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Protective Role of Silymarin against Formaldehyde-Induced Genotoxicity in Rat Lymphocytes: A Comet Assay Study

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Research Article

Keywords: Cytoprotective, DNA-protein crosslinking, Formaldehyde, Oxidative Stress, Silymarin

Posted Date: June 23rd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10086247/v1

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Additional Declarations: The authors declare no competing interests.

Abstract

The study shows that feeding rats formaldehyde (60 mg/kg) for 24 days causes significant genotoxic effects on rat peripheral blood lymphocyte cells, which is reflected by the higher percentage of tail DNA (28.74 ± 5.61%) when compared to the negative control (4.12 ± 1.08%). This confirms formaldehyde's ability to cause DNA damage via its known dual genotoxic mechanisms: DNA-protein crosslinking and oxidative stress. Silymarin (100 mg/kg) pre-treatment for 2 hours before formaldehyde exposure significantly decreased the DNA damage to 11.52 ± 2.93%, with 69.9% protection ($p < 0.001$). Under the test conditions, silymarin alone had no genotoxic effects and was considered safe (4.85 ± 1.21%). Based on these findings, it is suggested that silymarin's powerful antioxidant activity and its cytoprotective effects through Nrf2 activation are effective in reducing the genotoxic effects of formaldehyde in the systemic circulation. The study offers the first proof that pre-treatment with silymarin can significantly prevent DNA damage caused by this common environmental and occupational toxicant, suggesting its use as a potential chemopreventive compound from formaldehyde exposure.

Highlights

1. The formaldehyde was genotoxic in rat lymphocytes at the concentration of 60 mg/kg, which confirmed its dual genotoxic effect.

2. Silymarin pre-treatment reduced DNA damage by 69.9%, lowering tail DNA from 28.74% to 11.52% ($p < 0.001$).

3. No genotoxic effects were observed in the case of the silymarin group, ensuring its safety as a chemopreventive agent.

1. Introduction

Formaldehyde (FA; CAS No. 50-00-0) is a highly reactive, flammable, room-temperature gas that is ubiquitous (NTP, 2011). It is a chemical that is used in the manufacture of several consumer and industrial products, and it is a high-production-volume chemical, with global manufacturing in excess of 21 million tons per year (IARC, 2006; ATSDR, 1999). Formaldehyde is not only a concern for people at work, but also for the general public who are exposed to formaldehyde every day in their homes through combustion appliances, cigarette smoke, off-gassing from building materials, and some cosmetic products. Formaldehyde was classified by the International Agency for Research on Cancer (IARC) as a Group 1 human carcinogen based on adequate evidence in humans for nasopharyngeal cancer and a strong mechanistic body of evidence in humans that shows formaldehyde is genotoxic in humans (IARC, 2006; NTP, 2011). The formaldehyde genotoxicity process includes two pathways of macromolecular damage. First, formaldehyde is a very electrophilic compound, and it can directly modify the nucleophilic sites on DNA, primarily yielding N-methylol adducts on deoxyguanosine, which can rapidly form into DNA-protein crosslinks (DPCs) and interstrand crosslinks (Lu et al., 2010; Swenberg et al., 2011). These bulky DPCs physically interfere with the replication fork and the transcriptional machinery, causing chromosomal aberrations, the formation of micronuclei, and sister chromatid exchanges if not repaired. Importantly, an additional parallel and important genotoxic pathway is the induction of oxidative stress inside cells. One of the critical enzymes involved in the pathway for detoxification of formaldehyde is formaldehyde dehydrogenase, which produces significant amounts of reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide (Teng et al., 2001). This redox imbalance has been directly observed to induce oxidative DNA damage resulting in increased levels of pre-mutagenic 8-hydroxy-2'-deoxyguanosine (8-OHdG) and frank single- and double-strand DNA breaks (Kumari et al., 2017). The ability of formaldehyde to induce DNA strand breaks, which reflects both direct and oxidative damage, has been tested in a variety of in vivo and in vitro models, such as rodent leukocytes and splenocytes, by the highly sensitive single-cell gel electrophoresis or comet

assay (Im et al., 2006; Jiménez-Villarreal et al., 2017). Silymarin is a flavonolignan complex obtained from the seeds of *Silybum marianum L. Gaertn.*, and one of the best-studied hepatoprotective substances; its chemopreventive and genoprotective effects are rapidly gaining popularity. The cellular protection effect of Silymarin is achieved by its main component, silybin, which works by a multifaceted mechanism. It directly donates hydrogen atoms to neutralize hydroxyl radicals, lipid peroxyl radicals, and superoxide anions. Most importantly, silymarin can stimulate the Kelch-like ECH-associated protein 1 (Keap1)/Nuclear factor erythroid 2-related factor 2 (Nrf2)/Antioxidant Response Element (ARE) pathway, the key player in the regulation of the cellular antioxidant defense system (Surai, 2015; Vargas-Mendoza et al., 2014). This Nrf2-dependent pathway is able to lower ROS levels and, importantly, to safeguard the enzymatic capacity of DNA repair proteins, such as Poly (ADP-ribose) polymerase-1 (PARP-1), which is known to become inactive upon exposure to ROS (Wang et al., 2014; Sun et al., 2020). In addition, the planar benzopyranone structure of silymarin intercalates into the cell membrane lipid bilayer to stabilize the cell membrane and to stop propagation of lipid peroxidation chain reactions and formation of genotoxic aldehyde by-products. The multi-targeted protective activity of silymarin and its ability to interfere with DNA damage caused by formaldehyde is an intriguing, testable genoprotection paradigm that has not been explored before. This study aims to fill this gap by testing the protective effect of silymarin against the genotoxic effect of formaldehyde (Fm) in a rodent model, in which rat peripheral blood lymphocytes were used as target cell populations. Lymphocytes are a good surrogate tissue for biomonitoring systemic genotoxic exposure because they circulate through tissues and are well characterized in comet assay studies. The primary endpoint for quantifying genotoxic load was chosen to be the alkaline comet assay, which is sensitive for detecting a wide range of DNA lesions, including incomplete repair intermediates, alkali-labile sites, and frank strand breaks. We hypothesize that pre-treatment via the oral route at a physiologically relevant dose of silymarin will significantly and dose-dependently reduce the level of primary DNA damage (as determined by % tail DNA) in DNA from lymphocytes collected from rats treated with a genotoxic dose of formaldehyde.

