Chapter-4

Determination of the efficacy of different solvent extracts of *Andrographis paniculata* Nees on Cr (VI) induced tissue toxicity

Contents

4.0 Introduction

The occurrences of heavy metals in the environment and their enormous industrial use have led to an increase in the frequency of the human organ toxicity. Among different heavy metals, chromium is one of the important heavy metal for development of toxicities in both terrestrial and aquatic environments (Sadek, 2014). It is also a trace element which is extracted from chromate (Andleeb, 2014). Chromium is present in the environment in various oxidation states. Trivalent chromium [Cr (III)] is extensively used as supplement and also a good element for glucose/insulin homeostasis (Ghalehkandi, 2014); whereas tetravalent [Cr (IV)] and hexavalent chromium [Cr (VI)] is highly toxic for their easy permeation at physiological pH through the permease system (Ghalehkandi, 2014). Toxicity of liver and kidney are commonly observed in people with high risk of exposure to Cr (VI) owing to their occupation or in animals as a result of environmental exposure (Andleeb, 2014). The functional difference between Cr (III) and Cr (VI) is mainly dependent upon the selective permeability of cell membrane to particular ionic state of chromium (Wise et al, 2014). Metallic compounds of Cr (VI) are more permeable than trivalent chromium Cr (III) and easily transported across plasma membranes with the help of non-specific anion transporters (Yasmina et al, 2015). Crucial factor for development of Cr (VI) induced tissue toxicity is cell membrane damage (Dey et al, 2003). Intracellular Cr (VI) reduction, through generation of metabolic reactive intermediates like Cr (V) , Cr (IV) and stable Cr (III) (Sun et al, 2015), leads to excess accumulation of highly reactive oxygen species (ROS) and cause damages to liver, pancreas, kidney and cerebellum (Dey et al, 2016).

The mechanism behind the Cr (VI) mediated alteration of mitochondrial bioenergetics to develop organ toxicity is not clearly explained. Strongly oxidizing Cr (VI), during intracellular reduction, shunts electrons across electron transport chain and generates reactive chemicals like hydroxyl radical (-$OH^{\cdot}$) (Shi et al, 1990a). Trivalent chromium (III) hinders ATP synthesis forming stable complexes with precursors and enzymes required for generation of energy ATP (Debetto et al, 1982; Bianchi et al, 1982). Enhanced lipid peroxidation in mitochondria of rat brain and liver is observed after oral administration of low-dose of hexavalent chromium (Travacio et al, 2000).

Various traditional plants are used for preparation of folk medicines since ancient times. Active plant ingredients responsible for such therapeutic effects have created much research interest as potential anti-peroxidative agent (Lee and Park, 2002). *Andrographis paniculata* has been acclaimed worldwide as a useful plant source for preparation of many indigenous herbal elixirs for curing many illnesses. *Andrographis paniculata*, also known as 'Kalmegh', is used in the Ayurvedic system of medicine. *Andrographis paniculata* has wide areas of application of its pharmacotherapuetic properties in the Ayurveda, such as antipyretic, analgesic, wound healing, hepatoprotective agent (Vedavathy and Rao, 1992) and choleretic (Shukla et al, 1992). Many herbal preparations are assumed to exert their protective effects by strengthening antioxidant defence mechanism via detoxification of chemicals. They also potentiate immune system of the body by scavenging highly reactive free radicals and modulating carcinogen.

The present experiment intends to explore the anti-peroxidative activity of *Andrographis paniculata* extracts prepared indifferent solvents against oxidative damage in rat liver and lungs mitochondria exposed to Cr (VI). Different phytochemicals present in the plant are not equally soluble to one particular solvent. Therefore, to extract effective active substances from the plant, various solvents like water, methyl alcohol and petroleum ether are utilised to select a best suited solvent either single or most effective proportion of solvent mix.

4.1 Methodology

4.1.1 Chemicals

Required amount of various chemicals, reagents and consumables were collected as described in chapter II.

4.1.2 Collection, Identification and Preservation of Plant material

Fresh *Andrographis paniculata* plant was collected from the campus of Indian Institute of Technology, Kharagpur, Paschim Medinipur, West Bengal, India. Taxonomical identification was done by the Department of Botany, Vidyasagar University. Healthy and fresh leaves of the plant were cleaned and washed thrice to remove soil dirt and rinsed with sterile distilled water. Afterwards, leaves were air dried under a shed. Dried leaves were grinded into fine powder and stored at $4^{o}C$ in properly labelled airtight container for further use.

Andrographis paniculata Nees

Kingdom:	Plantae
(unranked):	Angiosperm
(unranked):	Eudicots
(unranked):	Asrerids
Order:	Lamiales
Family:	Acanthaceae
Genus:	*Andrographis*
Species:	*A. paniculata*

Figure1: Picture of *Andrographis paniculata* Nees plant

4.1.3 Preparation of Aqueous extracts

Collected and grinded fine plant powder amounting 2 gram was mixed with 20 ml of sterile distilled water and kept on a rotary shaker for 12 hours at 30^{o}C. Then, it was filtered by using of Whatman No. 1 filter paper. Then filtrate was centrifuged at 2000 rpm for 10 minute and supernatant was collected and stored at 4^{o}C for further use (Zhang and Tan, 1996). Supernatant was evaporated completely to make it dry. Then 20 mg of dry powder in water extract of *Andrographis paniculata* was mixed in 1 ml of double distilled water to prepare aqueous AP extract for administration to rats.

4.1.4 Preparation of Methanol extracts

10 gram of grinded powder of *Andrographis paniculata* plant leaves were soaked in 30ml of 70% methanol and were kept overnight at 30^{o}C on a rotary shaker for 12 hours. On the flowing day methanol was added to make the same volume and placed on a rotary shaker for another 12 hours at 30^{o}C. Then it was filtered through Whatman No. 1 filter paper and centrifuged at 2000 rpm for 10 minute. Then the supernatant was collected and stored at 4^{o}C for further use. Then supernatant was dried completely by evaporation. Then 30mg of dry powder extract was re-suspended in 1ml of 70% methanol to make final concentration of 30 mg/ml for supplementation in rats.

