Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.234

RESEARCH ARTICLE

Annona squamosa: A Promising Protective Approach for Mitigation of 1,4-Dioxane Induced Neurotoxicity in Male Sprague Dawley Rats

Mahpara Gilani[1], Salma Sultana*[1], Tayyaba Sultana[1] and Farhat Jabeen[1]

[1]Department of Zoology, Government College University Faisalabad, Pakistan
*Corresponding author: drsalmasultana@gcuf.edu.pk

ARTICLE HISTORY (25-411)

Received: May 05, 2025
Revised: July 29, 2025
Accepted: August 10, 2025
Published online: September 09, 2025

Key words:
A. squamosa
1,4-dioxane
Oxidative stress
Carcinogenic
Industrial hazards
Histological alterations.

ABSTRACT

1,4-dioxane (diethylene oxide) is a common industrial solvent which is extensively used in daily life. Being such common cyclic ether, it has emerged as serious water contaminant damaging human health at different levels. It has been classified as carcinogenic by Environmental Protection Agency. In this study, *Annona squamosa* (Custard Apple) leaves extract was used to investigate its role in mitigating the 1,4-dioxane induced toxicity. Twenty-five adult male Sprague Dawley rats were allocated into five groups; C (control group), and co-treated groups i.e. G1 (3000ppm 1,4-dioxane treated group), G2 (3000ppm 1,4-dioxane + 200mg/kg *A. squamosa* treated group), G3 (3000ppm 1,4-dioxane+ 400mg/kg *A. squamosa* treated group) and G4 (3000ppm 1,4-dioxane+ 600mg/kg *A. squamosa* treated group) in triplicates which were gavaged orally for 60 days. At the end of experiment, organs were collected to analyze the parameters (AchE level, somatic index brain, brain accumulation, cell viability), and oxidative stress markers (malondialdehyde, nitric oxide, superoxide dismutase, glutathione), and histological alteration. G1 exhibited the decrease in body weight, elevation in AchE activity, and oxidative stress. Comparatively, *A. squamosa* ethanolic leaves extract treated rats exhibited prominent improvement in all these parameters. Most significant improvement had been exhibited in G4 group establishing the protective effect of *A. squamosa* leaves extract in attenuating the toxicological damages and restoring the histological structure. Results have proved that *A. squamosa* leaves extract possess therapeutic as well as preventive capability against toxicity induced by 1,4-dioxane.

To Cite This Article: Gilani M, Sultana S, Sultana T and Jabeen F 2025. Annona squamosa: A promising protective approach for mitigation of 1,4-dioxane induced neurotoxicity in male sprague dawley rats. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2025.234

INTRODUCTION

Several neurological disorders are emerging due to occupational and environmental exposure to chemicals (Brown *et al*., 2005; Pearce *et al*., 2014). Organic solvents which are particularly used in car repair, furniture manufacturing, painting, and cleaning industries have been allied with various central nervous system (CNS) disorders. 1,4-dioxane is one such cyclic ether which is extensively utilized as industrial solvent, as a stabilizer in chlorinated solvents as well as in the manufacture of pharmaceuticals, veterinary drugs, and natural health products. The most significant source of 1,4-dioxane exposure to the general population is ingestion of contaminated drinking water. It is highly water-soluble, mobile in groundwater, and resistant to natural degradation, which allows it to persist in aquifers and municipal water supplies (Zenker *et al*., 2003; Adamson *et al*., 2014). Sites near industrial discharges, landfills, or wastewater treatment plants are particularly vulnerable (Mohr, 2020). 1,4-dioxane is also found as a contaminant in personal care and cleaning products due to its unintended formation during the ethoxylation process. 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). Dehydration, faintness, depression of CNS, headache, drowsiness, vomiting, skin cracking, irritation of skin and respiratory tract are possible signs of frequent contact to 1,4-dioxane. Even non-toxic concentrations of 1,4-dioxane (0.1, 0.25, 0.5, or 1%) visibly elevates oxidative stress, and abnormal phenotypic variations in *Drosophila melanogaster* (Turna Demir, 2022). In neurodegenerative diseases, ubiquitous role of oxidative stress is gaining momentum. Neuronal degeneration in the CNS is associated with oxidative damage to all bio-macromolecule types (Trofin *et al*., 2025).

In modern times, natural ingredients are of great importance for survival and betterment of humans and animals (Ahirwar *et al*., 2023). Recently, there has been rise in interest in exploration of pharmacological impact of herbal plants by utilizing different approaches (Lena *et al*., 2023). *Annona squamosa* (family: Annonaceae) known as sugar apple, custard apple, and sharifa is a tropical plant with substantial medicinal values rooted in traditional medicine systems globally. According to Dilworth *et al*. (2023), *A. squamosa* proved to be effective protective agent against oxidative damage in a time- and extract-concentration-dependent manner. Studies have shown that ethanolic extract of *A. squamosa* leaves possess flavonoids like hyperoside and rutin which have biological characteristics related to antioxidant mechanism (Kiranmayi *et al*., 2020). *A. squamosa* leaves proved beneficial for neuron protection based on experiment led by Somasekhar *et al*. (2020). Its leaves comprise various bioactive compounds such as flavonoids, alkaloids, saponins, tannins, phytosterols, phenolics and mainly quercetin and eugenol, all these can exhibit therapeutic properties. Sprague Dawley rats are an extensively recognized rodent model in neuro-toxicological research because of their genetic stability, well-characterized behavioral profiles, and close resemblance to human and animal neurophysiology. Selection of male rats is particularly important in such studies to reduce variability associated with the estrous cycle in females. Male Sprague Dawley rats exhibit dose- and time-dependent impairments following exposure to neurotoxic agents like manganese, reinforcing their validity for modeling different neurodegenerative and cognitive conditions (Ali *et al*., 2016). Therefore, male Sprague Dawley rats provide a robust, reproducible, and translationally relevant model for studying the neurotoxic effects of environmental or pharmaceutical agents. *A. squamosa* has never been used before against 1,4-dioxane. Focus of the present study is to evaluate the potential of *A. squamosa* 's leaves to recover the 1,4-dioxane induced neurotoxicity in male Sprague Dawley rats.

