Chapter-6

Exploration of ameliorative role of most effective mixed hydro-methanol solvent extract at the ratio 60:40 of *Andrographis paniculata* Nees against chromium (VI) induced toxicity

Contents

6.0 Introduction

Nowadays, various metal toxicities are widely prevalent and posing a serious threat towards survival of living beings. Liver and kidney toxicities are usually observed in workers or animals exposed to hexavalent chromium (Dey and Roy, 2010). The functional difference of Cr (VI) and Cr (III) induced toxicities is largely dependent on the ionic permeability across the plasma membrane (De Flora and Wetterhahn, 1989). Thus, cell membrane damage is one of the early crucial changes observed with Cr (VI) toxicity (Dey and Roy, 2010). Highly decreased level of ATP in cells exposed to Cr (VI) has been noticed in rat thymocytes (Lazzarini et al, 1985). The Cr (VI) mediated inhibition of the mitochondrial oxidative respiration is found in isolated rat liver mitochondria (Ryberg and Alexander, 1984); also in sub-mitochondrial particles of rat liver (Ryberg and Alexander, 1990). The mechanism of Cr (VI) which can interfere with the mitochondrial bioenergetics is not clear.

High dose and long term exposure of chromium can impair the body's immune system and cause genotoxicity and cytotoxicity. Till now it is not understood the mechanism of Cr (VI) exposed cytotoxicity. However, both *in vivo* and *in vitro* studies have reported that Cr (VI) induced cytotoxicity is most probably owing to oxidative stress through increased ROS production, modulation of intracellular oxidized states, DNA fragmentation, altered gene expression , activation of protein kinase C and apoptotic cell death (Bagchi et al, 2001).Apoptotic cell death has been observed in numerous skin inflammatory diseases. Moreover, there are few direct correlations among cell death, autophagy, antigen processing, immune responses and the inflammatory reactions (Pena-Sanoja and De Sanctis, 2013). During these processes, ROS-regulated redox-sensitive protein kinases and transcription factors

(Nuclear factor kB (NF-kB), Mitogen-activated protein kinase (MAPK) and Akt pathway) can affect the release of cytokines, such as tumour necrosis factor (TNF-α) and interleukin-1(IL-1) (Gaestel et al, 2009).

Andrographis paniculata is an Indian traditional herb and has been used as a medicine for various diseases. There are more than 20 different active bio constituents like flavonoids, phenols, alkaloids, glycosides, saponins and tannins are present in the *Andrographis paniculata*. Extract of *Andrographis paniculata* exhibits good anticancer, anti-bacterial and anti-fungal activities (Singha et al, 2003). *Andrographis paniculata* extracts contain principal active compound andrographolide. Methanol extract of this plant is more active in antioxidant activities (Lin et al, 2009).

Therefore, the present part of the research investigation is intended to ameliorate the Cr (VI) exposed tissue toxicity by supplementation with the mixed hydro-methanol solvent extract of *Andrographis paniculata* in the ratio of (60:40) *in vivo* rat model. During the study, certain parameters of alteration of structure and function of plasma membrane, mitochondrial electron transport chain (Mito–ETC) complexes, expression of certain cytokines, apoptotic signalling pathway and histopathological status of liver and lungs are observed in some detail.

6.1 Methodology

6.1.1 Chemicals

Procurement of necessary chemicals, reagents and other articles was described earlier in chapter-II**.**

6.1.2 Preparation of Mixed solvent extract

Preparation of hydro-methanol (60:40) mixed solvent extract from *Andrographis paniculata* was described in Chapter-V.

6.1.3 Animals and diet

Animals and their diet were described in Chapter-II.

6.1.4 Mode of animal treatment

Rats of almost equal average body weight (80-100 gram) were grouped into three. The animals of two groups were first injected with $K_2Cr_2O_7$ as described earlier (Dey et al, 2003). Remaining rats in third group were injected only (0.9% NaCl), served as control group. The rats of one of the chromium exposed group serving as the supplemented groups provided mixed hydro-methanol (60:40) solvent of *Andrographis paniculata* extract at a dose of 500 milligram per 100 gram body weight daily at an interval of six hours after injection of $K_2Cr_2O_7$ for 28 days and rats of other chromium exposed group were served as chromium control/only chromium treated group.

6.1.5 Animals sacrifice and collection of blood samples and tissues

Animal sacrifice, blood and tissue collection were described in Chapter-II.

6.1.6 Homogenization of tissues

Tissue homogenization was described in Chapter-II.

6.1.7 Isolation of crude plasma membrane

Fractions of membrane of tissues were isolated as per method described by Ghosh Chowdhuri et al, (1995).

6.1.8 Isolation of Mitochondria

Mitochondria isolation was described in Chapter-III.

6.1.9 Rat blood collection and Isolation of lymphocytes

About 2 millilitre of blood were drawn from hepatic vein and collected in heparin coated Vacutainers. Then the blood was mixed in a ratio of (1:1) with PBS. Thereafter density gradient centrifugation of PBS diluted blood sample was done in Histopaque 1077 (Sigma) at rotational force (400x g with1500 rpm) for 40 min at room temperature. Upper monolayer of buffy coat enriched in Lymphocytes was collected using a clean centrifuge tube and washed thrice in balanced salt solution. Collected blood lymphocytes were re-suspended with 10% FBS in RPMI complete media and incubated at 37°C for 24 hours in 5% CO2 incubator with 95% humid atmospheric air.

6.1.10 Analytical methods

A. Estimation of cholesterol and phospholipid of membrane

The levels of cholesterol and phospholipid from the isolated fractions of membrane were measured according to the method of Zlatkis et al, (1953) and Christopher and Ralph (1972), respectively.

B. Determination of activity of total ATPase and Na^+–K^+ ATPase

Activities of total ATP-ase and Na^+–K^+ ATPase were measured as per method of (Sen et al, 1981).

C. Assaying of activities of Mito–ETC complexes

The activity of the enzymes of individual electron transport chain complexes was performed spectrophotometrically. Activity of complex I (DPNH-coenzyme Q reductase) was measured by using the method of Hatefi and Rieske (1967). Activity of complex II (succinate dehydrogenase coenzyme Q reductase) was estimated according to the method of Ziegler and Rieske (1967). Activity of complex III (coenzyme Q cytochrome *c* reductase) was measured as per method of Rieske (1967).

D. Cytokine Assay from liver and lungs tissues

Liver and lung tissues were thawed on ice and about 0.2 ml glass beads and 1 ml PBS containing protease inhibitors were given to the frozen tissue to avoid degradation on thawing. Subsequently, both samples were homogenized in a Bullet-Blender for 5 minutes at 4°C and procedure was repeated thrice. Then homogenates were centrifuged by using 2 sequential centrifugation steps (164×*g*at 4°C for 10 minutes). After that, supernatants were stored at −80°C to analyse further for cytokine levels within seven days of collection of tissues. The cytokine assay of samples was carried out in duplicate as per the instructions provided with Mesoscale Discovery's Rat 7-Plex Ultra-Sensitive Kit (Gaithersburg, MD). Cytokine levels were estimated relative to total protein content of the sample, pg./mg protein.

E. The production of Intracellular Reactive Oxygen Species (ROS) measurement from Lymphocytes

Intracellular ROS measurement was undertaken using H_2DCFDA according to method of Dash et al, (2014). After the treatment period, lymphocytes were washed followed by incubation with 1 microgram per millilitre of H2DCFDA for 30 min at 37ºC. Thereafter lymphocytes were washed thrice by using PBS. DCF fluorescence was measured at 485 nm excitation and 520 nm emissions using a Hitachi F-7000 Fluorescence Spectrophotometer. All measurements were taken in triplicate.

