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HAEMATOLOGICAL AMELIORATION BY *ZIZYPHUS MAURITIANA* IN SILICA INDUCED TOXICITY IN WISTAR ALBINO RATS

Rudra Prasad Dutta and M. B. Patil*

University Department of Biochemistry, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur - 440033, Maharashtra, India.

Keywords:

Silicon dioxide, *Zizyphus mauritiana*, Proinflammatory cytokine, Hepatotoxicity, Flavonoids, Biomarkers

Correspondence to Author:
Dr. Mandakini B. Patil

Professor and Head,
University Department of Biochemistry, L. I. T Premises, Amravati Road, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur - 440033, Maharashtra, India.

E-mail: mbpatil@hotmail.com

ABSTRACT: This study was aimed to investigate the biochemical influence of aqueous extracts of roots, stem and leaves of *Zizyphus mauritiana* (ZM) on silica induced toxicity in rats, with changes in inflammatory markers in liver and kidney. To test the preventive effect of extracts of *Zizyphus mauritiana* on development of silica induced toxicity in wistar albino rats, animals were treated simultaneously with intraperitoneal administration of silica and oral feeding of extracts, for 21 days. At the end of the experiment, blood samples were withdrawn for biochemical analysis. The results revealed that animals administered with silica and simultaneous feeding of extracts of root, stem and leaves of *Zizyphus mauritiana* had a significant effect and decreased serum levels of ALT, AST, ALP, urea, uric acid, creatinine and LDH, as well as pro-inflammatory cytokines TNF-α and IL-6 as compared with normal control group. *Zizyphus mauritiana* extracts treated groups had shown recoupment in the body weight which was found decreased in only silica treated animals. In conclusion, this study indicated that extracts of bark of roots, stem and leaves of *Zizyphus mauritiana* have a preventive effect and could protect the animals from deleterious effects of silica as compared to other animals receiving only silica.

INTRODUCTION: Silicosis is a form of occupational lung disease caused by repeated or extended exposure to silica leading to acute or chronic respiratory ailments [1]. Reports indicate that about ten millions of Indian workers suffer from silicosis every year [2]. Silica is one of the most ubiquitous minerals on earth with widespread exposure in working and living environment [3]. Multiple exposure to silica increases health issues and mortality of those working in mines, thermal power station, ceramic industry, cement manufacturing company and rock drilling area. Silica may get into the human body *via* inhalation, ingestion, dermal penetration and injection [4].

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However, recent technological progresses have scaled up the use of silica with notable applications in biomedical fields, drug delivery system [5], sensor, and commercial field also [6]. Normally silica exposure has been reported to be associated with plethora of health diseases of heart, lung, liver, kidney and even cancer.

Liver is the major target organ for bio-transformation of xenobiotic compounds; evidences suggest that silica exposure causes liver cirrhosis, including particle accumulation and disturbances of metabolism [7]. It also damages the normal kidney function [8] due to silica accumulation which enhances free radical generation due to oxidative stress [9]. The free radicals thus formed disrupt the membrane integrity, marked by increase in the level of liver and kidney marker enzymes in the blood [10]. Currently, there is no effective treatment available for silica induced toxicity in humans. At present the treatment followed is mainly of a

supportive nature, comprising of administration of anti inflammatory drug to reduce pain. Such treatment often leads to adverse side effects with no relief from the disease. People working in mining area and industries are mostly the tribals and the poor labourers. Such economically backward class of people do not get acquainted with the appropriate treatment in time and therefore fall prey to diseases. Through ancient times India is known to follow the Ayurvedic way to cure many diseases. Uses of naturally occurring plant products lower down the possibility of reducing the side effects along with economic burden of treatment. For this reason we chose the herbal way to prevent the silica induced toxicity using a wild medicinal plant '*Zizyphus mauritiana*' (ZM).

This plant is found all over the world including drought prone regions and has been widely investigated for its medicinal properties without any adverse effects. *Zizyphus mauritiana* is a very rich source of alkaloids, ascorbic acid and other phytochemicals [11]. Aqueous extracts of root barks of *Zizyphus mauritiana* has analgesic effect in rats [12], whereas methanolic extracts of stem barks of *Zizyphus mauritiana* has anti-diarrheal effect. Compounds isolated from the root bark have shown anti allergic and anti-inflammatory activities [13]. Taking into consideration the medicinal properties of *Zizyphus mauritiana*, the present study has been designed to evaluate the therapeutic effect of aqueous extracts of bark of roots, stem and leaves and check for its preventive effect in silica induced toxicity in the animal models using wistar albino rats.

