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Silymarin Attenuates Chromate (VI)-Induced Toxicity on Human Peripheral Blood Lymphocytes *In- Vitro.*

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ABSTRACT

Hexavalent chromium compounds are well known human carcinogens. The effects of pretreatment with the flavanolignan silymarin (25, 50, 100 µg/ml) to counteract the toxic effects of Chromate (VI) (10 µg/ml) was studied on human peripheral blood lymphocytes cultured *in vitro*. Standard cell proliferation and cytotoxicity assays like MTT assay, NBT reduction test, trypan blue dye exclusion test, estimation of the levels of lipid peroxides and nitric oxide was performed to study the detrimental effect of chromate (VI) on the proliferation of the lymphocytes and to assess the degree of protection offered by pretreatment with silymarin. Chromate (VI) treatment (10µg/ml) resulted in statistically significant decrease in cell proliferation ($P < 0.001$), increase in cytotoxicity and increased oxidative stress ($P < 0.001$). Pretreatment with silymarin (100 µg/ml) for 15 minutes prior to chromate exposure was able to significantly prevent against chromate (VI)-induced toxicity which was confirmed by all the above mentioned assays. The results of our study give leads about the possible use of silymarin as a diet based prophylactic regimen to counteract the toxic effects of chromate in workers employed in chromium based industries.

Keywords: Chromate (VI), Cell proliferation, Oxidative stress, Prophylactic antioxidant, Silymarin.

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INTRODUCTION

Human cells are continuously subjected to physiological and external influences which can give rise to cytotoxic, genotoxic, and oxidative damage. However cells have sophisticated mechanisms for counteracting and minimizing these types of damages. In the recent years there has been increased understanding that dietary constituents and natural products can modulate these forms of toxicity in cells. Among these, polyphenolic antioxidants are receiving increasing attention in the recent years. One such naturally occurring polyphenolic antioxidant is silymarin. Silymarin derived from the milk thistle plant, *Silybum marianum,* is a flavonolignan shown to possess hepatoprotective, antioxidative, antilipid-peroxidative, antifibrotic, anti-inflammatory, anti-carcinogenic, membrane stabilising and immunomodulatory properties [1]**.** Its active constituent Silybin also known as Silibinin has been reported to be a potent antioxidant, scavenging free radicals and inhibiting lipid peroxidation. Studies also suggest that it protects against genomic injury, increases hepatocyte protein synthesis, decreases the activity of tumour promoters, and stabilizes mast cells and slows down calcium metabolism [2]. Silymarin is found to be effective in counteracting oxidative stress produced by acute or sub-chronic sodium arsenite exposure [3].

Chromium salts are well known carcinogens, teratogens and immunosuppressive agents. Exposure to chromium has been shown to cause asthma, ulceration of the nasal septum and subsequent perforation, pulmonary congestion, respiratory tract cancers, skin ulcerations, allergic and contact dermatitis, non-respiratory cancers with other health effects such as nephrotoxicity, hepatotoxicity, epigastric pain, erosion and discolouration of teeth, eye injury, leukocytosis, leukopenia and eosinophilia. Evidence of lung cancer is increased up to 15 times in workers exposed to dust of chromite and death from respiratory cancer increased 24 times for exposed individuals of all ages versus non exposed individuals [4].

As no previous studies have been undertaken to analyse the possible use of silymarin pretreatment as a prophylactic regimen against Cr (VI)-induced toxicity, in the present investigation an attempt has been made to study the effects of silymarin supplementation to ameliorate the toxic and immunosuppressive conditions induced by chromate (VI) on human peripheral blood lymphocytes (HPBL) *in vitro.* The present study is expected to give leads regarding the possible use of silymarin pretreatment to prevent occupational health hazards due to chromium toxicity and during clinical immunosuppressive conditions.

MATERIALS AND METHODS

Chemicals

Potassium dichromate was purchased from Hi Media Laboratories Limited, Mumbai, India and Silymarin was procured from Sigma Chemical Company (St. Louis, MO, USA). Other chemicals used for the investigation were of analytical grade and was purchased from local companies.

Isolation of Human Peripheral Blood Lymphocytes

Fresh human peripheral blood was drawn from healthy volunteers (age group between 18-25) following the Helsinki protocol through the median cubital vein of the forearm. Lymphocytes were isolated from the blood by following the method of Boyum [5] applying the technique of density gradient centrifugation by using lymphocyte separation media (LSM 001-Hi Media, India).

