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Mitigative Effect of *Vitex Negundo* against Fluoride-induced Oxidative Stress Mediated Cardio and Nephrotoxicity

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ABSTRACT

The present study investigated the mitigative effect of Vitex negundo against sodium fluoride (NaF) induced cardio and nephrotoxicity. The hydroalcoholic extract of Vitex negundo leaves (HAEVNL) was prepared by the maceration method. Group I, Group II, and Group III served as normal, toxic, and plant control groups in the treatment schedule. Group IV and V (200 and 400 mg/kg b.wt, p.o) served as treatment groups. Group II, IV, and V treated with NaF (100ppm) through drinking water for 4 weeks. Cardiac and kidney parameters such as LDH, CK-MB, Lipid profile, Creatinine, Urea, and Uric acid were estimated. The heart and kidney tissues LPO, GSH, SOD, and CAT levels and histopathological studies were performed. Phytochemical investigation showed the alkaloids, saponins, phytosterols, flavonoids, phenols, and tannins. Rats administered with NaF have demonstrated a significant rise in the LDH, CK-MB, TC, TG, LDL-C, VLDL-C, Creatinine, Urea, and Uric acid. Tissue LPO levels increased while there was a significant decrease in serum HDL-C and tissue SOD, GSH, and CAT levels. Treatment with HAEVNL showed effective recovery against NaF-induced cardio and nephrotoxicity. The histopathological evaluation also added to the benefits of the Vitex negundo leaves. The study concluded that Vitex negundo leaf extract showed a significant antioxidant and mitigative effect against fluoride- induced cardio and nephrotoxicity in rats.

Keywords: Vitex negundo; Sodium fluoride; Lipid profile; Kidney Profile; Oxidative stress

NOMENCLATURE

CAT	Catalase
CK-MB	Creatinine Kinase-MB
GSH	Reduced glutathione
HDL-C	High-density Lipoprotein-Cholesterol
LDH	Lactate Dehydrogenase
LDL-C	Low-density Lipoprotein-Cholesterol
LPO	Lipid Peroxidation
NaF	Sodium Fluoride
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TC	Total Cholesterol
TG	Triglycerides
VLDL-C	Very Low-density Lipoprotein- Cholesterol
WHO	World Health Organisation

1. INTRODUCTION

Fluorosis is a crippling disease caused by surfeit fluoride consumption through drinking water, edible products or industrial wastage over a prolonged period and affects all males and females.[1] WHO 2004, has specified that drinking water's maximum acceptable range of fluoride levels is ≤ 1.5 mg/L.[2] As per the Bureau of Indian Standards (BIS), the maximum permissible amount of fluoride in potable water is 1.5 mg/L.[3] Fluoride mainly exists as an ionic form in drinking water. So it is wholly absorbed in the intestine and hinders diverse metabolic pathways in animal and human beings.[1,4]

It is endemic and widely distributed globally, including in India, China, Pakistan, Egypt, South Africa, Jordan, Iraq, Turkey, Japan, Australia, and the USA.[4-6] India has been one of the most affected fluorosis countries since 1937 and 117 lakhs are suffering from high fluoride in drinking water.[7]

Excessive intake of fluoride is the leading cause of dental and skeletal fluorosis. Besides that, it has been showing toxic effects on the heart and kidneys. Fluoride induces oxidative stress by abnormally multiplying the reactive oxygen species formation and deteriorating the endogenous antioxidants. Oxidative stress damages the myocardial cell membrane and alters the antioxidant level, leading to cardiac diseases such as ischemic heart disease,

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hypertension, and atherosclerosis. High dose exposure to fluoride significantly increases inflammation and DNA destruction in the cardiac tissue.[8] NaF induces H9c2 cardiomyocyte cells apoptosis through a direct increase of intracellular ROS, downregulation of mitochondrial membrane potential, activation of caspases-3 and -9, altered Bcl-2/ Bax signaling pathway, and release of cytochrome c from mitochondria.[9] The fluid accumulation in the interstitial spaces, cloudy swelling, more vacuolisation of auricles, ventricle sarcoplasmic vacuolisation, fibrous necrosis, small hemorrhages and dissolution of nuclei in the myocardium after exposure to the high fluoride for two generations.[10]

The kidney is metabolically active and plays a dominant role in eliminating metabolites from the body. Exposure to excessive fluoride can damage kidney cell structure and proliferation, alter the mitochondrial respiratory chain complexes, and elevate the fusion of inner and outer mitochondrial membranes.[11] Sodium fluoride exposed rat's kidney histology showed destruction of the renal cortex, obligation of proximal and distal convoluted tubules, tubular cell vacuolisation and necrosis, and cell infiltration.[12] Abnormal expansion of renal capsule and tubule, glomeruli shrinkage, the altered epithelial structure of tubules with vacuolar cells, mild dilated interstitial small blood vessels, and development of interstitial capillaries with erythrocyte exudation were observed after exposure with fluoride.[13]

The fluoride-induced toxicity could be prevented by either reducing the ingestion or increasing the elimination of fluoride from the body. Defluoridation is the only existing option for removing fluoride concentration in water. Still, the techniques are costlier and unaffordable. Besides this, nutritional and medicinally active plant materials such as *Mangifera indica*[14], *Tamarindus indica*[15], *Limonia acidissima*[16], and a high protein diet[17] are dietary supplements for treating fluoride-induced changes in body metabolism. 43-kDa protein[18], quercetin[19], gallic acid[20], and curcumin[21] were reported for ameliorative effects against fluoride-induced oxidative stress. We reported the mitigative effect of cauliflower leaves[22] and *arthrospira platensis*[23] against fluoride-induced cardiotoxicity.

