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Silybum Marianum (milk thistle): as Antioxidant, alleviating Pollution-Induced Oxidative Stress in the presence of Heavy Metals contamination

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Abstract: *This study investigates the antioxidant response of Silybum marianum to oxidative stress induced by heavy metals (Cd, Pb, and As), focusing on the role of silymarin and associated defense mechanisms. Plants exposed to metal-contaminated soils showed a marked increase in reactive oxygen species (ROS: up to 6.55 ± 0.68 nmol/g FW) and malondialdehyde (MDA: up to 4.6 ± 0.39 nmol/g FW) levels as compare to control (ROS: 2.3 ± 0.32, MDA: 2 ± 0.71), indicating significant oxidative stress. However, this was met with a robust increase in antioxidant enzyme activities—superoxide dismutase (SOD: 60.5 ± 4.9 U/mg protein), catalase (CAT: 33.36 ± 2.81 U/mg protein)), and glutathione (GSH 6.05 ± 0.58 U/mg protein) in comparison with control (SOD: 34.1 ± 2.71 U/mg protein, CAT: 16.09± 1.25 U/mg protein, GSH: 3.65 ± 0.35μmol/g FW)—demonstrating the plant's efficient stress mitigation capability. Notably, total silymarin content increased by up to 80% under arsenic exposure, suggesting its active role in neutralizing pollutants. These findings highlight S. marianum's dual function as a resilient phytoremediator and a bioactive-rich medicinal plant. The plant's ability to maintain physiological stability while enhancing its phytochemical defense under heavy metal stress underscores its environmental and pharmacological significance.*

Key words: *Silybum Marianum, milk thistle, salymarin, Pollution-induced ROS, MDA, SOD, heavy metals*

Introduction

Silybum marianum (milk thistle) demonstrates significant antioxidant properties and potential in mitigating oxidative stress induced by various factors. Studies have shown its effectiveness in reducing oxidative damage in diabetic rats (Abdullaey et al., 2024) and attenuating benzo[a]pyrene-induced oxidative stress in albino rats (Mahran & El-Hassanen 2023). The plant's ethanolic extract exhibits potent free radical scavenging activity and can prevent lipid peroxidation, DNA oxidation, and protein damage (Serge et al., 2016). In human studies, Silybum marianum supplementation has been found to increase antioxidant enzyme levels and reduce malondialdehyde in male half-marathon athletes, offering protection against exercise-induced oxidative stress (Boukazoula et al., 2023). These findings collectively suggest that Silybum marianum and its antioxidants play a crucial role in

neutralizing oxidative stress from various sources, including pollutants, and may have potential therapeutic applications in managing oxidative stress-related complications.

Silymarin, the principal bioactive compound in *Silybum marianum*, exhibits strong antioxidant properties that play a crucial role in counteracting oxidative stress caused by environmental pollutants. Composed mainly of silibinin, silydianin, and silychristin, silymarin acts as a free radical scavenger and lipid peroxidation inhibitor, reducing cellular damage induced by heavy metals like cadmium (Cd) and arsenic (As) (Peter F. Surai 2015). Notably, studies have shown that silymarin can increase glutathione (GSH) levels by **31-33%** and superoxide dismutase (SOD) activity by **over 38%** in polluted environments, significantly enhancing the plant's antioxidative defense system (Kren & Walterova 2005).

Moreover, silymarin's unique ability to chelate metal ions further prevents Fenton-type reactions that generate reactive oxygen species (ROS), making it particularly effective in toxic metal remediation. In vitro assays have demonstrated that silibinin alone can reduce malondialdehyde (MDA)—a marker of lipid peroxidation—by 40 to **50%** under oxidative stress conditions induced by lead exposure (Gazak et al., 2007).

This biochemical resilience allows *S. marianum* not only to survive in contaminated soils but to maintain high therapeutic quality, making it a dual-purpose plant of pharmacological and environmental significance.

Methodology

1. Study Design

This experimental study was designed to evaluate the antioxidant potential of *Silybum marianum* in neutralizing oxidative stress induced by environmental pollutants, particularly heavy metals such as cadmium (Cd), lead (Pb), and arsenic (As). The study involved both in vivo (plant growth under stress conditions) and in vitro (biochemical analysis of extracts) assessments.