MATERIALS AND METHODS:

Chemicals: Silicon dioxide (SiO_2), ethanol, diethyl ether, chloroform, sodium carbonate, sodium bicarbonate, ethylene diamine-tetra acetic acid (EDTA), disodium phenyl phosphate, phenol, potassium ferricyanide, 4-amino antipyrine, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and sodium pyruvate were purchased from Sigma chem. Co. USA. All other chemicals were of analytical grade.

Animals: Healthy male Wistar albino rats (200-250g) were obtained from National Centre for Laboratory Animal Sciences (NCLAS), Hyderabad, India. Animals were maintained at standard conditions (temperature 25 ± 2 °C) with 12 h light / 12 h dark cycle and fed *ad libitum* with standard pellet diet (Hindusthan Lever) and purified water, with free access to food and water. All the norms prescribed by COPCEA, Government of India, were critically followed (vide the permission letter from Institutional Animal Ethics Committee, dated: 12/3/2015).

Collection of Plant Material: Barks of roots and stem and mature healthy leaves of *Ziziphus mauritiana* (ZM) tree were collected from a single tree growing in forest of Gadchiroli district of Maharashtra state (India) in the month of October 2014. The plant was authenticated at University Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur and voucher specimen no. 9138 was deposited in the herbarium. The plant was identified to be *Zizyphus mauritiana* Lam. (Family Rhamnaceae).

Preparation of Extracts: Dried barks were crushed in grinder and strained through the strainer to remove any hard part of the bark escaped during grinding. Leaves were air dried under shade in controlled condition and crushed to get powder. The powders were stored in air tight brown glass containers to protect from moisture and direct sunlight. Ten percent aqueous extract was prepared by boiling the powder in water for 30 min and passed through muslin cloth. The aqueous extracts were filtered and subjected to rotary vacuum evaporator (Superfit DB3135S).

Complete evaporation of water from extract was achieved by drying the extracts at room temperature under controlled conditions by spreading in clean glass petri plates. The dried scrapings were stored in sterilised airtight brown glass bottles until use. At the time of use the scrapings were carefully weighed on electronic balance and solubilised 0.1M sodium phosphate buffered saline (pH 7.0), vortexed for solubilisation before oral feeding to animals using gavage at 400mg/kg body weight [14].

Acute Toxicity Study: Acute toxicity study of silica was determined by up-and-down method [15], intraperitoneal administration of silica at LD_{50} (lethal dose) at 200mg/kg, and acute toxicity test of *Zizyphus mauritiana* extracts was determined by

using guide lines of Organization for Economic Corporation Development (OECD) [15]. Rats were orally administered daily with extracts of *Zizyphus mauritiana* and observed for the toxicity signs like mortality, loss of body weight and behavioural changes and obtained LD_{50} value at 4000mg/kg.

Experimental Design: Thirty six adult male rats were divided into six groups of six animals each. The animals were administered silica at the dose of 20mg/kg ($1/10^{th}$ of the LD_{50} value, solubilised in phosphate buffered saline, 0.1 M, pH 7.0), intraperitoneally followed by oral feeding of *Zizyphus mauritiana* extracts at 400 mg/kg ($1/10^{th}$ of the LD_{50} value) for 21 days.

Group 1: The animals were not given any treatment (normal control).
Group 2: The animals received only silica for 21 days (positive control).
Group 3: The animals received silica with extract of root bark (root preventive).
Group 4: The animals received silica with simultaneous extract of stem bark (stem preventive).
Group 5: The animals received silica with simultaneous extract of leaves (leaves preventive).
Group 6: The animals received only phosphate buffered saline (0.1M, pH 7.0) (vehicle control).

The doses were given to animals at particular time schedule daily before 9 AM from 0 to 21 days.

Collection of Blood and Serum Samples: At the end of the experimental schedule, the animals were given deep di-ethyl-ether (Merck) anaesthesia. After anaesthesia, blood was immediately collected from retro orbital plexus through capillary tube. The blood was allowed to clot for 30 min at room temperature followed by centrifugation using simple table-top centrifuge (Remi CM 12 Plus) at 1500 rpm for 10 min to obtain the serum. The colourless serum samples were stored at -80 ^{0}C until use. Blood samples were preserved at -40 ^{0}C until analysis.

Body Weight Measurement: Body weight of every rat in each group was carefully recorded before initiation of experiment and at the end of the experimental schedule and just before withdrawing the blood from animals (Essae Teraoka Ltd. FB200).

