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PROTECTIVE ROLE OF *MORINGA STENOPETALA* AGAINST SUB-CHRONIC EXPOSURE OF FLUORIDE IN SOFT TISSUE OF MICE.

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Jimma, Ethiopia

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Abstract

Background: *Excessive fluoride intake over a long period of time results in fluorosis. Skeletal and dental toxicity are known adverse effects of fluorides. Scientific data on the effect of excess fluorides on soft tissues is scarce. Studies implicated that among the mechanism by which fluorosis causes damage is through aggravating oxidative stress and inhibiting body anti oxidative systems. Moringa (Moringa stenopetala, Bac,) an endogenous tree to Ethiopia, is rich in antioxidants, and has potent anti-oxidative effect.*

Objective: *The aim of this study was to determine the effect of sub-chronic exposure to fluoride on histological status and function of Liver, kidney and pancreas, as well as protective role of Moringa stenopetala crude extract against soft tissue injury in adult mice.*

Materials and Methods: *Laboratory-based randomized control study with both quantitative and qualitative analyses was conducted using 7-8wks, 24 adult Swiss Albino mice with average weight 33.8±5.5g. All animals were allowed free access to standard pellet diet ad libitum and distilled water except when starvation was otherwise needed. After an acclimatization period of 7 days, the mice were randomly divided to six (five experimental and one control) groups of four animals each (two male and two females).*

The mice in the experimental groups were freely allowed to NaF in drinking water and orally administered a single daily dose of 100mg/kg Moringa extract, once a day at 24 hours intervals accordingly to feeding protocol for 90 days. Similarly, the control groups were also given 0.07 ppm of NaF in distilled water. After 3 months of exposure, on day 91, the mice were anesthetized and sacrificed by cervical dislocation for blood and organ collection, and histological preparation.

Result: *There was statistically significance difference in body weight gain between the treated and the control groups. Compared with the controls, weight of 100 ppm NaF treated group had significant weight decrease during 10^{th} and 12^{th} weeks of treatment with $F(5\ 23)= 5.19$, $P=0.04$, $\eta=0.59$ and $F(5\ 23)=10.25$, $P =0.000$, $\eta =0.74$ respectively. There was statistically significant difference in liver function tests between the treated and the control groups. Compared with the controls, plasma alkaline phosphatase of 100 ppm fluoride treated group had signicant plasma ALP level with $F(5\ 17)= 5.466$, $P=0.008$, $\eta=0.695$ and power of 0.917. Independent t test yields no significance difference between fluorotic without Moringa stenopetala and fluorotic with Moringa stenopetala groups ($P =0.168$). There was statistically significant difference in renal function tests(plasma urea) between the treated and the control groups. Compared with the controls, renal function of 100 ppm fluoride without Moringa treated group had significant increase in plasma urea level compared to control with $F(5\ 17)= 3.966$, $P=0.02$, $\eta=0.625$ and power of 0.801 independent t test yields no significance difference between fluorotic without Moringa stenopetala and fluorotic with Moringa stenopetala groups ($P=0.318$). There was severe histological change in liver, kidney and pancreas in group treated with F without Moringa.*

Conclusion and Recommendation: *The present study depicted that exposure to F alters the structures and function of organs in question. These are evidenced by necrosis of hepatocytes, glomeruli and pancreatic acinar cells, decreased renal urea clearance and increase in plasma alkaline phosphate level. Moringa has shown a protective role against F toxicity probably by its antioxidant role. Further large scale study is recommended.*

Key words: *Fluorosis, soft tissue, sodium fluoride, Liver, kidney, pancreas, Moringa stenopetala protective effect, histology.*

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TABLE OF CONTENTS

CONTENTS

LIST OF FIGURES

LIST OF TABLES

Acronyms

ACP	=	Acid Phosphatase
ALP/AKP	=	Alkaline Phosphatase
ANOVA	=	Analysis Of Variance
AST	=	Aspartate Aminotransferase
ALT	=	Alanine Aminotransferase
BUN	=	Blood Urea Nitrogen
Bu	=	Biluribin
CAT	=	Catalase
Cre	=	Creatinine
CNS	=	Central Nervous System
ER	=	Endoplasmic Reticulum
F	=	Fluoride
HE	=	Haematoxylin and Eosin
IFCC	=	International Federation of Clinical Chemistry
JU	=	Jimma University
L	=	Liter
LPO	=	Lipid peroxidation
LFT	=	Liver Function Test
MDA	=	Malondialdehyde
MO	=	Moringa Olifera
MS	=	Moringa Stenopetala
NaF	=	Sodium Fluoride
NBF	=	Neutral Buffered Formalin
NO	=	Nitric Oxide
NADPH	=	Nicotinamide Adenine Dinucleotide Phosphate
SOD	=	Superoxide Dismutase
GSH-Px	=	Glutathione Peroxidase
OSI	=	Organ-Somatic Index
ROS	=	Reactive Oxygen Species
RFT	=	Renal Function Test
LPO	=	Lipid Peroxidation
LDH	=	Dehydrogenase
UN	=	Urea Nitrogen
O2•−	=	Superoxide Anions
NOS	=	Nitric Oxide Synthase
NT	=	Nitrotyrosine
Ppm	=	Parts Per Million
WHO	=	World Health Organization

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Fluorine (F) is a common element that does not occur in the elemental state in nature because of its high reactivity. It accounts for about 0.3 g/kg of the Earth's crust (1) and exists in the form of fluoride ion, with an oxidation state of -1. Fluoride ions naturally occur in a number of minerals, including Fluorspar, Cryolite and Fluorapatite, and groundwater, from the breakdown of rocks and soils or weathering and deposition of atmospheric volcanic particles (2). Apart from their health impact, inorganic fluorine compounds are used in aluminum production and steel and glass fiber industries. They can be released to the environment during the production of phosphate fertilizers (which contain an average of 3.8% fluorine), bricks, tiles and ceramics. Fluor-silicic acid, sodium hex-fluorosilicate and sodium F are used in municipal water fluoridation schemes (3).

Below or above its recommended narrow safety ranges, fluoride is a double-edged sword, of adverse consequences. Prolonged ingestion of F in excess of the daily requirement is associated with Fluorosis, Similarly, inadequate intake of F in drinking water is known to cause dental caries (4). Excessive F intake over a long period of time results in a serious public health problem called fluorosis, which is characterized by dental mottling, skeletal manifestations such as crippling deformities, osteoporosis, osteosclerosis, and soft tissue damage (5). Skeletal and dental toxicity was most common because of fluorides, however soft tissues are also afflicted with damage by F (6). Soft tissues and organs are affected by the excess F, which is referred to as non-skeletal fluorosis. kidneys, pancreas spleen and liver are affected in non-skeletal fluorosis (7).

Globally, twenty eight countries face the problem of F concentration above 1.5 mg-F/L in drinking water. Many of these countries are confronted with the problems of skeletal and non-skeletal fluorosis (8). In US twenty-three percent of persons aged 6–49 had fluorosis(9). In China the prevalence rate of dental fluorosis increased significantly with the increase of water F concentration. The prevalence rates of dental fluorosis ranges from 40.82- 67.20% in areas where F concentration in drinking water was >4 mg/l (10). Where as in Brasil fluorosis was detected in approximately 36% of the volunteers examined in particalar research(11). In a study in india a

high prevalence of dental fluorosis 76.74% has been found with the F level ranging from 0.04 to 4.9 mg/l(12).

The East African Rift Valley which cuts through Ethiopia is geomorphologically still an active volcanic region. The volcanic rocks particularly in the young basalt contain high concentrations of F and Fluorapatite. Large fault systems in the Valley create conditions that allow very deep percolation of infiltrating surface water. The floor of the Rift Valley which is characterized by high hydrothermal activity accelerates the solubility of fluorite. The hot climate and high F water bed of the Rift Valley therefore favors the development of endemic fluorosis (8).

Over 14 million of people in the Ethiopian Rift Valley region are at risk of fluorosis (13). In Ethiopia F concentration in the water sample varied from 0.0 mg/L to 40 mg/L. Generally in water samples 38.5 % of the well water samples, 15.18 % of the spring water samples and 12.18 % of the taps water samples had F concentration higher than the maximum allowable concentration, which is 0.5-1.5 mg/dl (3,14). The prevalence of dental fluorosis ranges from 70 to 100%, with 35% having the severe form of the complication in children in Ethiopian Rift valley (8).

The main route for the incorporation of F into the human body is the digestive tract; 90% of the F ingested is absorbed in the stomach. In adults, some 10% of it is deposited in the bones, whereas in children, up to 50% is fixed to bone tissue. The maximum concentration of F in plasma is reached between 30 and 60 min after intake(15). Fluoride is absorbed from GI by simple diffusion readily.

Moringa *(Moringa stenopetala,* Bac.) a tree that belongs to the family Moringaceae is cultivated in Ethiopia mainly in the Zones and Special districts such as South Omo, Gamo Gofa, Kaffa, Sheka, Bench Maji, Wolaita, Dawaro, Bale, Borena, Sidama, Burji, Amaro, Konso and Derashe(16). Apart from being consumed as a vegetable *M. stenopetala* is also used to treat malaria, hypertension, stomach disorder and dysentery, asthma, diabetes, epilepsy and visceral leishmaniosis by local communities (17).

The *M. stenopetala* extract is rich in antioxidants including rutin and has potent anti-oxidative effect(18,19). Moringa (MS) Lam (Family: Moringaceae) is a highly valued plant in tropic and subtropical countries where it is mostly cultivated(20). The leaves are highly nutritious, being a good source of protein, β-carotene, vitamins A, B, C and E, riboflavin, nicotinic acid, folic acid,

pyridoxine, amino acids, minerals and various phenolic compounds(20,21). Moringa Olifera (MO) leaves are highly nutritious, being a significant source of beta-carotene, vitamins, protein, minerals, amino acids, sterols, glycosides, alkaloids, flavonoids and phenolic acids(21,22). Almost all the parts of these plants have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepato-renal disorders.

1.2 STATEMENT OF THE PROBLEM

About five billion people worldwide experience dental fluorosis presented in various forms of discomfort at different stages of its clinical presentation(23). The major health problems caused by excessive F include dental fluorosis, and skeletal fluorosis, deformation of bones in children and adults(24–26). The presence of dental fluorosis can be regarded as a marker for accumulation of F in the body(27).

