, is generally considered as a regulator of many pro-survival and anti-apoptotic proteins. On the other hand, Nrf2 is a transcription factor that regulates the expressions of many antioxidant enzymes. In Fig. 3.7A, the representative western blot images of the expressions of ERK1 and Nrf2 in PC12 cells showed that the expressions of both ERK1 and Nrf2 became significantly ($p < 0.05$) lessened due to the exposure to Cd^{2+} (10 μM) for 48 h. But, the band for co-exposed group was much more visible. The quantified expression levels of ERK1 and Nrf2 represented in Fig. 3.7B implies that upon co-exposure, carvacrol (100 μM) significantly ($p < 0.05$) alleviated the Cd^{2+} (10 μM)-induced reduction of ERK1 and Nrf2 protein expressions.

(A)



(B)

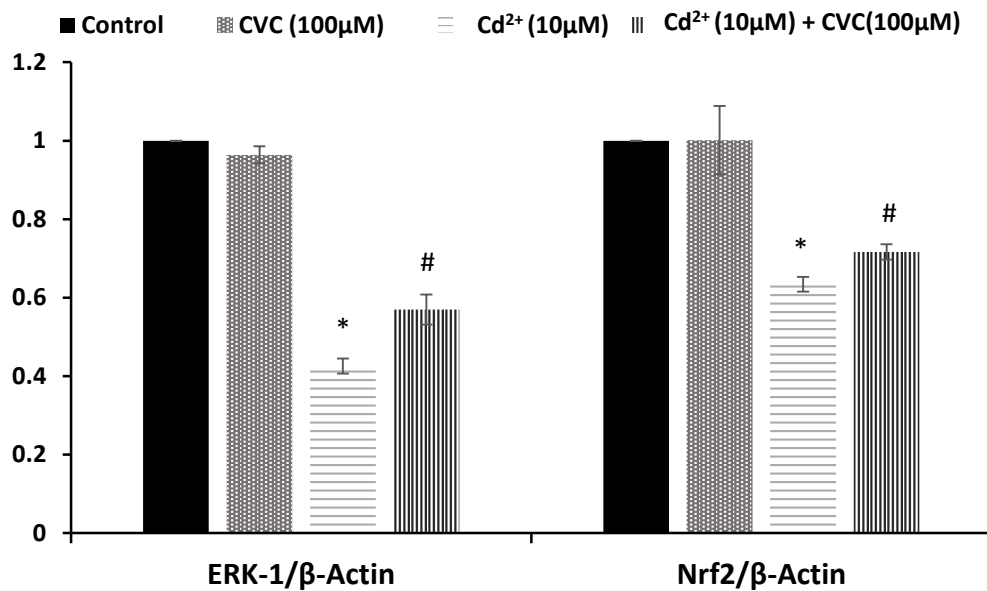


Fig. 3.7 Western blot analysis for the expression of extracellular signal-regulated kinase-1 (ERK1) and nuclear factor erythroid 2-related factor 2 (Nrf2) in PC12 cells exposed/ co-exposed with 10 μM cadmium (Cd²⁺) and 100 μM carvacrol (CVC) for 48 h. (A) The representative images (cropped) of immunoblotting for the expressions of ERK1 and Nrf2 in PC12 cells exposed/ co-exposed with Cd²⁺ and CVC for 48 h. (B) Relative density for the expression bands of ERK-1 and Nrf2 to β-actin in PC12 cells exposed/ co-exposed with Cd²⁺ and CVC for 48 h. Error bars indicate mean ± S.E.M. (n= 3), * and # indicate significant differences (P< 0.05) from the control group and the Cd²⁺-exposed group respectively.

3.3.8 Effects of carvacrol on Cd²⁺-induced cleavage of caspase 3

The activation of caspase 3 by cleavage is the final event in the caspase cascade reactions in apoptotic cellular execution.