MATERIALS AND METHODS

Plant Collection and Extract preparations: *A. squamosa* leaves were collected locally from Karachi, Pakistan in the month of October. The identification was verified by contemplating the authentic sample at University of Agriculture, Faisalabad, Pakistan (Specimen Voucher No. 315/21/04). After washing leaves thoroughly with fresh water to wipe off all inessential particles, they were desiccated at room temperature (20-25°C), pounded through mechanical crusher and sieved through mesh-40. Through Soxhlet's apparatus, 95% ethanolic extract was acquired from powder material. Extract was concerted under reduced pressure and semi-solid mass was further utilized.

Quantitative analysis of Ethanolic leaves extract of *A. squamosa*: Phenols, tannins and, flavonoids in *A. squamosa* leaves extract were assessed through standard protocols and their detailed concentrations are mentioned in table 1.

Table 1: Qualitative analysis of *A. squamosa* leaves extract

Compounds	Concentrations
Phenols	75.83±0.53
Tannins	47.00±0.03
Flavonoids	22.61±0.03

Total Phenolic Evaluation: Calorimetric methods were used to measure total phenolic contents of plant extract. Reaction mixture contained 0.5ml plant extract, 0.2mM $NaHCO_3$ 0.6ml and 0.2ml Folin Ciocalteu 's reagent. Incubation was done for 120min and then absorbance was noted at 765nm. Equivalent mass (mg/100g) was measured from standard solution of gallic Acid at range from 50-350mg/ml after standard preparation.

Determination of Tannins: Folin Ciocalteu method was utilized to evaluate tannin content. Plant extract was added to 750µl distilled water along with 500µl Folin Ciocalteu reagent and 35% 1000µl sodium carbonate. Mixture was diluted with 10ml of water, shaken well, incubated for 30min at room temperature and measured at 725nm. Total tannins content was calculated as TA/g dry matter, from a standard curve (50 - 100 mg/ TA) and absorbance was calculated at 700nm.

Determination of Total Flavonoids: Colorimetric method was used to determine the total flavonoids. Plant sample was extracted with methanol separately at room temperature. After that, filtration of sample through Whatman filter paper was done and filtrate was poured on the crucible to dry. Then 2% $AlCl_3$ was added in ethanol to dissolve plant extracts (ratio 1:1). After that, at room temperature, incubation was done for 1hour and absorbance was measured at 420nm through spectrophotometer (Unico Spectro Quest Model SQ2802).

Proximate analysis: Proximate analysis was conducted according to standard protocols of AOAC (2012). These protocols are mentioned briefly below (details in Table 2).

Table 2. Proximate analysis of *A. squamosa* leaves

Parameters	Concentrations
Ash content	7.50%
Fat content	3.18%
Moisture content	59.32%
Crude Protein	3.05%
Crude Fiber	21.01%

Ash content: Organic components of plant samples were burned through furnace (Nabertherm–L3) to determine the ash content. 2g plant sample was taken into pre weighed crucible and places in furnace at 450^0C for 12 hours until white ash was obtained. The ash contents were calculated by the following formula:

$$Ash\% = \frac{Weight\ of\ ash}{Weight\ of\ sample} \times 100$$

Crude Fat: Soxhlet apparatus (Selecta-0481090) was utilized to obtain the total crude fat from plant samples (i.e. Petroleum ether). Adequate petroleum ether was placed into a pre-oven dried flask (overnight at 60ºC). On the contrary, plant samples were put into thimble. Then extractor and flask was fixed into heating mantle and condenser. Flask was heated till the solvent boiled and this

extraction procedure continued for six hours before removal. At last, residual solvent was dried overnight at 60ºC and stored in desiccators to cool before weighing. Formula for fat content evaluation is:

Crude Fat (%) = (Weight of extracted fat / Weight of original sample) x 100

Moisture content: 10g of fresh plant sample was taken into a hot airtight oven (Model: DO-1-30/02, PCSIR, Pakistan) at 67^0C to 70^0C for 24 hours to determine moisture content. Moisture content evaluation formula is:

$$Moisture\% = \frac{Loss\ in\ weight\ of\ sample}{Weight\ of\ sample} \times 100$$

Crude Protein: Kjeldahl apparatus (model No: 808132) was utilized for crude protein evaluation while nitrogen percentage was estimated following protocol described by AOAC (2012) which was then used in the following formula for estimation of crude protein.

$$\%\ Crue\ Protein = 6.25 \times \%\ N$$

$$\%N\ \frac{(S-B) \times 0.014 \times D \times 100}{Weight\ of\ sample \times V}$$

Crude fiber: Acid digestion was done to evaluate crude fiber. Fat was removed from 1g of dried sample through soxhlet, which further digested separately in 1.26% NaOH and H_2SO_4. Then for 24 hours, samples were shifted to petri dishes and kept in oven at 105°C. Then sample was ignited at 600°C in muffle furnace after washing with distilled water to attain ash, which was utilized to evaluate crude fiber (Satti *et al*., 2024).

Ethical Approval: Prior to initializing the trial, Animal Ethical Committee (AEC) / Ethics Review Committee of Government College University Faisalabad approved the experiment (Ref no. GCUF/ERC/21/01A).

Animals: Twenty-five mature male Sprague Dawley rats 6-8 weeks old of weight 200-210g were acquired from Government College University Faisalabad for this experiment. Whole trial was carried out according to standard recommendations and appropriate protocols. Rats were placed in steel cages and acclimatized at room temperature (20-23°C) with 12:12h light: dark cycles in approximately 45-60% humidity as per standard laboratory rules.

Experimental Design: 1,4-dioxane (CAS NO 123-91-1 anhydrous, 99.8%) was purchased from Sigma Aldrich®. Rats were allocated into five groups randomly with each group containing five rats. Groups of male Sprague Dawley rats according to doses and treatments.

Groups	Treatment
C	Control (no treatment)
G1	3000ppm (1,4-dioxane)
G2	3000ppm 1,4-dioxane + 200mg/kg *A. squamosa*
G3	3000ppm 1,4-dioxane + 400mg/kg *A. squamosa*
G4	3000ppm 1,4-dioxane + 600mg/kg *A. squamosa*

Commercial rodent feed having 19% crude protein (feed formulation is given in Table 3) and distilled water were supplied to all groups *ad libitum*. Rats were given the defined doses through oral gavage for 60 days on alternating day (Hassan *et al*., 2023). Experimental design is shown in Fig. 1.