4.1.5 Preparation of Petroleum-ether extracts

10 gram of grinded powder plant leaf materials were mixed with 20 ml of petroleum-ether solvent and kept on a rotary shaker for 12 hours at 30^{o}C. Then, it was filtered by the using of Whatman No. 1 filter paper and centrifuged at 2000 rpm for 10 minute. Then the collected supernatant was allowed to dry completely by evaporation technique. Then 30mg of dry extract was re-suspended in 1ml of Petroleum-ether. So the final concentration of the extract was 30 mg/ml.

4.1.6 Animals and diet

Animals and diet were described in chapter-II

4.1.7 Mode of treatment: Rats were divided into eight groups of almost equal average body weight. The rats of seven test groups were injected (i.p.) with $K_2Cr_2O_7$ at a dose of 0.8 mg per 100 g body weight per day (20% LD_{50}) for 28

days, as described earlier (Dey et al, 2003). Remaining 8th group of the rats were given only the vehicle (0.9% NaCl), designated as vehicle control. Out of seven chromium injected groups,1st group of rats served as Chromium control, 2nd group as the supplemented AE-AP250 (250 mg/kg body weight/day of aqueous extract of AP), 3rd group as the supplemented AE-AP500 (500 mg/kg body weight/day of aqueous extract of AP), 4th group as the supplemented ME-AP250 (250 mg/kg body weight/day of methanol extract of AP), 5th group as the supplemented ME-AP500 (500 mg/kg body weight/day of methanol extract of AP), 6th group as the supplemented PEE-AP250 (250 mg/kg body weight/day of petroleum-ether extract of AP) and 7th group as the supplemented PEE-AP500 (500 mg/kg body weight/day of petroleum-ether extract of AP) supplemented daily with prepared extracts of *Andrographis paniculata* at an interval of six hours after treatment of $K_2Cr_2O_7$ for a period of 28 days.

4.1.8 Sacrifice of Animals and sample preparation

Sacrifice and sample preparation were described in chapter-II

4.1.9 Homogenization of tissues

Tissue homogenization were described in chapter-II

4.1.10 Isolation of Mitochondria

Mitochondria isolation were described in chapter-III

4.1.11 Analytical methods

Following methods like Lipid peroxidation, Conjugated dienes, NO release, SOD, GSH, GSSG, GPx, GR, GST and protein were described in chapter-II & III

4.1.12 Statistical Analysis

Statistical data analysis were described in chapter-II

4.2 Results

The results those are depicted in figures 2 and 3 (A, B & C) the changes of MDA and conjugated dienes concentration were elevated significantly in response to chromium; but after the supplementation of different doses of aqueous, methanol and petroleum ether extracts of *Andrographis paniculata*, it has been observed that AP 500 of aqueous and methanol extracts of *Andrographis paniculata* have the potent role to counteract the chromium toxicity in liver and lungs mitochondria.

The changes of Nitric Oxide production (NO) and the SOD activity in response to chromium and the supplementation role of different doses of aqueous, methanol and petroleum ether extracts of *Andrographis paniculata* are elaborated in figures 4 and 5 (A, B & C). The results showed that NO production has increased and SOD activity significantly decreased in response to chromium in liver and lungs mitochondria. On the other hand, AP_{500} of aqueous and methanol extracts of *Andrographis paniculata* have showed the potent role to counteract the chromium-induced toxicity.

GSH and GSSG activity level are markedly low in tested tissue mitochondria after chromium injection as per figures 6 and 7 (A, B & C), but after supplementation with the different doses of aqueous, methanol and petroleum ether extracts of *Andrographis paniculata,* it was found that AP 500 of aqueous and methanol extracts of *Andrographis paniculata* showed the maximum role to reduce the chromium induced tissue toxicity.

GPx, GR and GST activities have diminished noticeably after chromium treatment in both tissue mitochondria as noticed in figures 8, 9 and 10 (A, B & C). But it has been observed that the supplementation of AP 500 of aqueous and methanol extract of *Andrographis paniculata* in Cr (VI) injected rats, activity of the above enzymes regained towards normal control to some extent.