Biochemical Analysis:

Determination of Serum Alanine Transaminase and Aspartate Transaminase: Serum alanine transaminase (ALT) and aspartate transaminase (AST) were analyzed by the method of Reitman (1957) [16].

Determination of Serum Alkaline Phosphatase: Alkaline phosphatase (ALP) with its optimum pH in the alkaline range liberates inorganic phosphate; ALP was measured by the method of King *et al.*, [16].

Determination of Serum Lactate Dehydrogenase: Activity of oxidoreductase, that requires NAD^+ or $NADP^+$ as coenzyme was determined by measuring the rate of change of NAD^+ in system followed by production of lactate [17].

Determination of Serum Creatinine: Serum creatinine estimation was measured by alkaline picrate method [18].

Determination of Blood Urea: Blood urea concentration was determined by Nesslerisation method by the method of Cleon (1942) [19].

Uric Acid Level in Serum: Uric acid in the protein free filtrate of serum was made to react with phosphotungstic acid reagent, in the presence of alkaline medium to form blue coloured complex by the method of Martinek (1965) [20].

TNF-α and IL-6: Level of pro-inflammatory cytokines including TNF-α and IL-6 in serum was evaluated using commercially available multi-analyte ELISA kit (Qiagen). Absorbance was read at 450 nm using Thermo electron Corp. 358 ELISA plate reader.

Determination of Total Bilirubin: Total Bilirubin (TB) in serum was measured by Malloy and Evylin method (1937). Bilirubin reacts with diazotized sulphanilic acid to form purple colour azobilirubin. The intensity of the purple colour is proportional to the bilirubin concentration in the serum [21].

Haemoglobin Percentage: Haemoglobin level in blood was estimated by Sahli's Haemoglobino-meter by acid haematin method [22].

Estimation of Blood Glucose level: Blood sugar was estimated by Accurex Glu eco kit (Young, *et al*., 1975).

Statistical Analysis: The data obtained were expressed as Mean ± Standard error of mean (n = 6) and analyzed with one way ANOVA followed by Tukey Post hoc analysis comparison with each group using GraphPad Prism version 5.0. $P < 0.05$ was considered as statistically significant.

RESULTS: Body Weight Changes in Silica and *Zizyphus mauritiana* Treated Rats: Body weight of experimental animals before and after the experimental schedule has been presented in **Fig. 1**. The results indicate that rats from the normal control group (Group 1) followed a normal pattern of growth and attained a normal weight gain reaching to 230 ± 8.12g from average 205 ± 7.13 over 3 weeks. Animals in positive control (Group 2) suffered growth retardation and had a significantly ($P < 0.05$) lower weight than other groups. Among the treated groups (Group 3, 4, 5), treatment of animals by extracts of stem bark (Group 4) was more significant with respect to weight gain as compared to treatment with extracts of roots (Group 3) and leaves (Group 5).

The body weight of treated group was quite near to the group 1 animals *i.e.* a group where animals did not receive any treatment as compared to group 2 animals (cirrhosis positive control group rats). No significant change was observed in vehicle control (Group 6) as compared with the normal control group of rats' w. r. t the weight gain.

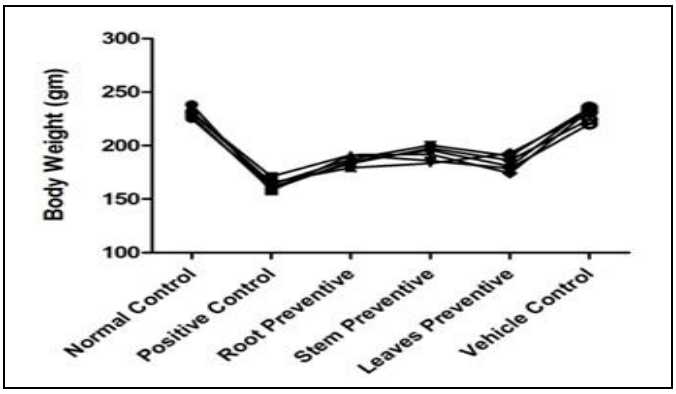
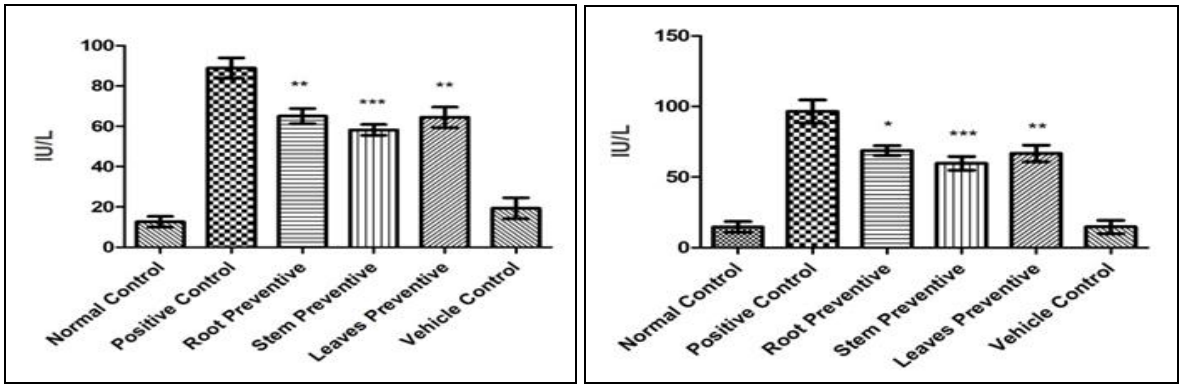