Lymphocyte preparations having >90% viability as assessed by trypan blue dye exclusion test was suspended in Iscove's Modified Dulbecco's Medium (AL 070A- Hi Media, India) supplemented with Antibiotic Antimycotic solution (A 002- Hi Media, India) and 10% fetal bovine serum (RM 1112- Hi Media, India).The cell count was adjusted to contain $5x10^6$ cells/ml and the cells were plated on to 96 well micro titer tissue culture plates (Hi Media) for the assays.

Experimental Set Up

Plated cells were pre-incubated with different concentrations of Silymarin (25, 50, 100µg/ml) 15 minutes prior to the addition of Chromate (10 µg/ml) for induction of toxicity and immunosuppressive conditions. Cell control wells, media control wells, and vehicle control wells were put up for comparison

purpose. The plates were incubated at 37°C with 5% CO_2 and 95% humidity in a carbon dioxide incubator (Forma scientific, USA) for 72 hours. After 72 hours of proliferation all specialized assays viz., cell growth and viability assays (MTT assay and NBT reduction test), cytotoxicity assay (trypan blue dye exclusion test), and assessment of oxidative stress (estimation of lipid peroxide and nitric oxide) were carried out.

Cell Growth and Viability Assay

MTT Assay

MTT assay was performed following the method of Mosman[6]**.** After 72 hours of proliferation 25 µl of MTT (5 mg/ml) in phosphate buffered saline (PH 7.4) was added to the wells. The plates were then incubated in a Carbon dioxide incubator for six hours. After six hours the formazan crystals produced were solubilized by adding 75 µl of Dimethyl sulfoxide to each well followed by vigorous mixing. The intensity of the colour developed was read at 570nm in a micro plate reader (Bio Tek system, USA). The intensity of the colour developed is directly proportional to the number of viable cells present.

NBT Reduction Test

NBT reduction test was performed following the method of Williams [7]**.** Briefly after 72 hours of proliferation, 10 µl of nitro blue tetrazolium chloride (5mg/ml) was added and incubated in a CO_2 incubator at 37°C for 5 hours. The cells were then washed three times with isotonic phosphate buffered saline and the NBT reduced was solubilised in 100µl of Isopropanol. The optical density of each well was measured at 570nm using a micro plate reader.

Cytotoxicity Assays

Trypan Blue Dye Exclusion Test

After the required period of proliferation, appropriately diluted cells (control, untreated and treated) were mixed with the dye trypan blue (0.1%) and the suspension was charged in to a haemocytometer. The number of viable cells present was counted in all the four corner squares of the haemocytometer under an inverted microscope and the final cell count was obtained by employing the formula:

Cell count/ml =Mean cell count X dilution factor X 10,000

Assessment of Oxidative Stress

Estimation of Lipid Peroxides

The levels of lipid peroxides were estimated by the method of Ohkawa[8] . Briefly after 72 hours of proliferation 1ml of the cell suspension of the treated and untreated cells were added to 0.5ml of 10% Thiobarbituric acid and centrifuged at 1500 rpm for 10 minutes. From this 700 µl of the supernatant was taken into a fresh vial and 500 µl of Thiobarbituric acid was added. The tubes were incubated at 87ºC for 45 minutes and centrifuged at 1500 rpm for 5 minutes. The supernatant was taken and the intensity of the colour developed was read at 540nm.

Estimation of Nitric Oxide Release

Nitric oxide released was estimated based on the Griess reaction following the method of Green [9]. Briefly Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine in 5% phosphoric acid) was added to an equal volume of cell culture supernatants and the absorbance at 546nm was measured after 10 minutes. The amount of nitric oxide produced was quantitatively measured using sodium nitrate as standard.

Statistical Analysis

All the experiments were carried out in triplicate on two different occasions and the statistical analysis of the data was determined by Student's t-test.