In China the prevalence rate of dental fluorosis increased significantly with the increase of water F concentration. The prevalence rates of dental fluorosis ranges from 40.82- 67.20% in areas where F concentration in drinking water was >4 mg/l (29).

Where as in Brasil fluorosis was detected in approximately 36% of the volunteers examined in particalar research(30). In a study done India a high prevalence of dental fluorosis 76.74%has been found with the Flevel ranging from 0.04 to 4.9 mg/l(31).

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Generally in water samples 38.5 % of the well water samples, 15.18 % of the spring water samples and 12.18 % of the taps water samples had F concentration higher than the maximum allowable concentration, which is 0.5-1.5 mg/dl(1,25). The prevalence of dental fluorosis ranges from 70 to 100%, with 35% having the severe form of the complication in children in Ethiopian Rift valley(25).

Dental as well as skeletal fluorosis is endemic in the Region of Ethiopian Rift Valley. This is because of the higher F level in the ground water sources of this region (14). The region is one of the worst affected by surface fresh water scarcity in the country and hence, the population in this region is dependent up on ground water sources. On the other hand, as this region is found in hot and dry climatic zone of the country, the daily intake of water and, therefore, that of F could be

much higher. Besides, drinking water may not be the only source of F. But, other sources like beverages and food consumption may increase the risk of fluorosis(32).

Ethiopian rift valley is one of the regions that are affected by naturally occurring F contamination in ground water. Kloos and Tekle Haymanot reported ground water sources with F concentration above WHO recommendation, 1.5 mg/L, are found in all parts of Ethiopia but the highest level of F levels are found in Rift valley, the lowland area of the country with recent volcanic activity(33). Rango and his colleague's study indicated that water wells in the Ziway-Shala basin of Rift valley have high F levels with average of 9.4 mg/L and range from 1.1 to 68 mg/L(34). They also found that 48 of 50 water wells they investigated in the basin have F levels that exceed the WHO drinking water guideline limit of 1.5 mg/L.

Another study by Rango and Tekle Haimanot et al showed that the fluoride level of drinking water collected from deep wells in the Rift Valley ranges from 1.5 mg/L to 36 mg/L with mean value of 10.0 mg/L(25). An intake of F above 20 parts per million is toxic and causes pathological changes in the bones and different tissues. Neurological disturbances are also common in this level of fluorosis(35).

Fluorosis is prevalent public health problem in Ethiopian Rift Valley as large number of people in the region depends on F contaminated ground water for drinking and domestic use. Beyond its impact of compromising living standard of the affected people it has huge economic impact as skeletal fluorosis impairs physical ability of people in productive age.

Moreover, many epidemiological studies and animal studies indicated other adverse health out comes due to chronic exposure to high level of F; A systematic review and meta-analysis study indicated that children in high F areas had significantly lower IQ scores than those who lived in low F areas(36).

Despite emerging data that indicate detrimental effects of chronic fluorosis on soft tissue organs World Health Organization (WHO) (2004) report indicated that the absence of sufficient evidence on effect of fluorosis on soft tissue organs including hepatic, renal, respiratory, gastrointestinal and reproductive system organs(3). Besides several studies recommended further studies are needed to understand the effect of F on soft tissues(37,38).

The purpose of this study was to determine changes in soft tissue as a result of exposure to F on soft tissue organs, and protective effect of *Moringa stenopetala* leaf crude extract against soft

tissue organs damage in adult mice as such study is very scarce worldwide and not done in Ethiopia.

This is indicative for need of more researches and data on effect of fluorosis on soft tissue organs. Specially, because of prevalent fluorosis problem in Ethiopia investigating its impact on organs and systems generates important data that will be used by affected community and stakeholders in tackling the problem.

1.3 SIGNIFICANCE OF THE STUDY

Ethiopia is of particular interest due to the predominance of extensive volcanic basalt flows in the lowland and highland regions. Exacerbated by low socioeconomic status wide spread occurrence of skeletal fluorosis in Rift Valley Regions and dental fluorosis highland areas, efforts are being on progress on fluorosis control recently.

Despite the extensive studies regarding dental and skeletal fluorosis worldwide concrete evidences are lacking about fluoride toxicity on soft tissues.

Moreover, *Moringa stenopetala,* a tree, which is used by some Ethiopian communities as food and tea, may have protective effect against tissue damaging impact of fluorosis because of its potent antioxidants content.

Thus, the outcome of the present study will contribute towards enriching local literature for scientific community as evidence for further study in large scales and to develop new view and evidence based strategies to tackle the huge impact of fluorosis; and also will generate scientific information to the affected communities on optional ways to minimize the adverse effects of fluorosis.

CHAPTER TWO: LITERATURE REVIEW

From various sources of exposure in human being, the main route for the incorporation of F into the body is the through digestive tract; 90% of the F ingested is absorbed in the stomach. Inhalational, topical through skin could be the possible routes. In adults, some 10% of it is deposited in the bones, whereas in children, up to 50% is fixed to bone tissue. The maximum concentration of F in plasma is reached between 30 and 60 min after intake. F is absorbed from gastrointestinal tract by simple diffusion readily(15).

2.1 EFFECT OF FLUORIDE ON LIVER

Fluoride causes serious health problems, as it is a well determined non-biodegradable and moderate pollutant. As a very active site of metabolism, the liver is especially susceptible to F intoxication(27). The histopathological results in the study indicate that exposure to NaF in high doses caused necrotic changes in hepatocytes and liver sinusoids(39).

Histopathological examination revealed increasing degrees of hepatocellular necrosis, degenerative changes, hepatic hyperplasia, extensive vacuolization in hepatocytes, and centrilobular necrosis in the liver of the exposed animals. The central vein and sinusoids of the liver were dilated and engorged with blood and were associated with small areas of hemorrhages. These effects were not observed in the control group(27). This hepatotoxicity in rabbit exposed to NaF might be due to oxidative stress. Histopathological changes in the liver interrupt the normal hepatic architecture(39). Histopathological disorders were observed in liver specimens of the animals exposed to chronic fluorosis depending on doses of chemicals given to the animals. Hyperemia, local necrosis, hydropic degeneration, vacuolar degenerations, and swelling on hepatocytes around the central vein were detected(40).

The study by Tao and et al indicated the mechanism of excess F on the impairment of soft tissues involved in lipid peroxidation and decreased the activities of some enzymes associated with free radical metabolism(41).

In thyroid, liver, and kidney tissues, the MDA level was significantly increased, and SOD and GSH-Px activities were significantly decreased. These results suggest that F induces excessive production of NO and reactive oxygen species (ROS), enhances lipid peroxidation, and disturbs

the body antioxidant system of pigs. Oxidative damage from oxidative stress could therefore be an important pathway for fluoride toxicity in soft tissues(40).

But there are some contrary reports. Reddy et al. reported finding which shows that no changes in lipid peroxides, GSH, and vitamin C levels, as well as in SOD, GSH-Px, and CAT activities in red blood cells of fluorotic humans and rabbits(41).

The study by Hanen Bouaziz, Sabeur Ketata et al showed a significant decrease in serum levels of total protein and albumin, a marked hypoglycemia and a significant decline in serum cholesterol and triglyceride levels in F-treated mice and their pups. Whereas globulin and bilirubin levels in serum were not significantly changed by NaF treatment(42). On the other hand, serum transaminase activities (aspartate transaminase; alanine transaminase), which well known as markers of liver function, were elevated indicating hepatic cells' damage after treatment with F. (43).

Xi Shuhua, Liu Ziyou, et el. Found that Fluoride Decreased the Cell Viability of BV-2 Cells, increase of ROS and LPO has been considered to play an important role in the pathogenesis of chronic F toxicity(44).

Anamika Jha and Komal Shah reported protein degradation in liver of rabbits during experimental fluorosis. Also, F is known to affect the rate of cellular protein synthesis, which is mainly due to impairment of peptide chain initiation(45).

According to the finding by Seema Zargar et, al, administration of quercetin two hours before CdF2 significantly reduced the biochemical alterations in reduced glutathione, ascorbic acid, lipid peroxidation, super oxide dismutase, catalase and total proteins(46).

2.2 EFFECT OF FLUORIDE ON KIDNEY

The kidney is well recognized for its histopathological and functional responses to excessive amounts of F(47). Moreover, this organ is the major route for removal of F from the body and thus is sensitive to damage(48). Fluoride caused various renal histological structure changes such as necrosis of glomeruli and tubules, atrophic glomeruli, glomerular capsule and tubules dilatation; moreover, severe tubular leakage(49).

Study by Xiu Zhan indicated that supplemental F-treatment caused severe renal histological changes as well as increased renal cell apoptosis. In kidney tissue, lactate dehydrogenase (LDH) activity was significantly increased whereas alkaline phosphatase (ALP) activity was significantly decreased. In the serum, significantly increased urea nitrogen (UN) was present and the serum had elevated creatinine (Cre) and decreased Na+. Their findings show that chronic excessive F exposure is deleterious to kidney structure and function of pigs(49).

According to Inkielewicz, Krechniak J. et al in all the tissues and organs the F content increased in a dose-dependent and a time-dependent manner. In animals receiving the higher dose of sodium Fluorides (NaF) the increase after 12 weeks of exposure was about two-fold in serum, seven-fold in liver and kidney, nine-fold in brain, and twelve-fold in testis. Urinary F also increased from the beginning of exposure in a dose-dependent manner(48).

Fluoride induces various degrees of damage to the architecture of proximal tubular epithelia, such as cell swelling and lysis, cytoplasm and mitochondria vaculaotion, nuclear membrane breakdown, cell shrinkage, nuclear condensation, apoptosis, and necrosis(49).

The NaF treatment caused a significant decline in organo-somatic Index, total protein, cholesterol, DNA, RNA, Acid phosphates (ACP) and ALP and a significant dose dependent increases in the level of glycogen, Urea and Cr which are indicative of membrane permeability, cell function and tissue damage(50).

2.3 EFFECT OF FLUORIDE ON PANCREAS

The pancreas plays an important role in the system of neuroendocrine regulation of metabolic processes(51). The impact mechanism of F on the pancreas and in particular on its hormonal function has not been researched sufficiently (52).Previous studies have emphasized the adverse impact of F on diabetic patients because they typically consume much larger quantities of water than average humans and have impaired kidney function leading to higher risk from the diverse toxic effects of F(53). At appropriate concentrations, sodium fluoride can be a direct cellular toxin which interferes with calcium metabolism and enzyme mechanisms by activating both proteolytic and glycolytic functions(53).