FIG. 1: EFFECT OF EXTRACTS OF *Z. MAURITIANA* ON THE BODY WEIGHT IN SILICA TREATED RATS

Effect on Serum Alanine Transaminase and Aspartate Transaminase Activity: Fig. 2a, 2b shows that in silica treated rats, increase in the enzymatic activities of ALT, AST was significant as compared to the control group. Oral administration of extracts of root and stem bark, and extract of leaves of *Zizyphus mauritiana* significantly reversed their level towards normal. The dose of three different extracts revealed more significant therapeutic effectiveness as compared to positive control. There is no significant change observed in vehicle control group when compared with normal group of rats.



A **B**

FIG. 2: EFFECT OF TREATMENT WITH *Z. MAURITIANA* EXTRACTS ON ALT (A) AND AST (B) SERUM ENZYME ACTIVITY OF SILICA TREATED RATS

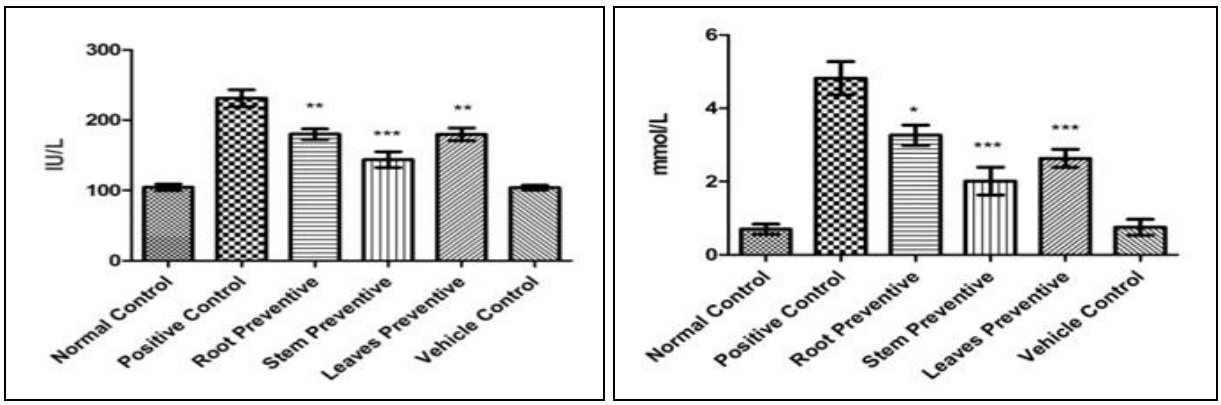
Data are expressed in Means ± S.E.M. $P < 0.001$ compared with the normal group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with positive control group.

Effect on Serum Alkaline Phosphatase (ALP) Activity: Fig. 3a demonstrates a higher serum ALP levels in silica treated positive group as compared to the normal control group. However, a significant decrease in ALP activity was detected in all *Zizyphus mauritiana* treated groups.

Effect on Serum Lactate Dehydrogenase (LDH) Activity: Fig. 3b shows that the positive control group of rats' have higher level of LDH activity than the normal control group. In all *Z. mauritiana* treated groups significantly decreased LDH activity was observed, however, vehicle control group did

not have significant changes in LDH level than the normal control group.