F. Morphological Analysis of Lymphocytes by Acridine Orange (AO)– Ethidium Bromide (ETBR) Double Staining method

To analyse the morphological alterations of cell apoptosis isolated lymphocytes are stained as per AO/EtBr double staining method. Collected rat lymphocytes were washed with PBS. 10 microliter of the cells were taken on a glass slide and mixed with 10 microliter of acridine orange (50 microgram per millilitre) and ethidium bromide (50 microgram per millilitre). Then stained lymphocytes were observed under a fluorescence microscope (NIKON ECLIPSE LV100POL) under 400X magnification (Ho et al, 2009).

G. Assessment of Nuclear Morphological Changes by DAPI nuclear fluorescence Staining

To examine the nuclear morphological changes of prepared lymphocytes, DAPI nuclear staining procedure was accomplished step by step according to the method of (Graziano et al, 2001) with few modifications. After proper washing, lymphocytes were fixed on a cover slip applying 2.5% glutaraldehyde for 15 min

and then permeabilized with 0.1% Triton X-100 for 5 minute. Then, diluted DAPI stain about 1 µg/ml were added and kept at 37^0 C for 5 min. At last lymphocytes were washed with PBS and changes of nuclear morphology are observed by fluorescence microscopy (NIKON ECLIPSE LV100POL).

H. Estimation of Cytokines from Lymphocytes

After the treatment of chromium and supplemented with hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata*, lymphocytes were used to estimate changes in cytokines level. To study the effect of chromium on cytokine production, ELISA test was performed for the estimation of tumour necrosis factor-alpha (TNF-α), tumour growth factor-beta (TGF-β), interleukin (IL-10) and interleukin (IL-12) production by using the manufacturer's protocol (e-Biosciences, San Diego, USA). The whole experiment was repeated thrice.

I. Estimation of Pro-apoptotic and Anti-apoptotic Markers

The pro-apoptotic factors like caspase-3, caspase-8 and anti-apoptotic factor pAKT levels were measured by using ELISA technique with some modification (Sharifi et al, 2015). After the treatment period is over, isolated lymphocytes were lysed and then centrifuged for the collection of supernatant. For estimation of pro-apoptotic and anti-apoptotic markers the supernatants were tested using ELISA kits according to instructions of manufacturer. The plates were coated with caspase-8 (50 µl per well), 50 µl per well cleaved caspase-3 (c-caspase-3) and capture antibodies (2mg/ml) diluted in 0.05M carbonate buffer (pH 9.6). Then after overnight incubation at 4 °C, plates were washed thrice with 0.15M PBS -0.05% Tween-20 (PBST) and blocked with 50µl per well PBS, 5% FBS, 0.05% Tween-20, 0.02%

sodium azide (PBSTN) at room temperature for 1 hour. Then plates were washed thrice with PBST and 100µl of samples were added to each well and then incubated for 2.5 hour at room temperature. Subsequently, the plates were washed four times with PBST and incubated with 50 µl per well of biotinylated anti caspase-8 and caspase-3 detection antibody at room temperature for 2 hour. After washing three times with PBST, 50µl per well of HRP-avidin solution (e- Biosciences) was added. After keeping 30 minutes at room temperature, the plates were washed two times in PBST and then100µl per well substrate buffer (e- Biosciences) was added and then incubated at room temperature for 30 minutes in dark. Optical densities were measured at 450 nm by using an ELISA reader (Bio-Rad, Singapore). All samples were analysed for three times.

J. Protein Estimation

Estimation of protein was described in Chapter-II.

K. Histopathological studies

After completion of experimental period, tissue samples (liver and lung) were collected afresh. The samples were immediately fixed with 4% paraformaldehyde (PFA) after rinsing with PBS. Then the PFA-fixed tissue samples were dipped serially in graded ethanol concentration (70, 80, 90, 95 and 100%) for dehydration. The samples were cleared in two changes of xylene to get rid of excess alcohol. Samples were impregnated with two changes of molten paraffin wax and embedded to prepare a paraffin blocked specimen. In next step the paraffin-blocked tissue specimen was cut into thin sections about thickness of 5.0-µm with the microtome. The 5.0- µm sections were stained with haematoxylin and eosin (H&E) and examined for any sign of histological impairment under a light microscope

using10X (low power), 40X (high power) and 100X (oil immersion) objectives and the photomicrographs were taken for further studies (Wu et al, 2016)

6.1.11 Statistical Analysis

Statistical data analysis was described in Chapter-II.

6.2 Results

Alterations of the phospholipid and cholesterol contents of cell membrane and the activities of total ATPase and Na^+–K^+ ATPase of the tested organs are presented in figures (1-4). It has been observed that increased level of membrane cholesterol, but decreased membrane phospholipid contents, activities of total ATPase and Na^+–K^+ ATPase in both tissue plasma membrane of the Cr (VI) treated group. While hydro-methanol (60:40) extract of *Andrographis paniculata* was supplemented to Cr (VI) administered rats; the cholesterol and phospholipid contents, the activities of total ATPase and Na^+–K^+ ATPase in plasma membrane of the tested organs were significantly recovered towards normal control in comparison with chromium treated group.

The alterations of the activity of mitochondrial ETC complexes I-III are focused in figures (5-7). The significantly decreased activity of the electron transport chain enzyme complexes (I, II, III) has been noticed in the chromium exposed rats. Supplementation with hydro-methanol (60:40) extract of *Andrographis paniculata* showed the significant regain of the specific activity of enzymes towards normal control in liver and lungs mitochondria.

In liver and lungs tissues, the Pro-inflammatory cytokines (TNF-α and IL-12) levels are significantly increased and decreased TGF-β and IL-10 (anti-inflammatory

cytokines) level in chromium treated rats as depicted in figures(8-11). On the other hand, hydro-methanol (60:40) extract of *Andrographis paniculata* plays a vital role to counteract such types of alteration.

ROS induction of lymphocytes is noted in the figure (12). It has been found that chromium injection to rats elevated the cellular ROS level significantly. Supplementation with hydro-methanol (60:40) extract of *Andrographi paniculata* to chromium treated rats; it has been noticed that the increased level of ROS induction of lymphocytes in response to chromium diminished significantly towards control group.

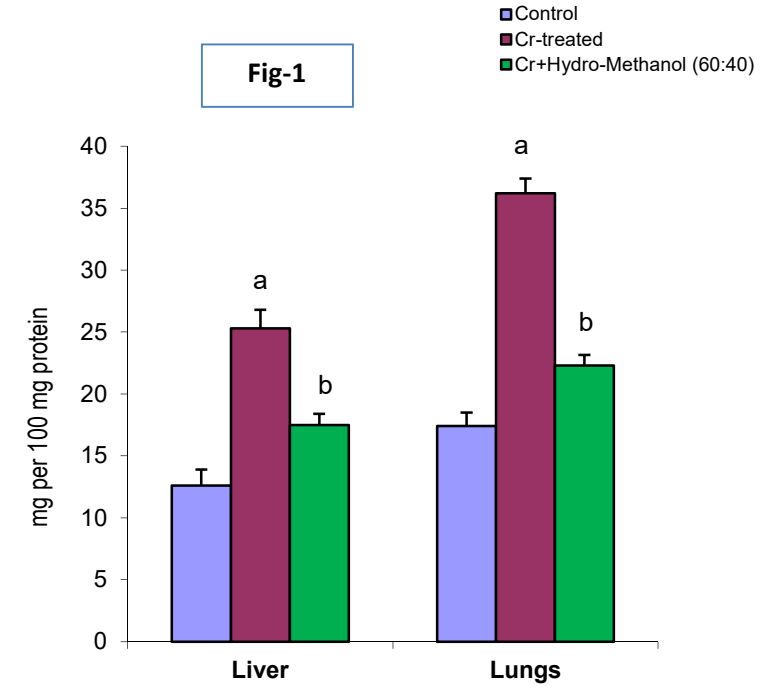
Study of the morphological changes of lymphocyte after chromium treatment, using Et Br-AO double staining is shown in figure (13). Observed findings show that chromium treatment is able to decrease the number of viable lymphocyte cells to a great extent, but after supplementation with hydro-methanol (60:40) extract of *Andrographis paniculata* to rats exposed to Cr (VI), no significant changes in lymphocyte number are observed. The structural changes with characteristics of apoptosis, such as chromatin condensation and nuclear fragmentation, are manifested in lymphocytes upon treatment with chromium as shown in the figure (14). Early apoptosis cells are coloured greenish yellow and late apoptosis cells are seen as orange. On the other hand, it has been found that no marked apoptotic changes are noticed in rat lymphocytes upon the co-administration of hydro-methanol (60:40) extract of *Andrographis paniculata* with chromium.

