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Cadmium-Induced Perturbation of Spleen Redox Status: Therapeutic Role of Pumpkin Seed Protein Isolate

Siddhartha Singh[1], Oly Banerjee[1], Ishita Saha[2], Sudipta Kundu[3], Alak Kumar Syamal[4], Bithin Kumar Maji[1] and Sandip Mukherjee[1*]

[1]*Department of Physiology, Serampore College, 9 William Carey Road, Hooghly - 712201, West Bengal, India; sm_kdc@yahoo.co.in*

[2]*Department of Physiology, Medical College and Hospital, Kolkata - 700073, West Bengal, India*

[3]*Department of Physiology, Kalka Dental College, Partapur By-Pass Road, Meerut - 250006, Uttar Pradesh, India*

[4]*Department of Physiology, Hooghly Mohsin College, Chinsurah - 712101, West Bengal, India*

Abstract

One of the major threats to humanity is from the exposure of heavy metals irrespective of its source. Cadmium is one of such heavy metals to which humans are exposed in their daily lives via food or environment. Regardless of this, there is no established or efficient way of recycling Cadmium. On the other hand, Pumpkin seeds have innumerable health aiding properties. The present study aims to understand the antioxidative and anti-inflammatory properties of Pumpkin Seeds Protein Isolate (PSPI) against Cadmium mediated oxidative stress in spleen. Twenty male albino rats were divided into four groups; Control, Cadmium treated, Cadmium treated and PSPI 1 supplemented, Cadmium treated and PSPI 2 supplemented. After completion of treatment period (21 days), oxidative stress parameters, ROS generation levels and proinflammatory cytokines were measured along with histopathological evaluations. PSPI supplementation was observed to have significant free radical scavenging activities as evidenced by decreased lipid peroxidation and nitric oxide generation simultaneously with increased glutathione level, activities of superoxide dismutase and catalase. Cadmium also caused an elevation in tumor necrosis factor and interleukin-6 as well as ROS generation levels which were substantially reduced upon supplementation with PSPI. Furthermore, cadmium-induced micro architectural changes in the spleen were also countered upon PSPI supplementation. In summary, both lower and higher doses of PSPI supplementation curtail the cadmium induced oxidative stress, ROS levels, proinflammatory cytokines and damage in the splenic tissue. The Results of this study necessitates further mechanistic study to establish key role of PSPI in amelioration of cadmium toxicity.

Keywords: Cadmium Toxicity, Cytokines, Oxidative Stress, PSPI, ROS, Spleen Histology

**Author for correspondence*

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1. Introduction

Considerable amount of Cadmium (Cd) is used in the production of alloys, pigments and batteries. Possible ways of Cd exposure to humans are via cigarette smoke, working in metal industries, intake of contaminated food or inhalation[1]. Earlier studies revealed that cadmium contributes to many health problems, including heart disease, cancer, diabetes, and osteoporosis[2]. Cd compounds may be reasonably described as carcinogenic[3]. Cd incites the production of metallothioneins and ROS by excessive oxygen metabolism[4]. As a result of reactive oxygen species formation, tissues undergo oxidative stress, which damages DNA, lipids, and proteins leading to cellular death[5,6]. The oxidative stress metabolism in humans, which leads to the many hazardous diseases and the ability of natural antioxidants to counter its effects[7] has attracted the interests of many researchers from all over the world.

In the last decades, *Cucurbita maxima* (pumpkin) has drawn lots of attention due to the health-alleviating actions of the bio compounds present in its seeds and fruits. Pumpkin has many health-ameliorating properties, such as hepatoprotection, antidiabetic, anticancer, and anti-obesity properties. Pumpkin seeds have also been reported to have an anti-depressant food score of 47%. Therefore, it reflects upon the significant anti-depressing activity of pumpkin seeds[8]. It is a marvelous source of oil and protein[9]. It has been reported that pumpkin seed extract has significantly declined the number of adult *H. nana* worms and their egg production and therefore shows an anthelmintic effect[10]. Furthermore, it has also been reported that pumpkin seed extract increases hypoglycemic and antidiabetic activity in the body through increasing insulin production from β-cells[11]. Pumpkin seed protein components have antiperoxidative properties, counters protein malnutrition and CCl_4 toxicity and are considered an alternative treatment for irritable bladder and benign prostatic hyperplasia[12].

There is very limited information on *C. maxima's* seed protein isolates ameliorative properties on the Cd-induced spleen toxicity in rats. Therefore, this study aims to understand the antioxidative, anti-inflammatory properties of *C. maxima* seeds protein isolate against Cd-mediated oxidative stress and proinflammatory cytokine production in the spleen, which leads to immunomodulation.

2. Materials and Methods

2.1 Preparation of Pumpkin Seed Protein Isolates (PSPI)

Fresh pumpkin seeds were collected from Serampore, West Bengal, India and verified by the Department of Botany, Serampore College, West Bengal, India. The seeds were initially washed under tap water and then in distilled water. By subsequent iso-electric precipitation and alkaline extraction PSPI was developed. Hexane was used to defeat the grounded pumpkin seeds (without hulls). At ambient temperature, the seeds were dispersed in 5 volumes of 1MNaCl solution and the pH was adjusted to 9.5 using 1MNaOH and stirred for 30mins. The mixture was centrifuged and the supernatant was filtered, pH was adjusted to 4.0 with 1NHCl. The protein was precipitated and dispersed in 50% ethyl alcohol (1:5), mixed for 30mins and then centrifuged for 20 mins. The precipitate was washed with distilled water and then left to dry in the air. This developed product was referred to as pumpkin seed protein isolate or PSPI[13].

2.2 Experimental Animals

The current study used twenty male albino rats (Wistar strain) weighing 110-125 g. The animals were acclimatized in the animal house for one week prior to the experiment. The animals were kept at 25 ± 3 degrees Celsius and relative humidity of 40%-45% with alternative 12 hrs light/dark cycles. Food and water were freely available for all animals, and good hygiene was maintained. All the experiments were conducted following the ethical guidelines provided by the Institutional Animal Ethics Committee (IAEC) of Serampore College, West Bengal, India (1946/PO/Re/S/17/CPCSEA).

