Alleviation of arsenic-induced toxicity by curcumin and D-pinitol, the main components of the herbs commonly used in Bangladesh, and their mechanism

バングラデシュで日常的に用いられているハーブの主成分であるクルクミンとD-ピニトールによるヒ素誘発毒性の緩和とそのメカニズム

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Course in Environmental Adaptation Science
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A dissertation in fulfillment of the requirement for the degree of Doctor of Philosophy (PhD) in Environmental Science

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DEDICATION

To my beloved parents, teachers and noble followers of religion who have passed away showing the mankind the right path of life

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CONTENTS

List of Abbreviations

ACP	Acid phosphatase
Akt	Protein kinase B
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
ARE	Antioxidant responsive elements
AST	Aspartate transaminase
ATCC	American Type Cell Culture
ATSDR	Agency for toxic substances and disease registry
CAT	Catalase
CD	Conjugated dienes
DTNB	5, 5′-dithiobis-2-nitrobenzoic acid
ECL	Enhanced chemiluminescence
ERK-1	Extracellular signal-regulated kinase-1
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione S-transferases
LDH	Lactate dehydorogenase
LH	Lipid hydroperoxides
LPO	Lipid peroxidation
MDA	Malondialdehyde
MMP	Mitochondrial membrane potential
MPTP	Mitochondrial permeability transition pore
mTOR	Mammalian target of rapamycin
NaF	Sodium fluoride
Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate buffered saline

PC	Protein carbonyl
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
TBARS	Thiobarbituric acid reactive substances
WHO	World Health Organization
XIAP	X-linked inhibitor of apoptosis protein
γGT	γ-glutamyl transferase

Abstract

Arsenic toxicity and arsenic-related health consequences are major public health concerns and a global problem affecting countries on all five continents. Arsenic contamination in the aquatic environment due to natural sources and anthropogenic activities is posing a dreadful threat to human health. Among various heavy metals, arsenic is one of the most toxic and carcinogenic substances that extensively contaminate the water bodies. Both acute and chronic arsenic exposure through drinking water and foods can induce cellular, metabolic and physiological toxicities which cause diseases involving tissues and organ systems such as injuries for skin, liver, kidney, lungs, gastrointestinal tract, and cardiovascular systems, diabetes mellitus, neurological disorders, Alzheimer disease and various forms of cancer. So far there is no particular cost-effective treatment available for arsenic-related diseases. Though chelation therapy is a well-known treatment for arsenic-related diseases; however, it is costly and showed several undesirable side effects. In the last few decades, the interest in the nutritional field has gone a step forward, searching for novel natural bioactive compounds with the capacity to reduce the risk of non-communicable diseases.

As hypothesis for the thesis, natural dietary supplements and a balanced diet can be a cost-effective and safe therapeutic approach against arsenic toxicity. Thus, the objective of this research has been set to investigate the ameliorative effects of natural dietary bioactive compounds on arsenic toxicity using molecular and cellular biological techniques. In the present research, natural dietary bioactive compounds; D-pinitol and curcumin have been used to detoxify arsenic toxicity in PC12 cells which has been well known as model cell line for fundamental molecular and toxicological study. D-pinitol is a natural dietary bioactive compound that has antioxidant properties and drawn great attention due to its diverse biological activities and therapeutic potential against many human ailments. In chapter 2, the ameliorative effects of D-pinitol on arsenic-induced toxicity has been investigated in PC12 cells. Obtained results demonstrated that co-exposure of D-pinitol (1, 5 and 50 µM) with arsenic (5 µM) increases cell viability, and decreases DNA damage and protects PC12 cells from arsenic-induced cytotoxicity by increasing glutathione (GSH) level and glutathione

reductase (GR). Protein expression of western blot analysis revealed that co-exposure of D-pinitol and arsenic significantly decreased arsenic-induced autophagy which further suppressed apoptosis through up-regulation of survival factors; mTOR, p-mTOR, Akt, p-Akt, NF-κB, Nrf2, ERK1, GR, and Bcl-x, and down-regulation of death factors; p53, Bax, cytochrome c, and LC3, although arsenic regulated those factors negatively. From the results, D-pinitol protects PC12 cells from arsenic-induced cytotoxicity.

Recently a naturally occurring polyphenol, curcumin which has been widely used in Bangladesh as a spice has also drawn great attention due to its diverse biological activities, strong antioxidant properties and therapeutic potential against many human diseases. Similarly, in Chapter 3, the protective effects of curcumin on arsenic-induced toxicity has been investigated using PC12 cells, because arsenic is a serious concern in Bangladesh. Arsenic (10 µM) treatment in PC12 cells for 24 h induced cytotoxicity by decreasing cell viability and intracellular GSH level and DNA fragmentation in PC12 cells and increasing lactate dehydrogenase (LDH) activity in the cell cultured medium. Also, arsenic caused apoptotic cell death in PC12 cells, which were confirmed from flow cytometry results. In addition, arsenic (10 µM) treatment significantly down-regulated the survival factors; mTOR, Akt, Nrf2, ERK1, Bcl-x and Xiap, up-regulated the death factors; ULK, LC3, p53, Bax, cytochrome c, caspase 9 and cleaved caspase 3, and eventually caused autophagic and apoptotic cell death. However, curcumin (2.5 µM) pretreatment (1 h) with arsenic (10 µM) protects PC12 cells from arsenic-induced cytotoxicity by increasing cell viability, GSH level and boosting the antioxidant defense system, and limiting the LDH activity and DNA damage. Furthermore, pretreatment of curcumin with arsenic expressively alleviated arsenic-induced toxicity and cell death by reversing the expressions of protein mTOR, Akt, Nrf2, ERK1, Bcl-x, Xiap, ULK, LC3, p53, Bax, cytochrome c, caspase 9 and cleaved caspase 3.

These findings indicated that curcumin showed antioxidant properties through the Nrf2 antioxidant signaling pathway and reduces arsenic-induced toxicity in PC12 cells *via* modulating autophagy/apoptosis. Considering a person's diet, both dietary compounds,

curcumin and D-pinitol are often consumed together; however, there is no information at all whether co-exposure of curcumin and D-pinitol has an additive, synergistic, or no effect on arsenic-induced toxicity. Thus, in chapter 4, we hypothesized that the combination treatment of curcumin and D-pinitol might have synergistic and strong protective effects against arsenic toxicity. As results, pretreatment of curcumin or D-pinitol, or their combined pretreatment with arsenic increases cell viability, decreases DNA damage and protects PC12 cells from arsenic-induced cytotoxicity by increasing GSH level and antioxidant defense. Protein expression of western blot analyses showed that pretreatment of curcumin or D-pinitol, or their combined pretreatment with arsenic significantly inhibited arsenic-induced cell death through up-regulation of survival factors; mTOR, Akt, Nrf2, ERK1, Bcl2, Bcl-x, and XIAP and down-regulation of death factors; p53, Bax, cytosolic cytochrome c, caspase 9 and cleaved caspase 3, although arsenic regulated those factors negatively. These findings indicated that curcumin and D-pinitol showed antioxidant properties and protects PC12 cells from arsenic-induced cytotoxicity. Furthermore, the effect of combined treatment with curcumin and D-pinitol was stronger than individual treatment.

Both chronic and acute exposure of arsenic possess serious health hazards and lack of a balanced diet could exaggerate the toxic effects. In short, a balanced diet and proper dietary intake of natural bioactive compounds promise to reduce the arsenic toxicity. The present study suggested that both natural dietary compounds; curcumin and D-pinitol have beneficial role against arsenic toxicity *in vitro* and the needs for future animal experiments were shown for practical application.

Chapter One: General Introduction

1.1 Introduction

The present thesis focuses on the ameliorative effects of natural dietary compounds against arsenic toxicity *in vitro*. Both acute toxicity of arsenic at high concentrations and chronic toxicity of arsenic for long-term exposure at low concentrations has been known about for centuries. Recently, several reports revealed that long-term exposure to markedly low concentrations of arsenic has a strong adverse health effects and affecting many millions of people in the world (Escudero-Lourdes, 2016; Ratnaike et al., 2003; Walvekar et al., 2007). The major source of arsenic for human intake is drinking water which comes from the wells drilled into arsenic-rich ground strata. In recent decades, the extensive presence of arsenic in groundwater to supply for drinking water in many countries has been reported, and it was suggested that many millions of people mostly in developing countries using drinking water with several times higher arsenic concentrations than the limit (10 µg/L) recommended by World Health Organization (WHO) (Petrusevski et al., 2007). Though arsenic contamination in drinking water is a worldwide problem, the most serious damage to health has taken place in Bangladesh.

Since arsenic is a ubiquitous element and present as many forms in the environment, it seems to be impossible for humans to avoid arsenic exposure. Arsenic may enter human body by ingestion, inhalation or skin absorption as major routes of exposure and can constantly spread in the body including the major organs such as skin, lungs, liver and kidneys (Abdul et al., 2015; Hong et al., 2014). Arsenic exposure is linked with many human diseases including arsenicosis, cardiovascular disease, pulmonary disorders, liver and kidney disorders, neurological diseases, diabetes mellitus, Alzheimer's disease and various forms of cancer (Naujokas et al., 2013; Sun et al., 2014). However, there is still now no particular cost-effective treatment available for arsenic-related diseases. Though chelation therapy is a well-

known treatment for arsenic-related diseases, it is expensive and showed several undesirable side effects. In the last few decades, a newly introduced research field 'nutrigenomics' indicates that diet influences genetic expressions and disease incidents. Recently, the interest in the nutritional field has gone a step forward, searching for novel natural bioactive compounds with the capacity to reduce the risk of non-communicable diseases. So, it is important to explore the novel natural bioactive compounds that can alleviate chemicals such as arsenic-induced toxicity and related diseases as cost-effective and safe therapeutic agents.

1.2 Arsenic

Arsenic is a ubiquitous chemical element with the atomic number 33, relative atomic mass 74.92 and a well-known environmental toxicant. Arsenic is classified as metalloid due to its chemical and physical properties. Arsenic is the 20th most abundant elements in the Earth's crust and it also widely distributed throughout the environment in rocks, soil, water, air, and in biota even in human body. Arsenic-enriched minerals, rocks, volcanoes and forest fires are the primary natural sources of arsenic in the environment whereas, numerous insecticides, herbicides, phosphate fertilizers, mining and smelting, coal combustion, industrial processes and timber preservatives are the anthropogenic sources (Bundschuh et al., 2011; Mondal et al., 2006; Singh et al., 2015). Historically, arsenic is widely used in wood preservatives, semiconductor, lead acid batteries, glass manufacturing, pesticides, herbicides, fungicides, crop desiccants, pharmaceuticals and food additives for cattle and poultry. The occurrence, distribution and mobility of arsenic depend on various geochemical factors including pH, reduction-oxidation reactions, other ionic species, aquatic chemistry and microbial activity (Shih, 2005). Arsenic can be released into water, soil and air via both natural and anthropogenic processes, and existed in some different chemical forms (inorganic or organic) and oxidation states (−3, 0, +3, +5) (Hughes et al., 2011). There are three major groups in arsenic compounds, such as, (i) inorganic arsenic compounds (ii) organic arsenic compounds

and (iii) arsine gas. In water, arsenite (As^{III}) and arsenate (As^{V}) are the most predominant inorganic arsenic compounds and in foods, particularly in sea foods, arsenobetaine, arsenocholine, and arsenosugars are the common organic forms of arsenic (Ahmed, 2014). Fig. 1.1 displays the common sources of arsenic.

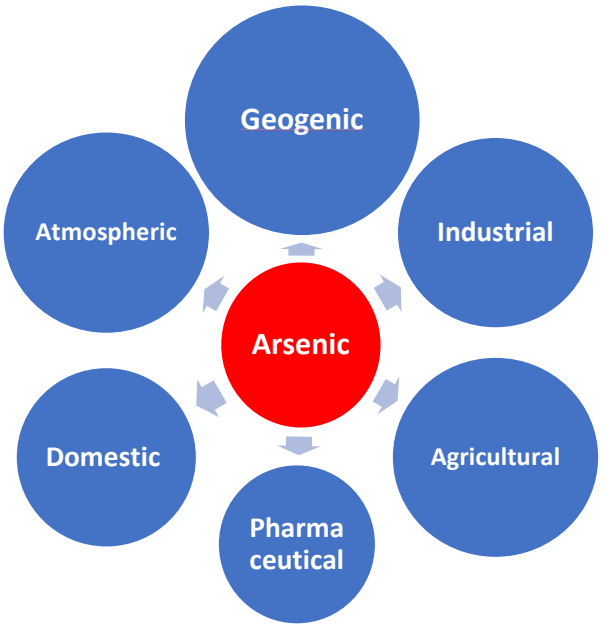

Fig. 1.1 Sources of arsenic.

Arsenic toxicity depends on its valency state and chemical form, and generally inorganic form of arsenic (As^{III} or As^{V}) is considered as the most toxic species of arsenic usually present in drinking water. Again, trivalent arsenic (As^{III}) is generally more toxic for humans and 4-10 times more soluble in water than pentavalent arsenic (As^{V}) (Petrusevski et al., 2007). However, arsenic contamination in groundwater, either from natural sources or anthropogenic with multiple social and health impacts, has now become a major concern for the environment in various parts of the world. More than 150 million of people are exposed to high levels of arsenic in many countries around the world through the intake of arsenic-rich groundwater (Shankar et al., 2014).

1.3 Arsenic problem: Global perspectives

Arsenic contamination in drinking water has been considered as one of the major global public health concerns of WHO since 2010 because many millions of people worldwide are exposed to arsenic and suffering from arsenic associated serious diseases (Fisher et al., 2017). Groundwater is widely used as a major source of drinking water in many regions of the world, especially in South Asian countries.

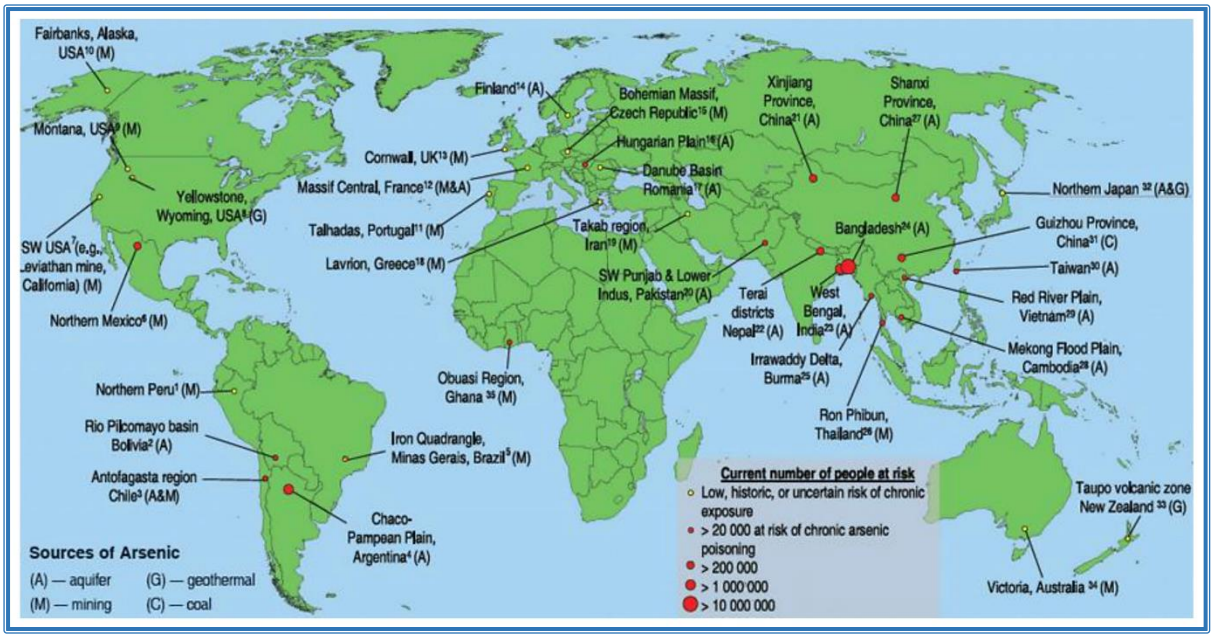

Fig. 1.2 Worldwide distribution of arsenic contaminated regions, showing source of arsenic and numbers of people at risk of chronic exposure. (Thakur et al., 2011)

Arsenic contamination in drinking water affects many countries of the world including Argentina, Bangladesh, China, India, Mexico, Myanmar, Nepal, Pakistan, Vietnam, and parts of the USA (Fig. 1.2). Depending upon the status and natural settings of each country, exposure to arsenic has come from natural sources as well as from various man-made sources such as industrial sources or food and beverages. The largest arsenic-related health consequences in the world have been taken place in Bangladesh, and West Bengal, India and potentially affecting many millions of people due to the contamination of drinking water

aquifers (Thakur et al., 2011). In addition, several countries have been reported localized groundwater arsenic pollutions and new cases are continuing to be discovered. More than 150 million people in the world have been exposed to drinking water with higher arsenic contamination levels than 10 μg/L; the WHO's recommended guideline (Shankar et al., 2014). And continuously new affected areas in various parts of the world are discovered (Ravenscroft et al., 2009). Many countries, especially developing countries, still use the 50 μg/L of arsenic as their national standard, because of lack of adequate test, removal and mitigation facilities for lower-level arsenic concentrations due to financial support. High arsenic levels in groundwater depend on the high arsenic concentration in the rocks or sediments, mobility, speciation and environmental conditions (Thakur et al., 2011), and subsequently arsenic can enter groundwater systems from weathering and leaching of arsenic minerals in the rock and soil.

Arsenic concentrations which are higher than the standards for drinking water have been reported in many countries in all five continents (Table 1.1).

Table 1.1: List of countries where arsenic concentrations have been reported higher than 10 μg/L in groundwater.

Asia	Bangladesh, Cambodia, China (including provinces of Taiwan and Inner Mongolia), India, Iran, Japan, Myanmar, Nepal, Pakistan, Thailand, Vietnam
Americas	Alaska, Argentina, Chile, Dominica, El Salvador, Honduras, Mexico, Nicaragua, Peru, United States of America
Europe	Austria, Croatia, Finland, France, Germany, Greece, Hungary, Italy, Romania, Russia, Serbia, United Kingdom
Africa	Ghana, South Africa, Zimbabwe
Pacific	Australia, New Zealand

(Source: Petrusevski et al., 2007).

1.4 Arsenic problem: Bangladesh perspectives

Though the arsenic pollution is recognized as a global public health concern, in Bangladesh and West Bengal millions of people drink water with high levels of arsenic (higher than 10 µg/L) from the alluvial and deltaic aquifer. The arsenic contamination in groundwater in Bangladesh is one of the largest poisoning of a population in history. The scale of this environmental poisoning disaster is considered to be greater than the accident at Bhopal, India, in 1984 (Pearce, 2002).

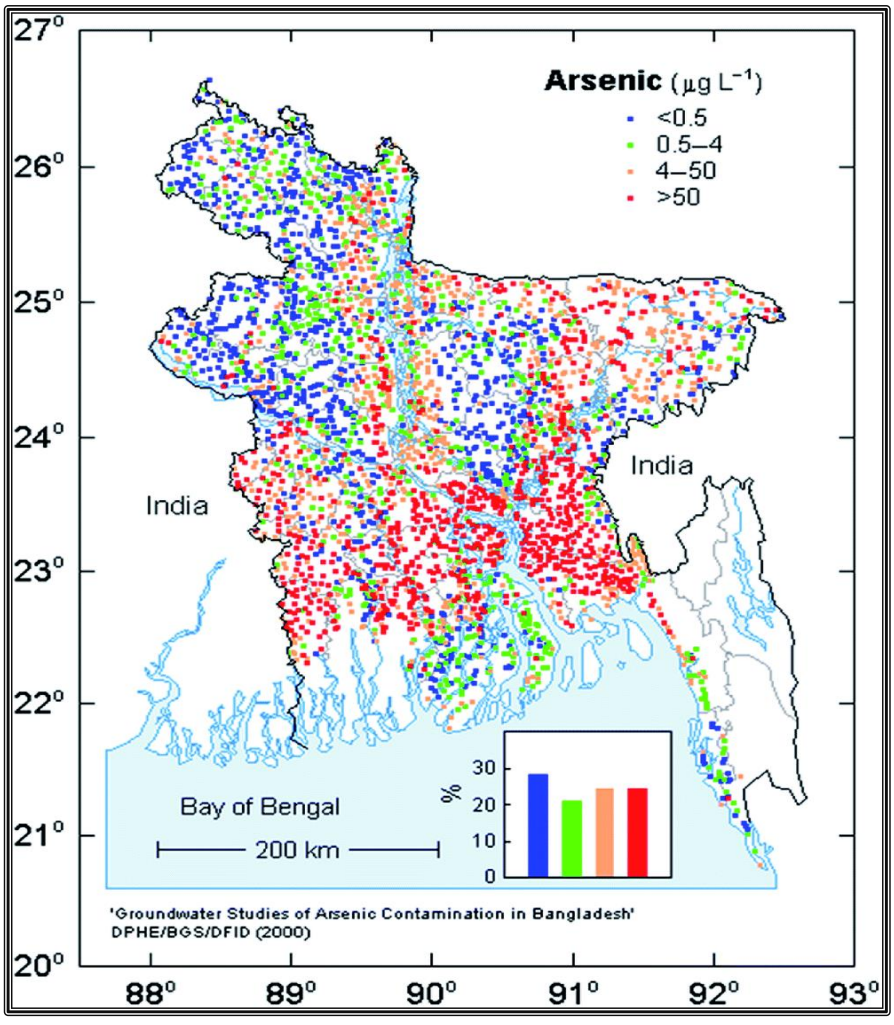

Fig. 1.3 Arsenic concentrations in groundwater in Bangladesh (BGS and DPHE, 2001).

Arsenic contamination was first detected in the tube-well water in 1993 in Chapai Nawabganj, a northern district of Bangladesh (DPHE, 1993). Within 12 years from the first detection of arsenic contamination, it was revealed that arsenic contamination of groundwater was present in 62 out of 64 districts of Bangladesh (Fig. 1.3). Tube-wells have been the main source of drinking water in rural areas of Bangladesh, and the arsenic-contaminated tube-wells are distributed in entire country (Ahmad et al., 2018). Around 97% of the population in rural areas of Bangladesh relies on tube-well water as their main source of drinking water (Flanagan et al., 2012). More than 50 million people in Bangladesh were routinely exposed to more than 10 μg/L (WHO guideline value). Arsenic exposures more than 50 μg/L and in the range of 10–50 μg/L could account for 24,000 and 19,000 adult deaths annually, respectively (Flanagan et al., 2012). In 2013, another survey research demonstrated that about 35-77 million people in Bangladesh are chronically exposed to high levels of arsenic through their drinking water which contains higher than WHO drinking water guideline value, 10 μg/L (Winston et al., 2013). In the dry season, farmers extensively use arsenic-contaminated groundwater for irrigation of the fields, and people are additionally exposed to inorganic arsenic via food, especially rice which is the main food of Bangladesh.

Various adverse health effects of chronic arsenic toxicity are already evident in Bangladesh such as cardiovascular diseases, various forms of cancer, diabetes, increased blood pressure, reproductive disorders, adverse pregnancy outcomes (stillbirth and preterm birth), and decreased intelligence quotient among the children. Cancer due to chronic arsenic exposure through consumption of contaminated water is now a major problem for Bangladesh. Still now, a greater percentage of individuals consume arsenic-contaminated water due to the absence of sustainable arsenic-free water supply (Ahmad et al., 2018).

1.5 Arsenic exposure and human diseases

Arsenic toxicity is a global health problem affecting millions of people in whole of the world and responsible for many human diseases. Arsenic is considered as one of the most hazardous chemicals in the world due to its worldwide occurrence, toxicity and carcinogenicity (ATSDR, 2017). Humans are exposed to arsenic through ingestion, inhalation, and dermal contact. Keratosis or skin lesions are some of the most common and earliest nonmalignant effects related to chronic arsenic exposure, and have been observed even at the exposure levels in range of 0.005-0.01 mg/L of arsenic in drinking water. Long-term (5–10 years) excessive inorganic arsenic exposure to human *via* drinking water and food may leads arsenicosis, skin disorders, skin cancers, cancers of internal organ (bladder, kidney, and lung), diseases of the blood vessels of the legs and feet, possibly diabetes, cardiovascular diseases, increased blood pressure and reproductive disorders (WHO, 2011; Santra et al., 2013).

In the 1980s, arsenic was officially recognized as class 1 carcinogenic substances and registered with the International Agency for Research on Cancer (IARC). Arsenic is the carcinogen known to cause cancer through respiratory exposure and gastrointestinal exposure. After the relationship between arsenic exposure and carcinogenicity was revealed, studies were conducted in the United States, Taiwan, Bangladesh, India, Argentina, and Chile to further examine this relationship, and their results supported carcinogenicity of arsenic revealed by previous reports (Kapaj et al., 2006). Many complications including dermatological effects (i.e. hyperkeratosis, pigmentation changes), cardiovascular effects, pulmonary disorders, liver and kidney disorders, reproductive effects and neurological effects have been reported due to arsenic exposure in adults and children specifically *via* drinking water. An outline of human diseases due to arsenic exposure is explicitly elucidated in Table 1.2.

Table1.2: Human diseases due to arsenic exposure.

Arsenic toxicity related diseases	**Affected organ/ cells**	**References**
Various types of cancer	Skin cancer	Rossman et al., 2004; Hsueh et al., 1997; Leonardi et al., 2012
	Lung cancer	Hopenhayn-Rich et al., 1998; Ferreccio et al., 2000; Smith et al., 2006; Chiu et al., 2004; Chen et al. 2004; Garcia-Esquinas et al., 2013
	Bladder cancer	Chiang et al., 1993; Gabriel et al., 2006; Gamble et al., 2007; Meliker et al., 2010
	Kidney cancer	Ahmed, 2014; Ferreccio et al., 2013
	Liver cancer	Morales et al., 2000; Baastrup et al., 2008; Lin et al., 2013a
	Prostate cancer	Bulka et al., 2016; Benbrahim-Tallaa and Waalkes, 2008
	Leukemia	Durant et al., 1995; Heck et al., 2014
Neurological disorders, memory, and intellectual function	Brain, Central Nerve System	Tsai et al., 2003; Wasserman et al., 2004; Mundey et al., 2013
Diabetes	Heart, blood vessels, nerves, eyes and kidneys.	Lai et al., 1994. Tseng et al., 2002; Nizam et al., 2013; Rahman et al., 1998
Dermal diseases	Whole body, specially hand, foot, face, back.	Ahsan et al., 2000; Ahsan et al., 2006; McCarty et al., 2007; Rahman et al., 2009

Cardiovascular diseases	Heart or blood vessels	Lee et al., 2002; Kapaj et al., 2006; Rahman et al., 2009
Respiratory diseases	Lungs	Argos et al., 2010; Parvez et al., 2010; Parvez et al., 2011
Liver diseases	Liver	Ratnaike, 2003; Kapaj et al., 2006; Das et al., 2012
Renal diseases	kidney	Madden and Fowler, 2000; Lokuge et al., 2004; Zheng et al., 2014
Endocrine disruption	Thyroid, Pancreas, gonads	Davey et al., 2008; Ciarrocca et al., 2012; Naujokas et al., 2013
Reproductive diseases	Reproductive system	Chattopadhyay et al., 2002; Chakraborti et al., 2003; Hopenhayn et al., 2003
Hematopoietic system disorders	Bone marrow, spleen and erythrocytes.	Hall, 2002; Zhang et al., 2014a

Arsenic associated health effects are mostly reported in adults; however, recently some researches revealed that arsenic has also detrimental effects on child health and development (Tsuji et al., 2004; Vahter, 2008). The risk of infant mortality, child growth restriction and lack of neurodevelopment of young children have been increased due to maternal chronic arsenic exposure during pregnancy. Maternal arsenic exposure during pregnancy has been related to increased risk of spontaneous abortion, stillbirth, preterm birth and low birth weight (Ahmed, 2014).

Numerous researches demonstrated that chronic exposure to inorganic arsenic, even at fairly low exposure levels, leads to increased risk of infectious diseases, particularly lower respiratory tract infections, diarrhea, bronchiectasis and tuberculosis in children and adults (Ahmed, 2014; Rahman et al. 2011; Raqib et al. 2009; Farzan et al. 2013; Smith et al. 2006; Smith et al. 2011). These relations are most likely due to arsenic-related alterations of the immune function.

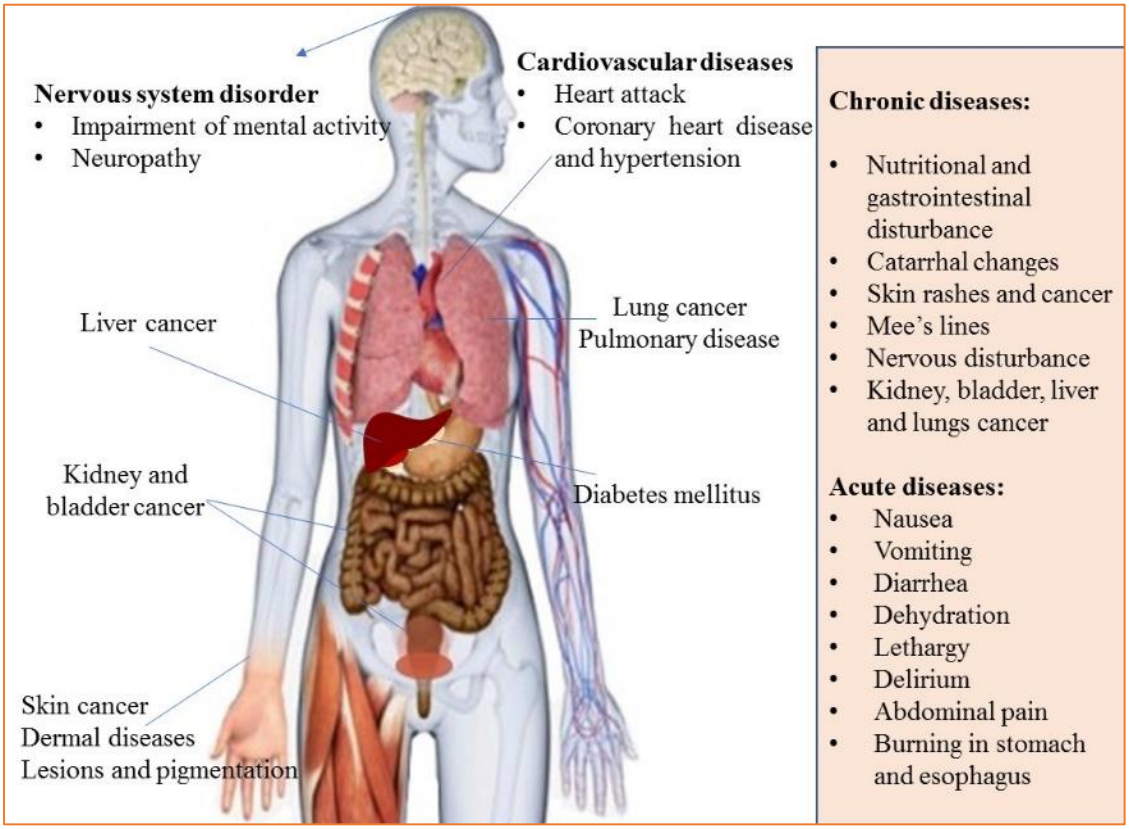

Fig. 1.4 Diseases associated with arsenic exposure (Shahid et al., 2018).

Acute arsenic exposure (high dose of arsenic with less exposure duration) has less hazardous effects on human health; however, chronic exposure (low dose with continuous exposure for long time) has more hazardous effects. Acute exposure of arsenic causes abdominal pain, vomiting, diarrhea, muscular pain, weakness, with flushing of the skin rashes, muscular cramping, hyperkeratosis, melanosis, black foot disease, damage of motor and sensory responses (Fig. 1.4).

1.6 Arsenic toxicity mechanisms in cellular systems

Although many basic studies have already investigated the arsenic toxicity mechanisms, the exact mechanisms are not yet clear. Arsenic toxicity varies depending on arsenic speciation, dose, duration of exposure, cell/tissue type, and metabolism. Recently a report demonstrated that arsenic induces cytotoxicity by increasing the production of reactive oxygen species (ROS) and accelerating the damage of biomolecules (DNA, lipid and proteins) (Susan et al., 2019). Excessive ROS generation through arsenic exposure negatively affects cellular functions by disrupting signaling pathways related to cell growth, proliferation, differentiation, DNA repair and other important cellular metabolic processes (Hughes, et al., 2011). A simplified mechanism of arsenic toxicity in a cellular system is illustrated in Fig. 1.5.

