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Mitigative effect of *Momordica cymbalaria* fruit extract against sodium fluoride induced hepatotoxicity in Wistar male albino rats

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Abstract

Objectives: The main objective of the present study is to evaluate the mitigative effect of hydroalcoholic extract of *Momordica cymbalaria* fruits against sodium fluoride (NaF) induced hepatotoxicity.

Methods: In this study, Wistar male albino rats were randomly divided into five groups of six rats each. Group I and II served as normal and toxic controls. Group III as plant control received extract at a dose of 400 mg/kg b. wt, *p.o* and Groups IV and V as treatment groups received extract at a dose 200 and 400 mg/kg b. wt, *p.o* for 30 days. All groups except Groups I and III received 100 ppm of NaF through drinking water. After completion of the study, blood collected for the estimation of liver blood serum biomarkers such as aspartate aminotransferases (AST), alanine aminotransferases (ALT), alkaline Phosphatase (ALP), direct and total bilirubin, total protein and albumin. The liver tissue homogenate was for estimation of lipid peroxidation, catalase, and reduced glutathione levels.

Results: The results showed that NaF intoxication caused elevation of liver blood serum levels and lipid peroxidation; decreased levels of serum total protein, albumin and liver reduced glutathione, and catalase observed. The treatment groups showed decreased elevated serum

biomarkers (ALT, AST, and ALP), liver lipid peroxidation and increased serum total protein and albumin, liver reduced glutathione and catalase levels in a dose-dependent manner. Histopathological studies also further strongly supported for mitigative effects of the plant.

Conclusions: In conclusion, our findings of the study indicated that *M. cymbalaria* fruits were a potential drug candidate in the treatment of NaF induced hepatotoxicity.

Keywords: catalase; lipid peroxidation; liver; *Momordica cymbalaria*; reduced glutathione; sodium fluoride.

Introduction

Fluoride (F⁻) is the element that does not occur in the elemental state in nature because of its high reactivity [1]. It accounts for about 0.3 g/kg of the Earth's crust and estimated to be the 13th most abundant element. It exists only in the form of fluorides in several minerals such as fluor-spar, cryolite, and fluorapatite [2]. At low concentrations, it is useful for normal mineralization of bone, dentin, and functions of teeth enamel. It is considered as the most productive prophylactic element as well as the treatment agent for dental caries and osteoporosis [3, 4].

Fluorosis is a public health related problem that is endemic all over the globe, including India. At the permissible levels (F⁻ ≤ 1.0 ppm), all ingested amount excreted in the urine but when it crosses the permissible levels, it causes dental, skeletal, and non-skeletal fluorosis in both animals as well as in human beings [5, 6]. The primary sources of fluoride intake include drinking water, dental products, food, processed beverages, tea, fluorinated pharmaceuticals, pesticides, and mechanically deboned meat [7, 8]. Referring to a common salt of fluoride as sodium fluoride (NaF), the lethal dose for most adults is estimated to be 5–10 g [9]. At acidic pH, ingested fluorides combine with hydrogen ions to form hydrogen fluoride which was passively entered in the circulation through the passive diffusion process and interferes with the major metabolic pathways [10].

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Liver is the one the major affected organ for potential fluoride toxicity after the kidney. Earlier studies have clearly shown that intake of high concentration of fluoride in the drinking water causes biochemical changes in the liver by alterations in the synthetic, metabolic, detoxification, excretion, homeostasis, increased lipid peroxidation, disturbed antioxidant defense system, and storage functions may cause hepatotoxicity. Also, histopathological changes reported in the liver after fluoride exposure included necrosis, infiltration of mononuclear cells, swelling of Kupffer cells, extensive vacuolization, hyperemia, ultra structural changes in hepatocytes, and increased apoptosis [11–16].

The plant *Momordica cymbalaria*, which is a vine of the *Momordica* genus originated from the Indian states of Andhra Pradesh, Tamil Nadu and Maharashtra, belongs to the Cucurbitaceae family. *M. cymbalaria* is economical, nutritional-rich, and also contains flavonoids and phenolic compounds as major secondary metabolites. It has been valued for its edible fruits, roots, and leaves as a vegetable [17]. It was already proved for its various medicinal purpose such as cardioprotective effect [18], anti-diabetic, and hypoglycemic activity [19], anti-diarrheal [20], anti-ulcer [21], anti-microbial [22], anti-implantation [23], anti-ovulatory [24], antiangiogenic, anticancer, and anti-tumor [25, 26], and nephroprotective [27] activity. The study aims to see the mitigative effect of hydroalcoholic extract of *M. cymbalaria* fruits [HAEMC] against NaF induced hepatotoxicity and oxidative stress in male Wistar albino rats.

