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Research Article

Ameliorative Role of Mixed Hydro-methanolic (60:40) Extract of *Andrographis paniculata* on Chromium (VI)-induced Immunomodulation in Male Albino Rats

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ABSTRACT

The immune system controls the body's homeostatic state and helps to defend against infections. Activation and expansion of many biomolecules are crucial for the homeostatic state. Chromium, a common heavy metal found in nature, is an active in its hexavalent state. The abundant use of chromium in paints, steel plants, intoxicated the workers and modulates their body homeostasis. In this article, we were intended to evaluate the role of *Andrographis paniculata* Nees extract on restoration of the body homeostatic state in terms of immune system. For the present investigation, a group of male albino rats (80–100 g) were induced by intraperitoneal injection of vehicle (0.9% NaCl), Potassium dichromate ($K_2Cr_2O_7$) (0.8 mg / 100 g body weight / day), $K_2Cr_2O_7$ plus mixed hydro-methanol solvent extract in the ratio of 60:40 at a dose of 500 mg/kg body weight daily at an interval of six hours after injection of $K_2Cr_2O_7$ for a period of 28 days. We found that Cr (VI) induces a hyper response of pro-inflammatory cytokines followed by apoptosis in liver and lung tissue. Excess production of reactive oxygen species (ROS) is controlling the whole phenomena. The *A. paniculata* Nees extract successively inhibit the ROS generation, as a result a significant quenching of pro-inflammatory cytokine production was noted. *A. paniculata* Nees extract prompted decrease amount of ROS and its associated inflammation provoke the cell survival and helps to maintain a homeostatic state of the body.

INTRODUCTION

High doses and long term exposure of chromium can increase cytotoxic and genotoxic reactions, affecting the body immune system. Till now it is not understood the mechanism of Cr (VI)-exposed cytotoxicity. However, *in vitro* and *in vivo* studies have noted that Cr (VI)-exposed oxidative stress through increased ROS production and genomic DNA fragmentation, modulation of intracellular oxidized states, activation of protein kinase C, apoptotic cell death and altered gene expression.^[1] Apoptotic cell death has been observed in numerous skin inflammatory diseases. In addition, there is a direct link among autophagy, cell death, antigen processing, and the generation of inflammatory and immune responses.^[2] During these

processes, ROS-regulated redox-sensitive protein kinases and transcription factors (for example, Nuclear factor κB (NF- κB), Mitogen-activated protein kinase (MAPK) and Akt pathway) can affect the cytokines release, such as tumor necrosis factor (TNF- α) and interleukin-1 (IL-1).^[3]

A. paniculata is an Indian traditional system used as a medicine for various diseases. There are more than 20 different active bio constituents like flavonoids, phenols, alkaloid, glycosides, saponins and tannins are present in the *A. paniculata*. The *A. paniculata* extract also exhibits good anti-cancer, anti-bacterial and anti-fungal activities.^[4] *A. paniculata* extracts contain the principal compound andrographolide. Methanol extract of this plant was more active in antioxidant activities.^[5]

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Therefore, the present investigation was intended to reduce the Cr-exposed cytotoxicity by using the mixed hydro-methanol solvent extract in the ratio of 60:40 of *A. paniculata* *in vivo* in terms of the expression of certain cytokines and apoptotic signaling pathways.

MATERIALS AND METHODS

Chemicals

Potassium dichromate ($K_2Cr_2O_7$) and other fine chemicals were purchased from Sigma Chemical Company, USA. All other chemicals and reagents were purchased from Sisco Research Laboratory Pvt. Ltd. (SRL), India, and were of analytical grade.

Animals and Diet

Adult male albino rats of Wistar strain of bodyweight 80–100 g were obtained. They were maintained following the guidelines of the Institutional Animal Ethics Committee of Vidyasagar University, Midnapore. They were housed in polypropylene cages and fed standard pellet diet (Hindusthan Lever Ltd., India) for one week and water *ad libitum*.