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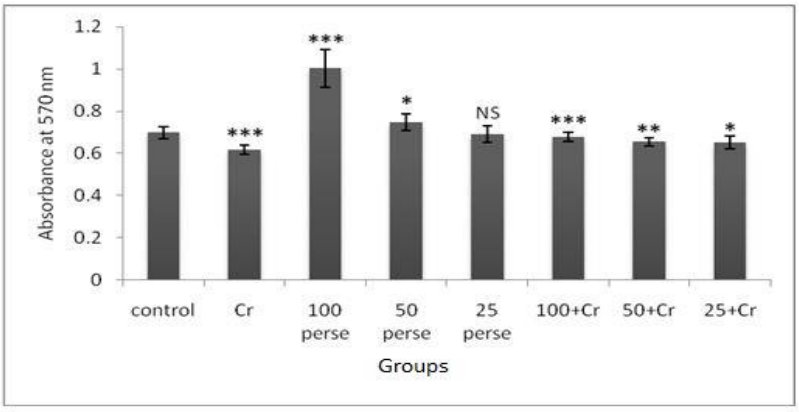
RESULTS

Effects of Pretreatment with Silymarin on MTT Assay (Figure 1)

Results shows the effects of pretreatment with silymarin (25, 50 and 100µg/ml) during chromate-induced immunosuppression on human peripheral blood lymphocytes by MTT assay. These dosages were fixed up after the preliminary studies with silymarin using concentrations ranging from 1.56 to 500µg/ml (Data not shown). Results indicate that chromate treatment resulted in significant inhibition of cell growth and proliferation ($P < 0.001$) as compared to the control. Cells treated with silymarin *per se* showed statistically significant increase in cell proliferation at the dosages of 100µg/ml ($P < 0.001$) and 50µg/ml ($P < 0.05$), but at the dosage of 25 µg/ml, non-significant increase was observed as compared to control. Pretreatment of the cells with all dosages of silymarin prior to chromate treatment resulted in statistically significant protection (100µg/ml= $P < 0.001$, 50µg/ml= $P < 0.01$ and 25µg/ml= $P < 0.05$) against chromate toxicity as evidenced by increase in cell proliferation as compared to chromate treated group.

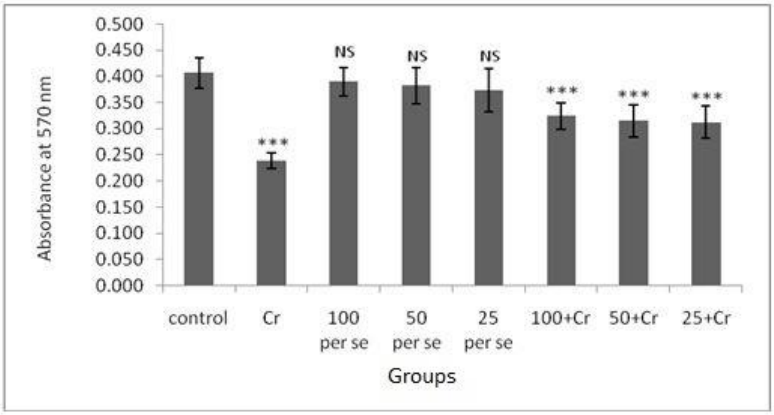
Figure 1: Effects of silymarin pretreatment on MTT assay



Values are expressed as mean ± standard deviation (n=6). Statistical comparisons are made between control vs. Cr and per se vs. control and silymarin+Cr vs. Cr. *** P< 0.001, ** P< 0.01, * P< 0.05 and NS nonsignificant

Effects of Pretreatment with Silymarin on NBT Assay (Figure 2)

Figure 2: Effects of silymarin pretreatment on nitro blue tetrazolium reduction assay



Values are expressed as mean ± standard deviation (n=6). Statistical comparisons are made between control vs. Cr and per se vs. control and silymarin+Cr vs. Cr. *** P< 0.001; NS: Non-significant.