Table 3: Components of diet provided to male Sprague Dawley rats.

Components	Concentration (g/kg)
Corn starch dextrinizade	132
Carbohydrate	629.5
Casein	200
L-Cysteine	3
Choline bitartrate	2.5
Fiber	50
Protein	205.5
Soybean Oil	70
Amido	397.5
Sucrose	100
Total Fats	70
Mineral mix	35
Vitamin mix	10
Energy content	3.97 Kcal/g
Mycotoxin sequestrants	0.05

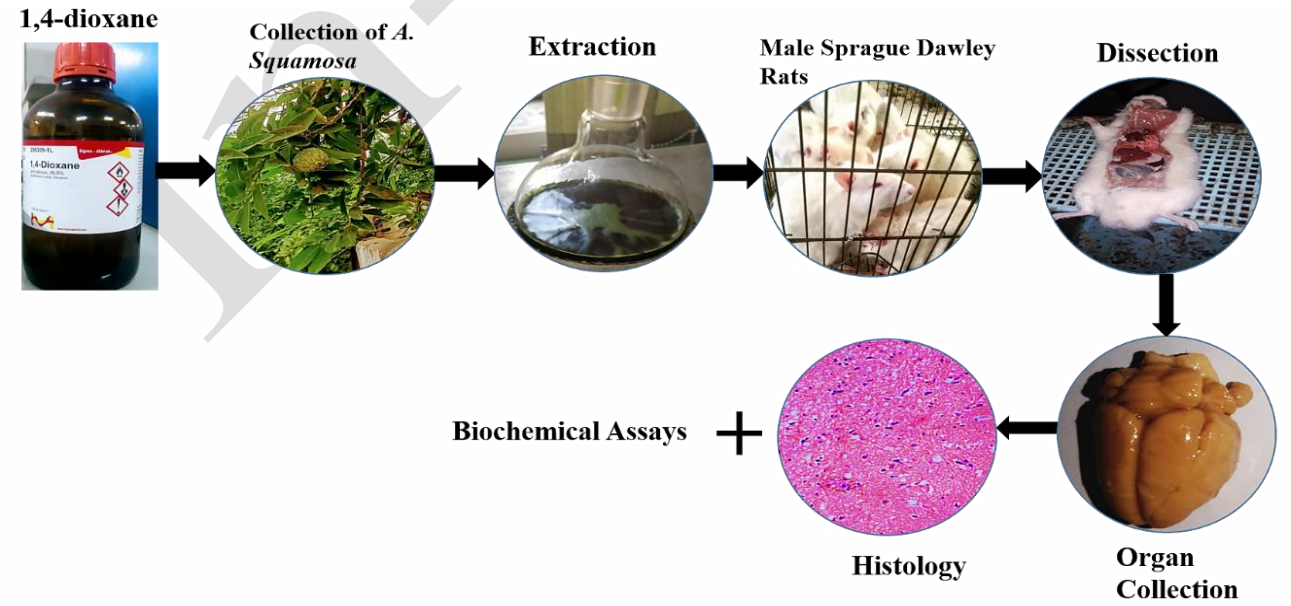

Fig. 1: Flow Diagram depicting Experimental Design.

Animal body weight: Weights of all rats were calculated on weighing balance before starting the trial. Proper feed and water were provided on regular basis. The selected dose of 1,4-Dioxane+*A. squamosa* were administered through oral gavage. Body weight was measured every 15 days throughout the trial. Body weight of all groups were compared thoroughly till the completion of trial and analyzed statistically.

Sample Collection: In fixative sera (formaldehyde: 30ml, ethanol: 60ml and glacial acetic acid: 10ml), small brain 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 in-filtering 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).

Somatic index of organs: At the end of experiment, individual weight of Sprague Dawley rats and weight of brain was noted to figure their brain-somatic index. The somatic index of brain was evaluated by formula (Pandit *et al*., 2019) given below:

SI = Weight of brain of SD rat/Body weight of SD rat x 100

1,4-Dioxane Accumulation: 100mg sample from each brain were digested in digesting solution (1.5ml perchloric acid and 3ml nitric acid) in heating digester (Velp Scientifica- D-6). Fumes were yellow in beginning and digestion was sustained till fumes became colorless and 1ml solution was left behind. Then it was filtered after adding distilled water to make 10ml solution. The samples were analyzed by Coupled Plasma Mass Spectrometry (Perkinelmer) (Vasantharaja and Ramalinggam, 2018)

Cell Viability: 0.2mg samples from brain were selected and homogenized for 10 minutes in bullet blender (Advance-BBY5E-CE). Eppendorf tubes were used for supernatants collection. 96 well plates were tagged with sample name and control. Then in sample tags wells of 96 well plate 100µl sample was transferred with micropipette excluding control tag wells and 20µl assay solution was also added in each well through micropipette. To gently blend, the reagent plate was shaken for 30min and incubated for 2hr at 37ºC in 5% CO_2 incubator (MCO-18AIC). After that sample bearing plate was removed from incubator and rinsed. Absorbance was measured on micro-plate reader at 570nm and 605nm ratio, Absorbance was measured on micro-plate reader (BioBase-EL10A) (Yu *et al*., 2013).

Cell viability percentage was calculated by following formula:
% Cell viability = $100 \times (R_s-R_o)/(R_c-R_o)$

R_s is the absorbance ratio of OD570/OD605 in the presence of the test compound. R_c is the absorbance ratio of OD570/OD605 in the absence of the test compound (vehicle control). R_o is the average background (non-cell control) absorbance ratio of OD570/OD605.

Biochemical Analysis: Using commercially available-ready to use kits (Nos1 ELISA kits); the following parameters were determined:

- Brain nitric oxide (MBS2884948) using a spectrophotometer.
- Brain malondialdehyde (MBS263626) using a spectrophotometer.
- Brain reduced glutathione (MBS724319) using a spectrophotometer.
- Superoxide dismutase (MBS036924) activity.
- Serum acetylcholinesterase (MBS283109) using a spectrophotometer (Hendawy *et al*., 2019).