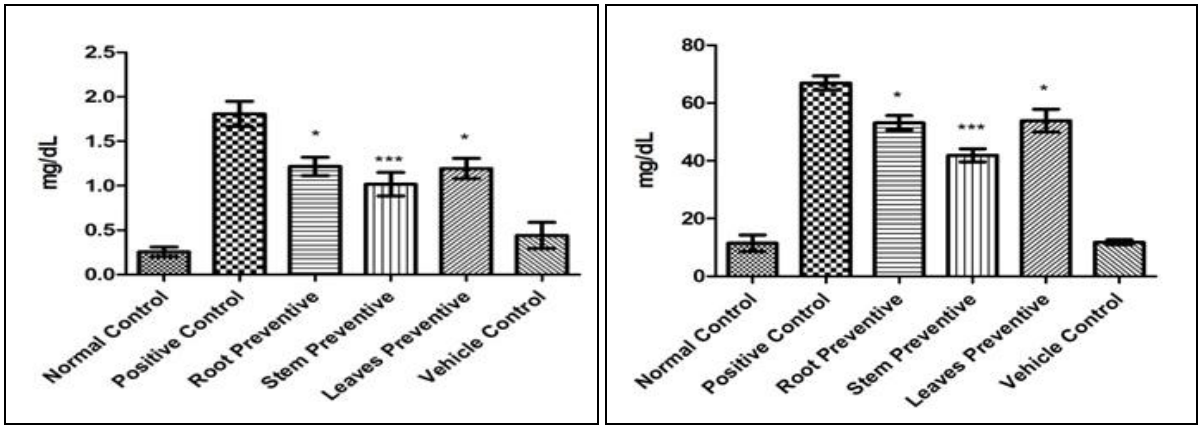
Effect on Serum Creatinine and Urea: Fig. 4a, 4b shows increased levels of serum creatinine and urea in silica induced rats as compared to the normal control group. On the other hand *Z. mauritiana* treated rats have significantly decreased level of creatinine and urea. Stem bark treated group has lower value of serum creatinine and urea as compared to the group treated with extract of root and leaves. No significant changes were observed in vehicle control group w. r. t creatinine and urea when the results were compared with that of normal control group. The rise in serum creatinine and urea in group 2 animals may be due to the kidney dysfunction.



A B

FIG. 3: EFFECT OF TREATMENT WITH *Z. MAURITIANA* EXTRACTS ON ALP (A) AND LDH (B) SERUM ENZYME ACTIVITY OF SILICA TREATED RATS

Data are expressed in Means ± S.E.M. P < 0.001 compared with the normal control group. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with positive control group.



A B

FIG. 4: EFFECT OF TREATMENT WITH *Z. MAURITIANA* EXTRACTS ON SERUM CREATININE (A) AND UREA (B) LEVELS OF SILICA TREATED RATS

Data are expressed in Means ± S.E.M P < 0.001 compared with the normal control group. *P < 0.05 and ***P < 0.001 compared with positive control group.

Effect on Serum Uric Acid: Fig. 5 shows the significantly elevated results of serum uric acid due to administration of silica, than the normal animals. Those animals receiving extract of root bark demonstrated much less content of uric acid than animals receiving extracts of stem and leaves. *Z. mauritiana* root bark treated group demonstrated significant reduction in serum uric acid value as compared to stem bark and leaves treated groups. No significant changes were observed in uric acid content in vehicle control group than the normal control group.

Effect on Serum Biomarker TNF-α and IL-6 of Systemic Toxicity: Fig. 6a, 6b shows that the positive control group animals have increased the level of inflammatory biomarker TNF-α and IL-6 as compared to normal control group, whereas *Z.*

mauritiana treated groups have significantly decreased levels of inflammatory biomarkers. Stem bark treated group displayed better decrease than those treated with root bark and leaves. Vehicle control rats showed slightly higher value of TNF-α level and almost similar IL-6 level to normal control animals.

Effect on Serum Total Bilirubin (TB), Glucose and Haemoglobin (Hb): Table 1 shows no significant difference in content of serum total bilirubin, glucose and haemoglobin as compared to silica treated group and all *Z. mauritiana* treated groups. The level of all the three parameters was found within a normal range.