The Pro-inflammatory cytokine (TNF-α) level is increased significantly and the anti-inflammatory cytokine (IL-10) is significantly reduced in rat lymphocytes by the treatment with chromium as per figure (15). After supplementation with hydro-

methanol (60:40) extract of *Andrographis paniculata*, it has been found that Pro- and anti- inflammatory cytokines levels are significantly restored.

After treatment schedule with Cr (VI) and co-supplementation with hydro-methanol (60:40) extract of *Andrographis paniculata*, lymphocytes are used to assay variations in levels of pro and anti-apoptotic markers using ELISA method. The observed results demonstrate that caspase-3 and caspase-8 increase significantly, and decreases pAKT level in response to chromium as seen in the figure (16). On the other hand, it has been noticed that such types of pro- and anti-apoptotic markers are significantly comes to normal level after co-supplementation of hydro-methanol (60:40) extract of *Andrographis paniculata* in chromium treated rats.

As per histology findings, section of liver shows gross inflammation of hepatocytes, loss of hepatic lobular architecture and peri-portal fibrosis indicating hepatic damage due to Cr (VI) toxicity as seen in the figure (17). The section of lung shows alveolar epithelial destruction, intra-alveolar haemorrhage and collapse of alveoli. Along with alveolar damage, bronchial epithelial hypertrophy and narrowing of bronchus are seen in Cr (VI) treated group as per figure (18). Hydro-methanol (60:40) extract of *Andrographis paniculata* administration has played a vital role to recover the histological changes of liver and lungs in chromium treated rats.



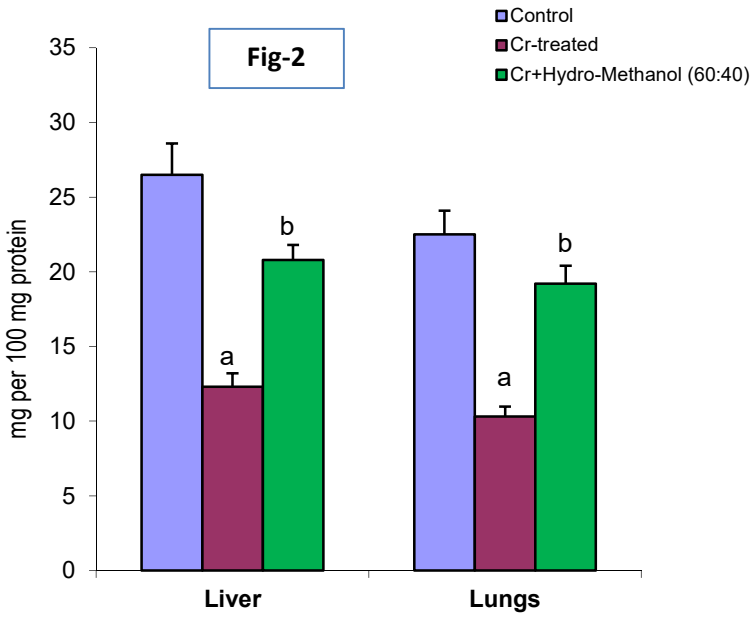
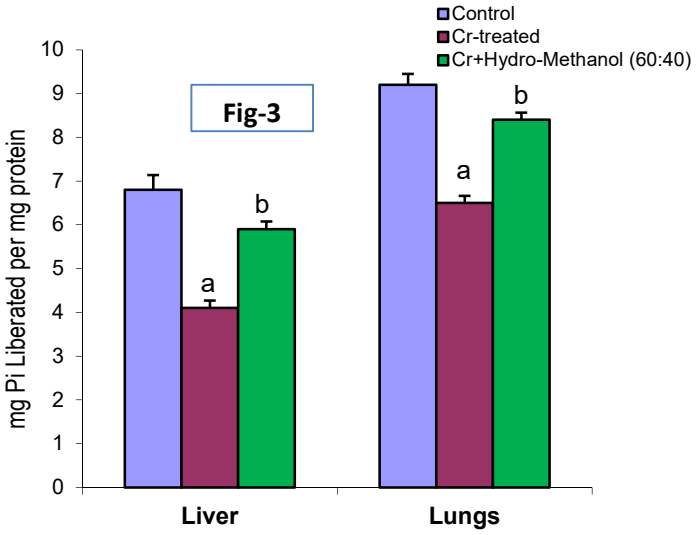


Figure 1 & 2: Shows the membrane cholesterol and phospholipids levels after supplementation with hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats. [a]$P < 0.05$ compared to control, [b]$P < 0.05$ compared to chromium.



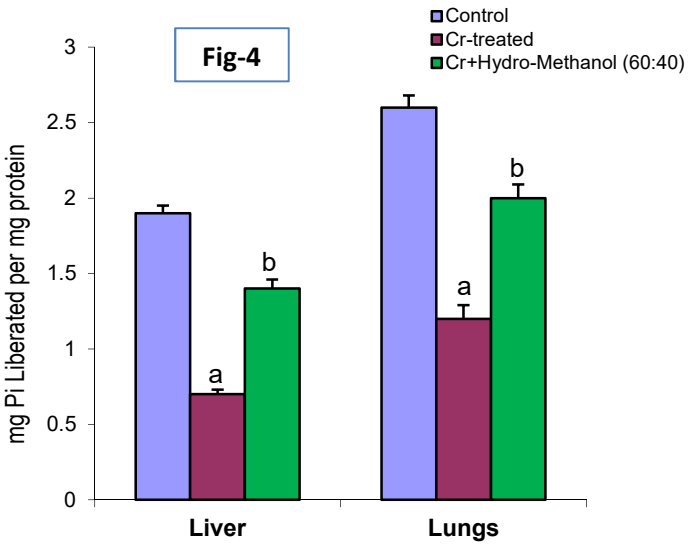
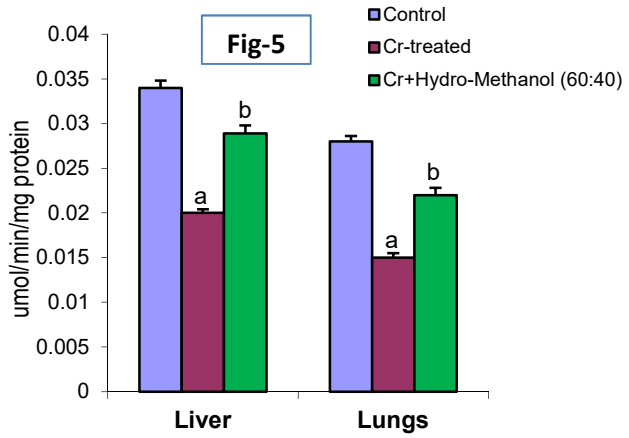
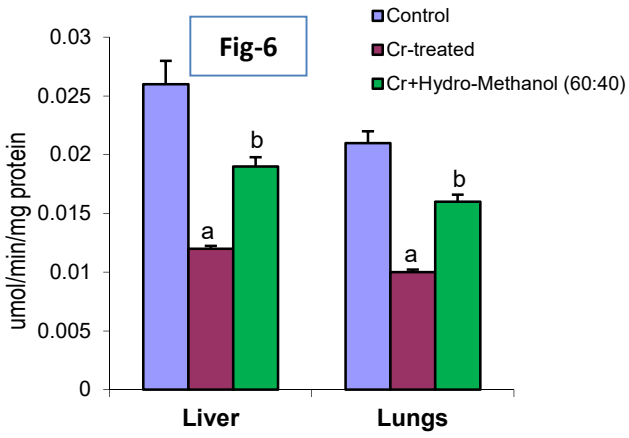