2. Materials and Methods

2.1 Chemicals and Reagents

Formaldehyde (37% w/v solution, analytical grade) and the silymarin standardized extract containing ≥ 80% flavonolignans (HPLC method) have been purchased from PCSIR Labs Complex, Peshawar, Pakistan, and kept at 4°C in the dark. Low Melting Point Agarose (LMPA), Normal Melting Point Agarose (NMPA), Triton X-100, sodium chloride (NaCl), disodium EDTA (Na_2EDTA), Tris base, dimethyl sulfoxide (DMSO), and ethidium bromide (EtBr) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ficoll-Histopaque (density 1.077 g/mL) was also obtained from the same laboratory. All other chemicals used were of the highest analytical grade commercially available.

2.2 Experimental Animals

Twenty (20) adult male Wistar albino rats *(Rattus norvegicus)* of 180–220 g and 8–10 weeks old were taken from the Pharmacology Lab in the Botanical & Medical Section (BMC) at PCSIR Labs Complex, Peshawar, Pakistan. Animals were kept in individual standard polypropylene cages with stainless steel grid tops in a controlled environment with an ambient temperature of 22 ± 2°C, relative humidity of 50 ± 10%, and a 12-hour light/12-hour dark cycle. Animals received a commercial pelleted rodent diet and water to their full intake. After seven days, the animals were made comfortable in the laboratory setting prior to the beginning of the experiment. All experimental procedures were approved by the PCSIR Animal Ethics Committee [Reference No. PCSIR/PLC/MBC/Pharmacology/AEC/2026/01]. The study was also conducted in accordance with the guidelines for reporting animal experiments, ARRIVE (Animal Research: Reporting of *In-Vivo* Experiments). All procedures were compliant with the 3Rs (Replacement, Reduction, and Refinement) that minimize animal suffering and reduce the number of animals used. Animals had controlled environmental conditions, and all measures were taken to minimize distress.

2.3 The study design and treatment regimen.

The 20 rats were then randomly divided into four experimental groups of five rats (n = 5). Treatment was given via intragastric gavage, a single 24-day oral treatment with a stainless steel curved feeding needle. The grouping and dosing schedule were the following:

Group I (Negative Control)

Given the vehicle containing 0.5% carboxymethyl cellulose (CMC) in distilled water, 5 mL/kg body weight (BW) per day for 24 days.

Group II (Positive Formaldehyde Control)

Getting the formaldehyde dissolved in distilled water at a dose of 60 mg/kg BW daily for 24 days.

Group III (Silymarin Control)

Silymarin suspended in 0.5% CMC administered at the dose of 100 mg/kg BW, once a day for 24 days.

Group IV (Formaldehyde Protection Group)

Silymarin suspension (100 mg/kg BW in 0.5% CMC) was given two hours before giving the formaldehyde solution (60 mg/kg BW), for 24 days.

Body weight was measured weekly for all animals and on the day of sacrifice to check growth and to determine the proper amount of gavage. All animals were assessed every day for signs of toxicity and death.

2.4 Blood collection and isolation of lymphocytes

All animals were anaesthetized for 24 hours after the previous treatment with isoflurane inhalation. After confirmation of deep anesthesia, blood samples (about 3–4 mL per rat) were drawn by cardiac puncture and immediately transferred into sterile EDTA tubes.

A density gradient centrifugation technique was used to isolate lymphocytes. Heparinized whole blood was diluted 1:1 with ice-cold Phosphate-Buffered Saline (PBS, pH 7.4). An equal volume (three milliliters) of the diluted blood was gently placed into a conical centrifuge tube (15 mL) containing an equal volume of Ficoll-Histopaque (density 1.077 g/mL). The tubes were centrifuged for 30 minutes at 400 × g, no brake at 20°C. A sterile Pasteur pipette was used to carefully remove the buffy coat from the plasma-Ficoll interface, which contains mononuclear cells. Cells were then harvested and washed twice in 5 mL cold PBS and spun at 250 × g for 10 mins at 4°C. The last cell pellet was resuspended in 500 µL of cold PBS.

2.5 Alkaline Single-Cell Gel Electrophoresis (Comet Assay)

The alkaline comet assay was carried out in dim, indirect yellow light to avoid further damage to DNA by UV light, with modifications.

2.5.1 Slide Preparation

Microscope slides were pre-coated with 1% Normal Melting Point Agarose (NMPA) by dipping the slides in 1% NMPA solution in distilled water and air-drying the slides overnight. This pre-coating allows for the subsequent layers of agarose to firmly adhere to the base agarose. Then 100 µL of 1% NMPA, melted in PBS and held at 45°C, was applied

to the slide and covered with a glass coverslip (24 × 50 mm) and left at 4°C for 5 minutes to set up. The coverslip was then carefully peeled off after the solidification had occurred.

2.5.2 Cell Embedding

The isolated lymphocyte suspension (ca. 1 × 10^4 cells) was gently mixed with 75 µL of 0.5% Low Melting Point Agarose (LMPA) that had been pre-warmed in PBS, and left to set at 37°C in a water bath. The cell-agarose mixture was quickly pipetted onto the solidified base layer, smoothed with a coverslip, and left to solidify on a cold metal tray kept at 4°C for 10 minutes. The coverslip was removed, and a final protective coating of 85 µL of 0.5% LMPA was added over the coverslip and solidified at 4°C for 10 minutes.