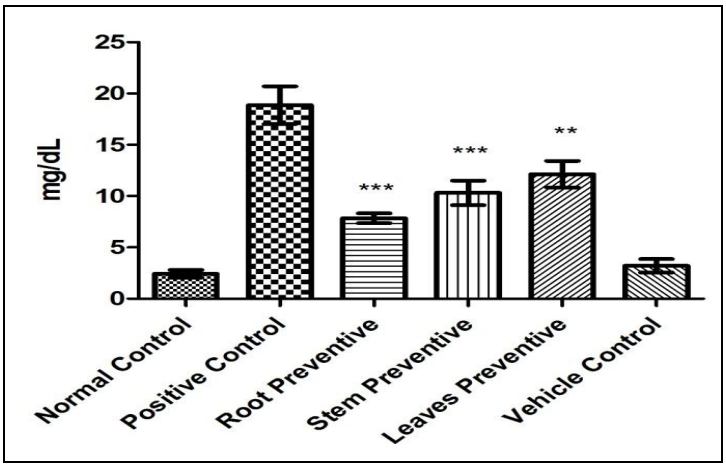
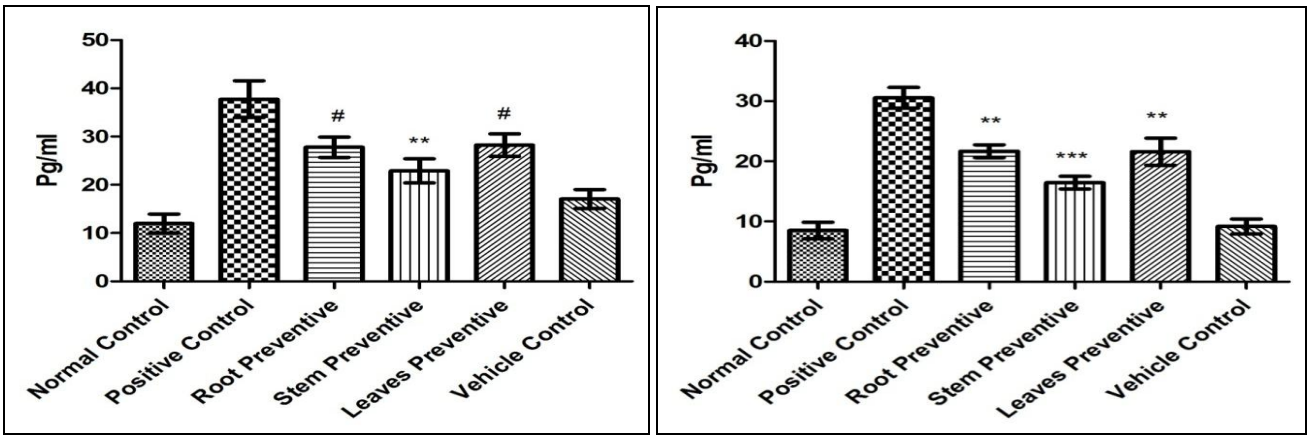


FIG. 5: EFFECT OF TREATMENT WITH *Z. MAURITIANA* EXTRACTS ON SERUM URIC ACID LEVELS OF SILICA TREATED RATS

Data are expressed in Means ± S.E.M P<0.001 compared with the normal control group. **P<0.01 and ***P<0.001 compared with positive control group.



A **B**

FIG. 6: EFFECT OF TREATMENT WITH *Z. MAURITIANA* EXTRACTS ON SERUM TNF- α(A) AND IL-6 (B) LEVELS OF RATS TREATED WITH SILICA

Data are expressed in Means ± S.E.M P<0.01 compared with the normal control group (Fig-a). P<0.001 compared with the normal control group (Fig-b). **P<0.01 and ***P<0.001 compared with positive control group. #P> 0.05 (Fig-a) compared with positive control group.

TABLE 1: EFFECT OF TREATMENT WITH *Z. MAURITIANA* EXTRACTS ON SERUM TB, GLUCOSE AND BLOOD Hb LEVELS OF RATS TREATED WITH SILICA

Group	Total Bilirubin (mg/dL)	Haemoglobin (g/dL)	Glucose (mg/dL)
Normal Control	0.36 ± 0.02	10.73 ± 0.56	80.1 ± 5.17
Positive Control	0.52 ± 0.06	11.18 ± 0.51	91.0 ± 6.56
Root Preventive	0.53 ± 0.09	11.17 ± 0.53	107.3 ± 8.34*
Stem Preventive	0.64 ± 0.11	10.08 ± 0.68*	111.0 ± 4.45**
Leaves Preventive	0.62 ± 0.06	11.12 ± 0.45	73.5 ± 3.12**
Vehicle Control	0.46 ± 0.08	10.91 ± 0.58	79.1 ± 6.47

Data are expressed in Means ± S.E.M *P< 0.05 and **P<0.01 compared with positive control group.

DISCUSSION: Silica is one of the abundant minerals found on the earth with varied use. Working with exposure to silica increases the rate of silica induced adverse health effects. The present study focuses on the preventative measure to silica induced toxicity. The plant in this study is a folk medicine used since ages to cure the diseases. Studies have demonstrated that silica induced toxicity mainly affects liver and kidneys.

