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Ameliorative effect of extract of *W. coagulans* (WCE) fruits against sodium fluoride-induced nephrotoxicity in mice

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ABSTRACT

Purpose: To determine the histopathological, biochemical and micrometric alterations in the kidney of mice exposed to sodium fluoride (NaF), alongside the ameliorative effects of *Withania coagulans* extract.

Methods: Forty-five male albino mice were utilized in this study being distributed into three groups: Control group: received saline and balanced diet without any added sodium fluoride. NaF and *Withania coagulans* combined treatment (NWCT) group: received *Withania coagulans* extract along with fluoridated drinking water. NaF group (NaF): was provided with fluoridated drinking water for 10 days. All animals were sacrificed on day 10 for organ retrieval. Kidney was removed from each group and was used for microscopic examination. Micrometric measurements were collected from different sections. Blood samples from each group were obtained for serum creatinine, urea and complete blood count (CBC) analysis to assess renal function and hematological parameters.

Results: Results revealed various histopathological alterations in the kidney including lesions in endothelial wall of Bowman's capsule and endothelial cell apoptosis following NaF exposure. Significant micrometric changes were also observed. Biochemical results and hematological parameters also displayed significant difference between the three groups. Reduction in damaged metrics of kidney was observed in NWCT group showing the ameliorative effect of *Withania coagulans*

Conclusions: Based on these findings, it is concluded that administration of NaF caused degenerative morphological changes and damage to the kidney and that *Withania coagulans* extract possesses a protective effect against NaF-induced toxicity.

Key-words: *Withania coagulans; Sodium fluoride; Nephrotoxicity; Hematology.*

INTRODUCTION

Sodium fluoride is one of the extensively studied fluorinated compounds. It is frequently found in dental hygiene products and municipal water fluoridation systems [1]. Additional sources of fluoride encompass food items, drinks that have been processed, and medications. Foods like fish, nuts, and dark green vegetables are notable for their high fluoride content, which they absorb from soil and water. Dental products such as gels, toothpaste, and mouthwashes predominantly contain fluoride as well [2]. Fluoride can rapidly pass through the intestinal mucosa the lining of the intestines because it exists in an ionic form in drinking water. Fluoride ions have small molecular weight and have an affinity for calcium ions (Ca^{2+}) in the body. Due to its small molecular weight and ionic nature, it easily enters cell membranes by simple diffusion and can interfere with metabolism and function of cell. It hinders with hydrogen bonding that is important for maintaining the structure and function of biological molecules must restrain many enzymes [3].

The impact of fluoride on cells varies by concentration, exposure duration, and cell type[4]. Surplus fluoride consumption for an extended duration can lead to a critical health problem called fluorosis[5]. Fluoride can be distributed to organs such as the liver, kidneys, skin, and reproductive system. Prolonged exposure to fluoride may cause significant gastrointestinal disturbances, neurological issues, and abnormalities in the reproductive system[6].The kidneys (followed by the liver) retain more fluoride compared to other organs in the body. Therefore, kidneys may be particularly sensitive to fluoride exposure, even in healthy individuals [7].NaF nephrotoxicity is marked by breakdown of tubular epithelium, shrinkage of glomeruli, death of the endothelial cells and convoluted tubules and infiltration of inflammatory cells in the interstitial tissue [8].

Plants possess medicinal compounds and have been utilized as traditional recovery remedies since ancient times. *Withania coagulans*, a member of the genus Withania, holds significant importance in the Ayurvedic medicinal system due to its significant phytochemical and pharmaceutical properties [9]. The drug has been documented to exhibit anti-inflammatory properties, as wellas cardiotonic activities, hepatoprotective effects, antifungal properties, hypoglycemic actions, hypolipidemic effects, wound healing activity, and potential benefits for diabetic nephropathy[10]. The objective of this study is to explore the shielding properties of extract of *W. coagulans* (WCE) fruits against sodium fluoride-induced toxicity in mice.

MATERIAL AND METHODS

Research animals and their management:

Forty-five mature male albino mice (*Mus musculus)* with weight ranging between 30-35 grams of reproductive age were utilized in present research work. These mice were placed in groups of three in three separate plastic cages gauzed with corrosion-resistant steel in animal house of Department of Zoology, University of Sargodha.