2.3 Experimental Design

The experiment was carried out for three weeks. The study used twenty male adult albino rats distributed into four groups each. Group A, control was treated with normal saline 10ml/kg body weight orally; Group B, received Cd Chloride ($CdCl_2$) 2ml/kg body[14-16] weight by injection intraperitoneally; Group C, received $CdCl_2$ 2ml/kg body weight by intraperitoneal injection and supplemented with 1ml/kg body weight of the PSPI in saline (20g/100ml)[11] by stomach tube (PSPI 1); Group D, received $CdCl_2$ 2ml/kg body weight in saline by injection intraperitoneally

and supplemented with 2ml/kg body weight of the PSPI in saline (20g/100ml) by stomach tube (PSPI 2). The animals were sacrificed after the completion of treatment period, i.e., 21 days[14,15], for the assessment of the effect of Cd exposure on spleen cells.

2.4 Sample Collections and Analysis

Following the treatment period, all rats were anaesthetized with light ether anaesthesia and sacrificed by cervical dislocation, which is one of the IAEC's approved physical ways of euthanasia. Spleen tissues were collected and then splenocytes were isolated and used for the analysis of oxidative stress parameters and intracellular ROS measurement. Additionally a part of the spleen tissue taken for preparation for histopatholgical evaluation. Furthermore, Blood was collected and serum was prepared for pro inflammatory cytokines measurement.

2.5 Isolation and Preparation of Spleen Cells

Following the collection of spleen tissue in PBS, the spleen tissues were disrupted using frosted glass slides. The spleen cells were then collected in (Roswell Park Memorial Institute medium) RPMI 1640 and centrifuged at 1200 rpm for 10 mins at 4°C. Further the cells were obtained by density centrifugation in Histopaque (Sigma Chemical CO) at 1200 rpm for 30 mins. After separation the cellular concentration was adjusted as per the assay requirement and the cells were kept in RPMI 1640 for further analysis[17].

2.6 Determination of Lipid Peroxidation (LPO)

The Thiobarbituric Acid (TBA) test[18] determines the quantity of Malondialdehyde (MDA) produced, which measures the degree of lipid peroxidation. Using the molar extinction coefficient, the data were represented as nanomoles of MDA per milligram of protein.

2.7 Estimation of Nitric Oxide (NO)

The Griess reaction was used to determine NO activity, which was expressed as nitrite accumulation[19]. Absorbance was measured at 550nm. Results were expressed as µM/mg of protein.

2.8 Estimation of Superoxide Dismutase (SOD)

SOD activities were determined using the Nitrobluetetrazolium (NBT) technique, which is based on the inhibition of NBT reduction by SOD[20]. At 560nm and 25°C, the absorbance of blue formazan was measured. The results were expressed in units per milligram of protein (U/mg).

2.9 Determination of Catalase (CAT)

The activity of catalase was measured using the Aebi[21] technique. At 240 nm and 25°C, the absorbance of blue formazan was measured. The results were expressed in units per milligram of protein (U/mg).

2.10 Determination of Glutathione

Following the approach of Sedlak and Lindsay[22] reduced GSH content was measured by a reaction with 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent). The results were represented in milligrams per milligram of protein.

2.11 Determination of the Pro-Inflammatory Cytokine Level

A 96-well microplate Enzyme-Linked Immunosorbent Assay (ELISA) technique was used to measure the levels of tumour necrosis factor-α and interleukin-6. Using a commercially available rat ELISA kit obtained from Raybiotech (Norcross, GA) and 5.7% and 6.3%, respectively was the intra-assay variation.

2.12 Histopathological Evaluations

After the completion of the treatment duration, spleen tissue samples were collected from the animals of each group. Then, they were immersed and fixed in neutral buffered formalin. Paraffin blocks were prepared, and the rotary microtome was used to cut 5µm thin sections from those paraffin blocks. After staining with haematoxylin and eosin, the tissue sections were mounted in DPX. Then the stained sections were examined under a microscope (Primostar model, Carl Zeiss Meditec Group, Dublin, CA) for histopathological abnormalities in spleen architecture.

2.13 Determination of Reactive Oxygen Species (ROS)

The spleen cells were incubated in the fluorescent probe DCFH-DA [20, 70-dichloro-fluorescin diacetate (DCFH-DA, Molecular Probes, Inc)] and then washed and placed in PBS. The cellular ROS generation levels were acquired on a BD Accuri Flow Cytometer, and emission was measured by 530/30-nm band-pass filter where excitation was at 485nm and emission was at 530nm.

2.14 Statistical Analysis

The mean and Standard Error of the Mean (SEM) were used to present all of the data. To see if the scores of different groups differed significantly, the Kruskal-Wallis non parametric ANOVA test was used. The Mann-Whitney U multiple comparison test was used to determine whether there was a significant difference between groups. If the difference was less than 0.05, i.e., $p < 0.05$, it was considered significant.

3. Results

The results of the current study revealed that there is a marked elevation of lipid peroxidation (Figure 1A) and nitric oxide production (Figure 1B), hallmarks of oxidative stress in the spleen tissue of the $CdCl_2$ exposed group when compared with an unexposed group ($p < 0.01$). This elevation was found to be minimized by the administration of both PSPI 1 ($p < 0.01$) and PSPI 2 ($p < 0.01$), which is a clear indication of inhibition of oxidative damage in the tissue.