Activities of serum amino transferases are normally considered as toxicity markers in hepatotoxicity studies even in silicosis. It has been demonstrated that intraperitoneally administered silica reaches the liver through portal vein circulation and gets deposited in liver, rupturing the hepatic cell membranes leading to the release of the enzymes from the cytoplasm into the blood circulation [4]. A significant rise in the level of serum ALT and AST in present case in group 2 animals may be due to the ruptured hepatic cells. The present study also revealed an increase the level of ALP in rats treated with silica, which is again an indication of obstructive damage in the hepatic tissue [23].

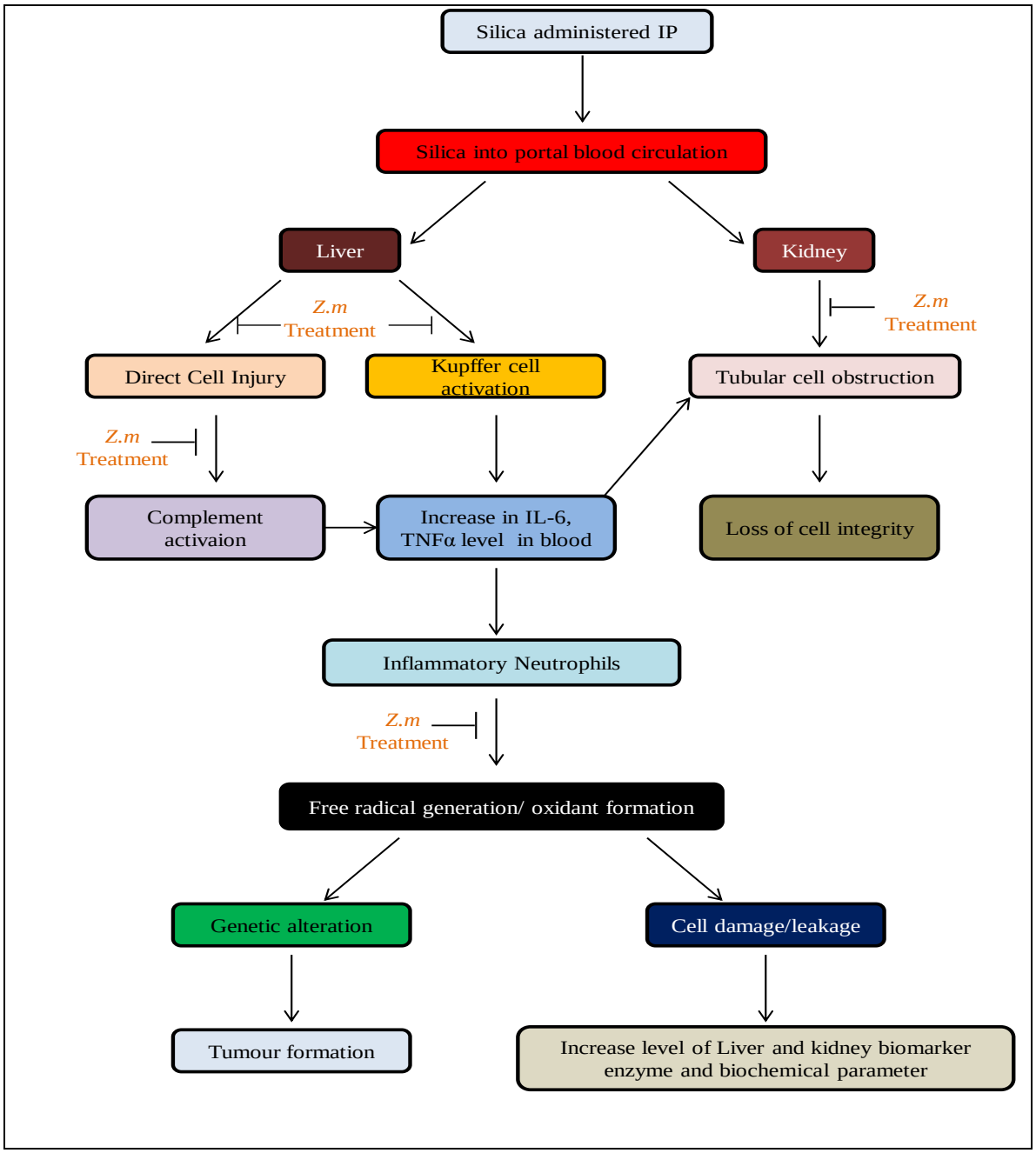


FIG. 7: A SCHEMATIC DIAGRAMMATIC REPRESENTATION SHOWING PROTECTING ROLE OF *ZIZYPHUS MAURITIANA* IN SILICA INDUCED TOXICITY

Increased levels of creatinine, urea and uric acid in silica treated animals than the control groups may be the result of injury and disturbances of renal functions, this may be due to dystrophic changes in kidney tissues, as a result the excretion of creatinine, urea and uric acid falls leading to the elevation in serum content of same [24]. Silica treated rats showed significant increase in the serum lactate dehydrogenase activity which again relates the damage and necrosis of liver and kidney tissues.