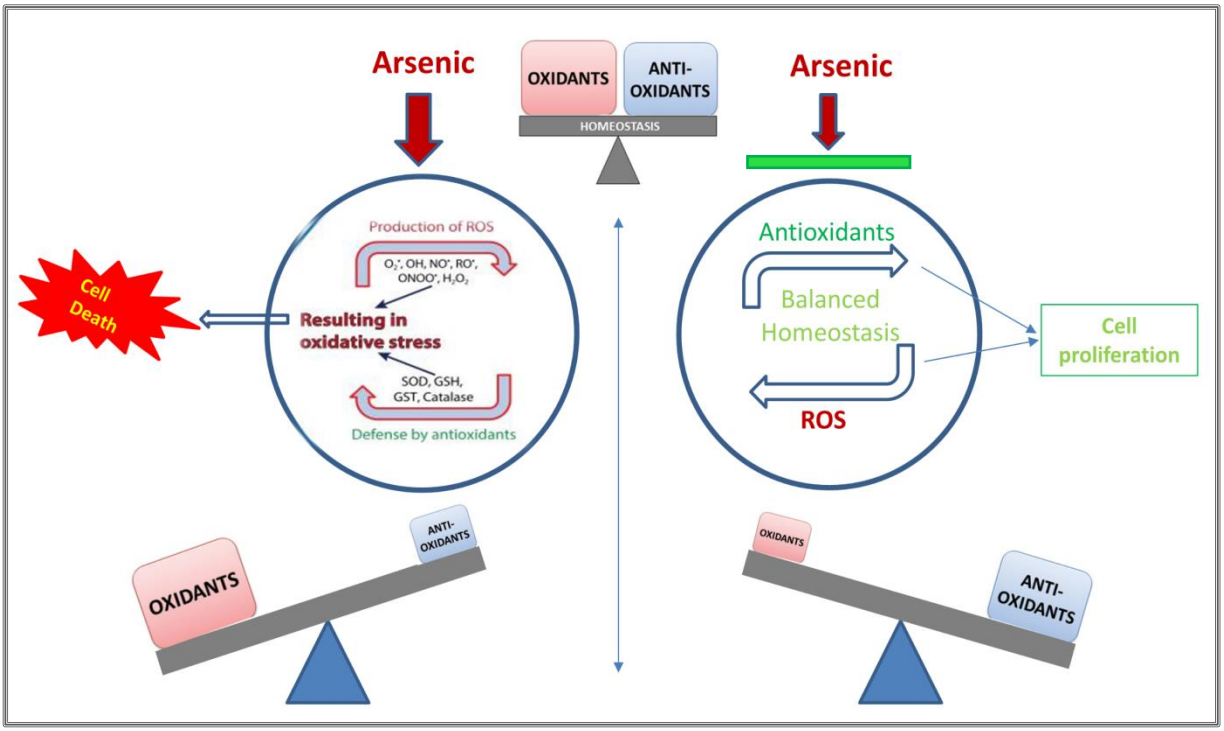

Fig. 1.5 Simplified mechanism of arsenic-induced toxicity in the cellular level.

ROS is continuously produced during regular aerobic metabolism and integral antioxidant defense systems of organisms play a vital role in the prevention of the probable damages triggered by overproduction of ROS (Fig. 1.5).

Arsenic increases ROS production through the suppression of cellular free radical scavenger enzymes such as superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT). Mechanism of Arsenic-induced cell death has been investigated widely in various cell lines (Perker et al., 2019; Rahaman et al., 2020; Wang et al., 2015) and it differ depending on arsenic concentration, exposure duration and cell types. However, most of the studies showed that arsenic induces cytotoxicity via ROS generation, direct binding with –SH groups, disruption of antioxidant defense system, lipid peroxidation, and damaging biomolecules (DNA, proteins), and finally caused cell death where oxidative stress plays the central role. Moreover, the elevated level of ROS inhibits the activity of survival proteins such as mTOR, Akt, Nrf2 and ERK1 which can promote DNA damage. Overproduction of ROS also responsible for the activation of autophagy related ULK1 and LC3 proteins and finally caused apoptotic cell death (Rahman et al., 2018; Rahaman et al., 2020; Roy et al., 2014a). A simplified molecular mechanism/s of arsenic-induced toxicity is shown in Fig. 1.6.

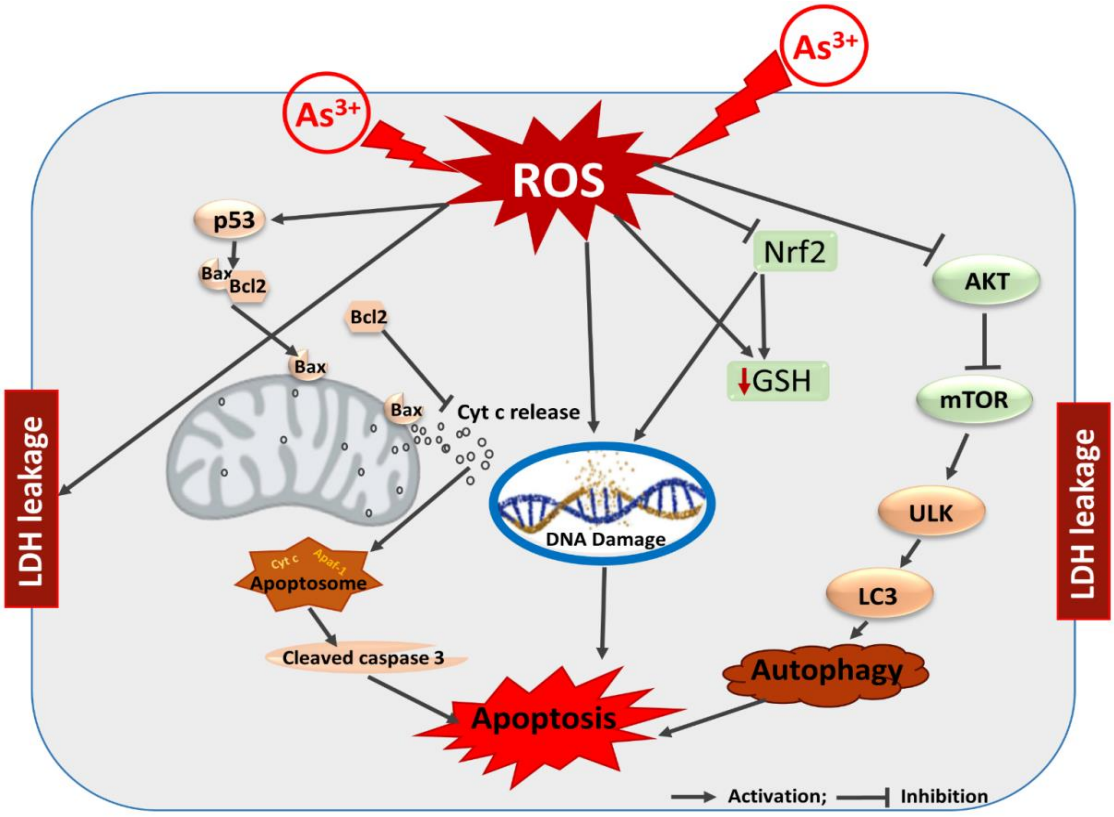

Fig. 1.6 Schematic diagram showing the molecular mechanism/s of arsenic-induced toxicity.

Different ROS including superoxide, hydrogen peroxide and hydroxyl and peroxyl radicals, are generated in the cells under normal and pathological or stressed conditions (Ataie et al., 2016). If ROS generation rate overcomes the ability of the antioxidant system in the cells, oxidative damage to DNA, proteins and lipids will occur (Sun and Cheng, 1999). Oxidative damage to the DNA has been associated to be the cause of cancer; aging and neurodegenerative diseases such as Alzheimer and Parkinson's diseases; cardiovascular diseases like arteriosclerosis, and is the primary cause of cell death and tissue damage resulting from heart attack and stroke (Perron and Brumaghim, 2009).

Besides the above-mentioned effects, arsenic also causes various biological effects on cells and tissues. In arsenic toxicity, metabolism of arsenic plays a pivotal role and this metabolism involves in methyltransferase mediated reduction to a trivalent state followed by oxidative methylation to a pentavalent state. It is well established that pentavalent arsenicals are less toxic than the trivalent arsenicals including those methylated. Arsenic-induced toxicity is mediated mostly via ROS generation, DNA damage, loss of mitochondrial membrane potential, up-regulation of Bax and caspase-3, and down-regulation of Bcl-2 (Fig. 1.6). Variations of these important factors from regular physiology might play eminent roles in cell death, carcinogenicity, cardiovascular, testicular and genotoxicity, diabetes, and nervous systems disorders. Potential mechanisms of various types of arsenic-induced cancer may include oxidative stress, co-carcinogenesis and tumor promotion, genotoxicity, altered DNA methylation, and altered cell proliferation. Genetic information damage inside the cell leads genotoxicity which ultimately leads to mutation. Genotoxicity occurs since ROS reacts with both deoxyribose and bases in DNA, causing base lesions and strand breaks. In addition, ROS are also involved in oxidation of DNA, alteration of DNA repair, gene regulation mechanism, and threatening of gene stability (Ramana et al., 1998).

1.7 Treatment/management options of arsenic toxicity

Since arsenic presents in many forms in the environment, it seems to be impossible for humans to avoid arsenic exposure. Once it enters into the body, it is difficult to find a cure for it. So far, no proper therapeutics is available to completely detoxify the arsenic toxicity in biological systems. To minimizing the human health impacts of arsenic, there are two main approaches such as (1) Prevention and (2) Cure. Prevention indicates staying safe from contaminated air, water and food or somehow staying away from all sorts of arsenic exposure. Cure indicates medication/therapy by using clinical chelation therapy or antioxidant-enriched proper nutrition and food. Probable ways to combat arsenic toxicity is shown in Fig. 1.7.

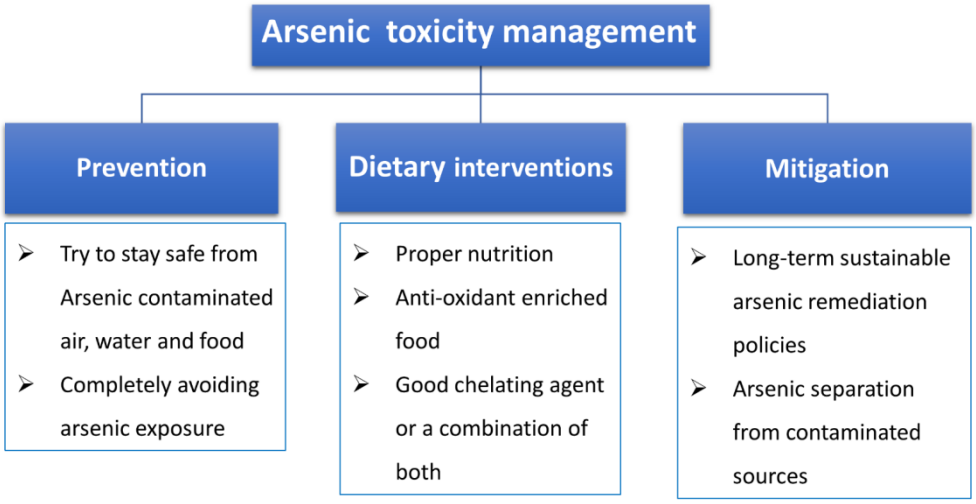

Fig. 1.7 Ways to combat arsenic toxicity.

Chelation therapy is the most efficient and well-known treatment of arsenic poisoning. British anti lewisite (BAL), sodium 2,3-dimercaptopropane-1-sulfonate (DMPS), meso 2,3-dimercaptosuccinic acid (DMSA) and D-penicillamine are familiar chelation therapeutic agents; however, they showed several undesirable side-effects (Flora et al., 2007). Many factors can affect the bio-accessibility, metabolism and toxicity of arsenic such as diet and nutrition. A high-protein diet with vitamin A, E and C-rich food helps recover from early

symptoms as well as medical additives in the form of vitamin A, E and C antioxidants are also beneficial.

Measures are urgently required to focus on reductions in arsenic toxicity, early diagnosis, and therapy of arsenic-induced cancers. Now, treatment options advocated are vitamin and mineral supplements and antioxidant therapy (Ratnaike, 2003).

Several studies have reported that intakes of some vitamins, jaggery, fruits, tea, and N-acetylcysteine, as well as high levels of glutathione, zinc, and selenium may reduce the arsenic-induced toxicity by reducing availability or formation of toxic mono-methylated species (Singh et al. 2008; Tseng, 2009; Sinha et al. 2010; Deb et al. 2013; Herrera et al. 2013; Sun et al. 2014; Yu et al. 2016). Chelating agent supplementation with antioxidants has been shown to be useful in attaining optimum impacts. Chelation therapy for arsenic toxicity is thought to be the specific therapy for relief of systemic clinical manifestations and reduction of arsenic stores in the body, reducing subsequent cancer risk. As a preventive and curative measure of arsenic toxicity, a combination therapies using antioxidants (N-acetylcysteine, α-lipoic acid, quercetin, etc.) and a thiol-chelating agent (with a proper chelator) can be considered as a better therapeutic approach than chelation therapy alone. However, much remains to be explored the ameliorative effects of natural compounds in prevention and curative measure for arsenic toxicity.

1.8 Natural bioactive compounds in detoxification of arsenic

Arsenic mainly induced toxicity via generation of excessive ROS and depletion of antioxidant defenses in a biological system. Therefore, it is considered that some devices can increase the antioxidant capacity of the cells and can be a therapeutic strategy in arsenic toxicity. This way could be accomplished by either reducing the possibility of the metal

interacting with critical biomolecules of the cells or by supplementation with antioxidant molecules from appropriate external sources.

Antioxidant can be defined simply as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell and Gutteridge 2007). Depending on specific characteristics of antioxidants, it can be divided into two categories, (i) enzymes such as SOD, catalase, GR, and glutathione peroxidase (GPx) and (ii) non-enzymatic metabolites such as GSH, uric acid, vitamins, and polyphenols. Regarding their origin, various antioxidants can be synthesized *in vivo* such as GSH, uric acid, catalase and SOD, whereas others, namely, polyphenols and β-carotene, are obtained from food.

Some natural compounds and antioxidants such as high-selenium lentil and other dietary selenium, mineral supplements, natural bioactive compounds like quercetin, resveratrol, α-tocopherol, natural chelators; alpha lipoic acid (α-LA) and its reduced form dihydrolipoic acid (DHLA) have ability to alleviate the arsenic toxicity. They are showed significant ameliorative effects on arsenic-induced toxicity (Balakumar et al., 2010; Bhattacharya, 2017). Recently a well-studied review report showed that 34 medicinal plants and 14 natural products exhibited significant protection against arsenic toxicity, mostly in preclinical trials and a few in clinical studies (Bhattacharya, 2017).

It is well established that arsenic is responsible for various human diseases by inducing oxidative stress in human body. Since, natural bioactive compounds and their derivatives have been used for curing oxidative stress-involved diseases (Pandey and Rizvi 2009), and these bioactive compounds have gained interest in their potential benefits largely due to their potent antioxidant activities.

Natural bioactive compounds can be described as components of foods that have an impact on physiological or cellular activities in the humans or animals. Natural bioactive compounds

are mainly found in fruits and vegetables as well as in milk, eggs, meat, fish, soy, wheat, broccoli, rice, and many more. Flavonoids, anthocyanins, tannins, betalains, carotenoids, plant sterols and glucosinolates are common bioactive compounds which poses antioxidant, anti-inflammatory, and anti-carcinogenic properties; and can be protective against various diseases and metabolic disorders (Walia et al., 2019). Such beneficial effects make them good candidates for management/treatment of arsenic toxicity. Some of the important natural bioactive compounds with antioxidant properties that have been explored for their role in reversing arsenic-induced oxidative stress and related organ dysfunctions have been shown in Table 1.3.

Table 1.3 Natural bioactive compounds ameliorating arsenic toxicity.

Name	**Organ/system /cell**	**Molecular Mechanism/(s) (salient inferences)**	**References**
All-trans retinoic acid	Uterus	Upregulated the estrogen receptor and cellular proliferation by boosting antioxidant defense.	Chatterjee and Chatterji, 2011
Arjunic acid	Liver, heart, brain, kidney, testes	Increased antioxidant enzymes SOD, CAT, GSTs, GPx, GR and prevent DNA damage; Scavenging free radicals.	Manna et al., 2007, 2008a, 2008b; Sinha et al., 2008a, 2008b
Ascorbic acid	Liver, testes, Kidney, Haematogical	Restored GSH, SOD, CAT, AcP, AlkP and GRD levels; increased epididymidal sperm counts and testicular weights.	Chang et al., 2007; Banerjee et al., 2009
Biochanin	Kidney, heart, Liver	Lowered plasma hepatic markers, increased GSH, SOD, CAT; reduced oxidative stress.	Jalaludeen et al., 2015, 2016
Diallyl trisulfide	Erythrocytes and lymphocytes	Decreased the levels of plasma marker enzymes (AST, ALT, ACP, ALP, LDH, MDA), and increased antioxidants (GSH, SOD, CAT, GPx, GST, GR)	Prabu and Sumedha, 2014

Epigallocate chin gallate (EGCG)	H9c2 cells, testes, liver	Decreased the level of LDH, CK, CK-MB, AST; inhibits ROS; regulates calcium homeostasis; reverses myocardial toxicity; mitigates sodium arsenite induced immune-suppression, inflammation and apoptosis in vitro; reduces oxidative stress through the activation of Nrf2 signaling.	Han et al., 2017; Guvvala et al, 2017
Genistein	Heart, brain	Inhibited the phosphorylated JNK and p38-MAPK (pp38-MAPK), cardiomyocytes apoptosis and blocked $\Delta\psi m$ collapse; Neuroprotection by modulating the JNK3 mediated apoptosis, ERK1/2 mediated autophagy and TNFα associated inflammatory pathways.	Fan et al., 2013; Saha et al., 2018
Leutin	Testes, liver	Increased antioxidants (GSH, SOD); upregulated the mRNA expression of Nrf2, HO-1, NQO1, and GST.	Niu et al., 2015; Li et al., 2016
Naringenin	Liver, kidney	Increased antioxidants (GSH, SOD, CAT, GPx, GST) and reduced serum bilirubin, urea, uric acid and creatinine levels; prevented DNA fragmentation.	Mershiba et al., 2013; Roy et al., 2014b.
Quercetin	Liver, brain, testes	Reduced ROS, increased antioxidant levels such as SOD, CAT, GR etc.; Increased in liver and brain cell membrane microviscosities.	Mishra and Flora., 2008; Ghosh et al., 2009; Jahan et al., 2015
Resveratrol	H9c2 cells, Kidney	Decreased LDH release; acted as a cardioprotective agent; reduced ROS in H9c2 cells; lowered blood urea nitrogen, creatinine and insignificant renal tubular epithelial cell necrosis.	Zhao et al., 2008; Zhang et al., 2014b
Rutin	Behavioral, brain	Increased nerve conduction velocity; attenuated neurofunctional alteration.	Sárközi et al., 2015;

Silibinin	Kidney, liver	Prevented renal damage by decreasing levels of serum urea, uric acid, creatinine; inhibited tubular cell apoptosis; Cardioprotection by reducing oxidative stress markers in heart, plasma total cholesterol (TC), triglycerides (TG), phospholipids (PL), free fatty acids (FFA).	Prabu and Muthumani, 2012; Muthumani and Prabu, 2014
Silymarin	CHO-K1 cells	Reduced conjugated diene formation and Hsp70 activity; decreased lipid peroxidation; regulated GGT activity.	Bongiovanni et al., 2007.
α-Tocopherol	Liver, kidney	Decresed ROS generation, maintained pro-oxidant/antioxidant balance, and reduced body arsenic store.	Mittal and Flora, 2007
β-Carotene	Liver, kidney	Normalized body weights, organ weights, hematological profiles, serum biochemical profile and significantly modulated all the hepatic and renal biochemical parameters and DNA fragmentation.	Das et al., 2015

1.9 D-pinitol and its bioactive properties

D-pinitol (3-O-methyl-chiro-inositol) is a naturally occurring dietary bioactive compound predominantly found in soybean seeds, pinewood, alfalfa and legumes. According to recent reports Pinaceae and Leguminosae family plants are major sources of D-pinitol (Gao et al., 2015; Negishi et al., 2015). Vegetable soybean (*Glycine max*) is well established legume in the human diet in all over the world including Bangladesh. Mature and dried soybean seeds contain up to 1% of D-pinitol (Lin et al., 2013b).

D-pinitol is also known as an alicyclic polyalcohol from the group 'cyclitols' which possesses diverse biological, pharmaceutical and therapeutic properties. Recently, D-pinitol gained a great attention for its potential health benefits mostly due to its strong antioxidant properties. Besides antioxidant properties, it also possesses feeding stimulant, antiviral, anti-inflammatory, antidiabetic, antihyperlipidemic, larvicidal, cardioprotective, anticancer and creatine retention promotion properties (Liu et al., 2012). The diversified biological activities of D-pinitol are shown in Fig. 1.8.

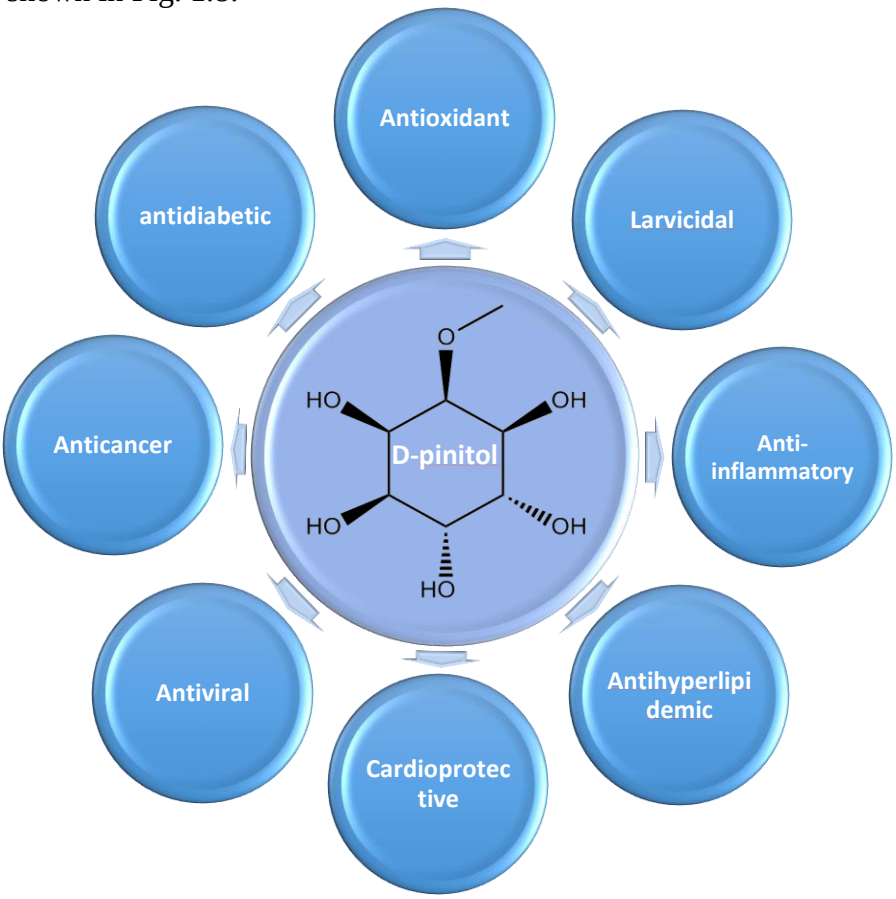

Fig. 1.8 Diversified biological activities of D-pinitol.

Numerous *in vivo* and *in vitro* studies already investigated the diversified biological activities of D-pinitol in various experimental models (Fig. 1.8). D-pinitol is well known in community of researchers mainly for its antidiabetic activities because it has been appearing to act downstream in the insulin signaling pathway to mimic the effects of insulin.

As mentioned above, it is well established that oxidative stress is one of the main causes of cell/tissue damage. Excessive production of free radicals resulting from oxidative stress can damage macromolecules such as DNA, proteins and lipids. These oxidative stress stimulated damages play a significant pathological role in human diseases and linked to numerous degenerative diseases. On the other hand, antioxidants have capacity to interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also acting as oxygen scavengers. However, antioxidant supplement may be used to help human body to reduce the oxidative stress stimulated damage. Rengarajan et al. (2015) revealed that D-pinitol treatment significantly increased the antioxidative enzyme activities, GSH levels and decreased oxidative stress marker, MDA and LPO levels in carbon tetrachloride (CCl_4)-treated rats. These findings indicated that D-pinitol acts as a protective agent by decreasing metabolic activation of xenobiotics through its antioxidant actions. Other important biological activities like antiviral and antidepressant effect of D-pinitol were reported by Zhan et al. (2006) and Alonso-Castro et al. (2019), respectively. D-pinitol showed excellent anti-inflammatory effects on K562 cells by significantly reducing Cox-2 protein expression (Eser et al., 2017) and acted as perfect antidiabetic agent through its insulin-like properties (Gao, et al., 2015).

It has already been reported that D-pinitol has preventive efficacy against prostate cancer (Lin et al., 2013b) and breast cancer (Rengarajan et al., 2014), and it is worth to mention that the work of Song et al. (2015) was patenting the use of D-pinitol for cancer treatment. D-pinitol also exerts hepatoprotective effect (Lee et al., 2019), cardioprotective effect (Kim et al., 2005) as well as acted as an excellent antihyperlipidemic (Geethan and Prince, 2008) and larvicidal agent (Chaubal et al., 2005). Recently a well-studied review report exhibited in detail information regarding various beneficial and health-promoting effects of D-pinitol (López-Sánchez et al., 2018).

1.9 Curcumin and its bioactive properties

Curcumin is a natural dietary bioactive compound usually known as turmeric which is found in the rhizomes of the herb *Curcuma longa* belonging to the Zingiberaceae family (Altenburg et al., 2011; Kim et al., 2018). In the Indian subcontinent including Bangladesh, curcumin has been commonly used spice for ages, and it also often used in ayurvedic medicine and traditional Chinese medicine as a therapeutic agent (Boyanapalli and Kong, 2015). Curcumin is a yellow color phenolic compound which exhibits many medicinal properties and diversified biological activities including antioxidant, antimicrobial, anti-inflammatory, antiviral, anticancer and antifibrotic effects (García-Niño and Pedraza-Chaverrí, 2014) as shown in Fig. 1.9.

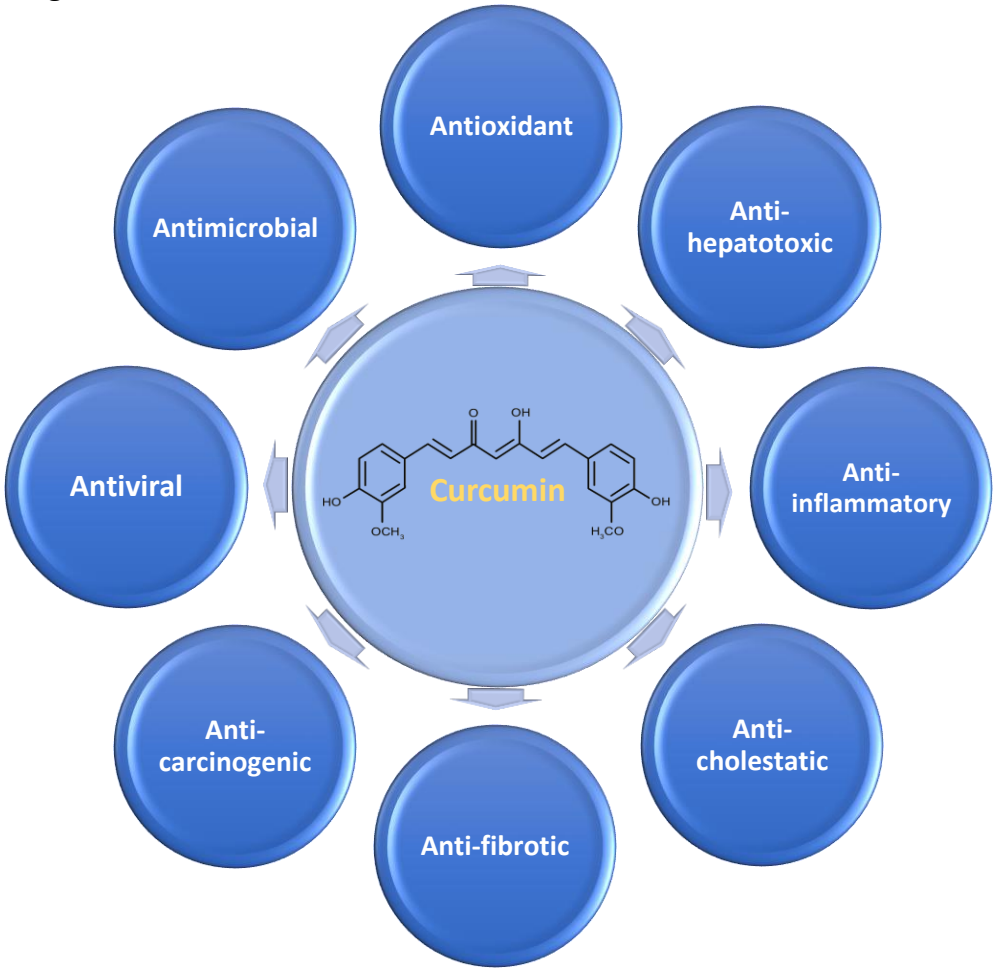

Fig. 1.9 Various biological activities of curcumin.

Day by day treatment and prevention of human diseases based on traditional medicine is becoming very popular globally due to inexpensive properties and lack of side effects. Recent findings support the effect of *Curcuma longa* and its main constituent's curcumin in a broad range of diseases cure via modulation of physiological and biochemical process. In addition, various studies based on animal model and clinical trials showed that curcumin does not cause any adverse complications on liver and kidney function and it is safe at high dose (Rahmani et al., 2018). Curcumin have also been approved by the US Food and Drug Administration (FDA) as "Generally Recognized As Safe" (GRAS) (Gupta et al., 2013).

Thus, curcumin is considered as a promising medicinal agent, because it regulates several key molecular signaling pathways that modulate survival, pathways governing anti-oxidative properties. It shows roles in the prevention of pathogenesis due its effective scavenger of ROS and reactive nitrogen species (Al-Jassabi et al., 2012). The effective anticancer property of curcumin is attributed to its antioxidant effect that control DNA damage and free radical-mediated lipid peroxidation (Shukla et al., 2003).

Results from numerous former study have already confirmed that curcumin has a therapeutic potential as antiviral (Kutluay et al., 2008), antioxidant (Al-Jassabi et al., 2012), anti-inflammatory (Bereswill et al., 2010), antimicrobial (De et al., 2009), antifibrotic (Pinlaor et al., 2010) and anticancer (Tomeh et al., 2019). Recently published several review reports demonstrated the various important role of curcumin in disease prevention and treatment as well as numerous beneficial health effects of dietary curcumin were also well-informed (Hewlings and Kalman, 2017; Rahmani et al., 2018).

1.11 Aims and objectives

Establishing a cost effective and safe treatment for arsenic detoxification method is a pressing issue for the people of Bangladesh. In this thesis, it was hypothesized that natural bioactive compounds D-pinitol and curcumin may greatly attenuate the toxicity of arsenic. Of course, various pharmacological activities of D-pinitol and curcumin have already been reported; however, much effort is still needed in order to further determine the other important beneficial effects of these natural dietary compounds. So far, there is no comprehensive investigation on the protective mechanism by which D-pinitol and curcumin can cause cytoprotection against arsenic toxicity. Thus, the main objective of this study is to explore the possible beneficial and ameliorative effects of D-pinitol and curcumin on arsenic induced toxicity in PC12 cells. To accomplish the objectives, the 1st experiment was performed to investigate the protective effects of D-pinitol on arsenic-induced toxicity in PC12 cells. Then, the 2nd experiment was done to check the protective effects of curcumin against arsenic-induced toxicity in PC12 cells. Further investigation of the combined effect of curcumin and D-pinitol on arsenic toxicity in PC12 cells also determined as 3rd experiments. Figure 1.10 exhibited the schematic diagram of research design of this study.

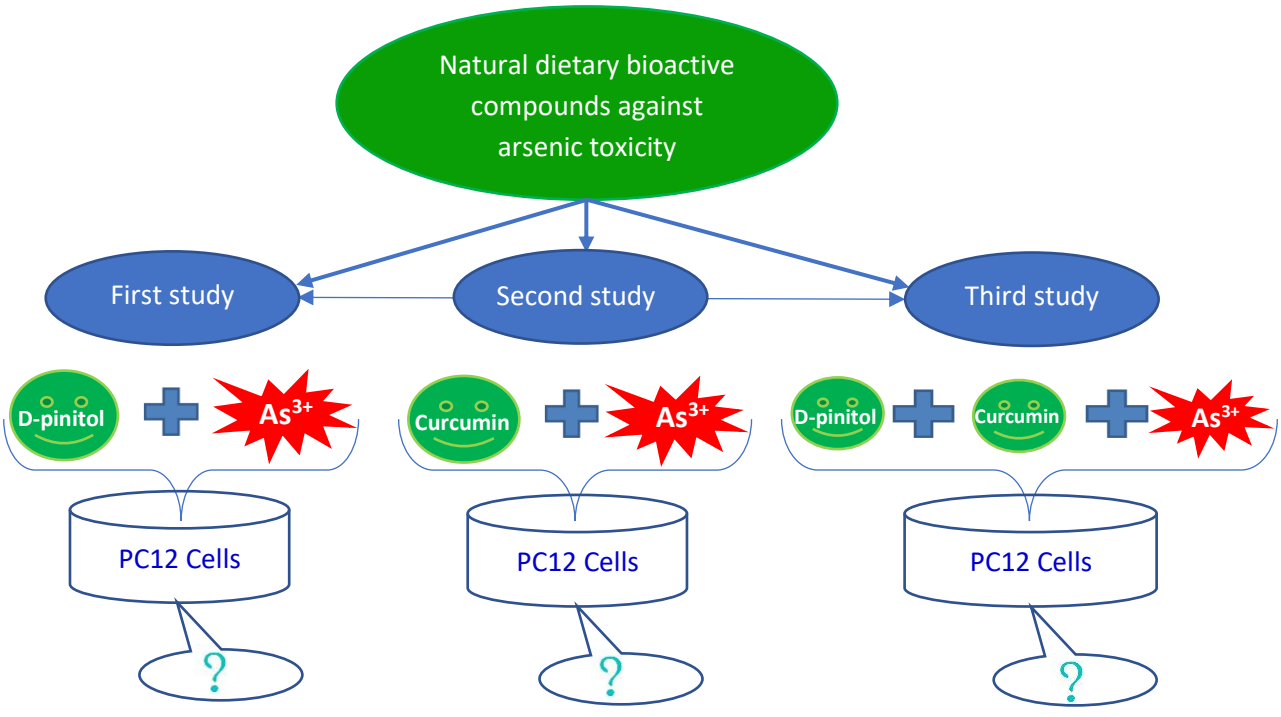

Fig. 1.10 Schematic diagram of research design.