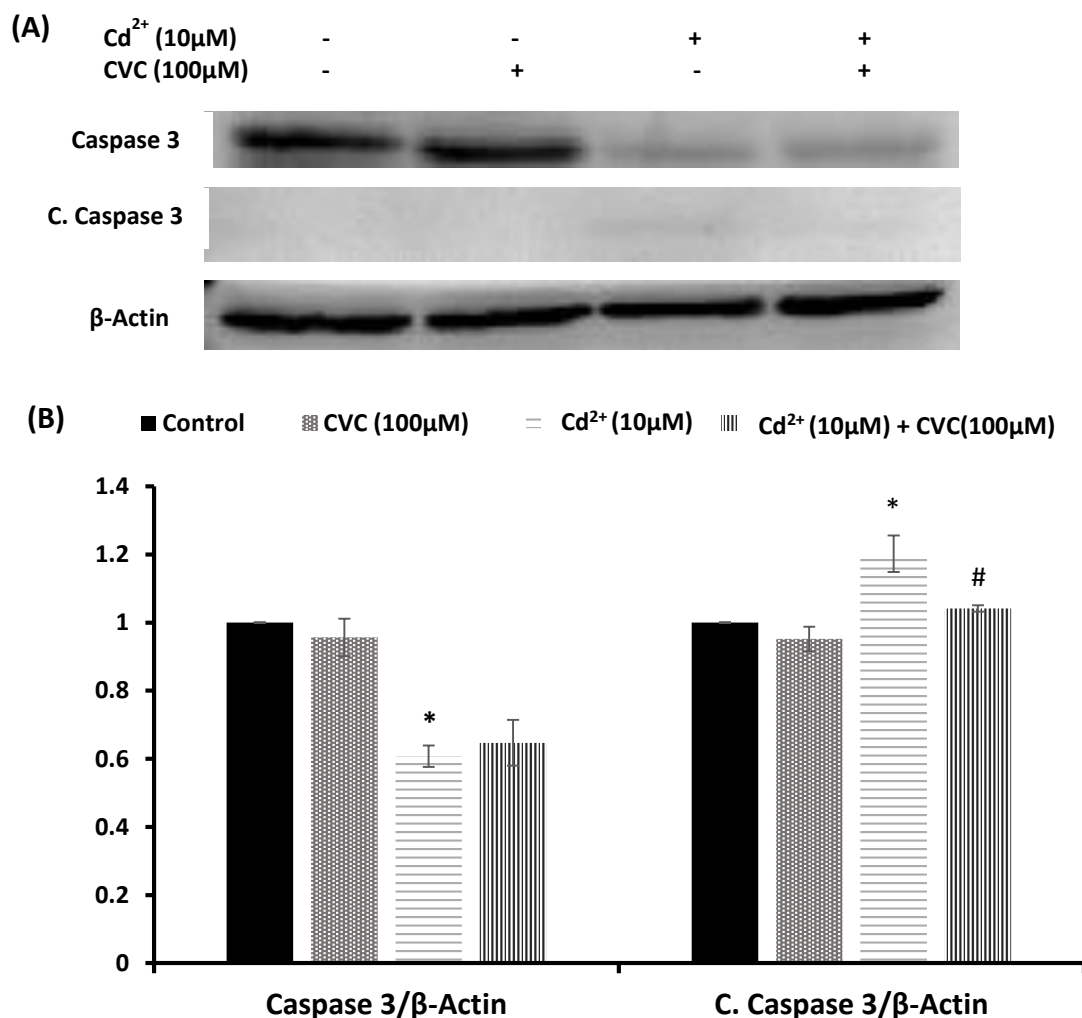


Fig. 3.8 Western blot analysis for the expression of caspase 3 and cleaved caspase 3 (c. caspase 3) in PC12 cells exposed/ co-exposed with 10 μM cadmium (Cd²⁺) and 100 μM carvacrol (CVC) for 48 h. (A) The representative images (cropped) of immunoblotting for the expressions of caspase 3 and c. caspase 3 in PC12 cells exposed/ co-exposed with Cd²⁺ and CVC for 48 h. (B) Relative density for the expression bands of caspase 3 and c. caspase 3 to β-actin in PC12 cells exposed/ co-exposed with Cd²⁺ and CVC for 48 h. Error bars indicate mean ± S.E.M. (n= 3), * and # indicate significant differences (P< 0.05) from the control group and the Cd²⁺-exposed group respectively.

PC12 cells were exposed to Cd²⁺ (10 μM), or carvacrol (100 μM), or both Cd²⁺ (10 μM) and carvacrol (100 μM) for 48 h. A substantial ($p < 0.05$) uplift was found in cleaved caspase 3 level in Cd²⁺ (10 μM) exposed cell group. Whereas, this level of cleaved caspase 3 was found to be

lowered in cells exposed to combined Cd^{2+} (10 μM) and carvacrol (100 μM) (Fig. 3.8A and B). On the other hand, the expression of caspase 3 in Cd^{2+} (10 μM) exposed cells became down-regulated significantly but showed no difference with co-exposed cell group. Therefore, from the results it was ascertain that carvacrol saves PC12 cells from Cd^{2+} (10 μM)-induced apoptosis by hindering the cleavage of caspase 3.

3.3.9 Effects of carvacrol on Cd^{2+} -induced release of cytochrome c and AIF from mitochondria into the cytosol

The release of the hallmark protein cytochrome c, from the mitochondria into the cytosol, is essential to initiate the caspase cascade reaction leading to apoptosis.