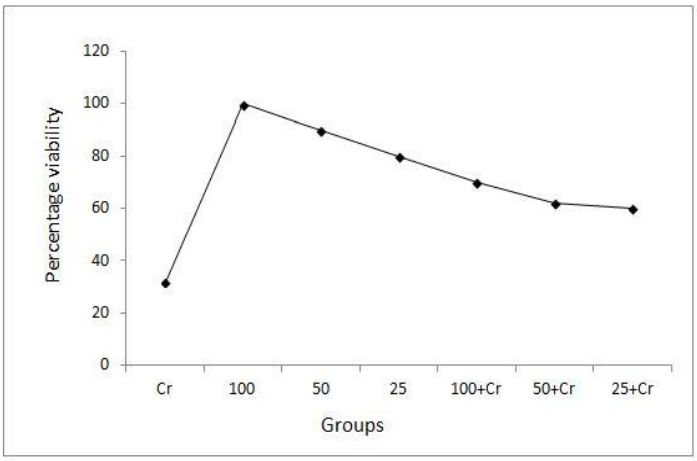
In agreement with the MTT assay, chromate treatment resulted in significant immunosuppression as evidenced by the significant decrease in the reduction of nitro blue tetrazolium ($P < 0.001$). This gave an indication about the strong immunotoxic effects of chromate and inhibition of cell proliferation. Cells treated *per se* with silymarin at all three dosages showed statistically non-significant change in cell proliferation as compared to control. Pretreatment with silymarin at all three dosages showed appreciable protective effects against chromate toxicity. This is evident from the statistically significant increase in the NBT reduction (P <

0.001) at all dosages employed as compared to the chromate treated group.

Effects of Pretreatment with Silymarin on Trypan Blue Dye Exclusion Test (Figure 3)

Effects of silymarin on chromate-induced cytotoxicity as observed by the trypan blue dye exclusion test. Chromate treatment resulted in significant cytotoxicity as observed from decreased cell survival (33%) due to inability of the cells to exclude the dye trypan blue as compared to control (100%). Silymarin treatment *per se* at the dosage of 100µg/ml showed a 100% cell survival whereas the dosages of 50µg/ml and 25µg/ml showed a percentage survival of 93.54 % and 82.60 % respectively. This is in agreement with the cell proliferation assay (MTT assay) where in the survival rates were maximum for the dosage of 100µg/ml (143.69%) followed by 50µg/ml (107.16%) and 25µg/ml (98.997%) as compared to 100% control.

Figure 3: Effects of silymarin pretreatment on trypan blue dye exclusion test

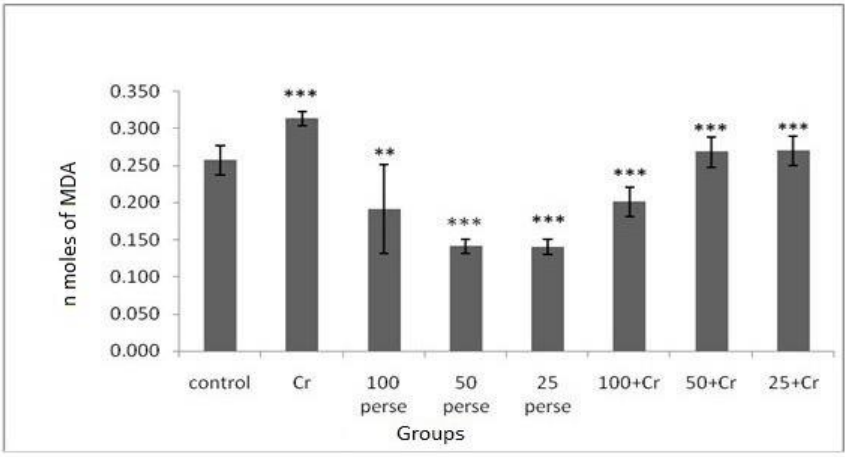


Values are expressed as mean ± standard deviation (n=6). Statistical comparisons are made between control vs. Cr and per se vs. control and silymarin+Cr vs. Cr

Silymarin pretreatment exhibited significant protection and preserved membrane integrity against chromate toxicity as indicated by enhanced cell survival (100µg/ml (68.96%); 50µg/ml (65.11%); 25µg/ml (61.53%) as compared to the chromate treated group (33%).

Effects of Pretreatment with Silymarin on Lipid Peroxidation (Figure 4)

Figure 4: Effects of silymarin pretreatment on lipid peroxidation



Values are expressed as mean ± standard deviation (n=6). Statistical comparisons are made between control vs. Cr and per se vs. control and silymarin+Cr vs. Cr. *** P< 0.001; ** P< 0.01