The plant *Vitex negundo* L. belongs to the Verbenaceae family and is popularly known as five leaved chaste trees in English and Nirgundi in Hindi. It is native to tropical Africa and Asia and is an aromatic small, slender tree of about 2-8 meters in height with quadrangular branches. It has been used extensively to prepare various ayurvedic formulations such as Vat Gajankush Ras, Mahavat Vidhwansan Ras, Dashamoola Taila, Ykrtptihara Lauha, Nirgundi Taila, Visatinduka Taila, and Trivikram Rasa.[24] No scientific report is available on *Vitex negundo* leaves significance against NaF-induced cardio and nephrotoxicity.

2. METHODOLOGY

2.1 Chemicals

Chemicals were procured from Himedia, Sigma Aldrich, SRL chemicals, and Research lab, India. Diagnostic kits from Coral diagnostics, India were used to estimate Heart and Kidney biochemical parameters.

2.2 Collection and Authentication

We collected *Vitex negundo* plant leaves from the Kushaiguda region, ECIL, Medchal District, Telangana State, India. Dr. L. Rasingam, Botanical Survey of India, Deccan Regional Centre, Hyderabad authenticated the plant material (Voucher number: BSI/DRC/2019-20/ Tech./293).

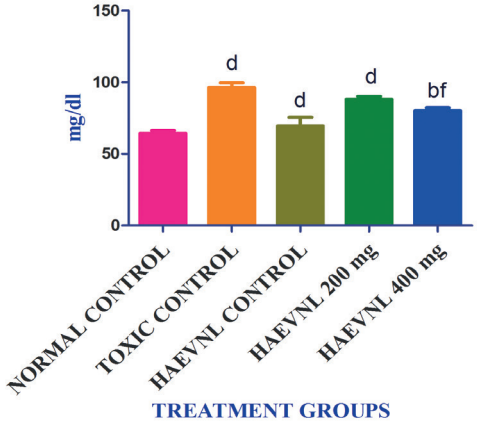
2.3 Extraction of *Vitex negundo* Leaves

The *Vitex negundo* leaves were cleaned with Milli-Q water and shade dried. The leaf powder was mixed with different water and ethyl alcohol ratios at 30:70, 50:50 and 70:30 respectively for seven days.

2.4 Phytochemical Evaluation

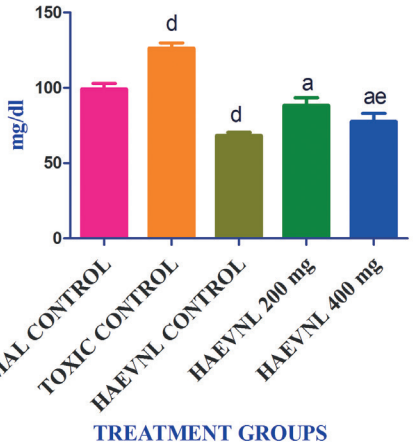
Table 1. Effect of HAEVNL treatment on change in body weight, food intake, and heart and kidney weights

S. No	Name of the group	Body weight (g)		Food intake (g/day)		Heart weight (g)	Kidney weight (g)
		1st day	28th day	1st day	28th day		
1	**Normal control**	204±4.17	235±5.48	15.04±0.32	18.07±0.56	0.77±0.01	1.49±0.06
2	**Toxic control**	231±6.3	185±5.8	18.70±0.45	7.79±0.17	0.68±0.03	1.15±0.06
3	**HAEVNL control**	215±8.06	259±4.79	15.78±0.21	18.63±0.32	0.75±0.02	1.65±0.10
4	**HAEVNL 200 mg**	215±4.83	241±7.26	15.71±0.45	18.58±0.22	0.70±0.02	1.61±0.05
5	**HAEVNL 400 mg**	224±9.17	254±4.17	16.41±0.36	19.03±0.51	0.74±0.02	1.68±0.11



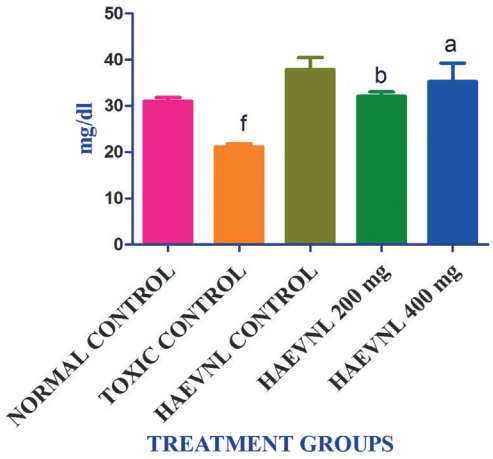
(a)

(a) Effect of HAEVNL on Serum Tiglycerides



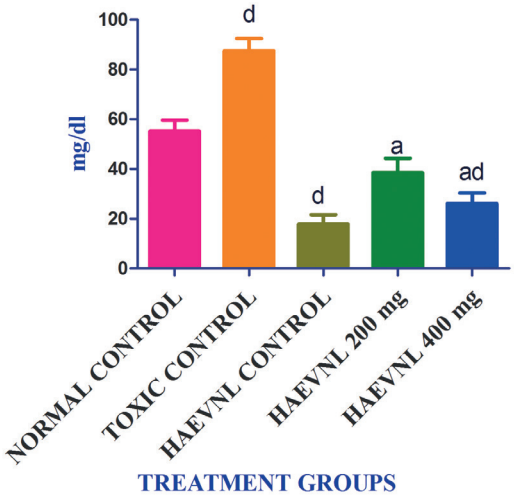
(b)