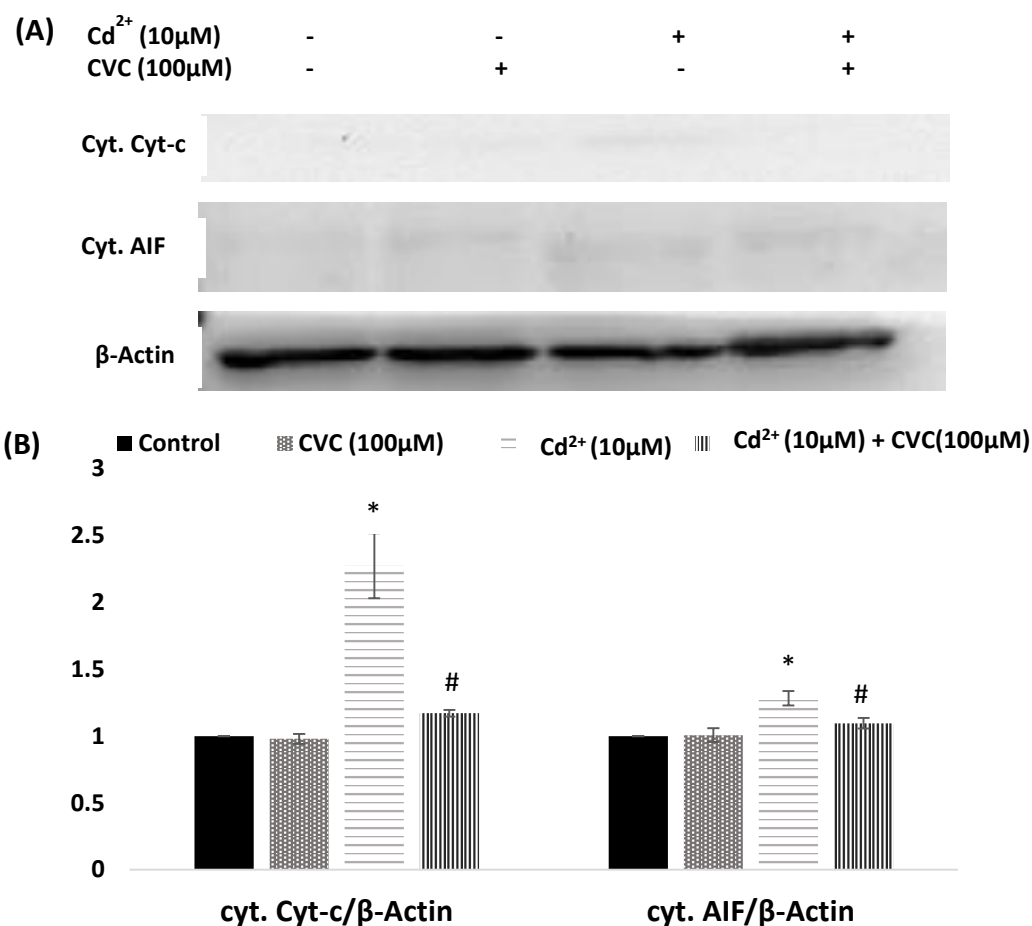


Fig. 3.9 Western blot analysis for cytosolic cytochrome c (cyt. Cyt-c) and cytosolic apoptosis inducing factor (cyt. AIF) in PC12 cells exposed/ co-exposed with 10 μM cadmium (Cd^{2+}) and 100 μM carvacrol (CVC) for 48 h. (A) The representative images (cropped) of immunoblotting for cyt. Cyt-c and cyt. AIF in PC12 cells exposed/ co-exposed with Cd^{2+} and CVC for 48 h. (B) Relative density for cyt. Cyt-c and cyt. AIF to β -actin in PC12 cells exposed/ co-exposed with Cd^{2+} and CVC for 48 h. Error bars indicate mean $\pm$ S.E.M. (n= 3), * and # indicate significant differences ($P < 0.05$) from the control group and the Cd^{2+} -exposed group respectively.

On the other hand, the mitochondrial membrane protein, AIF takes part in chromatin condensation and DNA fragmentation; and is translocated to the nucleus via cytosol during apoptosis. The results show a significant ($p < 0.05$) increase in cytosolic cytochrome c and AIF after exposing PC12 cells with Cd^{2+} (10 μM), which became considerably ($p < 0.05$) lesser for Cd^{2+} (10 μM) and carvacrol (100 μM) co-exposed groups (Fig. 3.9A and B).

3.3.10 Effects of carvacrol on Cd^{2+} uptake and MT expressions in PC12 cells

After exposing cells to Cd^{2+} (10 μM) and/or carvacrol (100 μM) for 48 h, the Cd^{2+} uptake by PC12 cells was measured using an ICP-MS. Although not significant, the Cd^{2+} uptake was higher in cells co-exposed to Cd^{2+} (10 μM) and carvacrol (100 μM) (Fig. 3.10) in comparison to cells exposed to Cd^{2+} (10 μM) only.

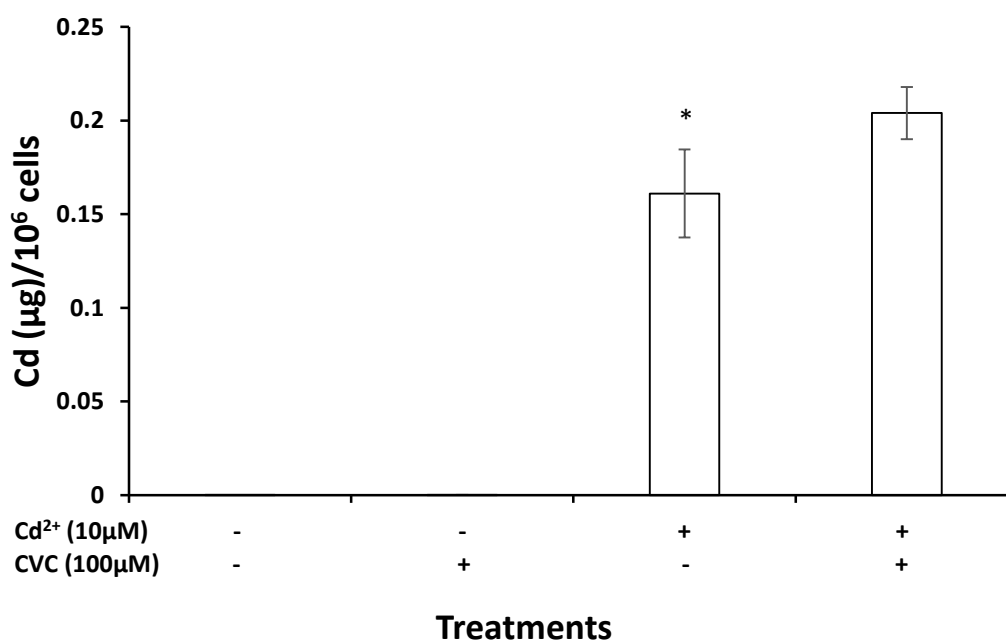


Fig. 3.10 Effects of carvacrol (CVC) on cadmium (Cd^{2+}) uptake in PC12 cells exposed/ co-exposed with 10 μM Cd^{2+} and 100 μM CVC for 48 h, measured by using ICP-MS analysis. Error bars indicate mean $\pm$ S.E.M. ($n = 3$), * and # indicate significant differences ($P < 0.05$) from the control group and the Cd^{2+} -exposed group respectively.