In addition to this SOD, CAT act as the major antioxidant enzymes. SOD dismutase's the O_2^- into less toxic intermediates like H_2O_2, which is further decomposed by CAT into water or oxygen. The present study revealed that $CdCl_2$ administration caused significant depreciation ($p < 0.01$) in the activity of SOD (Figure 2A) as compared to the control group. PSPI1 supplementation provided protection ($p < 0.05$) against this reduction in the activity of SOD, whose value was restored towards the value as that of the control group ($p < 0.01$) upon the administration of PSPI2. Likewise, the CAT activity (Figure 2B) was also compromised ($p < 0.01$) in the $CdCl_2$ exposed group, and in contrast, PSPI1 administration attenuated ($p < 0.01$) the $CdCl_2$ induced decrease of CAT activity. Further, PSPI 2 supplementation simultaneously with $CdCl_2$ significantly ($p < 0.01$) recovered the suppressed activity of CAT towards the control level.

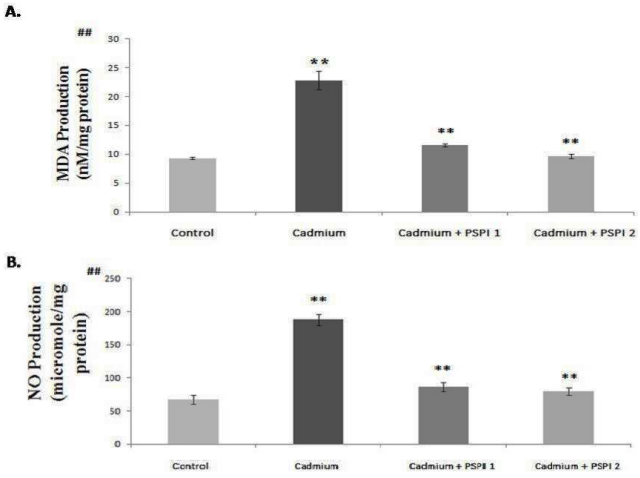

Figure 1. Effect of PSPI 1 and PSPI 2 supplementation on **(A)** MDA production (nM/mg protein), **(B)** NO production (micromole/mg protein) in spleen of Cd treated rats (n = 5); values are expressed as mean ± SE; significance based on Kruskal Wallis test ($p < 0.01$, $p < 0.01$; respectively). Significance based on Mann-Whitney U multiple comparison tests: Control vs. Cd treated ($p < 0.01$, $p < 0.01$; respectively), Cd treated vs. Cd treated + PSPI 1 supplemented ($p < 0.01$, $P < 0.01$; respectively), Cd treated vs Cd treated + PSPI 2 supplemented ($p < 0.01$, $p < 0.01$; respectively), Cd treated + PSPI 1 supplemented vs. Cd treated + PSPI 2 supplemented ($p < 0.01$, $p < 0.01$; respectively).

GSH maintains cellular integrity by protecting the cell from oxidative damage. $CdCl_2$ exposure induced a marked reduction ($p < 0.01$) in the GSH levels (Figure 2C) of the spleen as compared to exposed group. On the other hand, the $CdCl_2$ induced decrease in the tissue GSH levels of the spleen was reversed insignificantly by the administration of PSPI 1. However, co-administration of PSPI 2 and $CdCl_2$ significantly countered ($p < 0.05$) the suppressed levels of GSH.

The inclination in cytokines levels is considered to be one of the predominant causes of improper functioning and damage of various organs leading to organ failure[23]. So the level of both pro-inflammatory cytokines, IL-6 (Figure 3A), as well as TNF-α (Figure 3B), was measured. Both IL-6 and TNF-α levels were enhanced ($p < 0.01$) in $CdCl_2$-exposed rats. PSPI 1 supplementation blunted the $CdCl_2$ induced enhancement in both IL-6 and TNF-α

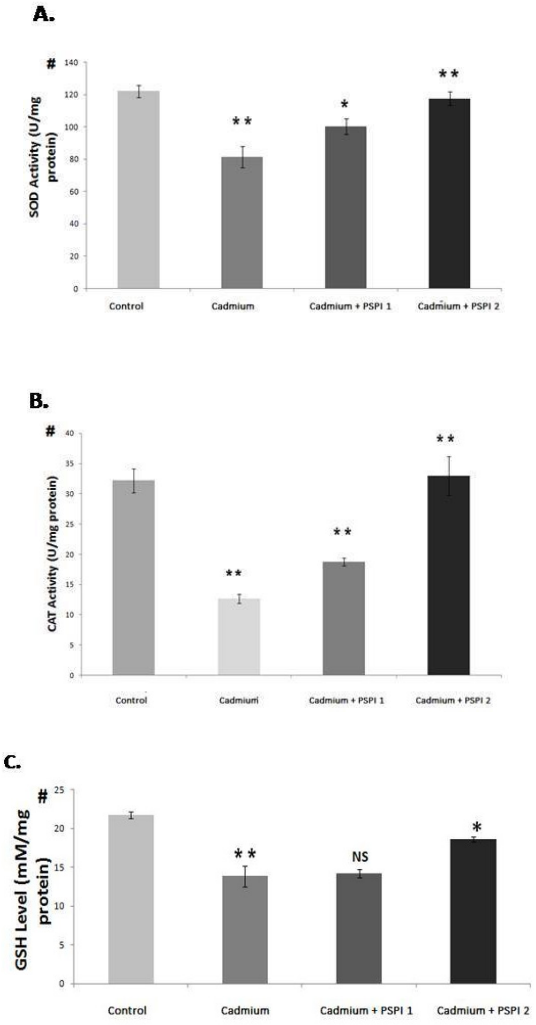

Figure 2. Effect of PSPI 1 and PSPI 2 supplementation on **(A)** SOD activity (U/mg protein). **(B)** CAT activity (U/mg protein). **(C)** GSH levels (nM/mg protein) in spleen of Cd treated rats (n = 5); values are expressed as mean ± SE; significance based on Kruskal Wallis test ($p < 0.05$, $p < 0.05$, $p < 0.05$; respectively). Significance based on Mann-Whitney U multiple comparison tests: Control vs. Cd treated ($p < 0.01$, $p < 0.01$, $p < 0.01$; respectively), Cd treated vs. Cd treated + PSPI 1 supplemented ($p < 0.05$, $p < 0.01$, not significant; respectively), Cd treated vs. Cd treated + PSPI 2 supplemented ($p < 0.01$, $p < 0.01$, $p < 0.05$; respectively), Cd treated + PSPI 1 supplemented vs. Cd treated + PSPI 2 supplemented ($p < 0.01$, $p < 0.01$, $p < 0.01$; respectively).