(b) Effect of HAEVNL on Serum Total Cholestrol



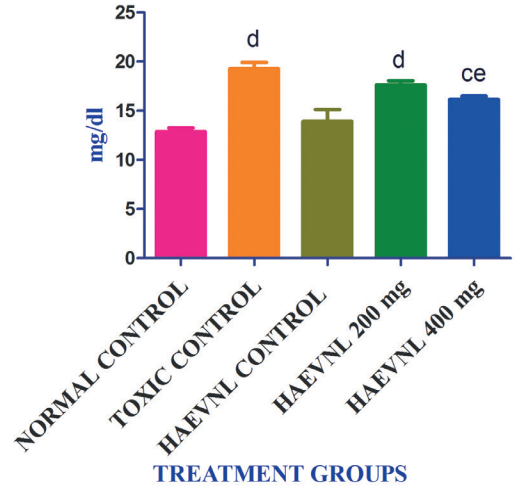
(c)

(c) Effect of HAEVNL on Serum HDL-C



(d)

(d) Effect of HAEVNL on Serum LDL-C



(e)

(e) Effect of HAEVNL on Serum VLDL-C

Figure 1. HAEVNL effect on serum lipid profile.

The Values were represented as Mean ± SEM. Statistical analysis performed using one way ANOVA followed by post hoc Dunnett's multiple comparison test. [a]p<0.001, [b]p<0.01 and [c]p<0.005 Vs NaF control; [d]p<0.001,[e]p<0.01and [f]p<0.005Vs Normal control.

The selected hydroalcoholic extract of *Vitex negundo* leaves (HAEVNL) was subjected to qualitative phytochemical evaluation to identify phytoconstituents.

2.5 Experimental Animals

The 200-230 gm weight (8-9 wks age) healthy male Wistar albino rats were selected and housed as per the CPCSEA guidelines. IAEC approved the experimental protocol (IAEC approved number: CPCSEA/1657/IAEC/CMRCP/COL-19/74).

2.6 Acute Toxicity Studies

The toxicity study was conducted for the selected extract as per OECD guidelines 425.

2.7 Experiment Protocols

Rats were arbitrarily divided into five groups (n=6) by blocking technique after the completion of 14 days of familiarisation.

Group I: Normal control group, drinking water (F^-< 1.5 ppm) for 4 weeks, *p.o.*

Group II: Toxic control group, drinking water (F^-:100 ppm) for 4 weeks, *p.o.*

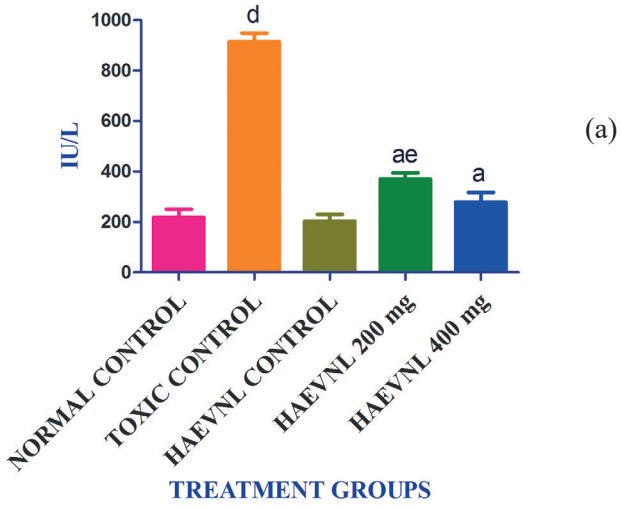
Group III: The plant control group, HAEVNL

(400 mg/kg, b. wt *p.o*) for 4 weeks.

Group IV: HAEVNL 200 mg/kg b. wt, *p.o* with drinking water (F^-:100 ppm) for 4 weeks.

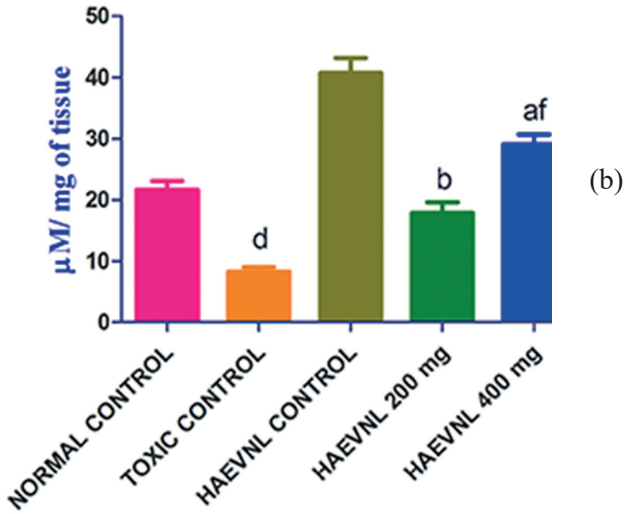
Group V: HAEVNL 400 mg/kg b.wt, *p.o* with drinking water (F^-:100 ppm) for 4 weeks.

2.8 Estimation of Biochemical Assays



(a)

(a) Effect of HAEVNL on Serum CK-MB



(b)

(b) Effect of HAEVNL on Serum LDH

Figure 2. HAEVNL effect on Heart Profile.

The values were represented as Mean ± SEM. Statistical analysis performed using one way ANOVA followed by post hoc Dunnett's multiple comparison test. [a]p<0.001, [b]p<0.01 and [c]p<0.005 Vs NaF control; [d]p<0.001,[e]p<0.01and [f]p<0.005Vs Normal control.

CK-MB, LDH, TG, TC, LDL-C, VLDL-C, HDL-C, Creatinine, Urea, and Uric acid parameters were estimated from blood samples. Heart tissue was isolated and wet weight was measured. The heart and kidney were separated into two portions; the first portion of each tissue was placed in 10 per cent formalin for histopathological study. The remaining part was used to estimate LPO, SOD, GSH, and CAT.[25-28]

2.9 Histopathological Studies

The histopathological changes in heart and kidney samples (4-5 µm) were estimated by the hematoxylin and eosin dye method.