Preparation of Mixed Solvent Extract

Hydro-Methanol (60:40) mixed solvent extract prepared by using the aqueous and methanol extract from *A. paniculata* Nees.

Mode of Treatment

Rats of almost equal average body weight were divided into three groups. The animals of two groups were injected with $K_2Cr_2O_7$ as described earlier.^[6] The rats of one of the chromium exposed groups serving as the supplemented groups injected with mixed hydro-methanol (60:40) solvent extract at a dose of 500 mg/kg body weight daily at an interval of six hours after injection $K_2Cr_2O_7$ for 28 days. The remaining group of rats have received only the vehicle (0.9% NaCl), served as control.

Animals Sacrifice and Collection of Blood Samples and Tissues

After drug treatment the animals were kept in fasted overnight before sacrifice. The intact liver and lungs were dissected, and blood and tissue fluid were blotted dry weighted. All the samples were kept at -20°C for further analysis.

Homogenization of Tissues

A weighted portion of different tissues was homogenized in an ice cold 0.2 M phosphate buffer saline, PBS (pH 7.4) using glass homogenizer. Homogenized tissues were used for analytical assessment.

Isolation of Rat Blood Lymphocytes

Blood samples were collected from hepatic vein in a heparin-coated Vacutainers. Then the blood diluted 1:1

with PBS. Histopaque 1077 (Sigma) was used for density gradient centrifugation at 400 X g (1500 rpm) for 40 min at room temperature. Lymphocytes comprising the upper monolayer of buffy coat were collected using a clean centrifuge tube and washed three times in balanced salt solution. Supplemented with 10% fetal bovine serum (FBS), the peripheral blood lymphocytes (PBL) were re-suspended in RPMI complete media and incubated for 24 hours in a 95% air 5% CO_2 atmosphere in CO_2 incubator at 37°C .

Cytokine Assay from Liver and Lungs Tissues

Tissues were thawed on ice, and approximately 0.2 mL glass and 1-mL PBS containing protease inhibitors were added to the frozen tissue to avoid degradation on thawing. Subsequently, both samples were homogenized 3 times for 5 minutes at 4°C using a Bullet-Blender (Next Advance, Averill Park, NY). Then homogenates were centrifuged using 2 sequential centrifugation steps ($164\times g$, 4°C , and 10 minutes). After that, supernatants were stored at -80°C to analyze for cytokine levels. The cytokine assay of samples was carried out in duplicate as per the instructions provided with Mesoscale Discovery's Rat 7-PlexUltra-Sensitive Kit (Gaithersburg, MD). Cytokine levels were estimated relative to the total protein content of the sample, i.e., pg/mg protein.

Production of Intracellular Reactive Oxygen Species (ROS) Measurement from Lymphocytes

Intracellular ROS measurement was performed using H2DCFDA according to Dash et al.^[7] After the treatment period, lymphocytes were washed followed by incubation with 1 $\mu\text{g/mL}$ H2DCFDA for 30 minutes at 37°C . Then lymphocytes were washed three times by using PBS. DCF fluorescence was determined at 485 nm excitation and 520 nm emission using a Hitachi F-7000 Fluorescence Spectrophotometer. All measurements were performed in triplicate.

Analysis of the morphology of lymphocytes by Acridine Orange (AO)-Ethidium Bromide (ETBR) Double staining

To know the probable cell death pathway, we analyzed lymphocytes by EtBr-AO double staining method. After the period of treatment, lymphocytes of rats were collected washed with PBS. 10 μL of the cells were kept on a glass slide and mixed with 10 μL of acridine orange (50 $\mu\text{g/mL}$) and ethidium bromide (50 $\mu\text{g/mL}$). Then cells were observed under a fluorescence microscope (NIKON ECLIPSE LV100POL) with 400X magnification.^[8]

Assessment of Nuclear Morphological Changes by DAPI Staining

To observe the nuclear morphological changes of prepared lymphocytes, DAPI staining was performed by an specific method^[9] with some modifications. After the treatment, lymphocytes were fixed with 2.5% glutaraldehyde for

15 min and permeabilized with 0.1% Triton X-100. Then, stain with 1- μ g/mL DAPI at 37°C for 5 min. After that lymphocytes were washed with PBS and observed by fluorescence microscopy (NIKONECLIPSE LV100POL).

