

## RESEARCH ARTICLE

### Remedy from Leaves: *Ziziphus mauritiana* Mitigates Neuronal and Reproductive Damage Induced by 1,4-Dioxane in Male Sprague Dawley Rats

Mahpara Gilani<sup>1</sup>, Salma Sultana<sup>\*1</sup>, Tayyaba Sultana<sup>1</sup> and Farhat Jabeen<sup>1</sup>

<sup>1</sup>Department of Zoology, Government College University Faisalabad, Pakistan

\*Corresponding author: [drsalmasultana@gcuf.edu.pk](mailto:drsalmasultana@gcuf.edu.pk)

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#### ABSTRACT

Plants have been utilized for therapeutic purposes since ancient times due to their effective healing properties without much side effects. Bioactive compounds present in medicinal plants are responsible for treating and preventing several diseases. *Ziziphus mauritiana* (family *Rhamnaceae*) also known as Indian jujube, possess antioxidant, anti-inflammatory, anti-cancer, neuroprotective and other biological activities. The main purpose of current work was to investigate protective effect of *Ziziphus mauritiana*'s leaves extract against 1,4-dioxane induced toxicity. 1,4-Dioxane, a cyclic diether, is an extensive water contaminant inducing severe toxicity and damaging human/animal health at many levels. Twenty-five male Sprague Dawley rats were distributed into five groups, C (control group), G1 (1,4-dioxane 3000ppm treated group), G2 (1,4-dioxane+ *Z. mauritiana* 3000ppm+ 120mg/kg treated group), G3 (1,4-dioxane+ *Z. mauritiana* 3000ppm+ 240mg/kg treated group) and G4 (1,4-dioxane+ *Z. mauritiana* 3000ppm+ 360mg/kg treated group) in triplicates which were gavaged orally for 60 days. After trial completion, brain and testis were excised, cleansed, and preserved in 10% neutral buffered formalin for bioassays and histological observations. Declined body weights, oxidative stress induction, elevated AchE activity, hormonal imbalance (testosterone, FSH and LH) and decreased sperm count besides deteriorated histological structures were observed in G1. However, *Z. mauritiana* treated groups exhibited clear improvement in these parameters. Results indicated that G4 group showed maximum improvement in body weight, AchE inhibition, hormonal balance, sperm count and histological structures underscoring the mitigating potential of *Z. mauritiana* 's leaves extract in attenuating the neurological and reproductive toxicities induced by 1,4-dioxane.

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#### INTRODUCTION

Medicinal plants and plants derived products have played a critical role in several scientific fields due to the renewed interest in natural drugs, which has driven the evolution and developments of phytochemical studies in recent years. New natural substances have proved great potential as therapeutic agents, and in drug synthesis, as the discovery of plant-based medicines has become quite significant in the modern world (Ersoy *et al.*, 2022). According to an estimation, herbal medicines and drug derived from them subsidize more than 60% of clinical drugs across the globe (Al-Saeedi *et al.*, 2017). Medicinal plants have been studied comprehensively because of their nutritional (Naseer *et al.*, 2019; Kamran *et al.*, 2020) and pharmaceutical values (Rehman *et al.*, 2018; Farhat *et al.*,

2020). Approximately 400 to 600 out of 5700 species of medicinal plants are considered to be present in Pakistan (Ahmad *et al.*, 2007) from which, several plants are extensively utilized as fruits and vegetables here (Rehman *et al.*, 2018).

*Ziziphus mauritiana* (family: *Rhamnaceae*) also known as Indian jujube, desert apple and Indian plum, is a tropical fruit tree and native to Pakistan, India, Afghanistan, Africa and Kenya (Plastina *et al.*, 2012). The leaves contain a complex mixture of flavonoids, polyphenolic hydrolysable tannins, mucilage, sterol, triterpenoids, cardiac glucosides, and leucoanthocyanidins (Prakash *et al.*, 2021). These phytochemicals act as antioxidative agents through scavenging free radicals, and anti-inflammatory through inhibiting pro-inflammatory cytokines (Shin *et al.*, 2020) & by downregulating NF-κB

pathway (Laurindo *et al.*, 2023), COX-2 and iNOS and maintain reproductive parameters by preserving testosterone level through inhibition of aromatase enzyme (CYP19A1) and reduce oxidative stress by scavenging ROS to prevent Leydig cells impairment and sperm damage (Faki and Er, 2021). Detected phytochemicals in *Z. mauritiana* leaves extract are as following: Chlorogenic acid which is a phenylacrylate polyphenol compound produced by the shikimic acid pathway and reported previously for its anti-tumor, anti-oxidant (through scavenging free radicals), anti-inflammatory (inhibition of pro-inflammatory cytokines), neuroprotective activities and hormonal balancing as far as reproductive hormones are concerned (Wang *et al.*, 2022; Zheng *et al.*, 2024). P-coumaric acid is a compelling phenolic compound and possess different biological activities like anti-oxidant, anticancer, neuroprotective, anti-inflammatory, anti-diabetic, repro-protective, cardioprotective, and hepatoprotective activities (Kaur and Kaur, 2022). Gallic acid (GA) is a phenolic molecule and possess several health benefits (Bhuia *et al.*, 2023). Similarly, ferulic acid is a crucial active component of many traditional Chinese medicines as it has diverse biological activities such as anti-oxidant, anti-inflammatory, and neuroprotective activities (Li *et al.*, 2021).

*Z. mauritiana* proves to be an auspicious phytotherapeutic avenue for mitigating neurotoxicity and reproductive toxicity induced by environmental pollutants. Its strong antioxidant, anti-inflammatory, hormonal regulatory, and anti-apoptotic characteristics make it a potent candidate for future nutraceutical or phytopharmaceutical development. Its ethanolic extract at oral administration in adult male rats showed significant improvement in testosterone and sperm count (Sukirti *et al.*, 2013). *Z. mauritiana* extract significantly reduced acetylcholinesterase activity, and malondialdehyde level (Kasher *et al.*, 2024).