Animal groups and treatment profiles:

Control group*:*

Received saline and balanced diet without any added sodium fluoride for days 1 to 10.

NaF and Withania coagulans combined treatment (NWCT) group:

Received 0.1ml of pure *Withania coagulans* extract twice a day corresponding to an approximate dose of 333.33 mg/kg body weight/day and fluoridated drinking water from the 1st day until day 10.

NaF treated group (NaF):

Mice were provided fluoridated drinking water from the 1st day until day 10. Sodium fluoride (NaF) was administered in drinking water at a concentration of 100 ppm. This corresponds to an approximate dose of 16.67 mg/kg body weight/day, assuming an average water intake of 5 mL/day for a 30 g mouse.

Preparation of Withania coagulans extract:

Paneer Dodi (*W. coagulans* fruit) was purchased from the market. Standard protocols were used to extract *W. coagulans.* The aqueous extract of the plant was prepared at a 1:4 ratio by mixing 20g of finely powdered plant material with 80ml of distilled water. The mixture was placed in a covered flask and set in a circulating water bath at medium to high speed for 4 hours at 65°C. Afterward, the solution was transferred to Falcon tubes and centrifuged at 7000 rpm for 25 minutes. The supernatant was collected and sonicated at 65°C for 20 minutes in a digital ultrasonic cleaner. Finally, the mixture was filtered through Whatman filter paper no. 1 to remove any undissolved particles. The prepared aqueous extract was then stored in an airtight vial to prevent contamination.

Preparation of NaF:

NaF was gained in the form of powder from market. 100ppm stock solution of sodium fluoride was prepared by dissolving 50 mg of NaF in 500 ml of water.

Animal dissection and organ recovery:

On the 10th day, animals were subjected to dissection to extract kidney specimens. The retrieved organs were then immersed in acidified formyl ethanol for 48 hours for fixation. Subsequently, the fixed organs were subjected to procedures for embedding in wax, sectioning, and staining with Hematoxylin and Eosin to produce permanent slides for histological and micrometric examinations.

Blood Collection:

After the mice were sacrificed, blood samples from each group were obtained for complete blood count (CBC) to evaluate hematological parameters as well as serum urea and creatinine levels to perform renal function test (RFTs).

Digital photography and processing:

Histological slides of kidney from each group were inspected using a trinocular research microscope at magnifications of 10x and 40x. Observations were documented through photographs and subsequent adjustments were made for color, contrast, cropping, and labeling.

Micrometric studies:

10X and 40X images of randomly selected potions of kidney from every group were used. Measurements were recorded from different areas for each section using digital scales in CORALdraw11. The micrometric data was utilized to compute group means ± SEM values. Thus, measurements were taken for CSA of glomeruli, thickness of peri glomerulus space and mean number of podocytes and glomeruli. CSA was calculated utilizing the given formula:

$$CSA = (length \times width /4)\ \pi$$

Statistical analysis:

Analysis of resulted was performed using SPSS 17.0 software. Significance was calculated using one way. ANOVA test followed by post -hoc analysis using Tukeys HSD. The results were taken into account and considered statistically insignificant when the P value was greater than 0.05, and significant when the P value was less than 0.05.

RESULTS

Histopathalogical Analysis:

The control group displayed typical features of normal kidney tissue under microscopic examination. This included the glomeruli being appropriately positioned with a thin well-defined gap separating them from the Bowman's capsule. The endothelial lining of the Bowman's capsule was intact and there were no indications of cell death or apoptosis. Glomeruli were of normal size and shape indicating healthy nephron. Blood capillaries and arterioles were also visible, distributed between the outer margins of adjacent tubular sections. No abnormalities in tissue structure were observed (Fig.1A and 1B).

The Group treated with NaF displayed several noticeable pathological changes in contrast to control group that include increased space between glomeruli and Bowman's capsules, endothelial damage in bowman’s capsule with excessive endothelial cell death compromising the endothelial layer. Additionally, there was shrinkage of glomeruli that indicates reduction in glomeruli size and potential loss of nephrons. Kidney tissues showed the damage signs including the formation of vacuoles in the tubular epithelium and damage to the brush border of microvilli on the luminal surface of PCT epithelial cells (Fig.1C and 1D).

