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Ameliorative Role of the Extract of *W. coagulans* (WCE) Fruits Against Sodium Fluoride-Induced Hepatotoxicity in Mice

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Areeja Fatima[1], Aima Iram Batool[1*], Javaria Ikram[1], Uswa Shoaib[1], Aiman Zahra[1], Muhammad Fayyaz-ur-Rehman[2], Naima Huma Naveed[3], Iram Inayat[1], Namra Tariq[1],Hakim Bibi [1], Syed Skinadar Habib[1], Syed Gohar Taqi Kazimi[4], Muhammad Ali Kanwal[1]

[1]Department of Zoology, University of Sargodha, Pakistan

[2]Department of Chemistry, University of Sargodha, Pakistan

[3]Department of Botany, University of Sargodha, Pakistan

[4] Government Ambala Muslim Graduate College, Sargodha, Pakistan

***Corresponding author:**
Dr. Aima Iram Batool
Department of Zoology
University of Sargodha
40100
Sargodha, Pakistan
Phone: +92 305 7600599
E-mail: aima.iram@uos.edu.pk

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ABSTRACT

Purpose: To investigate the potential protective effects of *Withania coagulans* extract along with the histological, biochemical, and micrometric alterations in the liver of mice exposed to NaF.

Methods: Forty-five male albino mice were distributed into three groups: control group was given saline and a balanced meal without any additional NaF. The NaF and *Withania coagulans* combination treatment (NWCT) group was given the *Withania coagulans* extract in addition to fluoridated drinking water. Sodium fluoride treated group (NaF) was provided with fluoridated drinking water for 10 days, after which they were euthanized. After 10 days, every animal was killed in order to remove their organs. Each group's liver was taken out and examined under a microscope. Micrometric measurements were also taken from various sections of each group. Additionally, blood samples from each group were also obtained to estimate the level of bilirubin and alkaline phosphatase in order to perform liver function tests (LFTs), along with complete blood count (CBC) to evaluate hematological parameters.

Results: In NaF treated group various histopathological alterations in the liver (such as enormously enlarged hepatocytes, disruption of hepatic cords) were observed. Significant micrometric changes accompanied pathological signs. Biochemical results also displayed significant difference between the three groups. The group treated with *Withania coagulans* exhibited milder pathological changes in contrast to the NaF group.

Conclusions: These findings lead to the conclusion that administration of sodium fluoride caused degenerative morphological changes and damage to the liver and that *Withania coagulans* extract possesses a shielding effect against NaF-induced hepatotoxicity.

Key-words: *Sodium fluoride; Withania coagulans; Hepatotoxicity; Hematology*

INTRODUCTION

Because of their high electronegativity fluoride ions are highly reactive in the environment. Sodium fluoride (NaF) is widely recognized and mostly used fluorinated compound in production of different products especially for dental hygiene.[1] Sources of fluoride can be natural or manmade, including fluoridated foods, pesticides, groundwater, medicines and released vapors from fluoride-using industries. Intermediate level of fluoride ingestion promotes bone development and helps prevent tooth decay. But ingestion of high dose of fluoride may have detrimental effects on well-being of humans and animals.[2] The biological effects of fluoride depend upon quantity, exposure duration and metabolic processing of fluoride consumed.[3] After the fluoride is ingested, it is absorbed by gastrointestinal tract, circulates in body and mainly rises by mineralized tissues and to a lesser degree by soft tissues. The residual amount is removed in urine. Plasma concentrations of fluoride increase within 10 minutes of absorption reaching highest peak after one hour and then take few hours to return to baseline levels. Fluoride is then stored in the skeleton or removed by the kidneys. Once fluoride assimilate into bone it is slowly excreted with a half-life of 120 weeks in adults and 70 weeks in children.[4]

Fluoride poisoning causes disturbances in many biochemical parameters.[5] Fluoride consumption not only weakens bones and teeth but also interferes with organ function.[6] One target organ that is influenced by high level of fluorides is liver. It is particularly vulnerable to fluoride toxicity because it is active site of metabolism.[7]

