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ANTIGENOTOXIC EFFECTS OF SILYMARIN AGAINST NICKEL CHLORIDE MEDIATED DNA DAMAGE IN CULTURED HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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ABSTRACT: DNA damage induced by Nickel chloride in lymphocytes has been studied *in-vitro*. The sister chromatids exchange and single-cell gel electrophoresis (SCGE), or comet assay were used to measure the level of DNA damage in terms of SCE frequency and comet parameters. Silymarin is a natural protective compound, isolated from *Silybum marianum* seeds and reported for its anti-genotoxic properties. In this study, the peripheral blood lymphocytes were administrated along with silymarin and Nickel chloride ($NiCl_2$) to evaluate the antigenotoxic effect of silymarin against $NiCl_2$ mediated genotoxicity. A significant ($P<0.05$) increase in the tail moment and SCE frequency indicating DNA damage was observed in $NiCl_2$ (129.59 µg/mL) treated samples. Simultaneously, a gradual decrease was observed in the tail moment, and SCE frequency in silymarin-treated samples against $NiCl_2$ induced DNA damage. Results showed that silymarin at a concentration of 40 µg/mL against $NiCl_2$ induced DNA damage showed a significant ($P< 0.05$) decrease in genotoxicity.

INTRODUCTION: Nickel and nickel compounds are associated with a multitude of health problems such as contact dermatitis, respiratory diseases (asthma), inflammation, pulmonary fibrosis, pulmonary edema and bronchitis [1]. According to the maximum allowable concentration (MAK) and biological tolerance (BAT) value report [2] nickel chloride is categorized as substances that can cause cancer or can be assumed to contribute to cancer risk.

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Many studies have reported that $NiCl_2$ induced ROS in different cell lines, including kidney cells of rats and human lymphocytes [3]. Its genotoxicity may be connected to the excess generation of DNA-damaging ROS and curtailment of DNA repairs [4]. ROS may arise from oxidative burst or mitochondrial transport chain in different cell types and as a byproduct of some enzymatic reactions [5].

It has been observed that many nickel salts induce chromosomal abnormalities in the bone marrow and spermatocyte mice which may lead to heritable changes in the genome and increase the risk of cancer [6]. However, the exact underlying mechanism of nickel-induced genotoxicity is still under investigation. Silymarin (SM), a natural hepatoprotective drug isolated from seed extract of *Silybum marianum* (Common name- Milk thistle,

Ayurvedic name –Dudhiya Kantkari) is reported to show antigenotoxic activities by reducing ROS formation in cells. Silymarin is a mixture of four isomeric flavonoids such as silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B [7, 8]. Silymarin has shown a protective effect against methyl methanesulfonate (MMS) induced genotoxicity by upregulating the gene expression of PTEN, BCL2 and downregulation of BAX and ABL1 expression in human blood cells [9].

Single-cell gel electrophoresis (Comet assay) is a simple, sensitive and economically favorable technique widely adopted to measure double and single DNA strand breaks, alkali labile sites and DNA repair impairs in a wide range of cells such as blood cells, cancerous cells, yeast and bacterial cells, *etc.* [10]

Sister chromatid exchanges (SCEs) are reciprocal exchanges of chromatin segments between homologous chromosomes that occur at low frequency in normal cells as a mechanism of homologous DNA repair. The frequency rises with the exposure of genotoxic agents. SCE assay is an excellent and widely used tool for the evaluation of genetic damage [11].

The study of silymarin-mediated improvement in the integrity of DNA in stress conditions is of great interest. Hence, the study focuses on the effect of SM against $NiCl_2$ mediated DNA damage in human PBLs.

Methodology:

Materials and Reagents: Nickel chloride (CAS: 7718-54-9) and silymarin (CAS: 65666-07-1) were obtained from Sigma Aldrich. All the experimental and culture-related reagents, chemicals, and solutions were purchased from Himedia.

Subject Recruitment: After taking consent, venous blood (4-5 mL) was aspired from healthy participants (Age 25-30 years) in heparin-coated vacutainer tubes (BD Vacutainer) under aseptic conditions. Ethical clearance for this research was accorded by the Institutional Human Ethics Committee, Kurukshetra University, Kurukshetra, vide No. IHEC/17/421.