The group treated with *Withania coagulans* after exposure to NaF exhibited ameliorative changes compared to the NAF group. Although some similarities were observed, such as increased space between glomeruli and Bowman's capsules, damage to the glomerular basement membrane and alterations in glomerular structure the severity of these effects was notably diminished in the NWCT group. Furthermore, endothelial cell apoptosis was present in the NWCT group; however, the extent of apoptosis and the widening of central spaces containing endothelial cell debris were less pronounced in contrast to the group treated with NaF. Additionally, while vacuolization in the tubular epithelium and damage to the brush border of microvilli on the luminal surface of PCT epithelial cells were noticed in the NWCT group, these effects were mitigated in contrast to the NaF-treated group (Fig.1E and 1F).

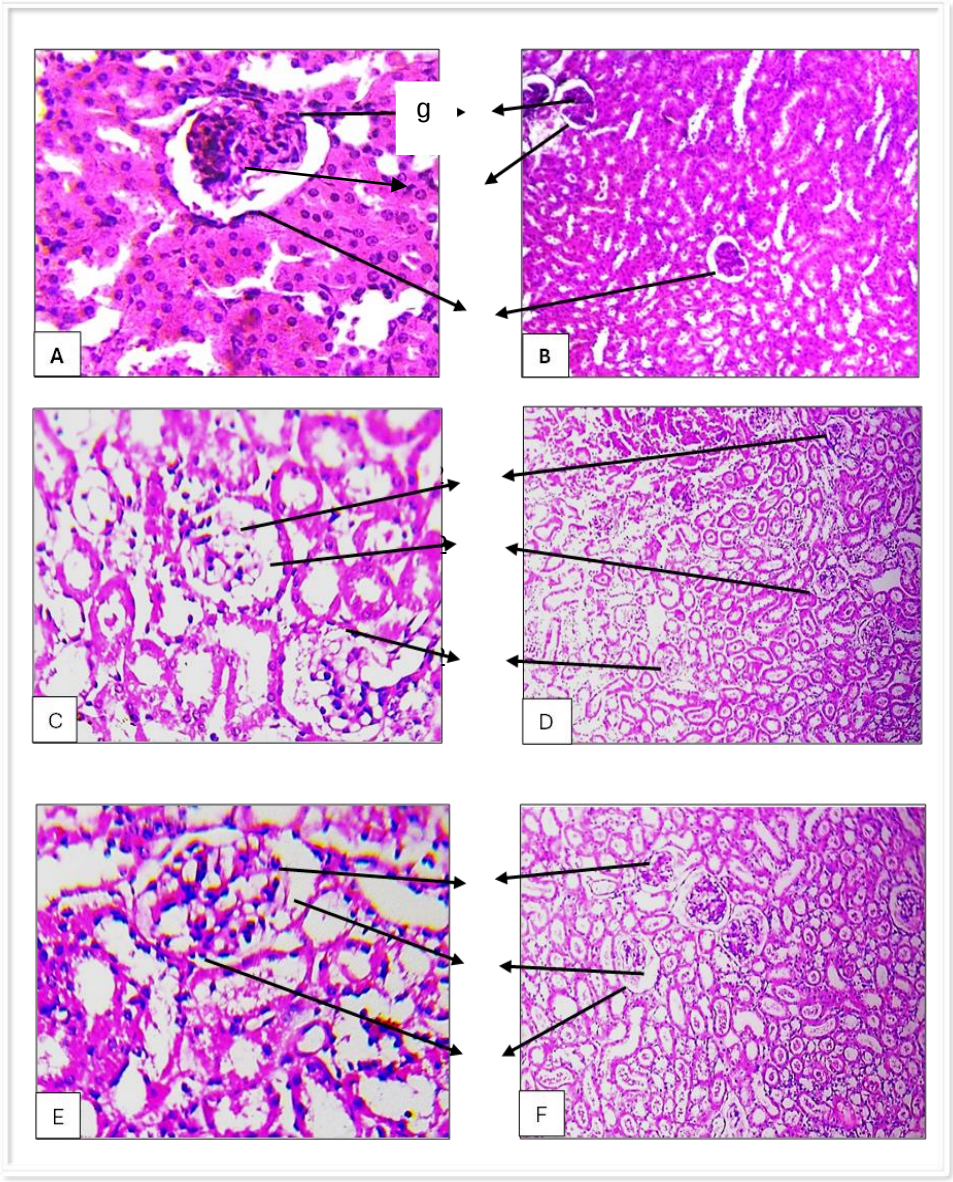

Figure 1. Microscopic images of a section from mice kidney A(Co), C(NaF), E(NWCT) at 40x magnification and B(Co), D(NaF), F(NWCT) at 10x magnification. g: glomeruli of normal size and shape, g1: shrunken glomerulus, g2 rehabilitated glomerulus. S: Thin well defined peri glomeruli space, S1: widened peri glomeruli space, S2: recovered peri glomeruli space. b: Intact endothelial lining of bowman's capsule, b1: cell death in endothelial lining of bowman's capsule, b2: recovered endothelial lining of bowman's capsule

Micrometric results:

