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Ameliorative Effect Of *Calendula officinalis* Against Arsenic Induced Toxicity In Charles Foster Rats

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Abstract:

The current work aims to prevent the adverse effects of arsenic poisoning in animal models by using a medicinal plant extract. The animals (Charles Foster rats) were exposed to Sodium arsenite at a dose of 8 mg per kg body weight for 90 days in order to develop an arsenic model. Leaf extract of *Calendula officinalis* at a dose of 200 mg per kg body weight was administered to these arsenic treated rats for 60 days to study the preventive effects of this plant extract. The study found that arsenic poisoning had an adverse effect on rats at the haematological, biochemical, and histopathological levels, but there was considerable normalization in the animal at all of these levels after the leaf extract administration of *Calendula officinalis*. As a result, it has ameliorative qualities against arsenic-induced toxicity and may be used therapeutically as a preventative medication.

Keywords: Sodium arsenite, Calendula officinalis, preventive effect, Charles Foster Rats

Introduction

Pollution levels have been steadily rising over the last several decades, with groundwater contamination emerging as a particularly pressing issue in recent years. Arsenic poisoning in groundwater is a growing concern that poses health risks to people across the world. Arsenic poisoning affects an estimated 300 million people worldwide, including 70 million in India and 10 million in Bihar's Gangetic plains. (Shaji et al., 2021; Hassan, 2018; Kumar et al., 2022[a]; Richards et al., 2021, 2020). People in the state of Bihar are particularly vulnerable to the harmful effects of arsenic poisoning. The exposed population exhibit severe arsenicosis symptoms with skin manifestations such as keratosis, melanosis, gastrointestinal disorders, loss of appetite, cardiovascular disorders, neurological disorders, diabetes, hormonal disorders etc. and disease of cancer (Kumar et al., 2021[a,b,c,d], 2019[a,b]). In the recent times there has been immense increase in the disease burden among the arsenic exposed population in the state of Bihar. Among the types of disease – cancer diseases such as liver cancer, renal cancer, colorectal cancer, bladder cancer, breast cancer, gallbladder cancer etc. has been reported in the state (Kumar et al., 2020[a,b]; Kumar et al., 2021[a,b,c,d], Kumar et al., 2020, Kumar et al., 2016, Kumar et al., 2015; Kumar et al., 2023[a,b]).

In Indian Medicine System called as *Ayurveda*, there are plethora of plant-based drugs available which has potent effect against various types of diseases, but for arsenic poisoning, there are only meager medicinal plants documented in the recent times (Kumar et al. 2022[b]). Hence, there is need of bioremedial approach to cater this complex problem. *Calendula officinalis* or the Marigold plant, has been used as drug to cure various diseases of skin infections and others. Its medicinal properties are well documented as it contains the active ingredients such as carotenoids, flavoxanthin, lutein, rubixanthin, beta carotene, lycopene, flavonoids, triterpenoids, saponins etc. It has antioxidant effects, genotoxic and chemopreventive effects, anti-tumour therapeutic effect, anti-inflammatory effects (Preethi & Kuttan, 2009; Abdel-Aziem et al., 2014; Hormozi et al., 2019; Lashkary et al., 2021; Kumar et al., 2010; Givol et al., 2019; Buzzi et al., 2016; Cruceriu et al., 2018).
The inhabitants of Bihar are especially susceptible to the devastating impacts of arsenic poisoning.

Materials and Methods

Ethics statement: This investigation got the necessary approvals from the relevant authorities in India before it was conducted in the animal house of the Mahavir Cancer Sansthan and Research
Centre in Patna, Bihar. The relevant registration numbers are CPCSEA Registration. The Institutional Animal Ethics Committee (IAEC) with the number 2021/1E-06/10/21 gave their approval to this study.

Animals: The animal house of the Mahavir Cancer Sansthan and Research Centre in Patna, Bihar, India, which is registered with the CPCSEA, Government of India (CPCSEA Reg. No. 1129/PO/ReBi/S/07/CPCSEA), provided the male Charles Foster rats (n=24) for this research. The rats were 8 weeks old and weighed around 150-180g. Two weeks prior to the start of the trial, the animals were allowed to acclimate. The laboratory was kept at a constant temperature of 22 ± 2°C with controlled humidity, and there was a 12-hour light-dark cycle. Both food and water were available to the animals at all times.