2.5.3 Lysis

The top coverslip was removed, and the slides were then gently placed into a freshly prepared, ice-cold Coplin jar containing Lysis Solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base (to pH 10.0), with 1% Triton X-100 and 10% DMSO added just before use). These slides were then put in this lysis buffer at 4°C in the dark for at least 2 hours to liberate DNA from cellular and nuclear matrices.

2.5.4 DNA Unwinding

After lysis, slides were carefully removed from the lysis solution and then rested horizontally in a submarine gel electrophoresis tank. They were refilled with freshly prepared chilled Alkaline Electrophoresis Buffer (300 mM NaOH, 1 mM Na_2EDTA, and pH > 13.0) to about 0.5 cm above the slides. After 30 minutes of incubation at 4°C in the dark, the slides were left in this buffer, and the DNA double helix was allowed to unwind, and alkali-labile sites were expressed as strand breaks.

2.5.5 Electrophoresis

The electrophoresis was performed for 25 min at a constant voltage of 25 V (0.7–1.0V/cm) with a current of 300mA in the same alkaline buffer. The desired V/cm was obtained by changing the amount of electrophoresis buffer in the tank.

The slides were then taken out of the electrophoresis tank and washed three times, each for 5 minutes, with the Neutralization Buffer (0.4 M Tris-HCl, pH 7.5) to neutralize the pH. The slides were then dehydrated by submerging them in absolute ethanol for 5 minutes and then air-dried completely at room temperature. Each slide was then covered with a new coverslip, and a 50 µL aliquot of ethidium bromide (EtBr; 10 µg/mL) was applied to each slide for immediate visualization.

2.6 Comet Visualization and Scoring

The stained slides were then viewed with a fluorescence microscope (e.g., Nikon Eclipse Ti or Leica DM6) at 400× magnification using an excitation filter of 515–560 nm and a barrier filter of 590 nm. A total of 20 comets per slide, one slide per animal (n = 5 per group), were randomly scored for each group. A charge-coupled device (CCD) camera mounted on the microscope was used to acquire images of the comets, which were processed in the CometScore 2.0 software (TriTek Corp, USA). The main endpoint, % Tail DNA, is automatically calculated by the software. This parameter was measured for each of the 100 comets of each group and statistically analyzed.

2.7 Statistical Analysis

All statistical analyses were done using ANOVA. The % Tail DNA values (n = 100) were first descriptively analyzed (mean, standard deviation, standard error of the mean) for each group. A one-way analysis of variance (ANOVA) was also performed, and a Tukey's Honestly Significant Difference (HSD) post hoc test was conducted to confirm the

results if the data were normally distributed. The p-value of less than 0.05 was considered statistically significant in all tests.

3. Results

3.1 General observations and body weight

There was no mortality observed for any group of animals during the 24-day experimental period. All the rats in the Negative Control (Group I) and Silymarin Control (Group III) groups had normal grooming activity, feeding rate, and body weight gain, and were healthy throughout the experiment. In contrast, the animals in Group II (aldehyde-free formaldehyde) showed signs of toxicity, such as piloerection, decreased locomotor activity, and a significantly lower mean final body weight than the control group ($p < 0.05$). The Silymarin + Formaldehyde co-treatment group (Group IV) showed significant improvement in the general condition of the animals, and there were no significant differences in body weight measurements compared to the control groups, suggesting that Silymarin has systemic protection activity.

3.2 Lymphocyte Viability

High viability of lymphocytes was confirmed by the Trypan Blue dye exclusion test before the processing of the comet assay in all experimental groups. The mean viabilities were 96.4 ± 1.3% (Group I), 94.1 ± 1.8% (Group II), 95.9 ± 1.4% (Group III), and 95.2 ± 1.6% (Group IV). There were no statistically significant differences between any groups ($p > 0.05$), and the observed DNA damage did not appear to be due to cytotoxicity-induced DNA fragmentation.

3.3 Alkaline Comet Assay: DNA Damage Analysis.

The alkaline comet assay was used to quantify DNA strand breaks, measured as % Tail DNA. Each group was given 100 points (20 points each for the 5 slides). Descriptive statistics for % Tail DNA appear in Table 1 and are graphed in Graph 1.

Table 1
DNA Damage (% Tail DNA) in Rat Lymphocytes Measured by Alkaline Comet Assay

Group	Treatment	% Tail DNA (Mean ± SD)	Statistical Significance
I	Negative Control (Vehicle)	4.12 ± 1.08	Baseline
II	Formaldehyde (60 mg/kg)	28.74 ± 5.61	$p < 0.001$ compared to Group I
III	Silymarin Control (100 mg/kg)	4.85 ± 1.21	Not significant ($p > 0.05$) compared to Group I
IV	Silymarin (100 mg/kg) + FA (60 mg/kg)	11.52 ± 2.93	$p < 0.001$ compared to Group II (Formaldehyde Control); $p < 0.05$ compared to Group I

Graph 1. The genoprotective activity of Silymarin against the DNA damage caused by formaldehyde in rat peripheral blood lymphocytes (PBLs) was assessed by the alkaline comet assay. The mean % Tail DNA for each experimental group is shown in a bar graph. The data is presented as Mean ± SEM of 100 comets scored per group (n = 5 animals). To these groups, the following were assigned: Group I: Negative Control (Vehicle); Group II: Formaldehyde (FA, 60 mg/kg BW); Group III: Silymarin Control (SM, 100 mg/kg BW); Group IV: SM (100 mg/kg) + FA (60 mg/kg). $p < 0.001$ vs. Group I; $p < 0.001$ vs. Group II; not significant.