Materials and methods

Chemicals

All chemicals used in the present study were of analytical grade purchased from Sigma–Aldrich Pvt Ltd., Himedia Pvt Ltd., SRL Pvt Ltd., and Research Lab Pvt Ltd., Mumbai, India. Diagnostic kits for the measurement of aspartate aminotransferase (AST), alanine aminotransferases (ALT), alkaline Phosphatase (ALP), direct and total bilirubin, total protein, and albumin purchased from Coral Diagnostics, Mumbai, India.

Collection and authentication of plant material

The fruits of the plant *M. cymbalaria* collected from the local market of YSR Kadapa, Kadapa district, Andhra Pradesh, India. The plant material authenticated by Dr. Manohar Rao, Taxonomist, Department of Botany, Professor Jayashankar Telangana State Agricultural University, Hyderabad, Telangana state, India.

Preparation of the plant extract

The freshly collected *M. cymbalaria* fruits washed with distilled water, shade dried, and powdered. The fruit powder was mixed in three different concentrations of 50:50, 30:70, 70:30 (water:ethyl alcohol) and kept aside for one week with occasional stirring. After that, stirred for 20 min and filtered. The filtrates dried in a rotary evaporator. The suitable extract was selected based on the percentage of yield obtained and stored in the refrigerator at 4 °C for further studies.

Preliminary phytochemical screening

The selected hydroalcoholic extract of *M. cymbalaria* fruits was subjected to qualitative phytochemical screening for the identification of phytoconstituents [28].

Experimental animals

In the experiment, we used adult male Wistar albino rats weighing 200–230 g (8–9 weeks of age). The animals were housed under the conditions of controlled temperature and 12 h day/night cycle and fed with standard pellet raw chaw and water ad libitum during the study. The experimental protocol used in this study reviewed by the Institutional Animal Ethics Committee (IAEC). IAEC NO: IAEC/1657/CMRCP/T2/PhD-16/46.

Acute toxicity studies

Acute toxicity study was performed according to the guidelines of OECD 425.

Experiment protocol

The rats were randomly divided into five groups of six animals in each group as follows [29]:

Group I (normal control): the rats received drinking water for 30 days.

Group II (toxic control): the rats received NaF (100 ppm) through drinking water for 30 days.

Group III (HAEMC control): the rats received HAEMC at a dose of 400 mg/kg *p.o* for 30 days.

Group IV (HAEMC 200 mg): the rats received a low dose of HAEMC 200 mg/kg *b. wt.*, *p.o* with NaF (100 ppm) for 30 days.

Group V (HAEMC 400 mg): the rats received a high dose of HAEMC 400 mg/kg *b. wt.*, *p.o* with NaF (100 ppm) for 30 days.

At the end of the study, blood was collected from overnight fasted animals by retro-orbital puncture method. Blood serum used for the estimation of various liver biomarkers such as AST, ALT, ALP, direct and total bilirubin, total protein and albumin. After blood collection, animals sacrificed by cervical dislocation and were cut open to isolate the liver and weighed immediately. Then, the liver was divided into two portions. One portion was stored in 10% formalin for histopathological study and remaining portion for the estimation of *in vivo* antioxidant studies.

Preparation of tissue homogenate

Tissue was homogenized in chilled 1.15% potassium chloride using a homogenizer. The homogenate was centrifuged at 800 rpm for 5 min at 4 °C (REMI C-24) to separate the molecular debris. The supernatant so obtained was centrifuged at 10,000 rpm for 20 min at 4 °C (REMI CM-12) to get the post mitochondrial supernatant (PMS), used for estimation of lipid peroxidation, reduced glutathione, and catalase levels.