Furthermore, we measured the MT expressions in cells to explain the phenomenon of Cd^{2+} uptake increase in the co-exposed group.

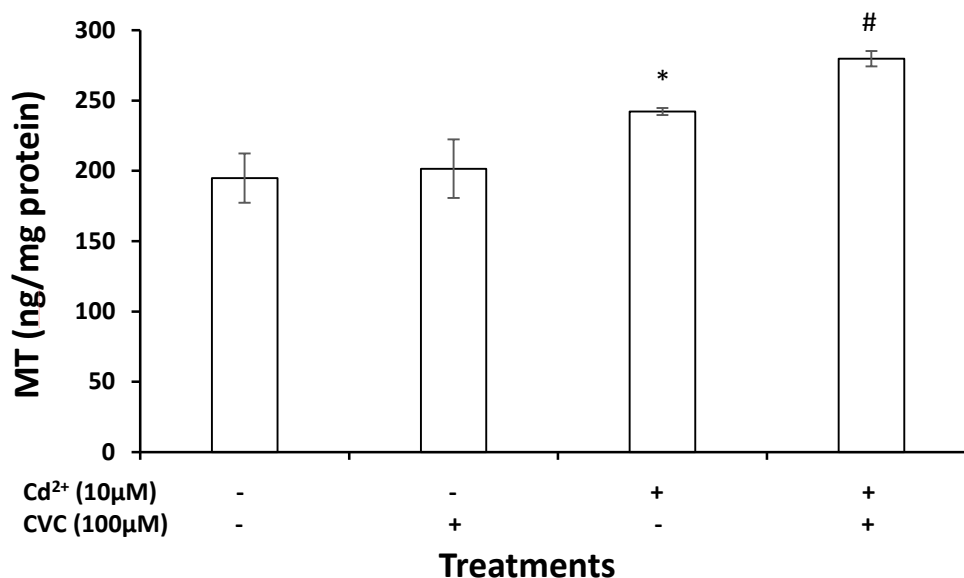


Fig. 3. 11 Effects of carvacrol (CVC) on metallothionein (MT) content in PC12 cells exposed/ co-exposed with 10 µM cadmium (Cd²⁺) and 100 µM CVC for 48 h, determined by enzyme-linked immunosorbant assay (ELISA). Error bars indicate mean ± S.E.M. (n= 3), * and # indicate significant differences (P< 0.05) from the control group and the Cd²⁺-exposed group respectively.

Fig. 3.11 shows that the expression of MT was significantly increased in co-exposed cells in comparison to only Cd²⁺ (10 µM) exposed cells. Thus, the results indicate that upon co-exposure, carvacrol increased the MT expressions in PC12 cells which increased the Cd²⁺ uptake by MT binding.

3.4 Discussion

In recent times, natural antioxidants have been attentively examined since these can act as potential preventive mechanisms against many toxicants to humans. In line with this, carvacrol, a food component possessing strong antioxidant activity, is believed to be a promising candidate (Aeschbach et al., 1994). Therefore, the objectives of this study were to define the roles of carvacrol on Cd²⁺-induced cytotoxicity and to interpret the mechanisms behind those roles through cellular and molecular perspectives. In this study, it was found that carvacrol effectively lessens the immensity of Cd²⁺-induced oxidative stress and apoptosis in PC12 cells. The underlying molecular mechanisms involved in the prevention Cd²⁺-induced toxicity in PC12 cells were also elucidated for the first time.

Numerous studies in a variety of cell lines suggest that Cd^{2+} toxicity is often associated with oxidative stress due to the over-generation of ROS causing cell-cycle degradation, deterioration of biological macromolecules and ultimately leading to apoptosis (Hu et al., 2015; Oh and Lim, 2006; Zhou et al., 2009; Chatterjee et al., 2009). In mammalian cells GSH plays a key role in combating ROS. ROS also interacts with classical antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and GR. The efficiency of GSH to regenerate depends on the redox state of the glutathione disulfide-glutathione couple (GSSG/2GSH) (Nemmiche, 2017). In line with this, the rate of the conversion of GSSG to GSH depends on the efficiency of the key enzyme GR. In this study, upon treatments and co-treatments, the intracellular GSH levels and the expressions of GR in PC12 cells were determined as oxidative stress markers (Fig. 3.3A and B). The results in this research demonstrated that Cd^{2+} (10 μM) exposure negatively affected the levels of both markers. Thus, results were in agreement with the results found in other cell lines such as CRL-1439 normal rat liver cells (Ikediobi et al., 2004). On the other hand, carvacrol (100 μM) significantly boosted GSH and GR levels upon co-exposure with Cd^{2+} (10 μM). Thus, carvacrol saves PC12 cells from Cd-induced oxidative stress by replenishing two vital components of antioxidant defense mechanisms; the low-molecular weight thiol compound, GSH and the enzyme reducing GSSG to GSH, GR. Results also exhibited that the acute oxidative stress posed by Cd^{2+} (10 μM) exposure for 48 h also caused the significant number of PC12 cell death (Fig. 3.1B) and increased the level of LDH activity in culture media (Fig. 3.2). These effects were reversed by the action of carvacrol (100 μM) after co-exposure. Here, it has been indicated that carvacrol (100 μM) can reduce the cell death by alleviating oxidative stress exerted by Cd^{2+} (10 μM). The oxidative stress ameliorating activity of carvacrol found in this research can be supported by previous findings. For instance, a recent study showed that carvacrol reduces oxidative stress by increasing the GSH levels against paracetamol-induced toxicity in HepG2 cells (Palabiyik et al., 2016).