Chemicals: The chemical used for making the arsenic models in Charles Foster rats was sodium arsenite (98.5% concentration), which was manufactured by Sigma-Aldrich in the USA (CAS Number: 7784-46-5; S7400-100G), Lot# SLBH5736V, PCode 1001683292. In accordance with the prior research, the arsenic model was prepared using a reference dosage of sodium arsenite of 8 mg/kg/body weight daily for 90 days (Kumar et al., 2022[d]).

Preparation of leaf ethanolic extract of *Calendula officinalis*: Upon collecting the *Calendula officinalis* leaves from the Patna Women's College Garden, a botanist from the institution verified their identity. After being rinsed under running water three times in sequence, the plant leaves were placed in an incubator and dried at 37°C. Next, the dehydrated leaves were ground into a fine powder. Following a 48-hour soaking in 100% alcohol, the 250g of fine powder was extracted using Buchi's Rota Vapour. The last step was a 48-hour drying period in the incubator for the fine extract. The substance was now used for the aim of experimentation. After estimating the LD_{50}, which was 3000 mg/Kg b.w., the ethanolic extract dosage of the leaf extract was determined. The 200 mg/kg dosage was used in the research because it was the LOAEL level and because it was one fifteenth of the LD_{50} value. The rats that were treated with sodium arsenite were given a fixed dose of 200 mg/kg body weight for 60 days after the dosage was adjusted. (Kumar et al., 2022[d]).

Experimental Design: With six rats per group, the experimental animals were classified into four main categories:-Group-I -There was a normal control group, Group-II - an arsenic group where rats were given 8 mg/kg body weight of sodium arsenite orally (Gavage technique) for 90 days, and Group-III- where rats were given a *Calendula officinalis* leaf extract at the dose of 200 mg/kg of body weight orally (Gavage technique) for 60 days, rats were pretreated with 8 mg/kg of body weight of sodium arsenite daily for 90 days. The animals in each group underwent the appropriate anesthesia and then sacrificed after the treatment was accomplished. The blood samples were obtained from the dissected animals using orbital puncture in order to separate the serum. They were subsequently stored adequately for biochemical testing, including tests for liver function, kidney function, and lipid peroxidation. In addition, the liver and kidney tissues were skillfully preserved in a 10% formalin fixative before processing.

Haematological study: The Neubauer's chamber was used for haematological tests, including red blood cell and white blood cell counts, and the hemoglobinometer was used for hemoglobin estimate using the Sahli's technique.

Biochemical analysis: Spectrophotometer (UV - Vis) (UV-10, Thermo Fisher, USA) was used to conduct the biochemical investigation according to the standard kit procedure (Coral crest). In this research, biochemical parameters such as serum glutamic oxidase (SGOT) and serum glutamic pyruvate transaminase (SGPT) were assessed using the Liver Function Tests using the methods of (Reitman & Frankel, 1957), Alkaline Phosphate (ALP) assay by the method of (Kind & King, 1954), total bilirubin activity by method of (Jendrassik & Grof, 1938). The Kidney Function Tests (KFT) were assayed as urea by the method of (Fawcett 1960, Berthelot 1859), creatinine assay by the method of (Toro and Ackermann 1975), and uric acid assay by the method of (Bones and Taskuy, 1945).

Histopathological study: After the liver and kidney samples were fixed in 10% formalin for 24 hours, they underwent a number of alcohol processing steps before being embedded into paraffin blocks. Fine sections, about 5µm thick, were cut using the digital microtome (Themo-Fisher) and subjected to the double staining procedure with hematoxylin and eosin (H&E). Under the light microscope, the dyed slides were examined for the microscopic examinations (Cardiff et al., 2014).

Lipid Peroxidation (LPO): Thiobarbituric acid reactive substances [TBARS] are the most reliable indicators to use when evaluating the lipid peroxidation procedure. Based on the premise of spectrophotometric measurement of color generated during the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), the test was assessed using the double heating technique (Draper and Hadley, 1992). Partially heated in a water bath at 90°C for 15 minutes after centrifuging 2.5 ml of a 100gm/L solution of trichloroacetic acid (TCA) with 0.5 ml of serum was used for this experiment. Once the combination had cooled to ambient temperature, it was spun again in a centrifuge at 3000 rpm for 10 minutes to collect the liquid on top. After measuring 2 mL of the supernatant, 1 mL of a newly made TBA solution with a concentration of 6.7 grams per liter was added to the test tube. The mixture was then heated in a water bath at 90 degrees Celsius for 15 minutes before being allowed to cool to room temperature. A UV-visible spectrophotometer (UV-10, USA: Thermo Scientific) was used to measure the final absorbance at 532 nm.