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Chapter Two

Investigating the protective actions of D-pinitol against arsenic-induced toxicity in PC12 cells and the underlying mechanism

Abstract

Arsenic is an environmental toxicant responsible for many human diseases all over the world. On the other hand, D-pinitol is a naturally occurring bioactive dietary compound having antioxidant properties. Recently it has drawn a great attention due to its diverse biological activities and therapeutic potential against many human ailments. The purpose of this study is to elucidate the protective effects of D-pinitol on arsenic-induced toxicity and investigate its regulatory role in biomolecular mechanisms. Results showed that co-exposure of D-pinitol with arsenic increases cell viability and protect PC12 cells from arsenic-induced oxidative stress by increasing the level of glutathione (GSH) and glutathione reductase (GR). Subsequently, arsenic-induced DNA damage was also reduced by D-pinitol co-exposure with arsenic. Protein expression of western blot analysis showed that co-exposure of D-pinitol and arsenic significantly inhibited arsenic-induced autophagy which further suppressed apoptosis through up-regulation of survival factors; mTOR, p-mTOR, Akt, p-Akt, NF-kB, Nrf2, ERK1, GR, Bcl-x and down-regulation of death factors; p53, Bax, cytochrome c, LC3, although arsenic regulated those factors negatively. Since, no significant change occurred in the expression of the protein caspase 3 and caspase 9. Thus, the present study demonstrated a new route of caspase-independent apoptosis followed by autophagy. Whereas, D-pinitol reformed oxidative stress activating the antioxidant promoter protein Nrf2 and hinder excessive autophagy by promoting starvation autophagy and subsequently defend apoptosis. Finally, the results obtained in this study clearly indicated that D-pinitol protects PC12 cells from arsenic-induced oxidative stress and cytotoxicity.

2.1 Introduction

Arsenic is a well-known ubiquitous toxic and poisonous metalloid enriched in the environment from natural sources as well as anthropogenic sources (Abdul et al., 2015; Hughes et al., 2011; Jomova et al., 2011). Arsenic-enriched minerals, rocks, volcanoes and forest fires are the primary natural sources of arsenic in the environment whereas, numerous insecticides, herbicides, phosphate fertilizers, mining and smelting, coal combustion, industrial processes, timber preservatives are the anthropogenic sources (Bundschuh et al., 2011; Mondal et al., 2006; Singh et al., 2015). Arsenic exposure poses a major health problem and catastrophic toxic effects has already been reported in many parts of the world; in Argentina, Bangladesh, Cambodia, Chile, India, Myanmar, Nepal, Taiwan, Mongolia, Philippines, Vietnam, China, Afghanistan, Pakistan, Mexico, United States and so on. (BGS; DPHE, 2001; Brinkel et al., 2009; Chowdhury, 2004; Khan et al., 2003). Bangladesh has suffered the highest arsenic poisoning disaster in human history (Khan et al., 2003).

Arsenic exist in the environment mainly in inorganic and organic form where inorganic trivalent arsenic is considered as one of the main form to induce epidemiological toxicity by the production of reactive oxygen species (ROS) (Shi et al., 2004; Wang et al., 2001; Wu et al., 2019) and it also employs genotoxicity (Gentry et al., 2010; Rahman et al., 2018). Arsenic enters into the food chain mainly *via* arsenic contaminated water and finally enters in the human body through the ingestion of contaminated food and water (Jones, 2007; Rosen and Liu, 2009; Smedley and Kinniburgh, 2002). Inorganic arsenic exposure is linked to bladder, lung and skin cancer. It also caused many human diseases including arsenicosis, cardiovascular disease, pulmonary disorders, liver and kidney disorders, neurological diseases, diabetes mellitus and Alzheimer's disease (Naujokas et al., 2013; H.-J. Sun et al., 2014).

Although organic arsenic exposure is less toxic, some of organic arsenic like monomethylarsonate (MMA) and Dimethylearsinate (DMA) also has toxicity and health consequences. Chronic exposure to DMA enhanced tumor development in the kidney, liver and urinary bladder of F 344 rats (Yamamoto et al.,1995).

Previous researches have already identified existence of hydrogen peroxide and superoxide anion production upon arsenic exposure in various cell lines (Flora, 2011; Shi et al., 2004). Arsenic induces oxidative stress through ROS formation, and which is responsible for the inhibition of DNA repairing (Faita et al., 2013; Wu et al., 2019). Oxidative stress is considered as a universal mechanism causing cell death (Wąsik and Antkiewicz-Michaluk, 2017). Trivalent arsenic-induced oxidative stress hinders glutathione production, which is an important antioxidant that protects cells from oxidative damage (Jomova et al., 2011; Miller Jr et al., 2002). Arsenic induces cytotoxicity in various cells through numerous pathways mostly through the production of ROS depending on the degree of exposure. It also induces NADPH oxidase which leads excessive ROS generation. It is already recognized that ROS can causes cytotoxicity *via* mitogen activated protein kinases (MAPKs) pathway as well as tumor necrosis factor (TNF). These pathways play pivotal role to regulate various important cellular activities and functions including cell growth, propagation, differentiation, cell death (Rahman et al., 2018; Wang et al., 2015). In addition, arsenic also can create cytotoxicity by regulating the functions of tumor-suppressor protein 53 (p53) and nuclear factor-κB (NF-κB), which ultimately regulates the normal growth of cell, cell development, proliferation, cellular differentiation, apoptotic cell death and so on (Rahman et al., 2018; Wang et al., 2015).

However, natural compounds and their bioactive molecules have gained great interest in their potential health benefits mostly due to their strong antioxidant activities. Still now, balanced diet is the most cost-effective preventive approach against many diseases (Mehta et al., 2018; Pandey and Rizvi, 2009; Zhao, 2009).

D-pinitol is a naturally occurring bioactive compound that poses antioxidant properties by scavenging ROS, stimulate the GR production, boost of antioxidant defense (Moreira et al., 2018; Rengarajan et al., 2015). Recently some reports showed that satisfactory amount of D-pinitol has been found in Soybean, Pinaceae and Leguminosae plants (Anderson et al., 1952; Gao et al., 2015; Negishi et al., 2015; Poongothai and Sripathi, 2013). D-pinitol is also known as sugar alcohol or cycloalkane polyols and draw contemplation for several pharmaceutical properties (Negishi et al., 2015).

D-pinitol also has gained a great attention for its various important biological activities such as antioxidant, antidiabetic (Gao et al., 2015), antiviral, anti-inflammatory, antihyperlipidemic, larvicidal, cardioprotective, and anticancer (Rengarajan et al., 2013; Sethi et al., 2008; Singh et al., 2001; Sivakumar et al., 2010). Although the antioxidant and cancer preventive properties of D-pinitol has recently received a great interest, but mechanism behind D-pinitol exerted antioxidant potentiality is still insufficient. Thus, in the present study we hypothesized that, combined-treatment of D-pinitol with arsenic could show protective and critical regulatory actions against arsenic-induced cytotoxicity in PC12 cells through the regulation of cellular processes and biomolecular mechanisms. PC12 is a clonal cell line of rat pheochromocytoma has chosen in our study to use as a model cell line for toxicity assessment as it was used in several previous molecular biology and toxicity studies as a model cell line for toxicity assessment (Hossain et al., 2018; Magalingam et al., 2014; Rahman et al., 2018, 2017).

Thus, we aimed to clarify cytoprotective actions of D-pinitol against sodium arsenite-induced toxicity and the underlying mechanisms. To investigate the protective actions of D-pinitol against arsenic-induced toxicity in PC12 cells and the underlying molecular mechanisms, cell viabilities, DNA damage, lactate dehydrogenase (LDH) activity, intracellular of GSH levels, and expressions of autophagy and apoptosis related proteins using western blotting were measured.

2.2 Materials and Methods

2.2.1 Chemicals and antibodies

PC12 cells were bought from the American Type Culture Collection (USA and Canada). Cell culture medium; Dulbecco's modified Eagle's medium (DMEM), ethidium bromide, ribonuclease A (RNase), and peroxidase-conjugated avidin, $NaAsO_2$ (As^{3+}), and D-pinitol ($C_7H_{14}O_6$) were bought from Sigma (St. Louis, MO, USA). From HyClone (Rockville, MD, USA), Fetal bovine serum (FBS) was obtained. From Roche Diagnostics (Mannheim, Germany), Proteinase K were bought. From Amersham Pharmacia Biotech (Buckinghamshire, England), Biotinylated goat anti-mouse IgG whole antibody and ECL western blotting detection reagent were obtained. Polyclonal antibodies against βeta-actin (#4967s), Akt (#4691s), mTOR (#2972s), p-Akt (#4058s), p53 (#2524s), Caspase 9 (#9508s), LC3B (#83506s) were purchased from Cell signaling Technology. Bcl-x (#610211, BD Biosciences), ERK1 (#610030, BD Biosciences), Bax (B-9) (#sc7480, Santa Cruz Biotechnology), p-mTOR (#sc293133, Santa Cruz Biotechnology), NF-κB (#ab16502, abcam), Nrf2 (PM069, MBL), Glutathione reductase (#ab16801, abcam), Cytochrome c (#JA5204, Merk-Millipore), Caspase 3 (GTX110543, GeneTEX) were obtained. 0.4 % trypan blue solution was bought from Bio-Rad (Hercules, CA, USA). From Agilent Technologies (Waldbronn, Germany), The DNA 7500 assay kits were bought. Entire chemical used in experiments were of analytical reagent grade.

2.2.2 Cell culture and treatment

In a humidified incubator, PC12 cells were grown in DMEM medium with 10 % FBS supplementation at 37 °C with 5 % CO_2 in flasks of 25 cm^2. After 48 h preincubation, medium was substituted by serum comprising DMEM and at the same time cells were treated with or

without As^{3+} (5 µM) as ($NaAsO_2$) and different concentrations (0.5, 5, 50,100, 150, 250 and 500 µM) of D-pinitol ($C_7H_{14}O_6$) for 48 h treatment incubation. Finally, treatment concentration of As^{3+} was selected as 5 µM according to the estimation of Petrusevski et al. (2007) for low level arsenic exposure to human, whereas D-pinitol was used at 1, 5 and 50 µM for co-treatment with As^{3+}.

2.2.3 Cell viability

Cell viability of As^{3+} and D-pinitol treated PC12 cells was examined by trypan blue exclusion assay method formerly reported by Rahman et al. (2017). PC12 cells (1×10^6 cells per 25 cm^2 flask) were seeded and cultured to confluence for 48 h pre-incubation. Cells were incubated in serum- comprising DMEM with As^{3+} (5 µM) and D-pinitol (0.5, 1, 5, 50, 100, 150, 250 and 500 µM) separately for 48 h; moreover, cells were co-treated with As^{3+} 5 µM and D-pinitol (1, 5, and 50 µM). After 48 h treatment incubation, 0.25 % trypan blue solution was used to stain harvested PC12 cells. A Bio-Rad automated cell counter (Hercules, CA, USA) was used to count total cells and trypan blue-stained cells numbers and viability results expressed in percentage of total cell number. To assure biological reproducibility and statistical reliability, cell viability experiment was done five times.

2.2.4 Lactate dehydrogenase (LDH) activity assay and visualization of cell-membrane integrity

Lactate dehydrogenase (LDH) activity assay was performed in the treatment medium to assess cell-membrane integrity using a nonradioactive cytotoxicity assay kit (Promega, WI, USA) as formerly reported by Kihara et al. (2012) and Akter et al. (2018). PC12 cells (1×10^6 cells per 25 cm^2 flask) were maintained in the serum-comprising DMEM medium with/without As^{3+} (5 µM) or As^{3+} 5 µM + D-pinitol (1, 5 and 50 µM) for 48 h incubation. Cells were harvested after the 48 h incubation and 50 µL medium was collected into a 1.5 mL tube, then 50 µL tetrazolium

salts-comprising substrate mixture was added to the same tube and kept for 30 min at room temperature (25 °C). After that, 50 µL stop solution was added to stop the reaction. Finally, a DU-65 spectrophotometer (Beckman, CA, USA) was used at 490 nm absorbance to measure the quantities of formazan dye formed. Result of LDH activity assay was showed as LDH activity/1×10^6 cells. This experiment was performed in triplicate to confirm reproducibility.

2.2.5 Measurement of intracellular free-sulfhydryl (SH) levels

A well studied method described by Rahman et al. (2018) was followed to measure the intracellular free-SH levels of PC12 cells. Cells were exposed to As^{3+} (5 µM) or As^{3+} 5 µM + D-pinitol (1, 5 and 50 µM) for 48 h. After harvesting 1× phosphate-buffered saline (PBS) was used to wash the cells. By adding 150 µL of lysis buffer, cells were kept for incubation at 4 °C for 10 min. Two freeze-thaw sonication cycles were applied for rupturing the cell membranes, and 10 min long centrifugation of raptured solution was done at 1500 rpm. After centrifugation supernatant was collected, and protein assay dye reagent (Bio-Rad, Hercules, CA, USA) was used to determine the total protein contents spectrophotometrically in collected supernatant. An iMark™ immunoplate Reader (BioRad; Hercules, CA, USA) was used to measure intracellular free-SH levels in the cell lysate using 2.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm wavelength. A molecular coefficient factor of 13,600 per cell number (1×10^6) was used to calculate the free-SH concentration in PC12 cells. The experiment was conducted three times to assure reproducibility.

2.2.6 Genomic DNA isolation from PC12 cells

The genomic DNA of As^{3+} and D-pinitol treated PC12 cells were isolated using high pure PCR template preparation kit (Roche Diagnostics; Germany) followed by the protocol reported by Kawakami et al. (2008) and Hossain et al. (2018). The cells were washed with 1 × PBS after harvesting. For recovering genomic DNA, ethanol precipitation method was performed followed by RNAase incubation. The concentration of DNA was determined by adding 1 × Tris/Borate/EDTA (TBE) using a Personal Spectrum Monitor (Gene Quant Pro, GE, USA).

2.2.7 Electrophoresis of genomic DNA

Agarose gel electrophoresis was performed to examine DNA fragmentation and degradation of As^{3+} and D-pinitol treated PC12 cells. About 3-5 µg DNA including 2 µL loading dye was used for 1.5% agarose gel electrophoresis at 100 V for 30 min using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). In dark condition, electrophoresed gel was soaked for 15 min in ethidium bromide solution. After that, a ChemiDoc XRS (Bio-Rad, USA) was used for visualizing DNA through UV illumination and capturing images. Intact DNA density level was measured by using a software named Image J. Electrophoresis experiment was replicated more than three times for assuring reproducibility.

2.2.8 Western blot analysis

According to the manufacturer instructions, Cytosolic cytochrome c was analyzed using cytochrome c release apoptosis assay kit (Merk-Millipore, Germany). As^{3+} and D-pinitol treated PC12 cells were harvested after 48 h treatment and suspended in ice-cold 1 × PBS. Supernatants were removed followed by the centrifugation at 1500 rpm for 5 min, the cells were suspended again in 150 µL of cytosol extraction buffer mix. After keeping 10 min on ice,

the mixture with cells were sonicated two cycles for 30s using a Sonicator 250 (Branson). For removing unbroken cells, 10 min centrifugation at 3000 rpm of lysate cells was performed. Then a 1.5 mL tube was used to collect the supernatant and again centrifuged at 10,000 rpm for 30 min. Finally, collected supernatant as cytosolic fraction was used for cytochrome c analysis.

For β-actin, Akt, Bax, Bcl-x, p-Akt, mTOR, p-mTOR, ERK, NF-κB, Nrf2, Glutathione reductase (GR), Caspase 3, Caspase 9, LC3B and p53 protein analysis, lysis buffer (2 mM HEPES, 100 mM NaCl, 10 mM EGTA, 0.1 µM PMSF, 1 mM Na_3VO_4, 0.1 mM Na_2MgO_4, 5 mM 2- glycerophosphoric acid, 10 µM $MgCl_2$, 2 mM DTT, 50 µM NaF, and 1% triton X-100) was used for collecting fraction of protein. Cell lysate were sonicated two cycles and centrifuged at 1500 rpm for 10 min. Protein assay dye reagent (Bio-Rad, Hercules, CA, USA) was used to measure the concentration of protein spectrophotometrically. Thereafter, approximately same amount protein (20 µg) was applied and separated *via* 12.5-15 % polyacrylamide gel electrophoresis.

Then, a semidry blotting system type-AE6678 (ATTO, Tokyo, Japan) was used to transfer the electrophoresed proteins onto nitrocellulose membranes. After overnight incubation in 5 % skimmed milk at 4 °C, membranes were sequentially incubated along with desired primary antibodies as well as secondary antibodies for antibody reaction. Finally, expression of protein was photographed in the way of protein bands using an enhanced chemiluminescence imaging system (ChemiDoc XRS, Bio-Rad, USA). All experiments were replicated three times to confirm reproducibility.

2.2.9 Arsenic contents measurement in PC12 cells

Arsenic content in As^{3+} and D-pinitol treated PC12 cells was determined using an inductively coupled plasma mass spectrometry (ICP-MS) (ICPE-9000, Shimadzu Corporation, Kyoto, Japan) as formerly described by Watanabe et al. (2011). Acid digestion of PC12 cells were done with 1 M of nitric acid at 70 °C for 1 h. A membrane of 0.45 μm pore was used to filter the digested samples and diluted for measurement with deionized water. The experiment was performed three times for assuring reproducibility.

2.2.10 Statistical analysis

All the experiments were performed at least in triplicate. Significant difference was investigated using single-factor analysis of variance (ANOVA) followed by unpaired Student's t-test in MS excel 2016 program. P values ≦ 0.05 were considered to indicate statistically significant differences. All data are expressed as the mean ± standard error of mean (SEM).

2.3. Results

2.3.1 Cell Viability

To check the effects of D-pinitol on PC12 cells, cells were exposed to 0.5, 5, 50, 100, 150, 250 and 500 μM of D-pinitol for 48 h (Fig. 2.1A). The cell viability result showed no significant change. It was indicated that there is no toxic effect after treatment with 0.5, 5, 50, 100, 150, 250 and 500 μM of D-pinitol compared with the control group. In the same way, cell viability in the case of combined treatment (As^{3+} and D-pinitol) was shown in Fig. 2.1B. Cell viability of PC12 cells treated only with 5 μM of As^{3+} showed a significant reduction compared to the control group.

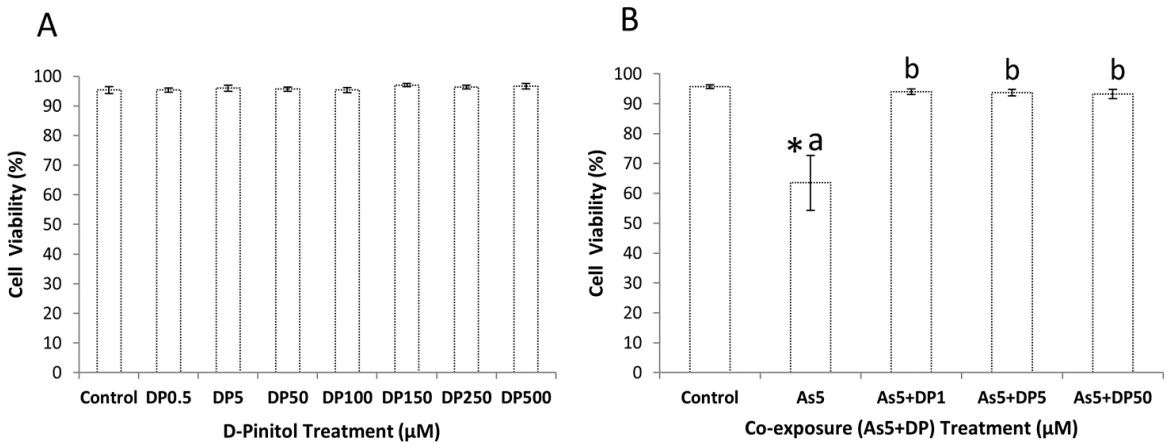

Fig. 2.1. Cell viability of PC12 cells after 48 h treatment using trypan blue exclusion method. **(A)** PC12 cells treated with different concentrations of D-pinitol. **(B)** PC12 cells treated with As^{3+} (5 µM) and co-treatment with As^{3+} (5 µM) and D-pinitol (1, 5, 50 µM). Each experiment was conducted 5 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=5), asterisk * indicates significance at $p < 0.05$ compared to the control group, and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and D-pinitol co-treatment groups.

However, co-exposure of D-pinitol (1, 5 and 50 µM) and As^{3+} (5 µM), for 48 h showed a significant increase of the cell viability when compared to the As^{3+}-treated group alone (Fig. 2.1B).

2.3.2 Analysis of cell-membrane integrity

Degree of cell membrane damage was measured *via* LDH activity assay. LDH leakage into the cell culture medium is an indication of cell-membrane disruption which further leads cells to death. PC12 cells were co-exposed with As^{3+} (5 µM) and D-pinitol (1, 5 and 50 µM) for 48 h. Result revealed that LDH activity was significantly increased in As^{3+}(5 µM)-treated cell culture medium compared to those in control group (Fig. 2.2).

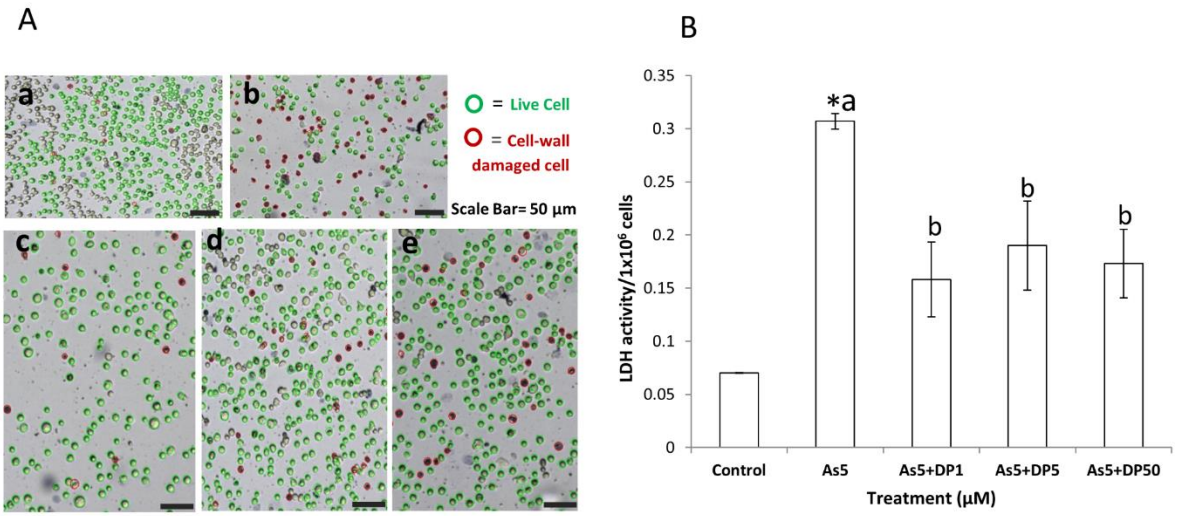

Fig. 2.2. Detection of cell membrane integrity of PC12 cells upon exposure to arsenic and D-pinitol. **(A)** Visualization of trypan blue staining after different treatments; a. control, b. As^{3+} (5 µM), c. As 5+DP 1, d. As 5+DP 5, e. As 5+DP 50. In the photograph, green and red colors mean intact and damage cell walls, respectively. **(B)** LDH leakage into the culture medium of PC12 cells after 48 h co-treatment of As^{3+} (5 µM) and D-pinitol (1, 5, 50 µM) measured by non-radiative cytotoxicity assay kit. Each experiment was conducted at least three times for reproducibility. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and D-pinitol co-treatment groups.

The combined exposure of As^{3+} (5 µM) and D-pinitol (0, 1, 5 and 50 µM) showed a significant reduction in LDH activity compared to only As^{3+}(5 µM)-treated culture medium. Results suggest that D-pinitol rescued cells from the toxic effects induced by As^{3+} (5 µM).

2.3.3 Effect of D-pinitol and arsenic on DNA

Effect of D-pinitol and arsenic on DNA of PC12 cells was investigated by using agarose gel electrophoresis after co-treatment of D-pinitol (1, 5, 50 µM) and As^{3+} (5 µM) for 48 h (Fig. 2.3A). The 1.5% agarose gel electrophoresis analysis showed a substantial reduction of the intact DNA in the group treated with As^{3+} (5 µM) when compared with no treatment group (control). But, the combined treatment of D-pinitol (1, 5, and 50 µM) with As^{3+} (5 µM) showed

a significant increase ($P < 0.05$) of intact DNA comparing to As^{3+} (5 μM) treatment group. From these results it was indicated that As^{3+} induces DNA injury or damage.

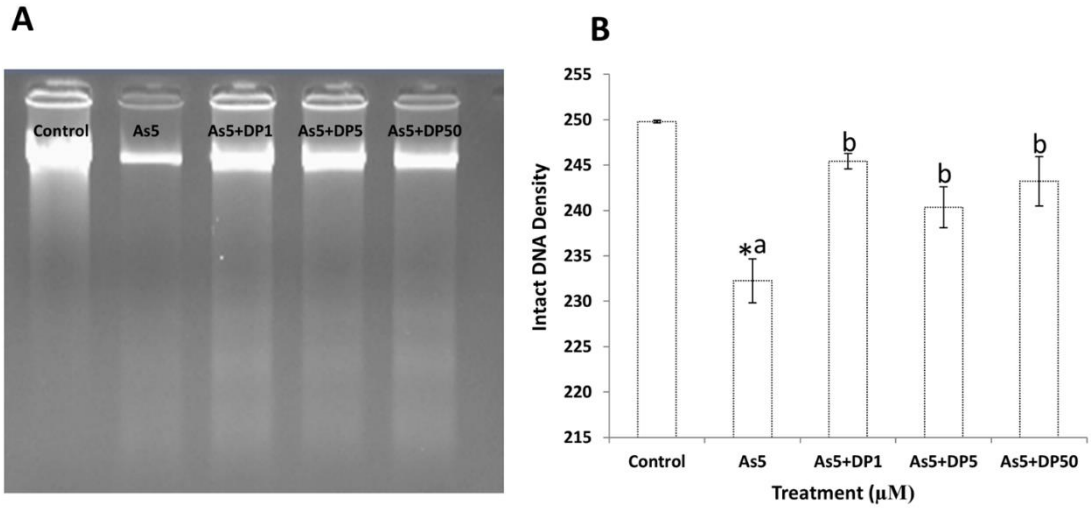

Fig. 2.3 Agarose gel electrophoresis of genomic DNA extracted from PC12 cells treated with and/without As^{3+} (5 μM) and/D-pinitol (1, 5, 50 μM) for 48 h. This experiment was done three times independently for reproducibility. **(A)** DNA electrophoresis after treated with As^{3+} (5 μM) and/D-pinitol (1, 5, 50 μM). **(B)** DNA band intensity for fragmentation detection. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and D-pinitol co-treatment groups.

The results of DNA degradation by As^{3+} was in good agreement with the results of cell viability (Fig. 2.1B) and LDH leakage (Fig. 2.2). However, D-pinitol significantly recovered the amounts of intact DNA upon co-exposure in PC12 cells (Fig. 2.3). These results recommend that D-pinitol repressed As^{3+}-induced DNA damage in PC12 cells.

2.3.4 Intracellular free-sulfhydryl (SH) levels and Glutathione Reductase (GR)

Intracellular GSH level in As^{3+} (5 μM)-treated PC12 cells as well as D-pinitol (1, 5, 50 μM) and As^{3+} (5 μM) combinedly treated cells was measured for 48 h (Fig. 4). GSH level in the cell treated with (5 μM) of As^{3+} alone showed a significant decrease ($P < 0.05$) compared to that in

the control group (Fig. 2.4A). However, simultaneous co-exposure of As^{3+} and D-pinitol showed a significant increase in GSH levels compared to the group treated with only As^{3+} (5 μM) (Fig. 2.4A).

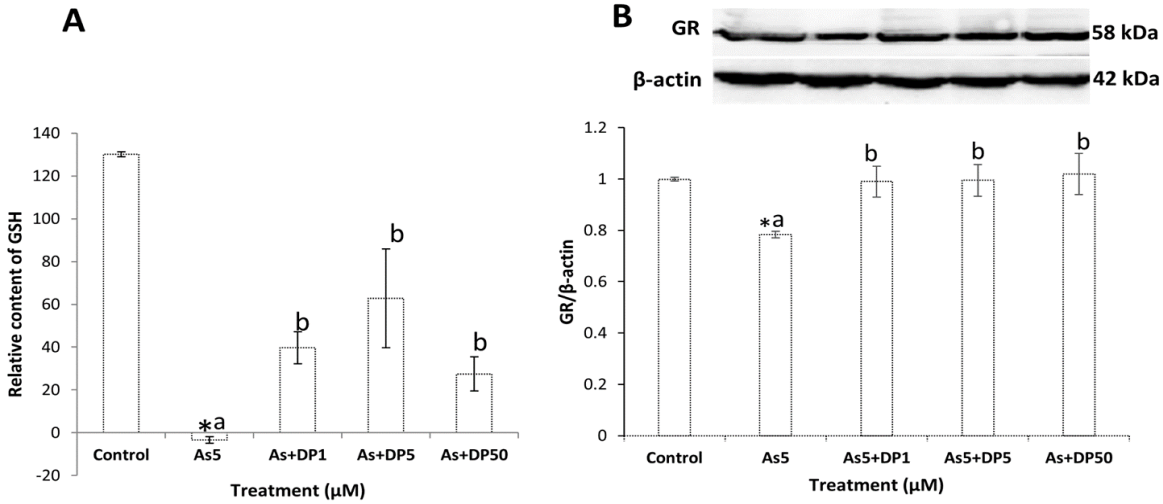

Fig. 2.4. Oxidative stress marker in PC12 cells after 48 h treatment with As^{3+} (5 μM) and different concentrations of D-pinitol (1, 5, 50 μM). **(A)** Intracellular glutathione (GSH) level in PC12 cells after exposure of As3+ (5 μM) and/D-pinitol (1, 5, 50 μM) *via* DTNB assay. **(B)** Expression of antioxidant enzyme Glutathione Reductase (GR) of PC12 cells after 48 h co-treatment of As^{3+} (5 μM) and D-pinitol (1, 5, 50 μM) through western blot analysis. Each experiment was conducted at least three times for reproducibility. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and D-pinitol co-treatment groups.

These results suggest that As^{3+} (5 μM)) induces oxidative stress in PC12 cells. On the other hand, D-pinitol in combined treatment with As^{3+} (5 μM) significantly decreased the oxidative stress caused by As^{3+}. In addition, glutathione reductase (GR) which catalyzes the reduction of glutathione disulfide (GSSG) to GSH, was also measured by western blotting analysis (Fig. 2.4B). When PC12 cells were treated with As^{3+} (5 μM) alone, it was found that GR level was significantly ($p < 0.05$) reduced compared to that in control group. Whereas combined treatment with D-pinitol (1, 5, 50 μM) and As^{3+} (5 μM) showed significant ($p < 0.05$) increased of GR level compared to that cells treated with only As^{3+} (5 μM) (Fig. 2.4B). There is an

excellent agreement among these GSH results and results revealed in Figs. 2.1 to 2.3 and suggested that D-pinitol alleviates the toxic effects of arsenic in PC12 cells.

2.3.5 Western blotting Analysis

As it was not known whether arsenic caused apoptosis from the result of Fig. 2.3, the expression of key proteins/factors related to autophagy and/or apoptosis induced by As^{3+} were analyzed *via* western blotting analysis.