Decreasing of the insulin concentration and increasing of the CR-peptide level in blood serum of workers of cryolite industries detected by radioimmunilogical method. These changes were

caused by the fluorine intoxication of workers(51). These results indicate that excessive F in the diet can inhibit pancreatic digestive enzyme activities and cause ultrastructural changes which may lead to a series of biochemical and pathological abnormalities(54). Although fluorosis has been investigated for many years, there are relatively few studies of its effect on the digestive system such as the pancreas, a small organ located near the lower part of the stomach and the beginning of the small intestine. Enzyme secretions of the exocrine pancreas are required for hydrolysis of nutrients present in food and feed(55). Activities of pancreatic lipase and protease (but not amylase) were significantly decreased. Pancreatic acinar cells showed markedly swollen mitochondria and loss of mitochondrial cristae. Endoplasmic reticulum (ER) was markedly dilated and its folds were irregular(54). *Moringa stenopetala* has been used in traditional health systems to treat diabetes mellitus(35).

2.4 MECHANISM OF FLUOROSIS

Many studies implicated that among the mechanisms by which fluorosis cause damage to tissue cells is through aggravating oxidative stress. Increasing oxidative stress, interference with the balance of anti-oxidase activity and morphological changes in soft tissue organs, and decrease in cell viability and excessive oxidative stress in primary cell culture are indicated by animal model and cell culture studies(44). It is concluded that F has accumulated in the blood circulatory system and dietary F in the range of 800 - 1200 mg/kg could significantly induce abnormalities of bone, liver and kidney, inhibit the synthesis of protein, enhance lipid peroxidation and disturb the anti-oxidative system of broiler chickens(32). The generation of ROS and LPO has been considered to play an important role in the pathogenesis of chronic F toxicity and study found that NaF of concentration from 5 to 20 mg/L can stimuli BV-2 cells to change into activated microglia displaying upregulated OX-42 expression.(44).

Excessive F may cause central nervous system (CNS) dysfunction, and oxidative stress is a recognized mode of action of F toxicity. In CNS, activated microglial cells can release more ROS, and NADPH oxidase (NOX) is the major enzyme for the production of extracellular superoxide in microglia. ROS have been characterized as an important secondary messenger and modulator for various mammalian intracellular signaling pathways.

Fluoride increased JNK phosphorylation level of BV-2 cells and pretreatment with JNK inhibitor SP600125 markedly reduced the levels of intracellular $O2\cdot-$ and NO. NOX inhibitor apocynin

and iNOS inhibitor SMT dramatically decreased NaF-induced ROS and NO generations, respectively. Antioxidant melatonin (MEL) resulted in a reduction in JNK phosphorylation in F-stimulated BV-2 microglial cells. The results confirmed that NOX and iNOS played an important role in F inducing oxidative stress and NO production and JNK took part in the oxidative stress induced by F and meanwhile also could be activated by ROS in F-treated BV-2 cells.(56)

The generation of ROS and LPO has been considered to play an important role in the pathogenesis of chronic F toxicity. Intracellular superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), ROS, superoxide anions ($O2^{\bullet-}$), nitric oxide synthase (NOS), nitrotyrosine (NT) and nitric oxide (NO), NOS in cell medium were determined for oxidative stress assessment. SOD activities significantly decreased in F-treated BV-2 cells as compared with control, and MDA concentrations and contents of ROS and $O2^{\bullet-}$ increased in NaF treated cells. Activities of NOS in cells and medium significantly increased with F concentrations in a dose-dependent manner. NT concentrations also increased significantly in 10 and 50 mg/L NaF-treated cells compared with the control cells. Toxic effects of F on the CNS possibly partly ascribed to activating of microglia, which enhanced oxidative stress induced by ROS and reactive nitrogen species.(44)

After an oral dose, F- may inhibit the respiratory chain, increasing the production of superoxide radical (F- treatment significantly inhibited complex IV and partially decreased (30% inhibition) complex I-II and complex I-III activities) and thereby of hydroxide peroxide and peroxynitrite. Antioxidant enzymes activities cannot prevent increased free radical formation. Therefore, there is an increase in ROS that finally produce oxidations in membranes and damage the cell macromolecules (as seen by the increase in lipid peroxidation) and may be the cause of the inflammatory foci observed in the bone. Therefore, inflammatory foci could be enhanced via ROS-dependent activation of pro-inflammatory genes.(57)

2.5 POTENTIAL PROTECTIVE ROLE OF MORINGA STENOPETALA

Moringa *(Moringa stenopetala,* Bac.) known as "The Miracle Tree" is a tree that belongs to the family Moringaceae, is cultivated in Ethiopia mainly in the Zones and Special districts such as South Omo, Gamo Gofa, Kaffa, Sheka, Bench Maji, Wolaita, Dawaro, Bale, Borena, Sidama, Burji, Amaro, Konso and Derashe(44). Moringa stenopetala is a drought tolerant and fast growing tree, and an indigenous vegetable tree native to southern Ethiopia, grown mainly for its

food value. Millions of people in southern Ethiopia consume its leaves as a staple vegetable. It has potential medicinal and industrial applications and significant economic contributions (24,58). Moringa olifera (MO) Lam (Family: Moringaceae) is a highly valued plant in tropic and subtropical countries where it is mostly cultivated(59). The leaves are highly nutritious, being a good source of protein, β-carotene, vitamins A, B, C and E, riboflavin, nicotinic acid, folic acid, pyridoxine, amino acids, minerals and various phenolic compounds(20,59,60). MO leaves are highly nutritious, being a significant source of beta-carotene, vitamins, protein, minerals, amino acids, sterols, glycosides, alkaloids, flavonoids and phenolic(20,22). Almost all the parts of these plants have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders(21). Moringa possesses antitumor, anti-inflammatory, antihypertensive, cholesterol lowering, antioxidant, antidiabetic and hepatoprotective activities. M. oleifera is very important for its medicinal value. Various parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods act as cardiac and circulatory stimulants, possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities, and are being employed for the treatment of different ailments in the indigenous system of folk medicine(61,62). The plant has also been reported to be hepatoprotective against anti-tubercular drug and acetaminophen(63,64).

Moringa stenopetala contains essential amino acids, carbohydrates, fats, vitamins (A, C & E), minerals (Na, K, P, Ca, Fe, Zn, Mg, Mn), and have antimicrobial, antioxidant, antihypertensive and antidiabetics effects(65). Among these: vitamins C and E, carotenoids, and antioxidant enzymes containing Mn, Fe or Zn are involved in antioxidant activity(58).

CHAPTER THREE: OBJECTIVE

3.1 GENERAL OBJECTIVE

The purpose of this study was to evaluate protective role of *Moringa stenopetala* against sub-chronic exposure of fluoride in soft tissue of mice.

3.2 SPECIFIC OBJECTIVES

1. To determine the effect of sub chronic exposure to F on body weight of mice.
2. To detect effect of subchronic exposure to F on histology of liver, kidney and pancreas in mice.
3. To investigate the effect of subchronic exposure to F on liver & renal functions in mice.
4. To evaluate protective role of *Moringa Stenopetala* against soft tissue injury in mice.

CHAPTER 4: MATERIALS AND METHODS

4.1 FLUORIDE PREPARATION

NaF was used to sub-chronically expose the mice to fluoride. NaF with 99.5% purity was purchased from LOBA Chemie laboratory reagents and fine chemicals Mumbai, India. A stock solution of 2000ppm fluoride was prepared by dissolving 4.42g of NaF in 1L of distilled water; and stored at 4-8 °C for 1 week. The stock solution was diluted with distilled water to prepare different test doses of fluoride at concentrations of 0.07ppm, 60ppm and 100ppm. To prepare working solution with 0.07ppm fluoride, 0.035ml of stock solution was diluted by 1L of distilled water; 60ppm fluoride: 30ml of stock solution diluted in 1L distilled water; and 100ppm fluoride was obtained by diluting 50 ml of stock solution by 1L distilled water. New batches of test solutions, not older than one week were used for administration (50).

4.2 PREPARATION OF MORINGA STENOPETALA AQUEOUS EXTRACT

The fresh leaves of *Moringa stenopetala* were collected from Jimma agricultural research center and confirmed by experts. The leaves were rinsed with tap water to clean off extraneous materials and air-dried under shade without sunshine exposure in shadow room, then powdered. The dried leaves were then cut into pieces and ground to a fine powder using a sample mill. The powdered substance was then weighed using an analytical balance and stored at room temperature until extraction.

Aqueous extract of MS leaves powder was used for these studies. This was in accordance with the traditional way of preparation and use of the plant for tradition medicine. One hundred gram of the powdered plant material was soaked in 1000 ml of distilled water in conical flask at room temperature and stirred intermittently for 72 hours on Orbital Shaker. The material will then filtered using sterile Whatman No.1, 15 cm filter paper into a clean conical flask. The filtrates were freeze-dried in a lyophilizer to yield a crude extract. From 500 g dry leaf, which was dissolved in a total of 5000ml distilled water (1:10), 105g (10.03%) of crude extract was obtained. The crude extract was kept in desiccators at -20^{o} C until used.

4.3 STUDY DESIGN

Experimental (Randomized Controlled study) were used. The study has multiple end points and for determining sample size resource equation method were used. This method is best when it is not possible to assume about effect size, to get an idea about standard deviation as no previous findings are available or when multiple endpoints are measured or complex statistical procedure is used for analysis.

$$sample\ size = \frac{Resource\ at\ hand}{cost\ per\ single\ sample} = 21$$

By taking existence possible attrition into consideration (10%) = 21(10%) =2.2 ~3 Total sample size =21+3= 24 According to resource equation E (degree of freedom) is measured as follow: E=Total number of animals – Total number of groups.

E = 24 – 6; E= 18

Where E: is the degree of freedom of analysis of variance (ANOVA). E between 10 and 20 is considered as adequate. E larger than 20 implicate more than adequate animals are used (66).This implicates as we intended to utilize more than adequate animals, but this is justifiable as the sample of blood that can be obtained from a mice is limited, only 0.75ml and increasing the number of animals used is obligatory for this study. Moreover, some procedures during data collection necessitate avoiding of the mice from other procedures to be done, so this also influence to increase the number of animals that will be used in the study.

First 24 Swiss Albino Mice were selected from adult healthy mice population randomly, and then these mice were randomly assigned into five experimental and one control group.