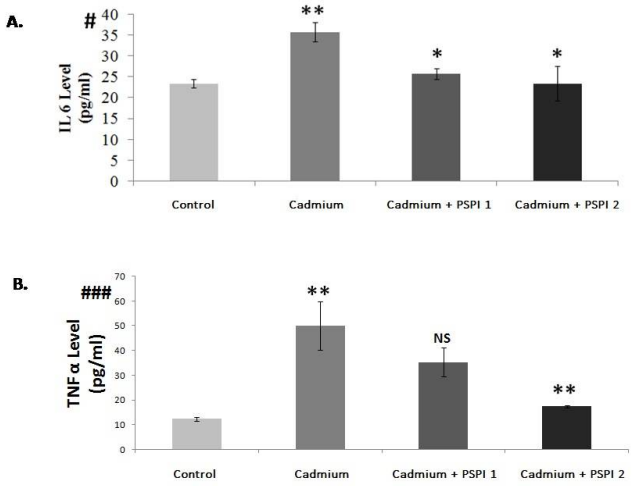

Figure 3. Effect of PSPI 1 and PSPI 2 supplementation on **(A)** IL 6 levels(pg/ml). **(B)** TNF α levels (pg/ml) in spleen of Cd treated rats (n = 5); values are expressed as mean ± SE; significance based on Kruskal Wallis test ($p < 0.05$, $p < 0.001$; respectively). Significance based on Mann-Whitney U multiple comparison tests: Control vs. Cd treated ($p < 0.01$, $p < 0.01$; respectively), Cd treated vs. Cd treated + PSPI 1 supplemented ($p < 0.05$, non-significant; respectively), Cd treated vs. Cd treated + PSPI 2 supplemented ($p < 0.05$, $p < 0.01$; respectively), Cd treated + PSPI 1 supplemented vs. Cd treated + PSPI 2 supplemented (not significant, $p < 0.01$; respectively).

($p < 0.05$, not significant; respectively). However, PSPI 2 supplementation significantly counteracted the $CdCl_2$-induced increase in both the IL-6 ($p < 0.05$) and TNF-α levels ($p < 0.01$).

In this study, the eosin-hematoxylin stained spleen tissue (Figure 4) sections revealed that $CdCl_2$ treatment destroyed the splenic cellular microarchitecture. In addition, significant damage was also observed in the marginal zone and marginal sinus region upon $CdCl_2$ treatment (Figure 4A). This damage was perceived to be countered upon PSPI 1 (Figure 4C) administration as there was an increase in the cellularity of PALS (T cell area) simultaneously with the restoration of spleen microarchitecture. This restoration was further significantly enhanced upon PSPI 2 (Figure 4D) supplementation, as the marginal zone and marginal sinus structure was observed to be more prominent along with the PALS.