2.10 Statistical Analysis

The data were given as Mean± SEM. One-way ANOVA was used for the statistical analysis, followed by a post hoc Dunnett's test using Graph Pad Prism 5 and statistical significance where, $p<0.05$. [a]$p<0.001$, [b]$p<0.01$ and [c]$p<0.005$ Vs NaF control; [d]$p<0.001$,[e]$p<0.01$and [f]$p<0.005$Vs Normal control.

3. RESULTS

3.1 Percentage of Yield

Water and ethyl alcohol ratios were found to provide varying percentage yields at 30:70 (23.1%), 50:50 (26.7%), and 70:30 (25%), respectively.

3.2 Phytochemical Investigation

The secondary metabolites of HAEVNL were confirmed as alkaloids, phytosterols, flavonoids, saponins, phenols, and tannins.

3.3 Acute Toxicity Studies

2 g/kg body weight of HAEVNL was a nontoxic maximum limit. The doses that are 1/10th and 1/5th of the maximum limit were chosen for further research.

3.4 Body Weight and Food Intake

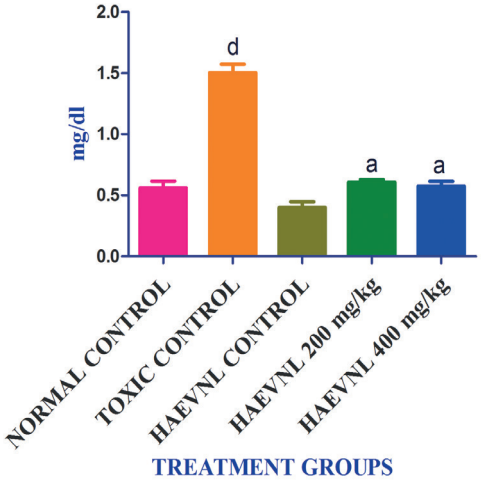
The animal's weight was noted one time a week. The obtained results were specified in Table 1. The NaF-treated rats had a substantial decrease in weight and food intake when compared to normal control group. Compared to toxic control animals, the animals treated with HAEVNL (200 and 400 mg/kg body weight) exhibited increased weight and food intake.

3.5 Organ Weights

Table 1 show the heart and kidney weights for distinct groups. Toxic control animals showed a slight decrease in organ weights, as seen with the normal control animals. There was no significant change in heart weight between normal control group and HAEVNL- treated groups.

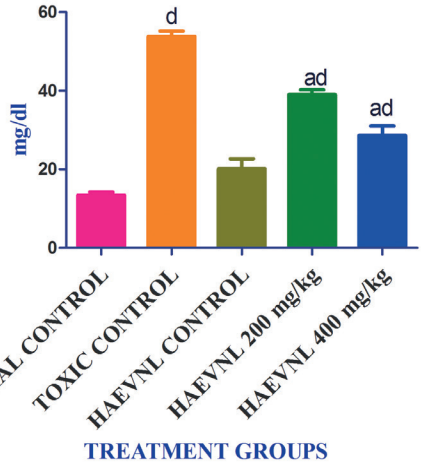
3.6 Lipid Profiles

The estimated serum lipid profile is presented in Figure 1. All lipid parameters were significantly increased in toxic control group but HDL-C levels were decreased in the toxic control group compared with the normal control group. At a dose of 200 and 400 mg/kg b. wt of HAEVNL treatment showed a decrease in lipid parameters along with an increase



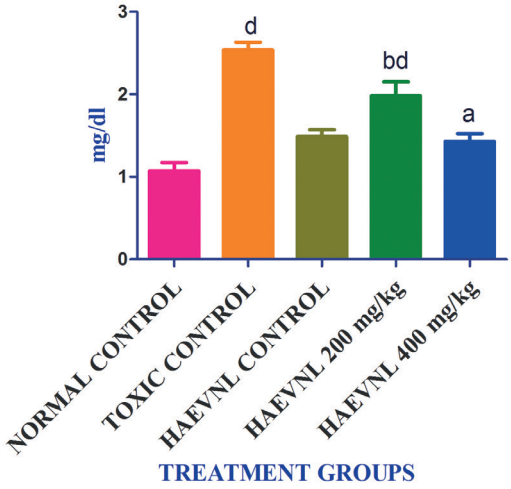
(a)

(a) Effect of HAEVNL on Serum Creatinine Level



(b)

(b) Effect of HAEVNL on Serum Urea Level



(c)

(C) Effect of HAEVNL on Serum Uric Acid Level

Figure 3. Effect of HAEVNL treatment on kidney markers. The Values were represented as Mean ± SEM. Statistical analysis performed using one way ANOVA followed by post hoc Dunnett's multiple comparison test. [a]p<0.001, [b]p<0.01 and [c]p<0.005 Vs NaF control; [d]p<0.001,[e]p<0.01and [f]p<0.005Vs Normal control.

in serum HDL-C levels.

3.7 Heart Profile

In HAEVNL-treated animals, the heart weights did not significantly differ from the normal control animals. HAEVNL (200 and 400 mg/kg b. wt) treated animals demonstrated significant restoration of LDH and CK-MB serum levels compared to the toxic control animals. The estimated values were represented in Figure 2.

3.8 Kidney Profile

Creatinine, urea, and, uric acid levels in serum of the toxic control animals were found to be considerably higher than those of a normal control animals. Compared to the toxic control animals, the HAEVNL (200 and 400 mg/kg b.wt) treated animals demonstrated a considerable normalisation of creatinine, urea, and uric acid serum levels. The values were represented in Figure 3.