Higher degree of fluoride concentration in drinking water elevates the serum level of liver function enzymes and causes serious histological alterations in liver.[8]

Researchers are seeking for natural active compounds which can exhibit powerful biological activity and therapeutic effects against damage induced by a toxicant.[5] The fruit extract of *Withania coagulans*, which belongs to the Solanaceae family, have attracted considerable attention due to their effectiveness in treating chronic diseases. It is commonly known as Indian rennet, vegetable rennet (English), paneer dodi (Hindi) and Ning gu shui qie (Chinese). The fruit extract has demonstrated potential activities, including anticancer, wound healing, antihyperglycemic, and hypolipidemic effects.[9] The aim of this study was to investigate the toxic effect of sodium fluoride on liver of albino mice by using histopathological, biochemical and micrometric study and to investigate the possible ameliorative effects of *Withania coagulans* during fluoride exposure if it is protective or not.

MATERIAL AND METHODS

Preparation of NaF solution

NaF was purchased in the form of powder from market. 100ppm stock solution of sodium fluoride was prepared by dissolving 50mg of NaF in 500mL of water which was then used for administration to the mice.

Preparation of the Withania coagulans Aqueous Fruit 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 4hours 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.

Animals

Forty-five mature and healthy male albino mice (*Mus musculus*), weighing between 30-35grams and of reproductive age, were purchased for this study. These animals were placed under standard laboratory conditions in separate plastic cages gauzed with corrosion-resistant steel in the animal house of Department of Zoology, University of Sargodha.

Experimental design

The mice were subdivided into 3 groups as following:

Group 1 (control group)

This group was given saline and balanced diet for 10 days.

Group 2 (NaF and *Withania coagulans* combined treatment group)

Mice were treated with 0.1mL of pure *Withania coagulans* extract twice a day and fluoridated drinking water for 10 days.

Group 3 (NaF only group)

Mice were administrated fluoridated drinking water from the 1st day until day 10 after which they were killed. Following the 10-day experimental period mice were killed and the liver was instantly removed and dissected out carefully to obtain the fresh specimens.

Preparation for microscopy

The liver specimens were then fixed in acidified formyl ethanol for 48 hours. After following standard histological protocols, the fixed organs were subjected to procedures for embedding in wax, sectioning, and staining with Hematoxylin and Eosin to produce permanent slides for microscopic examinations.

Blood Collection

After the mice were killed, blood samples from each group were collected for complete blood count (CBC) to evaluate hematological parameters and to perform liver function tests to assess the levels of, bilirubin, globulin, albumin, ALT (Alanine aminotransferase), AST (Aspartate aminotransferase) and Alkaline phosphatase.

Digital photography and processing

Histological slides of liver from each group were inspected using a trinocular research microscope at magnifications of 10X and 40X. Images were further edited to enhance clarity. Labels were also added for better understanding of the images.

Micrometric studies

10X and 40X images of randomly selected sections of liver from each group were used. CORAL draw 11 was used for micrometric analysis. Acquired data was utilized to evaluate group mean ± SEM values. Measurements were taken for CSA of central vein, CSA of hepatocytes and CSA of nuclei of hepatocytes. Formula used for calculating CSA is as follows:

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

Statistical analysis

Analysis of resulted was performed using SPSS 17.0 software. Significance was calculated using ANOVA test followed by post-hoc analysis using Tukey's HSD. Results were 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 Results

In the control group, normal liver histology was evident. This included the presence of hepatic cords, consisting of a single layer of hepatocytes, alternating with sinusoidal spaces radiating from a central blood vessel to the edges of the hepatic lobules. Hepatocytes showed no significant changes, with no evidence of cell death, degenerative changes, hypertrophy, or cytoplasmic vacuolations (Fig.1A and 1B).

The NaF group displayed several noticeable pathological alterations. Hepatocytes showed signs of damage that include cytoplasmic vacuolations and hepatocellular hypertrophy, which is indicated by an increase in the size of hepatocytes. The central vein appeared dilated or enlarged with a prominent lumen. Moreover, the examination also showed vascular degeneration and the dilation of sinusoidal spaces, coupled by a significant disruption of hepatic cords, leading to the formation of vacuolated spaces within the liver tissue. Additionally, numerous hepatocytes showed nuclear destruction, that is the clear evidence of cellular necrosis (Fig.1C and 1D).