Estimation of Cytokines from Lymphocytes

After the treatment of chromium and supplemented with hydro-methanol (60:40) mixed solvent extract of *A. paniculata*, lymphocytes were used to estimate cytokines level. To study the effect of chromium on cytokine production, an ELISA was used for the estimation of tumor necrosis factor-alpha (TNF- α) and interleukin (IL-10) production by using the manufacturer's protocol (eBiosciences, San Diego, USA). The whole experiment was repeated by three times.

Estimation of Pro-apoptotic and Anti-apoptotic Markers

The pro-apoptotic factors caspase-8 and caspase-3, and anti-apoptotic factors pAKT levels were measured by using ELISA.^[10] After the treatment period, lymphocytes were lysed and then centrifuged to collect supernatant. To estimate pro-apoptotic and anti-apoptotic markers, the supernatants were used using ELISA. The plates were coated with caspase-8 (50 μ l per well) and cleaved caspase-3 (c-caspase-3) (50 μ l per well) capture antibodies (2mg/ml) diluted in 0.05M carbonate buffer pH 9.6. Then incubated overnight at 4°C, plates were washed three times with 0.15M PBS-0.05% Tween-20 (PBST) and blocked with 50 μ l per well PBS, 5% FBS, 0.05% Tween-20, 0.02% sodium azide (PBSTN) at room temperature for 1 hours. Then plates were washed three times with PBST and 100 μ L of samples were added to each well and then incubated at 2.5 hat room temperature. After that, plates were washed four times with PBST and incubated with 50 μ l per well of biotinylated anti caspase-8 and caspase-3 detection antibody at room temperature for 2 hours. After three times washes with PBST, 50 μ l per well of HRP-avidin solution (e Biosciences) was added. After 30 min at room temperature, the plates were washed twice in PBST and then 100 μ L per well substrate buffer (e Biosciences) was added and then incubated for 30 min at room temperature in the dark. Optical densities were measured at 450 nm using an ELISA reader (Bio-Rad, Singapore). All samples were analyzed in triplicate.

Protein Estimation

Protein estimation was done according to other researches^[11] taking BSA as a standard.

Statistical analysis

All the parameters were repeated at least three times. The data were presented as mean $\pm$ SEM. By performing ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060, USA), the means of control and treated group was compared by multiple comparison t-test having $P < 0.05$ as a limit of significance.

RESULTS

In liver and lungs tissues, the Pro-inflammatory cytokines (TNF- α and IL-12) levels significantly increased and decreased the Anti-inflammatory cytokines (TGF- β and IL-10) levels in chromium-treated rats (Figs. 1-4). On the other hand, Hydro-Methanol (60:40) extract of *Andrographis Paniculata* plays a vital role in counteracting such alteration types.

ROS induction of lymphocytes is noted in the Fig. 5. It was found that treatment with chromium elevated the cellular ROS level significantly. Supplementation with Hydro-Methanol (60:40) extract of *Andrographis. Paniculata* on intracellular ROS induction in lymphocytes found that lymphocytes' increased level of ROS induction in response to chromium is recovered significantly.

Study the lymphocyte morphology after chromium treatment using Et Br-AO double staining is shown in Fig. 6. From these results, it was observed that chromium treatment decreased the number of viable lymphocyte cells tremendously. After supplementation with Hydro-Methanol (60:40) extract of *A. Paniculata*, no significant