The analytical result of kidney parameters showed significant differences between the Control, NaF, and NWCT groups, as indicated by ANOVA and Tukey HSD tests (Table1).

CSA of glomeruli:

The highest mean CSA of glomeruli was recorded in the control group (2358.90 ± 123.38 µm²), followed by the NWCT group (2346.17 ± 121.60 µm²), and the lowest in the NaF group (1452.24 ± 96.23 µm²). Statistical analysis (ANOVA) showed significant variation among the groups ($P \leq 0.0001$). The post hoc analysis indicated non-significant difference ($P > 0.05$) between the NWCT and Control groups (mean difference = 12.73 µm², $P = 0.997$). However, there was a highly significant difference ($p \leq 0.0001$) between the NaF and Control groups (mean difference = 906.67 µm², $P = 0.000$) (Fig.1, Table1).

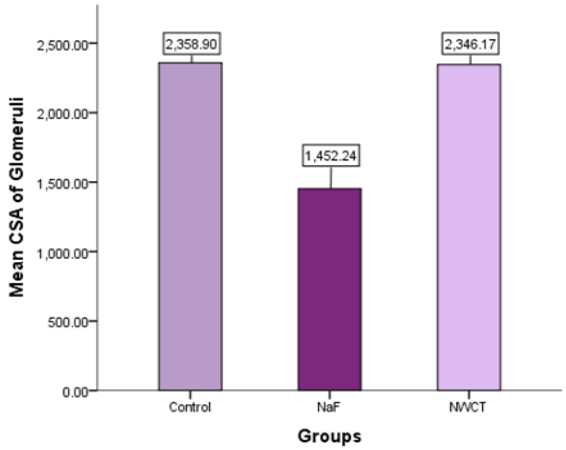

Figure 2. Mean CSA of Glomeruli among various groups.

Thickness of Periglomerular Space:

The highest mean thickness of the periglomerular space was recorded in the NaF group (14.99 ± 0.59 µm), followed by the NWCT group (7.69 ± 0.35 µm), and the lowest in the Control group (6.75 ± 0.30 µm). Statistical analysis (ANOVA) showed significant variation among the groups (P ≤ 0.0001). The post hoc analysis indicated no significant difference (P > 0.05) between the NWCT and Control groups (mean difference = 0.94 µm, P = 0.282). However, there was a highly significant difference (P ≤ 0.0001) between the NaF and Control groups (mean difference = 8.25 µm, P = 0.000) (Fig.2, Table1).

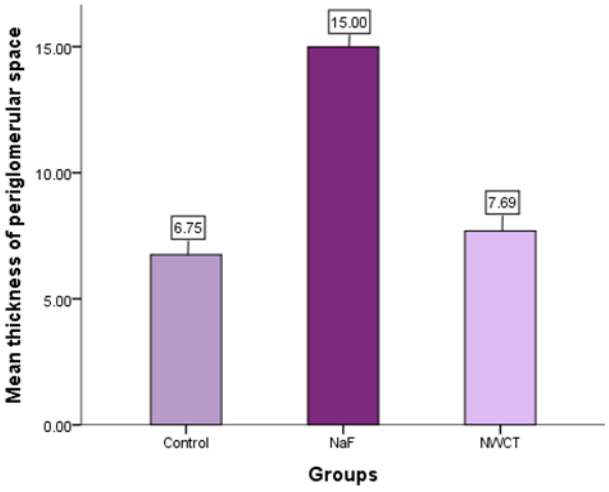

Figure 3. Mean thickness of periglomerular space among various groups.