Figure 3 & 4: Changes in membrane Total ATPase and Na^{+}-K^{+} ATPase activities after supplementation of hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats. Data represents mean±SE, [a] $P < 0.05$ compared to control, [b] $P < 0.05$ compared to chromium.





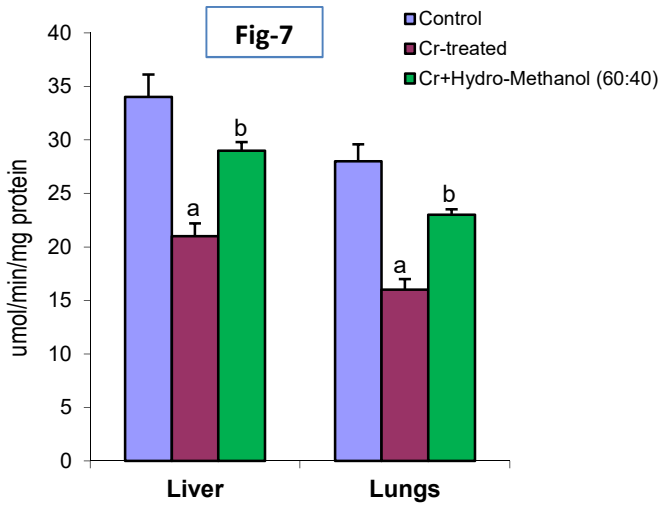
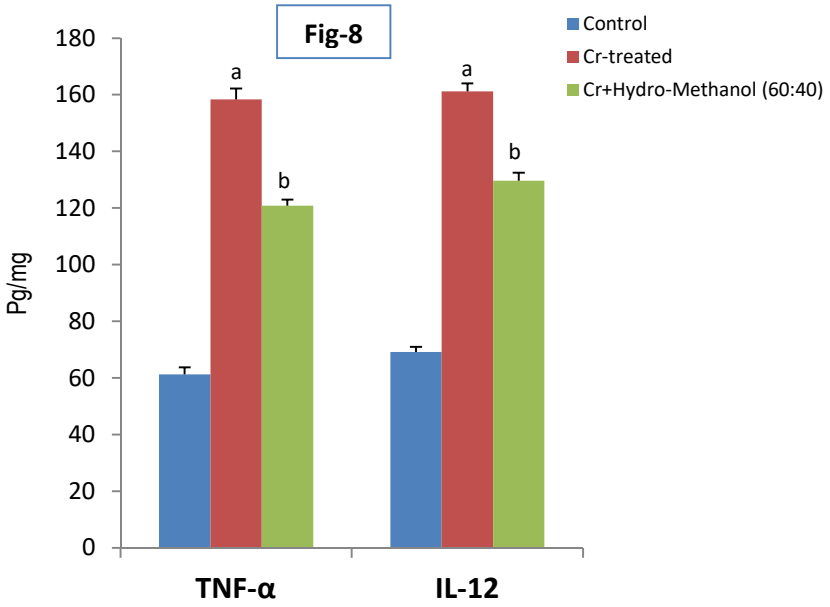


Figure 5, 6 & 7: Effect of hydro-methanol (60:40) extract of *A. paniculata* on Mito ETC Complex-I (DPNH-coenzyme Q reductase), Mito ETC Complex-II (succinate dehydrogenase coenzyme Q reductase) and Mito ETC Complex-III (coenzyme Q cytochrome c reductase) respectively in chromium-induced rats. [a] $P < 0.05$ compared to control, [b] $P < 0.05$ compared to chromium.



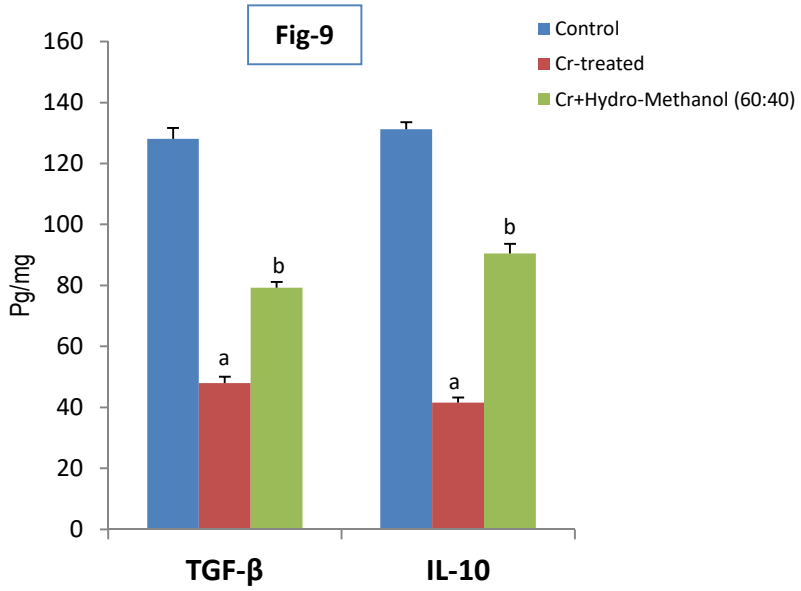
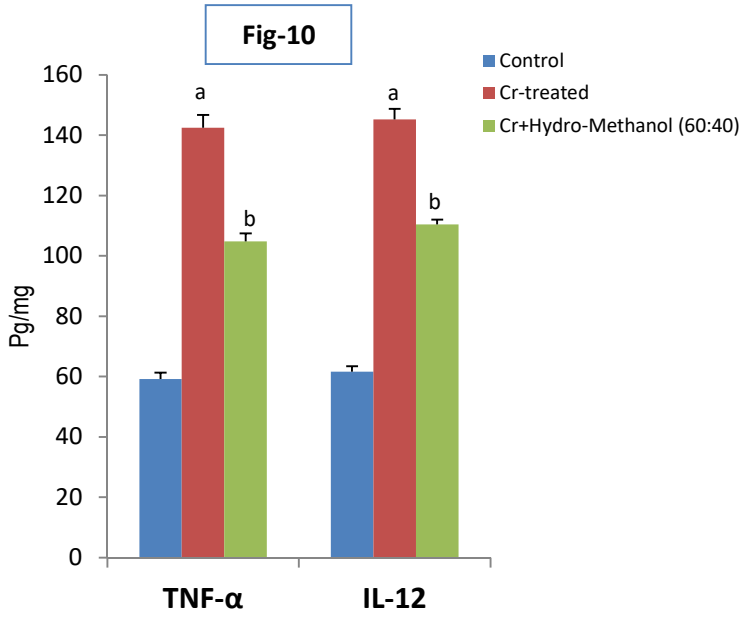


Figure 8 and 9: Changes of the Pro and Anti-inflammatory cytokines level in liver after supplementation of hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats. [a] $P < 0.05$ compared to control, [b] $P < 0.05$ compared to chromium.