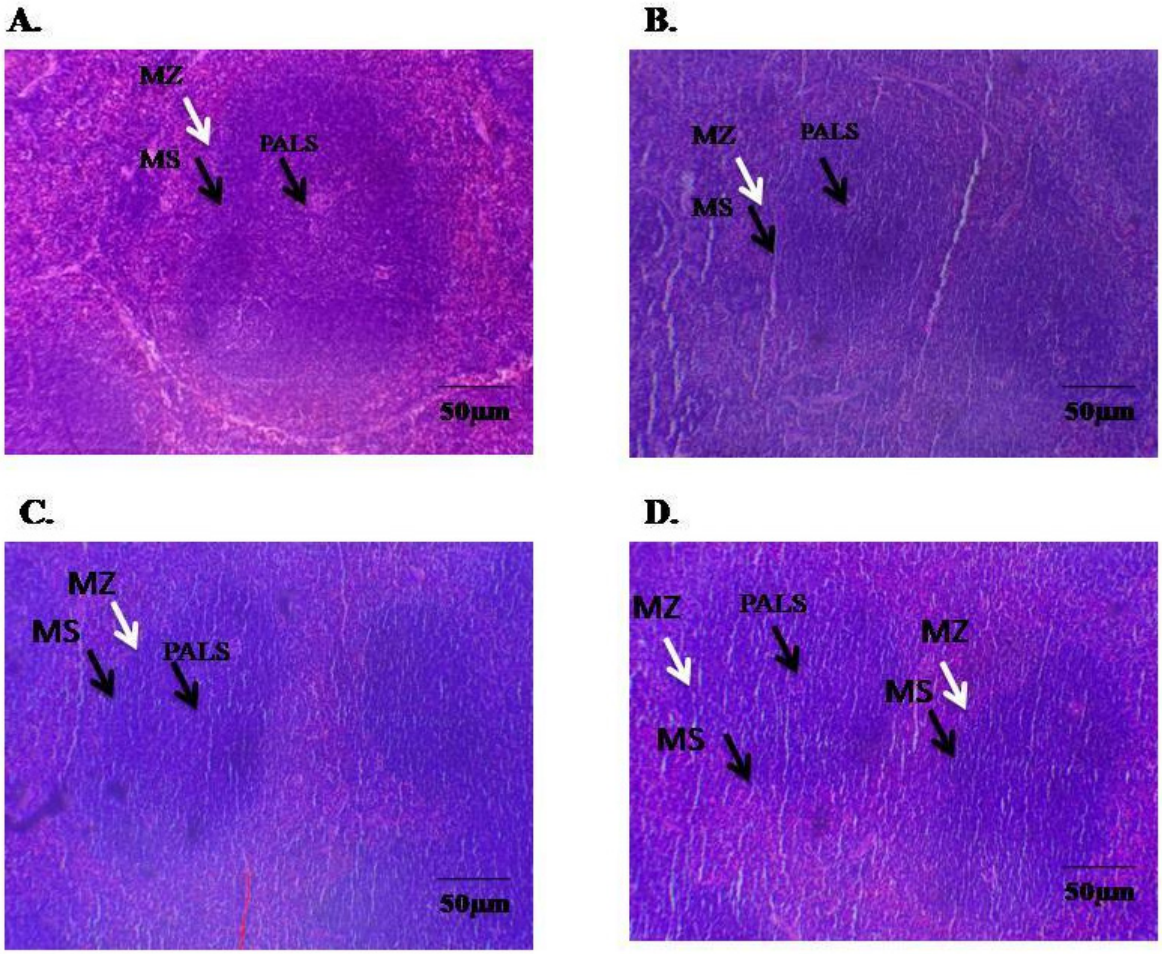

Figure 4. Photomicrographs of hematoxylin and eosine (H&E) stained splenic sections of rats upon PSPI 1 and PSPI 2 supplementation. **(A)** H&E stained section of spleen from the control group showing normal micro architecture having dark Periarteriolar Sheets (PALS), uniform Mariginal Zone (MZ) and much clear Marginal Sinus region (MS). **(B)** H&E stained section of spleen treated with Cd chloride ($CdCl_2$) 2ml/kg body weight showed a significant disorganization in the cellular microarchitecture (PALS) and a damage in the marginal zone and marginal sinus region is also clearly observed. **(C)** H&E stained section of spleen treated with Cd chloride ($CdCl_2$) 2ml/kg body weight and supplemented with 1ml/kg body weight of the PSPI in saline (20g/100ml) (PSPI 1) showed a recovery has been initiated in the splenic architecture and a more prominence in both MS and MZ structure were also observed. **(D)** H&E stained section of spleen treated with Cd chloride ($CdCl_2$) 2ml/kg body weight and supplemented with 2ml/kg body weight of the PSPI in saline (20g/100ml) (PSPI 2) showed a much enhanced recovery in the splenic architecture and the MZ and MS structures.

One of the most important trademark marks of oxidative stress is ROS generation. A higher level of ROS generation initiates a cascade of pathways that leads to severe damage of DNA, protein and lipids leading to cellular death or apoptosis[25]. In the present study (Figure 5 A, B), there was a significant elevation in ROS generation upon $CdCl_2$ treatment ($p < 0.05$), which was decreased upon PSPI 1 supplementation (NS). It was observed to be further decreased upon PSPI 2 supplementation ($p < 0.05$).

4. Discussion

The present study demonstrated that supplementation of PSPI 1 and PSPI 2 significantly the Cd-induced oxidative stress and splenotoxicity. However, a higher dose of PSPI, i.e., PSPI 2 was found to be more effective in counteracting the Cd-induced alteration in spleen tissue.

The over production of reactive oxygen species in a cellular compartment in comparison to under production of intrinsic antioxidant defense

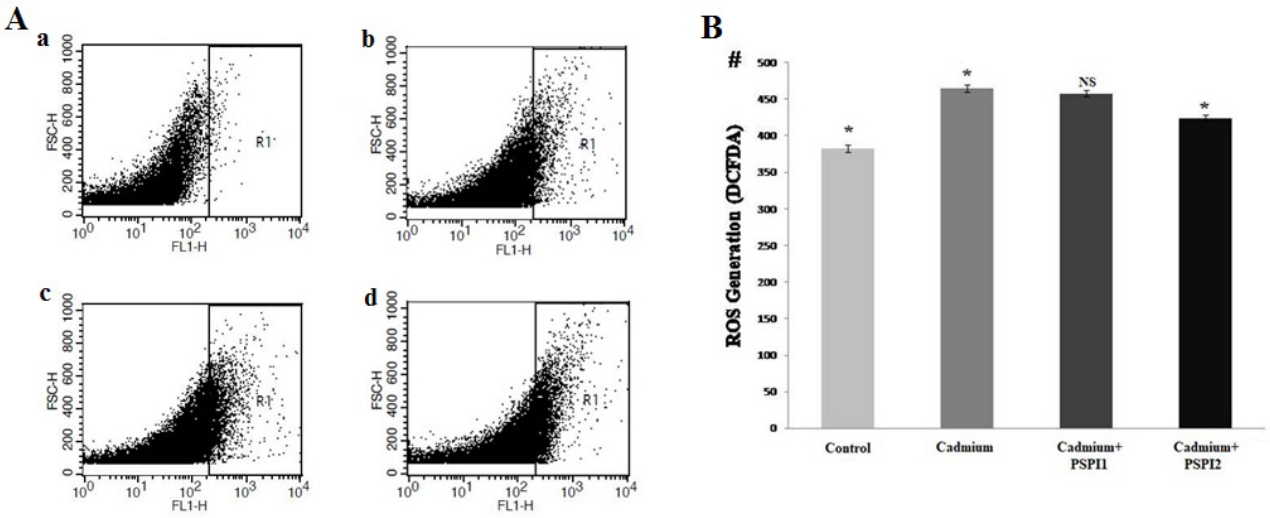

Figure 5. **(A, B)** Effect of PSPI 1 and PSPI 2 supplementation on changes in ROS generation, which was detected using a fluorescent probe 20, 70-dichloro-fluorescin diacetate (DCFH-DA), data presented as (Figure 5A) forward scatter plot using BD Accuri Flow Cytometer, (a) Control (b) Cd chloride ($CdCl_{2)}$ treated (c) Cd chloride ($CdCl_2$) treated + PSPI 1 supplemented (d) Cd chloride $CdCl_2$ treated + PSPI 2 supplemented. (Figure 5B) ROS generation: Values are expressed as mean ± SE; significance based on Kruskal–Wallis test ($p < 0.05$). Significance based on Mann–Whitney *U* multiple comparison tests- Control vs. Cd treated ($p < 0.05$), Cd treated vs. Cd treated + PSPI 1 supplemented (NS), Cd treated vs. Cd treated + PSPI 2 supplemented ($p < 0.05$).s