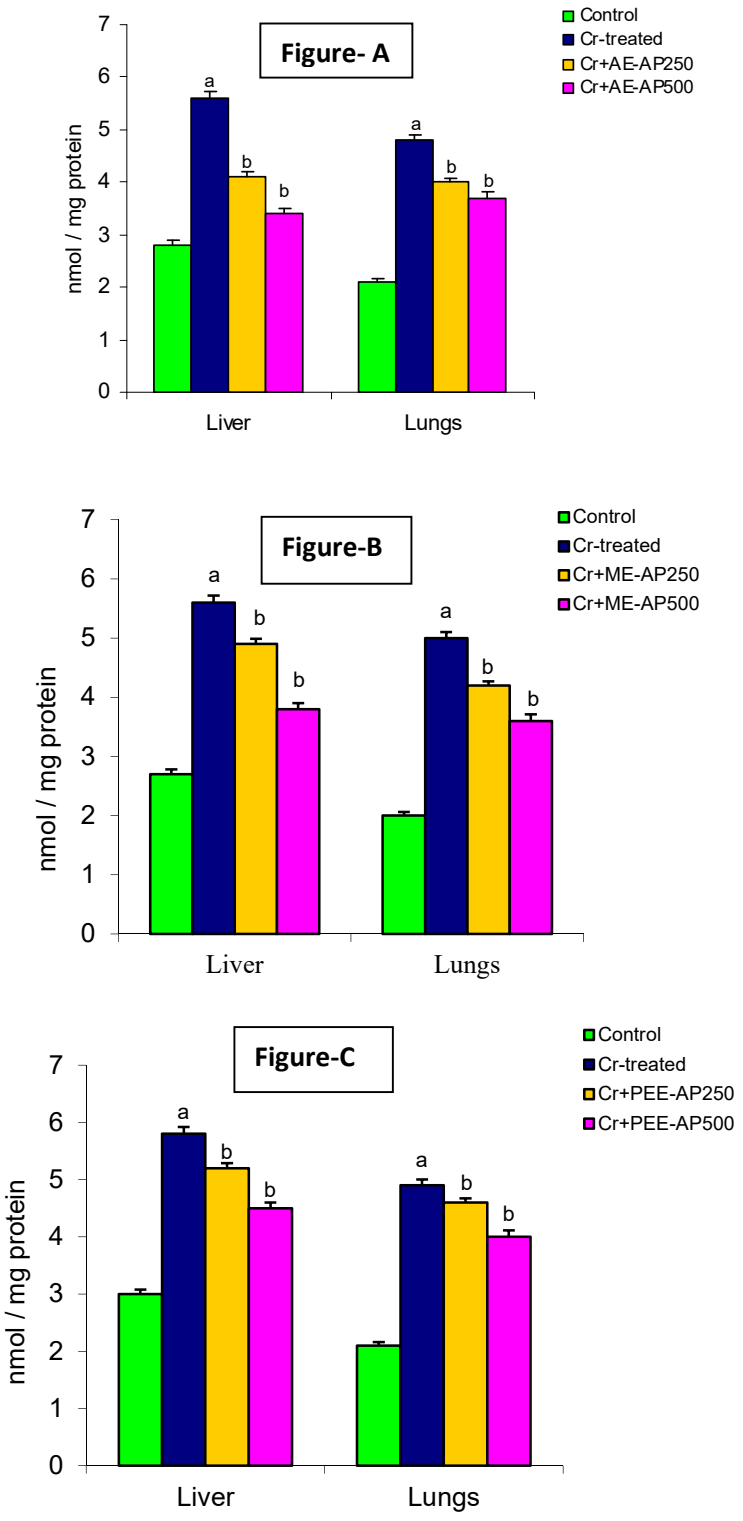


Figure 2 (A, B & C): Shows the MDA level in liver and lungs mitochondria after administration of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 & 500 in chromium treated rats. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.

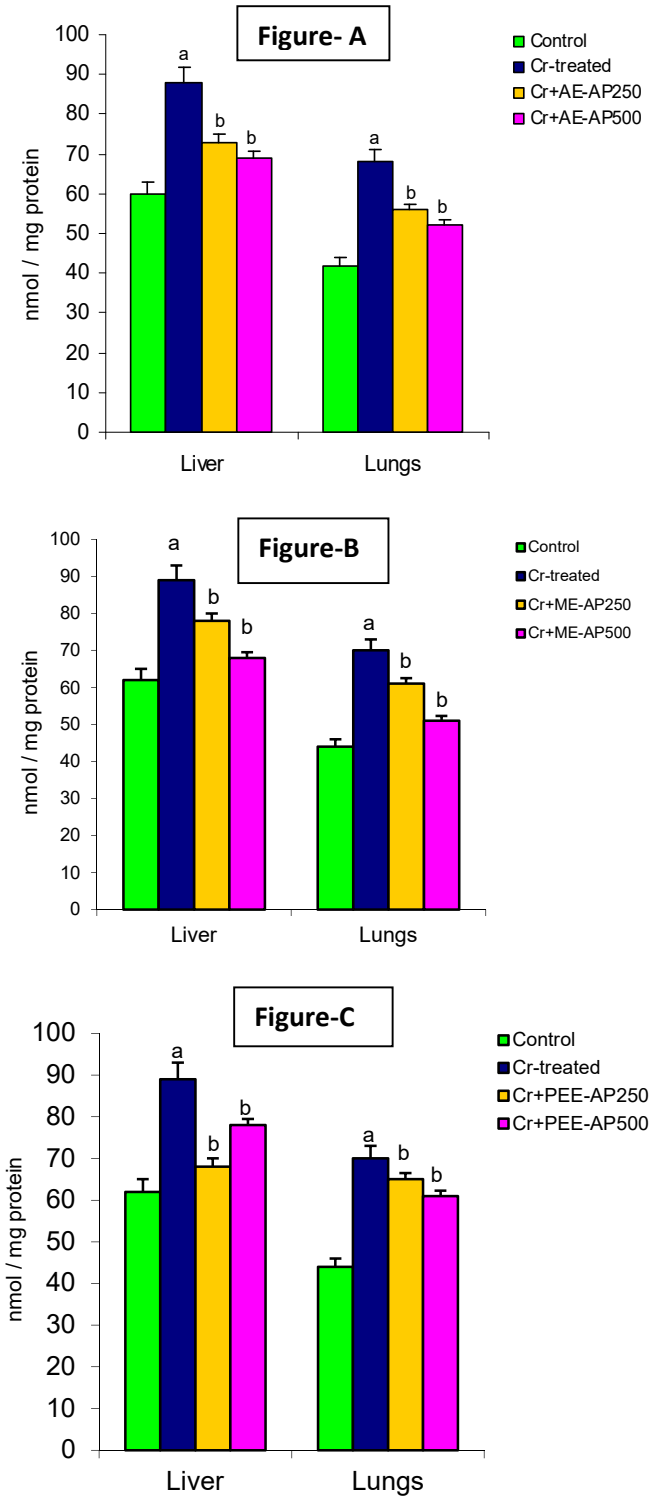


Figure 3 (A, B & C): Represent variations of the conjugated dienes content in liver and lungs mitochondria after supplementation of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 &500 in chromium treated rats. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.