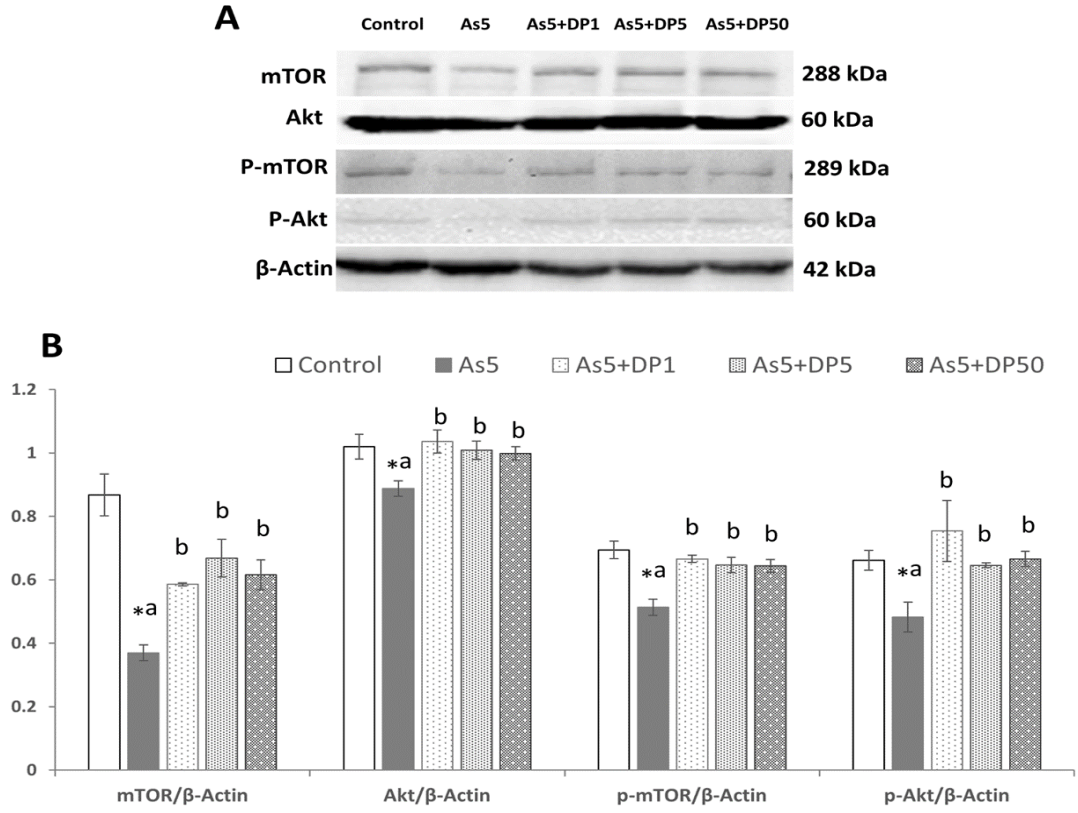

Fig. 2.5. Key proteins expression related to autophagy in PC12 cells after being exposed to As^{3+} (5 μM) and/D-pinitol (1, 5, 50 μM) for 48 h. **(A)** Western blot analysis for treatment with and/without As^{3+} (5 μM) and/D-pinitol (1, 5, 50 μM). This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. (B) Relative density of each protein band to β-Actin. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and D-pinitol co-treatment groups.

In other words, for understanding the molecular mechanisms associated with As^{3+}-induced cytotoxicity and the cytoprotective effect of D-pinitol in PC12 cells, protein expressions of mTOR, Akt, p-mTOR, p-Akt, NF-κB, ERK, Nrf2, p53, Bax, Bcl-x, cytochrome c, caspase 9, caspase 3 and LC3B were measured as shown in Figs. 2.5-2.8. A significant ($p < 0.05$) decreases of cell growth factor proteins, mTOR, p-mTOR, Akt and p-Akt (Fig. 2.5) and survival factors, NF-κB, Nrf2, ERK and Bcl-x (Figs. 2.6 and 2.7) were observed in As^{3+} (5 μM)-treated PC12 cells when compared with the control cells. Whereas, combined treatment of D-pinitol (1, 5 and 50 μM) and As^{3+} (5 μM) showed completely opposite scenario due to the protective/antioxidant effects of D-pinitol.

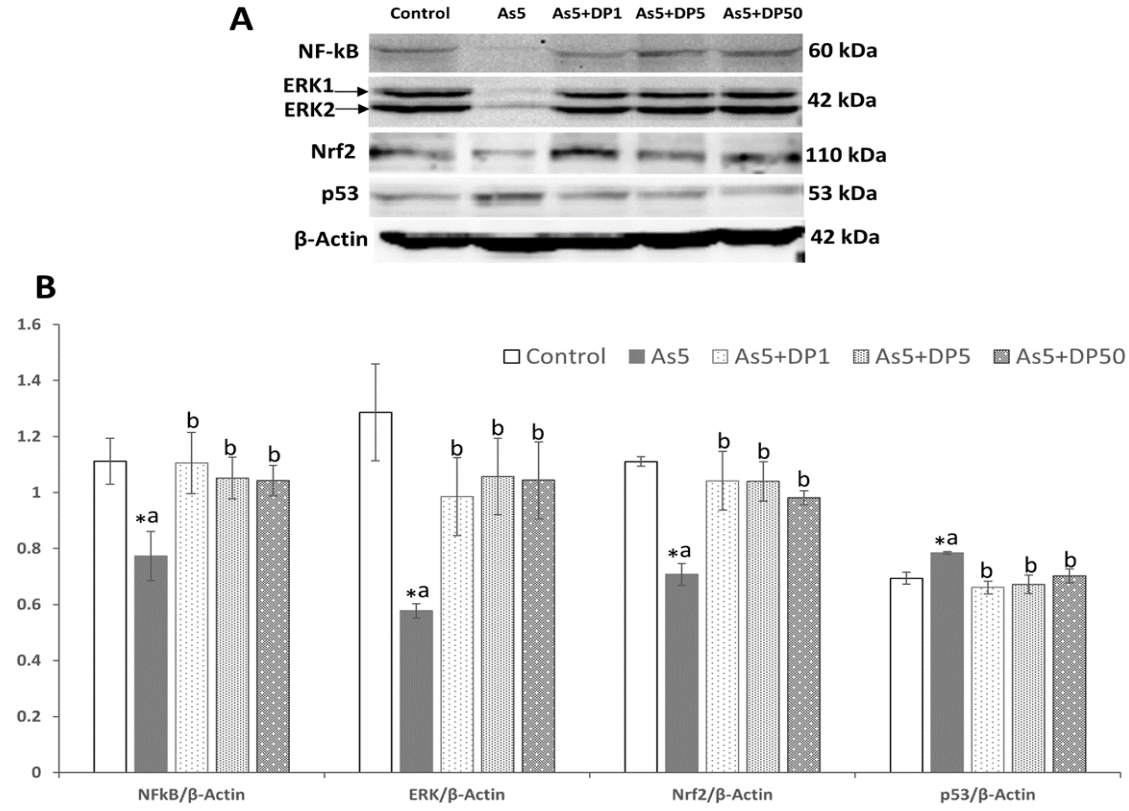

Fig. 2.6. Proteins expression in PC12 cells after being exposed to As^{3+} (5 μM) and/D-pinitol (1, 5, 50 μM) for 48 h. **(A)** Western blot analysis for treatment with and/without As^{3+} (5 μM) and/D-pinitol (1, 5, 50 μM). This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. **(B)** Relative density of each protein band to β-Actin. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and D-pinitol co-treatment groups.

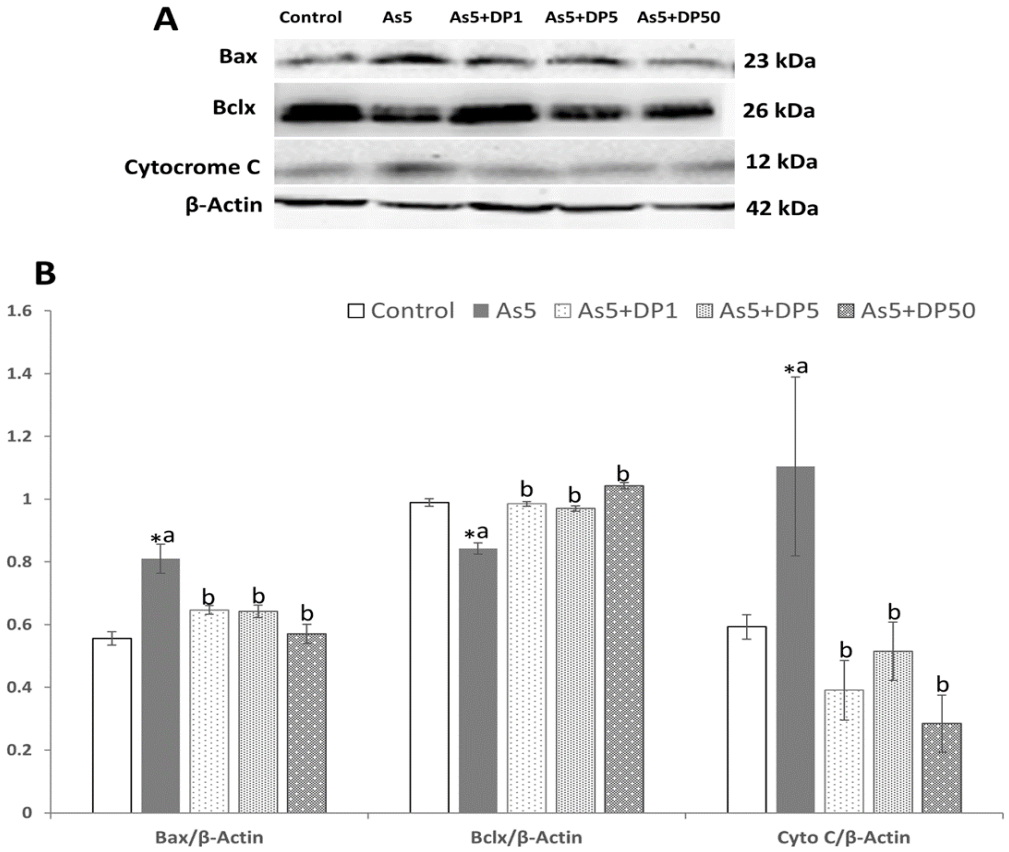

Fig. 2.7. Proteins expression in PC12 cells after being exposed to As^{3+} (5 μM) and/D-pinitol (1, 5, 50 μM) for 48 h. **(A)** Western blot analysis for treatment with and/without As^{3+} (5 μM) and/D-pinitol (1, 5, 50 μM). This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. **(B)** Relative density of each protein band to β-Actin. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and D-pinitol co-treatment groups.

In addition, there was a significant ($p < 0.05$) increase in the expressions of pro-apoptotic, Bax, cytochrome c, p53 (Figs. 2.6 and 2.7) and autophagic factor, LC3-II (Fig. 2.8) in only As^{3+} (5 μM)-treated PC12 cells when compared with control. The combined treatment of D-pinitol (1, 5 and 50 μM) with As^{3+} (5 μM) showed reverse effect due to the protective actions of D-pinitol.

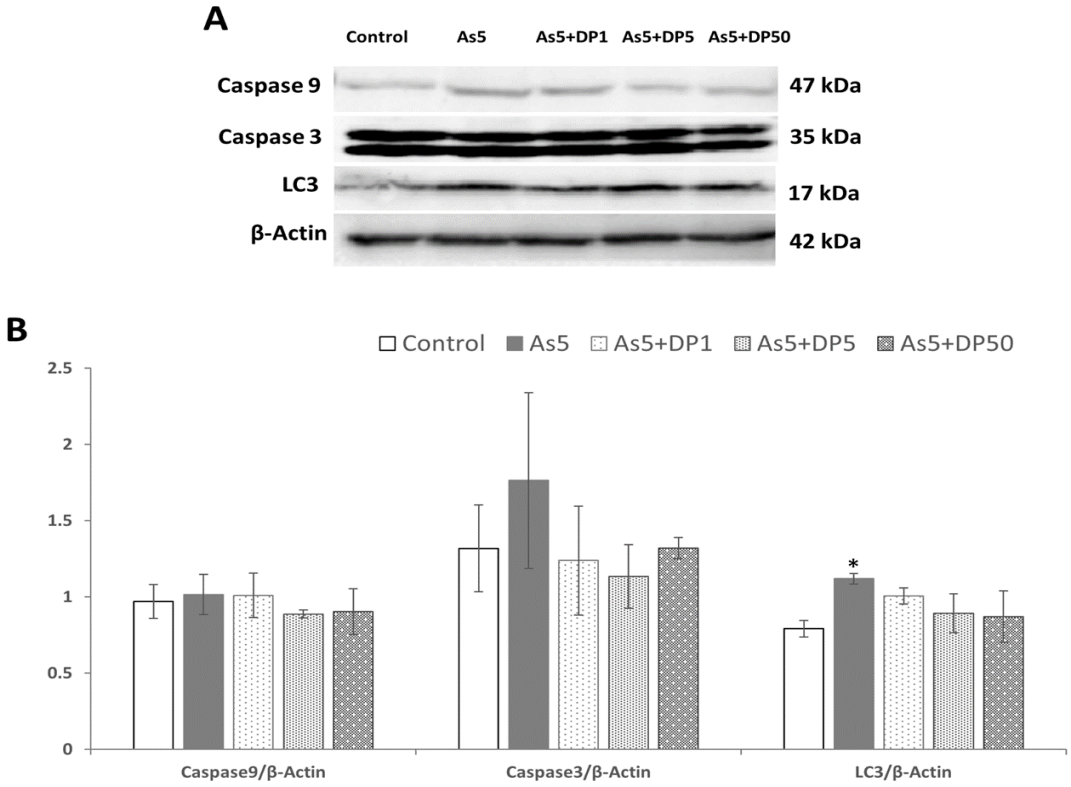

Fig. 2.8. Proteins expression in PC12 cells after being exposed to As^{3+} (5 µM) and/D-pinitol (1, 5, 50 µM) for 48 h. **(A)** Western blot analysis for treatment with and/without As^{3+} (5 µM) and/D-pinitol (1, 5, 50 µM). This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. **(B)** Relative density of each protein band to β-Actin. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group.

It was suggested that co-exposure of D-pinitol showed opposite effects with As^{3+} by regulating autophagic and apoptotic proteins. However, expressions of pro-apoptotic caspase 9 and caspase 3 were unchanged in all treatment groups (Fig. 2.8).

2.3.6 Effects of D-pinitol on arsenic uptake in PC12 cells

PC12 cells treated with As^{3+} (0, 5 µM) or As^{3+} (5 µM) + D-pinitol (1, 5 and 50 µM) for 48 h. Then, the cellular intake of arsenic was determined by ICP-MS and the results shown on Fig. 2.9.

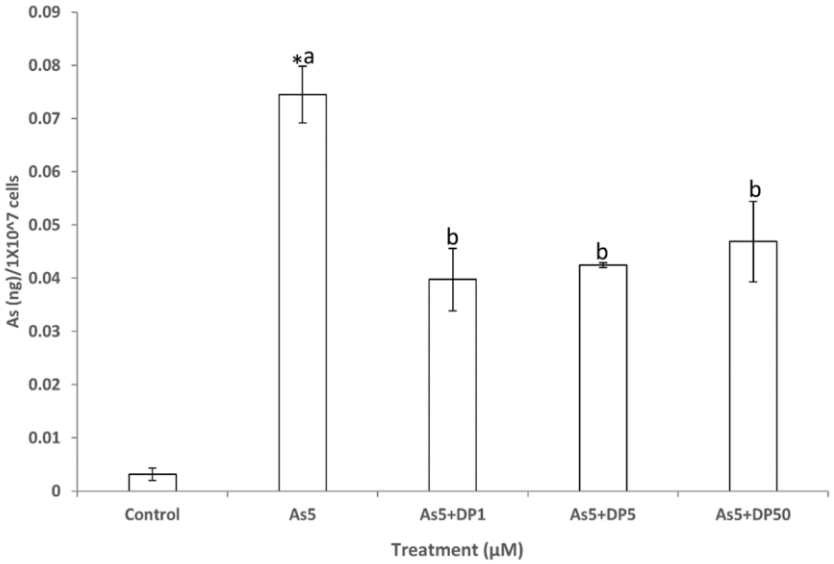

Fig. 2.9. Effects of D-pinitol on PC12 cell uptake of arsenic *via* ICP-MS analysis. PC12 cells exposed to As^{3+} (5 µM) and/D-pinitol (1, 5, 50 µM) for 48 h. This experiment was carried out three times for reproducibility. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < .05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and D-pinitol co-treatment groups.

Co-exposure of D-pinitol (1, 5, 50 µM) significantly repressed the As^{3+} uptake into the PC12 cells compared to As^{3+} treated group. These findings indicated that D-pinitol protected PC12 cells *via* inhibiting cellular arsenic uptake and antioxidant properties.

2.4 Discussion

In the present study, we showed that D-pinitol, a bioactive cyclitol present in soybean, pinaceae and leguminosae plants has protective effects against oxidative stress stimulated cellular damage induced by As^{3+} in PC12 cells. Sodium arsenite (As^{3+}, 5 µM) created autophagic situation and leads PC12 cells to death. With the treatment of As^{3+} (5 µM), cell viability decreased and LDH activity increased significantly in PC12 cells. However, co-exposure of D-pinitol (1, 5 and 50 µM) and As^{3+} (5 µM) interestingly improved cell viability and decrease the level of LDH leakage (Figs. 2.1B and 2.2). It was demonstrated that D-pinitol can reduce As^{3+}-

induced cell death in the PC12 cells. These results were predicted that D-pinitol improves the intracellular oxidation state caused by As^{3+}.

As shown in Fig. 2.4, it was proved a decrease level of GSH and GR with treatment of As^{3+} alone whereas those levels were significantly increased with the co-exposure of D-pinitol and As^{3+}. GSH is considered as one of the most important antioxidants due to its protecting capacity of cells and cellular components from reactive oxygen species (ROS). Excessive generation of ROS induces oxidative stress in cells, and GSH level decreases by the conversion of GSH to GSSG due to decrease of oxidative stress. GR is one of a chain of enzymes which plays an important enzymatic role in recycling GSSG back to GSH (Couto et al., 2016). In consequence, this study demonstrates antioxidant effect of D-pinitol against As^{3+}-induced oxidative stress.

I confirmed that As^{3+} stimulates the autophagic pathway and ultimately causes cell death. Autophagy is generally a biological process of starvation; however, autophagy could also indorse cell death due to the excessive consumption of critical cellular organelles/components (Dikic and Elazar, 2018; Mizushima, 2005). As^{3+} suppressed to activate the pro survival proteins mTOR and Akt (Fig. 2.5) and initiate a well-known intra-cellular starvation process-autophagy. It is believed that mTOR (mammalian target of rapamycin) is phosphorylated and thereby acts as a crucial regulator for regulation of nutrient sensing, cell metabolism and cell proliferation. TOR kinase (phosphorylated mTOR) acts in downstream of Akt kinase (phosphorylated Akt) and functions to inhibit autophagy under growth-endorsing conditions. The downregulation of phosphorylated mTOR and Akt by As^{3+} not only regulated the phosphorylation but also suppressed the expressions of mTOR and Akt themselves (Fig. 2.5). Autophagy consists of four conjugated stages; (i) initiation (ii) autophagosome formation (iii) maturation and finally (iv) lysosomal breakdown of cytoplasmic materials (Chun and Kim, 2018; Parzych and Klionsky, 2014). Hence, LC3II keeps pivotal role in autophagosome formation and subsequently formation of autolysosome (Mizushima et al., 2011). Thus, up

regulated expression of protein LC3II was predicted formation of autolysosome in PC12 cells with the treatment of As^{3+} (Fig. 2.8). Finally, it seems that formation of excessive autophagosome induced autophagic cell death.

It was well established that As^{3+} induced apoptotic cell death depending on mitochondrial intrinsic pathway where ROS plays a crucial role (Rahman et al., 2018). However, in this study, to overcome As^{3+}-induced oxidative stress intracellular starvation process or autophagy was started, subsequently mitochondrial intrinsic apoptotic pathway stimulated although finally caspases 3 and 9 were not changed (Figs. 2.7 and 2.8). As^{3+} exposure stimulate excessive ROS generation which plays a critical role to inhibit the activity of mTOR and Akt (Roy et al., 2014; Thangapandiyan et al., 2019). This inhibition triggers pro-apoptotic Bax upregulation and/or downregulation of antiapoptotic Bcl-2 and Bcl-x (Singh et al., 2012). Although it is still unclear whether this cell death mechanism is ultimately necrotic or apoptotic, one possibility is considered as caspase independent but Bax / Bcl-2 dependent partial apoptosis in PC 12 cells (Broker et al., 2005; Kim et al., 2014; Y. Sun et al., 2014).

Cell death process shown in this study further promoted through p53, NF-κB and cytochrome c release into the cytosol (Figs. 2.6 and 2.7). p53 is a tumor suppressor protein, promote apoptotic process through transcription-dependent and independent mechanisms (Fridman and Lowe, 2003; Tan et al., 2018) and can directly regulate activity of pro-apoptotic protein Bax, which further facilitate mitochondrial membrane permeabilization and apoptosis (Chipuk et al., 2004). NF-κB is an anti-apoptotic protein complex which poses controlling capability on DNA transcription, cytokine production, cell survival and has regulating role in the expression of several members of the Bcl-2 family (Wang et al., 1998). Thus, upregulated expression of pro-apoptotic proteins, p53 and Bax, cytochrome c release in cytosol, and down-regulated expression of Bcl-x, NF-κB, ERK1 anti-apoptotic proteins supported strongly that As^{3+} induced apoptotic cell death in PC12 cells.

Against such lethal effects of As^{3+}, D-pinitol exhibited a beneficial role to overcome stressed condition induced by As^{3+} in PC12 cells. Since, co-exposure of D-pinitol with As^{3+} showed an efficient reduction of As^{3+}-induced cell death through the downregulation of pro-apoptotic proteins, p53 and Bax, cytochrome c release into the cytosol, and upregulation of anti-apoptotic proteins, Bcl-x, NF-κB, and ERK1. Upregulation expression of Nrf2 with co-exposure of D-pinitol and As^{3+} might be a key factor of mechanism behind D-pinitol indicated antioxidant activities in PC12 cells. Nrf2 is an antioxidant promoter protein directly affects the ROS homeostasis by controlling the antioxidant defense systems *via* several mechanisms including induction of catabolism of superoxide and peroxides and regeneration of oxidized cofactors and proteins (Ma, 2013). Thus, in As^{3+}-induced stressed condition, D-pinitol was considered to promote the activity of Nrf2 to regulate homeostasis of ROS. Therefore, As^{3+}-induced oxidative stress reduced by the activation of Nrf2; results prohibition of excessive autophagy and cell death. Moreover, we also found that D-pinitol co-exposure with As^{3+} can significantly decrease the cellular uptake of arsenic in PC12 cells (Fig. 2.9). D-pinitol may be reducing cell death by hindering the cellular uptake of arsenic.

In summary, As^{3+}-induced autophagic cell death as well as partial apoptotic cell death was shown in PC12 cells. The mechanism of the cell death was considered to be the possibility of caspase independent but Bax / Bcl-x dependent apoptosis from the variation of factors of the endogenous apoptotic pathway (Fig. 2.10). As^{3+} accelerates the generation of intracellular ROS which further initiates autophagy and finally apoptosis through the activation of proapoptotic proteins. Whereas, D-pinitol protects PC12 cells from As^{3+}-induced toxicity by maintaining homeostasis of ROS where Nrf2 keeps pivotal role. D-pinitol significantly increased the levels of GSH and GR which improved ROS induced oxidative stress and inhibited excessive autophagy and subsequently apoptosis.

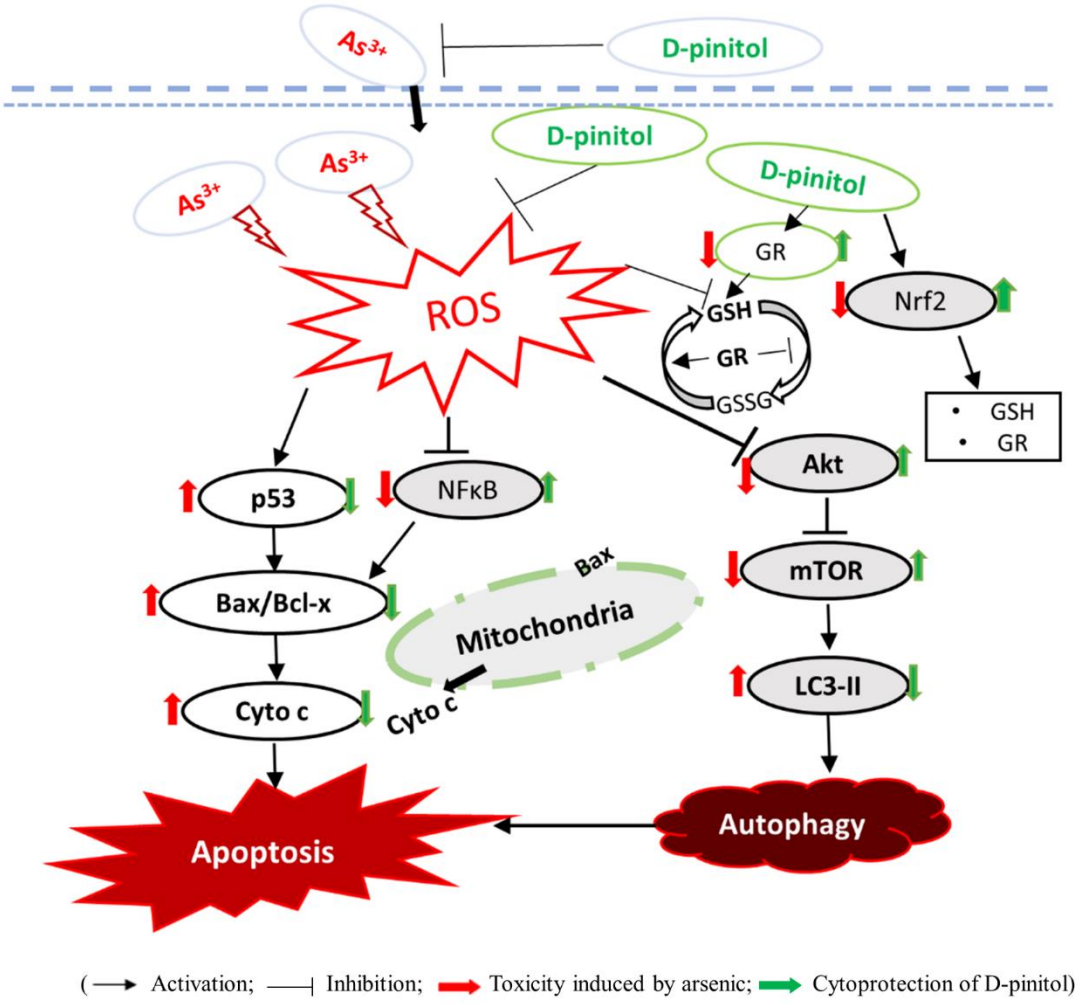

Fig. 2.10. Schematic diagram showing potential molecular mechanism/s of As^{3+}-induced toxicity and cyto-protection of D-pinitol in PC12 cells upon co-exposure.

This consideration was proved by the up-regulating expressions of proteins, mTOR, p-mTOR, Akt, p-Akt, Bcl-x, ERK1, NF-κB, Nrf2 and GR, down-regulating expressions of the proteins LC3, p53, Bax and cytochrome c with the co-exposure of D-pinitol and As^{3+} (Fig. 2.10).

2.5 Conclusion

Present study findings indicate that D-pinitol showed antioxidant properties and protected PC12 cells from As^{3+}-induced cytotoxicity by enhancing cell viability, repairing cell-membrane integrity, decreasing DNA damage, increasing GSH level, up-regulating the survival proteins mTOR, p-mTOR, Akt, p-Akt, Bcl-x, ERK1, NF-kB, Nrf2, GR and down-

regulating LC3, p53, Bax and cytochrome c release into cytosol. These results may assist in further research and understanding of the complex mechanisms of As^{3+}-induced toxicity and antioxidant actions of D-pinitol in organism.

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Chapter Three

Curcumin alleviates arsenic-induced toxicity in PC12 cells *via* modulating autophagy/apoptosis

Abstract

Arsenic is a recognized highly toxic environmental contaminant, responsible for numerous human diseases and affecting many millions of people in different parts of the world. Contrarily, curcumin is a natural dietary polyphenolic compound and the main active ingredient in turmeric. Recently it has drawn great attention due to its diverse biological activities, strong antioxidant properties and therapeutic potential against many human ailments. In this study, we aimed to explore the protective effects and the regulatory role of curcumin on arsenic-induced toxicity and gain insights into biomolecular mechanism/s. In this study PC12 cells, a well-known cell line for toxicity assessment was employed. Arsenic (10 µM) treatment in PC12 cells for 24 h induced cytotoxicity by decreasing cell viability and intracellular glutathione level and increasing lactate dehydrogenase activity and DNA fragmentation. In addition, arsenic caused apoptotic cell death in PC12 cells, which were confirmed from flow cytometry results. In addition, arsenic (10 µM) treatment significantly down-regulated the survival factors; mTOR, Akt, Nrf2, ERK1, Bcl-x, Xiap and up-regulated the death factors; ULK, LC3, p53, Bax, cytochrome c, caspase 9, cleaved caspase 3 and eventually caused autophagic and apoptotic cell death. However, curcumin (2.5 µM) pretreatment (1 h) with arsenic (10 µM) effectively saves PC12 cells against arsenic-induced cytotoxicity through increasing cell viability, intracellular GSH level and boosting the antioxidant defense system, and limiting the LDH activity and DNA damage. Furthermore, pretreatment of curcumin with arsenic expressively inhibited arsenic-induced toxicity and cell death by reversing the expressions of protein mTOR, Akt, Nrf2, ERK1, Bcl-x, Xiap, ULK, LC3, p53, Bax, cytochrome c, caspase 9 and cleaved caspase 3. Our findings indicated that curcumin showed antioxidant properties

through the Nrf2 antioxidant signaling pathway and inhibits arsenic-triggered toxicity in PC12 cells by regulating autophagy/apoptosis.

3.1 Introduction

Arsenic is a toxic heavy metal, chemically classified as a metalloid and unpleasantly familiar for its extreme toxicity. Arsenic toxicity is a worldwide public health concern and affects millions of people around the world (Ratnaike et al., 2003; Walvekar et al., 2007). As reported by the Agency for Toxic Substances and Disease Registry (ATSDR), arsenic is the most toxic substance and ranks the first position in "priority list of hazardous substances" due to its occurrence, frequency, toxicity, and potential for human exposure (Garbinski et al., 2019). By the report of the International Agency for Research on Cancer (IARC), arsenic is classified as Group 1 human carcinogen, and it can cause numerous types of cancer including skin, lung and bladder cancer. Besides carcinogenicity, arsenic also associated with cardiovascular diseases, neurological disorders, Alzheimer's disease, diabetes mellitus, liver disorders, chronic kidney disease and cerebrovascular diseases (Abernathy et al., 2003; Naujokas et al., 2013). Arsenic is an extremely hazardous contaminant ubiquitous in the environment and commonly presents in the earth's crust, seawater, groundwater as well as in the human body (Mandal and Suzuki, 2002). Since arsenic contamination may occur in the human food chain through both the natural biogeological processes and human activities (Abdul et al., 2015; Jomova et al., 2011), humans are regularly exposed to arsenic through food, drinking water, air, and soil (Ghosh and Sil 2015). Abdul et al (2015) reported also that arsenic-contaminated groundwater and chronic exposure of inorganic arsenic through drinking water pose a severe risk to human health.

In aqueous solution, the two most common forms of arsenic are pentavalent arsenate (As^{V}) and trivalent arsenite (As^{III}), where trivalent arsenic (arsenite) is much more toxic than pentavalent arsenic (arsenate) (Das and Chaudhuri, 2014). Furthermore, trivalent arsenic compounds are

highly lipid-soluble and more readily absorbed into cells than their pentavalent forms (Ellenhorn, 1997). Arsenic toxicity has been considered to be caused by generating excessive reactive oxygen species (ROS) in the cellular systems (Shi et al., 2004; Wu et al., 2019) and it is also responsible for DNA damage and mutation (Gentry et al., 2010; Rahaman et al., 2020). It meant that arsenic induces oxidative stress and cytotoxicity in different cell lines through numerous pathways mostly by producing ROS and triggering the oxidation of NADPH (Chou et al., 2004). Glutathione is an important antioxidant that maintains the antioxidant/prooxidant ratio and plays a vital role to protect cells from oxidative stress stimulated damage (Jomova et al., 2011; Miller Jr et al., 2002).

In recent years, numerous *in vivo* (Bhattacharya and Haldar, 2012; Firdaus et al., 2018; Singh et al., 2017) and *in vitro* (Perker et al., 2019; Wang et al., 2015; Zhang et al., 2017) studies reported arsenic cytotoxicity. Most of the studies showed that oxidative stress is one of the main causes of cell death (Wąsik and Antkiewicz-Michaluk, 2017).

Recently, several reports showed that natural bioactive compounds exhibit antioxidant properties and efficiently inhibit heavy metals induced toxicity by boosting up the antioxidant defense system (Badr et al., 2019; Banik et al., 2019; Cao et al., 2020; Donmez et al., 2019; Olaniyi et al., 2020), and considered as comparatively safe and cost-efficient preventive therapeutics against various human diseases and disorders (Mehta et al., 2018). Curcumin is a natural product commonly known as turmeric found in the rhizomes of the herb *Curcuma longa* belonging to the Zingiberaceae family (Altenburg et al., 2011; Kim et al., 2018). In the Indian subcontinent including Bangladesh, curcumin has been commonly used spice for ages and it also often used in ayurvedic medicine and traditional Chinese medicine as a therapeutic agent (Boyanapalli and Kong, 2015).