Cd^{2+} exposure induces apoptosis in a number of cell systems including PC12 cells (Rahman et al., 2017). The flow cytometry analyses in the current study showed that carvacrol (100 μM), when co-exposed with Cd^{2+} (10 μM), recovered more than half of the apoptotic PC12 cells (from >54% to about 26%) compared to only Cd^{2+} (10 μM) exposure (Fig. 3.5). One of the probable reasons behind this performance to rescue cells from apoptosis is the

ameliorating effects of carvacrol (100 μ M) on oxidative stress posed by Cd^{2+} (10 μ M). carvacrol also contributed to the inhibitory effect against oxidative DNA damage, as well as enhancing effect on the cell cycle progression, through inducing antioxidant protection. Previous studies have reported that Cd^{2+} exposure accelerates the ROS induced DNA damages, inhibits the repair of oxidative DNA damage, affects cell cycle progression, and induces apoptosis in various cell lines (e.g., HepG2 cells) (Rani et al., 2014; Skipper et al., 2016; Norbury and Zhivotovsky, 2004). In a recent study, Horvathova et al. (2007) established the inhibitory effects of carvacrol on H_2O_2 induced DNA damage in K562 cells. The intact DNA density was measured following agarose gel electrophoresis and a considerable amount of intact DNA was fragmented by exposure to Cd^{2+} (10 μ M). However, exposure to combined Cd^{2+} (10 μ M) and carvacrol (100 μ M) increased the amount of intact DNA significantly (Fig. 3.4A and B), thereby minimizing the DNA damage.

It has already been established that a leucine zipper transcription factor namely Nrf2 mediates an important signaling pathway leading to cellular protection against oxidative stress and electrophilic compounds (Loboda et al., 2016). Normally, Nrf2 is sequestered in a complex with kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. But, onset of oxidative stress disassociates and translocates Nrf2 into the nucleus to bind with antioxidant responsive element (ARE). This binding allows expressions of cytoprotective genes such as *HMOX1* and *NQO1* (Zhang et al., 2017; Taguchi et al., 2011). Even though the underlying molecular mechanism is still unclear, the inactivation of Nrf2 is necessary for the accomplishment of apoptosis (Méndez-García et al., 2019). In the current research, it was found that upon co-exposure, carvacrol (100 μ M) significantly lessened the Cd^{2+} -imposed inactivation of Nrf2 (Fig. 3.7A and B) and perhaps promoted the expressions of antioxidant enzymes. Additionally, the involvement of a variety of oxidative transcription factors like NF- κ B is responsible for achieving Cd^{2+} -induced apoptosis. It has been suggested that apoptosis due to Cd^{2+} toxicity in rat kidney proximal tubular epithelial cells, NRK-52E, involves the down regulation of NF- κ B which could facilitated oxidative stress (Xie and Shaikh, 2006). Oxidative stress also inhibits autophagy-related pro-survival proteins like mTOR and Akt in the PI3/mTOR/Akt signaling pathway, leading to the induction of Bax expression and suppression of Bcl-2 which ultimately activates caspase cascade to induce apoptosis (Rahman et al., 2018; Roy et al., 2014; Singh et al., 2012). As shown in Fig. 3.6A and B, Cd^{2+} (10 μ M) induced

downregulated expressions of NF- κ B, mTOR, and Akt, which were considerably increased by the co-exposure of Cd²⁺ (10 μ M) and carvacrol (100 μ M). Furthermore, the involvement of MAPKs pathways has already been elucidated in various cell lines undergoing *in vitro* apoptotic, as well as necrotic cell death induced by Cd²⁺. ERK1 belongs to MAPKs protein family which is known to be involved in cell proliferation, differentiation, and apoptosis. A contemporary research by Hu et al. (2015) has shown that Cd²⁺ encourages apoptosis in MG63 cells by increasing ROS and inhibiting ERK 1/2 pathway. Results also exhibited a significant down regulation of ERK1 by Cd²⁺ toxicity which was meaningfully recovered by combined exposure with carvacrol (100 μ M) (Fig. 3.7A and B). Again, the probable reason for ERK1 revival is that carvacrol could ameliorate the oxidative stress and placate the ROS activity.