3.9 Heart and Kidney Oxidative Stress Marker

The effect of HAEVNL on oxidative stress markers was summarised in Figure 4. Comparing the normal control group to the toxic control animals demonstrated a large increase in LPO levels and significant decreases in SOD, GSH, and CAT. Compared to the toxic control group, HAEVNL treatments of 200 and 400 mg/kg b. wt. resulted in a considerable reduction in LPO levels and an increase in SOD, GSH, and CAT levels.

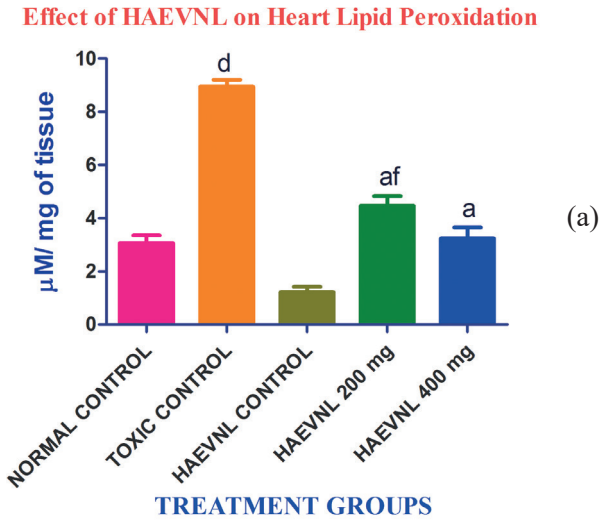
3.10 Histopathological Studies

Histopathological changes of HAEVNL on the heart and kidney tissues are specified in Figures 5 and 6. The normal and plant control animal tissues showed standard architecture of myocardial fibers in the heart. The toxic control group tissue showed focal chronic inflammatory cell infiltration. The low and high dose treatment groups showed complete protection from fluoride-induced focal chronic inflammatory cell infiltration.

The normal and plant control animal tissues showed standard nephron architecture in the kidneys. The toxic control animal tissue showed focal chronic inflammatory cell infiltration of lymphocytes in the cortex. The low and high dose treated animals showed complete protection.

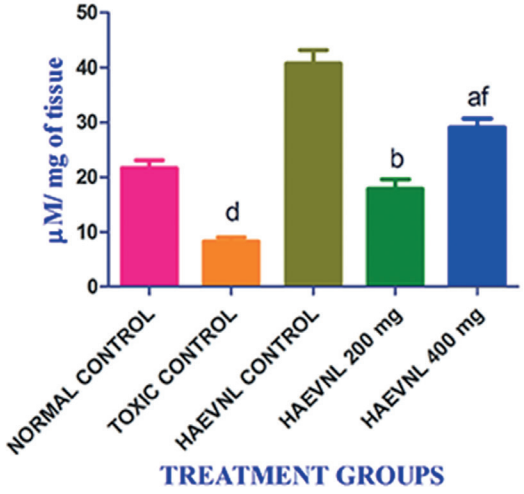
4. DISCUSSION

The mitigative effect of HAEVNL against sodium fluoride induced cardio and nephrotoxicity in male Wistar albino rats. Alkaloids, phytosterols, flavonoids, saponins, phenols, and tannins are identified secondary metabolites in the HAEVNL. The 50:50 (water: ethyl alcohol) concentration extract was chosen for further studies based on the per cent yield. Up to a dose of 2 g/kg, no changes in clinical observations or death were seen in HAEVNL's acute toxicity test. In order to test the mitigative effect against fluoride-induced cardiac and



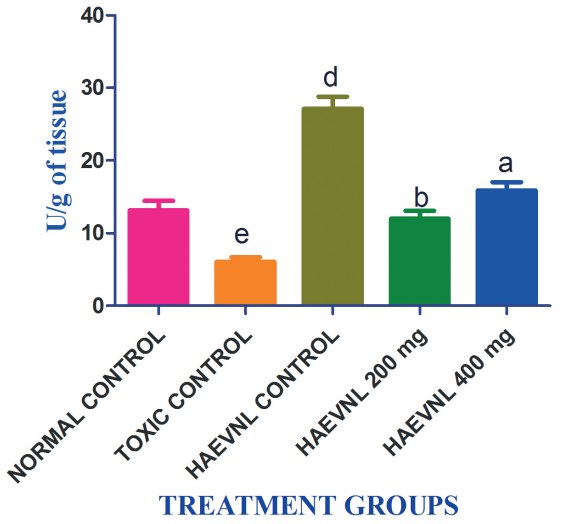
(a)

(a) Effect of HAEVNL on Heart Lipid Peroxidation



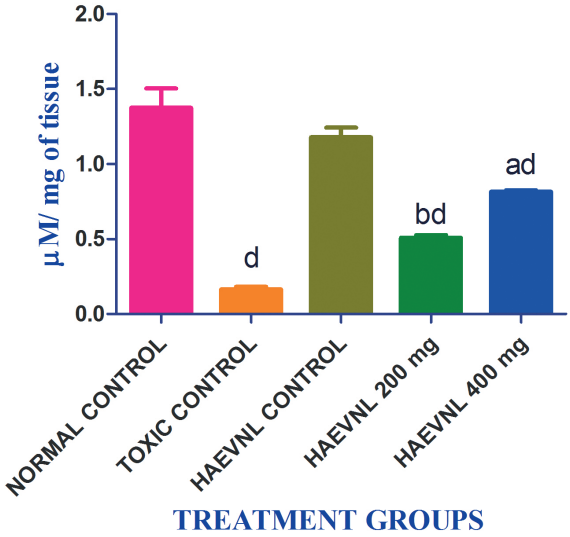
(b)