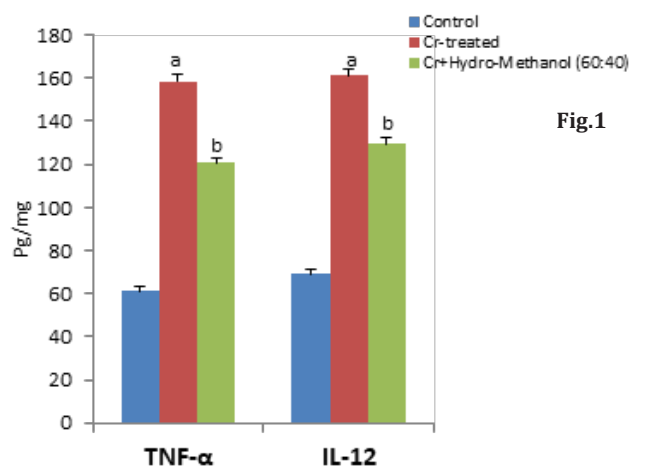


Fig.1

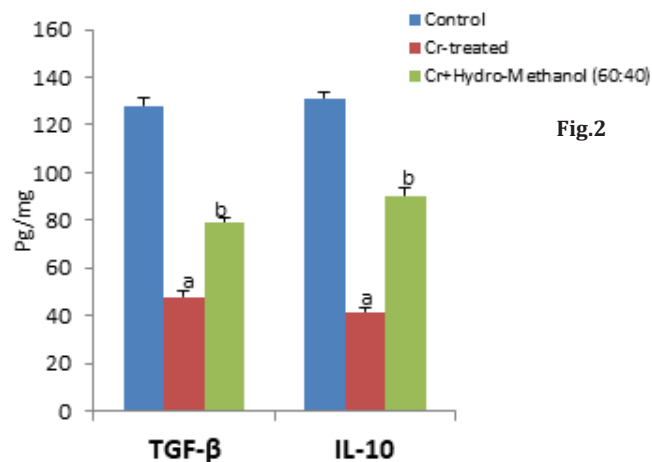


Fig.2

Figs. 1 and 2: Changes 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.



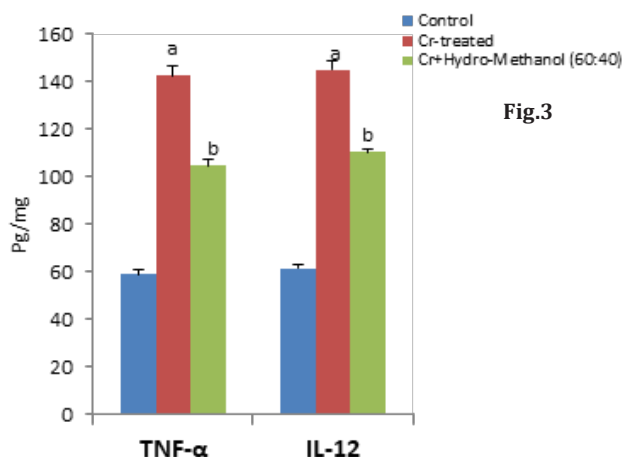


Fig.3

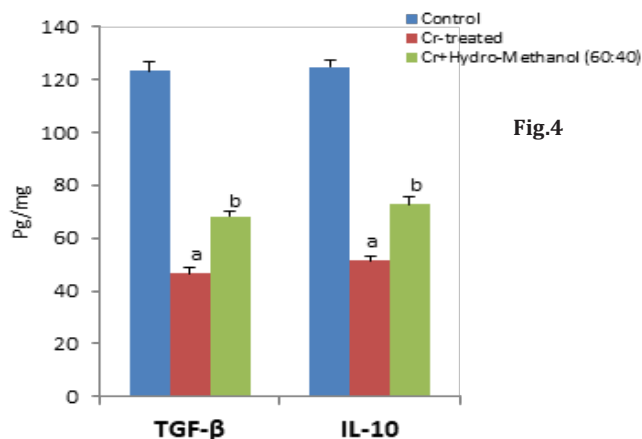


Fig.4

Figs 3 & 4: Changes 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.