metabolites gives rise to a phenomenon commonly referred to as oxidative stress[25,26]. NO production and lipid peroxidation are considered to be most efficient markers of oxidative stress. NO plays a dual role in the human body. On one hand, it is considered to be one of the determining factors of normal endothelial and vascular functioning, and on the other hand when produced in excess in association with ROS generation contributes significantly to oxidative stress leading to neurotoxicity and apoptosis[27]. In the present study, we observed a conspicuous rise in the NO production in the splenic tissue of the Cd treated group, which pointed out towards an induction of oxidative stress. In conjunction, exaggerated ROS generation in spleen tissue was observed in Cd-treated group. This ROS, in turn, can initiated oxidative damage in the form of lipid peroxidation[28]. MDA levels are considered to be the cellular toxic end products of lipid peroxidation and hence assessed to understand the amount of lipid peroxidation in a particular tissue or cell[29]. Lipid peroxidation initiates the mechanism of cellular damage by protein cross-linking or fragmentation along with deactivation of membrane enzymes[30]. Thus, NO production and lipid peroxidation combined with exaggerated ROS generation in splenic tissue of Cd-treated group leads to oxidative stress. Supplementation of PSPI 1 and PSPI 2 to the Cd treated groups showed a marked antioxidative response in the spleen tissue, as evidenced by decrease in the production of NO and MDA. Furthermore, PSPI 1 supplementation significantly countered the Cd induced ROS generation, which was again enhanced upon PSPI 2 supplementation. These results demonstrated a strong counteraction of Cd-induced oxidative stress in splenic tissue and highlighted the antioxidative capacity of PSPI.

Biological systems protect themselves against these damaging effects by several means, which includes free radical scavengers and enzymes that are found in our cells, such as SOD, CAT and non-enzymatic GSH system[31]. Any inhibition in the activity of these enzymes initiates and enhances the proneness to free radical induced cellular damage. Whenever the ROS production is initiated it further promotes the production of superoxide ($\underline{O}_2^-$) which is a reactive oxygen metabolite. It is then metabolized and converted to H_2O_2 by the antioxidative enzyme SOD[32]. Compared to control, SOD activity showed considerable decrease in Cd-exposed rats in this

study. So we can assume that higher amount of super oxide radical ($\underline{O}_2^-$) and H_2O_2 was released upon treatment with Cd. Catalase is considered to be a very important enzyme which has free radical degrading activities[33]. Catalase was also observed to decrease markedly in the splenic tissue of the rats treated with Cd leading to less decomposition of H_2O_2 to water and oxygen and enhancement of oxidative injury. Supplementation of PSPI 1 and PSPI2 to the Cd treated rats dose dependently blunted the Cd-induced alteration in both SOD and CAT activity. However, ameliorating potential of PSPI 2 against Cd induced alteration in activities of SOD and CAT was more grounded. The non enzymatic GSH system is considered to be another important antioxidant which potentially scavenge free radicals as well as the excess generated ROS in the cells and tissues through various enzymatic reactions, mainly by the NADPH dependent glutathione reductase, which reduces the oxidized GSH in the form of GSSG to GSH[34]. In the present study it was observed that Cd treatment also caused depletion of GSH stores in the spleen tissue making it prone and susceptible to oxidative injury, and this also reveals that GSH must have been used as an antioxidant for countering the toxic effects of the oxidative metabolites. On the contrary, it was observed that PSPI 1 and PSPI 2 supplementation reduced Cd-induced oxidative damage by balancing and maintaining GSH reserves in the spleen, indicating enhancement in antioxidant activities.

Inflammatory reactions are up regulated by several prominent proinflammatory cytokines such as IL-6, IL-1, TNF-α. and several others. Earlier, it has already been reported that a rise in the proinflammatory cytokine levels may be the result of onset of oxidative stress and immunomodulation[35]. In addition to that, TNF alpha is mainly secreted by macrophages in response to cellular damage caused by various intrinsic and extrinsic factors and is also known to possess cytotoxic effects[36]. Moreover, it has also been reported that NO production and accumulation is further increased by this cytokines, which ultimately leads to cellular toxicity and apoptosis[37]. Therefore by keeping in check the levels of proinflammatory cytokines might play a major role in restriction of spleen damage induced by Cd treatment. Also, they are known to modulate immune responses and are considered as trademarks of immune toxicity. In accordance with that in the present study, administration of PSPI 1 and PSPI 2 totally countered the increase in TNF-α and IL-6 levels in the Cd treated rats. Such results points out towards the anti-inflammatory properties of PSPI supplementation.

Histopathological evaluation in this study revealed that Cd administration initiated a process leading to destruction of the splenic micro architecture along with decrease cellularity in the PALS, which is associated with T cells. These findings pointed towards the immune suppressive effects of Cd[38], which was significantly countered upon PSPI administration in a dose dependent manner, as the Marginal Sinus (MS) and the Marginal Zone (MZ) regions were significantly restored along with the PALS. Thus, these results pointed out the ameliorative potential of PSPI upon Cd toxicity.

Results of the present study led us to speculate that Cd-induced high levels of ROS generation and NO production along with altered antioxidative enzyme activities and pro-inflammatory cytokines caused lipid peroxidation, which may be the key player for modulating the spleen's response to Cd toxicity. On the contrary, the PSPI treated groups had a significant increase in non-enzymatic GSH levels and SOD and CAT activities, as well as a decrease in ROS levels and pro-inflammatory cytokines IL6 and TNF-α, which may have contributed to the amelioration of Cd-induced oxidative stress and prevention of splenotoxicity. It has been already reported that *C. maxima* seeds are rich source of flavonoids, alkaloids, which are polyphenolic compounds[39]. *C. maxima* seeds also contain phytoconstituents such as tocopherols (e.g., α- and γ-tocopherol), carotenoid (e.g., β-carotene, β-cryptoxanthin, lutein, and zeaxanthin), glycosides, tannins, triterpenes and β sitosterol and saponins, some of which have powerful antioxidant properties[40]. The mechanism by which PSPI counteracts the oxidative stress and proinflammatory cytokines production leading to immunomodulation in rats is not better understood from this study, though it is tempting to speculate that one or more of the reported active constituent(s) of PSPI might add to its ameliorative potential by modulating the ROS and cytokine mediated oxidative responses and in turn leading to prevention of immunomodulation caused by Cd.