Statistical analysis: The study's findings were the Mean ± Standard Error (SE) for each group of six rats, as well as the total variance in the data set examined using one-way Analysis of Variance (ANOVA). At a confidence level of 99.9% ($p < 0.05$), Dunnett's 't' test was used to examine the changes in mean variance. With the help of GraphPad Prism Program 5.0 (San Diego, USA:
GraphPad Software, Inc.), the last computations were carried out.

Results:

Haematological Assay:
There were significant changes observed in the haematological parameters of the studied groups – **RBC Counts:** Compared to the control group, rats treated with arsenic had significantly lower red blood cell counts ($p<0.001$) in this research. However, after being given the *Calendula officinalis* leaf extract, the levels significantly restored to normal (Figure 1A).

WBC Counts: After administering the leaf extract of *Calendula officinalis*, there was a considerable consolidation in the WBC counts, however there was a significant fall ($p<0.001$) in the WBC counts in the arsenic treated group rats compared to the control group (Figure 1B). **Haemoglobin Percentage:** Rats in the arsenic treatment group had a significantly lower hemoglobin percentage compared to the control group ($p<0.001$), however this percentage was significantly normalized following the administration of *Calendula officinalis* leaf extract (Figure 1C).

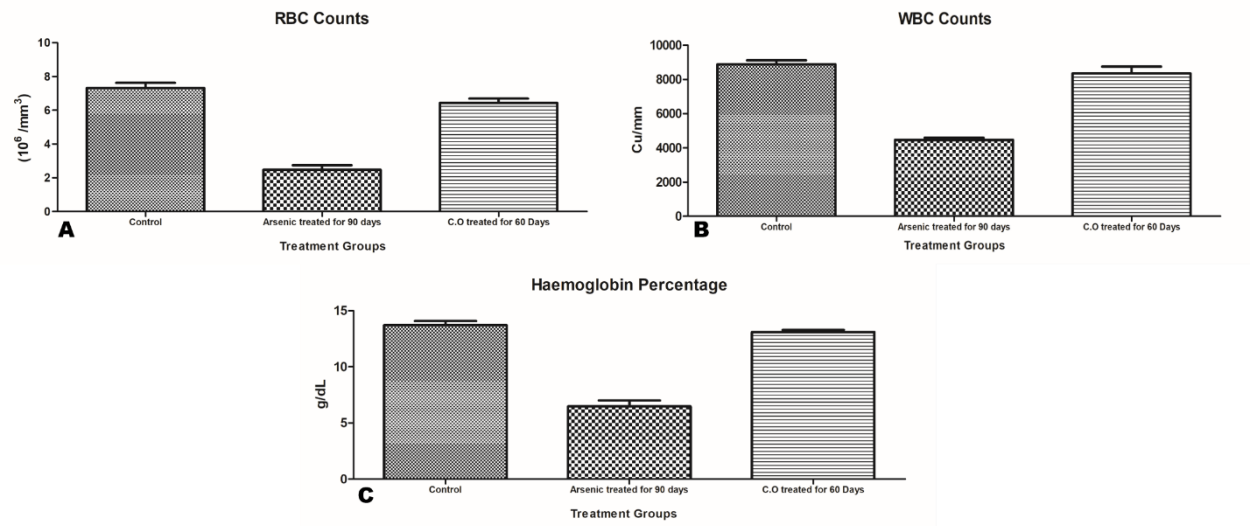

Figure 1. Haematological parameters of the treated groups (One way ANOVA Test in various group of rats (n=6) values displayed as Mean ± SE, p<0.001)

Biochemical Study: There were significant changes observed in the biochemical parameters of the studied groups – **Liver Function Tests:**

SGPT Assay: The SGPT levels in the rats treated with arsenic were significantly higher than in the control group ($p<0.001$), but they were significantly normalized following the administration of *Calendula officinalis* leaf extract (Figure 2A).

SGOT Assay: Rats in the arsenic-treated group had significantly higher SGOT levels than the control group ($p<0.001$), but these levels were significantly normalized following the administration of the *Calendula officinalis* leaf extract (Figure 2B).

Alkaline Phosphatase Assay: The alkaline phosphatase levels in the rats treated with arsenic were much higher than in the control group ($p<0.001$), however these levels were significantly normalized when the animals were given the *Calendula officinalis* leaf extract (Figure 2C).