1,4-Dioxane is a cyclic diether with extensive effect on drinking water sources around the globe. It is a synthetic industrial chemical utilized as stabilizer for chlorinated solvents in industrial processes, and as solvent in various products like grease, varnishes, waxes, dyes, detergents, deodorants and cosmetics (EPA, 2017). It has been listed in class 2B and classified as “likely to be carcinogenic to humans” over many exposure pathways according to United States Environmental Protection Agency. Its low mobility in clay soils contribute to a long half-life of 2-5 years in groundwater (Khan *et al.*, 2018; Pollitt *et al.*, 2019). Livestock such as cattle, as well as wildlife and other animals, are particularly vulnerable to environmental contaminants like 1,4-dioxane due to their recurrent and prolonged contact with contaminated water sources, soil, or fodder irrigated with polluted water. As a highly water-soluble and mobile compound, 1,4-dioxane can persist in groundwater and surface water, thus enhancing the risk of oral and dermal exposure in grazing animals (EPA, 2020). Metabolic byproducts of 1,4-dioxane, including  $\beta$ -hydroxyethoxyacetic acid (HEAA), can contribute to systemic toxicity in exposed animals. Though several toxicological investigations have focused on rodent models, the potential for bioaccumulation and chronic toxicity in ruminants raises apprehensions regarding animal health, food safety, and zoonotic transmission

pathways (Zhou *et al.*, 2021). Due to its high-water solubility, persistence, and poor biodegradability, it is classified as an emerging environmental toxicant with considerable risks to animal and human health.

Humans and animals can come in contact with 1,4-dioxane in different ways as through contaminated water ingestion and through product utilization by dermal absorption, inhalation, or accidental ingestion (EPA, 2020). Most significant source of 1,4-dioxane is ingestion of contaminated drinking water which persist in municipal water supplies and aquifers due to low degradation (Zenker *et al.*, 2003; Adamson *et al.*, 2014). Workers from solvent production, metal cleaning, textile processing, and resin manufacturing industries may be exposed to 1,4-dioxane through dermal absorption or inhalation (ATSDR, 2020). 1,4-dioxane can affect grazing animals through contaminated drinking sites and through getting effected diet from accumulated-soil irrigated crops near industrial areas. The higher concentrations of 1,4-dioxane cause significant decrease in testosterone level and other hormonal changes which leads to damage as necrosis, cellular coagulation, tumors and hemorrhage in gonads (Hashim *et al.*, 2018). 1,4-dioxane (100  $\mu$ g/kg body weight) in drinking water for rats disrupt body weight, malondialdehyde (MDA) peroxidation marker, and cause edema in the structure of brain and reproductive organs (Said *et al.*, 2016).

*Ziziphus mauritiana* 's protective role against 1,4-dioxane induced toxicity was explored for the very first time. *Ziziphus mauritiana* 's ethanolic extract significantly improved the 1,4-dioxane induced damage. Based on results this plant is highly recommended against 1,4-dioxane induced toxicity. Present study has highlighted the significance of *Z. mauritiana* to present the better cure for neurotoxicity and reproductive toxicity induced by 1,4-dioxane and to eliminate the hazardous effects of other chemicals and to make environment better.

## MATERIALS AND METHODS

**Plant Collection and Extract preparations:** *Z. mauritiana* leaves were collected from local areas and identification was verified by contemplating the authentic samples at University of Agriculture, Faisalabad, Pakistan (Specimen Voucher No. 315/21/05). The leaves were washed to wipe away inessential materials. The leaves were desiccated at room temperature, pounded by mechanical crusher and sieved through mesh-40. The 95% ethanolic extract was obtained by using Soxhlet 's apparatus through crushed powder. Under reduced pressure, extract was concerted. Therefore, semi-solid ethanol free mass was further utilized. Brief protocol is shown in Fig. 1.

**HPLC study of ethanolic extract of *Z. mauritiana* leaves:** Binary gradient solvent system was developed for HPLC and phenolic acids and flavonoids were separated at same time through C-18 column (250  $\times$  4.6mm internal diameter, 5 $\mu$ m particle size) at 0.8mL/min flow rate within 50min. Separation factors for all separated compounds were greater than 1 with more than 1.5 resolutions. Reliability for phenolic acids and flavonoids separation was also great with RSD < 2.00% (run-to-run) and 2.70% (day-to-day) for cohesive areas basis. This technique was utilized to

separate phenolic acids and flavonoids in one run from samples with diverse matrixes. Liquid chromatography comprised of phase C18 column (250 × 4.6mm internal diameter), having 5µm film thickness, accompanying an oven set at 30°C. Chromera HPLC system (Perkin Elmer, USA.) attached with Flexer Binary LC pump, UV/Vis LC Detector (Shelton CT, 06484 USA) controlled by software V. 4.2.6410 was used to analyze the data. The mobile phase consisted of solvent A (acetonitrile: methanol as 70:30) and solvent B (double distilled water with 0.5% glacial acetic acid). UV spectra were documented at 275nm. The analytes were identified by corresponding the retention times and spiking samples with quantifications and standards was based on an external standard technique. HPLC separation efficacy was evaluated by the separation factor and resolution (Hussain *et al.*, 2013).

**Animals Selection:** 210-220g weighed twenty-five mature male Sprague Dawley rats were obtained from Government College University Faisalabad for this experiment. Prior to initializing the trial, Animal Ethical Committee (AEC) / Ethics Review Committee of Government College University has approved the experiment (Ref no. GCUF/ERC/21/01A). Entire experiment was carried out according to appropriate protocols and recommendations. In steel cages, rats were kept and acclimatized at room temperature (20-23°C) with 12:12h light: dark cycles in approximately 40-60% humidity as per standard laboratory rules.

**Experimental Design:** 1,4-dioxane (CAS NO 123-91-1 anhydrous, 99.8%) were purchased from Sigma Aldrich. In the pilot experiment, LD<sub>50</sub> was evaluated according to standard protocol (APHA, 1998). The sub-lethal dose of 1,4-dioxane (3000mg/kg BW of rat) was selected @ 1/30<sup>th</sup> of 1,4-dioxane LD<sub>50</sub>. All rats were distributed into five groups randomly with each group containing five rats and ordained as:

Control (C) normal diet and free access to water given without any treatment, G1: rats were treated with 3000ppm 1,4-dioxane, G2: rats were treated with 120mg/kg *Ziziphus mauritiana* + 3000ppm 1,4-Dioxane, G3: rats were treated with 240mg/kg *Ziziphus mauritiana* + 3000ppm 1,4-Dioxane, G4: rats were treated with 360mg/kg *Ziziphus mauritiana* + 3000ppm 1,4-Dioxane.

Commercial rodent feed, 19% crude protein and distilled water *ad libitum* were supplied to all groups. Rats were given the defined doses through oral gavage for 60 days on alternating days (Hassan *et al.*, 2023). Complete experimental design is shown in Fig. 2.