Curcumin is a yellow phenolic compound exhibits many medicinal properties and diversified biological activities including antioxidant (Al-Jassabi et al., 2012; Dairam et al., 2008),

antimicrobial (De et al., 2009), anti-inflammatory (Bereswill et al., 2010) antiviral (Kutluay et al., 2008), anticancer (Tomeh et al., 2019), antifibrotic (Pinlaor et al., 2010). As a therapeutic agent, curcumin has many beneficial effects against human diseases without any side effects (Joe et al., 2004; Mohajeri et al., 2017). The collective evidence from both *in vitro* (cellular systems) and *in vivo* (animal) studies have already affirmed the antioxidant properties and protective effects of curcumin against oxidative tissue damage by inhibiting the ROS generation (Al-Jassabi et al., 2012; Dairam et al., 2008; Mohajeri et al., 2017). But, the protective effects of curcumin against arsenic-induced toxicity in a model cell line with biomolecular mechanisms have not been fully investigated yet.

Thus, we hypothesized that curcumin might have protective actions on arsenic-induced toxicity in cultured PC12 cells. The current study has been designed to evaluate the protective effects of curcumin against arsenic-induced cytotoxicity. Then, several cytotoxicity assessment methods and experiments (e.g.: cell viability, LDH activity, DNA fragmentation, glutathione content, western blotting) were conducted upon arsenic exposure in PC12 cells with/without curcumin-pretreatment.

3.2 Materials and Methods

3.2.1 Chemicals

Curcumin ($C_{21}H_{20}O_6$) was obtained from Wako Pure Chemical Corporation (Japan). Sodium arsenite ($NaAsO_2$), Dulbecco's Modified Eagle's Medium (DMEM), ethidium bromide, ribonuclease A (RNase), and peroxidase-conjugated avidin were provided by Sigma (St. Louis, USA). Biosera (Kansas City, MO, USA) and Roche Diagnostics (Mannheim, Germany) provided Fetal bovine serum (FBS) and Proteinase K, respectively. Anti-mouse IgG and anti-rabbit IgG antibodies were purchased and ECL western blotting detection reagent were

obtained from Promega (Madison, USA) and Amersham Pharmacia Biotech (Buckinghamshire, England), respectively. Cell signaling Technology (Danvers, USA) provided antibodies were used against βeta-actin (#4967s), Akt (#4691s), mTOR (#2972s), p53 (#2524s), Bax (#2772s), caspase 9 (#9508s), ULK1 (#8054s), XIAP (#2042s). Bcl-x (#610211, BD Biosciences), Bcl-2 (#MAB8272, R&D Systems), ERK1 (#610030, BD Biosciences), LC3 (M152-3, MBL),Nrf2 (PM069, MBL), cytochrome c (#JA5204, Merk-Millipore), Active caspase 3 (#NB 100-56113SS, NOVUS Biologicals) were obtained. Trypan blue solution (0.4%) and the DNA 7500 assay kits were obtained from Bio-Rad (Hercules, USA) and Agilent Technologies (Waldbronn, Germany), respectively. All the other chemicals used in experiments were of analytical grade.

3.2.2 Cell culture and treatment

PC12 cells (ATCC, USA) were grown in DMEM with FBS (10%) supplementation at 37 °C in a humidified incubator with 5% CO_2 in 25 cm^2 flasks. After 48 h pre-incubation, the cell culture medium was changed to new 10% FBS medium and simultaneously cells were treated with or without 10 µM $NaAsO_2$ (As^{3+}) and different concentrations (1, 2.5, 5, 10, 25, 50 and 100 µM) of curcumin for 24 h incubation. The final selected concentrations for As^{3+} and curcumin were 10 µM and 2.5 µM, respectively. These concentrations were selected depending on their best combination results. Curcumin (2.5 µM) was used as pretreatment 1 h prior to As^{3+} treatment in PC12 cells for 24 h incubation. Freshly prepared sodium arsenite ($NaAsO_2$) and curcumin ($C_{21}H_{20}O_6$) solution were used in each cell experiment.

3.2.3 Cell viability analysis

The cytotoxic effects of As^{3+} and the cytoprotective effects of curcumin on PC12 cells was determined by performing the trypan blue exclusion assay. Cells were cultured in DMEM for

48-hour as pre-incubation. After that, cells were incubated with As^{3+} (0, 10 µM) and curcumin (0, 5, 10, 25, 50, and 100 µM) separately for 24-hour; likewise, cells were co-exposed with As^{3+} (0, 10) µM and nontoxic concentrations of curcumin (0, 1, 2.5, 5 and 10 µM). After 24-hour treatment incubation, cells were harvested and resuspended in the appropriate volume of phosphate buffered saline (PBS). Then, the aliquot of suspended cells was stained using 0.25% solution of trypan blue. A TC10^{TM} Automated cell counter (Bio-Rad, USA) was used to count trypan blue-stained and total cell numbers. Cell viability was expressed as a percentage of living cells against total cell number.

3.2.4 Lactate Dehydrogenase (LDH) assay

Oxidative stress-induced cytotoxicity and cell membrane damage in As^{3+} and curcumin-treated cells were determined by measuring LDH level released into the culture medium as described in our former study (Rahaman et al., 2020). In brief, PC12 cells were cultured in DMEM (10% FBS) with/without As^{3+} (10 µM), curcumin 2.5 µM and the combination of curcumin 2.5 µM and As^{3+} 10 µM for 24-hour incubation. After the incubation, the cell culture medium (50 µL) was collected and LDH activity was measured after adding substrate mixture (encompassing tetrazolium salts). After stopping the enzyme reaction, the absorbance was obtained using an iMark^{TM} microplate reader (BioRad, USA) at 490 nm absorbance. The LDH activity assay result was expressed as "LDH activity/1×10^6 cells".

3.2.5 Intracellular GSH levels measurement

The intracellular GSH levels in PC12 cells was measured by following a well-established protocol previously described by Kihara et al. (2012). PC12 cells were exposed to As^{3+} (0, 10 µM), curcumin 2.5 µM and the combination of curcumin 2.5 µM and As^{3+} 10 µM for 24-hour incubation. After harvesting, cells were washed with PBS, 150 µL of lysis buffer was added

and cells were kept at 4 °C temperature for 10 min. After getting cytosol fraction, protein and free SH concentration in the fraction were determined by using protein assay dye reagent (Bio-Rad, USA) and 2.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), respectively according to the method reported by Kihara et al. (2012).

3.2.6 Agarose gel electrophoresis of genomic DNA

PC12 cells were cultured in DMEM with/without 10 µM of As^{3+} and/or 2.5 µM of curcumin for 24 h. After treatment incubation, cells were harvested and washed with PBS. The genomic DNA extraction in PC12 cells was performed using a high pure PCR template preparation kit (Roche Diagnostics; Germany) following the manufacturer's protocol as formerly explained by Kawakami et al. (2008). For electrophoresis, obtained DNA (3-5 µg) mixed with 2 µL of loading dye was subjected on 1.5% agarose gel. Subsequently, a submarine-type electrophoresis system (Atto, Tokyo, Japan) was used to perform the electrophoresis at 100 V for 30 min. After the electrophoresis, the gel was kept for 15 min in ethidium bromide solution in the dark condition. Then, the image of agarose gel was taken by ChemiDoc XRS (Bio-Rad, USA) under UV illumination. DNA fragmentation was measured by using Image J software.

3.2.7 Flow cytometry analysis

The flow cytometry experiment was performed to analyze the cell death process using Annexin V-fluorescein isothiocyanate (V-FITC) apoptosis detection kit (BioVision, USA) according to the protocol provided by the manufacturer. Briefly, PC12 cells treated with curcumin and/or As^{3+} were harvested for 24 h after incubation and washed with 1×PBS. After washing, the cells were suspended in 500 µL of 1× binding buffer, and both 5 µL of Annexin V-FITC and propidium iodide (PI) were added to the cells. Then, the resulting solution was restrained for 5

min in a dark condition. Finally, the PC12 cell samples were analyzed with a flow cytometer (BD FACSVerse^{TM}, BD Biosciences).

3.2.8 Western blotting

Protein extraction from the treated cells and electrophoresis was carried out following the same protocol described in our recent study (Rahaman, et al., 2020). Protein expressions were detected by western blotting methods. In brief, after the electrophoresis, proteins were relocated to a nitrocellulose membrane. Then, the membrane was blocked by 5% blocking buffer (skimmed milk) for overnight. After blocking, the membranes were washed for three times with a washing buffer containing 0.15% tween and incubated overnight with the desired primary antibody at 4 °C. After 3 times washing with 0.15% tween-containing buffer, each membrane was incubated with secondary antibodies for 1 h at 37 °C. Then, again each membrane washed for 5 times, and the protein bands were detected and analyzed by using a ChemiDoc XRS (BioRad, USA).

3.2.9 Statistical analysis

All the experiments were performed more than three times. All the data are exhibited as the mean ± standard error of the mean (SEM). The statistically significant difference between groups was analyzed using a single-factor analysis of variance (ANOVA) followed by two-sided Student's t-test in the Microsoft excel 2019 program. P values $\leqq 0.05$ were considered as statistically significant.

3.3 Results

3.3.1 Effects of curcumin and arsenic on the viability of PC12 cells

To evaluate the effects of curcumin on the cytotoxicity of PC12 cells, cells were treated with 0, 5, 10, 25, 50 and 100 μM of curcumin for 24 h. As displayed in Fig. 3.1A, curcumin treatment did not show cytotoxicity in PC12 cells up to 25 μM of curcumin; however, cell viability in the cells exposed to 50 and 100 μM of curcumin was significantly decreased compared as that in PC12 cells without any treatment. For combination treatment, in this study, we employed 2.5 μM of curcumin because the concentration was not toxic. Cell viability of combination treatment of As^{3+} (10 μM) and curcumin (1, 2.5, 5 and 10 μM) was shown in Fig. 3.1B.

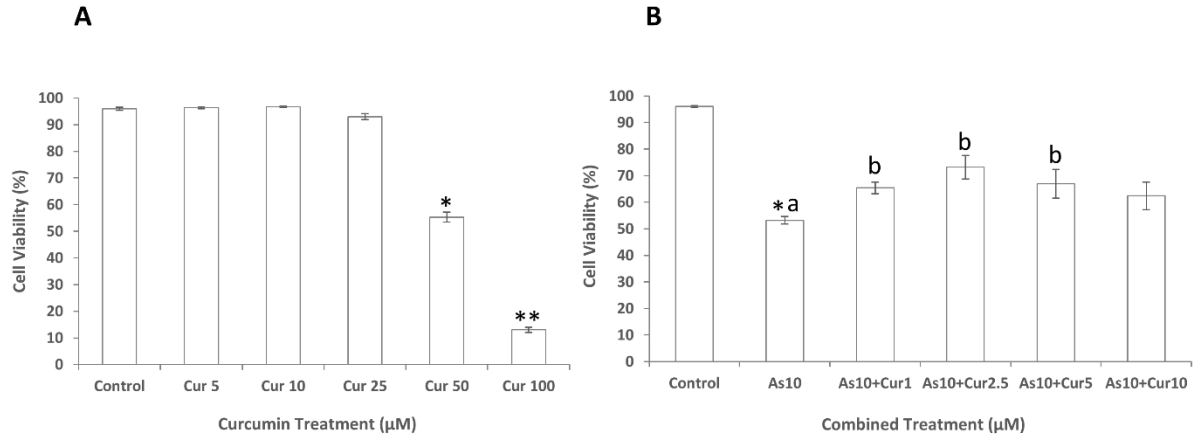

Fig. 3.1 Cell viability of PC12 cells after 24 h treatment using the trypan blue exclusion method. **(A)** PC12 cells treated with different concentrations of Curcumin. **(B)** Cell viability after As^{3+} (10 μM) exposure, with/without Curcumin (2.5 μM) treatment. Each experiment was conducted 6 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=6), the asterisk * indicates significance at $p < 0.05$ compared to the control group, and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

The cell viability results revealed that the viability of cells exposed to As^{3+} (10 μM) was significantly ($p < 0.05$) reduced compared to the control group. However, curcumin (1, 2.5 and 5 μM) and As^{3+} (10 μM) showed a significant ($p < 0.05$) improvement in cell viability compared to the As^{3+}-treated group alone for 24 h after incubation (Fig. 3.1B).

These results suggest that curcumin (1, 2.5 and 5 μM) exert cytoprotective effect against As^{3+} (10 μM)-induced toxicity in PC12 cells. In further experiments, curcumin 2.5 μM concentration has been used based on the lowest concentration of curcumin to provide the highest protection against As^{3+} (10 μM)-induced toxicity.

3.3.2 Curcumin protected membrane integrity against Arsenic in PC12 cells

LDH activity and trypan blue staining assay were performed to observe the cell membrane damage and analyze the cytotoxicity. Trypan blue stained PC12 cells were shown in Fig. 3.2A, where green and red colors indicate live cells and cell membrane damaged death cells, respectively. Curcumin (2.5 μM)-treated cells group did not show any noticeable change compared to the control group cells. Red-colored death cells were increased significantly ($p < 0.05$) only in the As^{3+} (10 μM)-treated cells group compared to the control group.

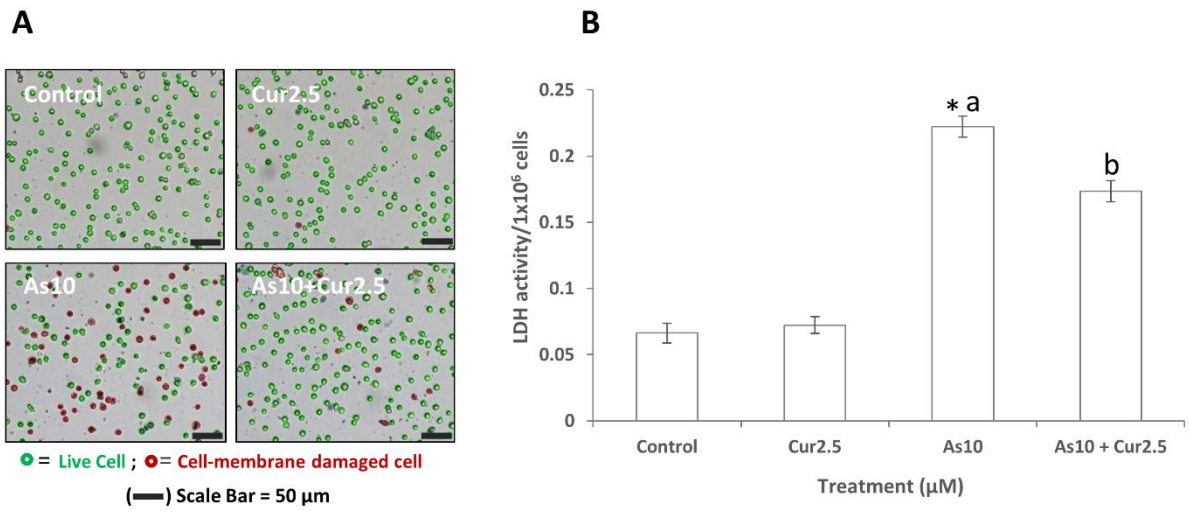

Fig. 3.2 (A) Detection of cell membrane integrity of PC12 cells upon exposure to As^{3+} (10 μM) and with/without Curcumin (2.5 μM). **(B)** LDH leakage into the culture medium of PC12 cells after 24 h combined-treatment of As^{3+} (10 μM) and curcumin (2.5 μM) measured by non-radiative cytotoxicity assay kit. Each experiment was conducted 6 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=6), the asterisk * indicates significance at $p < 0.05$ compared to the control group, and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

Cell membrane-damaged (red color) cells were reduced significantly when cells were co-treated with curcumin (2.5 μM) and As^{3+} (10 μM), compared to the cells group those were treated only with As^{3+} (10 μM) (Fig. 3.2A).

LDH is an important cytosolic enzyme and its leakage into the cell culture medium due to the cell membrane breakdown is an indication of toxicant-induced cell death. Leakage of LDH activity from PC12 cells co-exposed to As^{3+} (10 μM) and/or curcumin (2.5 μM) for 24 h was measured in their mediums. Curcumin (2.5 μM) treatment for 24 h showed no significant change in LDH activity compared to the control group (Fig. 3.2B). These results also revealed that LDH leakage was significantly ($p< 0.05$) increased in the medium for As^{3+}(10 μM)-treated cell culture compared to that for the control group (Fig. 3.2B). The combined exposure of As^{3+} (10 μM) and curcumin (2.5 μM) showed a significant ($p< 0.05$) reduction in leakage of LDH compared to exposure of only As^{3+}(10 μM) (Fig. 3.2B). These observations showed the same tendency with the cell viability results and suggest that curcumin rescued PC12 cells from As^{3+} (10 μM)-induced cytotoxicity.

3.3.3 Curcumin alleviated arsenic-induced DNA damage

The regulatory effects of curcumin on As^{3+}-induced cytotoxic effects guided us to investigate whether curcumin could alleviate DNA damage caused by As^{3+} exposure in PC12 cells. The effect of curcumin and As^{3+} on DNA injury in PC12 cells was inspected by performing agarose gel electrophoresis after the treatment of As^{3+} (10 μM) with/without curcumin (2.5 μM) for 24 h. As shown in Figs. 3.3A and B, DNA fragmentation was increased significantly in As^{3+} (10 μM)-treated cells group compared to the control group. Curcumin (2.5 μM) treatment had no damaging effects on DNA fragmentation compared to the control group (Figs. 3.3A and B).

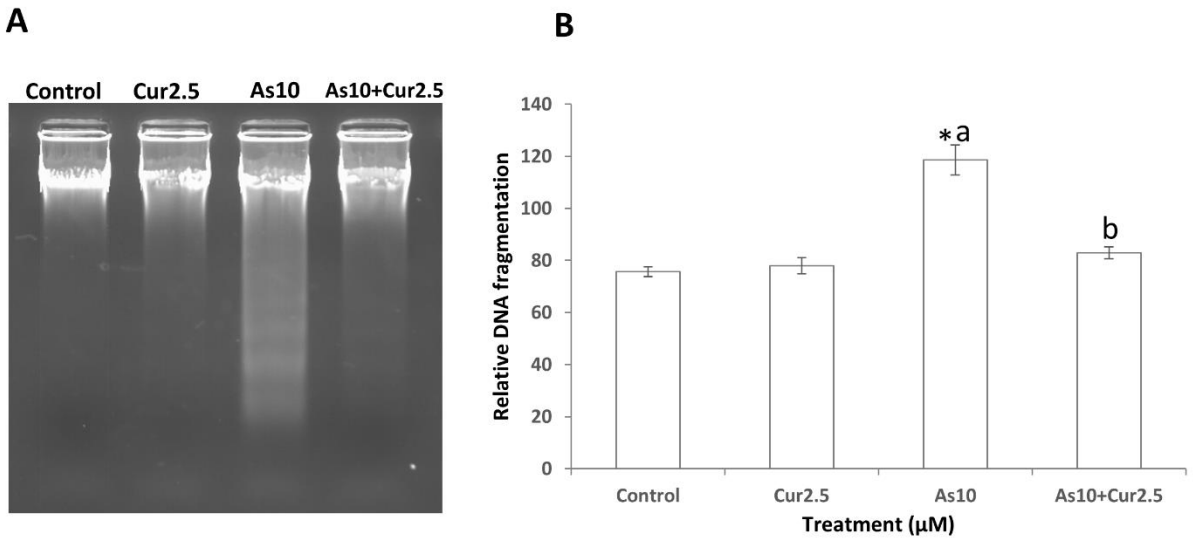

Fig. 3.3 Agarose gel electrophoresis of genomic DNA extracted from PC12 cells treated with As^{3+} (10 μM) and/or Curcumin (2.5 μM) for 24 h. This experiment was done three times independently for reproducibility. **(A)** DNA electrophoresis after treated with As^{3+} (10 μM) and/or Curcumin (2.5 μM). **(B)** DNA band intensity for fragmentation detection. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

However, the combined treatment of As^{3+} (10 μM) and curcumin (2.5 μM) showed a significant ($p < 0.05$) reduction in DNA fragmentation comparing to only As^{3+} (10 μM) treatment group (Figs. 3.3A and B). These results indicated that As^{3+} induces DNA damage and curcumin effectively blocked the DNA damage induced by As^{3+}. These results showed a similar tendency with our obtained previous cell viability results (Fig. 3.1B) as well as LDH activity results (Fig. 3.2B) and suggest that curcumin effectively alleviated DNA damage caused by As^{3+} in PC12 cells (Figs. 3.3A and B).

3.3.4 Effects of curcumin and arsenic on Intracellular free-sulfhydryl (SH) levels

GSH is an important non-enzymatic antioxidant substance that protects cells from oxidative damage. As shown in Fig. 3.4, GSH contents were significantly ($P < 0.05$) reduced in the cells exposed to only As^{3+} (10 μM) compared to the control group cells. GSH level in cells exposed

to only curcumin (2.5 μM) showed no significant difference from that in control cells. However, the GSH content in the cells exposed to combined treatment of As^{3+} and curcumin showed a significant ($P < 0.05$) improvement compared with that in the cells exposed to only As^{3+} (10 μM) (Fig. 3.4).

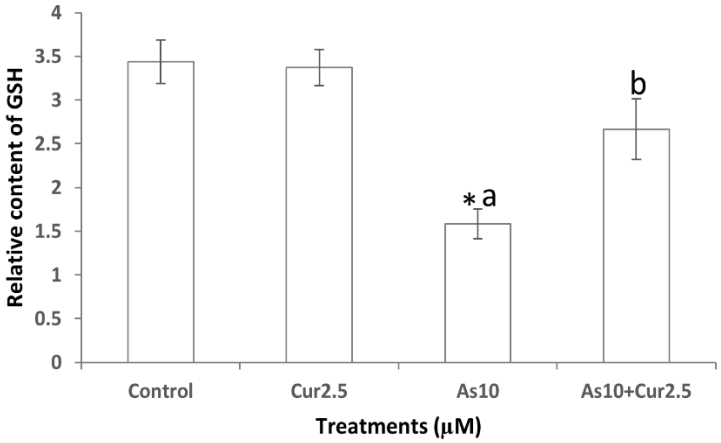

Fig. 3.4 Intracellular glutathione (GSH) level in PC12 cells after exposure of As^{3+} (10 μM) and/or Curcumin (2.5 μM) *via* DTNB assay. Each experiment was conducted at least three times for reproducibility. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

These results indicated that As^{3+} (10 μM) treatment in PC12 cells induces oxidative stress and breaks the cellular antioxidant defense system. On the other hand, treatment of curcumin (2.5 μM) combined with As^{3+} (10 μM) noticeably decreased the As^{3+}(10 μM)-triggered oxidative stress (Fig. 3.4). From the results, it was postulated that curcumin has antioxidant potentials and boosts the antioxidant defense system.

3.3.5 Curcumin inhibits apoptosis rate induced by arsenic

The flow cytometry assay using Annexin V-FITC/PI staining was performed to determine the apoptosis rate of PC12 cells exposed to As^{3+}(10 μM) and/or curcumin (2.5 μM) for 24 h.

As shown in Fig. 3.5, the apoptosis rates of the control group and the curcumin-treated group were 5.97 ± 1.2 % and 6.15 ± 1.99 %, respectively.

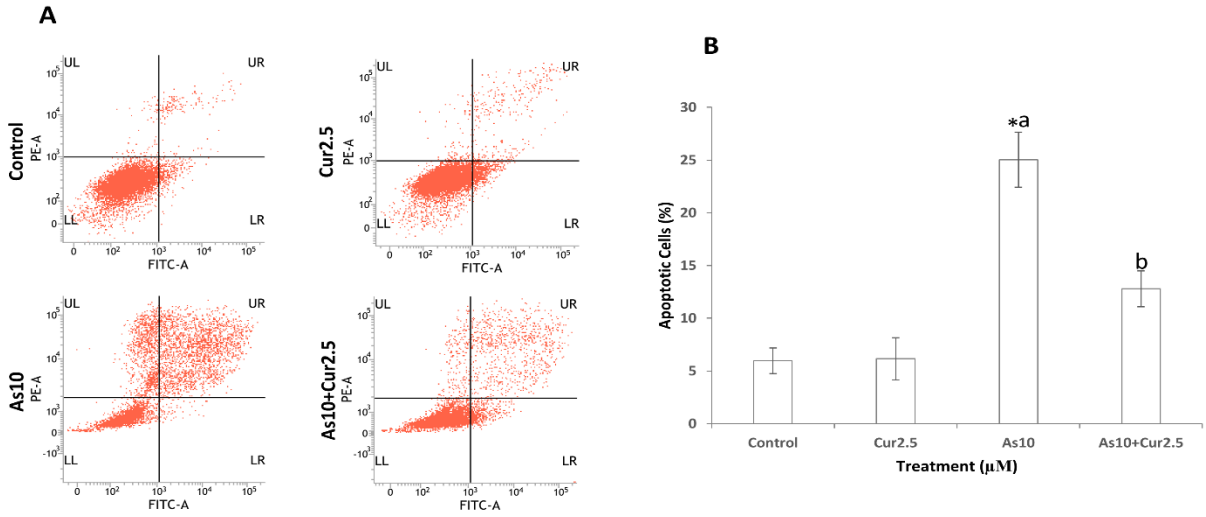

Fig. 3.5 The rate of apoptosis analyzed by flow cytometry (Annexin V-FITC/PI) of PC12 cells upon exposure to As^{3+} (10 µM) and with/without Curcumin (2.5 µM) for 24 h. (**A**) A representative Annexin V-FITC/PI strained flow cytometry result. (**B**) The rate of apoptosis of PC12 cells. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and a, b denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

The apoptosis rate of the cells exposed to only As^{3+}(10 µM) significantly arose to 25.04 ± 2.61 %, where that of cells co-exposed to curcumin (2.5 µM) and As^{3+}(10 µM) reduced significantly to 12.79 ± 1.71 % (Fig. 3.5). These results suggested that curcumin effectively protected PC12 cells against As^{3+}-induced apoptosis.

3.3.6 Effects of curcumin and arsenic on pro-survival protein expressions

To clarify the molecular mechanism/s involved in the cytoprotection of curcumin against As^{3+}(10 µM)-induced toxicity, the western blotting technique was employed. Some crucial proteins for cell growth and survival in PC12 cells were analyzed for 24 h after exposure to As^{3+}(10 µM) and/or curcumin (2.5 µM). The key regulator protein for cell, metabolism,

proliferation, and survival mammalian target of rapamycin (mTOR) was firstly checked as shown in Fig. 3.6. In As^{3+}(10 μM)-treated cells a significant ($P < 0.05$) reduction of mTOR was observed comparing in the control cells. Though mTOR content in the curcumin (2.5 μM)-treated cells showed no noticeable difference compared to that in the control group, mTOR content recovered successfully in the combined (curcumin+As^{3+}) treatment cells group (Fig. 3.6).

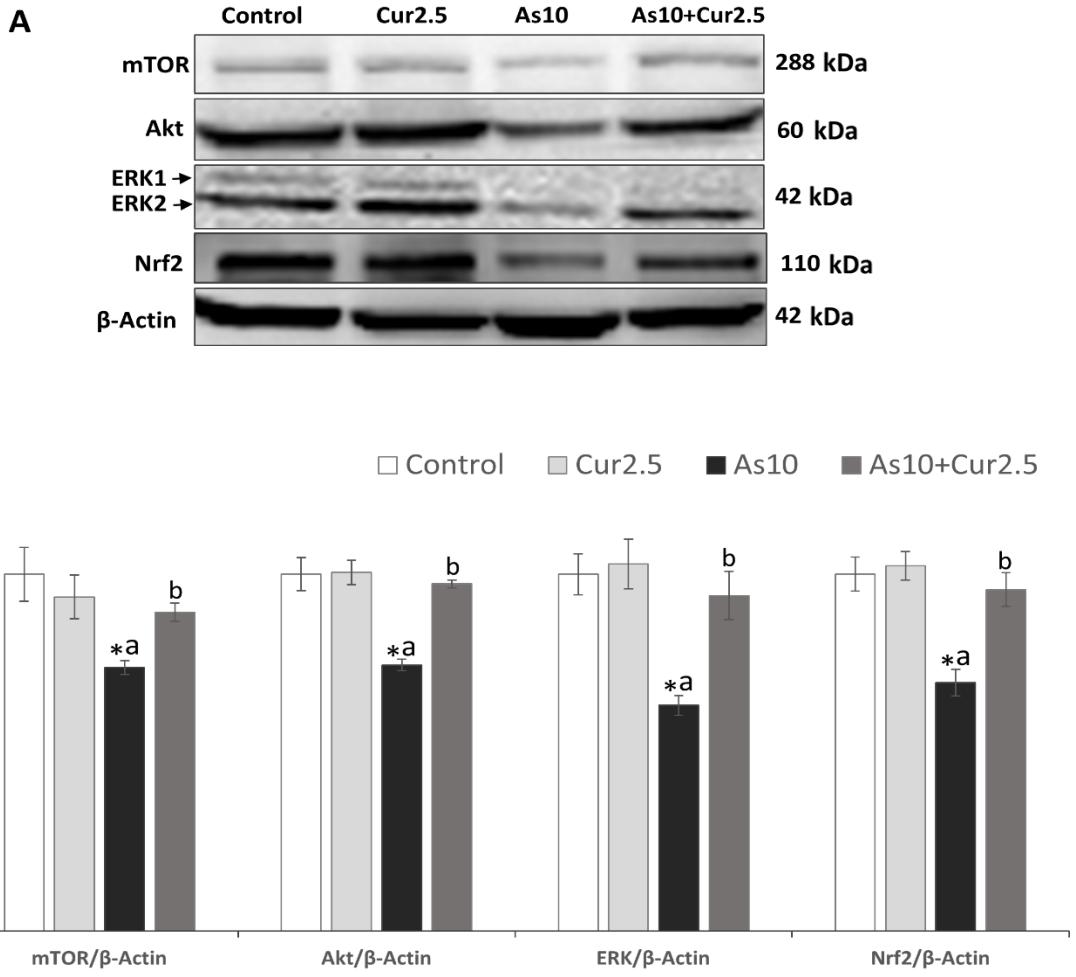

Fig. 3.6 Key pro-survival protein expressions in PC12 cells after being exposed to As^{3+} (10 μM) and/or Curcumin (2.5 μM) for 24 h. **(A)** Western blot analysis for treatment with As^{3+} (10 μM) and/or Curcumin (2.5 μM). This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. **(B)** The relative density of each protein band to β-Actin. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

Akt is considered as a key regulator protein for transcription, proliferation, glucose metabolism and cell survival. Only As^{3+}(10 μM) exposure significantly down-regulated Akt expression as comparison to control. The Akt expression in the cells exposed to As^{3+}(10 μM) with curcumin (2.5 μM) was significantly ($P < 0.05$) upregulated compared to that in the cells exposed to only As^{3+}(10 μM) (Fig. 3.6). These results support that curcumin (2.5 μM) treatment with As^{3+}(10 μM) enhanced the cell survival and rescued As^{3+}(10 μM)-induced oxidative damage.

3.3.7 Curcumin enhances ERK1 and Nrf2 expressions suppressed by Arsenic

Extracellular signal-regulated kinase (ERK1) is a member of the MAP kinase protein family and well-known for its pro-survival and anti-apoptotic role. Similarly, Nrf2 is an antioxidant booster protein and redox-sensitive transcription factor. Nrf2 positively regulates genes for antioxidant and anti-inflammatory, and controls the antioxidant defense systems. As shown in Fig. 3.6, the expressions of ERK and Nrf2 proteins significantly ($P < 0.05$) downregulated when cells were exposed to only As^{3+}(10 μM) compared to those cells were in the control group. A significant improvement in both ERK and Nrf2 protein expressions was observed in the cells co-exposed to curcumin (2.5 μM) and As^{3+}(10 μM) compared to the cells exposed to only As^{3+}(10 μM) (Fig. 3.6). These findings supported our previous results and indicated that curcumin exerts antioxidant properties.

3.3.8 Regulatory effects curcumin and arsenic on autophagy-related proteins

To understand molecular mechanism/s linked to As^{3+}-induced cell death in PC12 cells, We checked the autophagy initiator, ULK1 as well as responsible for autophagosomes formation (LC3-II) in the cells exposed to As^{3+}(10 μM) and/or curcumin (2.5 μM) for 24 h (Fig. 3.7).

The expressions of both proteins were significantly ($P < 0.05$) upregulated in the cells exposed to As^{3+}(10 μM). Co-exposure with curcumin (2.5 μM) and As^{3+}(10 μM) exhibited a significant downregulation of the ULK1 and LC3-II expressions when compared to the cells exposed only with As^{3+}(10 μM) (Fig. 3.7). From these findings, it proved that curcumin treatment significantly rescued PC12 cells from As^{3+} triggered autophagosomes and autophagy.