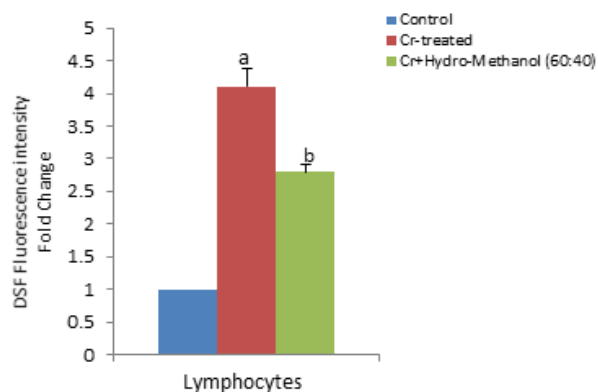


Fig. 5: Effects of chromium and its supplementation with Hydro-Methanol (60:40) extract of *A. Paniculata* on intracellular ROS induction in lymphocytes. ^a $P < 0.05$ compared to control, ^b $P < 0.05$ compared to chromium.

changes were observed upon treatment with chromium in rat lymphocytes.

The structural changes were occurring with characteristics of apoptosis such as chromatin condensation, nuclear fragmentation, and imagination of nuclei was manifest in

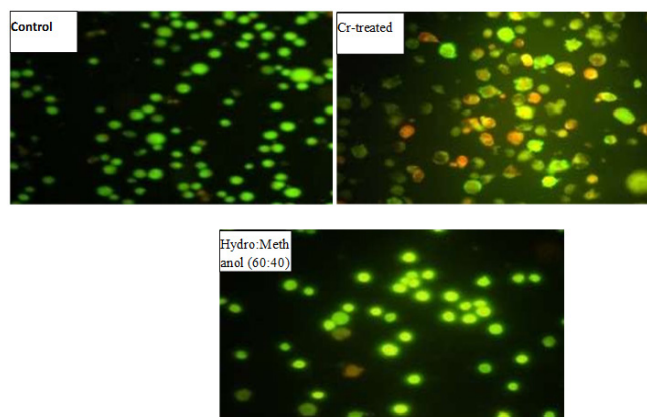


Fig. 6: Effects of chromium and its supplementation with Hydro-Methanol (60:40) extract of *A. Paniculata* on the morphology of lymphocytes. ^a $P < 0.05$ compared to control, ^b $P < 0.05$ compared to chromium.

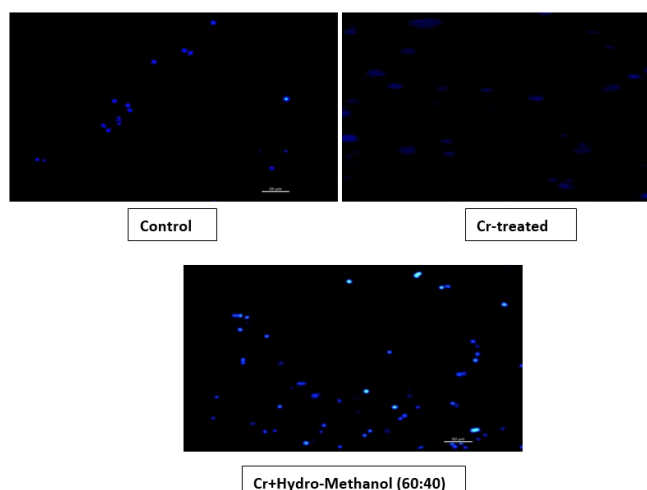


Fig. 7: Changes of nuclear morphological of lymphocyte after supplementation of Hydro-Methanol (60:40) extract of *A. paniculata* in Cr-treated rats. ^a $P < 0.05$ compared to control, ^b $P < 0.05$ compared to chromium.

lymphocytes upon treatment with chromium (Fig. 7). On the other hand, it was found that no significant apoptotic changes were observed in rat lymphocytes upon the co-administration of Hydro-Methanol (60:40) extract of *A. Paniculata* in response 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 (Fig. 8). After the supplementation of Hydro-Methanol (60:40) extract of *A. Paniculata*, it was found that pro- and anti-inflammatory cytokines levels were significantly restored.