**Animal body weight:** Weight of all rats were calculated on weighing balance before starting the trial. Proper feed and water were provided on regular bases. The selected dose of 1,4-dioxane+*Z. mauritiana* were administered through oral gavage. Body weight was measured after every 15 days throughout the trial. Weight of all groups were compared thoroughly till the completion of trial and analyzed significantly.

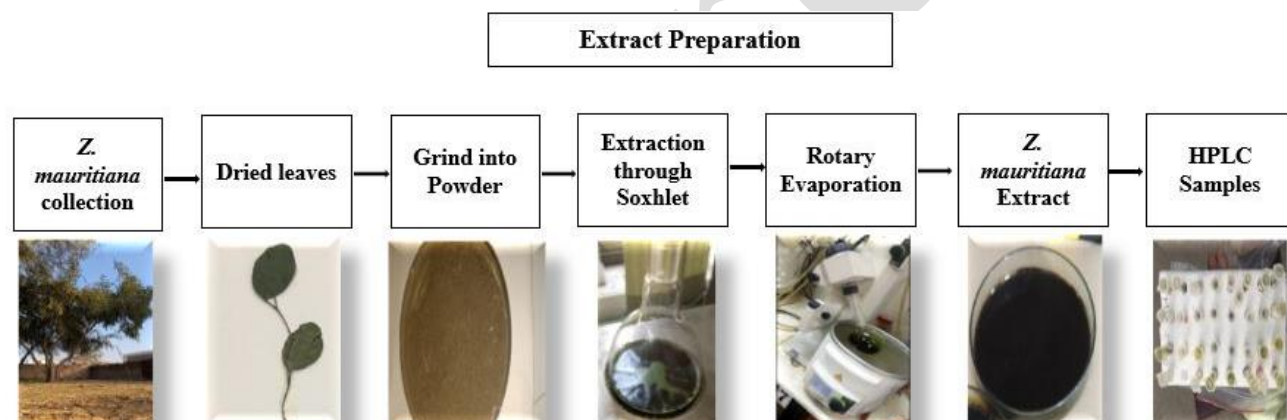


Fig. 1: Extraction from dried leaves of *Z. mauritiana* and analyzing bioactive compounds of extract.

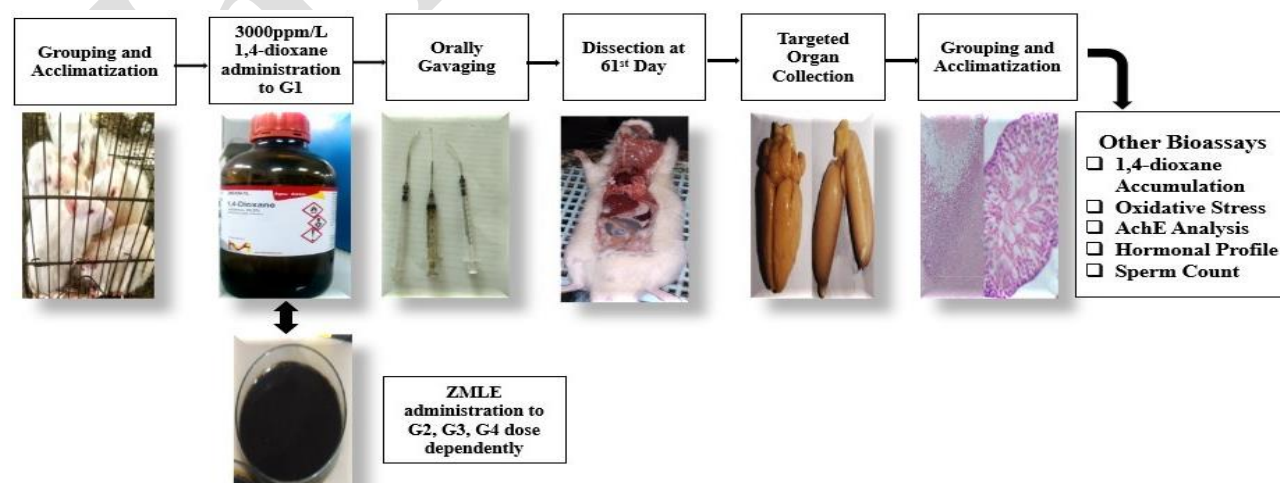
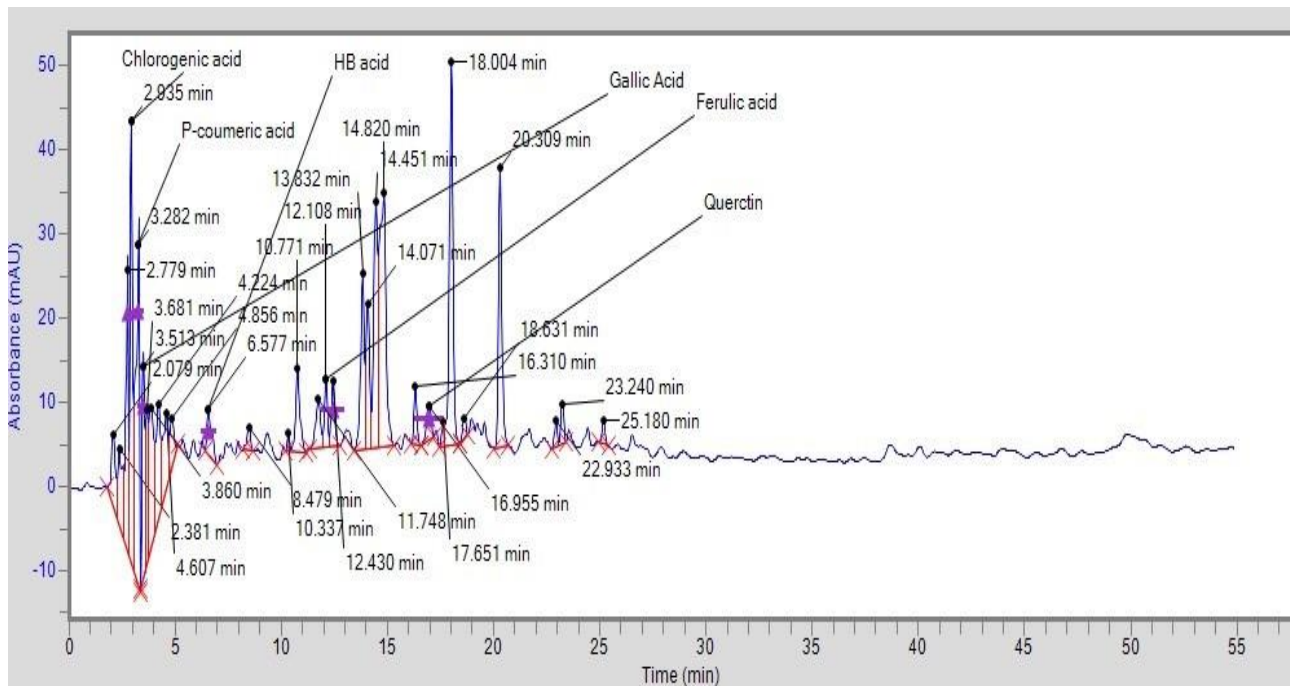


Fig. 2: Experimental Design.