Biochemical analysis

Estimation of lipid peroxidation [30]: In this method, 0.5 mL of PMS taken and added with 0.5 mL of Tris-HCl buffer (0.1 M, pH 7.4) and incubated at 37 °C for 2 h and then 1 mL of ice cold trichloroacetic acid (10%) was added and centrifuged at 1,000 rpm for 10 min. From the above, 1 mL of supernatant was taken and added to 1 mL of 0.67% w/v thiobarbituric acid (0.67%) and the tubes were kept in the boiling water bath for 10 min. One milliliter of distilled water added to the tubes after their removal and brought to room temperature. Absorbance measured at 532 nm by using a UV-Visible spectrophotometer and the lipid peroxidation is calculated using the following formula:

$$\frac{3 \times \text{Absorbance}}{50.156 \times (\text{mg of tissue taken})} = \mu\text{M/mg tissue.}$$

Estimation of reduced glutathione (GSH) [31]: In this method, 0.7 mL of PMS added to 0.75 mL of sulfosalicylic acid (4%). The precipitated fraction centrifuged (1200 rpm, 4 °C for 5 min). Half milliliter of supernatant was added to 4.5 mL of DTNB (0.1 mM in 0.1 M phosphate buffer, pH 8.0) solution. Absorbance measured at 412 nm by using a UV-Visible spectrophotometer. The amount of glutathione expressed as microgram per milligram of tissue and the reduced glutathione level calculated using the following formula:

$$\frac{3 \times \text{Absorbance of sample}}{13.6 \times (\text{mg of tissue taken})} = \mu\text{M of GSH/mg tissue.}$$

Estimation of catalase [32]: In this process, 0.05 mL of PMS, 1.95 mL of phosphate buffer (0.05 M, pH 7.0) and freshly prepared 1 mL of H₂O₂ (0.019 M, 50 mM phosphate buffer pH 7.0) were added. The absorbance recorded at 254 nm three times with 1 min interval against blank. Change in absorbance recorded. The results expressed as $\mu\text{M/mg}$ of tissue.

Histopathological studies

The liver samples (4–5 μm) were prepared and stained with hematoxylin and eosin dye.

Statistical analysis

The values were expressed as mean $\pm$ SEM and statistical analysis performed by using one-way analysis of variance (ANOVA) followed by post hoc Dunnett's multiple comparison test using Graph pad Prism 5. Results are considered statistically significant where $p < 0.05$.

Results

Percentage of yield obtained

The percentage yield of various proportions of water and ethyl alcohol obtained to be 70:30 (26.7%), 50:50 (30.3%), and 30:70 (39%).

Phytochemical investigation

The phytochemical constituents of the HAEMC found to be alkaloids, glycosides, steroids, flavonoids, phenols, and tannins as secondary metabolites.

Acute toxicity studies

The acute toxicity of the HAEMC doesn't show any morbidity and mortality upto the dose of 2,000 mg/kg b.wt; this is considered as a cut-off dose. Based on the cut-off dose, 1/10th (200 mg) and 1/5th (400 mg) of the cut-off doses were selected to study the mitigative effects of HAEMC against NaF induced hepatotoxicity.

Effect on body and liver weights

The change in body weights in the toxic control group on the 1st and 30th day indicates a significant reduction in the body weights compared to the normal control group. Animals treated with HAEMC showed increased body weight in a dose-dependent manner when compared to the toxic control group. Decreased liver weights were observed in the toxic control group compared to the normal control group and significant recovery was observed in the HAEMC treatment groups in a dose-dependent manner. The body and liver weight variations specified in Table 1.

Effect on liver serum biomarkers level

Excessive consumption of fluoride increases the blood serum levels of ALT (125.0 ± 9.85), AST (99.6 ± 6.65), ALP (73.3 ± 3.83), direct and total bilirubin (0.40 ± 0.04 and 3.44 ± 0.30) and decreased levels of total protein (6.40 ± 0.17) and albumin (2.093 ± 0.07) were observed in toxic control when compared to the normal control (35.19 ± 2.40 , 38.4 ± 3.09 , 34.0 ± 2.43 , 0.14 ± 0.01 , 1.25 ± 0.05 , 7.36 ± 0.16 , and 3.925 ± 0.08 , respectively). After treatment with HAEMC showed at a dose of 200 mg

Table 1: Effect of HAEMC treatment on body and liver weights against NaF induced toxicity.