(b) Effect of HAEVNL on Heart Reduced Glutathione



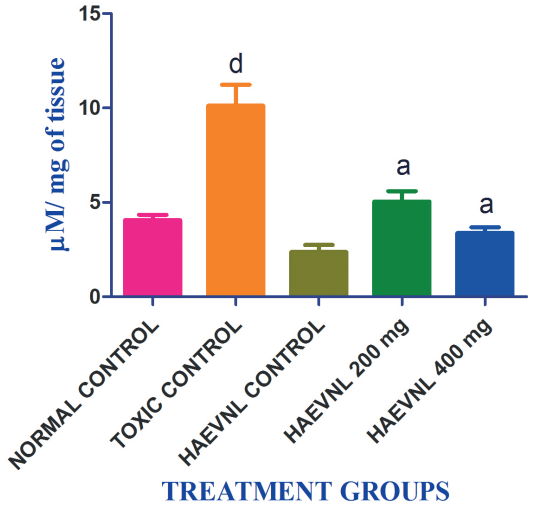
(c)

(c) Effect of HAEVNL on Heart Sodium Dismutase



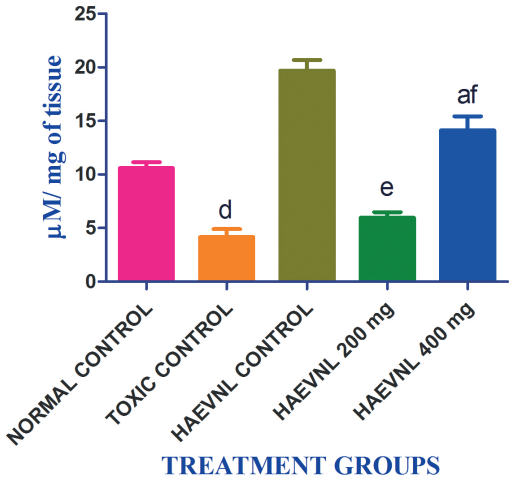
(d)

(d) Effect of HAEVNL on Heart Catalse



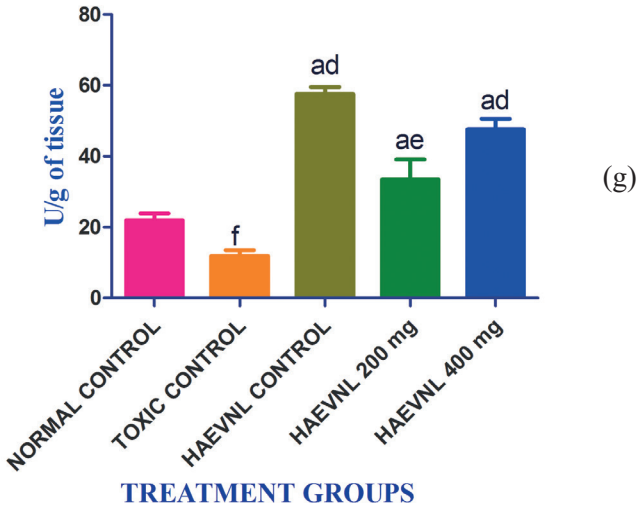
(e)

(e) Effect of HAEVNL on Kidney Lipid Peroxidation

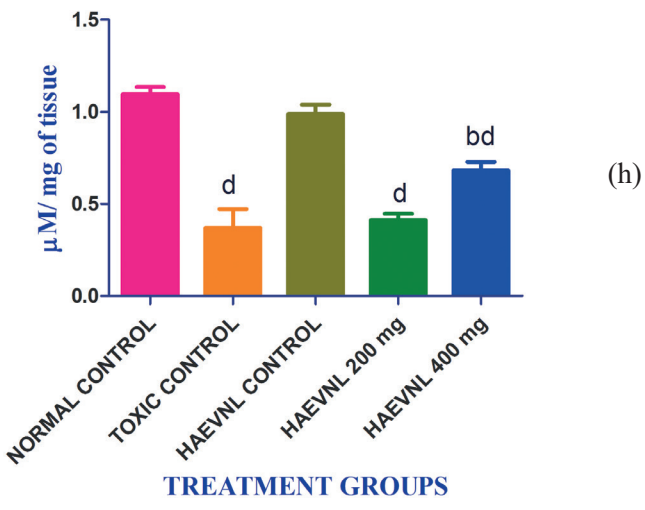


(f)

(f) Effect of HAEVNL on Kidney Reduced Glutathione



(g)



(h)

(g) Effect of HAEVNL on Kidney Sodium Dismutase

(h) Effect of HAEVNL on Kidney Catalase

Figure 4. HAEVNL effect on heart and kidney oxidative stress markers.

The Values were represented as Mean ± SEM. Statistical analysis performed using one way ANOVA followed by post hoc Dunnett's multiple comparison test. [a]p<0.001, [b]p<0.01 and [c]p<0.005 Vs NaF control; [d]p<0.001,[e]p<0.01and [f]p<0.005Vs Normal control.