After the treatment schedule, lymphocytes were used to quantify pro- and anti-apoptotic markers levels using ELISA. The results demonstrate that caspase-8 and caspase-3 increase significantly and decrease pAKT in response to chromium (Fig. 9). On the other hand, it was observed that such types of pro- and anti-apoptotic markers significantly come to normal level

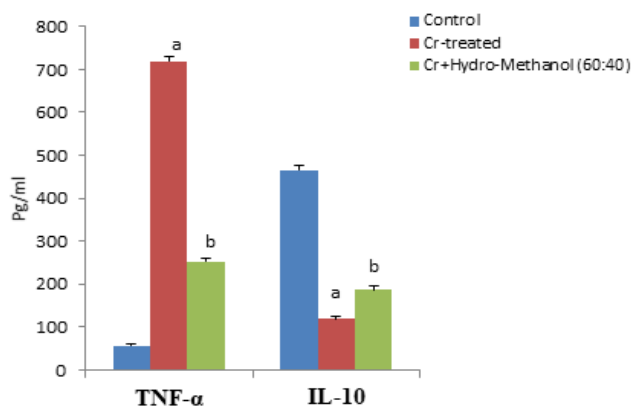


Fig. 8: Changes the cytokines level in lymphocytes after supplementation of Hydro-Methanol (60:40) extract of *A. paniculata* in Cr-treated rats. ^a $P < 0.05$ compared to control, ^b $p < 0.05$ compared to chromium.

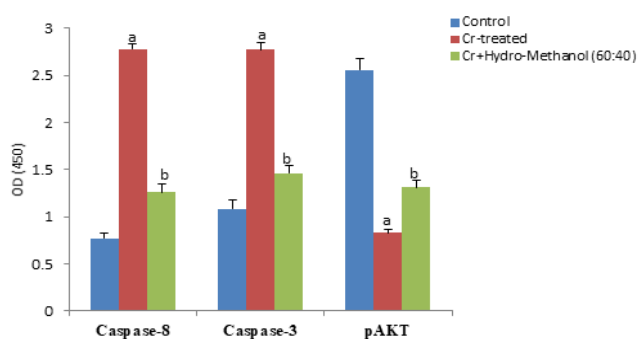


Fig. 9: Estimation of Caspase-8, Caspase-3 and pAKT from lymphocytes after supplementation of Hydro-Methanol (60:40) extract of *A. paniculata* in Cr-treated rats. ^a $P < 0.05$ compared to control, ^b $P < 0.05$ compared to chromium.

after co-exposure of Hydro-Methanol (60:40) extract of *A. Paniculata* in chromium treated rats.

DISCUSSION AND CONCLUSION

There is an urgent need to research the levels of pro-inflammatory cytokines (TNF- α and IL-12) and anti-inflammatory cytokines (TGF- β and IL-10) in liver and lung tissues in chromium-treated rats. It was found that TNF- α and IL-12 levels significantly increased, but the levels of TGF- β and IL-10 decreased significantly after chromium exposure (Figs. 1-4). On the other hand, Hydro-Methanol (60:40) extracts of *A. Paniculata* play a vital role in counteracting such alterations. From this point of view, it was observed that hydro-methanol (60:40) mixed solvent extract of *A. Paniculata* is more potent to regulate the pro- and anti-inflammatory cytokines in response to chromium in liver and lungs tissues.

Now it is important to describe the ROS. They are the molecules and ions containing unpaired valence shell electrons and being a free radical, it is very active and plays an important role in cell signaling regulation, leading to oxidative cell damage and ultimately cell death.^[12] The physiologically active cellular system normally develops

lower ROS during metabolism, which is effectively quenched by diverse antioxidant enzymes of the glutathione system. In addition, intracellular ROS production happens by the mitochondrial respiratory chain reaction, membrane-bound superoxide-generating enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase arachidonic metabolic reaction.^[13] In the present study the level of intracellular ROS was shown in Fig. 5. It was noted that the ROS level of lymphocytes was elevated significantly after chromium treatment. ROS played a crucial role for lymphocytic cell death. But after supplementation with hydro-methanol (60:40) mixed solvent extract of *A. Paniculata* plays a potent role for ROS inhibitor. It was noted that hydro-methanol (60:40), a mixed solvent extract of *A. Paniculata* treatment, effectively protected the lymphocytes from chromium-induced cytotoxicity.