Experimental Design: The culture of peripheral blood lymphocytes was done as per the protocol of Moorhead *et. al.,* with slight variations [12]. To each culture tube, RPMI 1640 medium (5 mL) including L-glutamine (1%), fetal bovine serum (10%), phytohemagglutinin (2%), penicillin (100 IU /L), and streptomycin (100 µg/mL) was added (HiMedia). To this 400 uL of freshly aspirated venous blood from healthy individuals was added. Different concentrations of $NiCl_2$ (16.19-129.59 µg/mL) were administered in the culture medium to evaluate its genotoxic potential. Similarly, silymarin at concentrations (8-40 µg/mL) was used to treat the cells to evaluate the antigenotoxic potential against $NiCl_2$ (129.59 µg/mL). Ethyl methanesulfonate (EMS) 2 µg/mL and dimethyl sulfoxide (DMSO) 20 µg/mL were used as positive and negative controls, respectively. Cell culturing was done at 37 °C in 5% CO_2 for 24 h for comet assay and 72 h for sister chromatid exchange assay in a humidified atmosphere.

Single-cell Gel Electrophoresis: The assay was performed directly with whole blood according to Singh *et al.,* with minor modifications [13]. In brief, after 24 h in culture, cells were isolated by centrifugation at 1,500 rpm and washed two times with phosphate buffer saline (PBS). Cells were mixed up in 0.5 % low melting agarose (LMA) and coated on a slide precoated with 1% normal melting agarose (NMA). The slides were immediately covered with coverslips and put for 10 min at 4 ºC.

Another layer was coated with low melting agarose to trap cells in the gel layers and allowed to solidify. Coverslips were removed and kept in chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM tris base, 1% Triton X-100 and 10% DMSO, pH 10) for 2 h at 4 ºC in the dark. After this, slides were arranged perpendicularly to electrodes in an electrophoresis tank and dipped completely in fresh cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH ≥ 13), and kept for 25 min. This was followed by electrophoresis (24 V and 300 mA) for 30 min.

After rinsing in distilled water, the slides neutralized for 5 min in neutralizing buffer (0.4 M tris buffer, pH 7.5). The slides were finally stained with ethidium bromide (EtBr) 10 µg/mL for 2 to 3 min and visualized under a fluorescent microscope for the appearance of comets

Comet Scoring: Visualization of the slides was done under a fluorescent microscope (Olympus CX41). The intact round cells indicated no DNA damage. Damaged cells appeared to have a comet-like shape. Fifty cells were analyzed randomly using comet assay IV software. The level of DNA damage was measured as % tail DNA (TD), tail length (TL), tail moment (TM), and olive tail moment (OTM). Tail length corresponds to the migration of DNA; the tail moment is the product of % tail DNA and tail length, while the olive tail moment is the product of % tail DNA and the distance between the head and tail centers of gravity [14].

Sister Chromatid Exchange Assay: The assay was performed as per the method of Wolff and Perry with slight changes [15]. Peripheral blood was cultured for 72 h as explained above. After 24 h of culture, 5'-bromodeoxyuridine (10 µg/mL) was added for two complete rounds of DNA replication. Colchicine (0.2 µg/mL) was added to cultures 35 min before harvesting to stop the cells at the metaphase stage.

Next, the cells were put in 8 mL 0.075 M KCl for 10–15 min at 37 ºC followed by fixing in ice-cold (3: 1 methanol to the acetic acid solution) fixative. Preparation of metaphase spreads was done on clear glass slides and followed by air drying of slides, 45 min staining with the Hoechst 33258, and 2 h long-wave UV light exposure. Slides were stained with Giemsa stain for 10 min and observed under microscope.

Chromosomes were analyzed under a light microscope with 100 × magnification for a permanent record of exchanges. Images were captured as explained above and metaphase spreads were observed for analysis of SCEs. Around fifty cells were observed, and SCE frequency was calculated as the number of exchanges/cells.

Statistical Analysis: Statistical analysis was done using SPSS 16 software. The results were expressed as Mean ± S.D. All the experiments were performed in duplicates. One-way analysis of variance (ANOVA) was carried out to compare the samples, and student's t-test was performed to compare paired samples. The values at $p<0.05$ were taken as significant.