Cd²⁺ encourages mitochondrial permeability transition pore (MPTP) opening, facilitates the release of apoptogenic proteins into the cytosol and thus intensifies the occurrence of multiple apoptotic pathways via caspase-dependent (releasing cytochrome c) or via caspase-independent (releasing AIF and Endo G) pathways. However, the activation of mitochondrial apoptotic pathways largely contributes to the manifestation of apoptotic death caused by Cd²⁺ toxicity (Liu et al., 2016b). Cumulative evidences from studies on a number of cell lines suggested that the Cd²⁺-induced oxidative stress diminishes the mitochondrial membrane potential (MMP), decreases Bcl-2/Bax ratio, releases cytochrome c into the cytosol, activates caspase 9, subsequently activates caspase3, and finally causes apoptosis (a process called intrinsic mitochondrial pathway) (Jiang et al., 2014; Lasfer et al., 2008). Cytochrome c is the most related and crucial hallmark molecule of mitochondrial apoptotic pathway; which after being released from mitochondria binds with Apaf-1 to form apoptosome and activates the caspase cascade (Choi et al., 2006). It was found that Cd²⁺ (10 μ M) exposure increased cytosolic cytochrome c level which was significantly lowered by the co-exposure of Cd²⁺ (10 μ M) and carvacrol (100 μ M) (Fig. 3.9A and B). Alternatively, Cd²⁺ can also induce apoptosis via extrinsic pathway. For instance, Pal et al. (2011) showed that Cd²⁺ can trigger extrinsic pathway of apoptosis by up-regulating Bid, Fas, and caspase 8 in murine hepatocytes. However, both the extrinsic and intrinsic caspase-dependent apoptotic pathways mediated by caspase 8 and 9, respectively, result in apoptosis via the cleavage and activation of the executioner caspase 3 (Wu et al., 2014). In current research, it was depicted that Cd²⁺ (10 μ M) significantly induces caspase 3 cleavage, which is in accordance with the

previous studies. However, this rise in cleaved caspase 3 level was considerably reduced by the co-exposure of Cd^{2+} (10 μM) and carvacrol (100 μM) (Fig. 3.8A and B). A recent finding also demonstrated that carvacrol provides neuroprotection on focal cerebral ischemia/reperfusion by reducing apoptosis via prohibiting caspase 3 cleavage (Yu et al., 2012). On the other hand, reports on various cell types (e.g., HEP3B cells) suggested that Cd^{2+} induces oxidative stress and subsequently releases AIF and endoG from the mitochondria into the cytosol causing caspase-independent apoptosis (Lemarié et al., 2004). Researchers also found AIF not only as an apoptotic executioner, but also as a survival protein (Sevriokova, 2011). Nevertheless, the translocation of AIF from mitochondria to the nucleus via cytosol is considered as a standard sign of caspase-independent apoptosis (Mao et al., 2011). A significant increase in the cytosolic AIF in PC12 cells was found after 48 h Cd^{2+} (10 μM) exposure which was significantly reduced by co-exposure with carvacrol (100 μM) (fig. 3.9A and B). Therefore, it can be deduced from the findings that carvacrol can ameliorate both caspase-dependent and caspase-independent apoptosis upon Cd^{2+} -induced oxidative stress.

MTs are divalent, cysteine-rich small metal-binding stress-proteins ubiquitously expressed in many tissues; but expressions are induced by a variety of factors especially by metal ions such as Cd^{2+} . It was recommended that cells synthesizing MTs are resistant, while cells not synthesizing MTs are sensitive to Cd^{2+} toxicity. It also have been reported that MTs protect cells from oxidative species reacting with sulfhydryl groups (Ruttkey-Nedecky et al., 2013). Although the physiological functions of MTs are still debated, a number of *in vivo* studies showed that MTs are induced in tissues upon Cd^{2+} exposure and is engaged in the metabolism and detoxification through Cd^{2+} binding (Lu et al., 2001). Likewise, a recent *in vitro* study on HEK293 cell system demonstrated that MTs overexpression protected cells against Cd^{2+} (10 μM) toxicity (Li et al., 2005). Similarly, we found the MTs were considerably overexpressed in PC12 cells after exposure to Cd^{2+} (10 μM) (Fig. 3.11). Surprisingly, it was also observed that the MTs expression level was significantly higher in cells co-exposed to Cd^{2+} (10 μM) and carvacrol (100 μM) in comparison to that of the Cd^{2+} (10 μM) exposed cells. A previous study supported this finding showing that carvacrol can boost the overexpression of MTs in tolerogenic dendritic cells (Spiering et al., 2012). Therefore, the higher uptake of Cd^{2+} (Fig. 3.10) by PC12 cells co-exposed with Cd^{2+} (10 μM) and carvacrol (100 μM) might be explained by the overexpression of MT and probable increase in Cd^{2+} -binding by MTs.

Overall, it can be summarized that carvacrol protected PC12 cells from Cd^{2+} -triggered toxicity by combating oxidative stress, increasing GSH levels, upregulating GR expression, and reducing DNA damage; as well as, stimulating ERK-1 MAPK expression, activating expressions of Nrf2, NF κ B, and autophagy related pro-survival proteins- mTOR and Akt, and promoting MT overexpression (Fig. 3.12). Moreover, CVC effectively defended cells against both Cd^{2+} -induced caspase-dependent and caspase-independent apoptosis through hindering caspase 3 cleavage, and prohibiting cytochrome c and AIF release into cytosol from mitochondria.