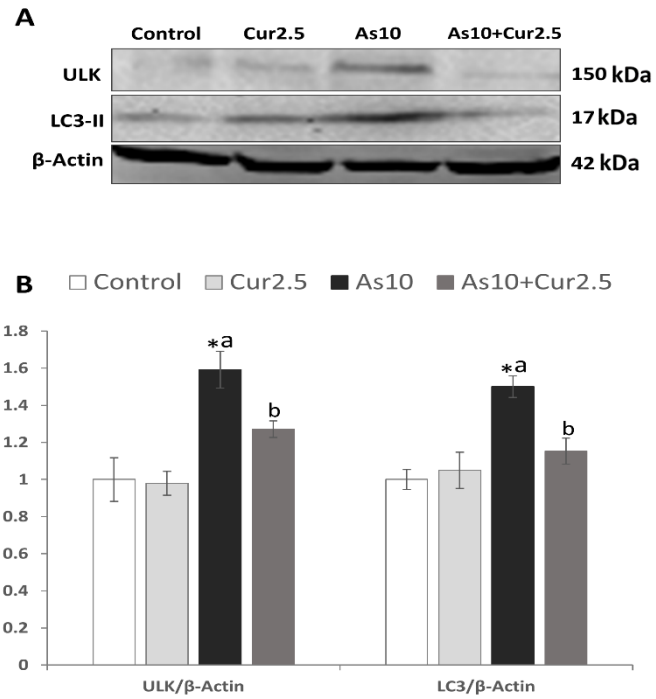

Fig. 3.7. Key proteins expression related to autophagy in PC12 cells after being exposed to As^{3+} (10 μM) and/or Curcumin (2.5 μM) for 24 h. **(A)** Western blot analysis for treatment with As^{3+} (10 μM) and/or Curcumin (2.5 μM). This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. **(B)** The relative density of each protein band to β-Actin. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

3.3.9 Regulatory effects of curcumin and arsenic on apoptosis-related proteins

To clarify the cell death process caused by As^{3+} in more detail, key proteins associated with apoptosis were also investigated in the cells exposed to As^{3+}(10 μM) and/or curcumin (2.5 μM). Pro-apoptotic proteins of p53, Bax, cytosolic cytochrome c, caspase 9, cleaved/active caspase

3 were significantly ($P < 0.05$) upregulated in the cells exposed to only As^{3+}(10 μM) compared to cells those were in the control group (Figs. 3.8 and 3.9).

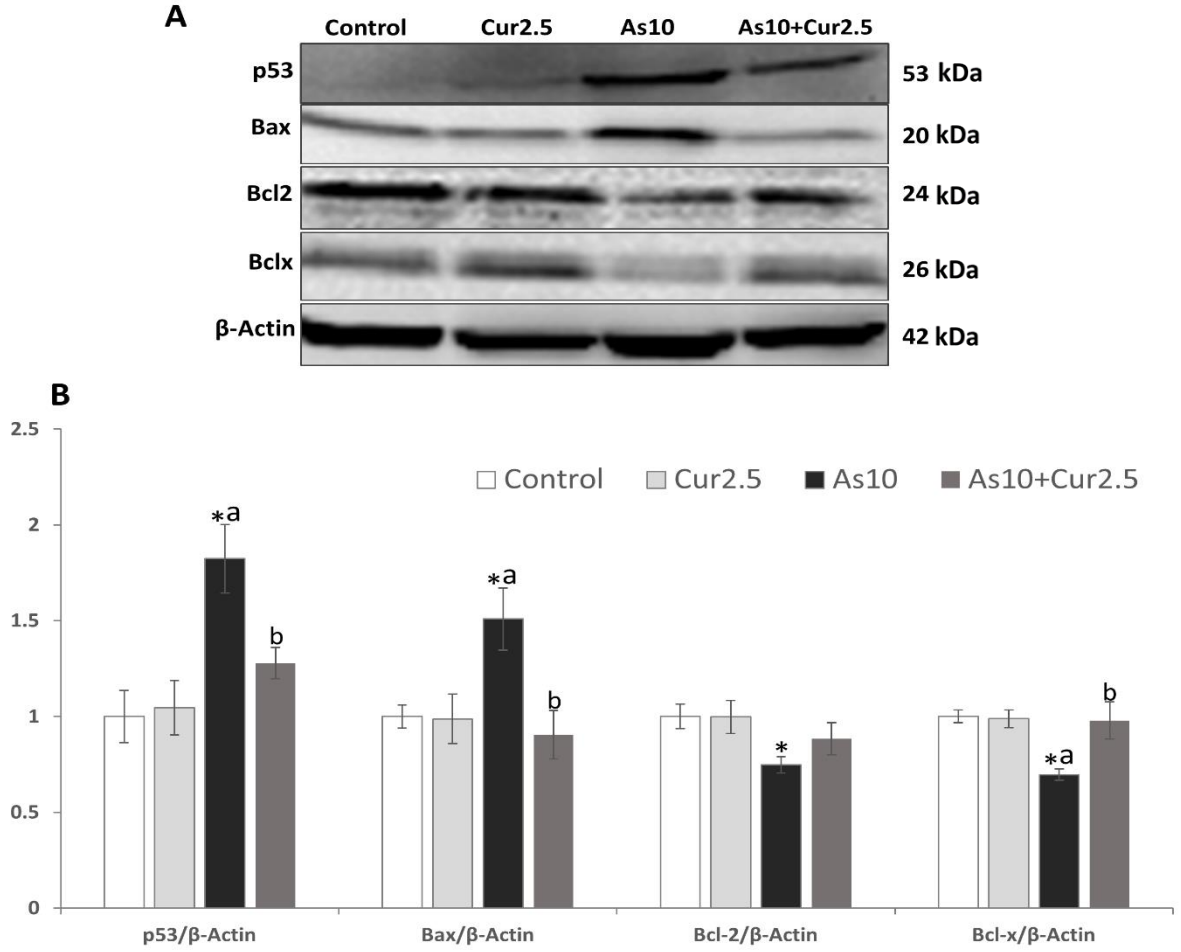

Fig.3.8 Key proteins expression related to apoptosis in PC12 cells after being exposed to As^{3+} (10 μM) and/or Curcumin (2.5 μM) for 24 h. **(A)** Western blot analysis for treatment with As^{3+} (10 μM) and/or Curcumin (2.5 μM). This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. **(B)** The relative density of each protein band to β-Actin. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

However, co-exposure with curcumin (2.5 μM) and As^{3+}(10 μM) exhibited significant downregulation of p53, Bax, cytosolic cytochrome c, caspase 9, cleaved/active caspase 3. Moreover, anti-apoptotic proteins, Bcl2, Bclx and XIAP were significantly ($P < 0.05$)

downregulated when cells were exposed to only As^{3+}(10 μM) compared to the cells those were in the control group. As shown in Figs. 3.8 and 3.9, co-exposure with curcumin (2.5 μM) and As^{3+}(10 μM) significantly recovered Bclx and XIAP protein levels which were suppressed by As^{3+}(10 μM).

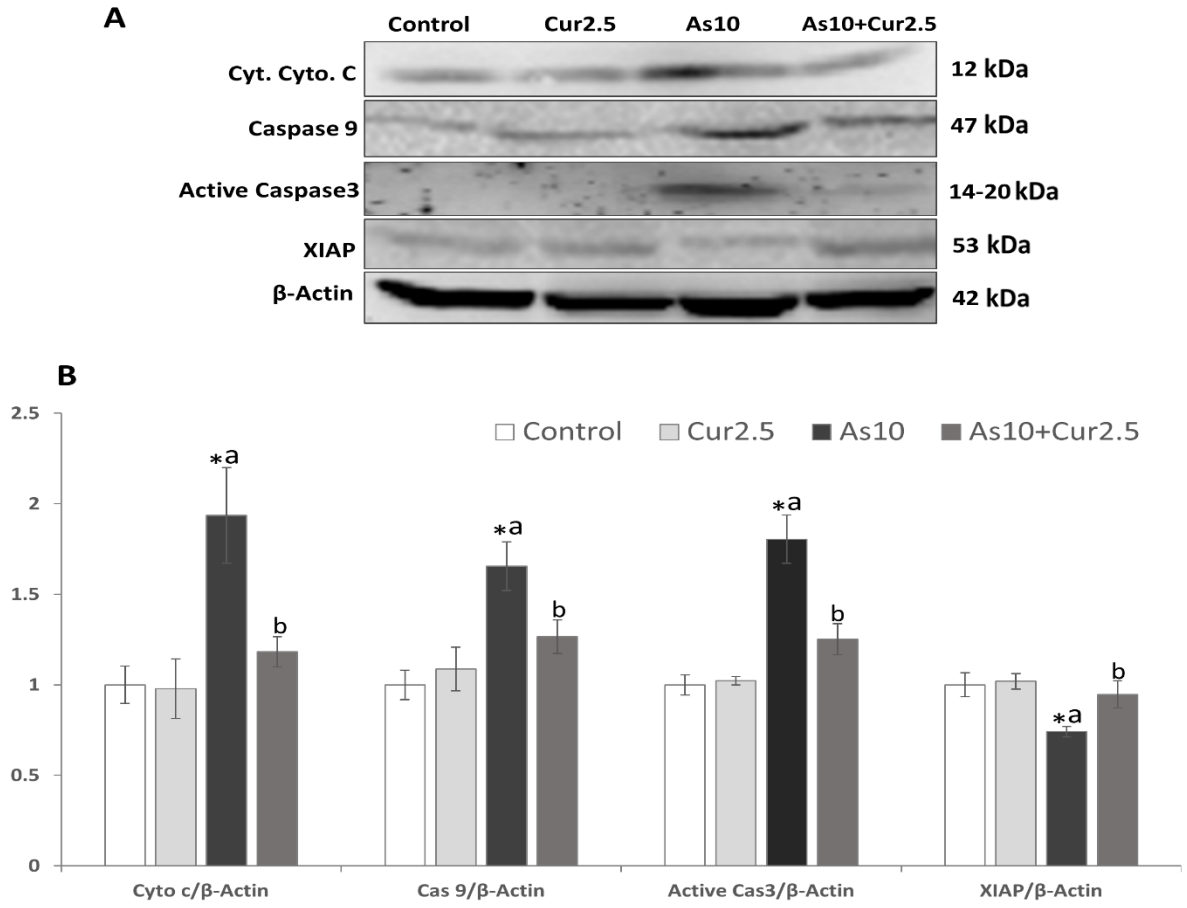

Fig.3.9 Key proteins expression related to apoptosis in PC12 cells after being exposed to As^{3+} (10 μM) and/or Curcumin (2.5 μM) for 24 h. **(A)** Western blot analysis for treatment with As^{3+} (10 μM) and/or Curcumin (2.5 μM). This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. **(B)** The relative density of each protein band to β-Actin. Error bars indicate mean ± SEM (n=3), * denotes significance at $p < 0.05$ compared to control group and $^{a, b}$ denotes significance at $p < 0.05$ between As^{3+} treated group and co-treatment groups (Curcumin treatment with As^{3+}).

These findings also completely support our previous cell viability (Fig. 3.1B), LDH activity (Fig. 3.2B), DNA fragmentation (Figs. 3.3A and B), GSH contents (Fig. 3.4), and other protein expression results, and proposed that curcumin effectively rescued PC12 cells from As^{3+}-triggered toxicity *via* modulating autophagy/apoptosis (Fig. 3.10).

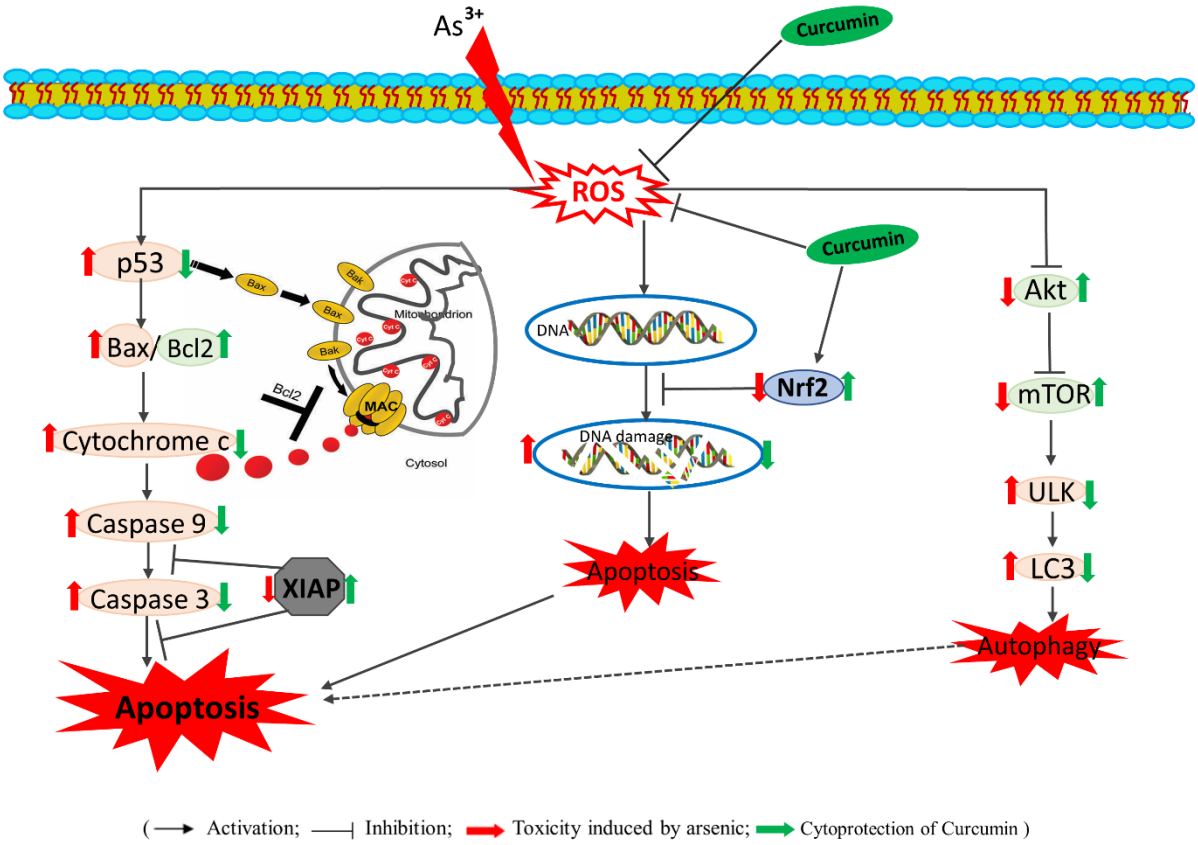

Fig. 3.10 Schematic diagram showing potential molecular mechanism/s of As^{3+}-induced toxicity and cytoprotection of Curcumin in PC12 cells upon co-exposure (Curcumin treatment with As^{3+}).

3.4 Discussion

Our obtained results provided evidence that curcumin could exert cytoprotective effects against As^{3+}-induced oxidative stress which is stimulated cytotoxicity and cellular damage in PC12 cells by boosting up the antioxidant defense system, improving cell membrane integrity, upregulating the antioxidant promoter, Nrf2 and finally modulating autophagy/apoptosis.

Therefore, our present study indicated that curcumin might be a potential candidate for the prevention and treatment of the toxicity posed by the toxic and poisonous metalloid As^{3+}.

Many researchers already reported that As^{3+}-induced toxicity in various cells mainly by inducing oxidative stress and leading cells to death (Shi et al., 2004; Wang et al., 2001; Gentry et al., 2010; Zhang et al., 2017). When cells exposed to any toxicant, cells systematically produce ROS that is concurrently neutralized by the cellular antioxidant defense systems (e.g.: GPx, GSH, vitamins, and flavonoids) for maintaining the cellular homeostasis (Urso and Clarkson, 2003). Reduced form GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) and the most important low molecular weight antioxidant. It also plays a regulatory role in nutrient metabolism, antioxidant defense, and regulation of cellular events (Wu et al., 2004). The present study demonstrated a significant decrease in the intracellular GSH levels in PC12 cells exposed to only As^{3+} compared to the control and a significant increase in those in the combined treatment of As^{3+} and curcumin (Fig. 3.4). These findings indicated that As^{3+} exposure damagingly affected the intracellular GSH levels in PC12 cells. Wang et al. (2015) also reported similar findings. They demonstrated that As^{3+} exposure negatively affected the intracellular GSH levels in the NB4 cell line. On the contrary, co-exposure of curcumin and As^{3+} significantly increased the intracellular GSH levels and boosted up the antioxidant capacity in PC12 cells (Fig. 3.4). Therefore, curcumin protects PC12 cells from As^{3+}-induced oxidative stress.

Sodium arsenite (As^{3+}) triggered cellular damage and disrupted the cellular oxidative homeostasis resulting in increases of stress markers such as the leakage of LDH into culture medium and even death of cells. Our study findings also revealed that 10 μM of As^{3+} induced acute oxidative stress and caused significant cell death in PC12 cells (Fig. 3.1B). The cell viability results (Fig. 3.1B) also demonstrated that co-exposure of curcumin and As^{3+} significantly increased the cell viability. Our results are in good agreement with the previous

findings in embryonic fibroblast cells (Perker et al., 2019). As shown in Fig. 3.2B, PC12 cells treated with only As^{3+} showed a significant increase of LDH levels due to the oxidative stress stimulated cell membrane damage. Orta Yilmaz et al. (2018) also reported that As^{3+} induced LDH release in Leydig and Sertoli cells. On the other hand, co-exposure of curcumin and As^{3+} significantly reduced the LDH levels in PC12 cells (Fig. 3.2B). It was demonstrated that curcumin can significantly reduce As^{3+}-induced LDH leakage from the PC12 cells. Zhao et al. (2008) was also reported that resveratrol reduced As^{3+} toxicity in H9c2 cardiomyocyte cells.

The ROS caused DNA damages as well as other biological macromolecules such as lipids, proteins and carbohydrates (Cai et al., 1997). The stability of DNA is an important matter for maintaining cellular integrity and avoiding genetic mutations. Several studies already reported that As^{3+} caused DNA damage and fragmentation by generating ROS (Akram et al., 2009; Khan et al., 2013; Rahman et al., 2018). Usually, cell could repair DNA fragmentation; however, As^{3+} may inhibit the cell-repair mechanisms. Our results also showed that only As^{3+} exposure significantly increases DNA fragmentation in PC12 cells (Figs. 3.3A and B). However, the co-exposure of curcumin and As^{3+} significantly reduced the DNA fragmentation induced by As^{3+} (Figs. 3.3A and B). Similarly, Khan et al. (2013) reported the preventive actions of curcumin against As^{3+}-induced oxidative DNA damage and DNA fragmentation in cultured murine Sertoli cells.

Arsenic can cause apoptotic and necrotic cell death in numerous cell lines based on exposure concentrations and duration (Wang et al., 2015; Zeng., 2001). There are two main pathways involved in the apoptotic cell death process. The apoptotic cell death process can be activated *via* the extrinsic pathway by the activation of death receptors or *via* mitochondria-mediated intrinsic pathway. Recently, Rahman et al. (2018) reported that As^{3+} caused both autophagic and mitochondria-mediated apoptotic cell death in PC12 cells. In this study, we also investigated the autophagy and apoptosis-related proteins. As results, As^{3+} exposure

accelerates autophagy and apoptosis-related proteins in PC12 cells (Figs. 3.6-3.9). As^{3+} treatment significantly increased the expressions of pro-apoptotic proteins, p53, Bax, cytosolic cytochrome c, caspase 9, and cleaved caspase 3, and decreased the expressions of anti-apoptotic proteins, ERK1, Bcl-2, Bcl-x, and Xiap in PC12 cells (Figs. 3.6, 3.8 and 3.9).

Pro-apoptotic p53 protein is normally known as tumor suppressor protein, which can promote the apoptotic cell death process through both transcription-independent and dependent mechanisms (Tan et al., 2018). In addition, the activation of pro-apoptotic Bax protein contributes mitochondrial membrane permeability (Chipuk et al., 2004). In the intrinsic apoptotic signaling pathway, Bcl-2 family members play a fundamental regulatory role in mitochondrial membrane permeability, and cytochrome c acts as a crucial regulator for activating caspase-dependent apoptosis. Depending on mitochondrial membrane integrity and mitochondrial membrane potential (MMP), cytochrome c release into cytosol from mitochondria (Rahman et al., 2017). In our study, we found a significantly upregulated expression of pro-apoptotic p53 and Bax, and downregulated expression of anti-apoptotic Bcl-2 and Bclx protein upon As^{3+} exposure in PC12 cells (Fig. 3.8). It was suggested that As^{3+} caused apoptotic cell death in PC12 cells *via* intrinsic partway. Similar findings in PC12 cells were also reported by Rahman et al. (2017) and Tan et al. (2018). Cytochrome c is considered as a hallmark protein in the mitochondria-mediated apoptotic signaling pathway. In cytosol, cytochrome c binds with Apaf-1 and forms apoptosome, then activates caspase cascade reactions and ultimately causes cell death (Banik et. al., 2019; Rahman et al., 2017). Our results showed that As^{3+} exposure increased cytochrome c in the cytosol and the upregulated the expression of caspase 9 and cleaved/active caspase 3 in PC12 cells and executed the intrinsic apoptosis (Fig. 3.9). Furthermore, As^{3+} treatment drastically suppressed the expression of a strong anti-apoptotic protein, X-linked inhibitor of apoptosis protein (XIAP) in PC12 cells. Similarly, Chen et al. (2012) reported a downregulated expression of XIAP upon As^{3+} exposure

in hepatocellular carcinoma. XIAP regulate the apoptosis process by binding directly to caspases (Chen et. al., 2012). However, co-exposure of curcumin and As^{3+} significantly downregulated the levels of cytosolic cytochrome c, caspase 9 and cleaved/active caspase 3 as well as efficiently recovered the expression of XIAP (Fig. 3.9). It was indicated that curcumin successfully saved PC12 cells from As^{3+}-induced apoptosis. This consideration strongly supported by our flow cytometry results (Fig. 3.5). Recently, Xu et al., (2019) showed that curcumin attenuates $A\beta_{25-35}$-induced oxidative stress in PC12 cells *via* modulating apoptosis. In addition, Motaghinejad et al., (2015) described the protective actions of curcumin against morphine-induced apoptosis in rat isolated hippocampus.

Previously, Roy et al. (2014) and Rahman et al. (2018) mentioned that apoptotic cell death can also be triggered *via* autophagy. In that case, ROS plays an important role to inhibit autophagy-related survival mTOR and Akt proteins. This inhibition of pro-survival mTOR and Akt protein results in the initiation of the autophagic process through the PI3K/mTOR/AKT pathway and subsequently the formation of autophagosomes (Roy et al., 2014). ULK1 is a serine/threonine-specific protein kinase that plays a central role in the initiation stage of autophagy (Wang et al., 2018). LC3 is considered as a central protein in the autophagic pathway to form autophagosome and autolysosome (Mizushima et al., 2011). We found that As^{3+} treatment significantly downregulated the expression of mTOR and Akt and upregulated the expression of ULK1 and LC3II (Fig. 3.7). These results suggested that As^{3+} triggered the formation of autolysosome in PC12 cells and the excessive formation of autophagosome caused autophagic cell death (Rahaman et al., 2020). Curcumin was found opposite effective against As^{3+}-induced autophagy as well as autophagy-mediated apoptosis.

Curcumin exerted antioxidative effects and showed a positive role to overcome As^{3+}-induced stressed conditions in PC12 cells as shown in Fig. 3.10. Recently, many researchers reported that curcumin showed its strong protective effects through the Keap1/Nrf2 signaling pathway

(Xu et al., 2019; García-Niño and Pedraza-Chaverrí, 2014). The nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is considered as an emerging cellular resistance regulator to oxidants in cells. In oxidative stress conditions, Kelch-like ECH-associated protein 1 (Keap1) and Nrf2 complex dissociate and Nrf2 bind with antioxidant responsive elements (ARE) upon translocation into the nucleus. The binding of Nrf2 and ARE triggered the cytoprotective antioxidant *HMOX1* and *NQO1* genes expression and controlled the antioxidant defense systems *via* positively regulating ROS homeostasis (Ma, 2013). Our results showed that As^{3+} exposure significantly downregulated expression of Nrf2, and co-exposure of curcumin and As^{3+} recovered the expression of Nrf2 in PC12 cells (Fig. 3.6). These results proposed that As^{3+}-triggered oxidative stress was weakened by the activation of Nrf2 upon exposure of curcumin in PC12 cells. Our findings were in agreement with the results reported by Xu et al. (2019). They demonstrated that curcumin protected PC12 cells from $A\beta_{25-35}$-induced oxidative damage by the activation of Nrf2.

In summary, our results demonstrated that As^{3+} induced cell death in PC12 cells through both mitochondrial apoptosis and autophagy. The mechanism of this apoptotic and autophagic cell death in PC12 cells may occur independently as well as cumulatively (Fig. 3.10). However, curcumin protects PC12 cells from As^{3+}-triggered toxicity *via* maintaining the oxidant/antioxidant homeostasis where Nrf2 plays the fundamental roles (Fig. 3.10).

3.5 Conclusion

In conclusion, we demonstrated that curcumin could efficiently protect PC12 cells from As^{3+}-induced oxidative damage and cytotoxicity possibly by limiting ROS generation and accumulation, boosting up antioxidant defense systems, and modulating both autophagy and apoptosis-related protein expressions in the protective and restoring way.

Thus, it indicated that the natural dietary compound curcumin worked as a strong antioxidant, antiapoptotic and anti-autophagic agents against As^{3+} toxicity. These findings recommend that curcumin will be potential and safe therapeutic agents to combat the As^{3+} toxicity in humans as well as in other biological systems. Further studies are essential to understand precisely the interactions and the protective mechanisms of curcumin against As^{3+}-induced toxicity.

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Chapter Four

Effects of curcumin, D-pinitol alone or in combination in cytotoxicity induced by arsenic in PC12 Cells

Abstract

Curcumin and D-pinitol both are naturally occurring bioactive dietary compounds that have antioxidant properties. Both are used to treat a broad variety of human diseases. Arsenic is a well-known potent toxicant affecting many millions of people all over the world by causing many human diseases including cancer. Thus, we hypothesized that Curcumin and D-pinitol may have synergistic effects against arsenic-induced toxicity in PC12 cells. PC12 cells were cultured in DMEM (+10% FBS) in 37°C with 5% CO_2 and pretreated with curcumin (2.5 µM), D-pinitol (5 µM) alone or in combination for 1 h, then exposed to sodium arsenite (10 µM) for 24 h. After 24 h incubation cell viability, DNA integrity, lactate dehydrogenase (LDH) activity, GSH levels, and expressions of proteins using western blotting were investigated. Results demonstrated that pretreatment of curcumin and D-pinitol and their combined pretreatment with arsenic increases cell viability, decreases DNA damage and protects PC12 cells from arsenic-induced cytotoxicity by increasing glutathione (GSH) level and antioxidant defense. Protein expression of western blot analysis showed that pretreatment of curcumin and D-pinitol and their combined pretreatment with arsenic significantly inhibited arsenic-induced cell death through up-regulation of survival factors; mTOR, Akt, Nrf2, ERK1, Bcl2, Bcl-x, XIAP and down-regulation of death factors; p53, Bax, cytosolic cytochrome c, caspase 9 and cleaved caspase 3, although arsenic regulated those factors negatively. Our findings indicated that curcumin and D-pinitol showed antioxidant properties and protects PC12 cells from arsenic-induced cytotoxicity. Furthermore, the effect of combined treatment with curcumin and D-pinitol was stronger than individual treatment.

4.1 Introduction

A naturally occurring ubiquitous metalloid element arsenic has been raising a global health concern due to its detrimental toxic effects and association with diverse human diseases including cancer (Escudero-Lourdes, 2016). Arsenic has been ranked the first position in the list of priority substances by the Agency for Toxic Substances and Disease Registry since 1997 (ATSDR, 2017) as well as it has been classified as a class-1 carcinogen by the International Agency of Research on Cancer (IARC) and considered as the most hazardous to human health among all of the toxicants (Alvarenga et al., 2020). Human may expose to arsenic through their drinking water which comes from the wells drilled into arsenic-rich ground strata. Food contaminated with arsenic is considered as another important source of arsenic exposure in humans (Saha et al., 1999). It is estimated that 40% of arsenic in the human body comes from contaminated food. Arsenic can be released into the environment (water, soil and air) via both natural processes and anthropogenic processes and existed in some different chemical forms (inorganic or organic) and oxidation states (−3, 0, +3, +5) (Hughes et al., 2011). Inorganic form of arsenic (As^{III} or As^{V}) is generally considered as the most toxic species of arsenic usually present in drinking water. An elevated level of arsenic contamination in groundwater has already been reported in many countries around the world and affected more than 140 million people (Shankar et al., 2014). The people of Bangladesh have been affected with high level of arsenic concentration (more than 50μg/L) and suffered the highest arsenic poisoning disaster in human history (Khan et al., 2003).

Arsenic may enter human body by ingestion, inhalation or skin absorption as major routes of exposure and can constantly spread in the body including the major organs; skin, lungs, liver and kidneys (Abdul et al., 2015; Hong et al., 2014). After entering the cells, arsenic binds with the sulphydryl (-SH) groups and affects the cellular energy generation process. Evidence proves that arsenic induces cytotoxicity by increasing the production of reactive oxygen species

(ROS) and accelerating the damage of biomolecules (DNA, lipid and proteins) (Susan et al., 2019). Excessive ROS generation through arsenic exposure negatively affects cellular functions by disrupting signaling pathways related to cell growth, proliferation, differentiation, DNA repair and other important cellular metabolic processes (Hughes, et al., 2011). Arsenic induced cell death mechanisms have been investigated widely in various cell lines (Perker et al., 2019; Rahaman et al., 2020; Wang et al., 2015) and it differ depending on arsenic concentration, exposure duration and cell type. But, most of the studies showed that arsenic induces cytotoxicity via ROS generation, antioxidant defense system disruption, lipid peroxidation, damaging biomolecules (DNA, proteins) and finally caused cell death where oxidative stress plays the fundamental role. Inhibition of these events may protect cells/tissue from arsenic toxicity.

Recently, numerous natural dietary bioactive compounds have been extensively studied for investigating their protective effects against various environmental toxicants to human including arsenic (Rahaman et al., 2020; Susan et al., 2019). Curcumin is a natural dietary food component predominantly found in turmeric (*Curcuma longa*) and well-known for its diverse biological and pharmacological activities (Sehgal et al., 2011). Reports demonstrated that curcumin has ameliorating potentials against many toxicants such as arsenic (Perker et al., 2019), acrylamid, cisplatin, cadmium and sodium fluoride (Hosseini et al., 2018), also it has therapeutic efficacy against many human ailments such as diabetes, rheumatoid arthritis, Alzheimer's disease, liver injury, atopic asthma, cystic fibrosis and cancer (García-Niño and Pedraza-Chaverrí, 2014). Another bioactive dietary compound D-pinitol, derived from soybean is drawn attention for its potential pharmaceutical properties (Negishi et al., 2015). A recent study demonstrated that D-pinitol has protective effects against arsenic toxicity (Rahaman et al., 2020). Cumulative evidence suggests that both curcumin and D-pinitol has protective effects against arsenic toxicity (Perker et al., 2019; Rahaman et al., 2020).

Considering a person's diet, both dietary compounds are widely consumed and coexposure to curcumin and D-pinitol may occur in dietary situations, but there is no combinational study of curcumin and D-pinitol available against arsenic toxicity. Thus, we hypothesized that the combination treatment of curcumin and D-pinitol might have synergistic and strong protective effects against arsenic toxicity.

Therefore, the present study aimed to investigate the synergistic or antagonistic effects of the combination treatment of curcumin and D-pinitol against arsenic toxicity in PC12 cells and gain insights into the molecular mechanism/s involved. For investigating the above-mentioned hypothesis, several cytotoxicity assessment techniques/methods have been employed to check the status of cell growth, oxidative stress, lactate dehydrogenase leakage, DNA damage, reduced glutathione (GSH) contents, and the possible chemical and cellular mechanism/s involved in cell death.

4. 2 Materials and Methods

4.2.1. Chemicals, reagents and antibodies

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM), ethidium bromide, ribonuclease A (RNase), and peroxidase-conjugated avidin, $NaAsO_2$ (As^{3+}), and D-pinitol ($C_7H_{14}O_6$) were obtained from Sigma-Aldrich, USA. Curcumin ($C_{21}H_{20}O_6$) was purchased from Wako Pure Chemical Corporation, Japan. Fetal bovine serum (FBS) and Proteinase K were bought from HyClone, USA and Roche Diagnostics, Germany respectively. Biotinylated goat anti-mouse IgG whole antibody and ECL western blotting detection reagent were obtained from Amersham Pharmacia Biotech, England. Cell signaling Technology (Danvers, MA, USA) provided βeta-actin (4967S), Akt (4691S), mTOR (2972S), p53 (2524S), Bax (2772S), Caspase 9 (9508S),

ULK1 (8054S), XIAP (2042S) antibodies. Bcl-x (610211, BD Biosciences), Bcl-2 (MAB8272, R&D Systems), ERK1 (610030, BD Biosciences), LC3 (M152-3, MBL), Nrf2 (PM069, MBL), cytochrome c (JA5204, Merk-Millipore), Active caspase 3 (NB 100-56113SS, NOVUS Biologicals) were purchased. 0.4 % trypan blue solution was bought from Bio-Rad, USA and the DNA 7500 assay kits were bought from Agilent Technologies, Germany. All the chemicals used in experiments were analytical reagent grade.