5. Conclusion

The present study demonstrated that Cd-induced oxidative stress and increased proinflammatory cytokines are pivotal in mediating splenic toxicity, and

supplementation of PSPI dose dependently imparts protection against the Cd-induced toxic changes in spleen tissue Considering multifaceted role of spleen in body, this study highlighted that spleen certainly deserves stronger attention in view of the occupational and environmental human exposure of cadmium. Moreover, pumpkin is easily available and more economical to the general population, and thus it may have a significant impact as a dietary intervention agent in a setting of heavy metal toxicity.

6. Acknowledgement

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8. References

1. Saoudi A, Zeghnoun A, Bidondo ML, Garnier R, Cirimele V, Persoons R, *et al*. Urinary arsenic levels in the French adult population: The French National Nutrition and Health Study, 2006–2007. Sci Total Environ. 2012; 433:206-15. https://doi.org/10.1016/j.scitotenv.2012.06.053 PMID: 22796411.
2. Åkesson A, Bjellerup P, Lundh T, Lidfeldt J, Nerbrand C, Samsioe G, *et al*. Cadmium-induced effects on bone in a population-based study of women. Environ Health Perspect. 2006; 114(6):830-4. https://doi.org/10.1289/ehp.8763 PMCID: PMC1480481.
3. Beyersmann D, Hartwig A. Carcinogenic metal compounds: Recent insight into molecular and cellular mechanisms. Arch Toxicol. 2008; 82(8):493-512. https://doi.org/10.1007/s00204-008-0313-y PMID: 18496671.
4. Mascagni P, Consonni D, Bregante G, Chiappino G, Toffoletto F. Olfactory function in workers exposed to moderate airborne cadmium levels. Neurotoxicology. 2003; 24(4-5):717-24. https://doi.org/10.1016/s0161-813x(03)00024-x PMID: 12900085.
5. Banerjee M, Banerjee O, Singh S, Mukherjee S. Protective effects of black tea (*Camellia sinensis*) extract on endosulfan induced oxidative stress, inflammation and hepatic damage in rats. Toxicol Int. 2022; 29(3):353-61. https://doi.org/10.18311/ti/2022/v29i3/29822
6. Kaur C, Kapoor HC. Anti-oxidant activity and total phenolic content of some Asian vegetables. Int J Food Sci. 2002; 37(2):153-61. https://doi.org/10.1046/j.1365-2621.2002.00552.x
7. Isabelle M, Lee BL, Lim MT, Koh WP, Huang D, Ong CN. Antioxidant activity and profiles of common fruits in Singapore. Food Chem. 2010; 123(1):77-84. https://doi.org/10.1016/J.FOODCHEM.2010.04.002
8. LaChance LR, Ramsey D. Antidepressant foods: An evidence-based nutrient profiling system for depression. World J Psychiatry. 2018; 8(3):97. https://doi.org/10.5498/wjp.v8.i3.97 PMCID: PMC6147775.
9. Batool M, Ranjha MM, Roobab U, Manzoor MF, Farooq U, Nadeem HR, *et al*. Nutritional value, phytochemical potential and therapeutic benefits of pumpkin (*Cucurbita sp*). Plants. 2022; 11(11). https://doi.org/10.3390/plants11111394
10. Alhawiti AO, Toulah FH, Wakid MH. Anthelmintic potential of cucurbitapepo seeds on hymenolepis nana. Acta Parasitol. 2019; 64(2):276-81. https://doi.org/10.2478/s11686-019-00033-z PMID: 30778840.
11. Kushawaha DK, Yadav M, Chatterji S, Srivastava AK, Watal G. Evidence based study of antidiabetic potential of *C. maxima* seeds - *In vivo*. J Tradit Complement Med. 2017; 7(4):466-70. https://doi.org/10.1016/j.jtcme.2016.12.001 PMCID: PMC5634717.
12. Makni M, Fetoui H, Gargouri NK, Garoui EM, Jaber H, Makni J, *et al*. Hypolipidemic and hepatoprotective effects of flax and pumpkin seed mixture rich in ω-3 and ω-6 fatty acids in hypercholesterolemic rats. Food Chem Toxicol. 2008; 46(12):3714-20. https://doi.org/10.1016/j.fct.2008.09.057 PMID: 18938206.
13. Mohamed RA, Ramadan RS, Ahmed LA. Effect of substituting pumpkin seed protein isolate for casein on serum liver enzymes, lipid profile and antioxidant enzymes in CCl4-intoxicated rats. Adv Biol Res. 2009; 3(1-2):9-15. ISSN 1992-0067.
14. Oliveira H, Spanò M, Santos C, Pereira Mde L. Adverse effects of cadmium exposure on mouse sperm. Reprod Toxicol. 2009; 28(4):550-5. https://doi.org/10.1016/j.reprotox.2009.08.001 PMID: 19695322.
15. Feng X, Jin X, Zhou R, Jiang Q, Wang Y, Zhang X, *et al*. Deep learning approach identified a gene signature predictive of the severity of renal damage caused by chronic cadmium accumulation. J Hazard Mater. 2022; 433. https://doi.org/10.1016/j.jhazmat.2022.128795 PMID: 35405588.
16. Nawrot TS, Staessen JA, Roels HA, Munters E, Cuypers A, Richart T, *et al*. Cadmium exposure in the population: From health risks to strategies of prevention. Biometals. 2010; 23(5):769-82. https://doi.org/10.1007/s10534-010-9343-z PMID: 20517707.
17. Puertollano MA, de Pablo MA, Álvarez de Cienfuegos G. Relevance of dietary lipids as modulators of immune functions in cells infected with Listeria monocytogenes. Clin Vaccine Immunol. 2002; 9(2):352-7. https://doi.org/10.1128/cdli.9.2.352-357.2002 .PMCID: PMC119940.