Table 1 Grouping and dosage of fluoride and *Moringa stenopetala*

Groups (N=4)	1	2	3	4	5	6
Fluoride Dose (ppm)	Control	60	100	60	100	0.07
Moringa Treated (mg/Kg)	-	-	-	100	100	100

4.4 ANIMALS AND ANIMALS HANDLING

Animals and management: The study was conducted using laboratory bred albino Mice. Twenty-four (12 male and 12 female) apparently healthy, 7- 8 weeks old mice weighing 33.8±5.5g, were collected from Tropical and Infectious Disease Research Center, Jimma University. The animals were housed in rectangular polypropylene cages (four mice per cage in order to provide ample spaces for movement and ambient temperature) with sawdust as bedding material and to soak the excretory fluids. Throughout the study period, the male and female mice were kept in separate cages to avoid breeding, and maintained under constant laboratory conditions of temperature (22 ± 2^{o}c) with 12 hours light/dark cycle. All animals were allowed free access to standard pellet diet *ad libitum* and distilled water except when starvation was otherwise needed. The food and water were changed daily and the cages were cleaned and the sawdust changed every three days. An acclimatization period of 7 days was allowed before experimentation, in order to minimize any non-specific stress as suggested by different scholars in various similar studies. Keeping all the environmental and other variables constant the outcome variables were effect of sodium F and effect of *Moringa stenopetala.*

4.5 BODY WEIGHT MEASUREMENT

Before the experiment selected mice baseline weight was measured using electronic balance (PA4102C, China). Individual weight of the experimental and control groups of animals was recorded as an initial weight before the test substance was administered and then weighed weekly afterwards until the end of schedules necropsy. At the end of the experimental period, animals weighed and humanely sacrificed under anesthesia by diethyl ether. Then blood sample, Liver, kidney and Pancreas carefully removed and weighed on electronic balance and weight of the organs per mg/l00gm of body weight calculated.

4.6 ANESTHESIA AND EUTHANASIA

At the end of the expriment, 12 hours before the Euthanasia animals deprived of food and drink. After fasting, the animals were weighed and recorded as the final body weight and then humanely sacrificed under anesthesia by diethyl ether. Immediately after anesthesia, blood sample were collected via cardiac puncture by the researcher followed by collection of the target organs for histopathological study. The liver, kidney and pancreas carefully removed, cleared off

surrounding tissue, washed by 0.9% NaCl, dried on tissue paper and weighed on electronic balance and weight of the organs per mg/g of body weight calculated.

Concentrated diethyl ether (99%) prepared, and the ether soaked with cotton. The mice were locked in biker with ether socked cotton until losing consciousness, then transferred to dissection board pined with pines to fix with the board. Five ml syringe was inserted anteriorly below xiphoid process directly to the heart to collect blood; after blood collection the abdominal cavity was opened, and then thoracic cavity to expose heart, 10ml of 10% formaldehyde was infused directly to the heart to fix the tissues. Liver, kidney and pancreas were collected.

4.7 TISSUE SAMPLE COLLECTION AND HISTOLOGICAL PROCESSING

The collected organs cleared of any surrounding tissues, cleaned with 0.9% NaCl solution and dried on clean tissue paper and weighed on electronic balance and the organ weight recorded.

Section of liver from largest lobe around major veins, longitudinal section of the Kidney and section of Pancreas removed and fixed for histological processing. The sampled tissues fixed in 10% neutral buffered formalin (NBF) overnight at room temperature. After fixation, the tissue sections washed with water to remove excess fixatives for about six hours and dehydrated with increased concentration of alcohol of 70% for two hours, 90% for two hours, absolute alcohol-I, II for one and half hours, and III overnight. The dehydrated tissues cleared in two changes of xylene (I and II) for one and half hours and two and half hours, respectively. The tissues were then infiltrated with three changes of paraffin wax (I, II and III) for one and half hours, two and half hours and overnight, respectively. Finally the tissues embedded in paraffin wax placed in square metal plates forming tissue blocks, where by each tissue block labeled and stored at room temperature till sectioned.

The tissue blocks sectioned in ribbons at a thickness of 6 µm with Leica microtome (Leica RM 2125RT Nussloch GmbH, Germany), the ribbons of the section collected and put onto the surface of a warm water bath of temperature of 400 ^{0}c. The floating ribbons over the surface of warm water mounted onto pre-cleaned slides spread with egg albumin. The slides containing paraffin wax arranged within the slide holder and placed in an oven with temperature of 400 c for about 20 minutes so as to fix the tissue to the slides and allowed to cool at room temperature for 30 minutes and stained regressively with routine Harris haematoxylin for 6 minutes and eosin for 17-20 second (H and E).

For routine H and E staining, two series of coupling jars prepared, one for paraffin removal and hydration and the other for dehydration and clearing. Sections placed in xylene- I for 5 minutes and xylene II for 2 minutes again to remove the paraffin from tissue and hydrated with decreasing concentrations of absolute I, II and 95% alcohol for two minutes each, 70% of alcohol for three minutes and 50% alcohol for five minutes. The tissue sections washed with tap water for five minutes and stained regressively with Harris haematoxylin for 6 minutes then washed under running tape water for five minutes again. Slides immersed in acidic alcohol for differentiation and controlling over stained haematoxylin for 1 second and then put in bluing solution (Sodium bicarbonate) until they became blue.

After bluing, slides counter stained with eosin for 17-20 seconds and then washed in tape water for two minutes. The sections dehydrated with increasing alcohol concentration of 50%, 70%, 95%, absolute I and II for two minutes each. The dehydrated sections cleared with xylene I and II for three minutes each and permanently mounted on microscopic slides using Dibutylphthalate Polystyrene Xylene and cover slips and then observed by light microscope for the investigations of any histological change, thereby the histology of the treated groups compared with histology of the control group(67).

4.8 MEASUREMENTS OF LIVER FUNCTION

The serum samples of mice investigated to assess AST, ALT, and ALP enzymes levels using automated clinical chemistry machine Humanstar 100. Test tubes were labeled in duplicates and arranged in assay rack. Then 50 µl pipette used for each tube of the standards, quality control sample and animal's sample. Enzymatic UV test was used for quantitative determination of AST in the mice serum. α-Ketoglutarate reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The indicator reaction uses the oxaloacetate for a kinetic determination of NADH consumption. According to IFCC standardized procedures for ALT determination, after optimization of substrate concentrations, the Tris buffers was used, a combined buffer and serum solution pre-incubated to allow side reactions with NADH to occur and substrate α-ketoglutarate react with optimal pyridoxal phosphate activation(68). ALP was measured by using photometric test with 2, 4-Dichloroaniline (DCA), biochemical assays was done using The Automated Humanstar100 clinical biochemistry analyzer.

4.9 MEASUREMENTS OF RENAL FUNCTION

The serum samples of mice investigated to assess Urea, Cre, and BUN levels using automated clinical chemistry machine Humanstar 100. Test tubes were labeled in duplicates and arranged in assay rack, and then 50 µl pipette used for each tube of the standards, quality control sample and animal's sample. Jaffe reaction, which is based on the work of Popper, Seeling, and Wuest was used to measure Cre in plasma of the mice. In an alkaline medium, Cre forms a yellow-orange-colored complex with picric acid. The rate of color formation is proportional to the concentration of Cre present and measured photo metrically(69).

Urea is hydrolyzed by urease to form CO2 and ammonia. The ammonia formed then reacts with α- ketoglutarate and NADH in the presence of glutamate dehydrogenase (GLDH) to yield glutamate and NAD. The decrease in absorbance due to consumption of NADH is measured kinetically. Urea is synthesized in the liver from ammonia produced as a result of deamination of amino acids. This biosynthetic pathway is the human body's chief means of excreting surplus nitrogen. BUN measurements are used in the diagnosis of certain renal and metabolic diseases. The determination of serum UN is the most widely used test for the evaluation of kidney function(68,70).

4.10 STATISTICAL ANALYSIS

After data collection and cleaning, data entered in to SPSS software version 20 for statistical analysis; results expressed as mean ± SD. Statistical data analysis was performed using paired t-test, independent t- test and one-way ANOVA with post hoc Bonferini test, where $p \leq 0.05$ considered statistically significant. Data from histological assessment triangulated with the quantitative data.

4.11 DATA QUALITY ASSURANCE

For maintenance of data quality well trained personnel assigned for laboratory animal handling, sample collection and sample processing. Moreover, by using recommended laboratory equipment, and adherence to scientific procedures and kit supplier companies guidelines the quality of the research and its outcome were maintained. In addition to these, to reduce bias in some subjective parameters histological analysis was performed by blinded histologist and pathologist.

4.12 ETHICAL AND ENVIRONMENTAL CONSIDERATIONS

In the administration of substances, appropriate techniques in a professional manner, enabling the achievement of the anticipated results whilst causing minimum distress to the animals' were applied. The physico-chemical properties of administered substances and their vehicle were in accordance with the biocompatibility criteria for the route of administration: local tolerance, temperature, sterility, osmolality, in order to guarantee the prevention of side effects such as irritation and traumatization during administration.

To minimize the study animals suffering humane procedures followed in handling the animals and during sample collection and euthanasia. Enriching of the cages in which the animals will be kept, using anesthesia during sample collection and scarification of the study animals will be among the steps to decrease the distress and suffering. Disposal of contaminated laboratory utensils, animal's tissues and waste chemicals handled according to laboratory safety guidelines. Ethical clearance was obtained from Jimma University Institutional Review Board.

4.13 DISSEMINATION PLAN

The research result report will be submitted to the Department of Biomedical sciences and, research and postgraduate coordinating office, Institute of health. The research results will be disseminated by peresenting on annual reviews of Jimma University, Ethiopian public health association and other nation and international research conferences. Attempts will be made to publish on national and international reputable journals.

4.14 LIMITATION OF THE STUDY

- This study does not measured fluoride consumption for individual mice,
- Did not measured plasma or specific tissue fluoride level and
- Did not measure urine fluoride level.

CHAPTER FIVE: RESULT

5.1 PLANT MATERIAL COLLECTION AND PREPARATION

The crude extract filtrate was lyophilized where 105g crude extract was obtained from 1045g powder of Moringa stenopetala leaves using distilled water as solvent and the percentage yield of the extract was about 10.05%. The crude extract was kept in a refrigerator at -20^{o}C until used.