4.2.2. Cell culture and treatment

PC12 cells were cultivated on 25 cm^2 flasks in DMEM medium with the supplementation of 10% FBS at 37 °C under 5% CO_2 in a humidified incubator. Following 48 h preincubation, medium was changed with new serum comprising DMEM and then cells were treated with As^{3+} (10 µM), curcumin (1, 2.5, 5, 10, 25, 50, 100 µM) and D-pinitol (0.5, 5, 50,100, 150, 250, 500 µM) separately as well as combinedly for 24 h treatment incubation. The final concentration of As^{3+}, curcumin and D-pinitol was selected based on the best combinational results for further experimentation. And the selected final concentration for As^{3+} was 10 µM, curcumin was 2.5 µM and D-pinitol was 5 µM. Curcumin and D-pinitol were used as pretreatment 1 h prior to the treatment of As^{3+} and cells were incubated for 24 h. In our present study we have 5 treatment groups; (1) no treatment control group, (2) As^{3+} treatment group, (3) curcumin+As^{3+} treatment group, (4) D-pinitol+As^{3+} treatment group and (5) curcumin+D-pinitol+As^{3+} combined treatment group.

4.2.3. Cell viability

Viability of PC12 cells treated with As^{3+}, curcumin and D-pinitol was inspected by performing trypan blue exclusion assay. Following 48 h preincubation, PC12 cells were treated with/without curcumin, D-pinitol and As^{3+} for 24 h. After the 24 h treatment incubation, cells

were harvested, and stained with 0.4 % trypan blue solution for checking the cell viability (total cells and trypan blue-stained cells) using a TC10^{TM} automated cell counter (Bio-Rad, USA).

4.2.4. Lactate dehydrogenase (LDH) activity assay and imaging of cell-membrane damage

To check the cell membrane integrity of curcumin, D-pinitol and As^{3+}-treated PC12 cells, LDH activity assay was performed in the culture medium using cytotoxicity assay kit (Promega, USA) according to the protocol indicated by the manufacturer. After 24 h treatment incubation, 50 µL culture medium from each treatment group cells were collected and subsequently mixed with 50 µL of substrate mixture (containing tetrazolium salts) in a 96 well plate for 0.5 h at room temperature. Then, 50 µL stop solution was added to stop the reaction and the absorbance at 490 nm was measured using an iMark^{TM} microplate reader (BioRad, USA). LDH activity assay result was presented as LDH activity/1×10^6 cells.

4.2.5. Determination of Intracellular Glutathione

Intracellular glutathione (GSH) was measured spectrophotometrically using 2.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to determine if oxidative stress is involved in As^{3+}-induced cell death. PC12 cells treated with curcumin, D-pinitol and As^{3+} and incubated for 24 h. After the treatment incubation, cells were harvested and washed with PBS, then 150 µL lysis buffer was added and kept at 4°C for 10 min. The cell-containing solution was sonicated in a two freeze-thaw cycle and then centrifuged at 1,500 rpm for 10 min. The protein content in the collected supernatants was measured using a protein assay dye reagent (BioRad, USA), spectrophotometrically (DU-65 spectrophotometer, USA). The intracellular GSH contents were determined followed by the addition of (DTNB, pH 7) to the cell lysate by measuring the absorbance at 405 nm using an iMark^{TM} microplate reader (BioRad, USA).

4.2.6. Determination of DNA fragmentation

Agarose gel electrophoresis technique was employed to examine the DNA fragmentation pattern (DNA ladder) previously described by Kawakami et al. (2008). At the end of treatment incubation, PC12 cells were harvested and washed with PBS. The isolation of the genomic DNA of As^{3+}, curcumin and D-pinitol treated PC12 cells were done using high pure PCR template preparation kit (Roche Diagnostics, Penzberg, Germany) followed by the manufacturer's instructions. DNA was recovered by using the ethanol precipitation method. About 3-5 µg DNA including 2 µL loading dye was used for 1.5% agarose gel electrophoresis at 100 V for 30 min using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). In dark condition, the electrophoresed gel was soaked for 15 min in ethidium bromide solution. After that, a ChemiDoc XRS (Bio-Rad, USA) was used for visualizing DNA through UV illumination and capturing images. Intact DNA density level was measured by using a software named Image J.

4.2.7 Flow cytometry analysis

Flow cytometric detection of apoptosis experiment was performed by using Annexin V-fluorescein isothiocyanate (V-FITC) apoptosis detection kit (BioVision, USA) following the manufacturer's instruction. Briefly, curcumin, D-pinitol and As^{3+}-treated PC12 were harvested after 24 h incubation and washed with PBS followed by 5 min centrifugation at 4° C. After centrifugation, the cells were suspended in 500 µL of 1× binding buffer, subsequently, cells were incubated at room temperature for 5 min with 5 µL of Annexin V-FITC and 5 µL of propidium iodide (PI) in dark condition. Then, the PC12 cell samples were analyzed to detect early and late apoptosis with a flow cytometer (BD FACSVerse^{TM}, BD Biosciences).

4.2.8. Western Blot Analyses

Curcumin, D-pinitol and As^{3+}-treated PC12 cells were harvested after 24 h of treatment and washed with PBS. Physical protein extraction and 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the same protocol described in our previous study (Rahaman et al., 2020). After electrophoresis, separated proteins were transferred on nitrocellulose membrane using a blotting system (AE6678, ATTO, Tokyo, Japan). Then the membranes were blocked with 5% nonfat milk at 4°C for overnight. After washing with 0.3% Tween, membranes were incubated with primary antibodies for overnight at 4° C. In the morning of the other day, membranes were washed three 3 times and incubated with secondary antibodies for 1 h at 37° C for antibody reactions. After that, membranes were washed 5 times and protein bands were detected by using enhanced chemiluminescence (ChemiDoc XRS, Bio-Rad, USA).

4.2.8. Statistical analysis

Every single experiment was repeated at least in triplicate for ensuring statistical validity. The statistically significant difference between treatment groups was determined by one-way analysis of variance (ANOVA), which was followed by unpaired Student's t-test. P values less than 0.05 or 0.01 were considered statistically significant. The data were presented as the mean ± standard error of mean (SEM).

4.3. Results

4.3.1 Cell viability of curcumin, D-pinitol and arsenic treated PC12 cells

To investigate the highest protective dose of curcumin and D-pinitol against As^{3+} (10 µM) in PC12 cells, cells were exposed to 1, 2.5, 5, 10 µM of curcumin and 1, 2.5, 5, 10 µM of D-

pinitol with As^{3+} (10 µM) for 24 h. Cell viability of PC12 cells after 24 h incubation with various concentrations of curcumin and D-pinitol against As^{3+} (10 µM) were shown in Fig. 4.1A and 4.1B, respectively. The cell viability result showed that curcumin (2.5 µM) and D-pinitol (5 µM) treatment has highest protection against As^{3+} (10 µM). For this reason, curcumin 2.5 µM and D-pinitol 5 µM concentration were chosen in combination experiment. Similarly, cell viability in the case of combination treatments (Curcumin+As^{3+}), (D-pinitol+As^{3+}) and (Curcumin+D-pinitol+As^{3+}) for 24 h, were also checked and shown in Fig. 4.1C.

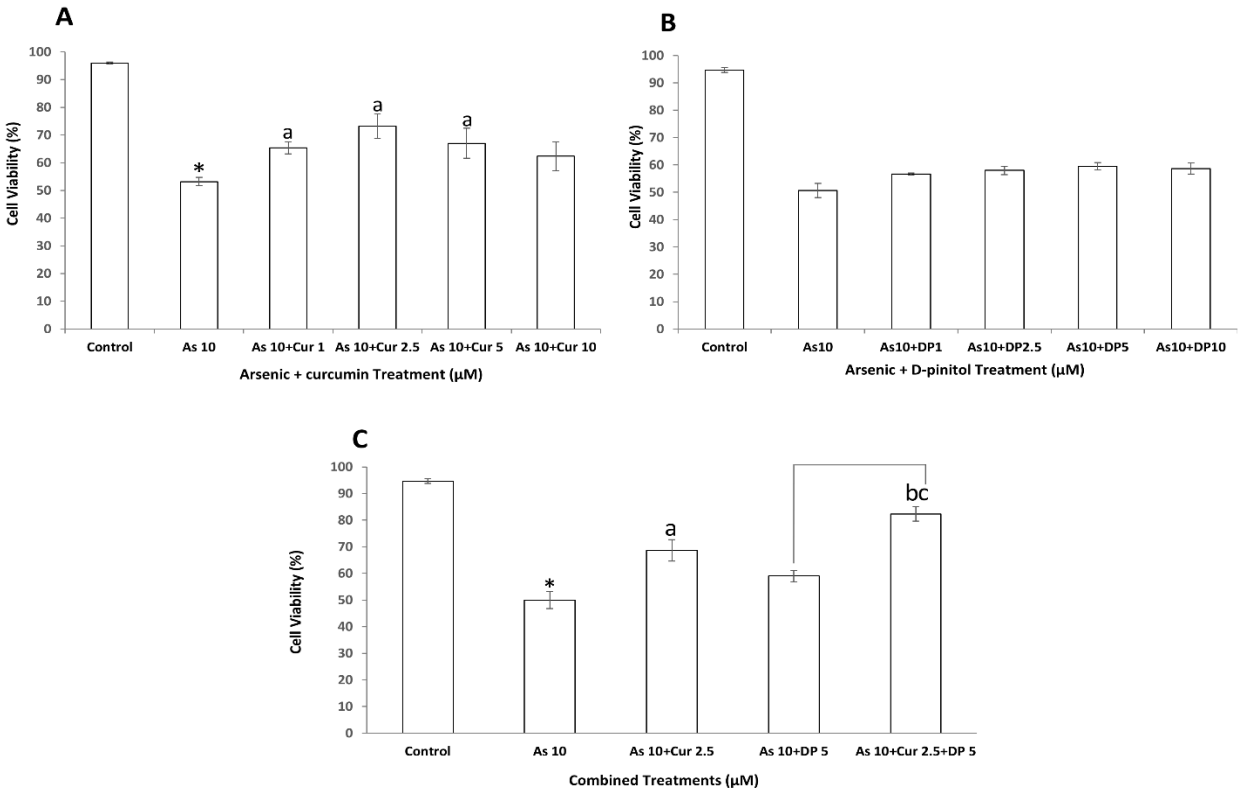

Fig. 4.1 Cell viability of PC12 cells after 24 h treatment using trypan blue exclusion method. **(A)** PC12 cells treated with different concentrations of D-pinitol and As^{3+} (10 µM). **(B)** PC12 cells treated with different concentrations of curcumin and As^{3+} (10 µM). **(C)** PC12 cells treated with curcumin (2.5 µM), D-pinitol (5.0 µM) and/or a combination of the both with As^{3+} (10 µM). Each experiment was conducted 5 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=5). * and ** denotes significance at $p<0.01$ compared to control group; [a] denotes significance at $p < 0.05$ compared to As^{3+} treated group; [b] denotes significance at $p < 0.01$ compared to As^{3+} treated group; [c] denotes significance at $p < 0.05$ among co-treatment groups (curcumin+As^{3+}, D-pinitol+As^{3+}, curcumin+D-pinitol+As^{3+}-treated groups). [Cur=curcumin, DP=D-pinitol]

Cell viability of PC12 cells treated only with 10 μM of As^{3+} showed a significant reduction compared to the control group. Treatment with curcumin (2.5 μM) in the background of As^{3+} (10 μM) exposure improved the cell viability at $p<0.05$ significant level and the combined treatment of curcumin (2.5 μM) and D-pinitol (5 μM) in the background of As^{3+} (10 μM) exposure showed higher improvement in cell viability at $p<0.01$ significant level comparing to only As^{3+} (10 μM) exposure cells (Fig. 4.1C). However, the treatment of D-pinitol against As^{3+} did not statistically alter the cell viability, but D-pinitol contributes in the cytoprotection of curcumin against As^{3+} (Fig. 4.1C). This cell viability results indicated that the combination of curcumin and D-pinitol exhibited stronger cytoprotection than individual treatment against As^{3+} toxicity.

4.3.2 Effect of curcumin, D-pinitol and arsenic on cell-membrane integrity

The status of cell-membrane integrity of curcumin, D-pinitol and arsenic treated PC12 cells was analyzed by measuring LDH activity in the cells culture medium. As^{3+} (10 μM) treatment significantly ($p<0.01$) increased the LDH activity in PC12 cells culture medium as compared to the control cells culture medium (Fig. 4.2A). D-pinitol (5 μM) treatment alone did not change the LDH activity, while curcumin (2.5 μM) treatment alone ($p<0.05$) or in combination treatment with D-pinitol (5 μM) in the background of As^{3+} (10 μM) exposure significantly ($P<0.01$) decreased the LDH activity as compared to only As^{3+} (10 μM) treated cells (Fig. 4.2A). Results suggested that As^{3+} (10 μM) treatment induces cytotoxicity in PC12 cells and curcumin alone or in combination with D-pinitol effectively reduces the As^{3+}-induced cytotoxicity. Moreover, the combined effect was stronger than that of D-pinitol alone and showed consistency with the cell viability results. Trypan blue staining assay was also performed to visualize the cell membrane damage.

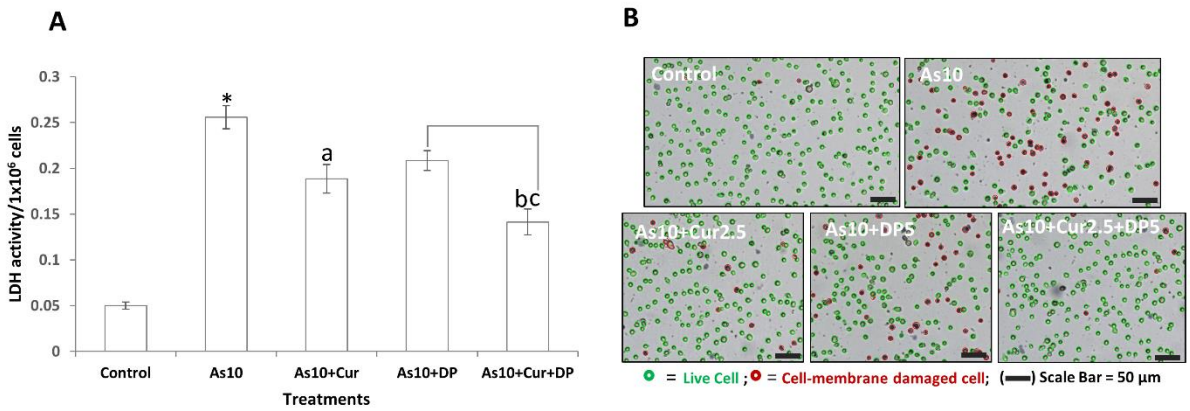

Fig. 4.2 (A) LDH activity of PC12 cells treated with curcumin (2.5 µM), D-pinitol (5.0 µM) and/or a combination of the both with As^{3+} (10 µM) for 24 h. **(B)** Detection of cell membrane integrity of PC12 cells upon exposure to curcumin (2.5 µM), D-pinitol (5.0 µM) and/or a combination of the both with As^{3+} (10 µM) for 24 h. Each experiment was conducted 3 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=3). * denotes significance at $p<0.01$ compared to control group; [a] denotes significance at $p < 0.05$ compared to As^{3+} treated group; [b] denotes significance at $p < 0.01$ compared to As^{3+} treated group; [c] denotes significance at $p < 0.05$ among co-treatment groups (curcumin+As^{3+}, D-pinitol+As^{3+}, curcumin+D-pinitol+As^{3+}-treated groups). [Cur=curcumin, DP=D-pinitol]

Trypan blue stained PC12 cells were shown in Fig. 4.2B, where green color represents live cells and cell membrane damaged death cells were represented by red color. Red-colored (cell membrane damaged) cells were increased significantly ($p< 0.01$) only in the As^{3+} (10 µM)-treated cells group as compared to the control group. However, combination treatment (curcumin 2.5 µM + D-pinitol 5 µM) in the background of As^{3+} (10 µM) exposure significantly ($p<0.01$) decreased the number of cell membrane-damaged (red color) cells compared to the cells group those were treated only with As^{3+} (10 µM) (Fig. 4.2B).

4.3.3 Effect of curcumin, D-pinitol and arsenic on DNA

Effect of curcumin, D-pinitol and arsenic on DNA of treated PC12 cells was examined by performing agarose gel electrophoresis and shown in Fig. 4.3. The agarose gel electrophoresis result showed that As^{3+} (10 µM) exposure in PC12 cells for 24 h significantly ($P<0.01$) increased the DNA fragmentation compared to the control group (Fig. 4.3).

Curcumin (2.5 µM) and D-pinitol (5 µM) alone or in combination in the background of As^{3+} (10 µM) exposure significantly reduce the As^{3+}-induced DNA fragmentation at the level of $p<0.05$ and $p<0.01$, respectively (Fig. 4.3).

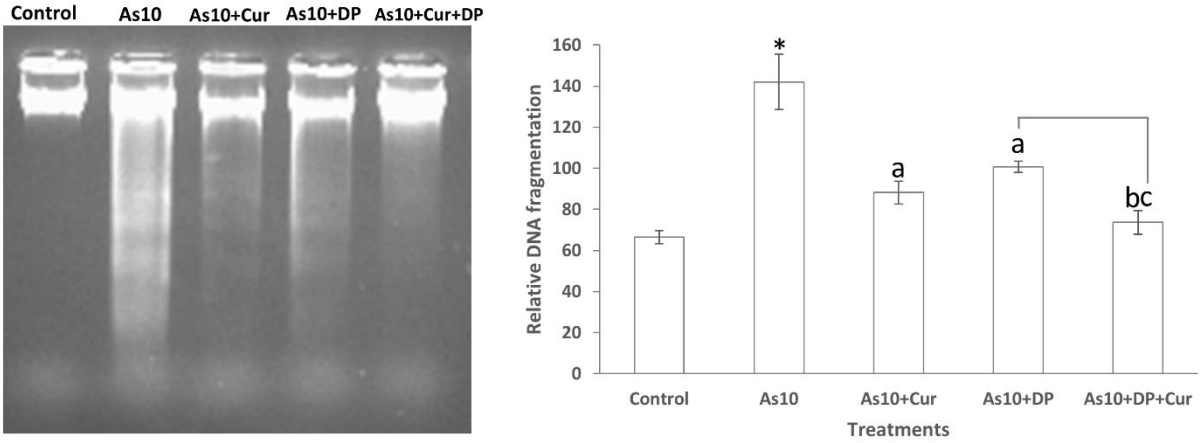

Fig. 4.3 Agarose gel electrophoresis of genomic DNA extracted from PC12 cells treated with curcumin (2.5 µM), D-pinitol (5.0 µM) and/or a combination of the both with As^{3+} (10 µM) for 24 h. **(A)** A representative electrophoresis image showing the DNA fragmentation. **(B)** DNA band intensity for fragmentation detection. This experiment was done three times independently for reproducibility. Error bars indicate mean ± SEM (n=3). * denotes significance at $p<0.01$ compared to control group; [a] denotes significance at $p < 0.05$ compared to As^{3+} treated group; [b] denotes significance at $p < 0.01$ compared to As^{3+} treated group; [c] denotes significance at $p < 0.05$ among co-treatment groups (curcumin+As^{3+}, D-pinitol+As^{3+}, curcumin+D-pinitol+As^{3+}-treated groups). [Cur=curcumin, DP=D-pinitol]

These electrophoresis results postulated that As^{3+} induces DNA fragmentation or damage which was effectively prevented by curcumin and D-pinitol. These results also indicated that the combined protective effect of curcumin and D-pinitol was higher than their individual treatment against As^{3+} caused DNA damage and showed a similar pattern with the previous cell viability (Fig. 4.1C) and LDH activity (Fig. 4.2A) results.

4.3.4 Total glutathione (GSH) levels in curcumin, D-pinitol and arsenic treated PC12 cells

PC12 cells were exposed to curcumin (2.5 µM) and/or D-pinitol (5 µM) in the background of As^{3+} (10 µM) exposure for 24 h and GSH level was measured and shown on Fig. 4.4.

As^{3+} (10 μM) treatment in PC12 cells showed a significant ($p<0.01$) reduction in GSH level as compared to the control (Fig. 4.4). Curcumin and D-pinitol independently with As^{3+} showed a significant ($p<0.05$) improvement in the GSH level compared to the As^{3+} treatment alone (Fig. 4.4).

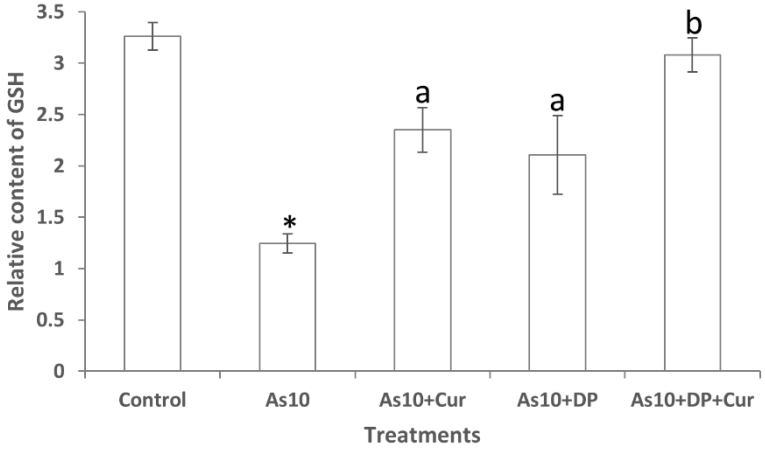

Fig. 4.4 Intracellular glutathione (GSH) level in PC12 cells after 24 h treatment with curcumin (2.5 μM), D-pinitol (5.0 μM) and/or a combination of the both with As^{3+} (10 μM). This experiment was done three times independently for ensuring reproducibility. Error bars indicate mean ± SEM (n=3). * denotes significance at $p<0.01$ compared to control group; **[a]** denotes significance at $p < 0.05$ compared to As^{3+} treated group; **[b]** denotes significance at $p < 0.01$ compared to As^{3+} treated group. [Cur=curcumin, DP=D-pinitol]

These results suggested that curcumin and D-pinitol have ameliorating potentials against As^{3+}-triggered oxidative stress in PC12 cells. However, the combined treatment of curcumin and D-pinitol against As^{3+} showed a higher recovery in GSH contents at $p<0.01$ significant level (Fig. 4.4).

4.3.5 Flow Cytometry analysis for detecting apoptosis rate in curcumin, D-pinitol and arsenic treated PC12 cells

To clarify whether As^{3+} (10 μM) treatment induced cell apoptosis in PC12 cells, Annexin V-FITC and PI double staining were performed. After the treatment of curcumin (2.5 μM) and D-pinitol (5 μM) alone and/or in combination in the background of As^{3+} (10 μM) exposure for

24 h, flow cytometry experiment was done and apoptotic rates were 5.04% ± 1.20%, 25.60% ± 2.61%, 12.79% ± 1.70%, 18.20% ± 1.87% and 8.10% ± 1.24% for control, As^{3+} (10 µM), (2.5 µM curcumin + 10 µM As^{3+}), (5.0 µM D-pinitol + 10 µM As^{3+}) and (2.5 µM curcumin + 5 µM D-pintol + 10.0 µM As^{3+}), respectively (Fig. 4.5).

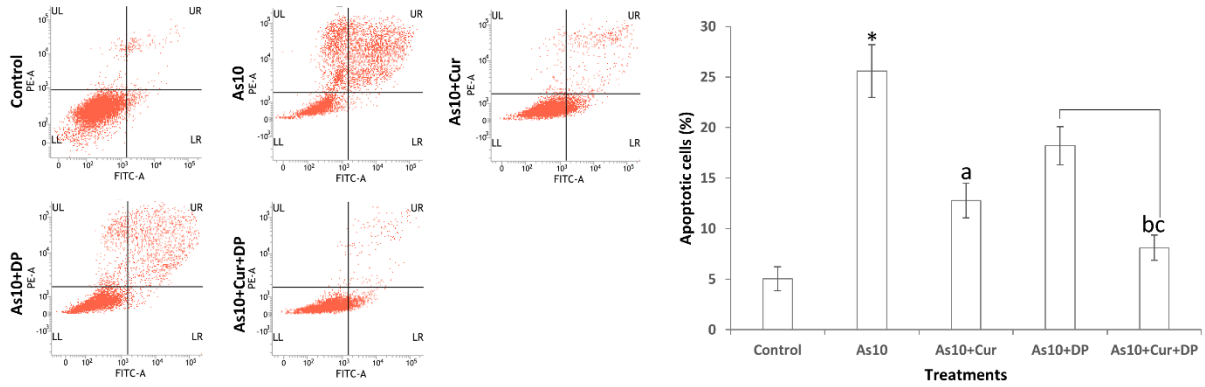

Fig. 4.5 Combination of curcumin and D-pinitol significantly halted apoptosis induced by As^{3+} in PC12 cells. PC12 cells were treated with curcumin (2.5 µM), D-pinitol (5.0 µM) or a combination of the both with As^{3+} (10 µM) for 24 h. Apoptosis rates were then measured by flow cytometry. **(A)** Representative Annexin V-FITC/PI strained flow cytometry results. **(B)** The rate of apoptosis of PC12 cells. Each experiment was conducted 3 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=3). * denotes significance at $p<0.01$ compared to control group; [a] denotes significance at $p < 0.05$ compared to As^{3+} treated group; [b] denotes significance at $p < 0.01$ compared to As^{3+} treated group; [c] denotes significance at $p < 0.05$ among co-treatment groups (curcumin+As^{3+}, D-pinitol+As^{3+}, curcumin+D-pinitol+As^{3+}-treated groups).
[Cur=curcumin, DP=D-pinitol]

The rates of apoptotic cells significantly ($p<0.01$) increased when cells were treated with only As^{3+} (10 µM) compared to the control cells and curcumin alone or in combination with D-pinitol effectively suppressed the apoptosis induced by As^{3+} exposure (Fig. 4.5). However, the combined effect was stronger than that of D-pinitol alone against As^{3+} triggered apoptosis and postulated that D-pinitol enhances the protective actions of curcumin, which was consistent with the previous results.

4.3.6 Effect of curcumin, D-pinitol and arsenic on pro-survival protein expressions

In general, toxicants induce toxicity and cause cell death in the cellular system by suppressing the pro-survival protein expressions. In the present study, several key pro-survival proteins expression were analyzed by western blotting in PC12 cells treated with curcumin (2.5 μM) and D-pinitol (5 μM) alone or in combination with As^{3+} (10 μM) for 24 h.

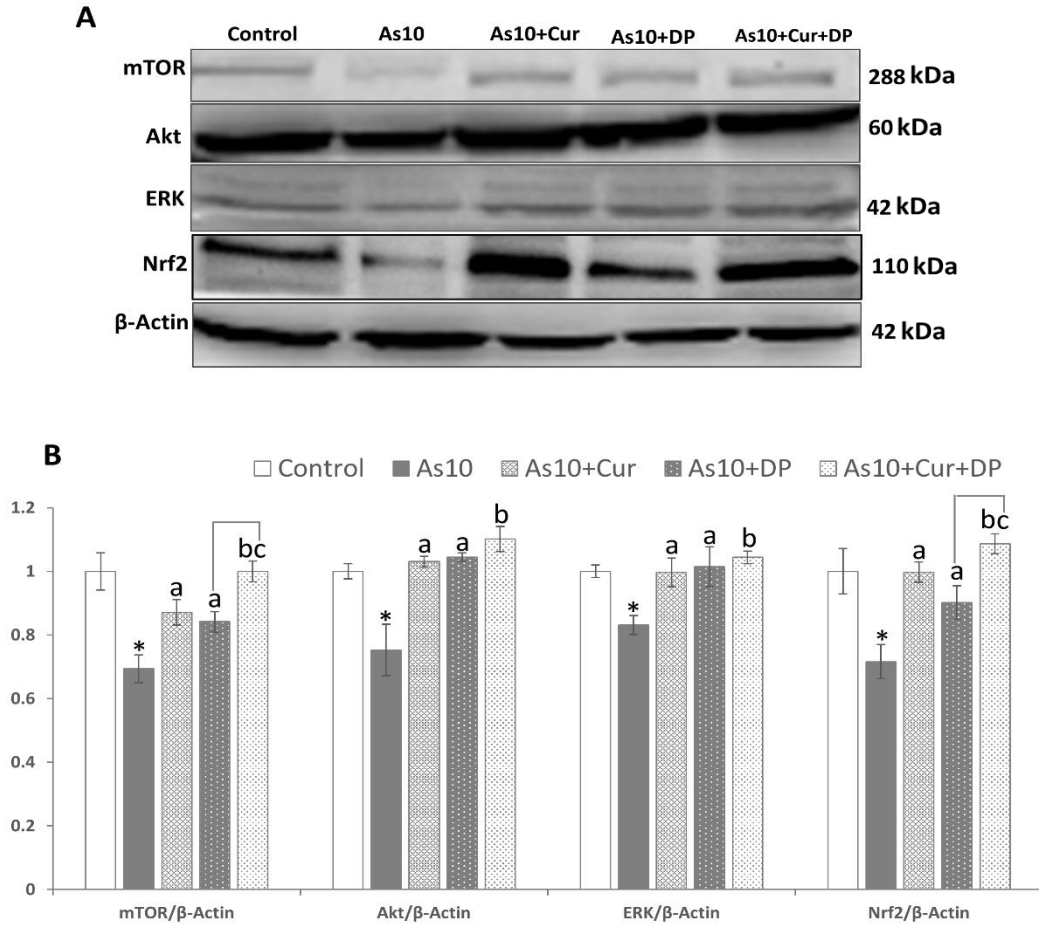

Fig. 4.6 Western blot analysis for the expression of pro-survival proteins in PC12 cells after being exposed to curcumin (2.5 μM), D-pinitol (5.0 μM) or a combination of the both with As^{3+} (10 μM) for 24 h. **(A)** The representative images (cropped) of immunoblotting for the expressions of mTOR, Akt, ERK1 and Nrf2. **(B)** Relative density of each protein band to β-Actin. Each experiment was conducted at least 3 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=3). * denotes significance at $p<0.05$ compared to control group; [a] denotes significance at $p < 0.05$ compared to As^{3+} treated group; [b] denotes significance at $p < 0.01$ compared to As^{3+} treated group; [c] denotes significance at $p < 0.05$ among co-treatment groups (curcumin+As^{3+}, D-pinitol+As^{3+}, curcumin+D-pinitol+As^{3+}-treated groups).
[Cur=curcumin, DP=D-pinitol]

Western blotting results revealed that As^{3+} (10 μM) exposure significantly ($P<0.05$) suppressed the expression of the vital pro-survival mTOR and Akt protein which are responsible for maintaining cellular metabolism, cell growth, proliferation as well as cell survival (Fig. 4.6). Curcumin and D-pinitol individually, and combinedly with As^{3+} showed a noticeable upregulated expression of mTOR and Akt protein expression as compared to only As^{3+} treated group at $p<0.05$ and $p<0.01$ significant level, respectively (Fig. 4.6). These results postulated that curcumin and D-pinitol individually and combinedly enhanced the survival rate and rescued PC12 cells from As^{3+}-induced cell death. However, the combined protective effect was stronger than that of D-pinitol alone against As^{3+} triggered cell death and showed consistency with the previous results.

4.3.7 Effect of curcumin, D-pinitol and arsenic on ERK1 and Nrf2 protein expressions

ERK1 is considered as an important member of mitogen-activated protein (MAP) kinase protein family and inhibits cell death. It plays a vital role in the major oxidative stress-sensitive signal transduction pathways and regulates cell growth and survival. Conversely, Nrf2 is a crucial cytoprotective protein and a transcription factor for the regulation of detoxifying enzymes and antioxidant status to counteract cellular oxidative stress. Western blotting results revealed that As^{3+} (10 μM) exposure in PC12 cells significantly suppressed the expression of both ERK1 and Nrf2 protein compared to the control (Fig. 4.6). However, curcumin (2.5 μM) and D-pinitol (5 μM) alone or in combination in the background of As^{3+} (10 μM) treatment effectively restored both ERK1 and Nrf2 protein expression as compared to the As^{3+} treated cells group (Fig. 4.6). From these results, it is postulated that As^{3+} exposure negatively affects the ERK1 and Nrf2 protein expression whereas curcumin and D-pinitol alone or in combination significantly upregulated these two essential proteins expression.

Furthermore, western blot results demonstrated that the combined effect of curcumin and D-pinitol was higher than their individual effect (Fig. 4.6).

4.3.8 Effect of curcumin, D-pinitol and arsenic on apoptosis related protein expressions

DNA fragmentation is considered as a hallmark of apoptotic cell death. Our obtained DNA fragmentation results (Fig. 4.3) and flowcytometry results (Fig. 4.5) suggested that arsenic cause apoptotic cell death in PC12 cells. Therefore, we analyzed key proteins related to apoptotic cell death and depicted in Figs. 4.7 and 4.8 for understanding of the molecular mechanism/s involved.