Number of Podocytes:

The highest mean number of podocytes was recorded in the Control group (35.93 ± 1.13), followed by the NWCT group (24.33 ± 1.12), and the lowest in the NaF group (17.07 ± 1.15). Statistical analysis (ANOVA) showed significant variation among the groups (P ≤ 0.0001). The post hoc analysis indicated a highly significant difference (P ≤ 0.0001) between the NWCT and Control groups (mean difference = 11.60, P = 0.000), and between the NaF and Control groups (mean difference = 18.87, P = 0.000) (Fig.4, Table1).

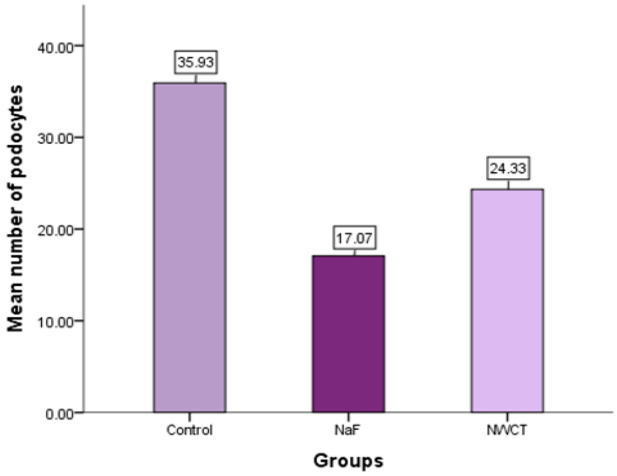

Figure 4. Mean number of podocytes of control, NaF and NWCT treated groups after 10 days of treatment of adult male mice**.**

Number of Glomeruli:

The highest mean number of glomeruli was recorded in the Control group (2.60 ± 0.13), followed by the NWCT group (2.47 ± 0.17), and the lowest in the NaF group (1.53 ± 0.19). Statistical analysis (ANOVA) showed significant variation among the groups (P ≤ 0.0001). The post hoc analysis indicated no significant difference (P > 0.05) between the NWCT and Control groups (mean difference = 0.13, P = 0.835). However, there was a highly significant difference (P ≤ 0.0001) between the NaF and Control groups (mean difference = 1.07, P = 0.000) (Fig.5, Table1).

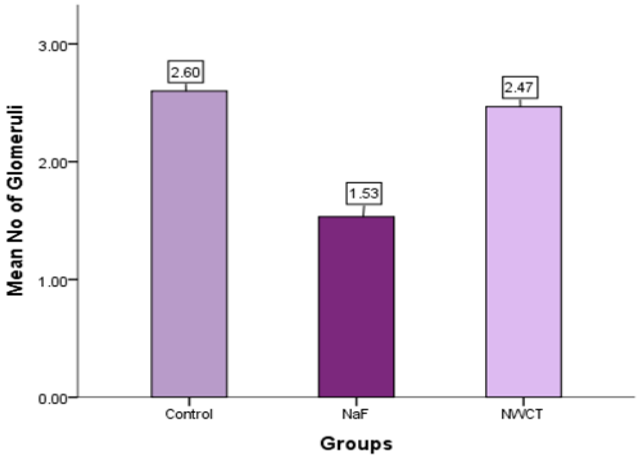

Figure 5: Mean number of glomeruli of control, NaF and NWCT treated groups after 10 days of treatment of adult male mice.

Table 1. Micrometric Variations of the Cross-sectional Area of Glomeruli, Thickness of Periglomerular Space, Number of Podocytes, and Number of Glomeruli per Unit Area in Mice Kidney.

Micrometric Parameters	Control	NaF	NWCT	ANOVA (P-value)
Mean CSA of glomeruli (µm²)	2358.90 ± 123.38[a]	1452.24 ± 96.23[b]	2346.17 ± 121.60[a]	P ≤ 0.0001***
Thickness of periglomerular space (µm)	6.75 ± 0.30[a]	14.99 ± 0.59[b]	7.69 ± 0.35[a]	P ≤ 0.0001***
Mean number of podocytes /unit area (2500 µm²)	35.93 ± 1.13[a]	17.07 ± 1.15[b]	24.33 ± 1.12[c]	P ≤ 0.0001***
Mean number of glomeruli /unit area (2500 µm²)	2.60 ± 0.13[a]	1.53 ± 0.19[b]	2.47 ± 0.17[a]	P ≤ 0.0001***

Values are Mean±SEM. N = 45. Groups sharing the same letter (a, b, c) are not significantly different (P > 0.05).