S. No	Name of the group	Body weight, g		Liver weight, g
		1st day	30th day	
1.	Normal control	221.85 ± 09.24	245.32 ± 05.27	8.86 ± 0.46
2.	Toxic control	216.66 ± 16.02	230.00 ± 15.24	6.54 ± 0.21
3.	HAEMC control	209.16 ± 13.92	249.166 ± 17.87	8.79 ± 0.19
4.	HAEMC 200 mg	224.16 ± 14.22	244.166 ± 13.32	7.26 ± 0.24
5.	HAEMC 400 mg	216.667 ± 15.21	245.833 ± 14.31	8.75 ± 0.07

Values were represented as mean ± SEM.

HAEMC, Hydroalcoholic extract of *M. cymbalaria* fruits.

(81.99 ± 3.64, 75.2 ± 2.80, 51.6 ± 2.69, 0.35 ± 0.03, 2.43 ± 0.16, 6.66 ± 0.031 and 3.215 ± 0.10) and 400 mg (61.15 ± 0.94, 52.3 ± 2.95, 36.6 ± 2.41, 0.21 ± 0.01, 1.85 ± 0.09, 7.94 ± 0.15 and 3.822 ± 0.14, respectively) dependent recovery from NaF induced hepatotoxicity. No significant variations were observed in HAEMC control (37.03 ± 1.46, 32.5 ± 1.78, 31.4 ± 2.01, 0.40 ± 0.04, 3.44 ± 0.30, 6.40 ± 0.17, and 2.093 ± 0.07, respectively) when compared to normal control. All values are specified in Figure 1.

Effect on lipid peroxidation and antioxidant profile of liver

Effect of HAEMC on the liver lipid peroxidation, reduced glutathione, and catalase levels were specified in Figure 2. There was a significant increase in the lipid peroxidation (5.24 ± 0.78) and decrease in the levels of reduced glutathione (4.82 ± 0.48) and catalase (0.24 ± 0.06) levels observed in toxic control when compared to the normal control (1.32 ± 0.35, 13.2 ± 0.85, and 1.18 ± 0.10). Treatment with HAEMC showed significant recovery from lipid peroxidation (3.17 ± 0.25 and 1.61 ± 0.15) and increased levels of reduced glutathione (8.89 ± 0.60 and 13.0 ± 0.80) and catalase (0.39 ± 0.10 and 0.81 ± 0.04) in a dose-dependent manner when compared to the NaF control. No significant variations observed in the tissue levels of lipid peroxidation (1.13 ± 0.91) and reduced glutathione (13.9 ± 0.46) and catalase (1.35 ± 0.20) in plant control when compared to the normal control.

Histopathological studies

Histopathological changes of the HAEMC on liver tissue specified in Figure 3. In this, normal control liver tissue

showed prominent hepatocytes with preserved cytoplasm, hepatic artery, portal tract, central vein, and normal blood vessels. The toxic control (NaF, 100 ppm) tissue showed distortion of hepatocytes, central vein and blood vessels, necrosis, and edema. The HAEMC control liver tissue is similar to the normal architecture of the liver tissue. Treatment control-I (HAEMC 200 mg) showed moderate destruction of hepatocytes, central vein, decreased fibrosis, and edema. Treatment control-II (HAEMC 400 mg) showed mild damage of hepatocytes, central vein, and decreased fibrosis and edema.

Discussion

The fluoride acts as the most productive agent for dental caries [33]. At the permissible levels ($F \leq 1.0$ ppm), all ingested amount excretes in the urine. But when it crosses the permissible levels, it causes dental, skeletal, and non-skeletal fluorosis in both animals and human beings [34].

Fluoride exhibits diverse metabolic, structural, and functional toxic effects. The toxic changes depend on the type of cell, duration, and concentration of exposure. It can bind to inhibit the function of Na^+/K^+ -ATPase leading to ATP depletion, alterations in the cell membrane potential, and inactivate function of various key enzymes involved in the glycolysis and the Krebs cycle [35]. At micromolar levels, fluoride inhibits the synthesis and/or secretion of protein, and thus various signaling pathways are involved in the cell multiplication and death, such as mitogen-activated protein kinase, p53, activator protein-1, and nuclear factor kappa pathways. At millimolar concentrations fluoride inhibits various enzymes, including phosphatases [36–38].