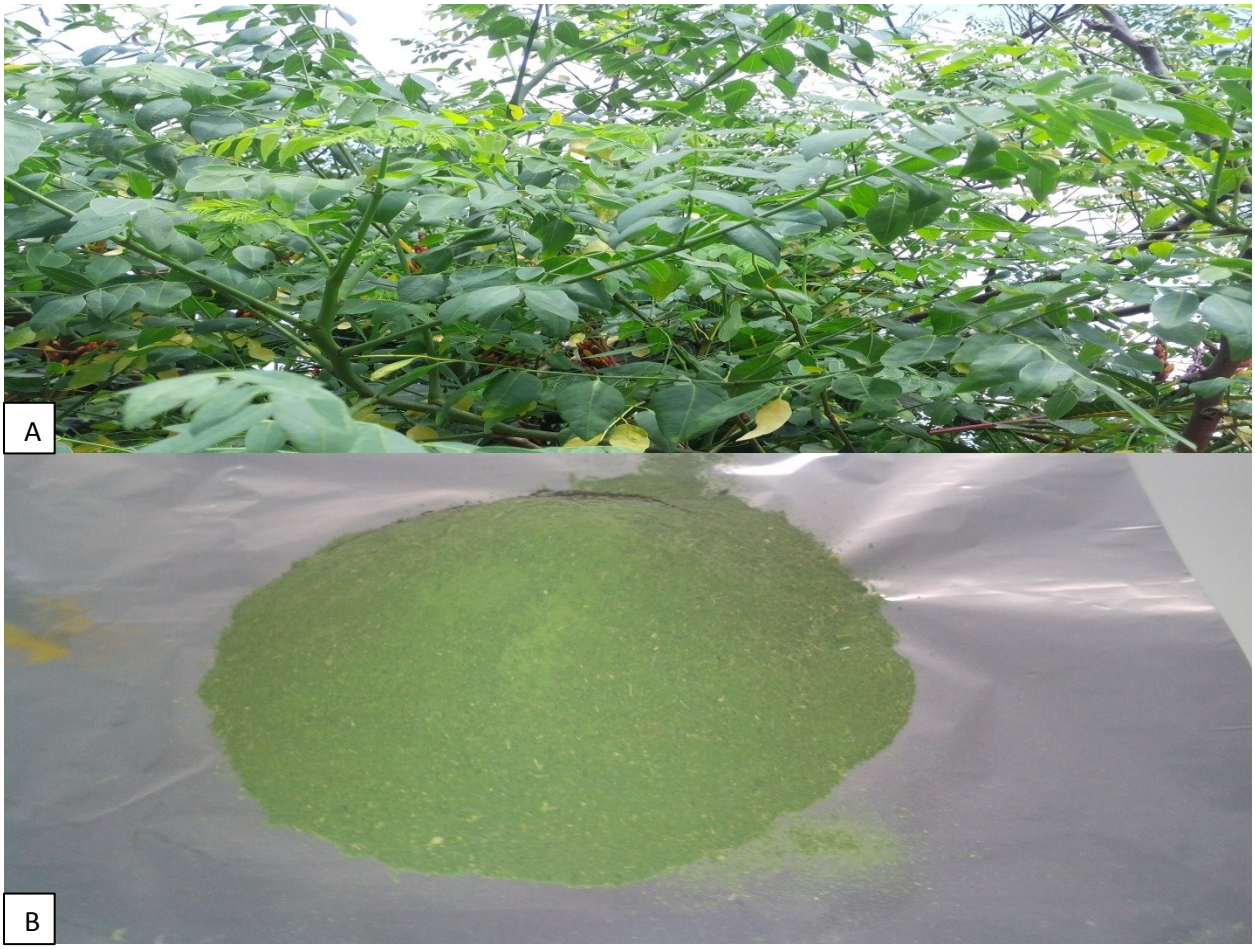


Figure 1 Moringa stenopetala tree; figure A (top) and powder after dried and grinded; figure B (bottom) in the laboratory without sunshine exposure.

5.2 SIGNS OF PHYSICAL TOXICITY OF FLUORIDE AND M. STENOPETALA

No animal showed signs and symptoms of toxicity at all doses of F and *Moringa stenopetala* as compared with the control. The parameters used to observe the physical signs of toxicity were food & water intake, appetite, pilo-erection, salivation, locomotion, lacrimation, diarrhea, urination, depression, breathing, excitement and alertness. No effects were observed in the given parameters in all groups as compared to control groups within 90 days of treatment.

5.3 EFFECTS OF FLUORIDE AND THE MORINGA EXTRACT ON GENERAL BODY WEIGHT

The mean weight for different treatment groups and control group is shown in table 2. The percentage of weight increase or decrease is different for diffrent treatment groups. From the intial weight the control group has 54.62% weight gain. Experimental group treated with 60ppm ppm NaF had showd slight reduction in weight which is 3.31%. In experimental group treated with 100ppm of NaF showed the pronounced decrease in weight which was 27.06%. There was slight increase of weight in experimental group co-treated with 60ppm+100mg/kg moringa which was 12.9% and very slight decrease in group co-treated with (100 ppm NaF + 100mg/kg moringa) which is 0.7%. Group 6 took moringa stenopetala crude extract with out F shows increment in weight by 28.96%.

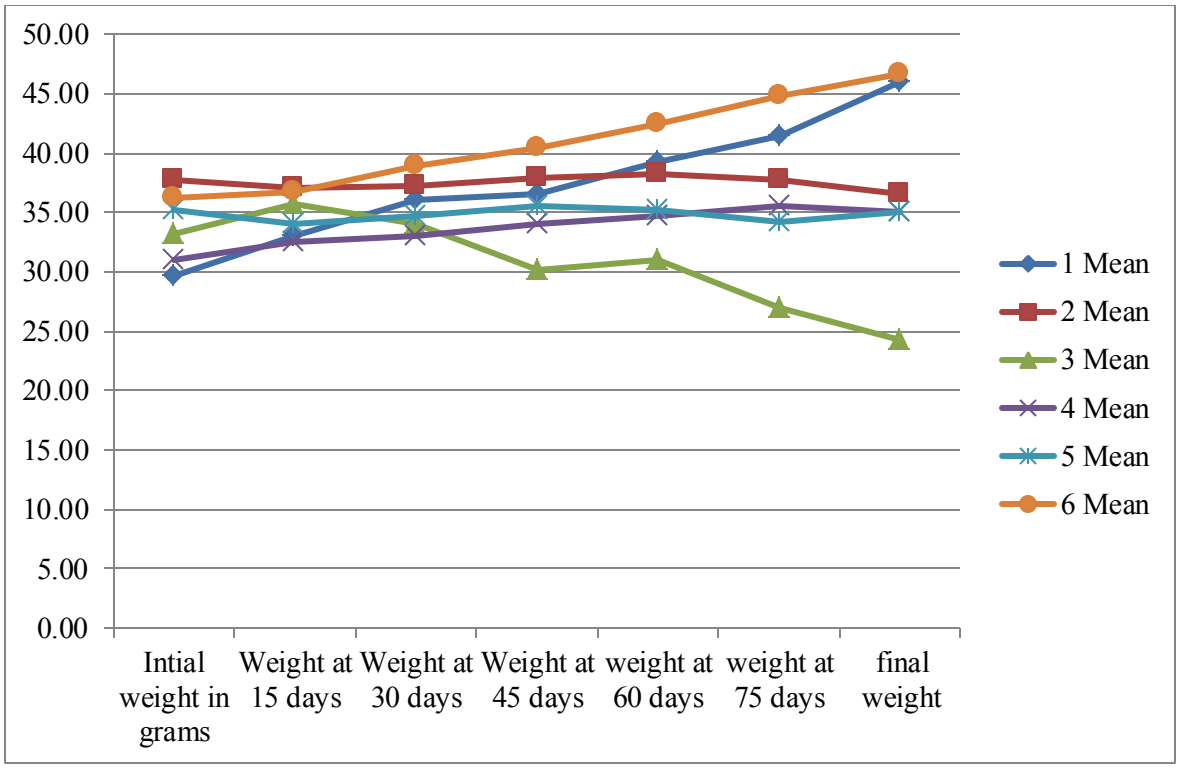
$$Percentage\ of\ weight\ gain = \frac{final - initial\ weight}{Initial} \times 100$$

Table 2 Mean body weight (in gram) of control and treatment groups of mice at different time interval, JU, May- August, 2017.

group	W0	Wt 15 days	Wt 30days	Wt 45 days	Wt 60 days	Wt 75 days	final weight
1	29.75±7.23	33.00±7.44	36.0±7.44	36.50±7.94	39.25±8.10	41.50±6.86	46.00±8.08
2	37.75±4.72	37.00±5.23	37.2±5.91	38.00±6.88	38.25±6.60	37.75±6.18	36.50±5.26
3	33.25±5.50	35.75±4.35	34.0±3.46	30.25±2.50	31.00±4.55	27.00±4.69*	24.25±4.19**
4	31.00±5.29	32.50±7.00	33.0±5.72	34.00±5.23	34.75±4.57	35.50±5.45	35.00±4.40
5	35.25±4.27	34.00±5.48	34.7±5.38	35.50±5.00	35.25±5.12	34.25±5.62	35.00±5.23
6	36.25±4.19	36.75±5.44	39.0±5.66	40.50±3.70	42.50±2.38	44.75±2.75	46.75±2.22

NB. * P-value <0.05 ** P-value <0.001

Group1=control, G2=60ppm NaF, G3=100ppm NaF, G4=60ppm NaF+100mg/kg moringa, G5= 100ppm NaF+100mg/kg moringa, G6= 100mg/kg moringa



NB. Group1=control, G2=60ppm NaF, G3=100ppm NaF, G4=60ppm NaF+100mg/kg moringa, G5= 100ppm NaF+100mg/kg moringa, G6= 100mg/kg moringa

Figure 2 Weight increase/reduction trends in control and different experimental groups of mice.

RELATIVES WEIGHT OF LIVERS, KIDNEYS AND PANCREAS

The subchronic effect of fluoride and aqueous extracts of the Moringa on organ weight of mice were illustrated in Table 3. Liver weight of control group was 3.18±0.90 (Table 3, Group I). For experimental group treated with 60ppm NaF the liver weight was reduced to 2.86±0.47. In group with 100ppm NaF treatment the value of Liver weight decreased to 2.87±0.73 compared to control. In the treatment group co-treated with 60ppm NaF+100mg/kg Moringa the value of Liver weight was 2.08±0.58. In group co-treated with 100ppm NaF +100mg/kg Moringa it was 1.49±0.86. In the treatment group with 100mg/kg Moringa alone it was 3.12±1.30.