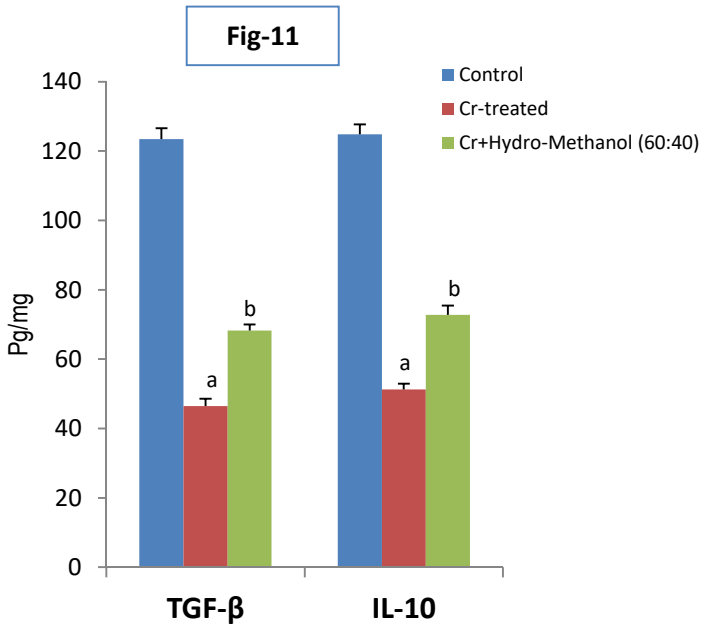


Figure 10 and 11: Changes of the Pro and Anti-inflammatory cytokines level in lungs after supplementation of hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats. [a] $P < 0.05$ compared to control, [b] $P < 0.05$ compared to chromium.

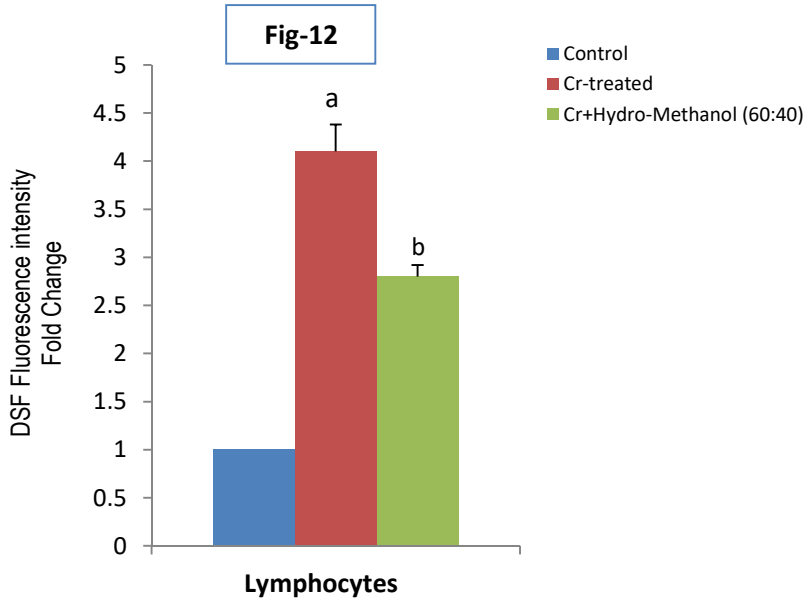


Figure 12: Effects of chromium and it's supplementation with hydro-methanol (60:40) extract *of A. paniculata* on intracellular ROS induction in lymphocytes. [a] $P < 0.05$ compared to control, [b] $P < 0.05$ compared to chromium.

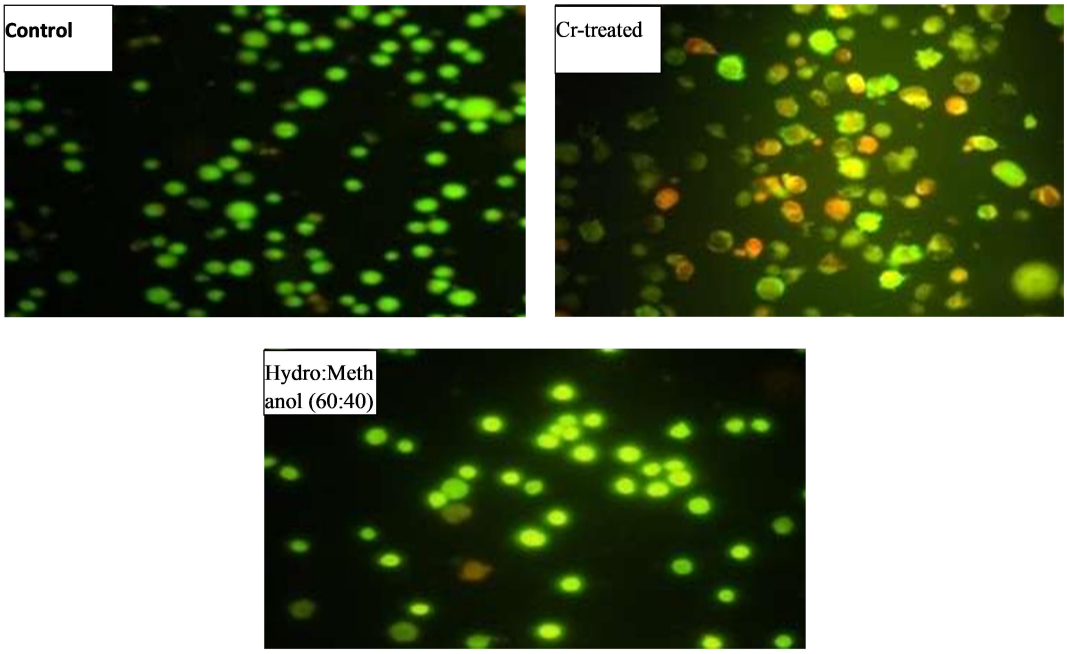


Figure 13: Effects of chromium and it's supplementation with hydro-methanol (60:40) extract *of A. paniculata* on morphology of lymphocytes.