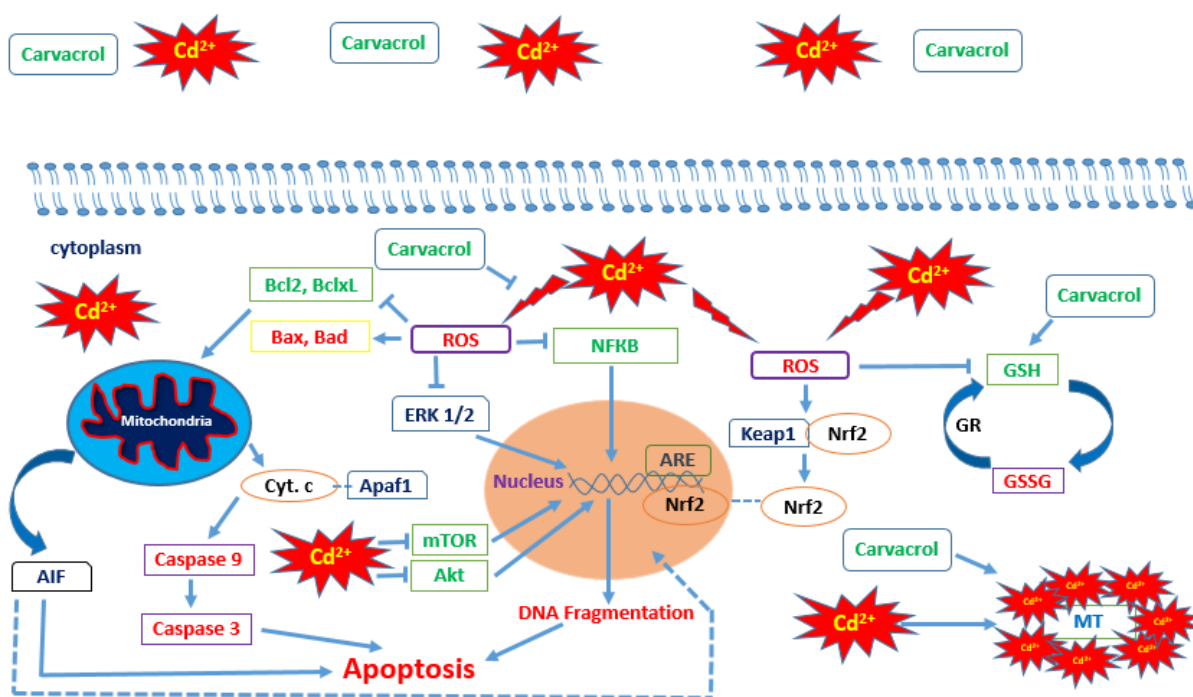


Fig. 3.12 A schematic representation of the proposed molecular mechanism of Cytoprotective and survival-enhancing effects of carvacrol against cadmium (Cd^{2+})-triggered oxidative stress and caspase dependent/independent apoptosis in PC12 cells.

3.5 Conclusion

It became certain that the natural antioxidant, carvacrol acted as a powerful anti-oxidative and anti-apoptotic agent against Cd^{2+} in PC12 cells. Finally, carvacrol is recommended as a potential and safe therapeutic agent against the toxicity posed by the toxic heavy metal, Cd^{2+} , in the biological system. However, further *in vivo* and *in vitro* researches are required for precise understanding of the effects, interactions, and

mechanism(s) of action of carvacrol against Cd²⁺-induced toxicity. To add, investigations are also necessary to understand the outcome of carvacrol treatment on other metal or non-metal toxicants in a diverse array of biological systems.

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Chapter 4: General conclusion

4.1 General Conclusion

In search of sustainable and protective measures against the Cd-induced toxicity in biological systems, the objectives of this research were set to understand the effects of ethanolic extract of ajwain and its phenolic compound, carvacrol on Cd-induced toxicity in PC12 cells. Moreover, exploration of the molecular mechanistic pathways behind the protections posed by these two agents was also under the coverage of the objective of the study. In a broader sense, this research is a part of the aim for expanding the spectrum of plant-based therapy in ameliorating the toxic effects of lethal toxicants, including heavy metals such as Cd. Cd contamination is a global environmental issue which is a great concern due to its long biological half-life and a high bioaccumulation tendency. Moreover, in modern life, the exposure to Cd is obvious due to the emergence of unavoidable tools for modernization and globalization, including must-used goods and must-doing practices in daily life. For instance, now we cannot think even a day without the Cd contaminating sources such as fossil fuel combustion, waste incineration, use of nickel-cadmium batteries, and phosphate fertilizer in agriculture. The concern is much more mounting in developing countries such as Bangladesh, which are overpopulated and have poor facilities for environmental management. Cd from the anthropogenic sources leaks out in three major environmental compartments, air, water and soils which afterwards spreads in the food chain to be taken up by all the organisms including human. Upon bio-accumulation in human, Cd exerts its toxicity which can lead to many diseases in various organ systems. Sometimes this toxicity appears as acute symptoms in both cases of occupational and chronic exposures. There is no effective drug or chelating agent against Cd toxicity. So, protective measures are necessary through less exposure or through the detoxification in sustainable and effective means to combat the toxic exposure. Researchers have been continuously working on the development of agents for remediating Cd-induced toxicity through unveiling the effectiveness of these agents and molecular mechanisms behind the remediation protocols in biological systems. In line with this, numerous researches have been carried out on the toxicity mechanisms posed by Cd and protection mechanisms posed by many antioxidant substances in various biological models. It has been shown that Cd can induce apoptosis in the biological system through caspase-dependent (both intrinsic and extrinsic or any one) and/or caspase-independent pathways. It

is also apparent that for ameliorating the toxicity of Cd, phyto-extracts and phytochemicals are the candidates for the right choice because of their availability, high antioxidant property, fewer side effects and low price. Many plant-based extracts and their phytochemicals have already been screened with molecular basis for the alleviation of Cd-induced toxicity in biological models; some examples of which have been represented in Tables 1.1 and 1.2. In line with this, this research work has been carried out to find the effects of ethanolic extract of ajwain and its phenolic compound, carvacrol on Cd-induced toxicity in PC12 cells with molecular clarifications.