Hematological parameters:

Many hematological alterations were observed during the experimental period. Following NaF exposure (10 days), significant decreases were noted in red blood cell (RBC) count, hemoglobin (Hb) percentage, packed cell volume (PCV), mean corpuscular volume (MCV). In contrast, mean corpuscular hemoglobin concentration (MCHC) and platelet Count increased significantly in the NaF group. Additionally, a significant increase in total leukocyte count (TLC) and neutrophil percentage was observed. Treatment with WCE significantly restored these values aligning closely with control values.

Table 2: Protective effects of *Withania coagulans* extracts on NaF induced pathologies on CBC.

Parameters	Control	NaF	NWCT
RBC (×10^6/ul) ***	8.453 ± 0.1919[a]	6.903 ± 0.1922[b]	8.035 ± 0.1638[a]
TLC (×10^3/ul) ***	7.465 ± 0.1826[a]	9.300 ± 0.8410[b]	5.057 ± 0.2479[c]
% Neutrophils ***	2.843 ± 0.0641[a]	12.915 ± 1.6623[b]	11.143 ± 0.8572[b]
% Lymphocytes***	71.590 ± 0.7959[a]	85.925 ± 1.7192[b]	80.000 ± 1.0801[a]
% Monocytes ***	0.500 ± 0.0540[a]	2.513 ± 0.2105[b]	3.575 ± 0.2175[c]
% Eosinophils ***	0.110 ± 0.0041[a]	2.263 ± 0.1546[b]	2.130 ± 0.1760[c]
Hb (g/dl) ***	13.450 ± 0.2108[a]	11.413 ± 0.2004[b]	13.593 ± 0.4639[a]
HCT (PCV) (%) **	43.393 ± 0.2605[a]	36.158 ± 1.3167[b]	43.098 ± 1.7240[a]
MCV (fl)***	56.005 ± 0.3456[a]	47.378 ± 0.6993[b]	50.703 ± 0.7711[b]
MCH (pg)*	17.583 ± 0.1625[a]	20.500 ± 1.3058[b]	16.258 ± 0.1897[a]
MCHC (g/dl) ***	31.313 ± 0.2175[a]	41.445 ± 2.3495[b]	32.528 ± 0.1909[a]
Platelet Count (×10^3/ul)***	699.428 ± 10.6522[a]	1895.150 ± 9.1464[b]	741.825 ± 24.3187[a]

Values are Mean±SEM. N = 45. Groups sharing the same letter (a, b, c) are not significantly different (P > 0.05).

Renal function test (RFTs):

Serum urea and creatinine levels were highest in the NaF group, followed by the NWCT and control groups. ANOVA showed significant variation (P ≤ 0.0001). Urea levels demonstrated significant differences between the Control and NaF groups (P = 0.000). Serum urea levels were reduced by 18% (25.46 mg/dL) in the NWCT group compared to the NaF group (28.69 mg/dL). Creatinine levels also differed significantly between the Control and NaF groups (P = 0.000). Serum creatinine was reduced by 26% (0.84 mg/dL) in the NWCT group compared to the NaF group (1.14 mg/dL), WCE treatment reduced urea and creatinine levels demonstrating a protective effect on kidney function.

Table 3: The comparison of mean values for serum urea and creatinine levels among the three studied groups.

Parameters	Control	NaF	NWCT	ANOVA (P-value)
Urea (mg/dL)	17.22 ± 1.24	28.69 ± 0.74	25.46 ± 0.39	P ≤ 0.0001
Creatinine (mg/dL)	0.36 ± 0.07	1.14 ± 0.11	0.84 ± 0.06	P ≤ 0.0001

Values are Mean±SEM. N = 45. Groups sharing the same letter (a, b, c) are not significantly different (P > 0.05).

DISCUSSION

Fluoride is one of the widespread toxicants that originates from natural and industrial sources [11]. Kidney is a region for possible fluoride toxicity, as it can be subjected to a comparatively high level of fluoride[8].