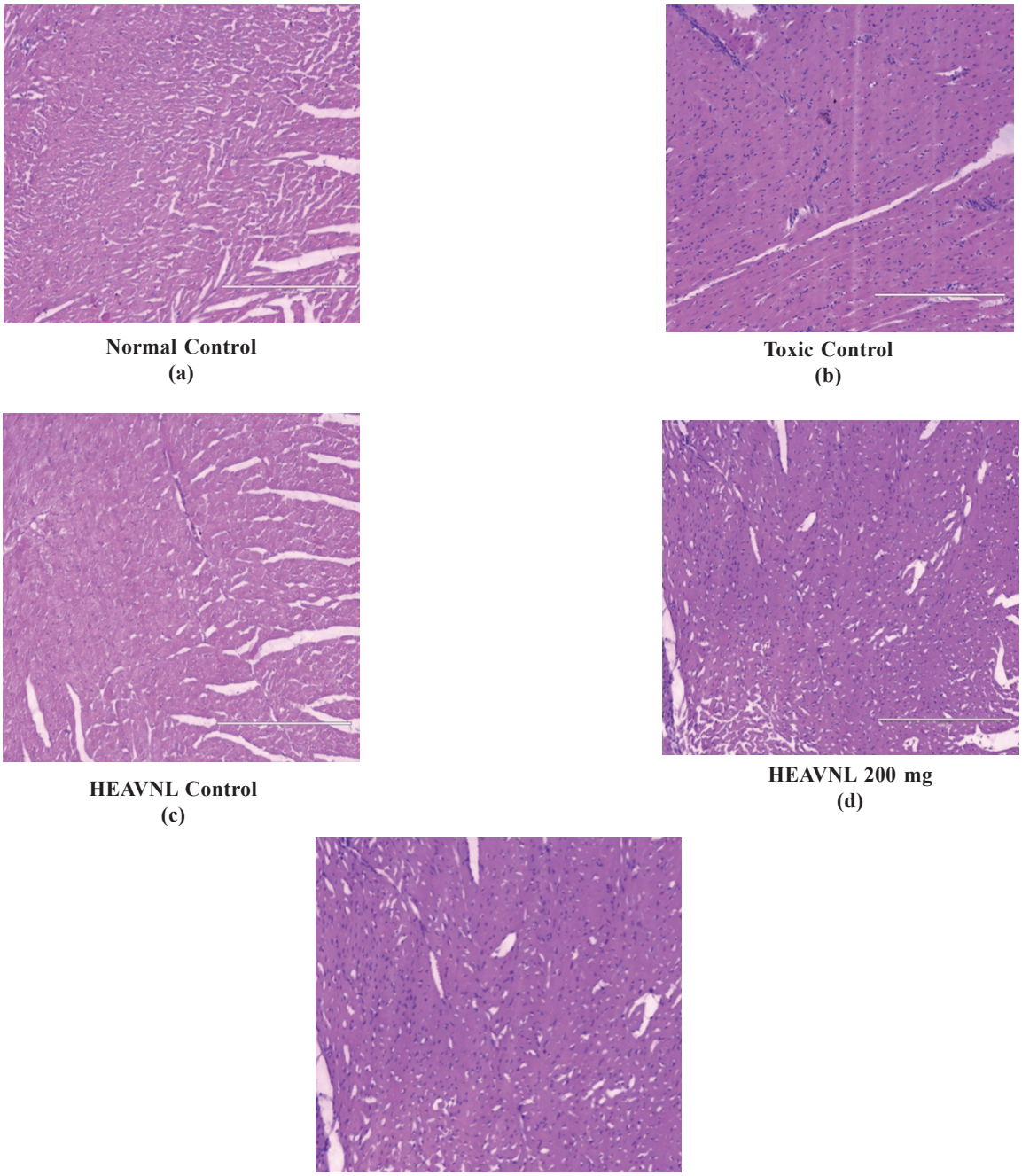
nephrotoxicity, 200 and 400 mg/kg were chosen from acute toxicity studies.

The *Crypinus carpio* fishes exposed to the high concentration of NaF showed a significant reduction in weight and food intake due to appetite loss; decreased total protein, albumin, and globulin levels[29] were observed. The animals exposed to NaF showed significant decrease in body weight. The weight decline could be attributed to altered GI absorption by atrophic gastritis; suppressed food intake and decreased digestibility of nutrients.[30] In the present study, significant decrease in body weight was observed in the toxic group and it was consistent with earlier reports.[31,32] The 400 mg/kg HAEVNL therapy led to a significant improvement in body weight indicated that *Vitex negundo* leaves to improve the appetite by normalizing the GI absorption and minimizing the excessive breakdown of cellular molecules. The Changes in the heart and kidneys weights were mild in the toxic group as well as in the animals treated with HAEVNL.

Fluoride-induced cardiotoxicity is associated with an abnormal increase in circulatory and cardiac lipids, which are the possible risk factors for myocardial dysfunction's pathogenesis. Apart from this, fluoride-induced hypercholesterolemia might be due to an abnormal increase in the uptake of LDL-C from the circulation by the myocardial cell membrane and hypertriglyceridemia by elevated levels of fatty acids and VLDL-C in the circulation.[8] The altered functions of lipase, unspecific esterase, and pyrophosphates are also one of the principal factors for the rise in serum TG levels.[33, 34] The increased LDL-C and VLDL-C levels in fluoride-treated groups could be directly linked to increased plasma TG levels. In this study, abnormally increased levels of lipid profiles and decreased HDL-C levels after exposure to high dose of fluoride results correlated with a report by Adamma Angela, et al. 2014.[35] Phytosterols inhibit the intestinal absorption of cholesterol. With their potential to precipitate cholesterol from micelles and obstruct the bile acids, saponins are potent antihypercholesterolaemic drugs that lower cholesterol levels in the plasma and liver.[36,37] The levels of lipid profile were significantly lower in animals given HAEVNL treatment. The ascorbic acid and flavonoids in the *Vitex negundo* leaves may cause elevated serum HDL-C levels in the HAEVNL treated rats. The anti-hyperlipidemic effects of phytosterols, saponins, dietary fiber, polyphenols, flavonoids, and ascorbic acid[38] are indicated by a substantial decrease in the lipid profile of high dose HAEVNL treated rats. The treatment results correlated with the earlier results.[15]

Estimating cardiac-specific markers (CK-MB, LDH) in the blood was the best way to approximate the extent of cardiac damage or myocardial ischemia.[39] Fluoride toxicity caused a significant elevation of cardiac markers in the circulation, resulting from increased lipid peroxidation, abnormal formation of superoxide anions, hydroxyl radicals, conjugated dienes, and protein carbonyls, and decreased levels of glutathione levels which cause damage to the myocardial cell membrane.[40] The present study result concurred with earlier reports after animal's exposure to fluoride.[41] Treatment with HAEVNL 400 mg/kg dose showed decreased CK-MB and LDH, indicating potential antioxidants and a membrane-stabilizing effect against fluoride-induced cardiac cell damage.