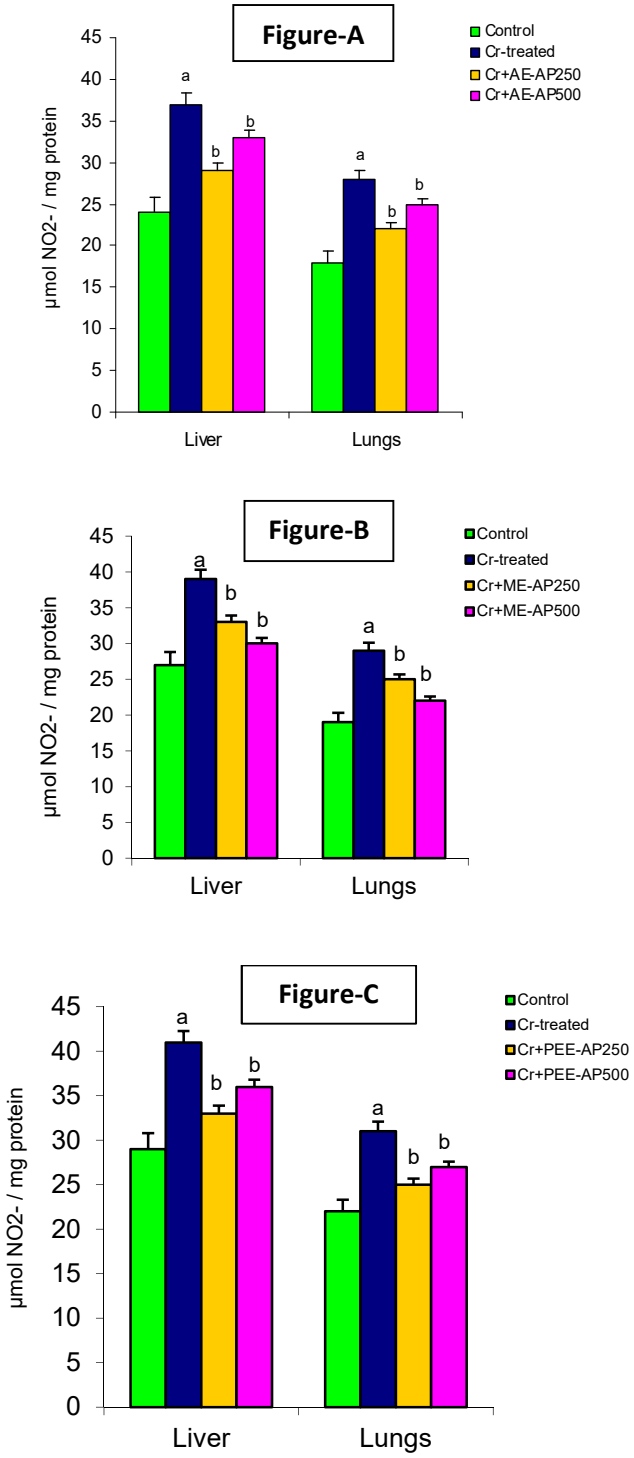


Figure 4 (A, B & C): Shows the production of Nitric Oxide (NO) in tissue mitochondria after co-administration of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 & 500 in chromium treated rats. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.

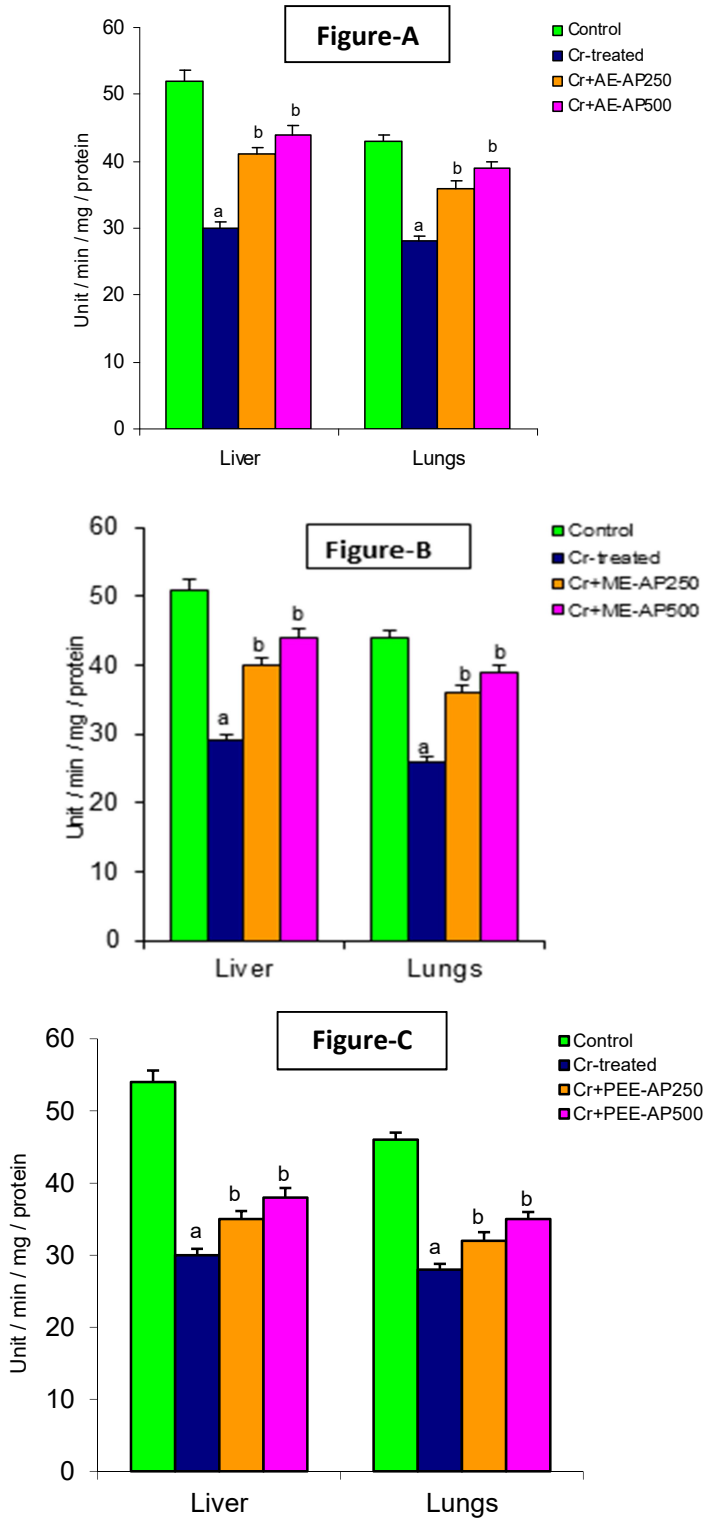


Figure 5 (A, B & C): variation of the SOD activity in liver and lungs mitochondria after administration of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 & 500 in chromium treated rats. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.