Histopathological examination: In fixative sera (formaldehyde: 30ml, ethanol: 60ml and glacial acetic acid: 10ml), small brain sections were fixed for 48h. Dehydration was done through graded series of ethanol (45min per step), then embedding was completed after clearing it through two changes of xylene and in filtering 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 haematoxylin and eosin was done to analyze them under microscope for further studies (Vasantharaja and Ramalingam, 2018).

Statistical Analysis: The results were statistically analyzed using SPSS Statistics 22 software. 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

Qualitative analysis: Results in Table 1 revealed the significant ($P<0.05$) concentrations of phenols, tannins, and flavonoids present in *A. squamosa* leaves extract.

Proximate analysis: Results suggested that significant ($P<0.05$) concentrations of ash (7.50%), fat (3.18%), moisture (59.32%), crude protein (3.05%) and crude fiber (21.01%) were present in *A. squamosa* leaves extract proving extract's quality and suitability for further use ($p<0.05$) (Table 2).

Body Weight: Table 4 exhibits body weight of rats administered with 1,4-dioxane and low to high concentration of *A. squamosa*. The results indicated that considerable ($P<0.05$) changes occurred in the body weight of Sprague Dawley rats for 60 days. As at start body weight was in accordance with control and co-treated groups. On the 15th day, body weight in G1 had started to severely decline due to 1,4-dioxane administration. On the contrary,

A. squamosa treated groups exhibited visible improvement in body weight as G4 (262.6±2.07) > G3 (242.2±1.92) > G2 (221.4±1.14). At 60th day, there was significant reduction in G1 body weight (130±1.58), while significantly elevation was observed in G4 (316.6±3.04) > G3 (303.4±2.40) > G2(292.8±1.92) groups treated with *A. squmosa* doses 600, 400, and 200mg/kg respectively. G4 exhibited body weight significantly (P<0.05) closer to control.

Table 4: Comparison of body weight of male Sprague Dawley rats supplemented with 1,4-dioxane and *A. squamosa* leaves 's extract

Days	C	G1	G2	G3	G4	*P-value*
0 Day	209.2± 1.09^{ab}	210.6± 0.54^{a}	209.8± 0.44^{ab}	209.6± 0.54^{ab}	210± 0.70^{ab}	0.047**
15 Days	220.8± 0.83^{c}	189± 0.70^{d}	221.4± 1.14^{c}	242.2± 1.92^{b}	262.6± 2.07^{a}	0.000***
30 Days	253.6± 0.54^{c}	173± 2.73^{d}	250± 2.23^{c}	265.2± 3.96^{b}	282± 1.58^{a}	0.000***
45 Days	277.6± 0.54^{c}	151.4± 0.54^{e}	273± 1.58^{d}	283.6± 3.04^{b}	298.6± 2.30^{a}	0.000***
60 Days	329.8± 1.92^{a}	130± 1.58^{e}	292.8± 1.92^{d}	303.4± 2.40^{c}	316.6± 3.04^{b}	0.000***

C=Control; G1= 1,4-dioxane (3000ppm); G2= 200mg/kg; G3=400mg/kg; and G4= 600mg/kg of *A. squamosa* leaves 's extract; means values varies significantly(P<0.05) in columns among different groups.

Somatic Index of Organs: Table 5 represents brain somatic index of rats administered with 1,4-dioxane and low to high concentration of *A. squamosa*. According to the results, there was prominent variations in brain somatic index. Conclusively, G1 exhibited drastic variation to control as (0.778±0.019) and there was significant (P<0.05) improvement in G4 (0.892±0.022) > G3 (0.848±0.029) > G2 (0.816±0.011).

Table 5: Comparison of Somatic Index of Brain in male Sprague Dawley rats supplemented with 1,4-dioxane and *A. squamosa* leaves 's extract

Groups	Somatic index brain	*P- value*
C	0.928±0.008^{a}	
G1	0.778±0.019^{c}	
G2	0.816±0.011^{b}	
G3	0.848±0.029^{b}	
G4	0.892±0.022^{a}	0.001***

C=Control; G1= 1,4-dioxane (3000ppm); G2= 200mg/kg; G3=400mg/kg; and G4= 600mg/kg of *A. squamosa* leaves 's extract; means values varies significantly(P<0.05) in rows among different groups.

Accumulation of 1,4-dioxane: Table 6 represents accumulation of 1,4-dioxane and effects of *A. squamosa* leaves extract against 1,4-dioxane accumulation in brain of Sprague Dawley rats. The 1,4-dioxane exposure induced severe damage to brain. 1,4-dioxane accumulation was calculated and results showed highest accumulation in G1 (6.676±0.07) while least accumulation was detected in G4 (1.89±0.01). *A. squamosa* leaves extract administration significantly (P<0.05) improved damage as G4 (1.89±0.01) > G3 (4.83±0.05) > G2 (5.95±0.04). G4 exhibited results closer to control.

Cell Viability: Cell viability was analyzed in brain and least significant values were detected in G1 (39.532±1.06) and it significantly improved in G4 (66.452±1.48) in comparison to other treated groups. *A. squamosa* leaves extract administration significantly (P <0.05) improved damage as G4 (66.452±1.48) > G3 (56.17±0.39) > G2 (43.80±0.69). Detailed results are described in Table 7.

Table 6: Comparison of 1,4-dioxane accumulation in male Sprague Dawley rats supplemented with 1,4-dioxane and *A. squamosa* leaves 's extract

Groups	Brain accumulation	
C	0.011±0.00^{e}	0.001***
G1	6.67±0.07^{a}	
G2	5.95±0.04^{b}	
G3	4.83±0.05^{c}	
G4	1.89±0.01^{d}	

C=Control; G1= 1,4-dioxane (3000ppm); G2= 200mg/kg; G3=400mg/kg; and G4= 600mg/kg of *A. squamosa* leaves 's extract; means values varies significantly(P<0.05) in rows among different groups.