18. Wills ED. Evaluation of lipid peroxidation in lipids and biological membranes in biochemical toxicology: A practical approach. Oxford: Oxford, IRL Press; 1987.
19. Raso GM, Meli R, Gualillo O, Pacilio M, Carlo RD. Prolactin induction of nitric oxide synthase in rat C6 glioma cells. J Neurochem. 1999; 73(6):2272-7. https://doi.org/10.1046/j.1471-4159.1999
20. Sun YI, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem. 1988; 34(3):497-500. https://doi.org/10.1093/clinchem/34.3.497
21. Aebi H. Catalase *in vitro*. Methods Enzymol. 1984; 105:121-6. 10.1016/s0076-6879(84)05016-3 PMID: 6727660.
22. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. 1968; 25:192-205. https://doi.org/10.1016/0003-2697(68)90092-4 PMID: 4973948.
23. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta. 2016; 1863(12):2977-92. https://doi.org/10.1016/j.bbamcr.2016.09.012 PMID: 27646922.
24. Bown MJ, Nicholson ML, Bell PR, Sayers RD. Cytokines and inflammatory pathways in the pathogenesis of multiple organ failure following abdominal aortic aneurysm repair. Eur J Vasc Endovasc Surg. 2001; 22(6):485-95. https://doi.org/10.1053/ejvs.2001.1522 PMID: 11735196.
25. Ray D, Jadav P, Panda P, Mukherjee S, Ghosh S. Protective role of capsaicin against sodium fluoride-induced oxidative damage of liver in rats. Toxicol Int. 2021; 28(1):31-8. https://doi.org/10.18311/ti/2021/v28i1/25920
26. Lubos E, Handy DE, Loscalzo J. Role of oxidative stress and nitric oxide in atherothrombosis. Front Biosci. 2008; 13. https://doi.org/10.2741/3084 PMCID: PMC2617738.
27. Kovacic P, Cooksy A. Iminium metabolite mechanism for nicotine toxicity and addiction: Oxidative stress and electron transfer. Med Hypotheses. 2005; 64(1):104-11. https://doi.org/10.1016/j.mehy.2004.03.048 PMID: 15533623.
28. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014; 2014. https://doi.org/10.1155/2014/360438 PMCID: PMC4066722.
29. Luqman S, Rizvi SI. Protection of lipid peroxidation and carbonyl formation in proteins by capsaicin in human erythrocytes subjected to oxidative stress. Phytother Res. 2006; 20(4):303-6. https://doi.org/10.1002/ptr.1861 PMID: 16557614.
30. Vázquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Domínguez-Bernal G, Goebel W, González-Zorn B, Wehland J, Kreft J. Listeria pathogenesis and molecular virulence determinants. Clin Microbiol Rev. 2001; 14(3):584-640. https://doi.org/10.1128/cmr.14.3.584-640.2001 PMCID: PMC88991.
31. Tretter L, Sipos I, Adam-Vizi V. Initiation of neuronal damage by complex I deficiency and oxidative stress in Parkinson's disease. Neurochem. Res. 2004; 29(3):569-77. https://doi.org/10.1023/b:nere.0000014827.94562.4b PMID: 15038604.
32. Gholami A, Ataei S, Ahmadimoghaddam D, Omidifar N, Nili-Ahmadabadi A. Pentoxifylline attenuates arsenic trioxide-induced cardiac oxidative damage in mice. Oxid Med Cell Longev. 2021; 2021. https://doi.org/10.1155/2021/6406318 PMCID: PMC7810555.
33. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004; 134(3):489-92. https://doi.org/10.1093/jn/134.3.489 PMID: 14988435.
34. Fischer R, Maier O. Interrelation of oxidative stress and inflammation in neurodegenerative disease: Role of TNF. Oxid Med Cell Longev. 2015; 2015. https://doi.org/10.1155/2015/610813 PMCID: PMC4365363.
35. Popko K, Gorska E, Stelmaszczyk-Emmel A, Plywaczewski R, Stoklosa A, Gorecka D, *et al*. Proinflammatory cytokines Il-6 and TNF-α and the development of inflammation in obese subjects. Eur J Med Res. 2010; 15(2):1-3. https://doi.org/10.1186/2047-783x-15-s2-120 PMCID: PMC4360270.
36. Elsabahy M, Wooley KL. Cytokines as biomarkers of nanoparticle immunotoxicity. ChemSoc Rev. 2013; 42(12):5552-76. https://doi.org/10.1039/c3cs60064e PMCID: PMC3665642.
37. Demenesku J, Mirkov I, Ninkov M, Aleksandrov AP, Zolotarevski L, Kataranovski D, *et al*. Acute cadmium administration to rats exerts both immunosuppressive and proinflammatory effects in spleen. Toxicology. 2014; 326:96-108. https://doi.org/10.1016/j.tox.2014.10.012 PMID: 25446329.
38. Dotto JM, Chacha JS. The potential of pumpkin seeds as a functional food ingredient: A review. Sci Afr. 2020; 10. https://doi.org/10.1016/j.sciaf.2020.e00575
39. Sapkota B, Devkota HP, Poudel P. *Citrus maxima* (Brum.) Merr. (Rutaceae): Bioactive chemical constituents and pharmacological activities. Evid Based Complement Alternat Med. 2022. https://doi.org/10.1155/2022/8741669 PMCID: PMC9170406.
40. Abou-Elella F, Mourad R. Anticancer and anti-oxidant potentials of ethanolic extracts of *Phoenix dactylifera*, *Musa acuminata* and *Cucurbita maxima*. Res J Pharm Biol Chem Sci. 2015; 6(1):710-20.https://doi.org/10.1016/j.sciaf.2020.e00575