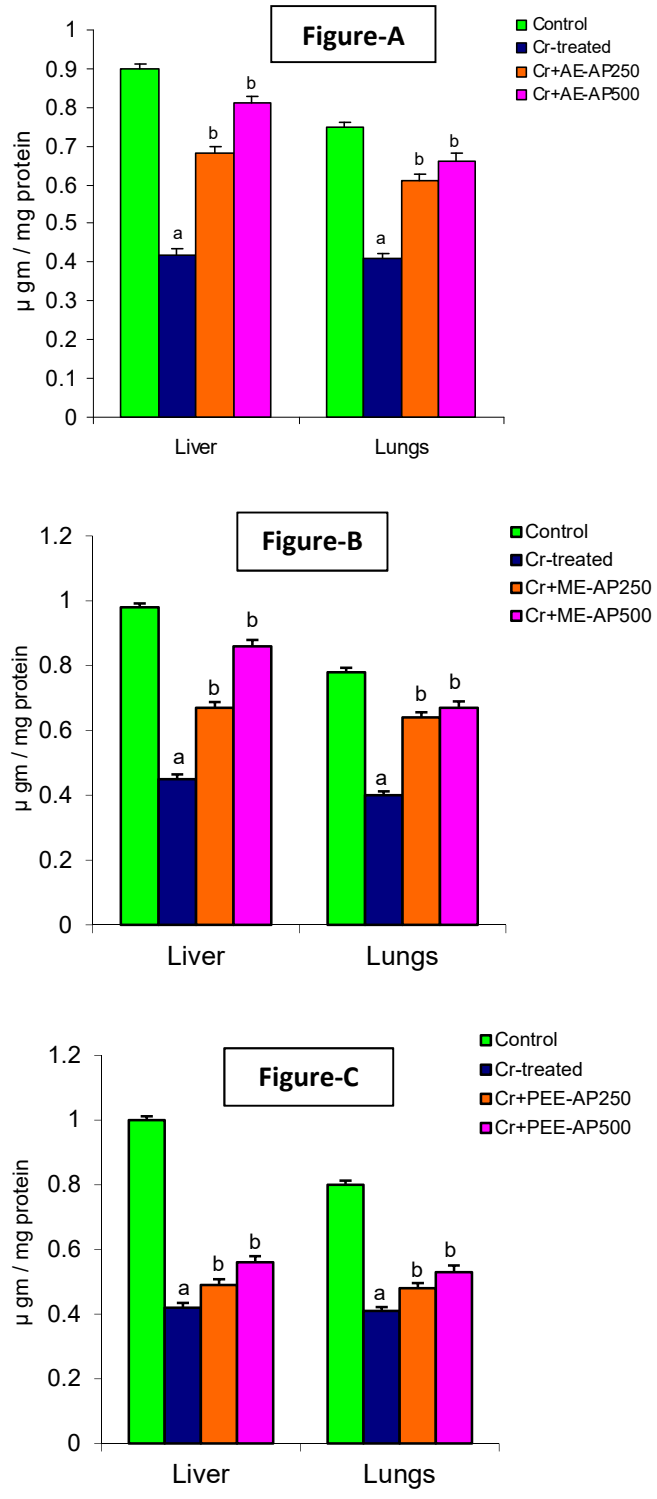


Figure 6 (A, B & C): Changes of the GSH level in liver and lungs mitochondria after co-administration of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 & 500 in chromium treated rats. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.

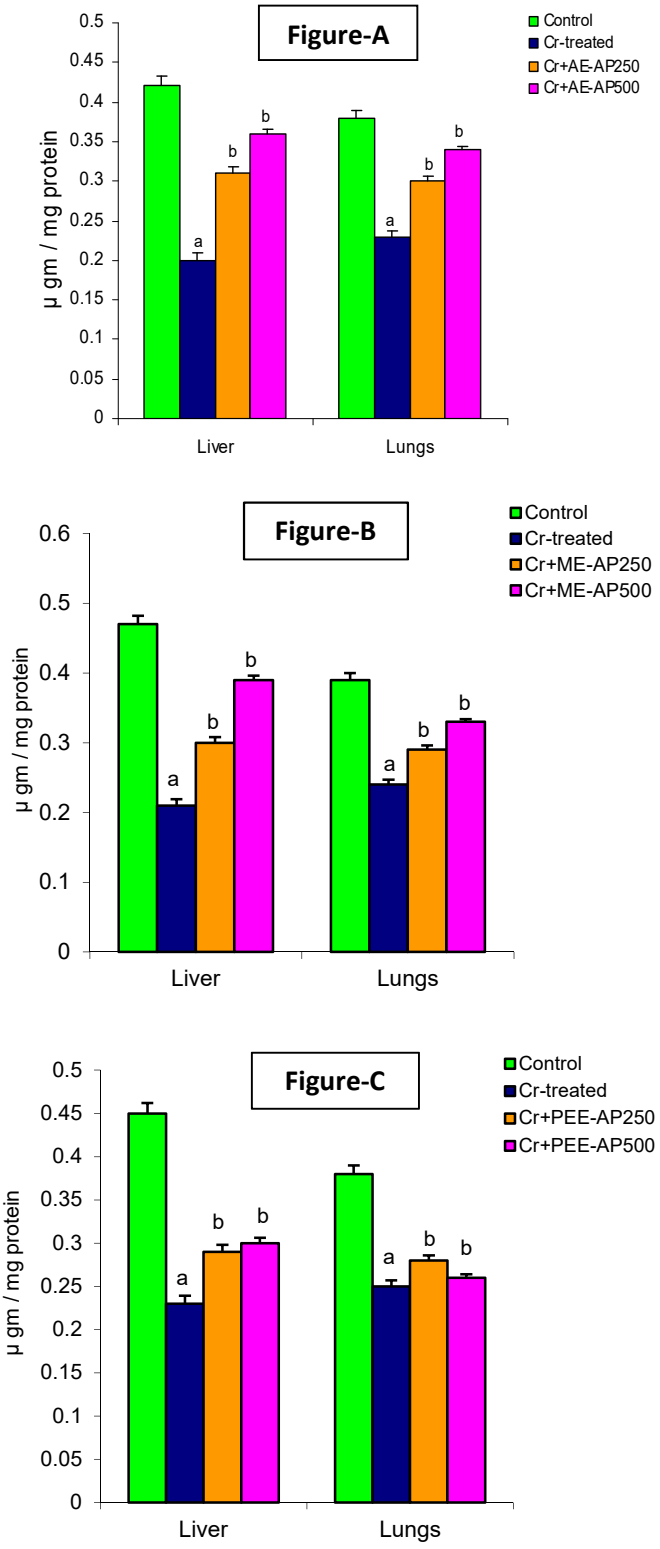


Figure 7 (A, B & C): Shows GSSG level in tissue mitochondria after co-administration of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 & 500 in chromium treated rats. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.