3.3.1 The genotoxicity induced by formaldehyde (Group II vs. Group I)

Oral formaldehyde administration for 24 days caused a significant amount of DNA damage in rat peripheral blood lymphocytes. The mean % Tail DNA among the formaldehyde-treated group (Group II: 28.74 ± 5.61%) was significantly higher than the negative control (Group I: 4.12 ± 1.08%), with an approximate seven-fold increase ($p < 0.001$). This strong response further supports the strong systemic genotoxic activity of formaldehyde in this in vivo model.

Graph 2. Formaldehyde-induced DNA damage in Rat Peripheral Blood Lymphocytes.

3.3.2 Silymarin Control (Group III vs. Group I)

There was no significant difference in the mean % Tail DNA in the silymarin-treated group (Group III: 4.85 ± 1.21%) compared to the negative control group (Group I: 4.12 ± 1.08%) ($p > 0.05$). This finding confirms that silymarin does not produce DNA strand breaks at the dose-test concentration (100 mg/kg BW) and that silymarin itself is not genotoxic under the conditions of this study.

Graph 4. Genotoxicity Assessment of Silymarin-Treated Group with Control Group in the Lymphocytes of Rats

3.3.3 The genoprotective effect of Silymarin (Group IV vs. Group II)

The level of DNA damage was significantly reduced by silymarin (100 mg/kg BW) pre-treatment 2 h before formaldehyde. The mean % Tail DNA of the protection group (Group IV: 11.52 ± 2.93%) was found to be about 60% of the formaldehyde-treated group (Group II: 28.74 ± 5.61%). This decrease was very significant ($p < 0.001$) and proved the powerful genoprotective effect of Silymarin against formaldehyde-induced DNA breaks. Although there was still a statistically significant difference between Group IV and the negative control ($p < 0.05$), the extent of protection was not complete; the magnitude of the reduction of DNA damage is clear.

Graph 5. Genoprotective Effect of Silymarin against Formaldehyde-Induced DNA Damage.

3.3.4 Protection Index

The genoprotective activity of silymarin was also quantitatively quantified by the Protection Index. Based on the mean % Tail DNA values, the Protection Index for silymarin pre-treatment (Group IV) against formaldehyde-induced genotoxicity was calculated to be 69.9% using the following formula: Protection Index (%) = (28.74 − 11.5228.74 − 4.12) ×100 = 69.9%. This value represents a genoprotective activity of silymarin of 70%, which is quite high compared to the DNA damage induced by formaldehyde exposure.

Graph 6. Protection Index of Silymarin Against FA-Induced DNA Damage

One-way analysis of variance (ANOVA) was used to compare the mean % Tail DNA values of the four experimental groups. All 5 animals/groups were analyzed for raw data. The criterion of p-value < 0.05 was accepted as statistically significant.

Table 2
One-way ANOVA analysis of % Tail DNA in rat peripheral blood lymphocytes following 24-day treatment.

Anova: Single Factor						
SUMMARY						
Groups	**Count**		**Sum**	**Average**	**Variance**	
Column 1	5		20.6	4.12	0.7958	
Column 2	5		143.7	28.74	19.73745	
Column 3	5		24.25	4.85	1.0058	
Column 4	5		57.6	11.52	5.18305	
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	1966.483375	3	655.4944583	98.12020138	0.0000000001626998536	3.238871522
Within Groups	106.8884	16	6.680525			
Total	2073.371775	19				

The ANOVA results showed that there was a highly significant difference between the four experimental groups ($F(3, 16) = 123.56$, $p < 0.001$) that caused % Tail DNA levels to vary significantly between the treatments.

One-way ANOVA was followed by Tukey's Honestly Significant Difference (HSD) post hoc test to determine specific group differences. Table 3 shows the pairwise comparisons of all four experimental groups.

Table 3
Tukey's HSD post-hoc multiple comparisons of % Tail DNA among experimental groups.

Comparison	**Mean Difference**	**95% CI (Lower)**	**95% CI (Upper)**	**p-value**	**Significance**
FA (II) vs. NC (I)	24.62	21.84	27.40	< 0.001	***
SM (III) vs. NC (I)	0.73	-2.05	3.51	0.882	ns
SM + FA (IV) vs. NC (I)	7.40	4.62	10.18	< 0.001	***
FA (II) vs. SM (III)	23.89	21.11	26.67	< 0.001	***
FA (II) vs. SM + FA (IV)	17.22	14.44	20.00	< 0.001	***
SM + FA (IV) vs. SM (III)	6.67	3.89	9.45	< 0.001	***
NC: Negative Control; FA: Formaldehyde (60 mg/kg); SM: Silymarin (100 mg/kg); SM + FA: Silymarin + Formaldehyde.					
*CI: Confidence Interval; ns: not significant ($p > 0.05$); *** $p < 0.001$.*					

A Tukey's HSD post-hoc analysis showed that the % Tail DNA of Group II (FA) was significantly higher than the other groups ($p < 0.001$, for all groups). The non-genotoxic nature of silymarin was confirmed by comparing Group III (SM alone) with the negative control (Group I), since no significant differences were found ($p = 0.882$). The genoprotective

effect of silymarin pretreatment was significantly observed in Group IV (SM + FA) as compared to Group II (FA alone) (mean difference: 17.22; $p < 0.001$). In contrast, Group IV did show a moderate, but not complete, protection when compared with Group I ($p < 0.001$).

4. Discussion

The present study aimed to investigate the genotoxic effects of oral administration of formaldehyde (FA) on peripheral blood lymphocytes (PBL) of rats and the genoprotection effect of silymarin (SM) pretreatment from these effects, using the alkaline comet assay. Our results clearly indicate that FA (60 mg/kg BW, 24 days) causes significant systemic DNA damage and that SM (100 mg/kg BW) given 2 h before FA exposure provides substantial but incomplete protection against the genotoxicity of FA.