The histopathological analysis of current study has shown that NaF is extremely toxic to the kidneys of exposed animals. It is observed that the implementation of sodium fluoride treatment to adult albino mice cause massive degenerative changes (if compared to normal mice) including glomerular degeneration (marked shrinkage with expanded Bowman's capsule). The findings of this study align with those of [12] who had illustrated that, in kidneys of adult albino rat treated with NaF showed atrophy of glomeruli with expanded Bowman's capsule. The present study also noticed damage signs including the formation of vacuoles in the tubular epithelium and apoptosis in endothelial cells. These damage signs have been detected by some authors [8] in mice exposed to NaF.

Additionally, many changes in various micrometric measurements, such mean cross-sectional area (CSA) of glomeruli, thickness of periglomerular space and mean number of podocytes and glomeruli per unit area were noticed. Several hematological changes were also observed including significant decreases in red blood cell (RBC) count, hemoglobin (Hb) percentage, packed cell volume (PCV) and mean corpuscular volume (MCV). Similar changes have also been noted in hematology of experimental animal on exposure to NaF [13]. In contrast, mean corpuscular hemoglobin concentration (MCHC) increased significantly in the NaF group which may suggest a compensatory response to the reduced red blood cell mass. Additionally, a significant increase in total leukocyte count (TLC) and neutrophil percentage was observed, reflecting an inflammatory response associated with NaF exposure.

Kidney profile tests have shown significantly elevated level of creatinine and urea in NaF treated group in contrast to control group. Resembling changes have also been noted in rat's Kidney upon exposure to NaF [14]. The aim of this study was to investigate the protective effects of *W. coagulans* during NaF exposure; whether it provides protection or not? As far as we know there is no previous study about the shielding effect of *W. coagulans* on the kidney tissue damage caused by NaF. However the histopathological and biochemical findings indicate that WCE plays a significant role in ameliorating kidney damage caused by NaF.These ayurvedic effects of WCE appear to be mediated through several potential mechanisms. Withania coagulans is rich in antioxidants like withanolides, flavonoids, and alkaloids, which neutralize harmful reactive oxygen species (ROS) and reduce oxidative stress. By protecting kidney tissues from sodium fluoride damage, it enhances antioxidant enzymes crucial for cellular defence. Sodium fluoride (NaF) causes kidney cell death, including glomerular shrinkage and endothelial damage. Withania coagulans helps protect against this by stabilizing mitochondria and reducing the activity of apoptosis-related enzymes like caspases, limiting cell damage[15]. The group treated with *Withania coagulans* (WCE) fruit extract exhibited milder pathological alterations as compared to group

treated with NaF. Although, both groups showed some similarity such as alterations in glomerular and tubular structures, the severity of these changes was notably milder in the WCE-treated group. Furthermore, *Withania coagulans* extract displayed significant protective effects on micrometric parameters, restoring glomerular size and structure closer to control levels. It reduced the thickening of the periglomerular space and helped preserve podocyte count, mitigating the damage caused by NaF exposure. *Withania coagulans* extract also showed a protective effect on hematological parameters by increasing RBC count, Hb levels, and reducing the abnormal increase in WBCs caused by NaF exposure. It helped restore parameters like HCT, MCV, MCH, and platelet count towards normal levels. Based on these results, it can be easily concluded that WCE might protect against renal damage caused by fluoride.

This study was conducted with a relatively small cohort of 45 mice which may limit the generalization of findings. The 10day exposure and treatment period might also not fully be able to assess the chronic effects of fluoride toxicity or long term WCE efficacy. Moreover these were specie specific response which might differ for humans and other animals due to physiological and metabolic differences.

CONCLUSIONS

Withania coagulans (WCE) fruit extract have potential nephroprotective effects against sodium fluoride (NaF)-induced toxicity in mice. Histological examination revealed that WCE treatment reduced the damage to kidney caused by NaF exposure. Micrometric and biochemical studies further supported these findings, showing significant improvements in parameters in the WCE-treated group compared to the group exposed to NaF. Future research could investigate the molecular mechanisms of WCE's protective effects and assess its potential to reduce fluoride toxicity in other organs.

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CONFLICT OF INTERESTS

None.

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