**Fig. 3:** Chromatogram of *Ziziphus mauritina* ethanolic leaves extract exhibiting detected phenolic acids.

**Sample Collection:** On 61<sup>st</sup> day rats were overnight abstained, sedated with ketamine hydrochloride and lacerated. Brain and testes were collected and stored after weighing with Sartorius weighing balance (Shimadzu, BL-2200H), were kept in plastic bags at -80°C for hormonal changes. Brain and testes were submerged in fixative sera (glacial acetic acid 10ml, formaldehyde 35ml, ethanol 55ml) for other histological procedures (by hematoxylin eosin staining method).

**Acetylcholine esterase Activity:** To analyze acetylcholine esterase level in brain, spectrophotometric method was used and a section of brain was added in falcon tube of bullet blender with homogenized solution (150mMNaCl, 1mM EDTA, 1mM EGTA, 100mMTris, 1mMPMSF, 0.5% sodium deoxycholate, 1% Triton X, pH 7.4) for membrane bound enzyme discharge and homogenized for 15min. Proceeding further, in each test tube 0.1Mm acetylcholine iodide solution was added and incubated again for 15min at 37°C with constant shaking. Test tubes were removed from incubator after 15min and 0.04% DTNB and 44% SDS were mixed in flask and 0.5ml of this solution was added in all test tubes. Then absorbance of each test was observed at 412nm at spectrophotometer (Hitachi, U-2800) at the end (Grissa *et al.*, 2016).

**Hormonal assays:** LH, FSH, and testosterone levels were estimated through rat specific ELISA Kit founded on the code of competitive binding and corresponding to the manufacturer's guidelines.

**Estimation of Oxidative Stress Markers:** Oxidative stress markers (MDA, NO, GSH, SOD) were determined through spectrometer with the help of available ready to use kits (Hendawy *et al.*, 2019).

**Sperm Count:** To measure the sperm count, 0.25g of left testis was taken and weighed. Tunica albuginea was

removed and weighed again. Then testis was homogenized in homogenizing solution (0.05% v/v) Triton X-100 and 0.25 mg sodium azide in physiological saline for 20 min. Each 0.2ml homogenate sample was diluted with 0.8ml Saline solution containing 1% trypan blue to count spermatids. Trypan blue was used to stain the spermatids. On Harwell chamber 10 µl of diluted homogenate samples were loaded. The average number of spermatids per sample was determined (Cao *et al.*, 2015). Following formula was used:

$$Y = \frac{X}{10 \times 100 \times 50 \times 5 \times 1000}$$

(DSP) = Y / Weight of de-capsulated testes

**Histology:** In fixative sera (formaldehyde: 30ml, ethanol: 60ml and glacial acetic acid: 10ml), brain and testis sections were fixed for 48h. Dehydration was done through graded series of ethanol (45 minutes per step), then embedding was completed after clearing it through two changes of xylene and infiltration with four changes of melted paraffin. Tissues were embedded in oil for transparent view at 25°C. Thick sections (5-6µm) were cut using a rotary microtome equipped with disposable steel knives and flattened further on a heated water bath, floated onto microscope slides and dried. Staining of slides with hematoxylin and eosin was done to analyze them under microscope for further studies (Vasantharaja and Ramalingam, 2018).

**Statistical Analysis:** The results were statistically analyzed by using software SPSS Statistics 22. One-Way ANOVA was implicated for data analysis and comparison between means. Then Post-hoc Tukey's test was also applied to compare the effect of means between groups. The variances among means were significant at P<0.05.



## RESULTS

**High Performance Lipid Chromatography (HPLC) of *Z. mauritiana* 's leaves:** Phytochemicals and polyphenols of *Z. mauritiana* 's leaves extract was analyzed by HPLC. Phenolic acids such as chlorogenic acid, ferulic acid, Hb acid, P-coumeric acid, gallic acid and a flavonoid (quercetin) were detected in extract. All detected compounds are briefly mentioned in Table 1. Chromatogram demonstrating these compounds is presented in Fig. 3. Quantitative and qualitative analysis is described in Table 2.

**Table 1:** High-performance liquid chromatography (HPLC) of ethanolic extract of *Z. mauritiana* leaves

Sr No.	Compound name	Concentration
1	Chlorogenic acid	55.88 mg/kg
2	P-coumeric acid	4.64 mg/kg
3	Gallic Acid	17.90 mg/kg
4	HB acid	10.22 mg/kg
5	Ferulic acid	6.86 mg/kg
6	Quercetin	43.41 mg/kg

**Table 2:** Qualitative and quantitative phytochemical analysis of ethanolic extract of *Z. mauritiana* leaves

Sr. No.	Phytochemical	Status	Concentration
1	Alkaloid test	-	-
2	Saponin test	+	-
3	Flavonoid test	+	91.15 ±0.28
4	Terpenoids test	-	-
5	Phenols	+	531.26±1.17
6	Tannins	+	214.37 ±1.10
7	Steroids	-	-

(Present= + or absent = -).

**Body Weight:** Body weights of all groups were measured regularly according to standard protocols, and a severe decline was noted in G1 group at 15<sup>th</sup> day due to 1,4-dioxane administration. While, in other groups there was a gradual maintenance in body weights. *Z. mauritiana* 's leaves extract has maintained body weight at different doses. High dose of *Z. mauritiana* 's leaves extract (360mg/kg) showed most significant improvement in body weights of rats from G4 group. Groups showed less significance at day 0 as compared to other Days. Detailed results are mentioned in Table 3.