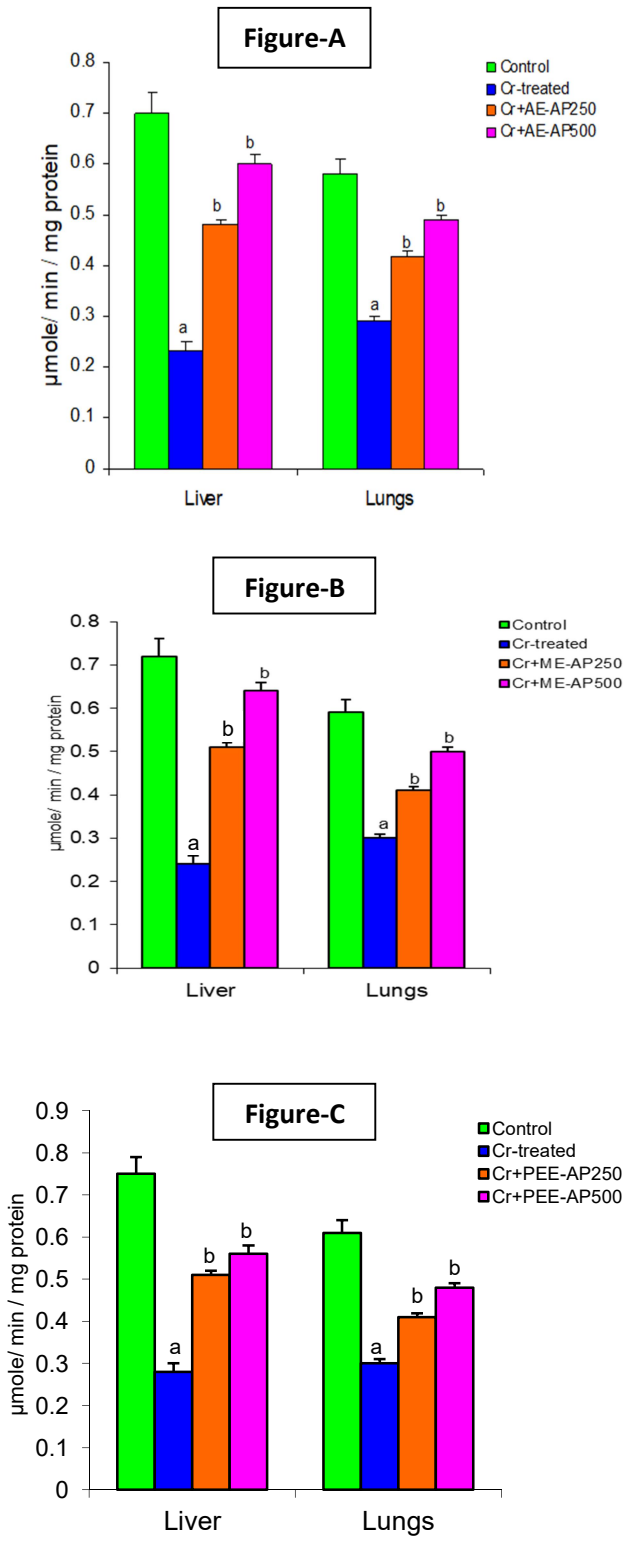


Figure 8 (A, B & C): Changes of the activity of GPx in liver and lungs mitochondria after co-administration of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 & 500 in chromium treated rats. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.