Bilirubin Assay: The rats in the arsenic treatment group had significantly higher bilirubin levels than the control group ($p<0.001$), however these levels were significantly normalized following the administration of the *Calendula officinalis* leaf extract (Figure 2D).

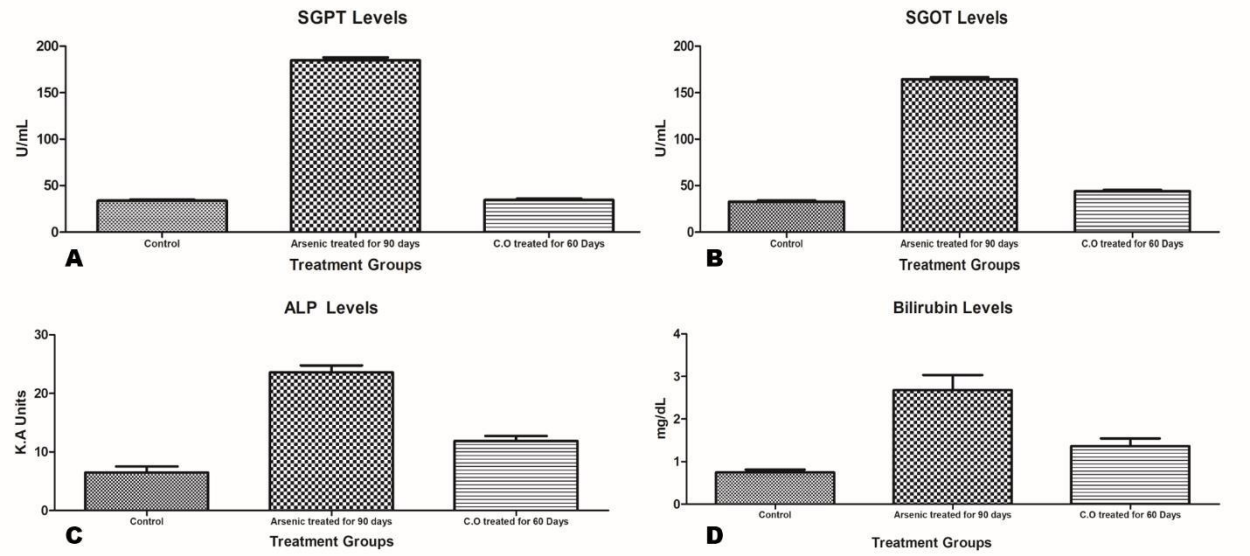

Figure 2. Liver Function Tests parameters of the treated groups (One way ANOVA Test in various group of rats (n=6) values displayed as Mean ± SE, p<0.001)

Kidney Function Tests:

Urea Assay: In the rats given arsenic, urea levels were much higher than in the control group ($p<0.001$). However, when the *Calendula officinalis* leaf extract was administered, the levels significantly returned to normal (Figure 3A).

Uric Acid Assay: After administering the *Calendula officinalis* leaf extract, there was a considerable normalization of uric acid levels, which had previously shown a significant rise ($p<0.001$) in the arsenic-treated group rats compared to the control group (Figure 3B).

Creatinine Assay: The rats in the arsenic treatment group had significantly higher creatinine levels than the control group ($p<0.001$), however these levels were significantly normalized following the administration of the *Calendula officinalis* leaf extract (Figure 3C).