**Table 3:** Comparison of body weight of male Sprague Dawley rats supplemented with 1,4-dioxane and *Ziziphus mauritiana* 'leaves extract

Days	C	G1	G2	G3	G4	P-value
0 Day	212.8±2.58 <sup>a</sup>	212.0±1.58 <sup>a</sup>	212.6±1.14 <sup>d</sup>	214.2±3.11 <sup>e</sup>	213.±2.50 <sup>e</sup>	
15 Days	221.6±3.13 <sup>d</sup>	192.8±1.92 <sup>b</sup>	207.0±1.58 <sup>e</sup>	222.4±3.97 <sup>d</sup>	236.4±1.51 <sup>d</sup>	
30 Days	254.8±3.11 <sup>c</sup>	171.0±4.00 <sup>f</sup>	224.2±1.92 <sup>c</sup>	236.6±1.67 <sup>c</sup>	247.0±1.58 <sup>c</sup>	0.001***
45 Days	277.2±2.58 <sup>b</sup>	151.6±1.51 <sup>d</sup>	230.4±1.51 <sup>b</sup>	251.6±2.07 <sup>b</sup>	273.2±2.04 <sup>b</sup>	
60 Days	300.2±1.87 <sup>a</sup>	130.2±2.58 <sup>f</sup>	247.8±1.92 <sup>a</sup>	264.3±1.08 <sup>a</sup>	295.2±3.11 <sup>a</sup>	

C=Control; G1= 1,4-dioxane (3000ppm); G2= 120mg/kg; G3=240mg/kg; and G4= 360mg/kg of *Z. mauritiana* leaves 's extract; means values varies significantly(P<0.05) in rows among different groups.

**Acetylcholine Esterase Activity:** Prominent increase in acetylcholine esterase level was observed in G1 (3000ppm 1,4-dioxane) group. Though treated groups with *Z. mauritiana* leaves extract (120, 240 and 360mg/kg body weight) exhibited a significant decline in AchE (P<0.05). Detailed results are mentioned in Table 4.

**Hormonal Profile:** Imbalance in hormones was observed in G1 after 1,4-dioxane exposure. Prominent decline in LH, FSH, and testosterone levels were exhibited. *Z. mauritiana* 's leaves extract evidently balanced the hormonal profile at different doses by improving LH, FSH, and Testosterone level in treated groups. High dose (360mg/kg) of *Z. mauritiana* 's leaves extract exhibited most significant improvement against 1,4-dioxane. Detailed results are mentioned in Table 5.

**Table 4:** Comparison of Acetylcholinesterase activity of male Sprague Dawley rats supplemented with 1,4-dioxane and *Z. mauritiana* 's leaves extract

Groups	Acetylcholinesterase	P-value
C	0.76±0.01 <sup>e</sup>	
G1	2.11±0.02 <sup>a</sup>	
G2	1.62±0.03 <sup>b</sup>	
G3	1.21±0.02 <sup>c</sup>	
G4	0.87±0.02 <sup>d</sup>	0.000***

C=Control; G1=1,4-dioxane (3000ppm); G2=120mg/kg; G3=240mg/kg; and G4=360mg/kg of *Z. mauritiana* leaves 's extract; means values varies significantly(P<0.05) in columns among different groups.

**Table 5:** Comparison of Testosterone, FSH, and LH Level of male Sprague Dawley rats supplemented with 1,4-dioxane and *Z. mauritiana* 's leaves extract

Groups	FSH	LH	Testosterone Level	P-value
C	0.26±0.01 <sup>d</sup>	1.46±0.01 <sup>e</sup>	2.18±0.01 <sup>a</sup>	
G1	0.43±0.01 <sup>a</sup>	2.21±0.03 <sup>a</sup>	1.86±0.02 <sup>e</sup>	
G2	0.40±0.01 <sup>b</sup>	2.15±0.01 <sup>b</sup>	2.00±0.02 <sup>d</sup>	
G3	0.36±0.01 <sup>c</sup>	1.91±0.02 <sup>c</sup>	2.06±0.01 <sup>c</sup>	
G4	0.29±0.01 <sup>d</sup>	1.53±0.02 <sup>d</sup>	2.14±0.01 <sup>b</sup>	0.000***

C=Control; G1=1,4-dioxane (3000ppm); G2=120mg/kg; G3=240mg/kg; and G4= 360 mg/kg of *Z. mauritiana* leaves 's extract; means values varies significantly(P<0.05) in columns among different groups.

**Sperm Count:** 1,4-dioxane administration severely declined sperm count in "G1" as compared to "C" control group. It was noted that *Z. mauritiana* 's leaves extract improved sperm count at low, medium, and high doses in other treated groups. 360mg/kg *Z. mauritiana* 's leaves extract exhibited most significant results in G4. Detailed results were described below in Table 6.

**Table 6:** Comparison of sperm count of male Sprague Dawley rats supplemented with 1,4-dioxane and *Z. mauritiana* 's leaves extract

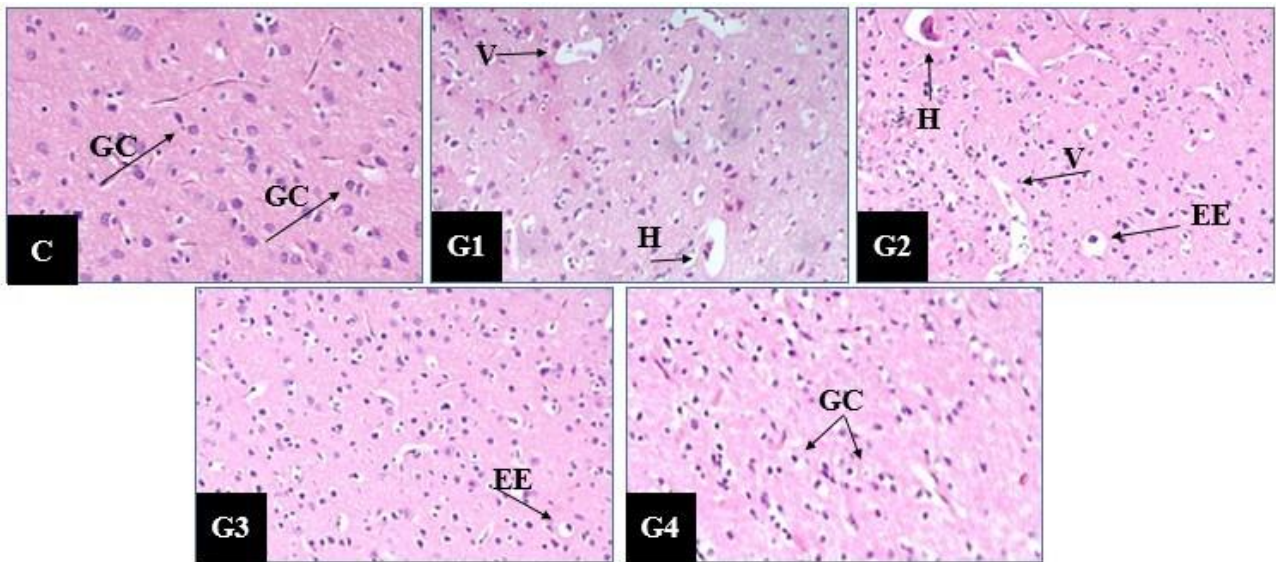
Groups	Sperm Count (10 <sup>8</sup> /ml)	P-value
C	28.03±0.05 <sup>a</sup>	
G1	9.92±0.02 <sup>e</sup>	
G2	13.53±0.40 <sup>d</sup>	
G3	19.37±0.37 <sup>c</sup>	
G4	27.50±0.32 <sup>b</sup>	0.000***