Gross examination of Liver of treated mice in high dose showed gross enlargement and 2 mice from total of 4 mice (100ppm NaF without Moringa) develop massive enlargement and visible hepatic tumor in right lobe of liver, however there is overall reduction in liver weight in groups receiving NaF only.

The mean kidney weight of control and experimental groups is present in Table 3. Mean kidney weight of experimental groups treated with 100 ppm NaF, 60 ppm NaF and 100ppm NaF +100mg/kg Moringa were found less than mean kidney weight of control group. Mean kidney weight of experimental group co-treated with 60 ppm NaF with Moringa, and the group treated with only Moring extract were found greater than mean kidney weight of control group. But the mean kidney weight difference between the groups was not statistically significant.

The mean pancreatic weight of control and experimental groups is given in Table 3. The mean pancreatic weight of experimental groups treated with 60 ppm NaF, 100 ppm NaF, and 100ppm NaF +100mg/kg Moringa were found less than mean pancreatic weight of control group. Mean pancreatic weight of experimental group co-treated with 60 ppm NaF with Moringa and the group treated with only Moring extract were found greater than mean pancreatic weight of control group. But the mean pancreatic weight difference between the groups was not statistically significant.

Table 3 The mean organ weight in gram for control group and treatment groups of mice after exposure for sub chronic F toxicity study, JU, May- August, 2017.

Group	weight of liver(gm)	weight of pancreas(gm)	weight of kidney(gm)
1	3.18±0.90	0.33±0.04	0.42±0.05
2	2.86±0.47	0.33±0.09	0.34±0.090
3	2.87±0.73	0.43±0.19	0.32±0.12
4	2.08±0.58	0.23±0.04	0.29±0.12
5	1.49±0.86	0.23±0.18	0.23±0.09
6	3.12±1.30	0.35±0.08	0.39±0.09

NB. Group1=control, G2=60ppm NaF, G3=100ppm NaF, G4=60ppm NaF+100mg/kg moringa, G5= 100ppm NaF+100mg/kg moringa, G6= 100mg/kg moringa

Kidney and Pancreas of treated mice showed no visible sign of toxicity as compared to control group. The mean absolute weights of kidney and pancreas showed reduction as compared to control mice, but the decrease in organs weight were statistically insignificant.

Compared to the control, there was gradual weight reduction of liver as we go from control group to experimental group treated with 100ppm NaF without Moringa extract.

Compared to the control group, there was slight reduction in relative weights of kidneys as the dose of NaF increases and, in high dose NaF (100ppm without Moringa) which is 5%, this decrease can be attributed to weight loss, degeneration of structure of kidneys.

In pancreas of the mice in our study as we go through the group from control to the experimental there was slight reduction except the group with the highest dose of NaF (100ppm without moringa) there was pancreatic weight increment which is may be paradoxical effect of F on pancreas. The results of this study showed that accumulation of excessive F induced abnormalities of livers, kidneys and pancreas in mice.

5.4 ORGANO-SOMATIC INDEX

The value of organosomatic index for liver in control group was 0.0690±0.0150 (Table 4, Group I). In the treatment group treated with 60ppm NaF without Moringa the value of hepatosomatic index was 0.0792±0.0147, it was increased by 14.78% compared to control. In the treatment group treated with 100ppm NaF without Moringa, Hepatosomatic index was 0.1219±0.0392 it was increased by 76% compared to control, since 2 of the mice out of 4 mice developed massive tumor which affected the indices. In the treatment group co-treated with 60ppm NaF +100mg/kg M. stenopetala it was 0.0588±0.0093. In the treatment group co-treated with 100ppm NaF +100mg/kg M. stenopetala it was 0.0420±0.0244. In the treatment group treated with 100mg/kg M. stenopetala alone it was 0 .0660±0.0246. Even though the organ-somatic index is affected by both animal weight and organ weight the increased in organ-somatic observed in experimental group treated with NaF is absent in experiments groups treated with NaF and Moringa extract, and group treated with only Moringa extract.

$$Organosomatic\ index = \frac{organ\ weight\ in\ mg}{final\ body\ weight\ in\ g}$$

Table 4 Organo-somatic indices of liver, kidney and pancreas of mice after treatment with F and M. stenopetala extract for subchronic toxicity study, JU, May- August, 2017.

group	organo somatic index for liver	organo somatic index for kidney	organo somatic index for pancreas
1	0.0690±.0150	0.0074±0.0011	0.0072±0.0011
2	0.0792±0.0147	0.0119±0.0033	0.0093±0.0031
3	0.1219±0.0392	0.0128±0.0039	0.0174±0.0065
4	0.0588±0.0093	0.0081±0.0027	0.0067±0.0013
5	0.0420±0.0244	0.0064±0.0021	0.0064±0.0053
6	0.0660±0.0246	0.0084±0.0017	0.0075±0.0015

NB. Group1=control, G2=60ppm NaF, G3=100ppm NaF, G4=60ppm NaF+100mg/kg moringa, G5= 100ppm NaF+100mg/kg moringa, G6= 100mg/kg moringa

5.5 LIVER FUNCTION TESTS

Plasma ALP of control group was 86.00±29.72 (Table 5, Group I). In experimental group treated with 60ppm NaF without Moringa extract ALP was increased to 150.33±118.51, the increment was 74.8% compared to the control group. In experimental group that took 100ppm NaF without Moringa extract it was significantly increased to 715.00±385.95 which was 7 fold increases compared to control. In experimental group co-treated with 60ppm NaF with Moringa extract value of ALP was 270.00±40.63, which was 3 fold increased. In experimental co-treated with 100ppm NaF with Moringa extract it was 152.00±99.24, which was 2 fold increases compared to the control. In experimental group that took only 100mg/kg of Moringa it was 185.00±16.82.

Glutamic pyruvic transaminase in plasma of control mice was 122.67±88.90. In experimental group treated with 60ppm NaF without Moringa extract it was decreased to 66.33±33.08 compared to control group. In experimental group treated with 100ppm NaF without Moringa extract it was 79.33±37.58. In experimental group co-treated with 60ppm NaF with 100mg/kg Moringa extract was 56.33±10.79. In experimental group co-treated with 100ppm NaF with 100mg/kg Moringa extract it was 79.00±49.49. In experimental group treated with Moringa extract alone it was 95.33±34.20.

The value of Aspartate aminotransferase in plasma of control group was 553.67±414.05 (Table 5, Group I). In experimental group treated with 60ppm NaF without Moringa extract it was decreased to 336.67±285.31. In experimental group treated with 100ppm NaF without Moringa extract it was decreased further to 222.33±202.23. In experimental group co-treated with 60ppm NaF with 100mg/kg Moringa extract it was 260.00±194.74, which shows recovery. In experimental group treated with 100ppm NaF with Moringa extract it was 186.33±93.52, decreased as dose of F increased. In experimental group treated 100mg/kg Moringa extract only it was 267.67±1.53.

Table 5 Liver function of mice after treatment with F and M. stenopetala extract for subchronic toxicity study, JU, May- August, 2017 (Mean ± SD).

Group	Plasma AST(U/L)	Plasma ALT(U/L)	plasma ALP(U/L)
1	553.67±414.05	122.67±88.90	86.00±29.72
2	336.67±285.31	66.33±33.08	150.33±118.51
3	222.33±202.23	79.33±37.58	715.00±385.95*
4	260.00±194.74	56.33±10.79	270.00±40.63
5	186.33±93.52	79.00±49.49	152.00±99.24
6	267.67±1.53	95.33±34.20	185.00±16.82

NB. *P<0.05, NB. Group1=control, G2=60ppm NaF, G3=100ppm NaF, G4=60ppm NaF+100mg/kg moringa, G5= 100ppm NaF+100mg/kg moringa, G6= 100mg/kg moringa

5.6 RENAL FUNCTION TESTS

Plasma Cre level of control group was 0.137±.021 (Table 6, Group I). In experimental group treated with 60ppm NaF without Moringa extract plasma Cre level was increased to 0.233±.121. In experimental group treated with 100ppm NaF without Moringa extract it was 0.143±.02.

In experimental group co-treated with 60ppm NaF with Moringa extract plasma Cre level was 0.133±.012. In experimental group co-treated with 100ppm NaF with 100mg/kg Moringa extract it was 0.120±.000. In experimental group that treated with 100mg/kg Moringa extract it was 0.120±.017.

Plasma Urea level of control group was 52.60±1.30 (Table 6, Group I). In experimental group that treated with 60ppm NaF without Moringa extract plasma Urea level was 55.17±8.72. In

experimental group that took 100ppm NaF without Moringa extract it was 103.90±45.06 and it was increased by 97.5%. In experimental group co-treated with 60ppm NaF with 100mg/kg Moringa extract plasma Urea level was 46.33±8.96 which shows 12% reduction. In group co-treated with 100ppm NaF + 100mg/kg Moringa it was 50.63±13.84. In group treated with only 100mg/kg of Moringa it was 37.10±10.74 which shows further reduction by 29.5% .

Blood Urea Nitrogen of control group was 25.966±2.185 (Table 5, Group I). In experimental group treated with 60ppm NaF without Moringa extract value of BUN level was 42.227±24.416. In experimental group treated with 100ppm NaF without Moringa extract it was 23.863±2.615. In the group treated with 60ppm NaF + 100mg/kg Moringa BUN level was 28.505±15.914. In group with 100ppm NaF + 100mg/kg Moringa it was 23.660±6.469. In experimental group treated with Moringa extract only it was 17.336±5.019.

Table 6 Renal function result after treatment with fluoride and M. stenopetala extract for subchronic toxicity study in mice, JU, May- August, 2017. (Mean ± SD)

Group	Cre(mg/dl)	Urea (mg/dl)	BUN(mg/dl)
1	0.1367±0.02082	52.6000±1.30000	25.9657±2.18496
2	0.1433±0.02082	55.1667±8.72028	23.8629±2.61529
3	0.2333±0.12097	103.9000±45.06407*	42.2274±24.41616
4	0.1333±0.01155	46.3333±8.96121	28.5047±15.91374
5	0.1200±0.00000	50.6333±13.84281	23.6604±6.46860
6	0.1200±0.01732	37.1000±10.74058	17.3364±5.01896

NB. *P<0.05, Group1=control, G2=60ppm NaF, G3=100ppm NaF, G4=60ppm NaF+100mg/kg moringa, G5= 100ppm NaF+100mg/kg moringa, G6= 100mg/kg moringa

5.7 HISTOLOGICAL EXAMINATION

Histopathological examination of the tissues was followed as per Humason (71).