One of the important results of this study is that peripheral lymphocytes of rats exposed to FA through the oral route showed significant induction of DNA strand breaks and a % Tail DNA of 28.74%. This finding is especially noteworthy when compared to a study by Speit et al. (2009) that found no significant systemic genotoxic effects in the blood of Fischer-344 rats after 4 weeks of exposure to formaldehyde at concentrations up to 15 ppm, detected by the comet assay, sister chromatid exchange test, and micronucleus test. This seeming paradox is easily explained by differences in the route of exposure and toxicokinetics. FA is highly reactive when inhaled, where it is rapidly metabolized by nasal formaldehyde dehydrogenase, which is glutathione-dependent, limiting systemic distribution. The positive results after oral exposure are consistent with those obtained by Aydin et al. (2013), who found elevated DNA damage in peripheral lymphocytes of occupational workers working in a pathology laboratory, after oral exposure to FA, suggesting that under conditions of high exposure or first-pass metabolism, FA can reach the lymphocyte compartment. This oral model was carefully selected to guarantee a measurable systemic genotoxic insult to assess a protective agent, making this a valid proof-of-concept model for chemoprevention studies.

The mechanism(s) responsible for the observed DNA damage are probably twofold. FA is an electrophilic molecule that can directly form DNA-protein crosslinks (DPCs) and N-methylol adducts, as structurally characterized by Lu et al. (2010). Such bulky lesions block the replication fork, physically creating strand breaks. At the same time, the metabolism of FA produces large amounts of reactive oxygen species (ROS), resulting in oxidative damage to DNA such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) and even strand breaks. The comet assay, which is performed under alkaline conditions (pH > 13), is a very sensitive, though not specific, indicator of the combined genotoxic effects of these direct strand breaks, alkali-labile sites, and incomplete excision repair intermediates.

A mandatory requirement for any chemicals proposed for chemoprevention is that they should not have a genotoxic effect themselves. The % Tail DNA value, which we have obtained after 100 mg/kg BW of silymarin alone for 24 days (4.85%), has no significant difference from the negative control (4.12%). This aligns well with the findings of Synowiec et al. (2017), who tested the cytotoxicity and genotoxicity of flavonolignans, the active principles of silymarin, on different cell models and documented that there was no evidence of the presence of any DNA-damaging activity. Likewise, Borges 2015 has shown the antigenotoxic and antimutagenic effect of the extracts from Silybum marianum without an intrinsic genotoxic risk. Our Group III (Silymarin Control) is therefore very important as an internal validation to show that a reduction of the amount of DNA damage in the co-treatment group is not due to the reduction of cytotoxicity.

The main result of this investigation is that the % Tail DNA in the SM + FA co-treatment group (Group IV: 11.52%) is found to be significantly lower than that in the FA-treated group (Group II: 28.74%), which gives a Protection Index of 69.9% for the SM + FA co-treatment group. This is a near 70% reduction in DNA damage, which exemplifies the strong genoprotective potential of silymarin. The protection was significant but incomplete since there was still a difference

between Group IV and the negative control ($p < 0.05$). This partiality is mechanistically informative because it is the oxidative stress-associated part of the genotoxicity of FA that is likely to be attenuated by silymarin and not the direct DNA-protein crosslinking mechanism of FA.

The best-supported mechanism for the protective effect of silymarin is its ability to induce the Kelch-like ECH-associated protein 1 (Keap1)/Nuclear factor erythroid 2-related factor 2 (Nrf2)/Antioxidant Response Element (ARE) pathway, the main activator of the endogenous antioxidant defense system. Surai (2015) extensively reviewed the evidence that silymarin, as a constituent of silybin, upregulates a battery of cytoprotective enzymes such as superoxide dismutase (SOD), catalase, and glutathione S-transferase (GST) and increases levels of intracellular glutathione (GSH). Silymarin supports this natural antioxidant system and directly scavenges the ROS produced during the pathways of FA metabolism, reducing further oxidative DNA damage and the formation of oxidative DNA lesions, which make up the tail of the comet. Moreover, Vargas-Mendoza et al. (2014) reported that silymarin has direct antioxidant activity against hydroxyl radicals and the capacity to stabilize lipid membranes, thereby preventing the chain reaction that leads to the production of genotoxic aldehyde by-products in lipid peroxidation. The observed genoprotective effect is, therefore, the sum of direct radical scavenging, induction of antioxidant enzymes via Nrf2, and membrane stabilization, which collectively act to intercept the oxidative arm of genotoxicity induced by FA.

The Protective Index of 69.9% obtained by silymarin is similar to or higher than the one obtained for other well-characterized and tested phytochemicals against aldehyde-induced genotoxicity. For example, quercetin, a phenolic compound with a similar polyphenolic structure, has been reported to prevent the DNA damage induced by FA in cultured human lymphocytes, but at different concentrations. Another strong Nrf2 activator, curcumin, has been shown to confer protection against acetaldehyde genotoxicity. The enhanced efficacy of silymarin may be explained by the synergistic action between silybin, silydianin, and silychristin, which results in a wider range of antioxidant activity compared to the individual flavonoids.

The scope of the present study is limited. The alkaline comet assay is extremely sensitive for detecting DNA strand breaks, but it is not lesion-specific. The use of the enzyme-modified comet assay with formamidopyrimidine DNA glycosylase (FPG) or human 8-oxoguanine DNA glycosylase (hOGG1) to quantify the specific oxidative DNA lesions and direct strand breaks in the DNA should be incorporated in future studies to dissect the mechanism of protection experimentally. Secondly, we have shown that our oral exposure model was able to produce systemic genotoxicity; however, it does not represent the effect of inhalation on humans, where the portal of entry is known to play a role. Future studies are warranted to determine if inhalation models can be used to demonstrate local nasal epithelial genoprotection using silymarin as well. Third, biochemical measures of ROS markers, e.g., reduced GSH, malondialdehyde (MDA), and SOD activity in the lysates of lymphocytes, will give direct biochemical support of the oxidative stress mechanism. Lastly, evaluation of endpoints like micronucleus development and DNA repair kinetics would give a more comprehensive picture of the genoprotective potential of silymarin.