Table 7: Comparison of cell viability % of male Sprague Dawley rats 's brain supplemented with 1,4-dioxane and *A. squamosa* leaves 's extract

Groups	Cell viability	P-value
C	76.98±0.49^{a}	0.001***
G1	39.53±1.06^{e}	
G2	43.80±0.69^{d}	
G3	56.17±0.39^{c}	
G4	66.45±1.48^{b}	

C=Control; G1= 1,4-dioxane (3000ppm); G2= 200mg/kg; G3=400mg/kg; and G4= 600mg/kg of *A. squamosa* leaves 's extract; means values varies significantly(P<0.05) in rows among different groups.

Table 8: Comparison of Oxidative stress parameters in male Sprague Dawley rats supplemented with 1,4-dioxane and *A. squamosa* leaves 's extract

Groups	MDA	NO	SOD	GHS	*P-value*
C	54.02±0.12^{e}	6.92±0.04^{e}	6.99±0.06^{a}	4.59±0.03^{a}	
G1	129.8±0.74^{a}	14.01±0.07^{a}	3.69±0.04^{e}	2.11±0.02^{e}	
G2	110.1±0.93^{b}	12.43±0.23^{b}	4.01±0.03^{d}	2.73±0.06^{d}	0.001***
G3	88.36±0.60^{c}	10.49±0.11^{c}	5.30±0.07^{c}	3.14±0.03^{c}	
G4	62.61±0.61^{d}	8.68±0.09^{d}	6.05±0.09^{b}	4.09±0.04^{b}	

C=Control; G1= 1,4-dioxane (3000ppm); G2= 200mg/kg; G3=400mg/kg; and G4= 600mg/kg of *A. squamosa* leaves 's extract; means values varies significantly(P<0.05) in rows among different groups.

Oxidative Stress Parameters: Oxidative stress was measured through analyzing parameters such as brain malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), and glutathione (GSH) through spectrometer and maximum oxidative stress was observed in G1 (1,4-dioxane administered group). Increase in malondialdehyde (MDA) and nitric oxide (NO) was observed up to 129.8±0.74, and 14.01±0.07 respectively while decrease in superoxide dismutase (SOD) and glutathione (GSH) was 3.69±0.04, and 2.11±0.02 respectively in G1 while G4 proved to be statistically more significant (P<0.05) among others. *A. squamosa* leaves extract administration significantly (P<0.05) reversed the damage as G4 (MDA: 62.61±0.61, NO: 8.68±0.09, SOD: 6.05±0.09, GSH: 4.09±0.04) > G3 (MDA: 88.36±0.60, NO: 10.49±0.11, SOD: 5.30±0.07, GSH: 3.14±0.03) > G2 (MDA: 110.1±0.93, NO: 12.43±0.23, SOD: 4.01±0.03, GSH: 2.73±0.06). Table 8 represents detailed results.

Table 9: Comparison of Acetylcholine esterase activity in male Sprague Dawley rats supplemented with 1,4-dioxane and *A. squamosa* leaves 's extract

Groups	Acetylcholine esterase (mU/mg)	*P-value*
C	0.772±0.014^{e}	0.001***
G1	2.106±0.019^{a}	
G2	1.75±0.015^{b}	
G3	1.166±0.020^{c}	
G4	0.868±0.019^{d}	

C=Control; G1= 1,4-dioxane (3000ppm); G2= 200mg/kg; G3=400mg/kg; and G4= 600mg/kg of *A. squamosa* leaves 's extract; means values varies significantly(P<0.05) in rows among different groups.

Acetylcholine esterase activity: Acetylcholine esterase level significantly increased in G1 (3000ppm) group. Though *A. squamosa* leaves extract administration significantly (P<0.05) balanced the AchE level in other treated groups as G4 exhibited highest improvement (0.868±0.019) > G3 (1.166±0.020) > G2 (1.75±0.015). Detailed results are described in Table 9.

Histological Profiling of Brain: Histological study clearly indicated deleterious effects of 1,4-dioxane on brain in G1 (3000ppm 1,4-dioxane treated group) as well as proved the protective effect of *A. squamosa* in other treated groups as shown in Fig. 2. Photomicrographs of brain of control group exhibited normal structure of cerebral cortex. Treated groups exhibited dose dependent abnormalities such as hemorrhage with asymmetrical pyramidal Neurons (APN) was clearly visible in G1 which was treated with 3000ppm 1,4-dioxane, while low dose of *A. squamosa* (200mg/kg) co-administered with 3000ppm 1,4-dioxane mildly controlled the damage as less hemorrhage and subdural hematoma was visible in G2. G3 showed significant recovery with medium dose of *A. squamosa* (400mg/kg) co-administered with 3000ppm 1,4-dioxane as compared to G2 by minimizing the pyramidal neurons degeneration. G4 with high dose of *A. squamosa* (600mg/kg) co-administered with 3000ppm 1,4-dioxane proved to be highly effective in terms of recovering the histopathological damage approximately closer to control by reducing the degeneration of pyramidal neurons and maintaining the normal glial cells shape. Hence this dose was most significant (P <0.05) as compared to low and medium doses.

The observed neuroprotective effects of *A. squamosa* leaves extract in 1,4-dioxane-exposed male Sprague Dawley rats could be attributed to several plausible molecular and cellular mechanisms, which collectively work to mitigate the toxic insult (Fig. 3).