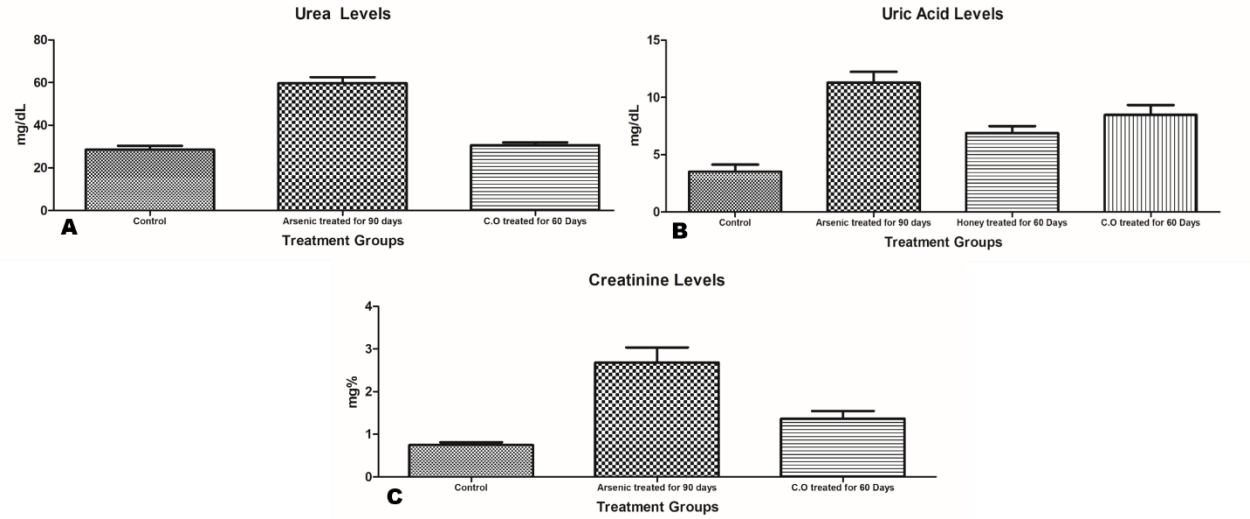

Figure 3. Kidney Function Tests parameters of the treated groups (One way ANOVA Test in various group of rats (n=6) values displayed as Mean ± SE; p<0.001)

Enzyme Assay:

Lipid Peroxidation Assay: The rats in the arsenic treatment group had significantly higher lipid peroxidation levels than the control group ($p<0.001$), however these levels were significantly normalized following the administration of the *Calendula officinalis* leaf extract (Figure 4).

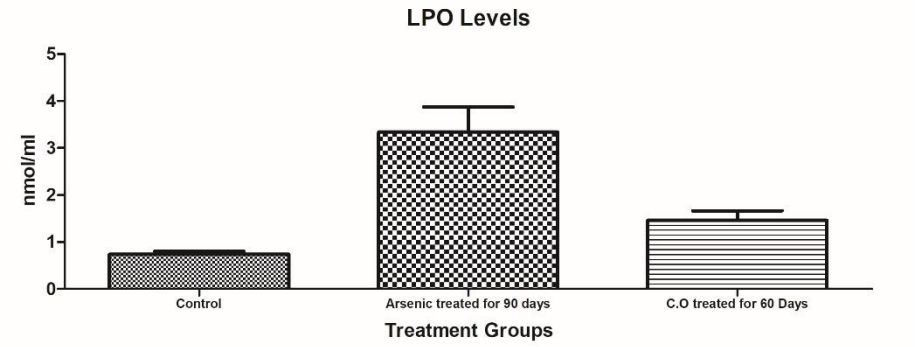

Figure 4. Lipid peroxidation levels of the treated groups (One way ANOVA Test in various group of rats (n=6) values displayed as Mean ± SE, p<0.001)

Histopathological Study: There were significant changes observed in the histopathological study in the studied groups –

The liver histopathological sections show normal architecture of hepatocytes with central vein. The hepatocytes are well arranged in the sinusoids denotes the normal functioning of the liver cells (Figure 5A). A significant degree of hepatocyte degradation and an increase in the number of Kupffer cells (69%, $p<0.005$) are seen in the arsenic-treated rat liver section. The presence of haemorrhages in the endothelial cells lining the central

venous membrane is another evidence of the extensive harm done to the hepatocytes. There are vacuolations in the sinusoidal spaces (Figure 5B). But after the administration with *C.officinalis*, there was significant amelioration in the hepatocytes, the central vein and the sinusoids and the Kupffer cells number (16%, $p<0.005$). Hepatocytes' Kupffer cell numbers dropped significantly, which is indicative of healthy cell activity (Figure 5C & 5D).

The kidney histopathological sections show normal architecture of glomerulus, Bowman's capsule, the convoluted tubules, and distal tubules (Figure 6A). The glomerulus and Bowman's capsule exhibit severe deterioration in the kidney part that was exposed to arsenic. A significant amount of blood leaking out of the kidneys is a sign of the serious damage that arsenic poisoning has caused (Figure 6B). The proper functioning of the nephrocytes was shown by the considerable amelioration in the nephrocytes following administration of *C.officinalis*, particularly in the glomerulus, Bowman's capsule, and convoluted tubules (Figure 6C & 6D).