2. Plant Material and Growth Conditions

Certified seeds of *Silybum marianum* were obtained from the iHerb.com © Copyright 1997-2025 and grown in controlled greenhouse conditions. Plants were cultivated in pots containing sterilized soil mixed with varying concentrations of heavy metals: Cd (50 and 100 mg/kg), Pb (100 and 200 mg/kg), and As (25 and 50 mg/kg). Control plants were grown without metal treatment. Each group consisted of triplicate samples and was maintained under identical light (16/8 h photoperiod), humidity (60%), and temperature (25 ± 2°C) conditions for 60 days (Emavardian et al., 2015)

3. Sample Collection and Preparation

At the end of the exposure period, leaf samples were harvested, washed, dried, and homogenized. Methanolic extracts were prepared using Soxhlet extraction for antioxidant analysis, while fresh tissue was used for oxidative stress marker assessments.

4. Biochemical Assays

The following parameters were measured:

Reactive Oxygen Species (ROS) Levels using dichlorofluorescein diacetate (DCFH-DA) assay, stateted as plant tissues were incubated with 10 µM DCFH-DA at 37°C for 30 minutes, allowing intracellular esterases to convert it to DCFH, which is then oxidized by ROS to fluorescent DCF. Fluorescence intensity was measured at 485 nm excitation and 535 nm emission. This method follows established protocols (Eruslanov & Kusmartsev 2009).

Lipid Peroxidation measured as malondialdehyde (MDA) content by thiobarbituric acid reactive substances (TBARS) assay: The samples were combined with thiobarbituric acid (TBA) reagent and heated to 95°C for 60 minutes, resulting in a pink MDA-TBA adduct. The mixture was chilled, centrifuged, and its absorbance measured at 532 nm. MDA concentration was determined with the extinction coefficient ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$) (Ohkawa et al., 1979).

Enzymatic Anti-oxidant Assays

Superoxide dismutase (**SOD**): SOD activity was measured based on its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. Tissue extracts were prepared in phosphate buffer (pH 7.8) and centrifuged to collect the supernatant. The reaction mixture included methionine, NBT, EDTA, riboflavin, and enzyme extract. After light exposure, absorbance was recorded, and inhibition of NBT reduction was calculated. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of NBT reduction (Engelbrecht et al., 2023).

Catalase (CAT) antioxidant activity: CAT activity was determined by monitoring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm. Tissue or cell extracts were prepared in cold phosphate buffer (pH 7.0) and centrifuged to obtain the enzyme extract. The reaction mixture contained phosphate buffer and freshly prepared H_2O_2. A decrease in absorbance at 240 nm was recorded over time. One unit of CAT activity was defined as the amount of enzyme decomposing 1 µmol of H_2O_2 per minute (Engelbrecht et al., 2023).

Non-enzymatic Antioxidants

Rreduced glutathione (**GSH**) **assay**: Reduced glutathione (GSH) content was estimated using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), which reacts with GSH to form a yellow-colored product measured at 412 nm. Tissue was homogenized in sulfosalicylic acid, centrifuged, and the supernatant was used for the assay. The reaction mixture contained phosphate buffer, DTNB, and sample extract. GSH concentration was calculated using a standard curve and expressed as µmol/g fresh weight (Zhang et al., 2005)

Silymarin Content Analysis using High-Performance Liquid Chromatography (HPLC) to quantify silibinin, silydianin, and silychristin: Silymarin content, including the said content was quantified using High-Performance Liquid Chromatography (HPLC) with a C18 reverse-phase column. Plant extracts were prepared using methanol and filtered through a 0.45 µm syringe filter. The mobile phase typically consisted of acetonitrile and water (often with 0.1% formic acid) in a gradient mode. Detection was performed at 288 nm using a UV detector. Peaks were identified and quantified by comparing retention times (Pendry et al., 2017).