The NWCT group exhibited some pathological changes similar to the NaF group, such as hepatocellular hypertrophy, characterized by increased hepatocyte size. However, the damage was less severe in NWCT group compared to the NaF group, suggesting that *Withania coagulans* treatment might have protective effect. Hepatocytes in the NWCT group still showed dedifferentiation, such as enlarged nuclei. While examination revealed dilation of sinusoidal spaces and disruption of hepatic cords, leading to the formation of vacuolated spaces within the liver tissue, the extent of these alterations seemed reduced in the NWCT group. Furthermore, although some hepatocytes exhibited nuclear destruction, indicating evidence of cellular necrosis, the prevalence of this phenomenon was diminished in the NWCT group (Fig.1E and 1F).

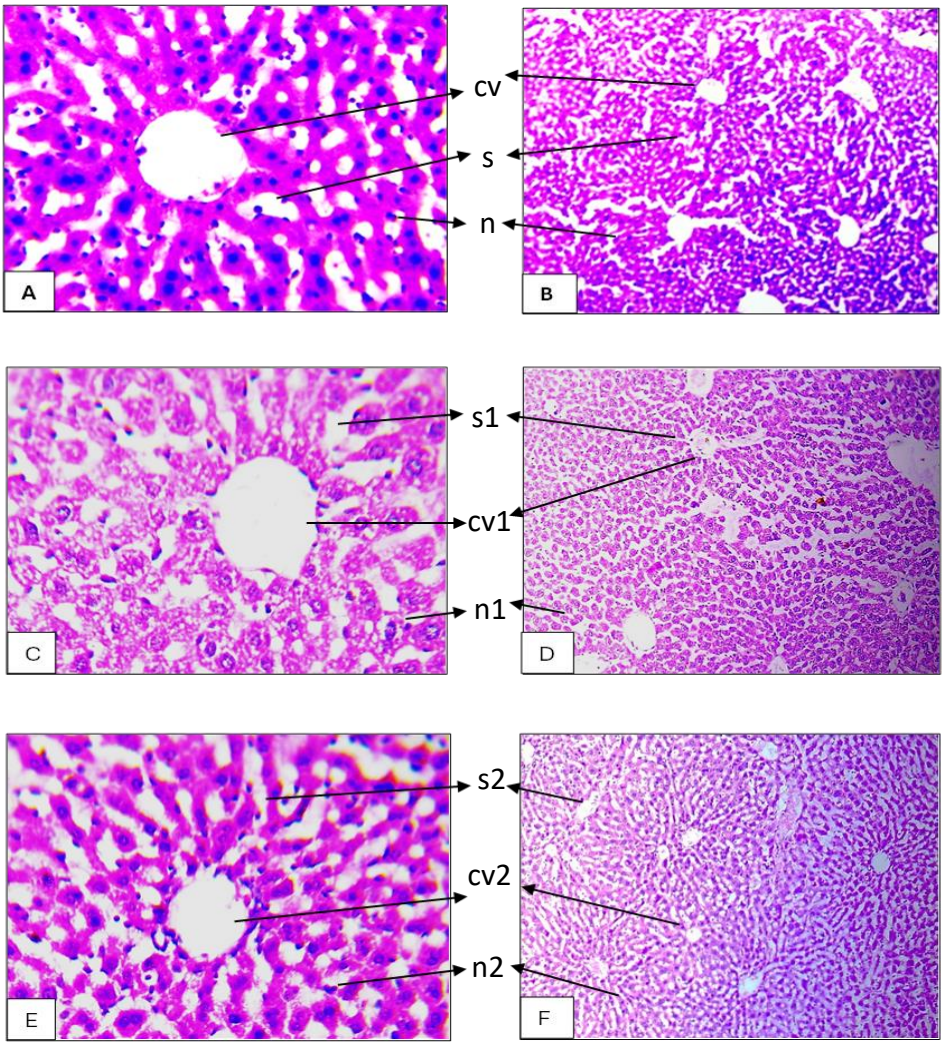

Figure 1. Microscopic images of a section from mice liver A(Co), C(NaF), E(NWCT) at 40x magnification and B(Co), D(NaF), F(NWCT) at 10x magnification. cv: normal central vein, cv1: enlarged central vein, cv2: reduced central