C=Control; G1= 1,4-dioxane (3000ppm); G2= 120mg/kg; G3=240mg/kg; and G4= 360mg/kg of *Z. mauritiana* leaves 's extract; means values varies significantly(P<0.05) in columns among different groups.

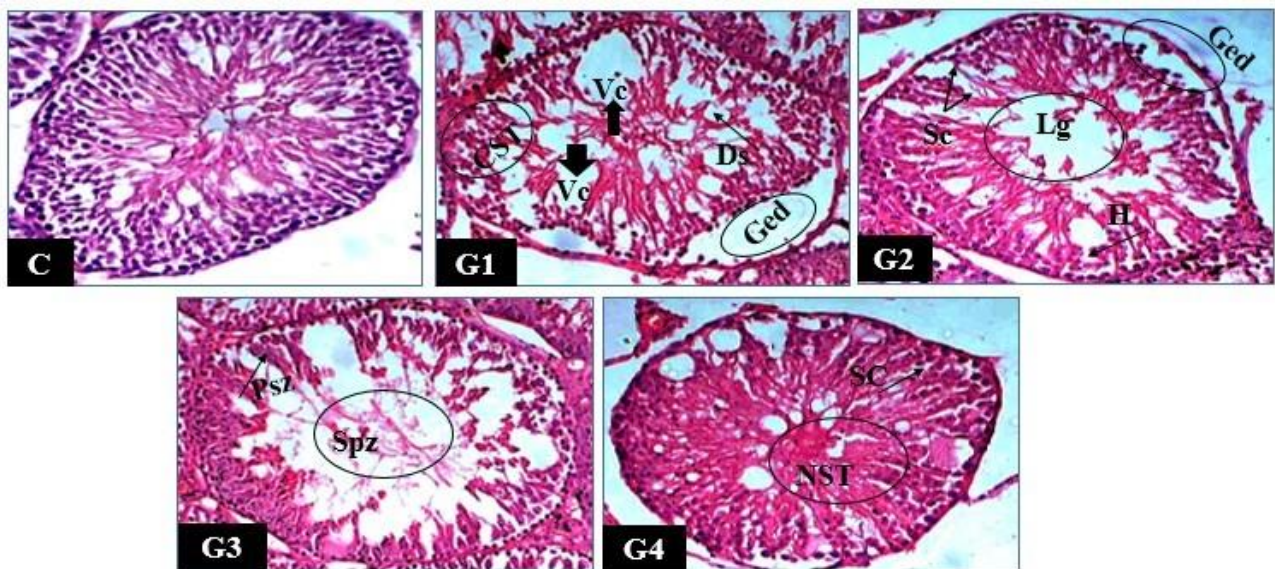
## Histological Profiling of Organs

**Brain Histology:** Histological alterations to rat 's brain induced by 3000 ppm 1,4-dioxane administration has been exhibited in Fig. 4. Dose-dependent effect of *Z. mauritiana* 's leaves extract were observed clearly.

**Testis Histology:** Histological alterations to rat 's testis induced by 3000ppm 1,4-dioxane administration has been exhibited in Fig. 5. Dose-dependent effect of *Z. mauritiana* 's leaves extract were clearly observed.



**Fig. 4:** Rat's Brain histopathology H and E × 40X. (C) Normal histological finding in control group showing normal Glial cells (GC) (G1) 1,4-dioxane (3000ppm) treated group depicting hemorrhage (H) and Vacuolation (V) (G2) 3000ppm 1,4-Dioxane + 120mg/kg *Z. mauritiana* treated group displaying hemorrhage (H), external edema (EE) and Vacuolation (V) (G3) 3000ppm 1,4-Dioxane + 240mg/kg *Z. mauritiana* depicting significant results in the term of recovery than G2 as neurons were more in shape and less external edema (EE), (G4) 3000ppm 1,4-Dioxane + 360mg/kg *Z. mauritiana* treated group showing most significant improvement in brain structures to recover neuronal degeneration, and Glial cells, hence this dose was most effective as compared to other doses.



**Fig. 5:** Photomicrographs of male Sprague Dawley rats, C) showing normal Testicular structure (G1) 3000ppm treated group's testes displaying congestion of sertoli and necrotic cells debris (CST), Vacuolation (Vc), degenerating seminiferous tubules (Ds), and germinal epithelium detachment (Ged) (G2) rats treated group with low dose (3000ppm 1,4-dioxane + 120mg/kg *Z. mauritiana* 's leaves extract) displaying loss of germ cells in lumen of seminiferous tubules (Lg), Sertoli cells (SC), Hemorrhage (H), and germinal epithelium detachment (Ged) (G3) rats treated with medium dose (3000ppm 1,4-dioxane + 240mg/kg *Z. mauritiana* 's leaves extract) displaying primary spermatozoa (Psz) and maturing spermatozoa (SPZ), and (G4) rats treated with high dose (3000ppm 1,4-dioxane + 360mg/kg *Z. mauritiana* 's leaves extract) exhibiting histological structure close to control group with normal seminiferous tubules (NST) and Sertoli cells (SC) exhibiting most significant improvement in testes 's structures to recover Vacuolation, seminiferous tubules degeneration, epithelium damage and Hemorrhage effective as compared to other doses.