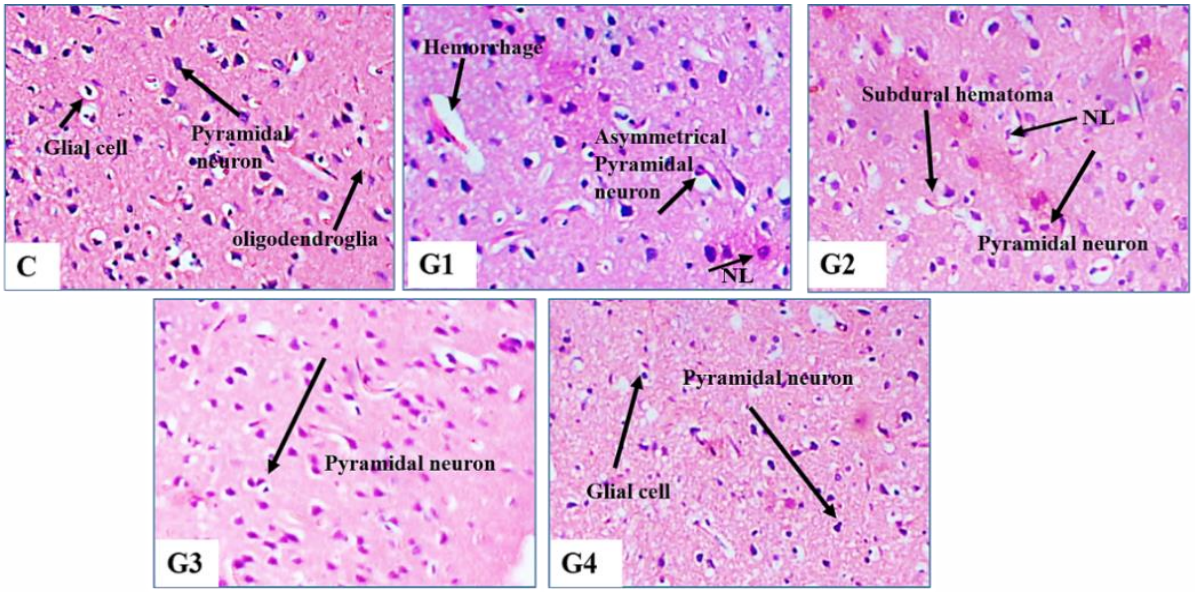

Fig. 2: Photomicrograph depicting Rat's Brain histopathology (Cerebral Cortex section) with HandE × 400X. (C) Normal histological (G1) showing hemorrhage and asymmetrical pyramidal neurons (APN), and neoplastic lesions (NL) in (3000ppm 1,4-dioxane), (G2) representing less degeneration, and reduced neoplastic lesions (NL) and subdural hematoma in (3000ppm 1,4-Dioxane + 200mg/kg *A. squamosa*), (G3) depicting quiet prominent recovery with more symmetrical pyramidal neurons (3000ppm 1,4-Dioxane + 400mg/kg *A. squamosa*), (G4) showing highly significant results in brain structure closer to control (3000ppm 1,4-Dioxane + 600mg/kg *A. squamosa*).

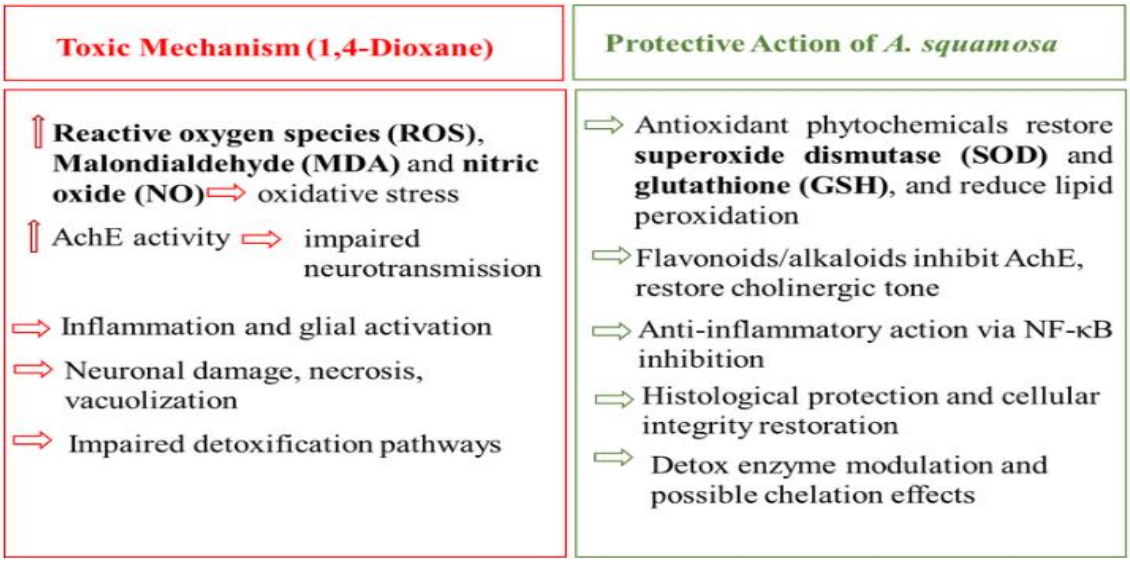

Fig. 3: Mode of action of *A. squamosa* leaves extract on 1,4 dioxane.

DISCUSSION

Organic solvents are broadly active in industry and are utilized in huge quantities across the globe. These industrial solvents are causing some serious health issues and are affecting CNS severely. Repeated and chronic exposure of these solvents can also produce reactive oxygen species, decrease neuronal plasticity, increase intracellular calcium, disturb nerve membrane integrity and induce neuro-inflammation causing long-term impairments potentially (van Thriel and Boyes, 2022). Plants have been receiving great public attention from ancient times and their role in disease treatments and solving health problems has been well documented throughout history. Many plants have some important phytochemicals which are really helpful in life saving procedures. Herbal and plant-based treatments are continuously swapping synthetic chemicals in many cultures (Baba *et al*., 2015). 1,4-dioxane is one of those chemicals which are widely used at industrial levels as well as in daily routines as in food, cosmetics, dyes and resins, and with all its uses it also becomes major water contaminant. In this study, the therapeutic potential of *A. squamosa* leaves extract was investigated against 1,4-dioxane induced neurotoxicity, oxidative stress, and histological alterations in male Sprague Dawley rats.

Our findings revealed that oral administration of 1,4-dioxane at 3000ppm triggered a sudden decline in body weight of rats as it falls within 2 weeks and at trial completion, lowest body weight was noted in G1 and it is also testified by comparing results with Kano *et al*. (2008) and Kasai *et al*. (2008) who evaluated substantial decline in body weight of male and female rats treated with 1,4-dioxane. Kano *et al*. (2009) observed 20% decrease in body weights of male and female rats treated with 5000ppm 1,4-dioxane with increased presence of malignant tumors in females. *A. squamosa* leaves extract administration improved body weight in all groups at low, medium and high doses. *A. squamosa* showed highest improvement at 600mg/kg by maintaining body weight significantly identical to control group. Present results were validated by comparing with previous study by Gupta *et al*. (2005) who treated streptozotocin-induced diabetic rats and alloxan-induced diabetic rabbits with ethanolic extract of *Annona squamosa* leaves. Kaleem *et al*. (2006) proved that *A. squamosa* with insulin for diabetic rats considerably reduced blood glucose level and increased body weight near to normal. *A. squamosa* extract administration improved the body weight of rats in all co-treated groups in a dose dependent manner and highest improvement in body weight was observed in G4.