vein enlargement. n: normal nuclei, n1: enlarged nuclei, n2: recovered nuclei, s: normal sinusoidal spaces, s1: Sinusoidal dilation, s2: less pronounced sinusoidal dilation.

Micrometric Results

The analytical result of liver parameters (CSA of hepatocytes, CSA of the nucleus of hepatocytes, and CSA of the central vein) showed significant differences between the Control, NaF, and NWCT groups, as indicated by ANOVA and Tukey HSD tests (Table1).

CSA of hepatocytes

The highest mean CSA of hepatocytes was recorded in the NaF group, followed by the Control group, and the lowest in the NWCT group. Statistical analysis (ANOVA) showed significant variation among the groups ($P \leq 0.0001$) (Figure 2) (Table1).

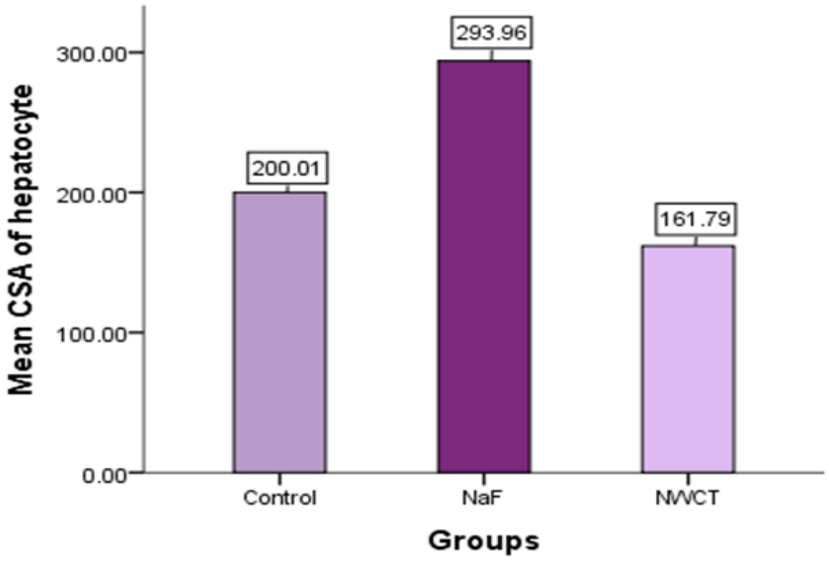

Figure 2. Mean CSA of Hepatocytes among various groups.

CSA of hepatocyte nuclei

For the CSA of hepatocyte nuclei, NaF group had the highest mean , followed by the Control group), and the lowest in the NWCT group (Figure 3). Statistical analysis (ANOVA) showed significant variation among the groups (P ≤ 0.001). (Table1).