RESULTS:

Genotoxicity of $NiCl_2$: Cells exposed to different concentrations of nickel (16.19-129.59 µg/mL) showed an increase in the appearance of comet parameters as compared to control. It was observed that at a dose of 129.59 µg/mL, nickel chloride caused a highly significant ($p<0.05$) rise in comet parameters such as tail DNA (TD %), tail length (TL µm), tail moment (TM arbitrary units), and olive tail moment (OTM arbitrary units) **Fig. 1**. However, increasing the concentrations beyond 129.59 µg/mL resulted in a decline in the cell population which was measured by trypan blue exclusion assay (data not shown). For different samples (n=30) treated with highest concentration of nickel (129.59 µg/mL) showed a significant increase in mean TD (37.7±9.3), TL (35.4±6.7), TM (12.8±2.3), and OTM (8.7±1.4) as compared to control (TD 3.4±1.8, TL 3.1±1.5, TM 0.3±1.2 and OTM 0.61±0.64) **Fig. 3**. The same concentration of nickel also resulted in a significant rise in the SCE frequencies (Mean SCE/Cell, 8.9 ± 0.42) as compared to control (Mean SCE/Cell 2.6 ± 0.30) **Fig. 4**.

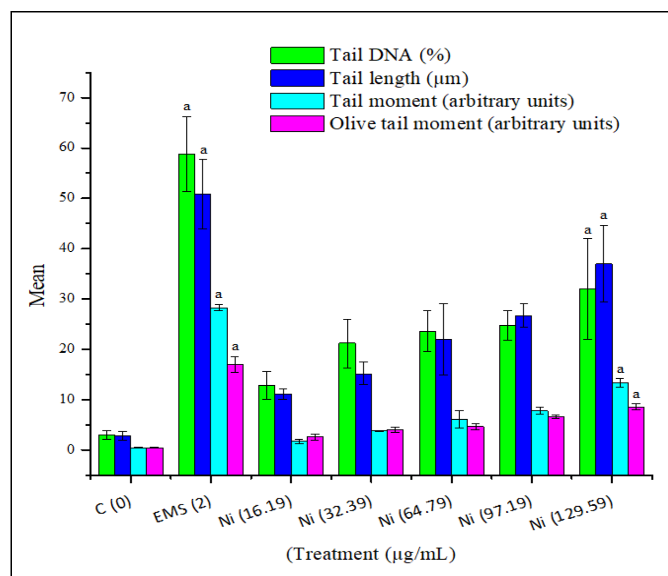

FIG. 1: EFFECTS OF DIFFERENT CONCENTRATIONS OF $NICL_2$ ON DNA DAMAGE IN CULTURED HUMAN PBLS ASSESSED BY COMET ASSAY PARAMETERS. C-Control, EMS -ethyl methanesulfonate, Ni –$NiCl_2$. $^{a}p<0.05$ significant as compared to control

Antigenotoxic Effects of Silymarin: To determine the antigenotoxic potential of silymarin, cells were treated with different concentrations of silymarin against the highest $NiCl_2$ concentration. Administration of silymarin at different concentrations (8-

40 µg/mL) in cultures along with nickel chloride (129.59 µg/mL) resulted in decreased % TD, TL, TM, and OTM **Fig. 2**. For different samples (n=30), cells co-cultured with 129.59 µg/mL nickel chloride and silymarin (40 µg /mL) showed a significant ($p<0.05$) reduction in the TD (25.9 ±5.9%), TL (22.4± 4.8), TM (5.6±1.3), and OTM (4.46±0.47) as compared with cells treated with only nickel chloride **Fig. 3**. Treatment with different concentrations of SM also leads to a dose-dependent decline in the SCE frequencies in cells treated with the selected dose of $NiCl_2$ **Fig. 4**. For different samples (n=30), treatment with silymarin (40 µg/mL) in combination with the highest dose of nickel chloride resulted in a significant decrease in mean SCE/cell (Mean SCE/Cell, 5.8 ± 0.70) **Fig. 5**. Individual treatment with silymarin alone did not cause a significant rise in the comet parameters and SCE frequency and thus indicates that silymarin is non-genotoxic.