In Chapter 2, the effects of ethanolic extract of ajwain (AE) was assessed to alleviate the Cd toxicity in PC12 cells. Ajwain has been chosen due to the fact that it has been historically used for the remedy of many pathological consequences such as inflammatory, toxic, neurological, genital or respiratory tract disorders. For the reasons, it can be expected to reduce Cd toxicity. PC12 was chosen due to their ease of culture with background knowledge on their proliferation, has versatility for pharmacological manipulations, and has been extensively used for toxicity studies. At first PC12 cells were exposed to various concentrations of AE to select the safe dosage. Then to reduce the Cd toxicity, cells were exposed/co-exposed to Cd (5 or 10 μ M) with/without non-toxic dose (240 μ g/mL) of AE for 24 h. AE upon co-exposure reduced LDH activity in the media and increased the cell viability and GSH contents in comparison to only Cd exposed cells. These phenomena indicated that AE reduced the cytotoxicity and oxidative stress posed by the exposure of Cd. The apoptosis caused by Cd has been confirmed from the ladder pattern detected by the agarose gel electrophoresis of genomic DNA in PC12 cells. The measurement of the intensity of the DNA image has shown that AE reduced the DNA fragmentation caused by Cd exposure. It was indicated that AE reduced the occurrence of Cd-induced apoptosis in PC12 cells. Reduction of the Cd-induced apoptosis caused by AE was further confirmed by the expressions of apoptotic and pro-apoptotic marker proteins detected by western blottings. AE reduced Cd-increased expression of Bax and up-regulated Cd-down-regulated expressions of Bcl-2, Bcl-xL and NF- κ B. Upon co-exposure AE reduced the cytosolic cytochrome c levels which is a hallmark protein of apoptosis. This indicates that AE might increase the mitochondrial membrane potentiality and prohibited the release of cytochrome c from mitochondria into the cytosol. AE also reduced the increased expression of the apoptotic executioner, caspase 3, ultimately

indicating the amelioration of Cd-induced apoptosis in PC12 cells. Thus, it was clarified that AE reduced Cd-induced apoptosis through the intrinsic mitochondrial pathway in PC12 cells.

Hence, to confirm whether the phenolic compound of AE, carvacrol can alleviate Cd-induced cytotoxicity and apoptosis, the following research was carried out in Chapter 3. The safe dose for this monoterpenic phenol present in AE was determined as 0-200 μM in PC12 cells. The dose of co-exposure of carvacrol with Cd (10 μM) was employed as 100 μM . Carvacrol was co-exposed with Cd in PC12 cells for 48 h to determine the cell viability, LDH levels, GSH levels and GR expression levels. Carvacrol reduced Cd-increased LDH level and increased Cd-decreased cell viability, GSH levels and GR expression level. These results indicate that carvacrol potentially reduced Cd-induced cytotoxicity and oxidative stress in PC12 cells. The flow cytometry result exhibited that the apoptosis rate is very high in Cd-exposed PC12 cells; however, carvacrol reduced apoptosis rate considerably caused by Cd. The expressions of survival proteins, mTOR, Akt, NF- κB , ERK-1 and Nrf2 were reduced significantly by Cd-exposure, which were revived by carvacrol co-exposures. Carvacrol reduced the Cd-induced cleavage of caspase 3 in PC12 cells. Additionally, the hallmark protein for intrinsic apoptosis, cytosolic cytochrome c and the hallmark protein for caspase-independent apoptosis, cytosolic AIF were considerably lowered by carvacrol co-exposure. Moreover, the expression of MT was increased in co-exposed cells, indicating that the MT bound Cd is higher in the cells of carvacrol co-exposed cells. From these results, it was summarized that carvacrol inhibited the Cd-induced caspase-dependent and -independent apoptosis in PC12 cells.

In conclusion, both AE and its phenolic molecule, carvacrol act as powerful anti-apoptotic agents upon Cd toxicity in PC12 cells. The anti-apoptotic effects of carvacrol (from the results of chapter 3) indicate that it might be the responsible compound of AE for the amelioration of Cd-induced apoptosis. This anti-apoptotic effect was achieved presumably by reducing the oxidative stress through the consequences of antioxidant properties of both agents. This research not only confirmed the anti-apoptotic effects of AE and carvacrol but also gained the apoptosis reducing dosage of the agents in the biological system. Additionally, AE and carvacrol can be recommended as potential Cd-induced toxicity remediating agents in other cell lines or in other animals. Moreover, the findings of this research will act as a source of baseline information for further *in vivo* and *in vitro* studies to unveil the effects,

interactions and mechanisms of these two agents against Cd or other organic and/or inorganic toxicants.