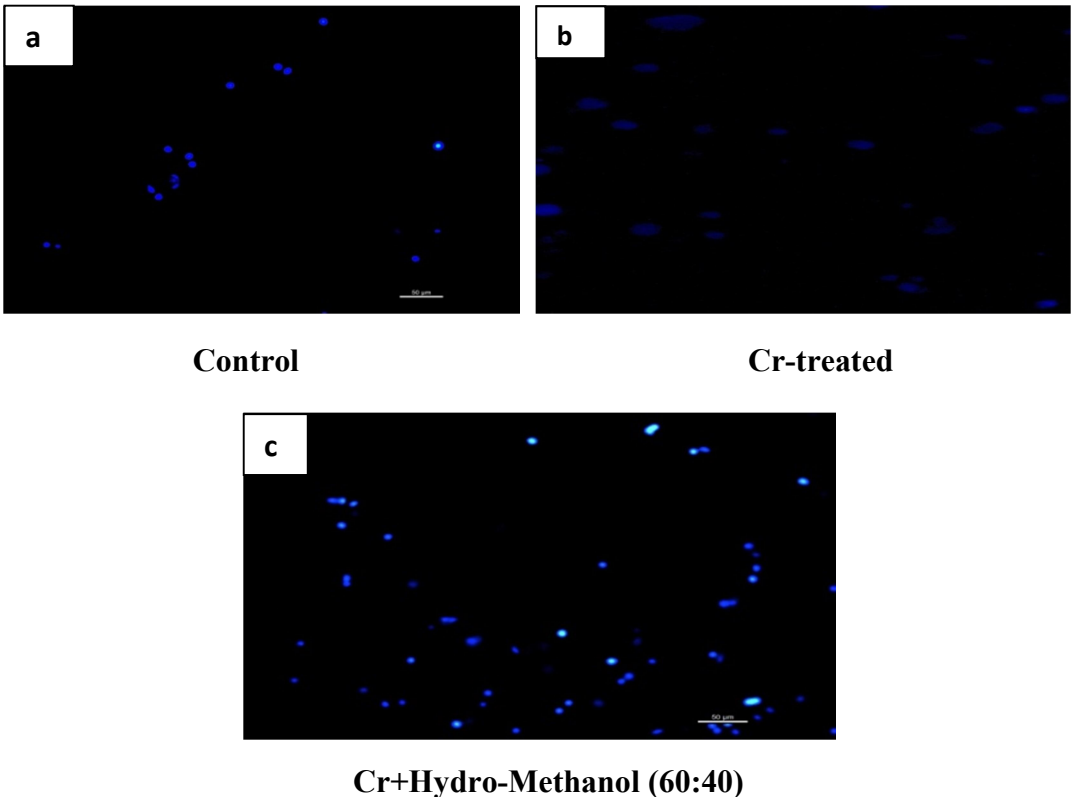


Figure 14: Nuclear morphological Change of lymphocyte (a) control, (b) Chromium treated and (c) after supplementation of hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats.

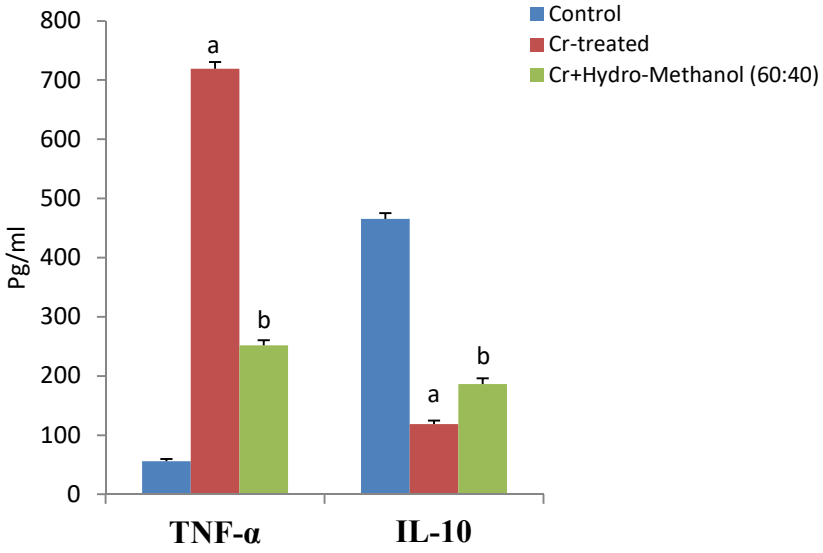


Figure 15: Changes of the cytokines level in lymphocytes after supplementation of hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats. [a]$P < 0.05$ compared to control, [b]$P < 0.05$ compared to chromium.

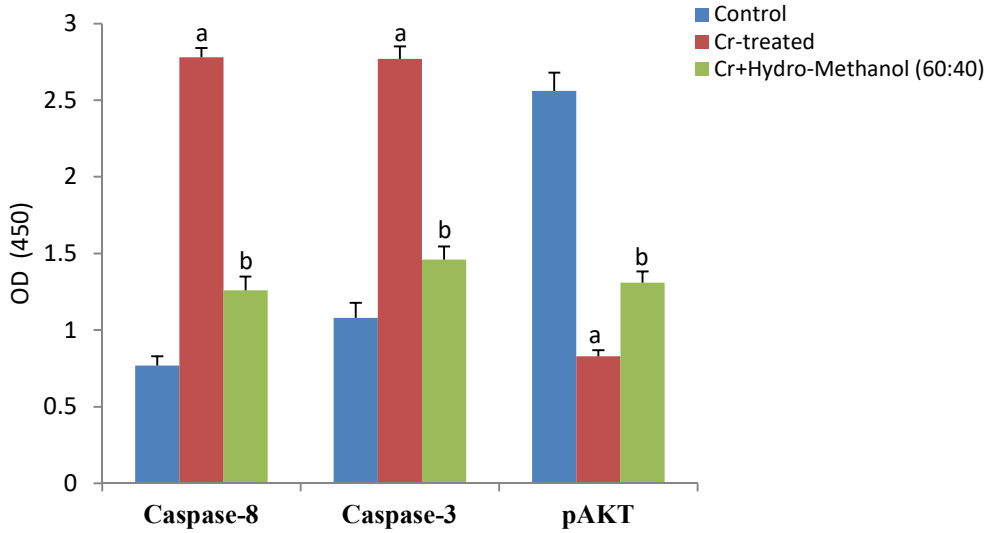
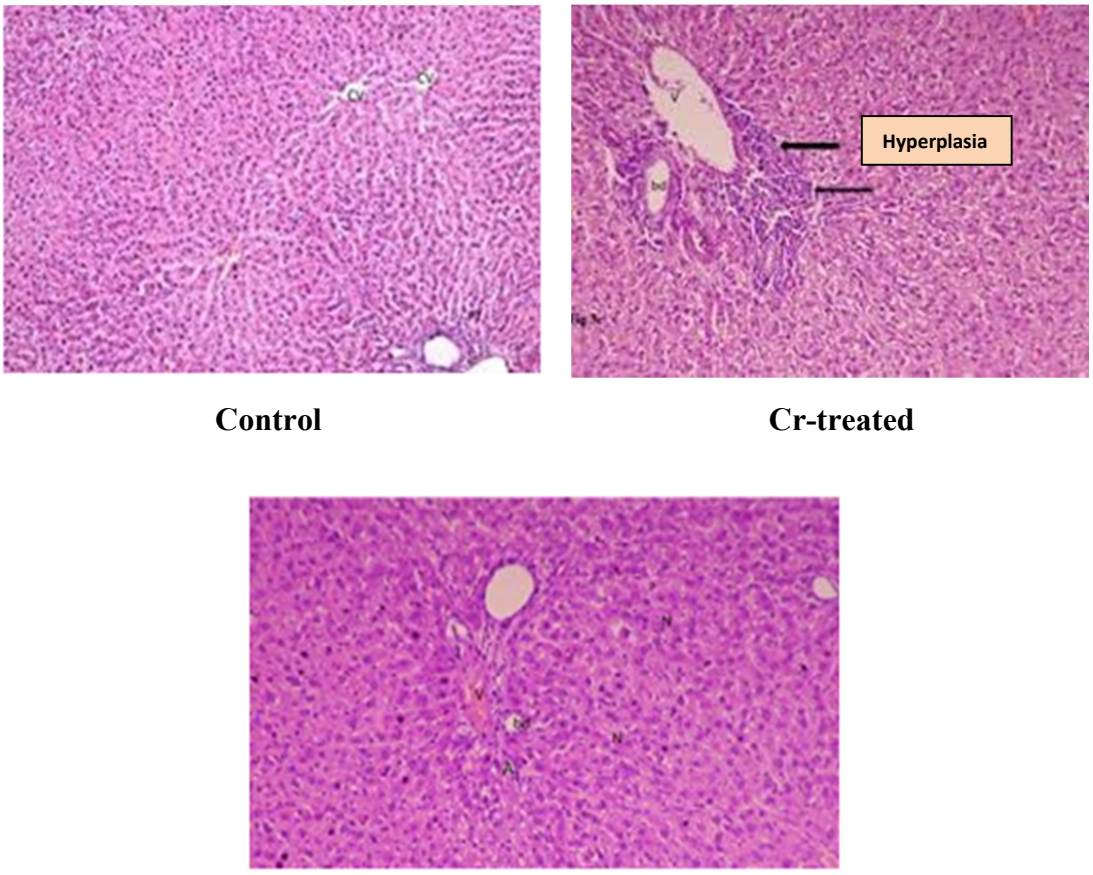


Figure16: Estimation of Caspase-8, Caspase-3 and pAKT from lymphocytes after supplementation of hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats. [a] $P < 0.05$ compared to control, [b] $P < 0.05$ compared to chromium.