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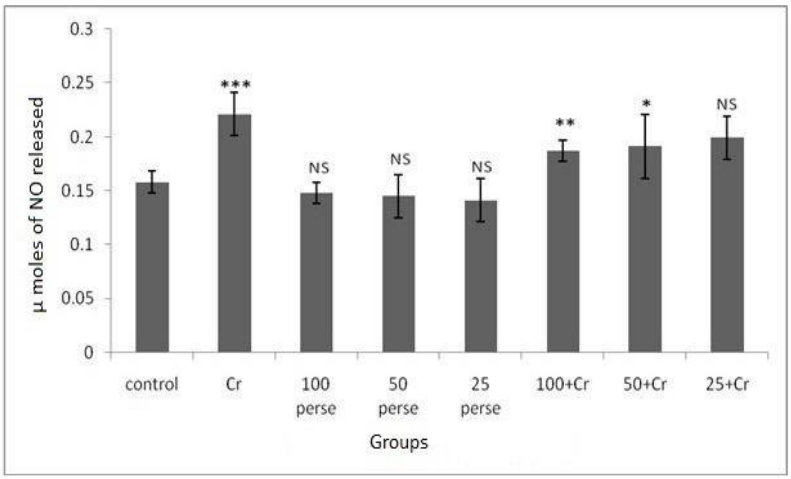
Results indicated that exposure to chromate (VI) resulted in the induction of oxidative stress as elicited by highly significant increase ($P < 0.001$) in the levels of lipid peroxides as compared to the control . Cells treated with silymarin *per se* showed significant decrease in the levels of lipid peroxides (100µg/ml- $P < 0.01$; 50µg/ml and 25µg/ml- $P < 0.001$) as compared to control. This indicates the antioxidant effects of the drugs *per se* at the dosages used in the study. Cells pretreated with silymarin prior to chromate exposure showed a statistically significant decrease in the levels of lipid peroxides at all the three dosages ($P < 0.001$) as compared to the chromate treated groups.

Effects of Pretreatment with Silymarin on Nitric Oxide Release (Figure 5)

Results shows the effects of silymarin on nitric oxide release during chromate-induced immunosuppression on human peripheral blood lymphocytes *in vitro*. Chromate-induced toxicity and immunosuppression was manifested by an increase in nitric oxide release which was found to be statistically significant ($P < 0.001$). Cells treated with silymarin *per se* at all the dosages showed statistically non-significant changes in the level of nitric oxide release as compared to control. Pretreatment with silymarin prior to chromate exposure showed significant decrease in nitric oxide release at a dosage of 100µg/ml ($P < 0.01$) and 50µg/ml ($P < 0.05$) whereas the dosage of 25µg/ml was found to be ineffective to prevent nitric oxide release as compared to the chromate-treated group.

Figure 5: Effects of silymarin pretreatment on nitric oxide



Values are expressed as mean ± standard deviation (n=6). Statistical comparisons are made between control vs. Cr and per se vs. control and silymarin+Cr vs. Cr. *** P< 0.001; ** P< 0.01; * P<0.05; NS: Non significant

DISCUSSIONS

The soluble hexavalent chromium is an environmental contaminant widely recognized to act as carcinogen, mutagen and teratogen towards humans and animals. Hexavalent chromium primarily enters the cells and undergoes metabolic reduction resulting in the formation of reactive chromate intermediates and reactive oxygen species together with oxidative tissue damage and culminating in a cascade of cellular events leading to cytotoxicity and apoptosis [10].

In the present study chromate treatment at a concentration of 10 µg/ml caused a significant inhibition of cell proliferation and exhibited significant cytotoxicity on human peripheral blood lymphocytes (HPBL) as evidenced by several tests like MTT assay, NBT reduction test and trypan blue dye exclusion test as compared to control. This can be attributed to the ability of chromate to enter the cell, cause mutagenic DNA lesions [11], arrests DNA replication and transcription resulting in S phase delay in the cell cycle [12-14] and finally cell death. Apart from causing DNA damage, Chromate (VI) is a potent inducer of cytokines TNF-α [15-16] and activates NF-κB that plays a vital role in triggering the inflammatory response, culminates in cytotoxicity and inhibition of cell proliferation.

Interestingly it has been reported that silymarin blocked activation of NF-κB and the translocation of p65 to the nucleus without affecting its ability to bind to the DNA. Silymarin also inhibited TNF-induced

activation of mitogen-activated protein kinase and C-jun N terminal kinase and abrogated TNF-induced cytotoxicity and caspase activation [17]. It also increases protein synthesis by its action on RNA polymerase I [18-20]. The structural similarity of silymarin to steroid hormones is believed to be responsible for its protein synthesis facilitatory actions [21]. All the above mentioned cumulative mechanisms could be responsible for the observed protective effects of silymarin pretreatment against chromate toxicity.