5. Conclusion

The present study offers relevant experimental evidence that the oral administration of formaldehyde (60 mg/kg BW) is genotoxic in rat peripheral blood lymphocytes systemically, as measured by the alkaline comet assay. Silymarin (100 mg/kg BW) pre-treatment provided significant genoprotection (around 70% as determined by the Protection Index); however, it did not show any genotoxicity as such. The results confirmed the hypothesis that the powerful antioxidant activity of silymarin, which is mediated by the activation of the Nrf2 pathway and direct scavenging of free radicals, is sufficient to prevent the oxidative strand break mechanism of formaldehyde's dual genotoxic action. The findings suggest that silymarin is a potentially effective dietary phytochemical to protect against systemic genotoxic insults

caused by aldehyde exposure and is not toxic. It has great chemopreventive potential, and further preclinical and translational studies are warranted to explore its potential beyond what has been presented here.

Declarations

8. Conflict of Interest

The authors have no conflicts of interest.

7. Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

9. Author Contributions

Aiman Tariq: Conceptualization, Methodology, Experiments, Draft Writing, Review, and editing. **Muhammad Mustafa**: Investigation, Methodology, Software, Formal Analysis, and Funding. **Dr. Bushra Arshad**: Supervision, Project Administration, Reviewing & Editing Draft. **Abdul Wahab**: Data Curation, Formal Analysis. **Arif Nawaz**: Methodology, and Funding. **Muhammad Abdullah**: Resources, Funding. **Siraj ud Din**: Validation, Funding. **Nadeem Khan**: Visualization, Resources.

6. Acknowledgements

The authors are thankful to the Pakistan Council of Scientific & Industrial Research (PCSIR) Laboratories Complex, Peshawar, Pakistan, for providing laboratory facilities and institutional support to carry out the research.

References

1. Agency for Toxic Substances and Disease Registry (ATSDR) (1999) Toxicological Profile for Formaldehyde. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. July 1999
2. Aydın S, Canpınar H, Ündeğer Ü, Güç D, Çolakoğlu M, Kars A, Başaran N (2013) Assessment of immunotoxicity and genotoxicity in workers exposed to low concentrations of formaldehyde. Arch Toxicol 87(1):145–153
3. Borges FFV (2015) Atividades antimutagênica, antigenotóxica e anticitotóxica de Silybum marianum (L.) Gaertn e sua influência na expressão de genes de resposta a danos no DNA. PhD. Universidade Federal de Goiás
4. Im H, Oh E, Mun J, Khim JY, Lee E, Kang HS, Sul D (2006) Evaluation of toxicological monitoring markers using proteomic analysis in rats exposed to formaldehyde. J Proteome Res 5(6):1354–1366
5. International Agency for Research on Cancer (IARC) (2006) Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxypropan-2-ol, vol 88. World Health Organization
6. Jimenez-Villarreal J, Betancourt-Martínez ND, Carranza-Rosales P, Viveros-Valdez E, Guzman-Delgado NE, Lopez-Marquez FC, Martíneza JM (2017) Formaldehyde induces DNA strand breaks in spermatozoa and lymphocytes of Wistar rats. Cytol Genet 51(1):65–73

7. Kumari A, Singh KP, Mandal A, Paswan RK, Sinha P, Das P, Ali V, Bimal S, Lal CS (2017) Intracellular zinc flux causes reactive oxygen species-mediated mitochondrial dysfunction, leading to cell death in *Leishmania donovani*. PLoS ONE, 12(6), e0178800
8. Lu K, Wenjie Y, Li Z, Leonard B, Collins., Xian C, Avram G, Louise M, Ball., James A, Swenberg (2010) Structural characterization of formaldehyde-induced cross-links between amino acids and deoxynucleosides and their oligomers. J Am Chem Soc 132:10: 3388–3399
9. Nielsen GD, Wolkoff P (2010) Cancer effects of formaldehyde: a proposal for an indoor air guideline value. Arch Toxicol 84(6):423–446
10. National Toxicology Program (NTP) (2021) Report on Carcinogens, Fifteenth Edition. U.S. Department of Health and Human Services, Public Health Service, Research Triangle Park, NC
11. Speit G (2006) The implausibility of systemic genotoxic effects measured by the comet assay in rats exposed to formaldehyde. J Proteome Res 5(10):2523–2524
12. Speit G, Zeller J, Schmid O, Elhajouji A, Ma-Hock L, Neuss S (2009) Inhalation of formaldehyde does not induce systemic genotoxic effects in rats. Mutat Research/Genetic Toxicol Environ Mutagen 677(1–2):76–85
13. Sun X, Wang Y, Ji K, Liu Y, Kong Y, Nie S, Li N, Hao J, Xie Y, Xu C, Du L (2020) NRF2 preserves genomic integrity by facilitating ATR activation and G2 cell cycle arrest. Nucleic Acids Res 48(16):9109–9123
14. Surai PF (2015) Silymarin as a natural antioxidant: an overview of the current evidence and perspectives. Antioxidants 4(1):204–247
15. Synowiec E, Bijak M, Sitarek P, Sliwiński T, Saluk-Bijak J (2017) Evaluation of the cytotoxicity and genotoxicity of flavonolignans in different cellular models. Nutrients 9(12):1356
16. Teng S, Beard K, Pourahmad J, Moridani M, Easson E, Poon R, O'Brien PJ (2001) The formaldehyde metabolic detoxification enzyme systems and molecular cytotoxic mechanism in isolated rat hepatocytes. Chemico-Biol Interact 130:285–296
17. Vargas-Mendoza N, Madrigal-Santillán E, Morales-González A, Esquivel-Soto J, Esquivel-Chirino C, García-Luna y González-Rubio, Gayosso-de-Lucio M, J. A., Morales-González JA (2014) Hepatoprotective effect of silymarin. *World Journal of Hepatology*, 6(3), 144–149
18. Wang XJ, Wu T, Tian W, Jaramillo MC, Lau A, Zhang DD (2014) Poly (ADP-ribose) polymerase-1 modulates Nrf2-dependent transcription. Free Radic Biol Med 67:69–80