A. squamosa exhibited positive results in treating cell proliferation in brain and reversed their cells and functions about equal to normal. Dilworth *et al*. (2023) has also revealed that 600 and 800µg/mL leaves extracts of *A. squamosa* were more effective for enhancing viability of damaged cells after 48hr of incubation.

Oxidative stress also has a crucial role in tissue damage (Soliman *et al*., 2022, Aboubakr *et al*., 2023a, Aboubakr *et al*., 2023b, Elsayed *et al*., 2024, Soliman *et al*., 2024). 1,4-dioxane administration also induced oxidative stress in rats from G1 group as increased malondialdehyde (MDA) and nitric oxide (NO) and declined Superoxide dismutase (SOD) and glutathione (GSH) were observed which clearly indicated the disturbance in brain 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 proved *A. squamosa* as an effective measure against 1,4-dioxane induced oxidative stress. Our results showed a substantial improvement in oxidative stress markers as malondialdehyde (MDA) and nitric oxide (NO) decreased after *A. squamosa* administration and glutathione (GSH) and Superoxide dismutase (SOD) increased as well. These improvements were clear signs of reversal of neuronal damage and improvement of brain functioning. Our findings align with prior studies signifying *A. squamosa* 's role in reducing oxidative stress as Dilworth *et al*. (2023) has also revealed that *A. squamosa* leaves extract has substantially increased Superoxide dismutase (SOD) and catalase activity in exposed cells after 24hr of incubation. Hendawy *et al*. (2019) indicated that *A. squamosa* leaves extract elevated SOD and GSH and notably decreased the MDA and NO levels in brain.

Acetylcholine-esterase (AchE) is a crucial enzyme for brain functioning. Increase in AchE activity can lead to decline in acetylcholine level which can further reduce cholinergic function, neuromuscular strength, and also induce Alzheimer's disease. Our findings bear novelty about the effects of 1,4-dioxane on AchE as there are no reports available on 1,4-dioxane effects on AchE in literature. But as 1,4-dioxane has classified as endocrine disruptor and oxidative stress was evidently induced which could disturb neuronal structure and activity. While, *A. squamosa* leaves extract showed stability in AchE level in other treated groups showing similarities to previous findings by Somasekhar *et al*. (2020) who proved that *A. squamosa* prominently reduced acetylcholinesterase due to presence of flavonoids, tannins and phenolic compounds. Results are consistent with prior findings that have indicated the great antioxidant ability of *A. squamosa* leaves extracts to inhibit AchE activity (Hendawy *et al*., 2019).

Histological alterations were also observed in 1,4-dioxane treated group. Brain histology exhibited hemorrhage, cell proliferation, and neuronal damage. Asymmetrical pyramidal neurons were clearly observed in G1 group histology. These alterations were in accordance with previous findings in male and female F344/DuCrj rats treated for 2 weeks with 2,960 and 2,750mg 1,4-dioxane/kg/day respectively, which exhibited vacuolar alterations in brain (JBRC, 1998). Kumar and Chadha (2025) has reported the histological variations in brain of Zebra fish exposed to 4-Bromodiphenyl Ether as vacuolization and low density of cells were observed in brain area of zebra fish larvae. (Somasekhar *et al*., 2020) suggesting that *A. squamosa* showed regeneration in brain tissues damaged by aluminum chloride in rats *A. squamosa* treated groups showed great improvement in damaged cells, neurons, maintained symmetrical pyramidal neurons and normal histological structure. 600mg/kg dose exhibited structure close to control group.

Conclusions: Current study explicates clearly the damage induced by 1,4-dioxane in male Sprague Dawley rats. Oxidative stress and AchE activity were counted as clear signs of neurotoxicity. *A. squamosa* leaves extract has significantly mitigated the toxicity induced by 1,4-dioxane, confirming its potential as a powerful measure against chemical induced toxic effects. Our study has raised serious concerns about uncontrolled exposure of 1,4-dioxane to human and animals and highlight the importance of plant based remedies against hazardous chemicals.

Credit Author Statement: Mahpara Gilani: Experimentation, Data collection, statistical analysis, writing original draft. Salma Sultana: Supervision, Conceptualization, Funding, Validation, Visualization. Tayyaba Sultana: Supervision, Reviewing, analyzing data, editing. Farhat Jabeen: Supervision, Conceptualization.

Acknowledgements: A great appreciation to Prof. Dr. Salma Sultana for her kind supervision and Dr. Shabana Naz for helping out in completion of research work and for her expert guidance for all necessary materials.

REFERENCES

Aboubakr M, Elbadawy M, Ibrahim SS, *et al*., 2023a. Allicin and lycopene possesses a protective effect against methotrexate-induced testicular toxicity in rats. Pak Vet J 43(3): 559-566

Aboubakr M, Elmahdy AM, Taima S, *et al*., 2023b. Protective effects of N acetylcysteine and vitamin E against acrylamide-induced neurotoxicity in rats. Pak Vet J 43(2): 262-268.

Ahirwar S, Jain S, Nisha P, 2023. *Annona squamosa L.*: A brief review on biological activities and their phytochemicals. Asian J Pharm Pharmacol 9(1): 13-19.

AOAC 2012. Official Method of Analysis: Association of Analytical Chemists. 19th Edition, Washington DC, 121-130.

ATSDR, 2020. Toxicological profile for 1,4-Dioxane. U.S. Department of Health and Human Services.

Adamson DT, Anderson RH, Mahendra S, *et al*., 2014. Evidence of 1,4-dioxane attenuation at groundwater sites contaminated with chlorinated solvents. Environ Sci Technol *48*(20): 12212–19.

Ali MM, Murthy RC, Chandra SV, 2016. Chronic exposure to manganese produces spatial memory impairments in rats: Role of oxidative stress. BMC Pharmacol Toxicol 17(1): 33.