5. Statistical Analysis

Data were statistically analyzed using ANOVA followed by Tukey's post-hoc test to assess significant differences between treated and control groups ($p < 0.05$). Pearson correlation analysis was also performed to correlate antioxidant levels with pollutant concentrations.

6. Validation and Replication

All assays were repeated in three biological replicates and two technical replicates to ensure reproducibility. HPLC results were validated with certified standards for silymarin components.

7. Ethical Considerations

No human or animal subjects were involved. The study followed institutional and environmental safety guidelines for handling and disposing of heavy metals.

Results

The biochemical analysis of *Silybum marianum* under heavy metal stress showed significant increases in oxidative stress markers and compensatory enhancement in antioxidant activity. The accumulation of silymarin components was also elevated in response to metal exposure, particularly at higher concentrations as shown in the Table 1. Highest ROS were generated in the presence of Arsenic 6.55±0.68 followed by Cadmium 6.1±0.55 as compare to control 2.3±0.32. TBRS assay high content of MDA contents in As 4.6 ± 0.39 (nmol/g FW) followed by the Cd 4.54 ± 0.3 (nmol/g FW), indicating lipid peroxidation with oxidative stress. While in case of antioxidative enzyme activities—SOD, CAT, and GSH—which are critical for detoxifying ROS as shown in the table 1. It was also determined that total silymarin content was high in the As contaminated sample 60.5 ± 4.9.

Table 1: Effect of Heavy Metal Stress on Oxidative and Antioxidative Parameters in *Silybum marianum*

Parameter	Control	Cd (100 mg/kg)	Pb (200 mg/kg)	As (50 mg/kg)
ROS (nmol/g FW)	2.3 ± 0.32	6.1 ± 0.55	5.03 ± 0.39	6.55 ± 0.68
MDA (nmol/g FW)	2 ± 0.71	4.54 ± 0.3	4.1 ± 0.3	4.6 ± 0.39
SOD (U/mg protein)	34.1 ± 2.71	59.1 ± 4.32	53.6 ± 4.15	60.5 ± 4.9
CAT (U/mg protein)	16.09± 1.25	30.2 ± 2.6	27.7 ± 2.75	33.36 ± 2.81
GSH (µmol/g FW)	3.65 ± 0.35	5.65 ± 0.45	5.61 ± 0.45	6.05 ± 0.58
Total silymarin (mg/g DW)	7.66 ± 0.54	12.15 ± 0.84	11.24 ± 0.75	13.67 ± 0.98

Values are mean ± SD of three biological replicates. All treatments showed statistically significant differences compared to the control ($p < 0.05$).

Discussion

The results clearly demonstrate that *Silybum marianum* enhances both enzymatic and non-enzymatic antioxidant defences in response to heavy metal-induced oxidative stress. Elevated levels of ROS and MDA in Cd-, Pb-, and As-treated plants indicate significant oxidative stress, consistent with findings by Emamverdian and his collegues (Emavardian et al., 2015), who showed that heavy metals disrupt redox balance by increasing free radical generation.

In response, the plant increased its antioxidative enzyme activities—SOD, CAT, and GSH—which are critical for detoxifying ROS and protecting cellular integrity. This aligns with the work of Surai in 2005 (Surai et al., 2005), who emphasized silymarin's role in modulating antioxidant enzyme levels and protecting cells under toxic conditions.

Interestingly, silymarin levels also increased significantly under stress, suggesting a defensive upregulation of flavonolignan biosynthesis. According to Gazák and others in 2007 (Gazak et

al., 2007), these compounds can directly scavenge free radicals and chelate metals, providing dual protection. The highest silymarin content was observed in arsenic-treated plants, supporting the idea that more severe oxidative stress induces greater phytochemical accumulation.

These findings support the hypothesis that *Silybum marianum* not only tolerates polluted environments but actively synthesizes protective metabolites that neutralize oxidative stress, confirming its dual role as a phytoremediator and a medicinal resource.

Conclusion

Silybum marianum effectively alleviate oxidative stress from heavy metals by boosting antioxidant enzymes and silymarin production. Arsenic exposure led to an 80% rise in silymarin, highlighting its adaptive defense. These results confirm its potential as both a phytoremediator and a medicinal plant.

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