The serum kidney profile are reliable and prophetic biomarkers for kidney function status.[42] Animals exposed to a high dose of fluoride caused a significant increase in the serum kidney profile, indicating increased protein catabolism, increased xanthine oxidase activity and



Normal Control
(a)

Toxic Control
(b)

HEAVNL Control
(c)

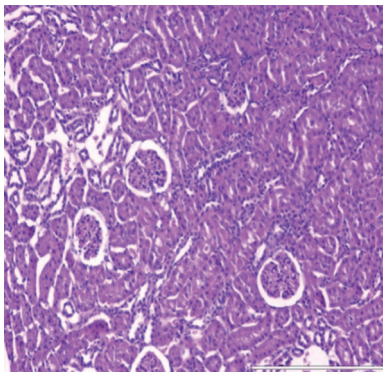
HEAVNL 200 mg
(d)

HEAVNL 400 mg
(e)

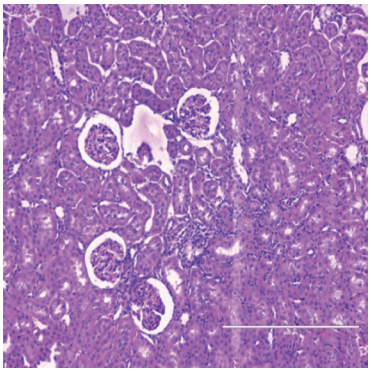
Figure 5. Histopathological changes of Heart.

glomerular as well as tubular cell damage.[43] The present results also showed abnormally increased serum levels of kidney profile. Treatment with HAEVNL 400 mg/kg dose showed a significant decrease in the serum kidney profile, indicating the shielding effect against fluoride-induced renotoxicity.

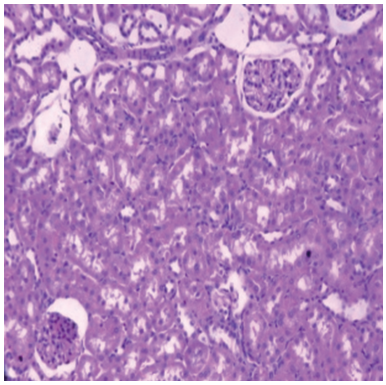
The severity of nephrotoxicity caused by fluoride is directly proportional to the number of fluoride ions diffused in the form of hydrogen fluoride.[12,44] Fluoride exposure causes the generation of excessive peroxynitrite, hydroxyl, superoxide, and hydrogen peroxide radicals. These radicals play a crucial role in activating various inflammatory pathways, resulting in soft tissue toxicity, including the heart[45] and kidneys.[12] It also slows down the SOD, CAT, and GPx and reduces the GSH levels. ROS production leads to oxidation of macromolecules, breakdown of membrane phospholipids, altered mitochondrial membrane potential, and apoptosis. Superoxide dismutase changes the superoxide radicals into hydrogen peroxide. Catalase converts the formed hydrogen peroxide to water and oxygen. For the putrefaction of hydrogen peroxide or other organic hydroperoxides into non-toxic compounds, the GPx uses the glutathione content.[46,47] HAEVNL treatment at 400 mg/kg dose showed a significant decrease in LPO levels and a significant increase in the SOD, GSH, and



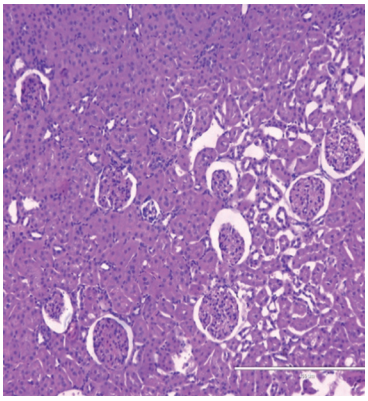
Normal Control
(a)



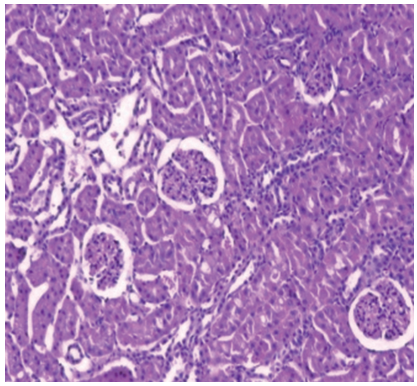
Toxic Control
(b)



HAEVNL Control
(c)



HAEVNL 200 mg
(d)



HAEVNL 400 mg
(e)

Figure 6. Histopathological changes of Kidney

CAT levels. The results of the study coincided with Bharti *et al*., 2014[48] and Ola-Davies and Azeez, 2018.[49]

5. CONCLUSION

High dose intake of fluoride significantly altered myocardial as well as kidney biochemical profiles and endogenous antioxidant levels. The *Vitex negundo* leaves plummet the formation of free radicals and improve the body's antioxidant status. Thereby, *Vitex negundo* leaves showed a significant mitigative effect against fluoride-induced cardiac and nephrotoxicity. This edible leaves would be of use in herbal formulation for effective treatment of fluoride-induced toxicity, particularly in endemic areas.

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