Baba SA, Malik SA, 2015. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume. J Taibah Univ Sci 9(4): 449-454.

Brown RC, Lockwood AH, Sonawane BR, 2005. Neurodegenerative diseases: an overview of environmental risk factors. Environ Health Perspect 113(9):1250-1256.

Chen Y, Wang Y, Charkoftaki G, *et al*., 2022. Oxidative stress and genotoxicity in 1, 4-dioxane liver toxicity as evidenced in a mouse model of glutathione deficiency. Sci Total Environ 806: 150703.

Dilworth LL, Stennett DK, Bailey DH, *et al*., 2023. Effect of *Annona squamosa* leaf extract on human promyelocytic leukemia cells subjected to oxidative stress. J Complement Integr Med 20(3): 612-620.

Elsayed A, Aboubakr M, Hassan FW, *et al*., 2024. Testicular Injury of Acrylamide in Rats and Potential Protection of Coenzyme Q10 and Rosuvastatin. Pak Vet J 44(2): 344-351.

Gupta RK, Kesari AN, Murthy PS, *et al*., 2005. Hypoglycemic and antidiabetic effect of ethanolic extract of leaves of *Annona squamosa L.* in experimental animals. J Ethnopharmacol 99(1): 75-81.

Hassan M, Rasul A, Shah MA, *et al*., 2023. Effect of PENN-DIABEX, a novel polyherbal formulation, in high fat diet streptozotocin-induced diabetic rats. Saudi J Biol Sci 30(11):103816.

Hendawy OM, ELBana MA, Abdelmawlla HA, *et al*., 2019. Effect of *Annona squamosa* ethanolic and aqueous leave extracts on aluminum chloride-induced neuroinflammation in albino rats. Biomed Pharmacol J 12(4): 1723-1730.

JBRC. 1998. Two-week studies of 1,4-dioxane in F344 rats and BDF1 mice (drinking water studies). Kanagawa, Japan: Japan Bioassay Research Center.

Kaleem M, Asif M, Ahmed QU, Bano, B, 2006. Antidiabetic and antioxidant activity of *Annona squamosa* extract in streptozotocin-induced diabetic rats. Singapore Med J 47(8): 670.

Kano H, Umeda Y, Kasai T, *et al*., 2009. Carcinogenicity studies of 1, 4-dioxane administered in drinking-water to rats and mice for 2 years. Food Chem Toxicol 47(11): 2776-2784.

Kano H, Umeda Y, Saito M, *et al*., 2008. Thirteen-week oral toxicity of 1, 4-dioxane in rats and mice. J Toxicol Sci 33(2): 141-153.

Kasai T, Saito M, Senoh H, *et al*., 2008. Thirteen-week inhalation toxicity of 1, 4-dioxane in rats. Inhal Toxicol 20(10): 961-971.

Kiranmayi GVN, Devi ST, 2020. Evaluation of anti-fatigue and antioxidant activities of ethanolic leaf extracts of *Annona squamosa* and *Annona reticulata*. Biochem. Biotechnol Res 8(3):34-42.

Kumar S, Chadha P, 2025. Toxic Effects of 4-Bromodiphenyl Ether (BDE-3) on Antioxidant Enzymes, Cell Viability, Histology and Biomolecules in Zebrafish Embryo-Larvae. J Appl Toxicol 45(2): 350-360.

Lena N, Jamil AS, Muchlisin MA, *et al*., 2023. Pharmacological Network Analysis of Dutch Teak Plant (Guazuma ulmifolia Lamk.) as an Immunomodulator. J Islamic Pharm 8: 1-6.

Mohr TK, DiGuiseppi WH, 2020. Regulation of 1, 4-Dioxane. Environ. Investig. Remed. 291-318.

Noaman E, Gharib OA, 2005. Early Signs of Oxidative Stress Induced by Environmental Pollutants and Radiation Exposure in Rats. Egypt J Radiat Sci Appl 18(2): 335-349.

Pandit DN, Gupta ML, 2019. Heapto-somatic index, gonado-somatic index and condition factor of Anabas testudineus as bio-monitoring tools of nickel and chromium toxicity. Int J Innov Eng Res Technol 12(3): 25-28.

Pearce N, Kromhout H, 2014. Neurodegenerative disease: the next occupational disease epidemic? Occup Environ Med 71(9):594-595.

Soliman A, Alkafafy M, Hassan FW, *et al*., 2024. Investigating the protective role of L-carnitine and thymoquinone against methotrexate-induced testicular damage in rats. Pak Vet J 44(2): 314-321.

Soliman MM, Aldhahrani A, Gaber A, *et al*., 2022. Ameliorative impacts of chrysin against gibberellic acid-induced liver and kidney damage through the regulation of antioxidants, oxidative stress, inflammatory cytokines, and apoptosis biomarkers. Toxicol Res 11(1): 235-244.

Somasekhar RK, Pradeepkumar B, Sudheer A, *et al*., 2020. Effect of against aluminium chloride induced Alzheimer's disease in rats. Egypt Pharm J 19(4): 330-337.

Trofin DM, Sardaru DP, Trofin D, *et al*., 2025. Oxidative stress in brain function. Antioxidants 14(3): 297.

Turna Demir F, 2022. In vivo effects of 1, 4-dioxane on genotoxic parameters and behavioral alterations in Drosophila melanogaster. J Toxicol Env Health Part A. 85(10): 414-430.

van Thriel C, Boyes WK, 2022. Neurotoxicity of organic solvents: An update on mechanisms and effects. In Advances in neurotoxicology (Vol. 7: pp. 133-202). Academic Press.

Vasantharaja D, Ramalingam V, 2018. Neurotoxic effect of titanium dioxide nanoparticles: Biochemical and pathological approach in male wistar rats. Int J Appl pharm 10(4): 74-81.

Yu Y, Zhang Y, Martin JA, *et al*., 2013. Evaluation of cell viability and functionality in vessel-like bioprintable cell-laden tubular channels. J Biomech Eng 135(9): 091011.

Zenker MJ, Borden RC, Barlaz MA, 2003. Occurrence and treatment of 1,4-dioxane in aqueous environments. *Environ Eng Sci 20*(5): 423–432.