5.7.1 LIVER

In this study, the general morphology of hepatocyte, nucleus and cytoplasm of hepatic cells in the control mice were in the normal range of healthy mice, figure 6 A.

The mice exposed to sodium fluoride separately and in combination with Moringa for 90 days have shown remarkable histological changes when compared to control (figure 6A to C and figure 7D to F). The liver tissue of mice exposed to 60ppm NaF without moringa(figure 6B) had moderate increase cell size, variability in number and vacuolated cells, hypertrophic hepatocytes, generally increased nuclear size with nuclear size variation, diffused cellular ballooning with loss of hepatic plate architecture, mainly in prominent in preportal areas were observed. The group treated with 100ppm NaF without moringa (figure 6C) had generally increase cell size, variability in number and had extensive vacuolar degeneration, hypertrophic hepatocytes, generally increased nuclear size with nuclear size variation, loss of hepatic plate arrangement, loss of lobular architecture, preportal eosinophiliosis of cytoplasm, hyperplasia with size variation were observed. In the group expose to 60ppm NaF with moringa (figure 7D) had mild increase in cell size, mild vacuolated cells, preserved lobular architecture with mild hypertrophy and hyperplasia, inconspicuous to distinct nucleus, mild vacuolar changes with preserved architecture were observed. In the group treated with 100ppm NaF with moringa (figure E) mild variation in cell size, mild hyperplastic response, and amphophilic cytoplasm with preserved architecture were observed. In the group treated with 100mg/kg moringa (figure 7F) uniform cell size, relatively uniform cell shape, and amphophilic cytoplasm with preserved architecture were observed.

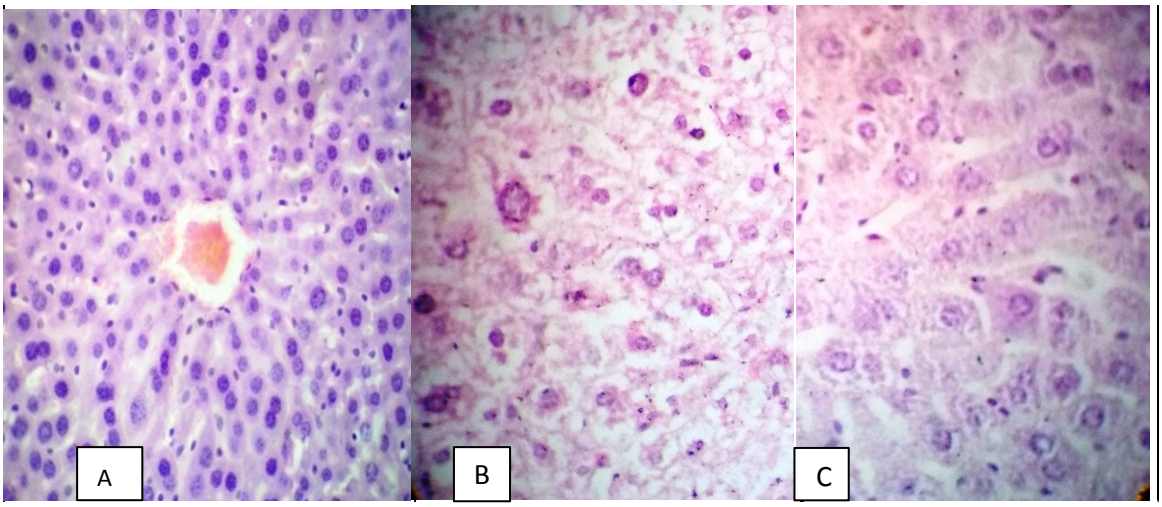


Figure 3 photomicrography of liver of control mice (A), treatment group with 60ppm NaF (B), treated group with 100ppm (C).

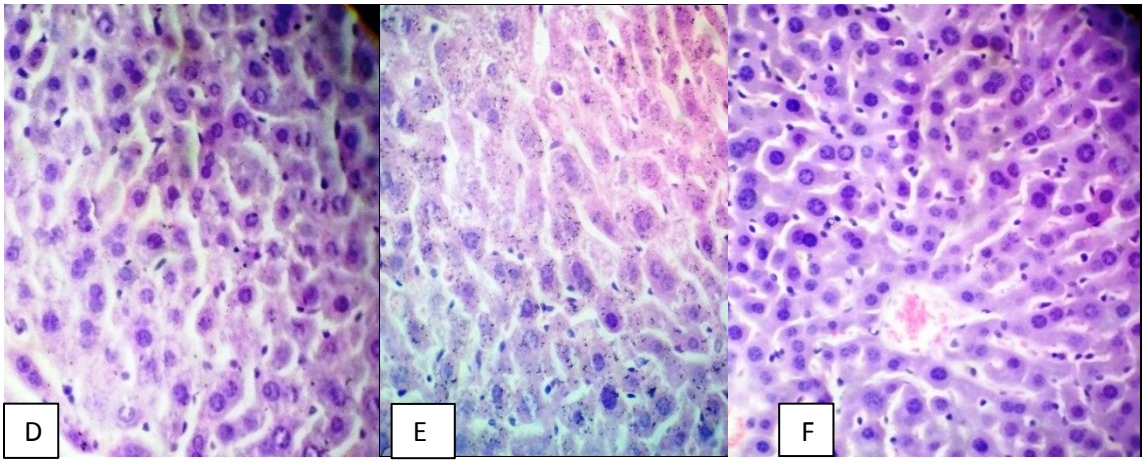


Figure 4 photomicrography of liver of treatment mice treated with 60ppm NaF+100mg/kg MS (D), treatment with 100ppm NaF+100mg/kg MS (E), treatment group with 100mg/kg MS only(F).

5.7.2 KIDNEY

In this study, the general morphology of glomeruli, basement membrane, size and shape, medullary and cortical portion cells in the control mice were in the normal range of healthy mice, figure 8G.

The mice exposed to sodium fluoride separately and in combination with Moringa for 90 days have shown remarkable kidney histological changes when compared to control (Figure 8G). The kidney tissue of mice exposed to 60ppm NaF without moringa (figure 8H) had moderate tubular

necrosis, edematous interstitium, destruction of glomeruli, moderate granulation, Eosinophilic changes observed. The group treated with 100ppm NaF without moringa (figure 8I) had extensive necrosis, interstitial hemorrhage and edema, extensive glomeruli destruction and lymphoid proliferation were observed. In the group exposed to 60ppm NaF with moringa (figure 9J) had diffused eosinophilia, glomerular necrosis, lymphocytic infiltration, mild hemorrhage and edema, and slight glomeruli destruction were observed. In the group exposed to 100ppm NaF with moringa(figure 9K) slight granulation, few lymphocytic infiltration, few focus of edema and hemorrhage and few focus of necrosis were observed. In the group treated with 100mg/kg moringa (figure 9K) very few focus of lymphatic infiltration, otherwise normal study were observed.

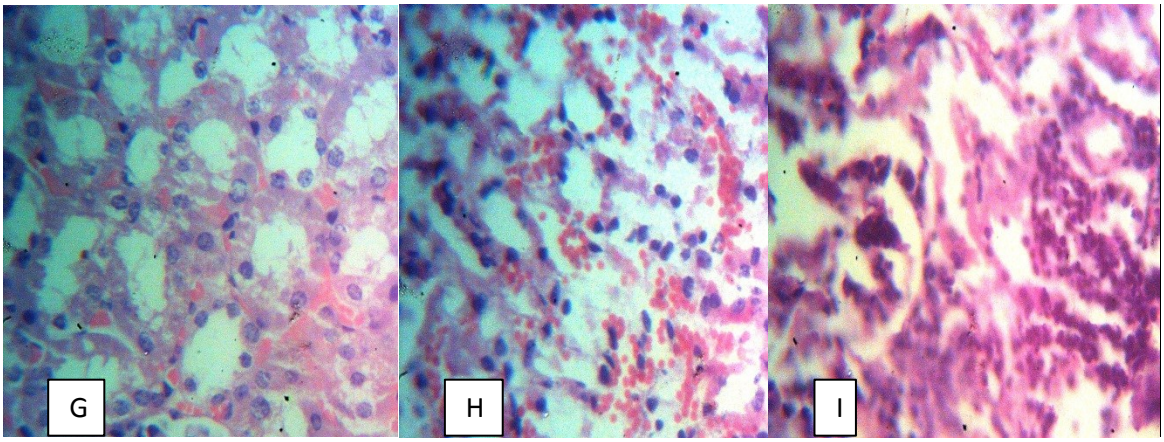


Figure 5 photomicrography of kidney of control group of mice (G), treatment group with 60ppm NaF (H), treatment group treated with 100ppm (I).

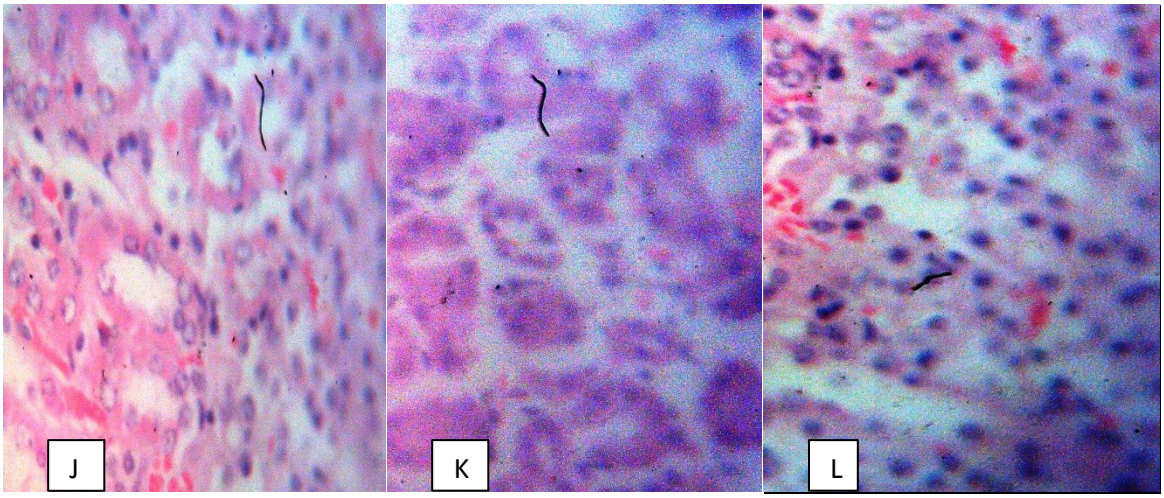


Figure 6 photomicrography of kidney of treatment mice treated with 60ppm NaF+100mg/kg MS (J), treated with 100ppm NaF+100mg/kg MS (K), treatment group treated with 100mg/kg MS only(L).