## DISCUSSION

Plants are most sustainable source for therapeutic purposes from ancient times and still gaining strong importance for treating various disorders across the globe. Traditional or corresponding medicine fields along with the presence of numerous folk and socio-religious actions aids majorly in healthcare across the subcontinent (Anand *et al.*, 2022). Present study aimed to evaluate the hazardous effects of 1,4-dioxane on human health as well as pharmaceutical properties of *Z. mauritiana* leaves

ethanolic extract against 1,4-dioxane induced toxicology. *Z. mauritiana* is commonly utilized in traditional medicines against various diseases including hypertonia, anemia, nephritis, nervous disorders and in stabilizing male functional fertility and sexual activity (Sukirti *et al.*, 2013). Its extract is rich in phytochemical constituents and have significant antioxidants and antimicrobial activities (Butt *et al.*, 2021). 1,4-Dioxane is a cyclic diether extensively contaminating drinking water and affecting human life. Aside being a carcinogenic solvent, it is also reportedly damaging tissues and organs, disrupting hormonal profiles,

inducing oxidative stress, disturbing histological structures and causing nervous system disorders at exposure (Said *et al.*, 2016; Hashim *et al.*, 2018).

Several compounds were detected through HPLC of *Z. mauritiana* leaves. Among these, phenolic compounds, chlorogenic acid, P-coumaric acid, ferulic acid, HB acid, gallic acid, and a flavonoid quercetin, was also detected. Presence of these compounds in *Z. mauritiana* leaves was also reported previously (Yahia *et al.*, 2020; Prakash *et al.*, 2021; Ramar *et al.*, 2022). Quercetin, a naturally occurring flavonoid exhibits antimicrobial, antioxidant, neuroprotective, and anti-cancer activity (Joseph *et al.*, 2013; Kumar *et al.*, 2017; Rauf *et al.*, 2018). It demonstrates anti-Alzheimer activity by decreasing oxidative stress and controlling neuro-inflammation (Poprac *et al.*, 2017; Xu *et al.*, 2019). Chlorogenic acid possess antioxidant, antibacterial, anti-tumor, anti-inflammatory activities as well as proves to be, neuroprotective compound (Wang *et al.*, 2022). It exerts multiple neuroprotective actions in several neuronal disorders by inhibiting neuro-inflammation and ROS production (Mira *et al.*, 2015; Gul *et al.*, 2016; Wang *et al.*, 2017; Yao *et al.*, 2019). P-coumaric acid has antioxidant, antimicrobial, anti-tumor, and neuroprotective effects (Zaman *et al.*, 2023; Chen *et al.*, 2024). Ferulic acid has reported to contain antioxidant, anti-inflammation activities (Alam *et al.*, 2013; Chowdhury *et al.*, 2019) and pivotal in treating testicular damage (Kassab *et al.*, 2020), and Alzheimer's disease (Montaser *et al.*, 2019). Gallic acid possesses antioxidant, anti-inflammatory activity (Xu *et al.*, 2021) and responsible in treatment of various neurological disorders, and neuroinflammation (Bhuia *et al.*, 2023).

Changes in body weight is a common indication of behavioral changes due to any environmental, physical, or mental stress. Our findings revealed the prominent decline in body weights of male Sprague Dawley rats which were orally exposed to 3000ppm 1,4-dioxane. As it was decreased to  $192.8 \pm 1.92$  from  $212.0 \pm 1.58$  in G1 within 2 weeks of 1,4-dioxane administration and at 60<sup>th</sup> day after trial completion it was recorded about  $130.2 \pm 2.58$  which was in accordance with Wang *et al.* (2024) who determined the decline in body weights of 1,4-dioxane treated male and female mice @ 5000ppm after 7 days to 3months. 20% decline in body weights of male and female rats administered with 5000ppm 1,4-dioxane was also reported with elevated presence of malignant tumors in females (Kano *et al.*, 2009). While the treatment with *Ziziphus mauritiana* 's leaves ethanolic extract has clearly maintained the body weights in G4 close to the control group. Our findings concur with previous literature by Ramar *et al.* (2022) who found similar body weight between control and *Ziziphus mauritiana* treated groups of male and female rats. Our results were also comparable with Mehdi *et al.* (2020) who reported prominent improvement in body weight of rats treated with 500mg/kg of *Z. mauritiana* leaves extract against standard amoebic drug.

Oxidative stress also has a pivotal role in several pathologies, diseases and tissue damage (Aboubakr *et al.*, 2023, Elsayed *et al.*, 2024, Soliman *et al.*, 2024) as well as in development of Alzheimer 's disease and brain aging (Ionescu-Tucker and Cotman, 2021) CNS is widely

exposed to oxidative stress as it consumes 20% of body's oxygen (Bonda *et al.*, 2014). Neuronal cells are highly vulnerable to ischemic or metabolic damage and associated oxidative stress (Jelinek *et al.*, 2012). Oxidative stress is responsible for male infertility as it determines fertility parameters by inducing oxidative damage to reproductive cells and intracellular components. Oxidative stress cause testicular damage in male Sprague Dawley rats and also induced apoptosis, inflammation, oxidative DNA damage and autophagy in testis (Cakmak *et al.*, 2023). Our results indicated the induction of oxidative stress in brain and testis of rats from G1 group as elevation in malondialdehyde (MDA) and nitric oxide (NO) and reduction in superoxide dismutase (SOD) and glutathione (GSH) were measured as a clear sign of disturbance in normal functioning. These results were in accordance with Noaman *et al.* (2005) who observed the elevation in malondialdehyde (MDA) only after 24hr, and prominent decrease in glutathione (GSH) after 1hr in 1,4-dioxane treated group. Chen *et al.* (2022) also proved that 1,4-dioxane administration evidently induced oxidative stress in mouse models. Our findings suggested that *Z. mauritiana* 's leaves extract substantially reversed the oxidative stress in other treated groups by reducing malondialdehyde (MDA) and nitric oxide (NO) and increasing superoxide dismutase (SOD) and glutathione (GSH). G4 has shown maximum restoration of these biomarkers close to control group values. All phenolic acids detected in leaves extracts are highly effective against oxidative stress as Quercetin is well-known for its free radical scavenging and transition metal ion binding ability. Our results exhibited similarities with findings of Dutta *et al.* (2018) who determined reduction in NO level and elevation in GSH level after *Z. mauritiana* administration against silica induced toxicity in Wister rats. Khanam *et al.* (2025) detected strong antioxidant activity of *Z. mauritiana* 's leaves than fruit extracts and proved its potential against oxidative stress. Alsuwat (2025) has also determined the antioxidant capacity of *Z. mauritiana* against free radicals.