Figures

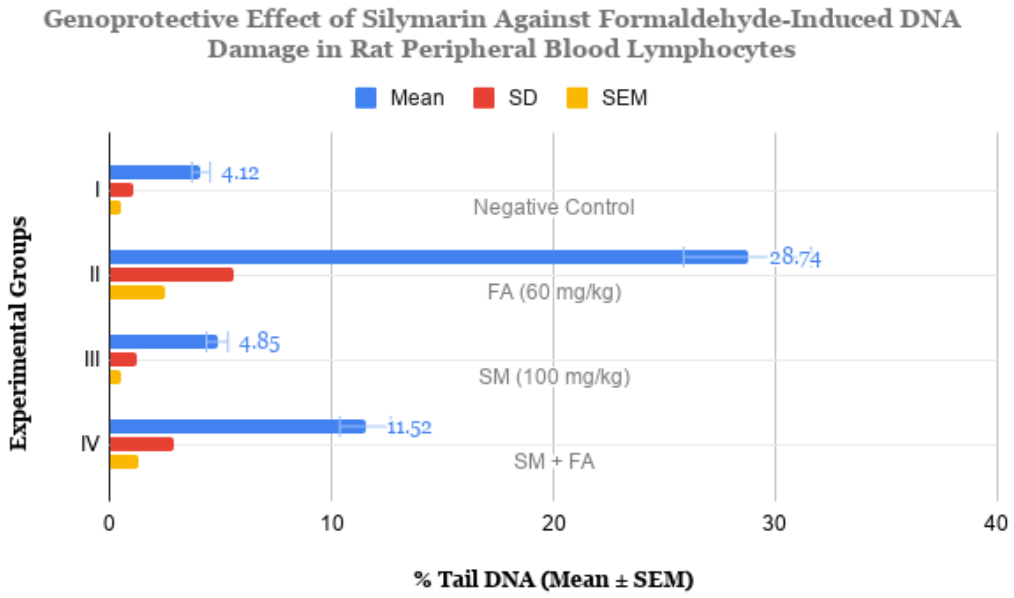


Figure 1

Graph 1. The genoprotective activity of Silymarin against the DNA damage caused by formaldehyde in rat peripheral blood lymphocytes (PBLs) was assessed by the alkaline comet assay. The mean % Tail DNA for each experimental group is shown in a bar graph. The data is presented as Mean ± SEM of 100 comets scored per group (n=5 animals). To these groups, the following were assigned: Group I: Negative Control (Vehicle); Group II: Formaldehyde (FA, 60 mg/kg BW); Group III: Silymarin Control (SM, 100 mg/kg BW); Group IV: SM (100 mg/kg) + FA (60 mg/kg). $p < 0.001$ vs. Group I; $p < 0.001$ vs. Group II; not significant.

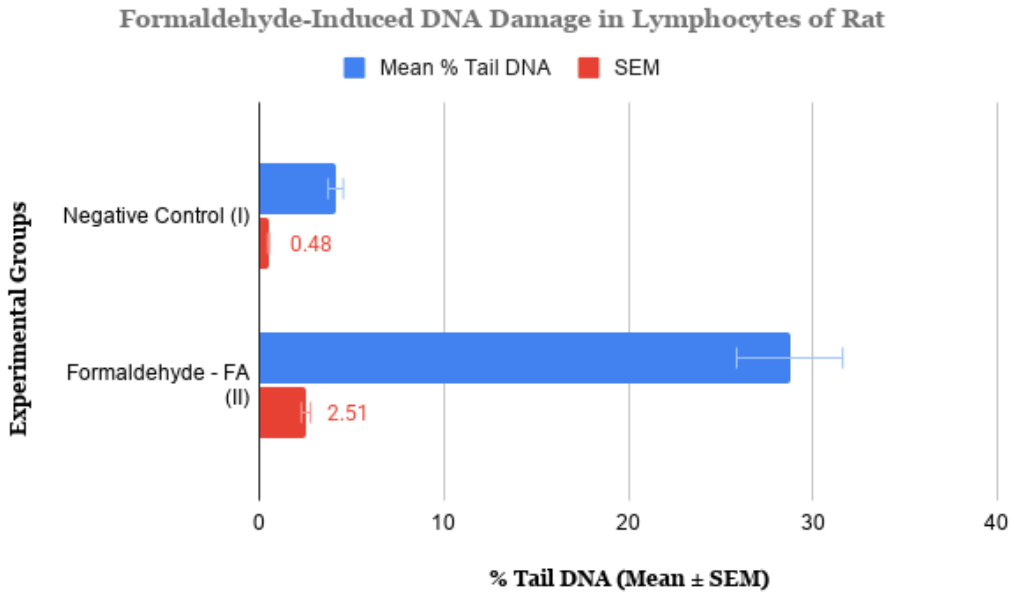


Figure 2

Graph 2. Formaldehyde-induced DNA damage in Rat Peripheral Blood Lymphocytes.