The extracellular appearance of LDH is considered as a sign to detect the cell necrosis or some organ dysfunctions [25]. Silica can generate free radicals and causes cytotoxicity in hepatic cells. The non parenchymal cells (kuppfer cells (KC), the resident macrophages, play a major role in defence by invading foreign particles *via* phagocytosis.

Activated KCs also play important role in maintenance of liver damage via the production of pro-inflammatory cytokines. Our results revealed that probably KCs are stimulated by silica exposure to release tumour necrosis factor (TNF-α) and IL-6 in the blood circulation. These bioactive substances cause harmful effects on hepatic cells.

We observed increase in the level of serum TNF-α and IL-6 in positive control group as compared with normal healthy group suggesting that inflammation might have occurred due to internalization of silica into macrophages thereby activating them to release pro-inflammatory cytokines. There are no significant changes in blood sugar; TB and Hb level in positive control group and *Z. mauritiana* extract treated groups indicating that silica does not affect these blood parameters [26]. Several studies have shown that high dose of silica or over exposure to silica increases the bilirubin level as it interferes with the conjugation of bilirubin with glucuronides in smooth endoplasmic reticulum in the liver [27]. *Z. mauritiana* extracts might be playing a major role in preventing the peroxidation of membrane lipids by inhibiting the free radical attack on the membranes. Previous phytochemical studies on this plant demonstrated the presence of various types of polyphenolic compounds. The bark of roots and stem and leaves of this plant has been shown to contain the abundant amount of alkaloids, flavonoids and many secondary metabolites [28].

The polyphenols help scavenge the generation of free radical and reduce the damage of cells. These compounds are having promising preventive role against the reactive oxygen species [29]. In this study we found that stem bark treated groups are more preventive of the harmful effect of silica as compared to animals receiving extract of root bark and leaves. The therapeutic preventive potential of *Z. mauritiana* extracts can be attributed to the presence of many –H and –OH groups of its phenolic and flavonoid components [30]. The presence of these groups helps to bind the free radical and hinder the formation of reactive oxygen species (ROS).

Z. mauritiana leaves contained excessive amount of ascorbic acid also which helps to neutralize the excessive free radicals [12]. Ascorbic acid plays major role to cure the diseases of male reproductive organs due to silica induced toxicity [31]. The present study reveals that oral administration of extracts of root, stem and leaves significantly decreases the liver and kidney biomarker enzymes ALT, AST, ALP, and LDH and biochemical parameters such as creatinine, urea and uric acid. Previous studies have also reported that polyphenolic compounds inhibit nitrosation of liver and kidney cells [32].

IL-6 has an ability to protect cells from free radical damage when repeated exposure of silica administration increases the level of IL-6 [33]. The phytochemical content of Stem bark, root bark and leaves may inhibit the activity of cyclooxygenase (COX) enzyme [34]. COX-2 form of this enzyme produces inflammatory prostaglandins (PGE2); this PGE2 is the potent inducer of IL-6 [35]. TNF-α is one of the major pro-inflammatory cytokine which increases when oxidative stress occurs on exposure to xenobiotics. *Z. mauritiana* (stem, root and leaves) treated groups manifested significantly decrease in level of TNF-α because *Z. mauritiana* contains flavonoids [36] which inhibit the NF-kβ. Several studies have also shown that quercetin, a flavonoid, has anti-inflammatory effects by down regulating the gene expression of NF-kβ1 by decreasing the phosphorylation of Ik-βα and Ik-ββ [37].

CONCLUSION: Aqueous extracts of *Zizyphus mauritiana* have demonstrated significant preventive role against silica induced toxicity.

Antioxidant effect of *Z. mauritiana* might be helping to maintain the structural and functional integrity of the cells. An anti inflammatory effect of *Z. mauritiana*, as observed in present investigation, may open up a promising area for the development of neutral-pharmaceutical agents for the treatment of various diseases due to inflammation.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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