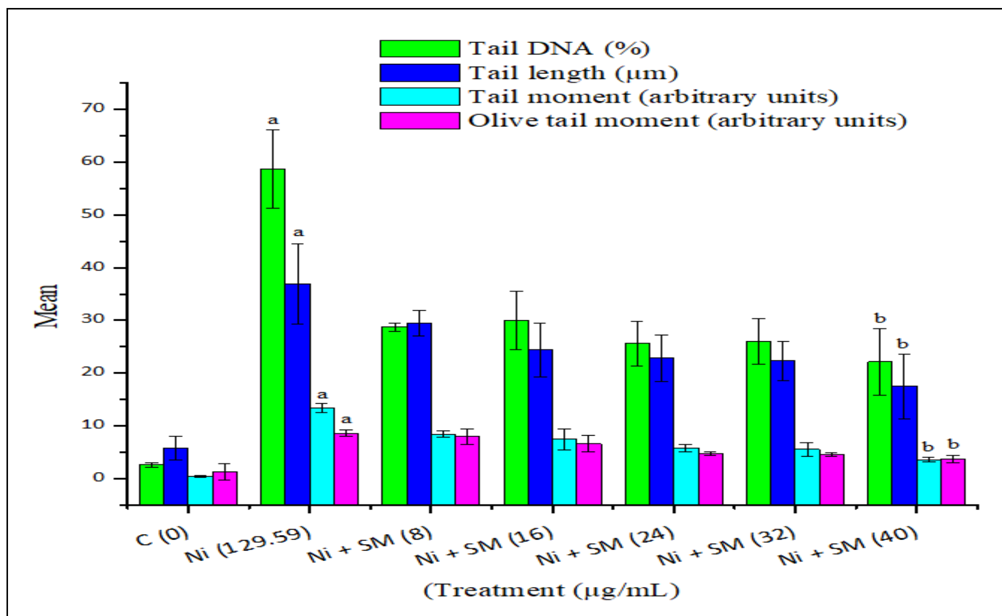

FIG. 2: EFFECTS OF DIFFERENT CONCENTRATIONS OF SILYMARIN AGAINST $NICL_2$ INDUCED DNA DAMAGE IN CULTURED HUMAN PBLS ASSESSED BY COMET ASSAY PARAMETERS. C- Control, Ni-$NiCl_2$, SM- Silymarin. [a]p<0.05 significant as compared control; [b]p<0.05 significant as compared to $NiCl_2$

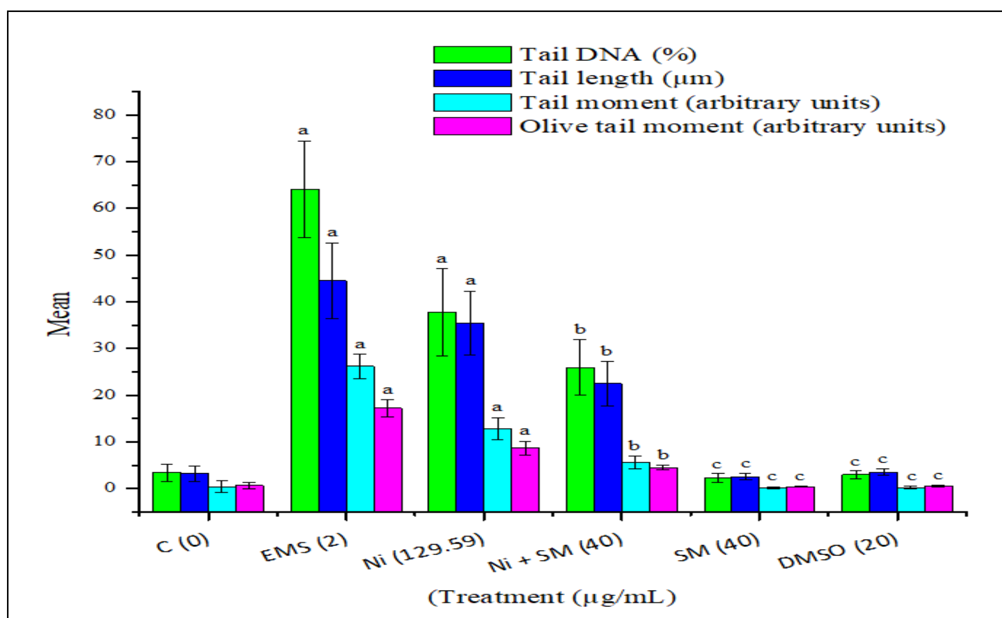

FIG. 3: EFFECT OF DIFFERENT TREATMENTS ON CULTURED HUMAN PBLS IN DIFFERENT SAMPLES (N=30) ASSESSED BY COMET ASSAY PARAMETERS. C- Control, Ni-$NiCl_2$, EMS -ethyl methanesulfonate, SM- Silymarin, DMSO-Dimethyl sulfoxide. [a]p<0.05 significant as compared to control; [b]p<0.05 significant as compared to $NiCl_2$; cp>0.05 non-significant as compared to control