Earlier studies discussed the relationship between fluoride and free radical production in various biological systems in natural as well as experimental cases of fluoride toxicity [16]. Antioxidants and chelating agents reduce the stress and/or toxicity against fluoride intoxication [7]. However, long term exposure to vitamins and minerals may not be safe due to their accumulation. Therefore, the study aimed not only mitigation of the fluoride induced toxicity in the liver but also safe, economical, and secure availability in nature. *M. cymbalaria* fruits are nutritional and unexplored plant material for the treatment of fluoride induced hepatotoxicity and oxidative stress.

Earlier studies clearly reported the variation in the extraction yield depends on the solvent nature and chemical nature of the sample [39]. Based on the percentage of yield obtained, selected 30:70 proportion of hydroalcoholic

Figure 1: Effect of HAEMC treatment on serum liver profile against NaF induced toxicity. The values are represented as mean $\pm$ 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. toxic control; ^d $p < 0.001$, ^e $p < 0.01$, and ^f $p < 0.005$ vs. normal control.

extract is used for further studies. The phytochemical constituents of HAEMC found to be a good source of alkaloids, glycosides, steroids, flavonoids, phenols, and tannins

as secondary metabolites. The acute toxicity test of HAEMC showed tolerance at 2000 mg/kg b. wt, *p.o.* All the animals in the treatment groups were normal in alertness and

Effect of HAEMC on Liver Lipid peroxidation Level

Effect of HAEMC on Liver Reduced Glutathione Level

Effect of HAEMC on Liver Catalase Level

Figure 2: Effect of HAEMC treatment on oxidative stress markers against NaF induced hepatotoxicity. The values are represented as mean $\pm$ 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. toxic control; ^d $p < 0.001$, ^e $p < 0.01$, and ^f $p < 0.005$ vs. normal control.

behavior up to 72 h of post administration. No mortality observed until the completion of the study.

The fluoride exposed rats showed a significant decrease in the body weight, it might be due to atrophic gastritis and poor absorption in the GIT by disturbed digestibility and decreased appetite was contributed to excessive breakdown of macromolecules causing weight loss. The decreased liver weight observed due to the degeneration of hepatocytes and altered antioxidant systems, which was the primary factor in fluoride toxicity [40]. The improved body and liver weights were found at the end of the study after treatment with HAEMC.

The abnormal increase in the concentration of aminotransferases (AST and ALT) and ALP levels in serum and altered protein metabolism was a clear indication of abnormal function of the hepatic tissue. Previous studies reported that a significant increase in AST, ALT, ALP, direct and total bilirubin levels and decreased levels of total protein and albumin in rats after NaF-intoxication [41, 42]. The increase in the serum bilirubin levels reflects the severity of hepatotoxicity [43]. Data obtained in the present

study also revealed that disturbed liver function in toxic control group reflected by increased levels of AST, ALT, ALP, and direct and total bilirubin and decreased levels of total protein and albumin. Treatment with HAEMC at a dose of 200 and 400 mg/kg b. wt showed normalized serum biomarkers and also increased total protein and albumin levels in a dose-dependent manner. The present results correlate with the findings of the Prakash et al. [44].

Fluoride induces hepatotoxicity by augmenting the free radical formation, which reacts with the polyunsaturated fatty acids to initiate lipid radical chain reaction leading to damage of the cell membrane. It can also reduce the functional capacity of antioxidant system in the hepatocytes cells [45]. The administered HAEMC showed a significant reduction in the lipid peroxidation level and a significant increase in the levels of reduced glutathione and catalase in a dose-dependent manner. These findings have coincided with the results of Perera et al. [12]. Histopathological studies also strongly supported the mitigative effects of HAEMC against fluoride induced hepatotoxicity and oxidative stress.

Figure 3: Histopathological studies of liver (magnification 10×).

Conclusion

In conclusion, the present study has proven that the hydro-alcoholic extract of *M. cymbalaria* fruit's effect on NaF induced hepatotoxicity by decreasing the level of free radical production and also by improving the liver function. We have also concluded that the present results strongly support further application in drug development for the treatment of fluoride toxicity. However, further detailed studies are necessary for a better understanding of changes in the molecular level and exact mechanism involved in the free radical scavenging activity of the plant material.

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