Chromium treatment resulted in statistically significant increase in the levels of lipid peroxides and nitric oxide release in the cells, thereby inducing oxidative stress and causing cell damage. Chromium reduction intermediates are believed to react with hydrogen peroxide to form the hydroxyl radical through a Fenton/Haber-Weiss reaction. This radical is capable of abstracting a hydrogen atom from a methylene group of polyunsaturated fatty acids enhancing lipid peroxidation [22-23] and may finally attack membrane lipids thereby disrupting cellular functions and integrity. This could be the reason for the increased levels of lipid peroxides and the subsequent cell damage observed in the cells treated with chromate as compared to the control wells.

Silymarin has been reported to protect against lipid peroxidation induced by several xenobiotic agents [24-25] and has been shown to be a strong antioxidant capable of scavenging free radicals by increasing cellular glutathione content, by preventing the oxidation and depletion of glutathione and by inducing the activity of superoxide dismutase [26-27]. In the present study treatment with silymarin *per se* showed statistically significant decrease in the levels of the lipid peroxides as compared to control which indicates the antioxidative property of silymarin. Like most antioxidants, it is a reducing agent due to its hydrogen and electron donating properties. All the three doses of silymarin employed in the study showed significant decrease in the levels of lipid peroxides as compared to chromate treated group and increased antioxidant effect was observed at the highest dose employed in the study (100 µg/ml).

In the current investigation, statistically significant increase in the levels of nitric oxide (NO) was observed in the cells treated with 10 µg/ml of chromate as compared to control. The effects of chromate in inducing the release of nitric oxide are mediated by two factors, activation of NF-κB and generation of reactive oxygen and nitrogen species. Accumulating evidence suggest that many metals are able to effect the activation or activity of NF-B transcription factor [28]. It has been well known that the hexavalent state of chromium is the strongest oxidizing form of chromium and is able to activate NF-κB even at lower concentrations [29]. This activation of NF-κB induced by hexavalent chromium in turn, up-regulates expression of inducible nitric oxide synthase (iNOS) gene through NF-κB/Rel family of proteins, which results in production of nitric oxide at high concentration inside the chromate treated cells. Also under oxidative stress the cytosolic calcium (Ca^{2+}) level increases and causes stimulation of nitric oxide synthase generate NO which inhibits complex IV (H^+ pump) enhancing reactive oxygen species output and finally induce apoptosis in human lymphocytes([29-30].

It has been demonstrated that silymarin inhibits NO production and iNOS gene expression in macrophages through the inhibition of NF-κB/Rel transcription factor by inhibition or suppression of both DNA binding activity and expression of NF-κB gene there by resulting in decreased levels of nitric oxide inside the cell [29]. Reactive oxygen species pathway is also believed to be involved in NF-κB/Rel activation, and it might be another target of silymarin [31]. The inhibition of NF-κB/Rel activation by silymarin is mediated by its radical scavenging and antioxidative activity [21-32] and this inhibition can reduce the nitric oxide levels in lymphocytes cultured *in vitro* during chromate-mediated oxidative stress. This could be the reason for the observed decrease in the levels of nitric oxide in the cells pre-treated with Silymarin prior to chromate challenge.

CONCLUSION

Thus based on this study, it could be revealed that Silymarin pretreatment at the concentration of 100µg/ml was found to be effective in reducing the inhibition of cell proliferation, cytotoxicity and oxidative stress induced by the carcinogen chromate on human peripheral blood lymphocytes. Silymarin is a potent immune-response modulator, with both immunostimulatory and immunosuppressive activities, which may be dependent on its concentration and/or treatment procedure. The dosages of the drug used, the dosage of chromate used, the period of pretreatment with the drug, and the duration of exposure to chromate, and the type of cells used in the study are all believed to play a vital role in determining the degree of protection against chromate toxicity. The study gives a preliminary idea about the potential efficacy of antioxidant

silymarin and the possibility of using it as a prophylactic regimen to reduce the risk of occupational hazards (chromate exposure) in humans and also the possibility of using it as an adjuvant therapy in patients who are under the risk of developing immunosuppression (HIV, HBV patients).

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