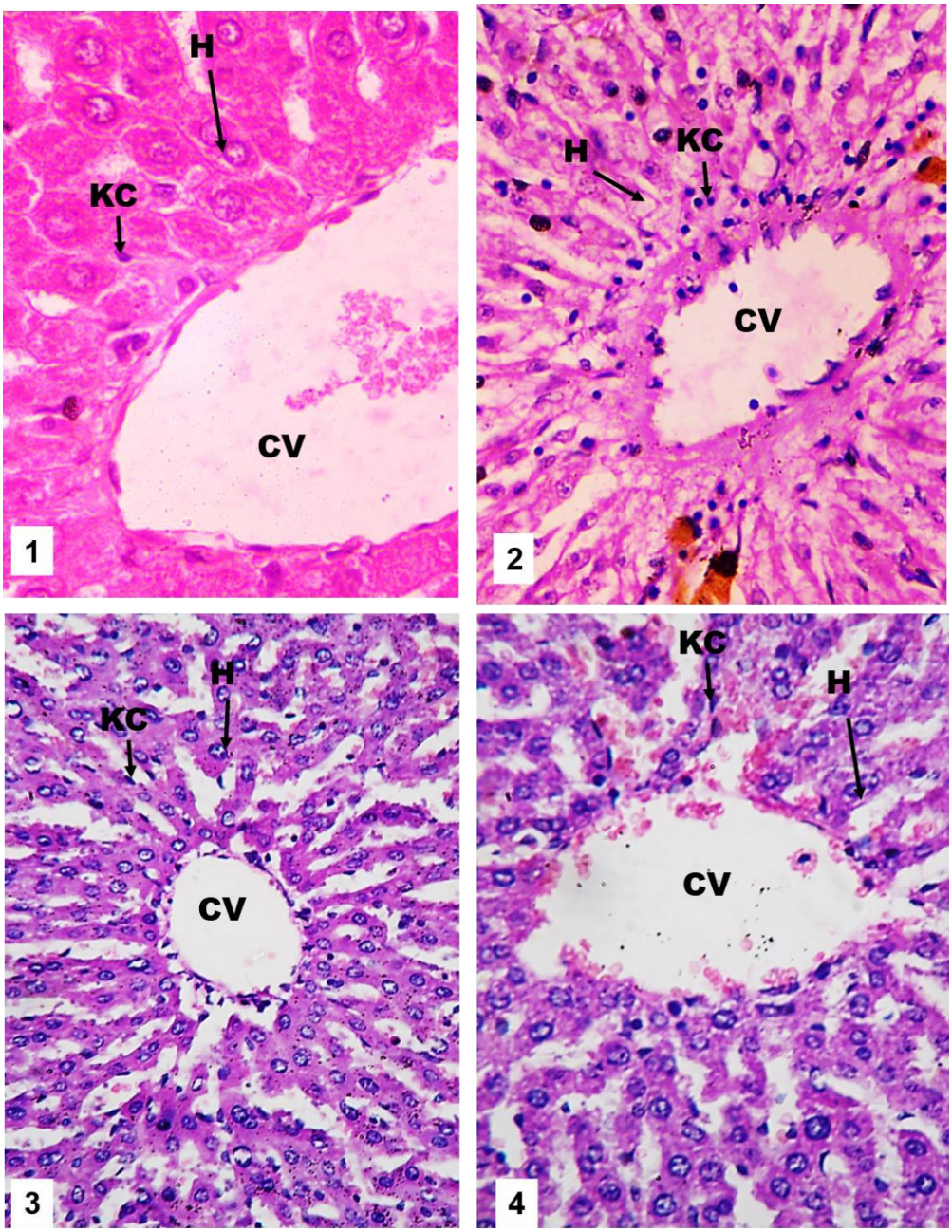

Figure 5: Microphotograph of rat hepatic sections stained with haematoxylin and eosin (H&E × 500). **[A]** Liver section of control rat showing normal architecture of hepatocytes (H), central vein (CV), with sinusoids and few Kupffer cells (KC). **[B].** Liver section of arsenic treated rat showing degenerated hepatocytes (H) with central vein (CV). The number of Kupffer cells (KC) has also increased many folds in the tissue denotes the magnitude of inflammation. **[C&D]** The *C.officinalis* administered rats shows significant normalization in the

hepatocytes (H) with central vein (CV). The hepatocytes are re-arranged in the sinusoidal spaces. The Kupffer cells (KC) numbers have relatively decreased. However, there is mild persistence of degeneration.