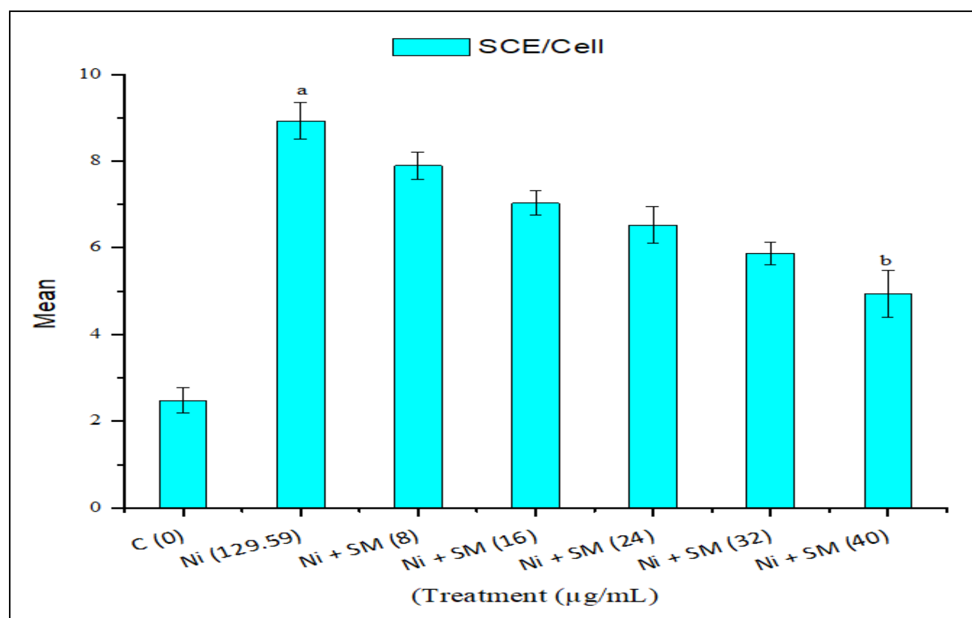

FIG. 4: EFFECT OF DIFFERENT CONCENTRATIONS OF SILYMARIN AGAINST NICL$_2$ - INDUCED SISTER CHROMATID EXCHANGES IN PBLs. C- Control, Ni-NiCl$_2$, SM- Silymarin. [a]p<0.05 significant as compared to control

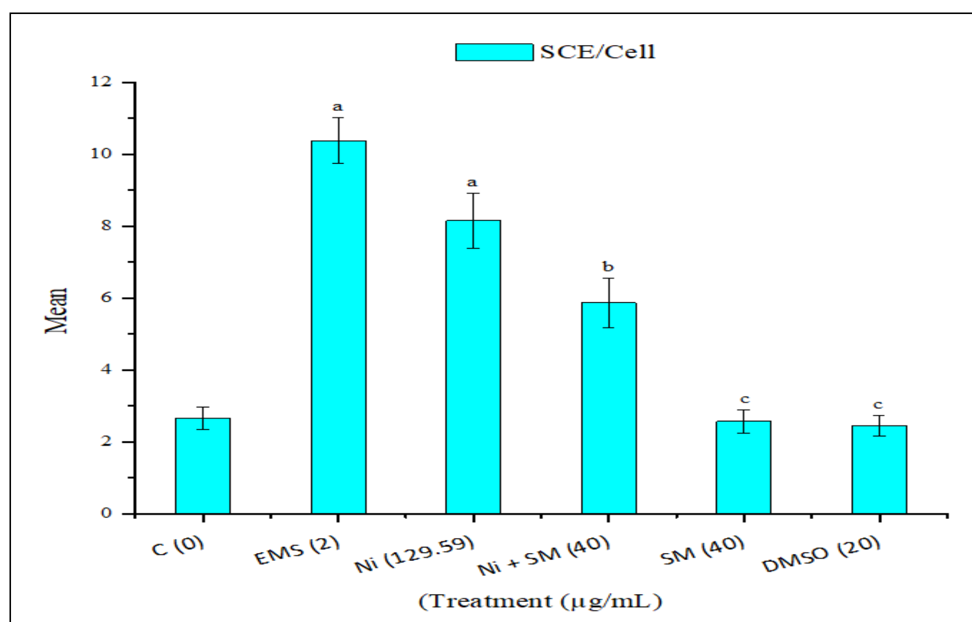

FIG. 5: EFFECTS OF DIFFERENT TREATMENTS ON CULTURED HUMAN PBLS IN DIFFERENT SAMPLES (N=30) ASSESSED BY SCE ASSAY. C- Control, EMS -ethyl methanesulfonate, Ni-NiCl$_2$, SM- Silymarin, DMSO- Dimethyl sulfoxide. [a]p<0.05 significant as compared to control; [b]p<0.05 significant as compared to NiCl$_2$; [c]p>0.05 non-significant as compared to control

DISCUSSION: The evaluation of lymphocytes with comet assay and sister chromatid exchange assay demonstrated that nickel chloride induces significant ($p<0.05$) genotoxicity in cultured peripheral human lymphocytes.