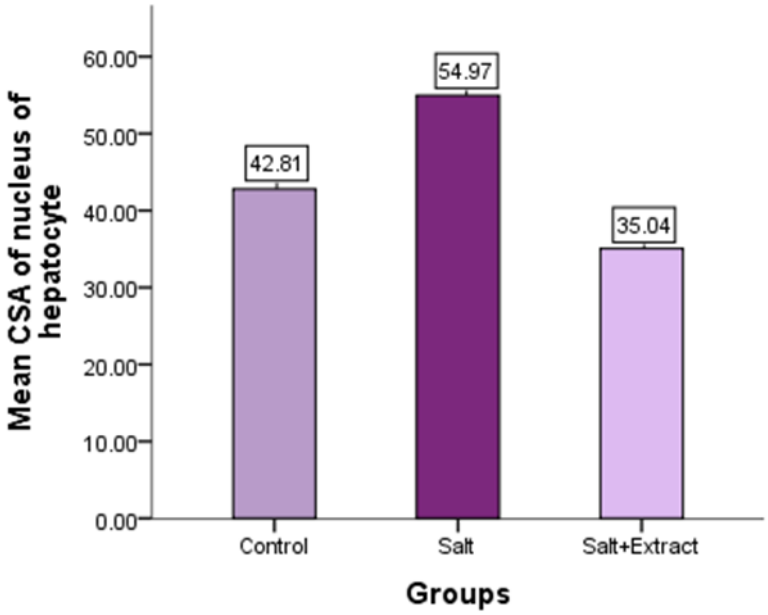

Figure 3. Mean CSA of Hepatocytes nuclei among various groups.

CSA of the central vein

The highest mean CSA of the central vein was recorded in the NaF group, followed by the NWCT group, and the lowest in the Control group (Figure 4). Statistical analysis (ANOVA) showed significant variation among the groups (P ≤ 0.0001). (Table1).

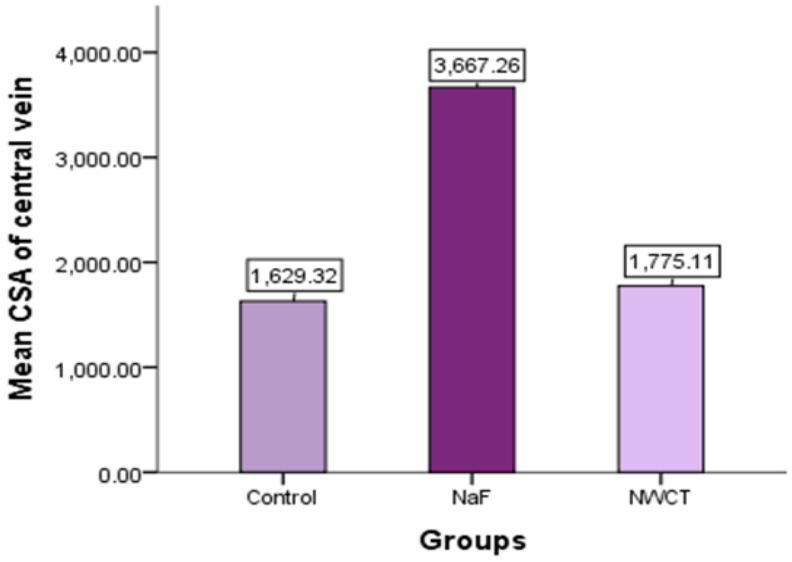

Figure 4. Mean CSA of Central vein among various groups.

Table 1. Micrometric Variations of the Cross-sectional Area of Hepatocytes, CSA of Nucleus of Hepatocytes (µm²) and

Micrometric Parameters	Control	NaF	NWCT	ANOVA (P-value)
CSA of Hepatocytes (µm²)	200.01 ± 6.51[a]	293.96 ± 21.12[b]	161.79 ± 9.55[a]	P ≤ 0.0001***
CSA of Nucleus of Hepatocytes (µm²)	42.81 ± 1.52[a]	54.97 ± 1.86[b]	35.04 ± 1.77[c]	P ≤ 0.0001***
CSA of Central Vein (µm²)	1629.32 ± 98.64[a]	3667.26 ± 306.94[b]	1775.11 ± 92.22[a]	P ≤ 0.0001***

CSA of Central Vein (µm²), in Mice liver.

Hematological parameters:

Many hematological alterations were observed during the course of the experiment. Following NaF exposure (10 days), significant decreases were noted in red blood cell (RBC) count, hemoglobin count (TLC) and neutrophil. (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

Table 2: Protective effects of Withania coagulans extracts on NaF induced pathologies on CBC (Complete blood cells).[1]

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]

[1]***= highly significant, **= highly significant, *= significant

Liver function tests

Serum bilirubin, ALT, AST, and alkaline phosphatase levels were highest in the NaF group, followed by the NWCT group, and lowest in the Control group. ANOVA showed significant variation among the groups ($P \leq 0.0001$). ALT levels were significantly different between the Control and NaF groups ($P = 0.000$), but not between the Control and NWCT groups ($P = 0.405$). AST and alkaline phosphatase levels were significantly higher in the NaF group compared to the Control ($P \leq 0.001$), with no difference between Control and NWCT. Total protein and albumin were lowest in the NaF group, with significant differences between groups ($P \leq 0.05$). Globulin was significantly higher in the NaF group compared to Control ($P = 0.021$), but there was no difference between the NWCT and Control groups (Table 3).