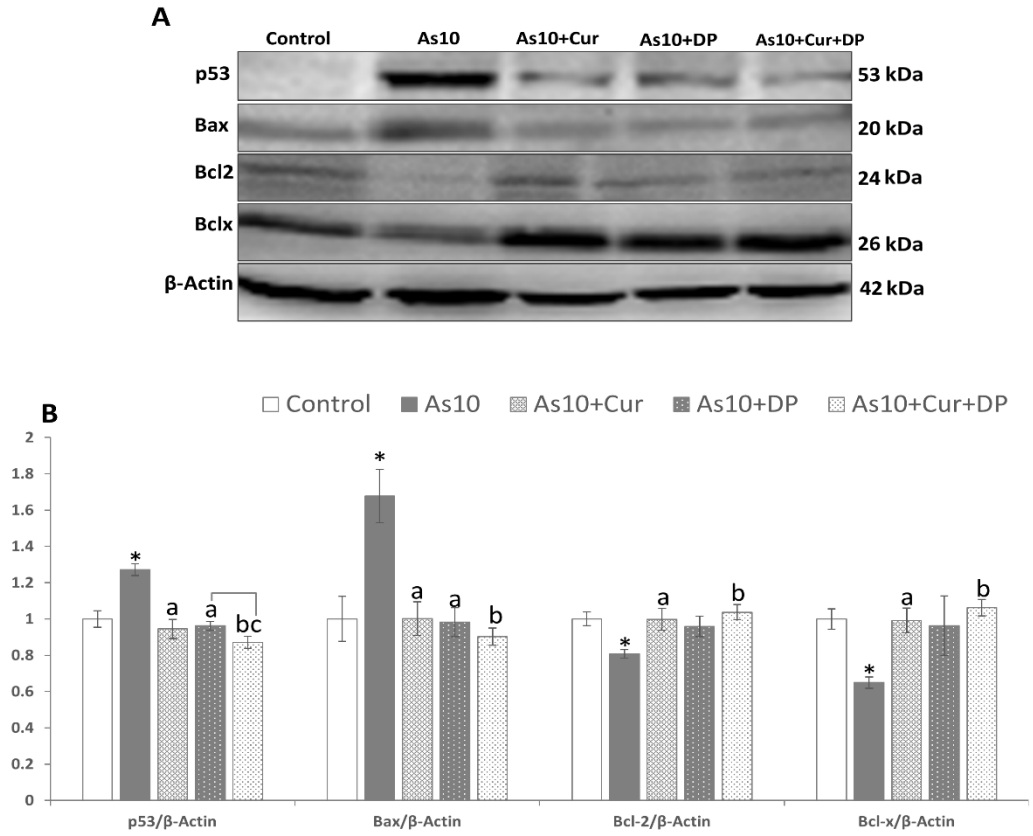

Fig. 4.7. Western blot analysis for the expression of apoptosis related proteins in PC12 cells after being exposed to curcumin (2.5 µM), D-pinitol (5.0 µM) or a combination of the both with As^{3+} (10 µM) for 24 h. **(A)** The representative images (cropped) of immunoblotting for the expressions of p53, Bax, Bcl2, Bclx. **(B)** Relative density of each protein band to β-Actin. Each experiment was conducted at least 3 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=3). * denotes significance at $p<0.05$ compared to control group; [a] denotes significance at $p < 0.05$ compared to As^{3+} treated group; [b] denotes significance at $p < 0.01$ compared to As^{3+} treated group; [c] denotes significance at $p < 0.05$ among co-treatment groups (curcumin+As^{3+}, D-pinitol+As^{3+}, curcumin+D-pinitol+As^{3+}-treated groups).[Cur=curcumin, DP=D-pinitol]

Results demonstrated that pro-apoptotic p53, Bax, cytosolic cytochrome c, caspase 9 and active/cleaved caspase 3 protein expressions were significantly upregulated in As^{3+} (10 μM) treated PC12 cells compared to the control group cells (Figs. 4.7 and 4.8). Interestingly, curcumin (2.5 μM) and D-pinitol (5 μM) alone or in combination against As^{3+} (10 μM) treatment significantly downregulated the expression of these pro-apoptotic proteins compared to the only As^{3+} (10 μM) treatment as shown in Figs 4.7 and 4.8.

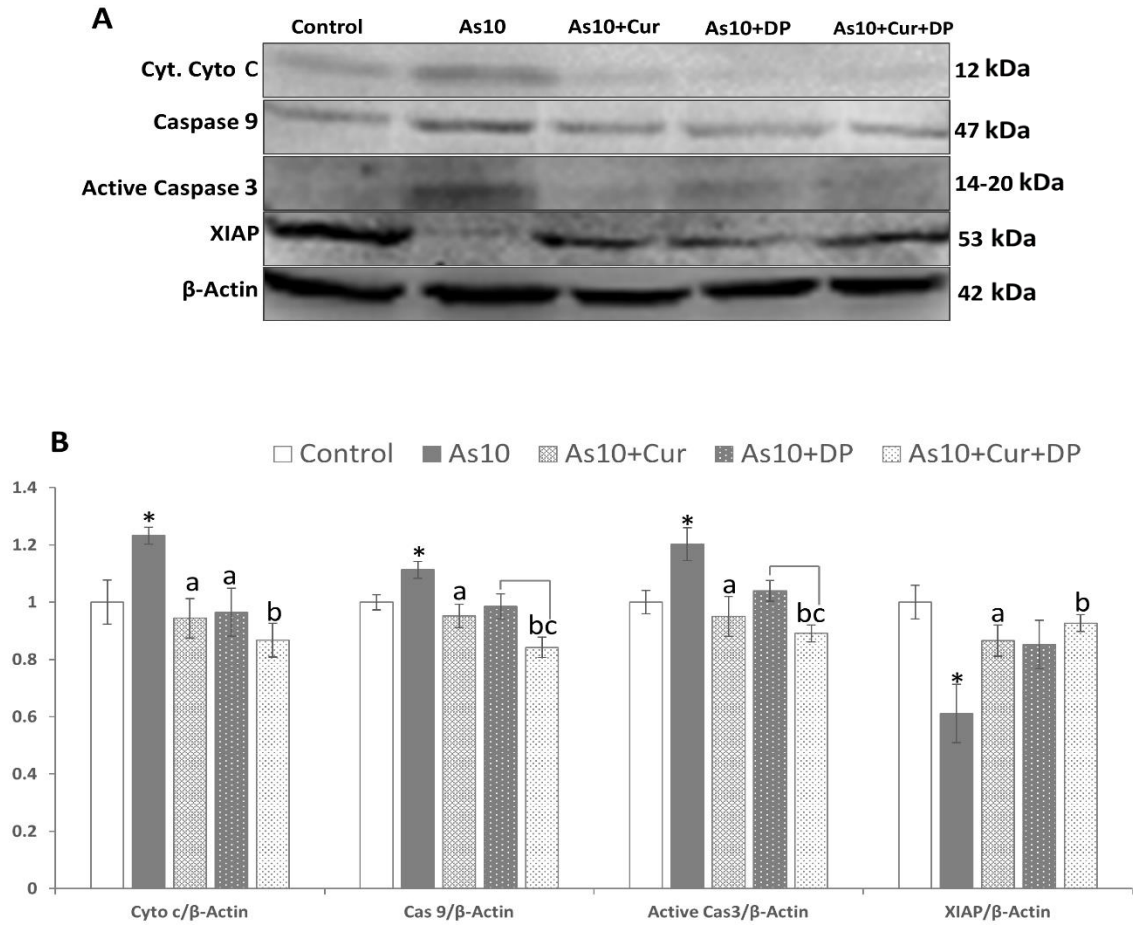

Fig.4.8 Western blot analysis for the expression of apoptosis related proteins in PC12 cells after being exposed to curcumin (2.5 μM), D-pinitol (5.0 μM) or a combination of the both with As^{3+} (10 μM) for 24 h. **(A)** The representative images (cropped) of immunoblotting for the expressions of cytosolic cytochrome c, caspase 9, active caspase 3 and XIAP. **(B)** Relative density of each protein band to β-Actin. Each experiment was conducted at least 3 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=3). * denotes significance at $p<0.05$ compared to control group; [a] denotes significance at $p < 0.05$ compared to As^{3+} treated group; [b] denotes significance at $p < 0.01$ compared to As^{3+} treated group; [c] denotes significance at $p < 0.05$ among co-treatment groups (curcumin+As^{3+}, D-pinitol+As^{3+}, curcumin+D-pinitol+As^{3+}-treated groups). [Cur=curcumin, DP=D-pinitol]

At the same time, As^{3+} (10 μM) exposure in PC12 cells destructively downregulated the expressions of anti-apoptotic Bcl2, Bclx and XIAP protein as compared to the control (Figs. 4.7 and 4.8). However, curcumin alone or in combination with D-pinitol against As^{3+} treatment magnificently restored the expressions of Bcl2, Bclx and XIAP compared to the As^{3+} treatment alone. These results indicated that curcumin alone or in combination with D-pinitol significantly protected PC12 cells from apoptotic cell death caused by As^{3+}. Furthermore, the combined protective effect of curcumin and D-pinitol against As^{3+} was stronger than their individual protection where D-pinitol enhances the cytoprotection of curcumin.

4.3.9 Effect of curcumin, D-pinitol and arsenic on autophagy-related protein expressions

For understanding the As^{3+} induced cell death mechanism/s more properly, we also checked key proteins related to autophagic cell death. After the treatment of curcumin (2.5 μM) and/or D-pinitol (5 μM) alone and in combination with As^{3+} (10 μM) in PC12 cells for 24 h, autophagy initiator ULK1 protein, as well as autophagy executioner LC3-II protein expressions, were checked by carrying out western blot analysis and depicted on Fig. 4.9. Results revealed that As^{3+} (10 μM) treatment in PC12 cells significantly upregulated the expressions of ULK1 and LC3-II protein as compared to the control cells group (Fig. 4.9). Curcumin and D-pinitol alone or in combination with As^{3+} efficiently downregulated the expressions of both ULK1 and LC3-II protein as compared to the only As^{3+}-treated cells group (Fig. 4.9). From these results, it is suggested that As^{3+} instigate autophagic cell death, and curcumin and D-pinitol successfully rescued PC12 cells from the autophagic condition. However, the combined protective effect of curcumin and D-pinitol was higher than that of their individual protection against As^{3+}-induced autophagy (Fig. 4.9).

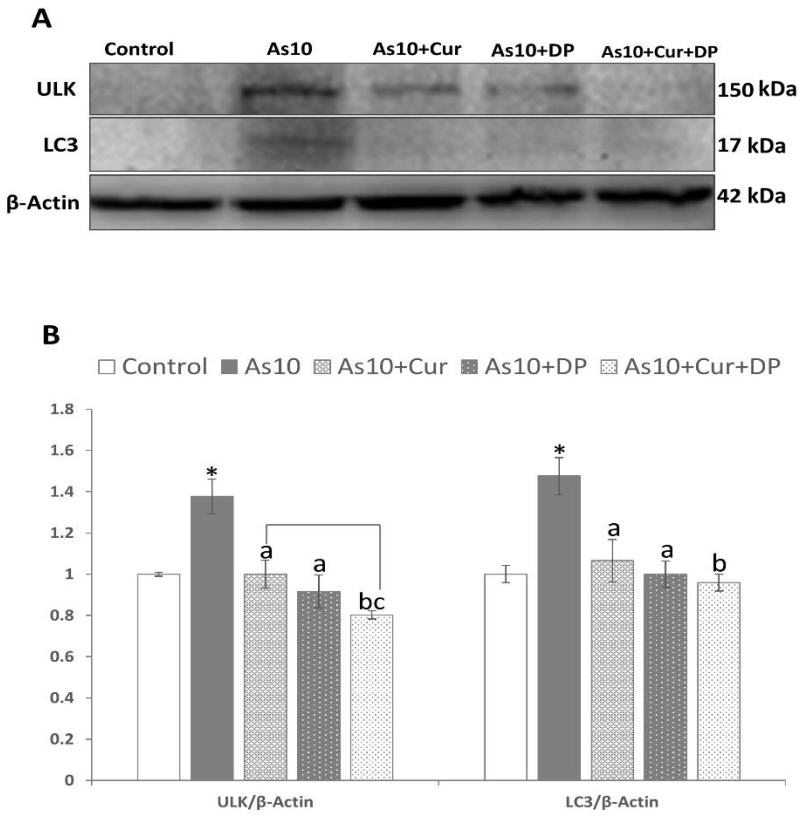

Fig. 4.9 Western blot analysis for the expression of autophagy related proteins in PC12 cells after being exposed to curcumin (2.5 µM), D-pinitol (5.0 µM) or a combination of the both with As^{3+} (10 µM) for 24 h. **(A)** The representative images (cropped) of immunoblotting for the expressions of ULK1 and LC3. **(B)** Relative density of each protein band to β-Actin. Each experiment was conducted at least 3 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=3). * denotes significance at $p<0.05$ compared to control group; [a] denotes significance at $p < 0.05$ compared to As^{3+} treated group; [b] denotes significance at $p < 0.01$ compared to As^{3+} treated group; [c] denotes significance at $p < 0.05$ among co-treatment groups (curcumin+As^{3+}, D-pinitol+As^{3+}, curcumin+D-pinitol+As^{3+}-treated groups). [Cur=curcumin, DP=D-pinitol]

4.4 Discussion

The results obtained during the present study indicate that the natural bioactive compound D-pinitol can contribute in the cytoprotection of natural dietary polyphenol curcumin against As^{3+}-induced toxicity in PC12 cells. Results also demonstrate that their combination cytoprotective effect against As^{3+} was higher than that of their individual effect and comparable to the control group. Recently, several reports showed that combination treatment of natural

bioactive compounds has strong ameliorating effects against environmental toxicants (Vetvicka and Vetvickova, 2012) and many human diseases (Ahmed et al., 2019; Zhang et al., 2015). For example, Sehgal et al. (2011) demonstrated that curcumin and piperine combinedly ameliorated benzo(a)pyrene induced DNA damage in lungs and livers of mice. Therefore, for the first time we explored the combined effect of curcumin and D-pinitol against As^{3+} toxicity in PC12 cells and investigated their regulatory roles in the underlying molecular mechanism/s. In this study, As^{3+} induced cytotoxicity and caused cell death in PC12 cells by reducing cell viability (Fig. 4.1C) and increasing LDH activity (Fig. 4.2A) significantly compared with the control. These findings were in agreement with the other *in vitro* studies that demonstrated As^{3+} exposure significantly decrease the cell viability and increase the LDH activity in embryonic fibroblast cells (Perker et al., 2019) and PC12 cells (Rahman et al., 2018). Interestingly, curcumin treatment in the background of As^{3+} exposure improved the cell viability and attenuated the LDH activity at $p<0.05$ significant level. The combined treatment of curcumin and D-pinitol in the background of As^{3+} exposure showed higher improvement in cell viability and effectively reduce the LDH activity at $p<0.01$ significant level comparing to only As^{3+} exposure (Figs. 4.1C and 4.2A). These results supported that curcumin and D-pinitol efficiently save PC12 cells from As^{3+}-induced cell death and cytotoxicity.

Our results also revealed that As^{3+} exposure in PC12 cells induced oxidative stress and negatively affect the total GSH content which is functioned as an essential antioxidant and oxidative stress marker (Fig. 4.4). Similar findings of the reduction of GSH content upon As^{3+} exposure was reported by Wang et al. (2015) and Hall et al. (2013). The significant reduction of the GSH content is an indication of oxidative damage in cells (Rose et al., 2012). Curcumin or D-pinitol individual treatment against As^{3+} exposure showed significant ($p<0.05$) improvement in GSH content compared to the As^{3+}-treated cells. However, the combination treatment of curcumin and D-pinitol against As^{3+} exposure showed higher improvement

($p<0.01$ against As^{3+}) in GSH content and anti-oxidative effect which were comparable to the control group (Control, 3.25±0.13 vs Cur+DP+As^{3+}, 3.07±0.16). Therefore, it was postulated that curcumin and D-pinitol could successfully save PC12 cells from As^{3+}-induced oxidative stress stimulated damage.

As^{3+} exposure results in excessive ROS formation and can cause damage in biomolecules including DNA (Hughes, et al., 2011; Susan et al., 2019). In our study, DNA fragmentation was investigated upon As^{3+} exposure in PC12 cells by carrying out agarose gel electrophoresis and the results demonstrated that As^{3+} treatment significantly increased the DNA fragmentation in PC12 cells compared to the control cells (Fig. 4.3). Similarly, As^{3+} caused DNA damage to ovarian cells were reported formerly by Akram et al., (2009). In another study, it was shown that As^{3+}-induced oxidative DNA damage and fragmentation in murine Sertoli cells (Khan et al., 2013). Curcumin or D-pinitol individually reduces the As^{3+}-induced DNA fragmentation at $p<0.05$ significant level and combinedly at $p<0.01$ significant level (Fig. 4.3). These results indicated that both natural dietary compounds have antigenotoxic effect and their combined effect was higher than their individual effect. The genoprotective effect of curcumin and D-pinitol was formerly reported by Khan et al. (2013) and Rahaman et al. (2020), respectively. In our study, curcumin and D-pinitol exhibited their excellent genoprotective effect against arsenic toxicity and showed a synergistic effect where D-pinitol enhances the protective effect of curcumin.

Previous studies already reported that As^{3+} exposure induced apoptotic cell death in various cellular systems (Akao et al., 2000; Mu et al., 2019; Roy et al., 2016; Wang et al., 2015). In the present study, we also checked As^{3+}-induced apoptotic cell death in PC12 cells by performing flow cytometry and results revealed that As^{3+} exposure significantly increased the apoptotic

cell death in PC12 cells as compared to control cells (Fig. 4.5). Curcumin alone or in combination with D-pinitol effectively suppressed the As^{3+}-induced apoptotic cell death.

D-pinitol alone did not significantly reduce the As^{3+}-induced apoptosis, but it showed a synergistic anti-apoptotic effect with curcumin and enhances curcumin's cytoprotection (Fig. 4.5). Flow cytometry results indicated that curcumin has strong antiapoptotic actions than D-pinitol against As^{3+}. Furthermore, protein expressions of western blotting analysis also further confirmed that As^{3+} persuaded apoptotic cell death in PC12 cells via mitochondrial pathway (Figs. 4.7 and 4.8). This consideration also supported by our obtained DNA fragmentation results (Fig. 4.3), because DNA fragmentation is considered as a hallmark of apoptosis (Arora and Tandon, 2015). Western blotting results revealed that As^{3+} exposure in PC12 cells significantly upregulated pro-apoptotic p53 protein expression, which further activated the pro-apoptotic Bax protein and the activation of Bax promotes mitochondrial membrane permeability (Chipuk et al., 2004) and cytochrome c release into cytosol from mitochondria (Rahman et al., 2017). Cytosolic cytochrome c is an important feature of mitochondria-mediated intrinsic apoptosis because it plays a critical role in apoptosome formation by binding with cytosolic Apaf-1 and activates caspase cascade reaction and finally cause caspase-dependent apoptotic cell death (Banik et. al., 2019; Fulda and Debatin,2006; Rahman et al., 2017). In this study, a significant upregulated expression of pro-apoptotic Bax, cytosolic cytochrome c, caspase 9 and cleaved caspase 3, and downregulated expression of anti-apoptotic Bcl2, Bclx and XIAP were observed upon As^{3+} exposure in PC12 cells (Figs. 4.7 and 4.8) which are in accordance with the previous studies. However, curcumin alone or in combination with D-pinitol in the background of As^{3+} exposure significantly downregulated the expression of pro-apoptotic Bax, cytosolic cytochrome c, caspase 9 and cleaved caspase 3, and effectively upregulated the expression of anti-apoptotic Bcl2, Bclx and XIAP (Figs. 4.7 and 4.8). Western blotting results suggested that As^{3+} exposure induced cell death in PC12 cells via mitochondria-

mediated intrinsic apoptotic pathway and curcumin and D-pinitol successfully protected PC12 cells from As^{3+}-induced apoptosis by regulating protein expression positively.

Our findings exhibited good agreement with recently reported anti-apoptotic effect of curcumin against $A\beta_{25-35}$-induced apoptosis (Xu et al., 2019) and anti-apoptotic effect of D-pinitol against As^{3+}-induced apoptosis (Rahaman et al., 2020) in PC12 cells.

Several reports demonstrated that apoptosis can also be initiated via autophagy (Rahman et al., 2018; Roy et al., 2014) where ROS plays a crucial role to inhibit pro-survival mTOR and Akt protein and initiate the autophagic process through the PI3K/mTOR/AKT pathway (Roy et al., 2014). In our study, we also found that As^{3+} exposure significantly inhibited pro-survival mTOR and Akt protein expression in PC12 cells, and curcumin and D-pinitol alone or in combination effectively restore those both protein expression (Fig. 4.6). We also further inspected whether As^{3+} could induce autophagy or not by checking autophagy initiator ULK1 and autophagy executioner LC3 protein. Interestingly, both autophagic protein expressions were significantly upregulated upon of As^{3+} exposure in PC12 cells, and curcumin and D-pinitol alone or in combination effectively inhibited the autophagic ULK1 and LC3 protein expressions (Fig. 4.9). These results suggested that curcumin and D-pinitol efficiently save PC12 cells from As^{3+}-triggered autophagic cell death as well as from autophagy-initiated apoptotic cell death.

Researchers already reported that curcumin and D-pinitol exhibited excellent antioxidant potentials via keap1/Nrf2 antioxidant signaling pathway (Dai et al., 2016; Rahaman et al., 2020; Xu et al., 2019). Previous report already has been established that transcription factor Nrf2 acted as an excellent cytoprotective factor by regulating the expression of genes coding for antioxidant and detoxifying proteins (Loboda et al., 2016). For the proper understanding of cytoprotection of curcumin and D-pinitol against As^{3+}, the expression level of Nrf2 antioxidant

promoter protein was investigated in treated PC12 cells. As^{3+} exposure in PC12 cells damagingly suppressed the expression of Nrf2 and subsequently disrupted the cellular antioxidant defense mechanism (Fig. 4.6), which is strongly supported by our obtained intracellular GSH results (Fig. 4.4). However, curcumin and D-pinitol alone or in combination significantly recovered the expression of Nrf2 and efficiently protected PC12 cells from As^{3+}-induced oxidative damage which is in agreement with the previous studies (Rahaman et al., 2020; Xu et al., 2019).

Previously, Fu et al., (2016) demonstrated that curcumin alleviated H_2O_2-induced toxicity via MAP kinase pathway where ERK plays a vital role and worked as a pro-survival protein. D-pinitol-mediated upregulation of ERK in PC12 cells already reported by Rahaman et al. (2020). ERK is considered as an important member of MAPKs protein family for its regulatory role in cell survival, proliferation, and apoptosis. Thus, in this study, the cytoprotection of curcumin and D-pinitol against As^{3+} and the possible involvement of MAP kinase pathway also investigated. Results revealed that As^{3+} exposure in PC12 cells detrimentally suppressed the pro-survival ERK protein expression, and curcumin and D-pinitol effectively restored the expression of ERK (Fig. 4.6). The probable reason for the ERK protein restoration is that curcumin and D-pinitol could attenuate the As^{3+}-induced oxidative stress. However, the combination cytoprotective effect of curcumin and D-pinitol was non-significantly higher than individual treatment against As^{3+} exposure in PC12 cells.

In summary, our results exhibit that As^{3+} induced oxidative stress in PC12 and triggered cell death via both mitochondria-mediated intrinsic apoptosis and autophagy. Autophagy initially started as a cell survival mechanism and prolonged stress finally leads cells to death. The autophagy and apoptosis involved in As^{3+}-induced PC12 cell death may happen independently and/or cumulatively (Fig. 4.10). However, curcumin and D-pinitol alone or in combination efficiently protect PC12 cells from As^{3+}-induced cytotoxicity by boosting up cellular

antioxidant defense system where Nrf2 plays an essential role, improving intracellular GSH level, reducing DNA fragmentation, inhibiting autophagy and apoptosis (Fig. 4.10).

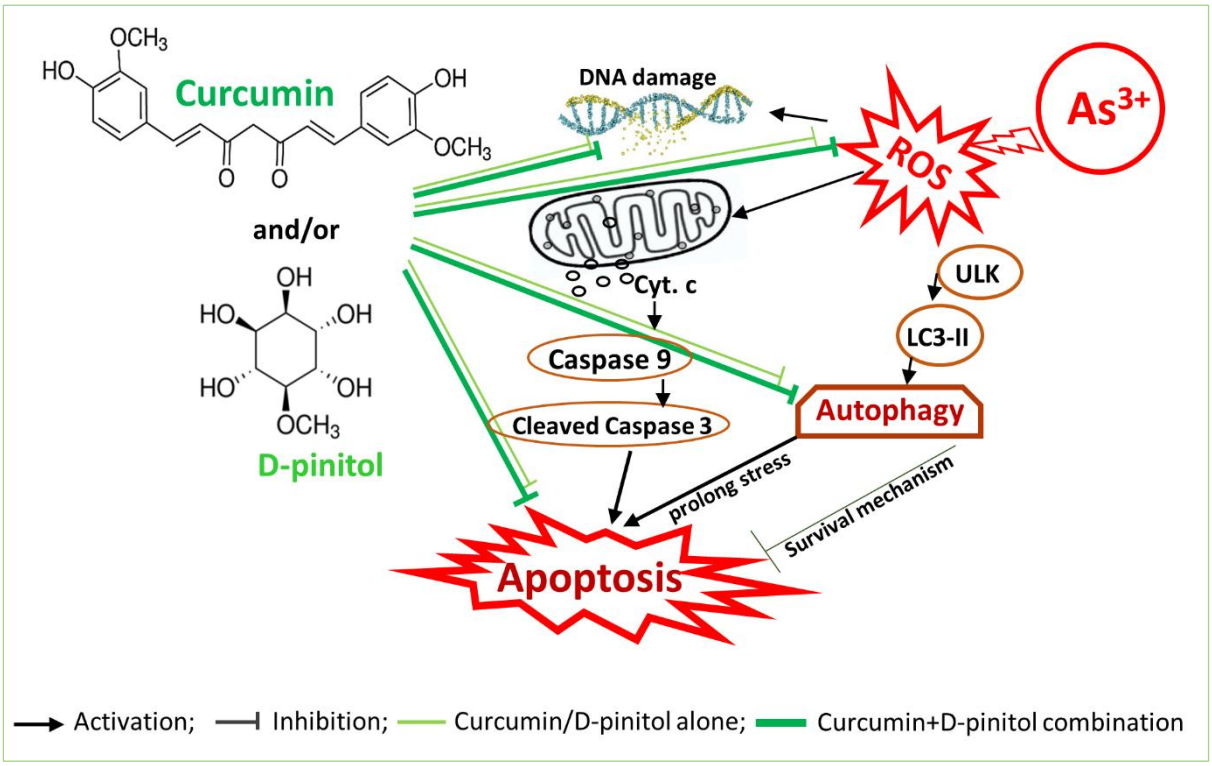

Fig. 10. Schematic diagram showing potential molecular mechanism/s of As^{3+}-induced toxicity and cytoprotection of curcumin and D-pinitol in PC12 cells upon co-exposure.

D-pinitol showed excellent cytoprotection against autophagic cell death caused by As^{3+} and curcumin showed effective cytoprotection against both programmed cell death mechanism of apoptosis and autophagy and has stronger apoptosis suppressive effect than D-pinitol. However, the combined cytoprotection of curcumin and D-pinitol against As^{3+}-triggered toxicity was higher than their individual cytoprotective effect.

4.5 Conclusion

The present study demonstrates that both natural dietary compound curcumin and D-pinitol acted as potential antioxidants and effectively inhibited As^{3+}-induced apoptotic and autophagic cell death where D-pinitol enhances the cytoprotection of curcumin against As^{3+}-induced

toxicity in PC12 cells. This study also explored the cellular alteration by As^{3+}-toxicity, and the complex mechanism/s involved in cytoprotection of curcumin and D-pinitol individually and combinedly in PC12 cells. Therefore, we may propose that natural dietary compound curcumin and D-pinitol will be a safe and potential therapeutic agent to combat As^{3+}-toxicity in the biological system. Our findings may provide useful information in further *in vivo* investigation for understanding the outcome of curcumin and D-pinitol treatment on other environmental toxicants.

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Chapter 5: Summary and general conclusion

In this study, the ameliorative effects of natural dietary bioactive compounds; Dpinitol and curcumin against arsenic toxicity were investigated using PC12 cells. Firstly, the protective effects of D-pinitol against arsenic toxicity in PC12 cells was examined. For this study, PC12 cells were treated with arsenic (5 µM) and/or different concentrations of D-pinitol (1, 5, 50 µM) for 48 h. The major findings in this study demonstrated that co-exposure of D-pinitol with arsenic increases cell viability, decreases DNA damage and protects PC12 cells from arsenic-induced cytotoxicity by incresasing GSH level and GR. Arsenic (5 µM) induced autophagic cell death, as well as partial apoptotic cell death in PC12 cells. The mechanism of the cell death was considered to be the possibility of caspase independent but Bax/Bcl-x dependent apoptosis on the basis of the changes of factors of the endogenous apoptotic pathway. Arsenic accelerates the generation of intracellular ROS which further initiates autophagy and finally apoptosis through the activation of proapoptotic proteins. Whereas, D-pinitol protects PC12 cells from arsenic-induced toxicity by maintaining homeostasis of ROS where Nrf2 keeps pivotal role. D-pinitol significantly increased the levels of GSH and GR which alleviated ROS induced oxidative stress and inhibited excessive autophagy and subsequently apoptosis. This consideration was proved by the up-regulating expressions of proteins, mTOR, p-mTOR, Akt, p-Akt, Bcl-x, ERK1, NF-κB, Nrf2 and GR, and down-regulating expressions of the proteins LC3, p53, Bax and cytochrome c with the co-exposure of D-pinitol and arsenic. Findings of this study suggested that D-pinitol protects PC12 cells from arsenic (5 µM) induced cytotoxicity.

Secondly, the protective effects of curcumin against arsenic toxicity in PC12 cells was investigated. For this investigation, PC12 cells were treated with arsenic (10 µM) and/or curcumin (2.5 µM) for 24 h. Obtained results from this study demonstrated that arsenic (10 µM) induced cell death in PC12 cells through both mitochondrial apoptosis and autophagy.

The mechanism of this apoptotic and autophagic cell death in PC12 cells may occur independently as well as cumulatively. However, curcumin (2.5 μM) protects PC12 cells from arsenic-induced toxicity via maintaining the oxidant/antioxidant homeostasis where Nrf2 plays the fundamental roles. These findings also demonstrated that curcumin could efficiently protect PC12 cells from arsenic-induced oxidative damage and cytotoxicity possibly by limiting ROS generation and accumulation, boosting up antioxidant defense systems, and modulating both autophagy and apoptosis-related protein expressions in the protective and restoring way. Thus, it indicated that the natural dietary compound; curcumin worked as a strong antioxidant, antiapoptotic and anti-autophagic agents against arsenic toxicity. These findings recommend that curcumin will be potential and safe therapeutic agents to combat the arsenic toxicity in humans as well as in other biological systems.

Thirdly, the combined effects of curcumin and D-pinitol against arsenic toxicity in PC12 cells was also investigated. In this study, PC12 cells were treated with curcumin (2.5 μM), D-pinitol (5.0 μM) or a combination of both with arsenic (10 μM) for 24 h. Results of this combination study exhibited that arsenic induced oxidative stress in PC12 and triggered cell death via both mitochondria-mediated intrinsic apoptosis and autophagy. Autophagy initially started as a cell survival mechanism and prolonged stress finally leads cells to death. The autophagy and apoptosis involved in arsenic-induced cell death may happen independently and/or cumulatively. However, curcumin and D-pinitol alone or in combination efficiently protect PC12 cells from arsenic-induced cytotoxicity by boosting up cellular antioxidant defense system where Nrf2 plays an essential role, improving intracellular GSH level, reducing DNA fragmentation, and inhibiting autophagy and apoptosis. D-pinitol showed excellent cytoprotection against autophagic cell death caused by arsenic and curcumin showed effective cytoprotection against both programmed cell death mechanism of apoptosis and autophagy and has stronger apoptosis suppressive effect than D-pinitol. Moreover, the

combined cytoprotection of curcumin and D-pinitol against arsenic-triggered toxicity was higher than their individual cytoprotective effect. However, the protective effect was not so strong synergistic as expected. In the future, it will be necessary to conduct a more detailed investigation about the ratio of exposure dose for the two compounds, D-pinitol and curcumin, and their exposure time.

Finally, the findings of this study demonstrated that both natural dietary compounds; curcumin and D-pinitol acted as potential antioxidants and effectively inhibited arsenic-induced apoptotic and autophagic cell death where D-pinitol enhances the cytoprotection of curcumin against arsenic-induced toxicity in PC12 cells. This study also explored the cellular alteration by arsenic-toxicity, and the complex mechanism/s involved in cytoprotection of curcumin and D-pinitol individually and combinedly in PC12 cells. From these comprehensive data, we may propose that natural dietary compound curcumin and D-pinitol will be safe and potential therapeutic agents to combat arsenic-toxicity in the biological system. Our findings may provide useful information in further *in vivo* investigation for understanding the outcome of curcumin and D-pinitol treatment on other environmental toxicants. The author will end the thesis with hope that the arsenic poisoning of people living in many arsenic-contaminated areas including Bangladesh will be alleviated.