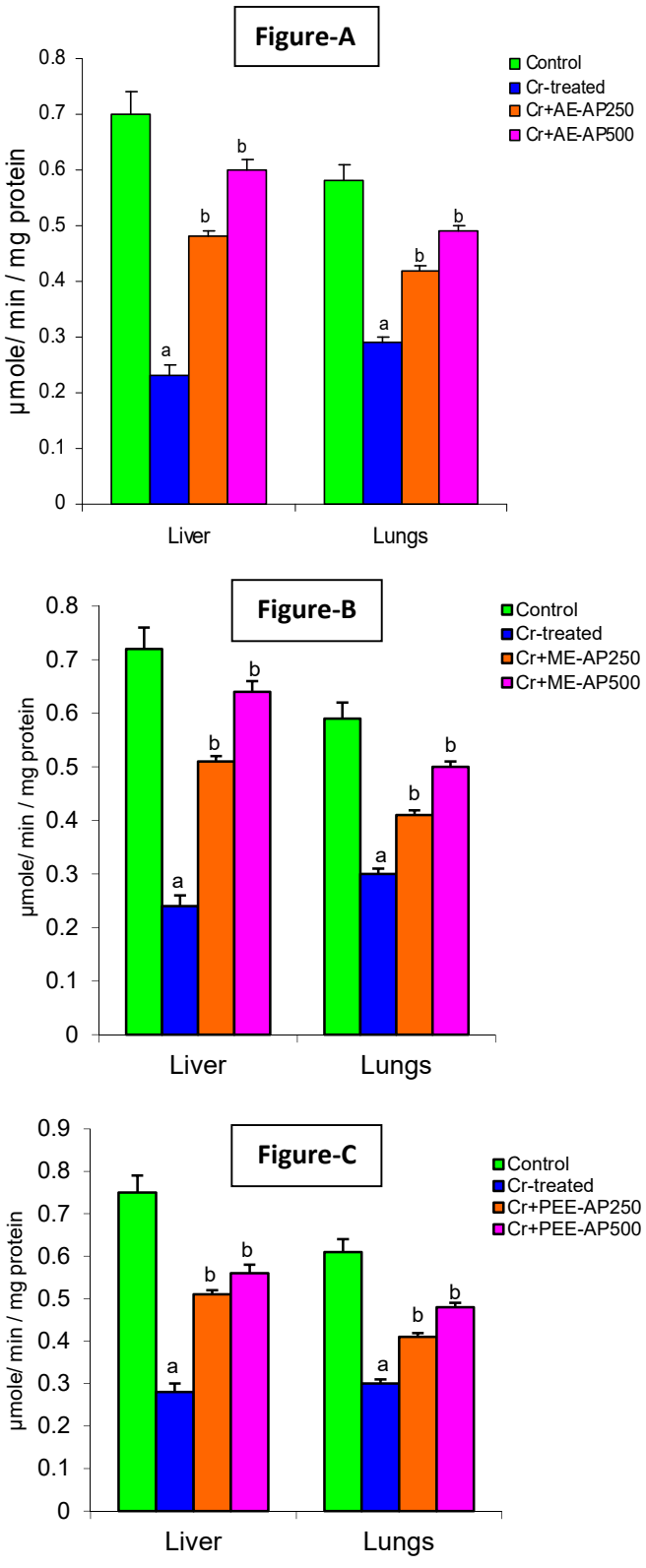
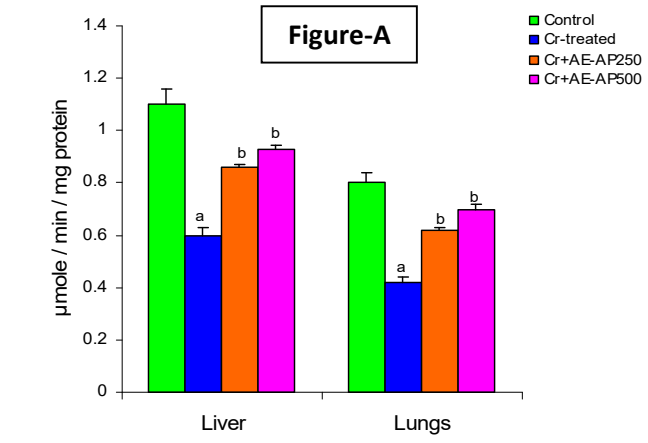
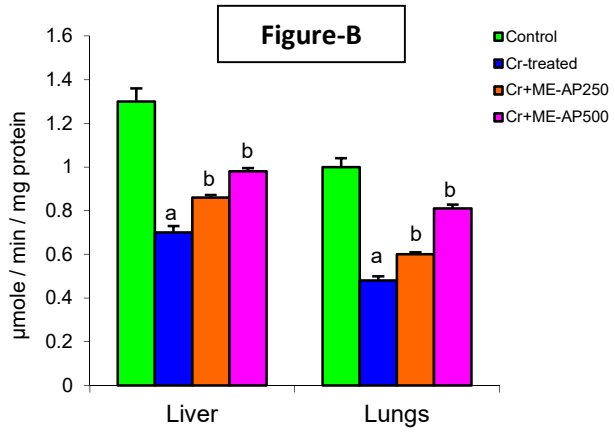


Figure 9 (A, B & C): Represent variation in GR activity in liver and lungs mitochondria after supplementation of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 & 500 in chromium treated rats. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.





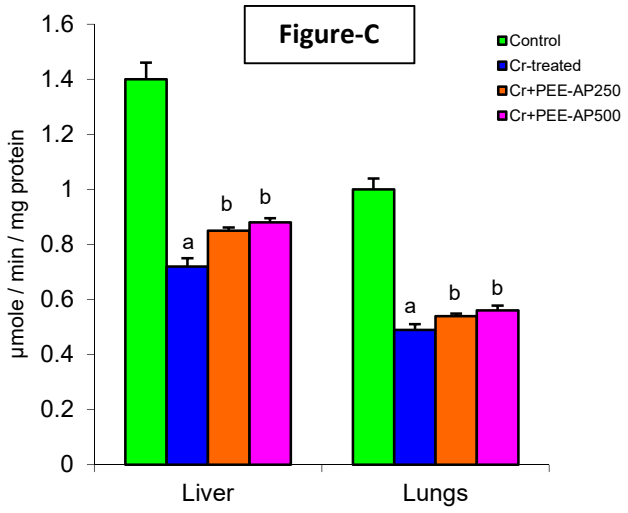


Figure 10 (A, B & C): Depicts the activity of GST in tissue mitochondria after co-administration of AE-AP250 & 500, ME-AP250 & 500 and PEE-AP250 & 500 in response to chromium. [a] noted significant difference ($P<0.05$). [b] $P < 0.05$ compared to chromium.

4.3 Discussion

Several research reports have well established the fact that metal induced oxidative stress is the principal mechanism behind development of metal toxicity. Mitochondria, as a major site of energy production through oxidative phosphorylation, are the potential source of oxidative stress. Compounds of Cr (VI) generating huge cellular oxidative stress and disrupting mitochondrial functions ultimately cause many tissue injuries (Lazzarini et al, 1985; Ryberg and Alexandar, 1990; Shi et al, 1991). Keeping in mind of the several reviewed research data about numerous clinical applications of *Andrographis paniculata* Nees, the role of this plant against various metal toxicities can be investigated further.