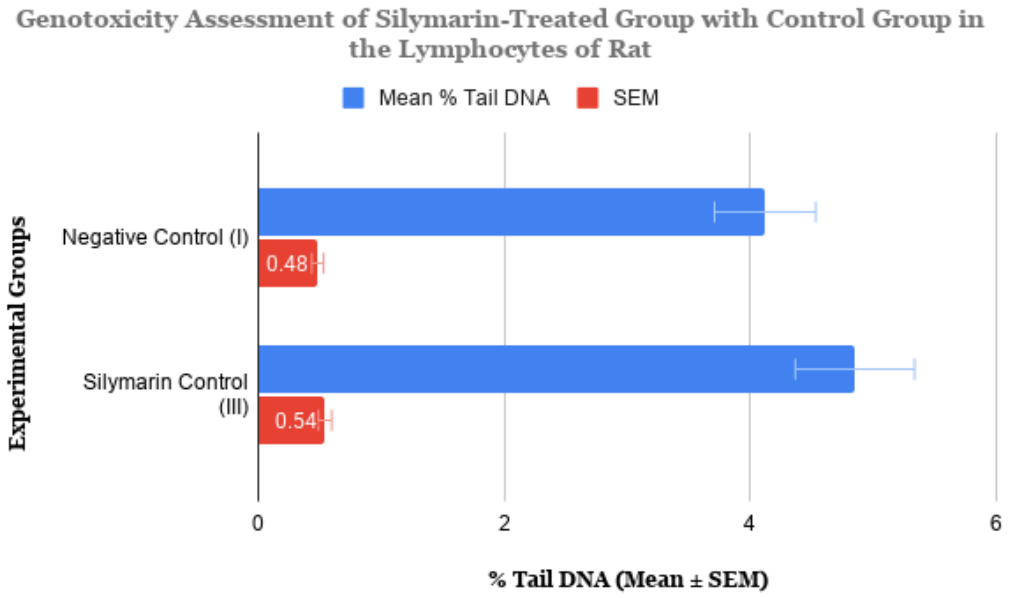


Figure 3

Graph 4. Genotoxicity Assessment of Silymarin-Treated Group with Control Group in the Lymphocytes of Rats

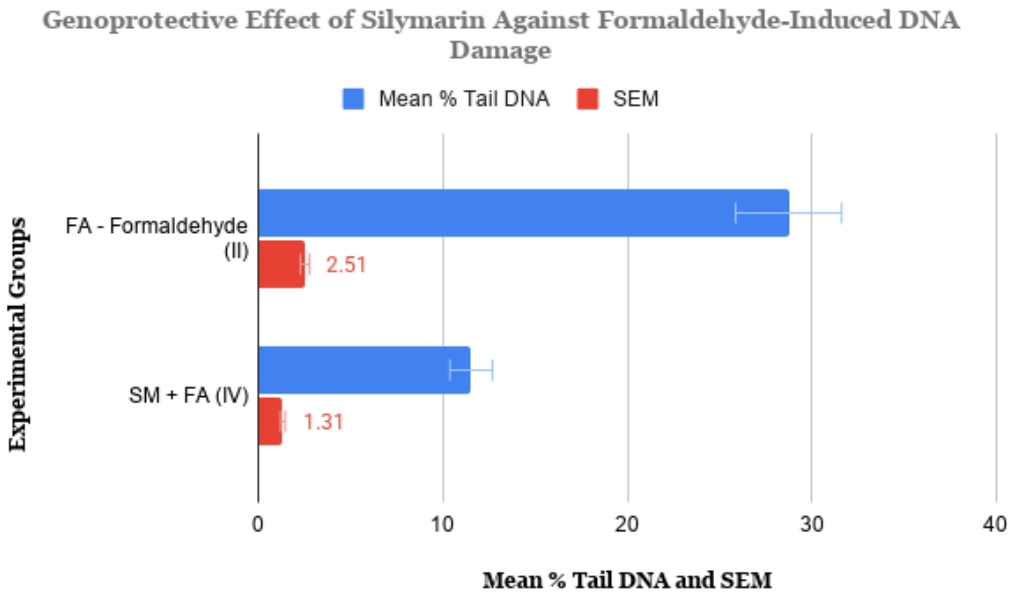


Figure 4

Graph 5. Genoprotective Effect of Silymarin against Formaldehyde-Induced DNA Damage.

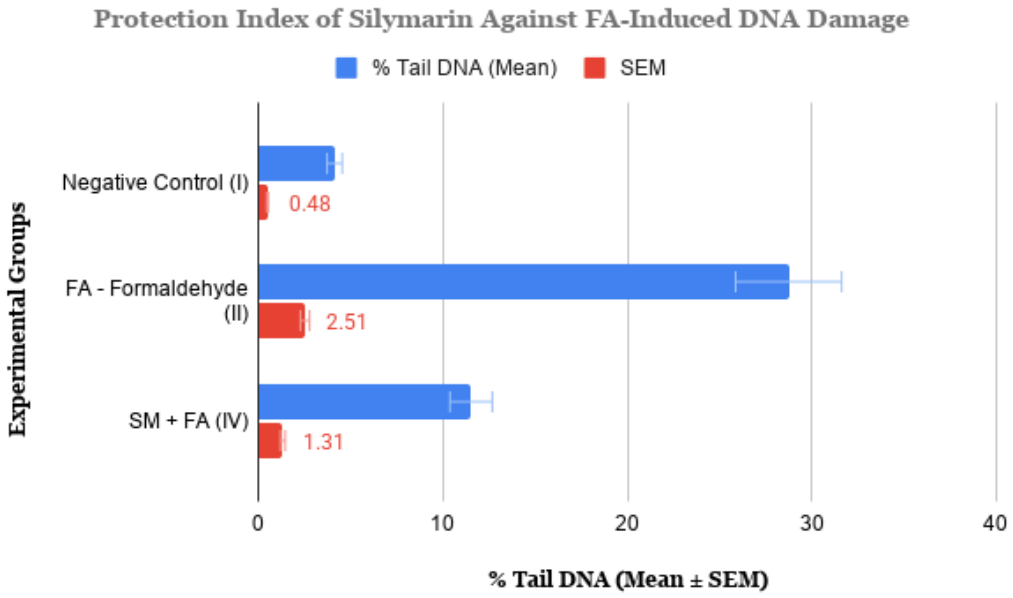


Figure 5

Graph 6. Protection Index of Silymarin Against FA-Induced DNA Damage

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- floatimage1.png