Brain functioning can be damaged through disruption in neurotransmitters and enzymes mechanisms. Acetylcholine-esterase (AChE) is an important enzyme for brain functioning. Elevated AChE induces inhibition of Acetylcholine level which could cause declined cholinergic function, neuromuscular strength, depression and could further induce AD (Alzheimer's disease). Our results revealed that 1,4-dioxane administration demonstrated a prominent increase in AChE activity in rats. Our findings considered novel as effect of 1,4-dioxane on AChE has not been reported yet in literature. But, being a classified endocrine disruptor and evidently oxidative stress inducer, 1,4-dioxane pointed out all reasons for disturbance in AChE activity. Histological alterations also visibly indicated the disturbance in neuronal functions. *Z. mauritiana* 's leaves extract has visibly balanced AChE activity in all treated groups and improved AChE in G4 close to normal values as per in control group. *Z. mauritiana* 's leaves extract administration improved the cholinergic fuctions and decreased the chances of AD (Alzheimer's disease) induction in rats. Results were comparable with Kausher (2024) who reported significant decline in AChE levels in Swiss albino mice treated with *Z. mauritiana* extract at 200 and 400mg/kg. All detected phenolic acids possess

neuroprotective ability as well as anti-depressant activity which also proved *Z. mauritiana* 's potential to balance the AchE level. Quercetin possesses strong inhibitory activity against acetylcholinesterase (Choi *et al.*, 2012). Chlorogenic acid exhibits strong inhibitory activity against acetylcholinesterase (AChE) activity in rat brains, proving its potential against cognitive impairments (Orhan *et al.*, 2004; Oboh *et al.*, 2013).

FSH and LH are both equally crucial hormones for male fertility and reproductive health. LH (luteinizing hormone) stimulates Leydig cells for testosterone production while FSH (follicle stimulating hormone) aids Sertoli cells for spermatogenesis and testosterone functioning for sperm production. 1,4-dioxane (3000ppm) administration severely reduced LH and FSH level in G1 which also indicated disturbance in pituitary gland brain functioning. Our findings were in accordance with Hashim *et al.* (2018) who reported a severe decline in testosterone level after 20<sup>th</sup> and 30<sup>th</sup> days of 1,4-dioxane administrations in rabbits. As testosterone decreased, spermatogenesis and sperm production have also deteriorated as our findings clearly indicated declined sperm count in G1. 1,4-dioxane classified as an endocrine disrupting chemical (USEPA, 2013; IARC, 1999; ATSDR, 2017). Qiu *et al.* (2019) detected endocrine disruption induced by 1,4-dioxane in mice. *Z. mauritiana* 's leaves extract visibly improved hormonal balance by elevating LH, FSH, and testosterone level as well as sperm count in treated rats. 360mg/kg *Z. mauritiana* 's leaves extract dose was most significant among other groups. Elevation in testosterone, LH, and FSH levels accounted for reversal of testicular damage and normal sperm production. Previously Sukirti *et al.* (2013) reported substantial increase in testosterone level, sperm count and fertility in male Wistar albino rats treated with *Z. mauritiana*. *Z. mauritiana* has also been reported previously for treating male infertility (Dubey and Dubey, 2011; Diatta *et al.*, 2020).

Histological alterations further emphasized on inimical effects of 1,4-dioxane exposure to organs. Brain sections revealed severe damage to neurons, vacuole formation, hemorrhage and edema. Vacuolation and damaged neurons were clear indication of induction of toxicity, cell death and deteriorated brain functioning. Edema has depicted the possibility of brain tumor. Testicular structure was also visibly damaged after 1,4-dioxane exposure. Distorted testicular structure, damaged seminiferous tubules, congestion and damage of Sertoli cells, vacuolation, hemorrhage, and germinal epithelium detachment were evidently visible in treated groups. Damaged Seminiferous tubules and Sertoli cells has evidently indicated the cause of low sperm count and disturbed hormonal profile after 1,4-dioxane exposure. Germinal epithelium detachment indicated the distortion in sperm morphology and quality, as well as indicated the possible infertility induced by 1,4-dioxane. Comparable histological alterations by 1,4-dioxane exposure have been reviewed previously by multiple studies (Kano *et al.*, 2008; Hashim *et al.* 2018; Aziz *et al.*, 2021). Histological preservation ability of *Z. mauritiana* has been reported several times in literature. *Z. mauritiana* 's leaves extract exhibited prominent improvement in histological damage at different doses. *Z. mauritiana* 's leaves extract at 360mg/kg exhibited maximum improvement in brain and

testis's histology and substantially reversed the damage close to normal structures and preserve organ 's normal functioning. Our results showed similarities with previous findings as Owolarafe *et al.* (2018) proved that *Z. mauritiana* has maintained normal histological structure of brain and testis. Mohebbati *et al.* (2021) evaluated that *Z. mauritiana* administration may significantly heal the damaged testicular tissues.

**Conclusions:** In recent times, with enhanced exposure to several hazardous chemicals, medicinal plants have a pivotal role in coping up with health risks. 1,4-dioxane emerged as a serious concern not only to humans because of its extensive utilization but also harmful to livestock, wildlife and other animals as during assessment of environmental and toxicological impact, it has proved precarious for both animal welfare and public health. Current work elucidates the 1,4-dioxane induced oxidative stress, neuronal and reproductive damage as well as protective potential of *Ziziphus mauritiana* 's leaves extract to prevent and attenuate the damage caused by 1,4-dioxane exposure. *Z. mauritiana* 's leaves contain such phytochemicals which are crucial for protective activity against this solvent. Significant improvements in biochemical, and histological, neuronal and reproductive parameters were detected in *Z. mauritiana* 's leaves extract treated groups. This study ascertained the *Z. mauritiana* as a natural remedy for protection against deleterious effects of environmental toxins such as 1,4-dioxane.

**Author's Contribution:** Salma Sultana and Farhat Jabeen designed the study. Mahpara Gilani collected the plant material and chemicals, analyzed the sample and initially prepared the manuscript. Tayyaba Sultana helped out in rephrasing the manuscript.

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