Our findings are in concordance with the other *in vitro* studies on the induction of DNA damage and chromosomal aberrations upon nickel treatment [16, 17, 18]. Previous studies indicate that nickel indirectly induces genotoxicity by elevating ROS and suppressing the antioxidant defense system. This effect may arise either by ROS production by Fenton type reaction or inactivation of anti-oxidative enzymes [19].

NiCl$_2$ is observed to cause oxidative intestinal harm in broilers, showing a dose- and time-dependent increase in malondialdehyde (MDA) content and a decline in glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) tissue and capability to inhibit

hydroxyl radicals in the intestines [20]. Nickel has been observed to significantly induce DNA damage measured as increased comets in cultured human lymphocytes [21, 22]. It has been reported that 1 mmol nickel has little effect on cell viability of human lymphocytes as compared to the highest dose of 10 mmol, which reduced the cellular viability by only 30% [22].

In a study, it has been shown that treatment with 0.05 to 5 mmol Ni for 48 h resulted in highly significant DNA damage, apoptosis and cytotoxicity in human Jurkat T cells at 5 mmol concentrations [23].

The elevated levels of ROS can attack DNA and oxidize nitrogen bases and result in modified nucleotides. It was observed that nickel (II) induced oxidation of DNA through an oscillatory formation of a modified nucleotide 8-oxoguanine (8-oxoG) from guanine in DNA [24]. The modified nucleotide, 8- oxo-dG is one of the most frequently present DNA damages resulted from DNA oxidation [25]. It is also observed that nickel ions can directly bind to ds-DNA and induces conformational changes that lead to helix destabilization. This renders the DNA prone to attack by oxidizing agents [26].

Many investigations have found that nickel interferes with DNA damage repair, cell proliferation and cell cycle. It was investigated the cytotoxic, apoptotic and DNA damaging effects of nickel nanoparticles (NiNPs) in A431 cells and found that NiNPs induced significant cytotoxicity, generation of ROS, and depletion of GSH, a significant rise of lipid peroxidation and caspase-3 activity in the cells [27].

Our results showed elevated levels of SCEs upon nickel exposure. In response to increased ds-DNA breaks homologous repair process gets activated, which leads to exchanges in chromosomal segments for the repair of DNA. Thus, increased SCEs indicate the presence of ds-DNA break [28].

In this study, the co-treatment of silymarin along with nickel chloride led to a significant reduction the DNA damage and SCEs in the PBLs. Studies also concluded that silymarin shows strong antioxidant potential and treatment with silymarin reduces the SCEs, abnormal metaphase, myxopapillary ependymoma (MPE), and aberrant diakinesis in mice [29]. It is found that silymarin has an antioxidant capability of scavenging free radicals by increasing cellular glutathione levels, suppressing the oxidation and reduction of glutathione and increasing the activity of superoxide dismutase [30]. It was demonstrated that silymarin administration increases the activity of important antioxidant enzymes such as SOD, CAT, GPx, and glutathione disulfide reductase (GSR), glutathione-s-transferase (GST) and decreased lipid peroxidation in erythrocytes and ultimately lead to reduced ROS formation [31]. Our study results indicated that silymarin exhibited antigenotoxic potential against $NiCl_2$ induced genetic damage in cultured PBLs by reducing tail moment and SCE frequency. We also observed that silymarin alone treatment showed non-significant effects in terms of genotoxicity in cultured PBLs. It is also found that silymarin alone treatment resulted in a non-significant change in the comet tail moments [32].

With the increasing concentration of nickel in our environment, the risk of health problems associated with its prolonged exposure is rising. This study suggests that silymarin treatment can lower nickel chloride-mediated genotoxicity. It is also reported that silymarin has a role in enhancing DNA repair in cells with UVB-induced DNA damage [33]. Silymarin is widely used for liver disease treatment and is commercially available [9, 34].

It may be used as a dietary supplement to complement the medical formulations against genotoxic and cancerous agents. More research is required to reveal the mechanism of action of silymarin against heavy metal-induced cellular risks, which may be beneficial to the exposed groups, especially the occupationally affected people.

CONCLUSION: The study concludes that Nickel chloride can induce DNA damage in human lymphocytes and silymarin has antigenotoxic potential against nickel and can be provided as a dietary supplement against nickel.

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