To examine the probable route of cell death occurs due to apoptosis or necrosis, the researcher observed the cells by EtBr-AO double staining method. The Cell morphology study was conducted after chromium treatment.

Et Br-AO double staining is observed in Fig. 6. These typical staining found that the viable cells with intact DNA and nucleus give bright green nuclei, whereas early apoptotic cells will have fragmented DNA and give some green colored nuclei. The late apoptotic and necrotic cell's DNA would be fragmented and stained orange and red.^[8] In our study, it is evident that chromium exposure drastically decreases the viable cells number. Most of the cells exhibited typical characteristics of apoptotic cells like plasma membrane blebbing and the formation of apoptotic bodies. A significant number of cells stained with orange color was elevated. But a small amount of cells was stained with red color. These indicate that most cells were not undergoing necrosis and cell death occurred primarily through apoptosis. In the case of hydro-methanol (60:40) mixed solvent extract of *A. Paniculata* supplementation, no significant apoptotic changes were observed upon chromium treatment.

To examine changes in the nuclear morphology of lymphocyte cells due to chromium treatment, the researcher stained the chromium-exposed lymphocytes by DAPI staining. It was noted that cells displayed significant morphological changes in nuclear chromatin after chromium treatment for an experimental period (Fig. 7). Chromatin condensation and fragmentation is one of the major characteristics of apoptosis.^[14] The nuclear morphological changes were happening with features of apoptosis such as chromatin condensation, the imagination of nuclei, and nuclear fragmentation. These were manifest in lymphocytes upon treatment with chromium. In the case of hydro-methanol (60:40) mixed solvent extract of *A. Paniculata* supplementation, no significant apoptotic changes were observed in response to chromium.

It was found that inflammatory cytokines, mainly TNF- α , are an established stimulator of the caspase-8-mediated apoptosis pathway.^[15,16] TNF- α , a major mediator of inflammation, has two chief signaling pathways. The



pro-survival/pro-inflammatory pathway activates NF- κ B and MAPK through TNF- α -induced signaling complex I. Another TNF- α signaling pathway is the pro-apoptotic pathway through TNF- α induced signaling complex II, in which ROS, a caspase cascade, and the mitochondria function as downstream mediators. The anti-inflammatory cytokine IL-10 has potent anti-inflammatory properties that play a vital role in limiting host immune response to pathogens, preventing damage to the host and maintaining homeostasis. This current research found that the concentration of TNF- α increased significantly, but the concentration of IL-10 decreased substantially in chromium-exposed rat lymphocytes (Fig. 8). 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.

The enhanced pro-inflammatory responses stimulated the cell death process. Caspase activation due to oxidative stress induces and begins a signaling pathway of apoptosis.^[17] Chromium can increase pro-apoptotic factors such as caspase 8 and caspase 3 (Fig. 9) in lymphocytes. ROS growth may directly suppress the pAKT (Fig. 9) and induce apoptosis in chromium-induced rat lymphocytes. But supplementation of hydro-methanol (60:40) mixed solvent extract of *A. Paniculata* acts as one of the most important medicinal plants, containing potent compounds that play a vital role in minimizing the chromium-induced changes of pro- and anti-apoptotic markers in rat lymphocytes.

All the findings noted that chromium exposure induces increased intracellular ROS and pro-inflammatory responses stimulate the cell death process were confirmed by PI staining and determination of pro-apoptotic and anti-apoptotic markers. Our results showed that pro-apoptotic markers were increased in the chromium-exposed rats. It was observed that chromium significantly induced cell death generated by ROS, which can induce TNF- α , serves as an important role in cell death by suppressing pAKT followed by caspase-8 and caspase-3. Hydro-methanol (60:40) mixed solvent extract of *A. paniculata* may be used as a potential therapeutic natural herbal product to improve chromium-mediated different diseases.

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