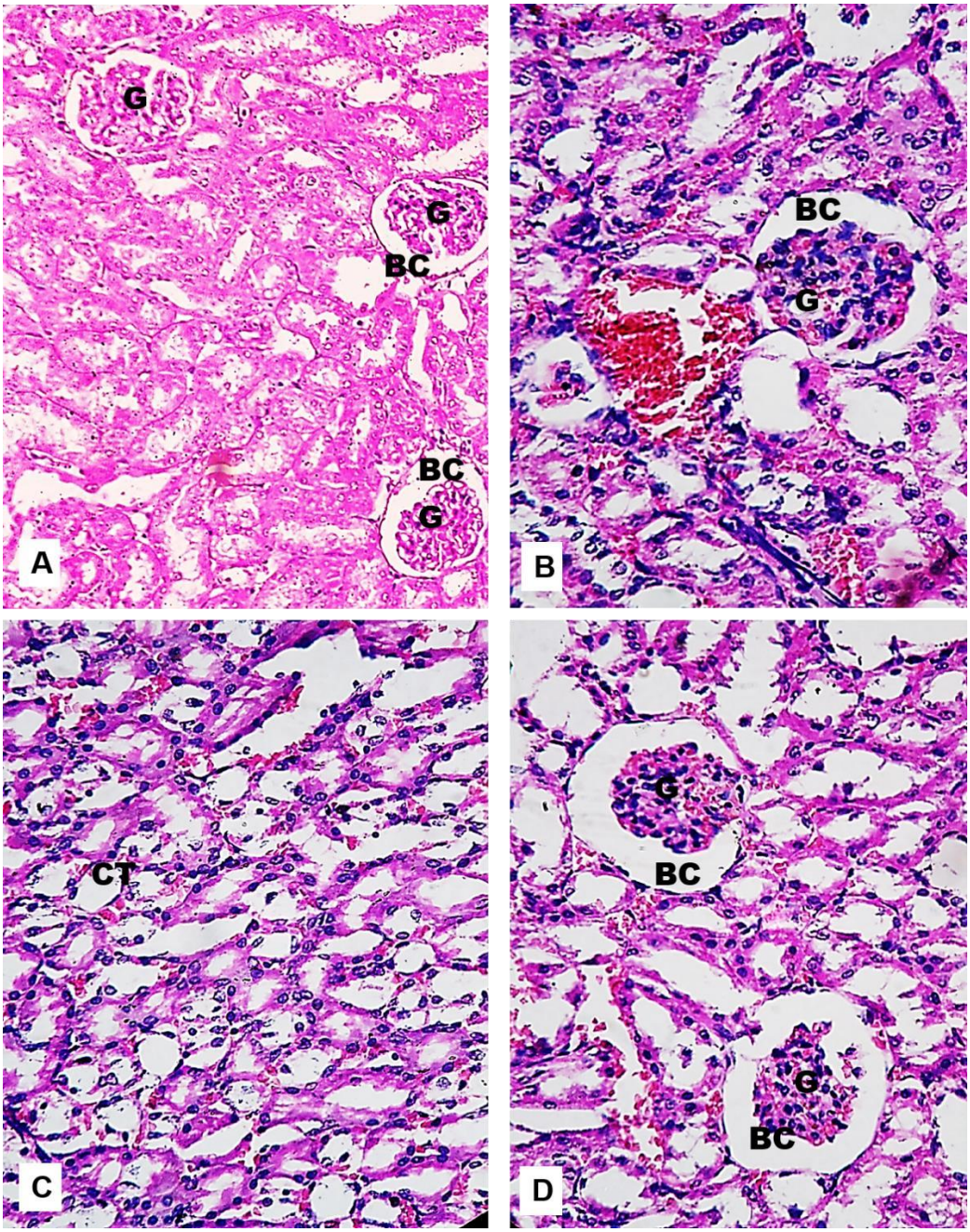

Figure 6: Microphotograph of rat renal sections stained with haematoxylin and eosin (H&E × 500). **[A]** The nephrocytes of control rats shows normal architecture of glomerulus (G) with Bowman's capsules (BC). The endothelial cells of convoluted tubules are also in normal architecture. **[B]** Kidney section of arsenic treated rats shows significant degeneration with haemorrhage at the entire tissue level (red coloured blood clots). The glomerulus (G) with Bowman's capsule (BC) are also in very degenerated condition denotes the level of toxicity in the liver. **[C&D]** The kidney section shows significant amelioration in the nephrocytes especially the glomerulus (G), Bowman's capsule (BC) and convoluted tubules (CT) after the administration of *C.officinalis*.