Histopathological studies of Liver:

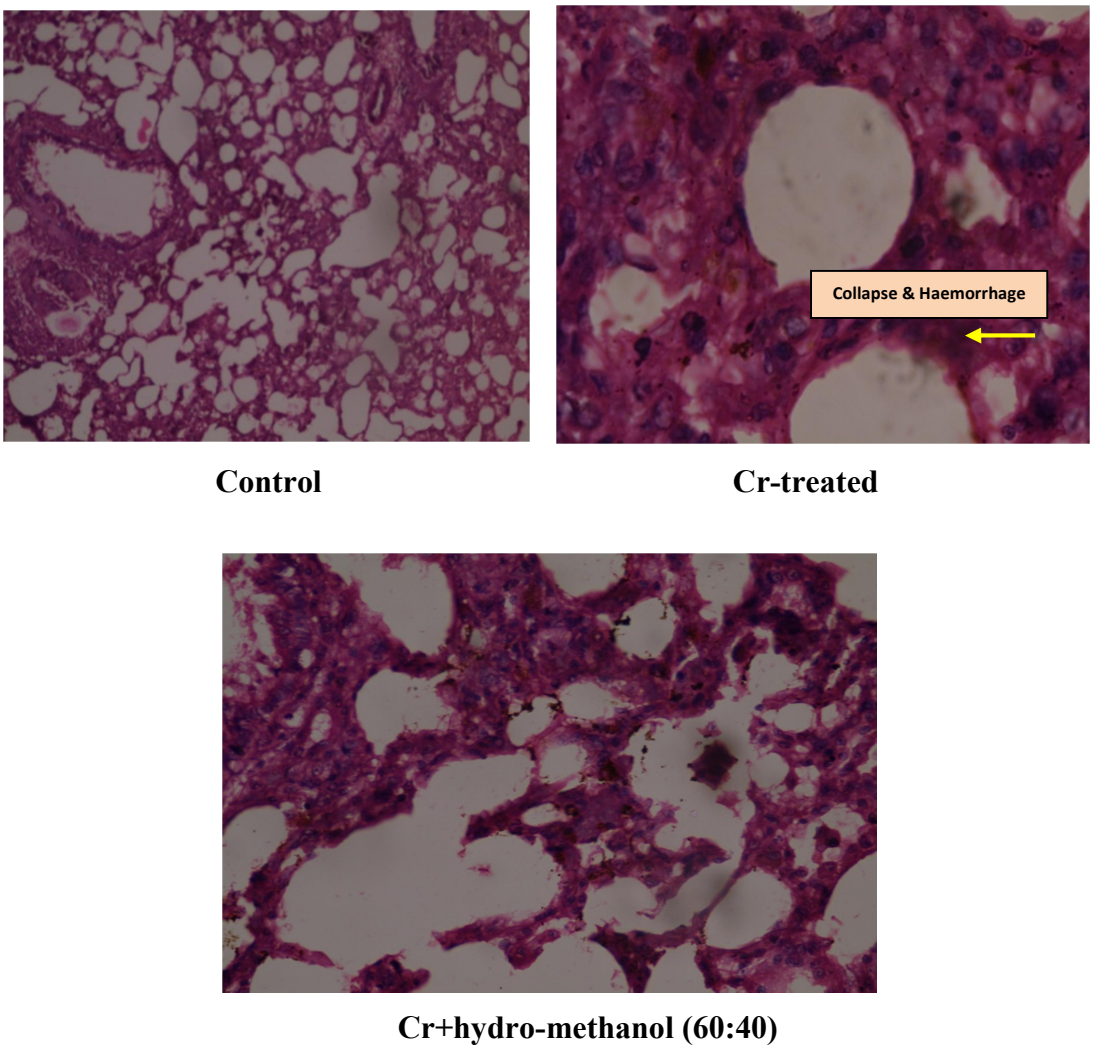


Control **Cr-treated**

Cr + hydro-methanol (60:40)

Figure 17: Study of the Histological changes in Liver tissues after co-administration of hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats.

Histopathological studies of Lungs:



Control **Cr-treated**

Cr+hydro-methanol (60:40)

Figure 18: Study of the Histological changes in Lungs tissues after co-administration of hydro-methanol (60:40) extract of *A. paniculata* in Cr-treated rats.

6.3 Discussion

Current study shows that the chromium-exposed membrane damage has been suggested by notable elevation of membrane cholesterol level in both tested liver and lung tissues (Figure1). This increased cholesterol level may be due to imbalance in cholesterol addition in the cell membrane. Thus impaired function of the enzyme lecithin cholesterol acetyl transferase might have developed such changes, in response to chromium. Decreased level of membrane phospholipids (Figure 2) indicates the structural damage of the cell membrane. The impact of chromium on destroying the lipoprotein cell membrane may possibly due to heightened lipolytic enzyme activity, as evidenced by increased excretion of urinary lipid metabolites in chromium exposure rats (Dey and Roy, 2010). This increased catabolism of lipids may be due to excess accumulation of acetyl-CoA which may cause increased cholesterol synthesis in the nonsteroid producing tissues. Thus, change in relative proportion of cholesterol and phospholipid, in challenge to chromium, might have caused structural damage of the plasma membrane. The effect of chromium on structural alterations of membrane cholesterol and phospholipids is observed to be reduced; when chromium treated rats are supplemented with hydro-methanol (60:40) extract of *Andrographis paniculata*. These findings certainly to be noted as a conclusive proof of definite ameliorative role of hydro-methanol (60:40) extract of *Andrographis paniculata* against Cr (VI) induced tissue toxicity.

The cumulative activities of membrane ATPase are notably decreased in the chromium-exposed group in both tested organs (liver and lungs). Hydro-methanol (60:40) extract of *Andrographis paniculata* co-supplementation may have exerted a protective effect on chromium-exposed alterations in the activity of total ATPase

(Figure3). Previously noted inhibition of energy production by the cytotoxic concentration of chromium (Dey and Roy, 2010); plays a vital role in the Cr (VI) mediated alterations of the ATPase activity. The Na^+–K^+ ATPase activity has been noticed to be decreased prominently in the chromium exposed group in both liver and lungs (Figure4). Observed results are supported by the findings on chromium-exposed reduction of cell membrane transport (Standeven and Wetterhahn 1991a, 1991b). But after supplementation with hydro-methanol (60:40) extract of *Andrographis paniculata*, it has been observed that the activity of Na^+–K^+ ATPase has been restored in plasma membranes of both liver and lung tissues in chromium-treated rats. Cholesterol/phospholipid ratio of the membrane is inversely related to the ATP hydrolysing activity of Na^+–K^+ ATPase (Yeagle, 1985). Hence, in the current observation, reduced Na^+–K^+ ATPase activity might be related to changes of cholesterol and phospholipid ratio, instead of direct effect of chromium.