Hence, the aim of this current experiment is to elucidate the comparative protective efficacy of different solvent extract of *Andrographis paniculata,* such as aqueous extract (AE-AP), methanol extract (ME-AP) and petroleum ether extract (PEE-AP) at doses (250 and 500 mg / kg body weight / day) on chromium-induced oxidative stress in liver and lungs mitochondria. In this study a significant rise of the MDA and conjugated dienes levels in liver and lungs mitochondria in chromium induced rats shown in figures 2 and 3 (A, B & C) indicates increased lipid peroxidation. Bagchi et al, (1995) reported that chromium (VI) increases lipid peroxidation in liver mitochondria and microsome, as a result of oxidative damage of mitochondrial membrane. Significant increase in NO production in liver and lungs as noticed in the figures 4 (A, B & C) and at the same time production of superoxide and peroxinitrite ($ONOO^-$) are accelerated. These highly reactive oxidants will disintegrate mitochondrial function and create oxidative imbalance (Poderoso et al, 1996; Radi et al, 2002). To avert oxidative tissue damage, activities of endogenous

antioxidant enzymes will be up regulated initially, but will be depleted at the end overpowered by excess accumulation of oxidative reactants. SOD, as a first line of antioxidant defence, catalyses the dismutations of super oxide anion radicals to form H_2O_2. SOD activity is diminished in chromium-induced rat liver and lungs mitochondria represented in figures 5 (A, B & C) and can be explained by excess utilisation of it for detoxification of huge amount of super oxide anions. On the other hand, it has been found that administration of water solvent extracts of *Andrographis paniculata* (AE-AP), as well as methanol extracted *Andrographis paniculata* (ME-AP) at the dose of 500mg/kg of body weight/day, have modulated the changes by replenishing the enzymes towards normal. But, petroleum ether extracted *Andrographis paniculata* has failed to show significant elevation of SOD activity in chromium induced tissues as per figures 5 (A, B & C) and supports these findings that solvent used for extraction of *Andrographis paniculata* will significantly influence the antioxidant activity (Qader et al, 2011). The particular solvent can better extract the active anti-oxidant compound present in *Andrographis paniculata* and establishes the fact of novel microwave assisted extraction of androgapholide (Vasu et al, 2010) also in our study that *Andrographis. paniculata* (ME-AP) extracted in methanol has more effective antioxidant property.

GSH and GSSG levels have markedly diminished in liver and lungs mitochondria of Cr (VI) injected rats as demonstrated in figures 6 and 7 (A, B & C), correlates the report of diminution of intracellular GSH in isolated rat hepatocytes after chromium (VI) treatment (Ueno et al, 1988).The decline in the activity of GPx in chromium treated rats in the present experiment as shown in the figure 8 (A, B & C) may be due to ROS mediated liver and lungs injury. Glutathione reductase (GR) is most

important for detoxifying ROS to reduce oxidative stress. In our present study, the activity of GR correlated in the figure 9 (A, B & C) and in the figure 10 (A, B & C) of GST level in liver and lungs mitochondria, have declined markedly in chromium treated rats in comparison with normal control. The decreased glutathione-S-transferase (GST) activity might be due to increased utilisation and depletion of it. Reduced (GST) activity is usually considered as a marker of oxidative stress (Lu et al, 2011). But it has been noticed that, after co-supplementation with *Andrographis paniculata* extracted in different solvents, there are elevation of all the above mentioned GSH, GSSG, GPX, GR and GST enzyme antioxidants counteracting oxidative stress in liver and lungs mitochondria.

Though ethanol extracted *Andrographis paniculata* show anti-oxidant potential (Rafat et al, 2009), but the aqueous extract has more antioxidant action (Lin et al, 2009). Administration of different solvent extracts of *Andrographis paniculata* at the dose of AP_{250} and AP_{500} have showed significant favourable changes in antioxidant enzyme activities protecting liver and lung tissues from oxidative injury. Thus, *Andrographis paniculata* may decrease the lipid peroxidation to stabilize the cell membrane and significantly reduce the activities of marker enzymes such as MDA and conjugated dienes in liver and lungs as depicted in figures 2 and 3 (A, B & C).

Solvent extracts of *Andrographis paniculata* especially methanol extracted AP (ME-AP) and water extracted AP (AE-AP) both have demonstrated more enhanced antioxidant status in liver and lungs mitochondria of chromium treated rats than petroleum-ether extracted (PEE-AP). This beneficial effect may be due to andrographolide that has been tested earlier as a protective agent against liver

toxicity experimentally produced by carbon tetrachloride (CCl_4) in mice (Kapil et al, 1993) and that andrographolide has also shown to function as chloretics (Shukla et al, 1992). Handa and Sharma (1990) have studied effects of pure andrographolide, whole plant extract in methanol and an andrographolide-free methanol extract of *Andrographis paniculata* against (CCl_4) induced liver damage in rats. Biochemical and histological evidences indicate that all three supplements prevented hepatic injury, but andrographolide is hepatoprotective against galactosamine and paracetamol induced rats (Handa and Sharma, 1990). Also antioxidant action of *Andrographis paniculata* against BHC induced toxicity of liver (Trivedi and Rawal, 2001) and protective effects in the activity of SOD, catalase, GPx, GR and the level of glutathione are reported (Trivedi and Rawal, 2001). Analysis of the above experimental results in detail, it clearly shows that the more antioxidant property lies in the (ME-AP500) and ($AE\text{-}AP_{500}$) in methanol and aqueous extract respectively than petroleum ether (PEE-AP) extract, in respect of free radical scavenging, anti-lipid peroxidation and replenishing endogenous antioxidant enzymes. Hydro alcoholic extract of *Andrographis paniculata* also protects isoproterenol induced lipid peroxidation and enhanced antioxidant enzyme activities, like catalase, super oxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione (GSH) in heart of three week old male albino Wistar rats (Ojha et al, 2009). The important observation of the present work shows the direction of further experiment and suggesting that *Andrographis paniculata* extracted in mixed water and methanol solvents may emerge as a preventive agent against liver and lungs tissue injury induced by Cr (VI).

4.4 Conclusion

Present investigation has suggested that chromium induced oxidative stress could be protected or minimized through the administration with various solvent extracts of *Andrographis paniculata* Nees herbal plant. Natural antioxidants by virtue of their strong ability to overcome the ROS mediated oxidative stress, play crucial role in disease prevention and management. *Andrographis paniculata* may have some natural antioxidant activity and most appropriate solvent is required to yield maximum active compound for therapeutic use. The protective action of this plant may be due to presence of active bitter substance, andrographolide. It may suppress the chromium induced ROS generation and ROS mediated oxidative stress in different tissues. This finding may explain that both the aqueous and methanol extract of *Andrographis paniculata*at the dose of 500 mg / kg body weight / day may have some important components, having the antioxidant property to diminish or prevent oxidative stress in liver and lungs mitochondria due to injurious effect of Cr (VI) compounds.