Discussion

The damaging effects of arsenic on the rats' essential organs were observed in this investigation. With respect to the body's bio-monitors, haematological markers are crucial. Modifications to the parameters has shown toxicological impact. Arsenic has severely damaged the bone marrow cells, since there was a significant reduction ($p<0.005$) in the red blood cell (RBC) count, white blood cell (WBC) count, and hemoglobin

percentage compared to the control. All of these hemoglobin measures were normalized after the administration of *C.officinalis*, indicating an enormous transformation. Similarly, when comparing the biochemical parameters to the control levels, there was an evident rise ($p<0.005$) in the levels of SGPT, SGOT, and alkaline phosphatase, indicating impairment to the liver function tests. A substantial rise ($p<0.005$) was also seen in the levels of urea, uric acid, and creatinine in the kidney function tests when compared to the control values. These metabolic markers, however, showed a significant decline after *C.officinalis* treatment. Histopathological examination of liver and kidney tissues revealed a rise in Kupffer cells, damage to nephrocytes, convoluted tubules, and distal tubules in the kidney cells, as well as hemorrhages in the hepatocytes and central veins and portal veins in the liver. The nephrocytes' and hepatocytes' architectures were significantly improved after *C.officinalis* administration. Significant reductions in cell necrosis and inflammation were also seen. It may be inferred that *C.officinalis* have a strong preventative effect, rendering arsenic hazardous effect. There have been similar models developed using other plants to combat the harmful effects of arsenic (Kumar et al., 2022[d]; 2021[a]; 2020[b];2015[b]).

When arsenic enters the body via the digestive system, it typically makes its way to every cell. Once there, it causes a cascade of free radical reactions, including lipid peroxidation, which disrupts cellular processes by removing membrane lipids. There is also a possibility that this may impair nuclear functions. Lipid peroxidation levels were found to be considerably higher ($p<0.005$) in the current investigation as well, but they were shown to be significantly lower after the administration of *C.officinalis* (An et al., 2021: Majhi et al., 2014; Yousefsani et al., 2018; Duan et al., 2016; Kumar et al., 2022[d], Kumar et al., 2020[b]).

Arsenic has caused severe damage to the haematological, biochemical and histopathological parameters. Moreover, our research studies have found protective effect of *Tinospora cordifolia* (Kumar et al., 2020[b]), *Coriandrum sativum* (Kumar et al., 2022[d]), and *Withania somnifera* (Kumar et al., 2015[b]), against arsenic induced toxicity in mouse and rat models.

The *C.officinalis* possesses active ingredients such as carotenoids, flavonoids, saponins, sterols, phenolic acids, lipids. Probably, the catenoids and flavonoids play the vital role in the normalization in the cellular functions in the arsenic induced toxicity. Quercetin is the major representative of the flavonoid which has protective effect against disease caused to the liver (Preethi et al., 2006). Moreover, the flavonoids also protect the kidneys by due to its diuretic, antioxidant, anti-inflammatory and antibacterial properties (Zeng et al., 2019). Hence, it protects the liver and kidneys by eliminating the arsenic from the body. The flavonoids also revitalize the damage caused by arsenic through the antioxidant mechanism normalizing the body functions at the haemotological levels, biochemical levels and histopathological level (Das et al., 2019; Jambor et al., 2021; Fonseca et al., 2010; Jiménez-Medina et al., 2006; Doligalska et al., 2013; Silva et al., 2007; Preethi et al., 2009; Preethi and Kuttan 2009).

Conclusion

The results of the research indicate that *C.officinalis* has antioxidant characteristics that protect rats from the harmful effects of arsenic poisoning. Further treatment studies against arsenic induced toxicity may be prompted through its therapeutic impact.

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Declarations

Competing interests

The authors declare that they have no conflicts of interest.

Consent for publish

All the authors provide their consent to publish this article.

Author contributions

The entire experimental work was conceptualized by M.K., S.S. and A.K. The manuscript's principal author M.K. contributed the majority of writing activities, but support was also provided by S.S, and A.K., Literature search was done by M.K.. Figures were developed by M.K. and A.K. The experimentation and data analysis were carried out by M.K. The figures were designed by M.K. and A.K. The statistics and data interpretation were done by M.K. The final manuscript writing was done by M.K. S.S. and A.K. All authors read and approved the final manuscript.

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Availability of data and materials

None of the data has been fabricated or manipulated (including image) to support this investigational study. Data supports the findings.

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