Table 3: The comparison between mean values of the three studied groups regarding liver function parameters.[2]

Parameters	Control	NaF	NWCT	ANOVA (P-value)
Bilirubin (mg/dL)	0.336 ± 0.004[a]	0.678 ± 0.011[b]	0.580 ± 0.014[c]	P ≤ 0.0001***
ALT (U/L)	29.33 ± 1.27[a]	38.86 ± 0.43[b]	31.00 ± 0.71[a]	P ≤ 0.0001***
AST (U/L)	67.93 ± 1.90[a]	87.60 ± 1.10[b]	67.22 ± 3.44[a]	P ≤ 0.0001***
Alkaline Phosphatase (U/L)	175.38 ± 2.41[a]	239.25 ± 7.82[b]	179.00 ± 11.17[a]	P ≤ 0.0001***
Total Protein (g/dL)	6.87 ± 0.19[a]	4.19 ± 0.18[b]	6.09 ± 0.08[c]	P ≤ 0.0001***
Albumin (g/dL)	3.03 ± 0.18[a]	2.30 ± 0.08[b]	2.92 ± 0.19[a]	P ≤ 0.05*
Globulin (g/dL)	2.67 ± 0.08[a]	3.22 ± 0.11[b]	2.81 ± 0.15[a]	P ≤ 0.05*

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

DISCUSSION

Fluoride's adverse effects causes severe impairment to the tissues of brain, liver, heart and lung.[10] Fluoride impairs liver function, leading to reduced metabolic activities, elevated serum levels in liver function tests, and inhibition of specific antioxidant enzymes and molecules.[11] Our study has shown that administration of fluoride increase the range of liver function enzymes in serum and induce harsh histological changes of the liver. Similar changes have also been noted in experimental animal on exposure to fluoride.[8,12]

The histological analysis of current study has shown that NaF cause severe damage to the liver of exposed animals. It is observed that the administration of sodium fluoride treatment to adult albino mice cause significant alterations (if compared to control mice) that include hepatocellular hypertrophy, dilated central vein. Moreover, vascular degeneration and the dilation of sinusoidal spaces were also observed. At cellular level, numerous hepatocytes displayed necrosis and cytoplasmic vacuolations.[13,14]

Additionally, many changes in various micrometric measurements, such mean cross-sectional area (CSA) of hepatocytes, CSA of the nucleus of hepatocytes, and CSA of the central vein 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.[15] 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.

Liver function tests have shown significantly elevated range of serum bilirubin, ALT, AST, globulin and alkaline phosphatase in the NaF treated group in contrast to control group. In contrast total protein and albumin levels were significantly lower in the NaF group as compared with control group. Our results are consistent with previous studies.[3,16]

The aim of this study was to determine the protective effects of *W. coagulans* against hepatotoxicity during NaF exposure. No previous study about the shielding effect of *W. coagulans* on the liver tissue damage caused by NaF. 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 hepatocellular hypertrophy, formation of vacuolated spaces and dilation of sinusoidal spaces 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 the structure of hepatocytes and the central vein closer to control levels. *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.

In the LFT results, *Withania coagulans* displayed a protective role by significantly lowering serum bilirubin, ALT, AST, globulin and alkaline phosphatase levels in the NWCT group compared to the NaF group. These results suggest that *Withania coagulans* mitigates liver damage induced by sodium fluoride, helping restore liver function to near-normal levels.

CONCLUSIONS

Withania coagulans (WCE) fruit extract have potential hepatoprotective effects against sodium fluoride (NaF)-induced toxicity in mice. Histological examination revealed that WCE treatment reduced the liver damage 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.

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CONFLICT OF INTERESTS

None.

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