Reduced activities of Mitochondrial ETC –Complex I, II and III during Cr (VI) treatment may be owing to a good amount of leakage of electrons through Mito-ETC, which could generate superoxide ions in liver and lung mitochondria.

The generation of superoxide ions through Complex -I can be modulated by inhibition of cytochrome- c and redox state of the cell (Kushnareva et al, 2002). Mitochondrion plays a vital role in multi-level regulatory process of apoptosis. Also, mitochondria are important site and major source of ROS production (Boveris and Chance, 1973). ROS generated in mitochondria has promoted release of pro-apoptic proteins and cytochrome-C that can initiate caspase cascade activation and apoptosis (Ott et al, 2007). Studies reported that loss of reduced glutathione will alter permeability of mitochondrial membrane and caspase activation (Cai and Jones,

1998). The hydro-methanol (60:40) extract of *Andrographis paniculata* supplementation has increased the activity level of Mitochondrial ETC-Complex I, II and III as shown in figures (5, 6 and 7).

There is an urgent need to research on the pro-inflammatory cytokines (TNF-α and IL-12) levels and anti-inflammatory cytokines (TGF-β and IL-10) levels in liver and lung tissues in chromium treated rats. It was noted that TNF-α and IL-12 levels significantly increased but the levels of TGF-β and IL-10 decreased significantly after chromium exposure as shown in figures (8-11). On the other hand, hydro-methanol (60:40) extracts of *Andrographis paniculata* has played a vital role to counteract such types of alterations. From this point of view it has been observed that hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* is more potent to regulate the release of pro- and anti- inflammatory cytokines in response to chromium in liver and lungs tissues.

Now it is important to describe the impact of extract of *Andrographis paniculata* on ROS generation. ROS are the molecules and ions containing unpaired electrons and being a free radical it is highly active. It plays an important active role in cell signalling pathway regulation, leading to oxidative cell damage and ultimately cell death (Rahman, 2007). The physiologically active cellular system normally develops lower amount of ROS during metabolism, which is effectively quenched by diverse antioxidant enzymes of glutathione system. In addition, intracellular ROS production is seen by the mitochondrial electron respiratory chain reaction, membrane-bound (NADPH) oxidase and arachidonic metabolic reaction (Thannickal and Fanburg, 2000). In the current study the level intracellular ROS has been shown in the figure (12). It is noted that the ROS level of lymphocytes has

been elevated significantly after chromium treatment. ROS has played crucial role for lymphocytic cell death. But supplementation of hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* has played a potent role as ROS inhibitor. It is noted that hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* treatment effectively may have protected the lymphocytes from chromium-induced cytotoxicity.

In order to examine the probable route of cell death due to apoptosis or necrosis, the researcher observed the cells by EtBr-AO double staining method. The Cell morphology study was conducted after EtBr-AO double staining in chromium treated rats as shown in the figure (13). By these typical staining, it shows that the viable cells with intact nucleus and DNA look round and bright green, whereas early apoptotic cells have fragmented DNA show yellow- green coloured nuclei. The late apoptosis cells are stained orange and necrotic cells as red (Ho et al, 2009). In our study, it is evident that chromium exposure drastically has decreased the number of viable cells. Majority of the lymphocytes shows typical yellowish- green colour of early apoptosis with characteristic plasma membrane damage and formation of apoptotic bodies. Significant number of lymphocytes has stained with orange colour indicates late apoptosis. But small numbers of lymphocytes are stained with red colour. These findings suggest that most of the cells are in the process of undergoing apoptosis but not much necrosis. In case of hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* supplementation, no significant apoptotic changes are observed towards chromium treated rat lymphocytes.

In order to examine changes of nuclear morphology of lymphocyte cells due to treatment of chromium, the researcher stained the chromium-exposed lymphocytes

by DAPI staining. It has been noticed that cells displayed significant morphological changes in nuclear chromatin after chromium treatment for an experimental period of 28 days (Figure-14). Chromatin condensation and fragmentation is one of the major characteristics of apoptosis (Lu et al, 2011). The nuclear morphological changes have manifested with features of apoptosis such as chromatin condensation, invagination of nuclei and nuclear fragmentation. These are manifested in lymphocytes upon treatment with chromium. In case of hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* supplementation, no significant apoptotic changes have been observed in response to chromium.

It is an established fact that TNF-α, one of the main inflammatory cytokines, stimulates the caspase-8 mediated apoptosis pathway (Pelagi et al, 2000; Wang et al, 2008). Out of the two major signalling pathways of TNF-α, first one is TNF-α - induced signalling complex I that is through NF-kB and MAPK activated pro-inflammatory pathway and other one is TNF-α signalling complex II pathway /TNF-α induced pro-apoptotic pathway. In the TNF-α mediated pro-apoptotic pathway, initially ROS activates caspase cascade system and finally alteration of the mitochondria function through caspases starts full-blown apoptosis. Interleukin-10 (IL-10), one of the potent anti-inflammatory cytokines, plays a vital role in controlling host immune response against pathogens, so that they can prevent the injury to the host tissues and maintain homeostasis. In this current research, it was found that concentration of TNF-α increased significantly, but the concentration of IL-10 decreased substantially in chromium exposed rat lymphocytes (Figure-15). On the other hand, hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* supplementation plays a crucial role to counteract such changes of pro-

and anti-inflammatory cytokines level in response to chromium.

Enhanced release of pro-inflammatory mediators stimulated the cell death process. Activation of caspase cascade due to oxidative stress induces a signalling pathway of apoptosis (Zhou et al, 2005). Chromium can escalate activation of pro-apoptotic factors such as caspase 3 and caspase 8 in lymphocytes (Figure-16). More ROS generation may directly suppress the activity of pAKT (Figure-16) and initiate apoptosis in chromium-induced rat lymphocytes. But supplementation of hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata*, has acted as one of the most important medicinal plants, containing potent compounds which play a vital role to minimize the chromium-induced changes of pro and anti-apoptotic markers in rat lymphocytes.

In regards to histology, section of liver shows gross inflammation of hepatocytes, loss of hepatic lobular structure and per portal fibrosis indicating hepatic damage due to Cr (VI) toxicity (Figure-17). The histology section of lung shows alveolar epithelial decimation, intra-alveolar haemorrhage and collapse of alveoli. Along with alveolar damage, bronchial epithelial hypertrophy and narrowing of bronchus are visible in Cr (VI) treated group (Figure-18). In the case of hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* supplementation, no significant hepatic damage and alveolar damage are seen in the presence of chromium.

6.4 Conclusion

All the findings established that chromium exposure induced structural and functional alteration in both the tested tissue plasma membrane of liver and lungs. These abnormalities of structure and function may be reduced by the effect of hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* supplementation. All such supplementary compounds acted as one of the most important agents against chromium-induced mitochondrial dysfunction in rat liver and lungs. Increased ROS production and pro-inflammatory cytokines mediated acceleration of the cell death process were confirmed by staining and estimation of pro and anti-apoptotic markers. Results from the study showed that pro-apoptotic markers were increased in the chromium-exposed rats. It was also observed that chromium significantly induced cell death by excess ROS generation. Oxidative stress augmented release of more TNF-α which served as a crucial role in cell death by suppressing pAKT followed by caspase-8 and caspase-3 activation. Thus, hydro-methanol (60:40) mixed solvent extract of *Andrographis paniculata* may be used as a potential herbal therapeutic product for improvement of chromium-mediated organ dysfunction and diseases.