5.7.3 PANCREAS

In this study, the general morphology of acinar cells (number and size), ductile system and cytoplasm in the control mice were in the normal range of healthy mice, figure 10M.

The mice exposed to sodium fluoride separately and in combination with Moringa for 90 days have shown remarkable pancreatic tissue histological changes when compared to control (Figure 10M to O). The pancreatic tissue of mice exposed to 60ppm NaF without moringa (figure N) had mild perivascular chronic inflammatory infiltrate, slight hyperplastic changes observed. The group treated with 100ppm NaF without moringa (figure O) had decrease in cell size and number, chronic inflammatory infiltrate, fibrosis and necrotic changes were observed. In the group exposed to 60ppm NaF with moringa (figure 11P) had few focal chronic inflammatory infiltrate were observed. In the group exposed to 100ppm NaF with moringa (figure 11Q) very few focus of chronic inflammatory infiltrate were observed. In the group treated with 100mg/kg moringa (figure 11R) normal study where observed.

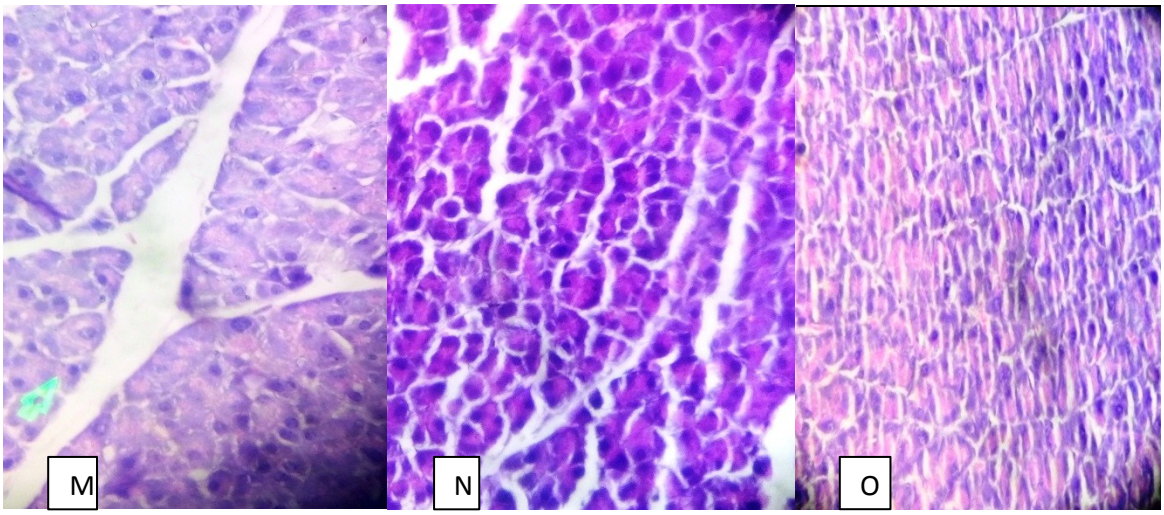


Figure 7 photomicrography of pancreas of control group (M), treated groups with 60ppm NaF (N), treatment group treated with 100ppm NaF (O).

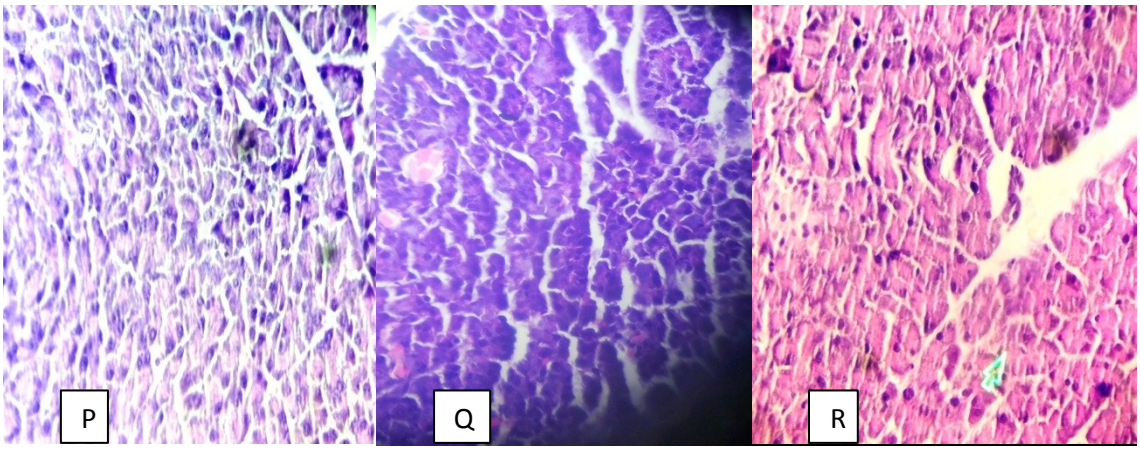


Figure 8 photomicrography of pancreas of treatment mice treated with 60ppm NaF+100mg/kg MS (P), treated group with 100ppm NaF+100mg/kg MS (Q), treatment group treated with 100mg/kg MS only(R).

CHAPTER SIX: DISCUSSION

6.1 GENERAL BODY WEIGHT

After checking the assumptions (skewness < |2| kurtosis <|9|, P=0.656 after Levene's Test of Equality of Variances) independent students t test, paired t test and one way ANOVA followed by *post hoc* Benferroni test were performed for mice weight. There was statistically significance difference in body weight gain between control and the treated groups. Compared with the control, weight of 100 ppm F treatment group had signicant weight reduction during 10th to 12th weeks of treatment with F (5 23) = 5.19, P=0.04, η=0.59 and F (5 23)=10.25, P =0.000, η =0.74 respectively. Paired t test for final and initial weights were performed there is no significant difference (P =0.072), independent t test also yields no significance difference between fluorotic without *Moringa stenopetala* and fluorotic with co-treatment with *moringa stenopetala* groups (P =0.17).

At the end of the experiment, the gain in body weight in all groups treated with NaF without Moringa was lower than those groups co-treatment NaF with Moringa which were similar to other finding by Samal U. et al. (1994) in which they reported that a decrease in mean body weight of both sexes was recorded in toads from the fluoride contaminated area(72). In laboratory studies, growth retardation has been observed in rats maintained for 6 months on a diet with a fluoride content of 3 mg kg/kg and drinking water with 100 mg/liter fluoride(73). In contrast to our finding the finding by Zhan X, et al. reported tha administration of 100 ppm of fluoride for 6 weeks starting in 3-month-old rats did not cause significant changes in body weight compared to controls (75). The difference may do to short treatment period in their study since effect of fluoride is dose dependent and duration dependent.

Our study results showing significant differences in body weight among the groups during 10^{th} and 12^{th} weeks were in contrast with some previous studies. In the study of Collins et al.(74), male and female rats exposed to fluoride for 10 weeks at levels up to 175 ppm in drinking water did not show any significant differences in body weight gain among groups. Mice exposed to 100 ppm fluoride in drinking water without Moringa showed a significant decrease in body weight gain compared to the controls. Relative organ weights were also not affected in this study.

The exposure to sodium fluoride in albino rats orally administrated with different doses for 15 days of duration showed the body weight of treated rats gain initially for 1 or 2 days and after that decreases significantly as compared with control rats(76).

According to the study by Tsunoda M, Aizawa Y et, al, oral administration of NaF led to a significant reduction in body weight gain (77).

Metabolic, functional and structural damage caused by sub chronic fluorosis have been reported in many tissues. Acute fluoride poisoning in the sheep has been associated with hypoglycemia. It has been suggested that the hypoglycemia is caused by blockade of the production of propionate and amino acids by fluoride (78). These substances are converted to glucose through the Krebs cycle and are the main source of glucose.

Apart from this, the blockage of Krebs cycle may well verify by a significant decline in epididymis succinate dehydrogenase (SDH) in NaF treated mice suggest a blockage in the conversion of succinate to fumarate. The mitochondrial structure and function are also likely to be disturbed, since SDH is a mitochondrial enzyme, and therefore oxidative metabolism will suffer(79). Research data strongly suggest that fluoride inhibits protein secretion and/or synthesis leading to weight reduction (80).

The significant decline in epididymal succinate dehydrogenase (SDH) in NaF treated mice suggests a block in Krebs cycle in the conversion of succinate to fumarate. The mitochondrial structure and function are also likely to be disturbed, since SDH is a mitochondrial enzyme, and therefore oxidative metabolism will suffer(4,28,81,82).

Research data strongly suggest that fluoride inhibits protein secretion and/or synthesis and that it influences distinct signaling pathways involved in proliferation and apoptosis including the mitogen-activated protein kinase (MAPK), p53, activator protein-1 (AP-1) and nuclear factor kappa B (NFB) pathways (83,84).

The rats orally administrated with different doses of NaF for 15 days of duration showed the body weight of treated rats gain initially for 1 or 2 days and after that decreases significantly as compared with control rats. Similarly the weight of the thyroid gland increased significantly as the doses increases.

The fluoride levels used in this study ranged from the 0.07-ppm concentration recommended for the prevention of dental caries adjusted for body size of mice to that known to induce kidney damage after several months in experimental animals(74). Since people in Ethiopian Rift Valley suffer from endemic fluorosis mainly when exposed to fluoride in their drinking water,(1,85) we conducted our study by exposing the mice to fluoride in their drinking water and giving 100mg/kg M. stenopetala accordingly for different groups. Among the group those treated with fluoride at 60ppm and 100 ppm in the water, their body weight was decreased compared to the control as well to experimental group co-treated with 60ppm NaF + 100mg/kg Moringa and 100ppm NaF + 100mg/kg Moringa. The results showing no significant differences in organ weight and relative organ weights among the groups are in agreement with those of previous studies study by Manocha et al (74).

6.1 ORGANOSOMATIC INDICES

The subchronic effect of fluoride and aqueous extracts of the plant on organ weight of mice were illustrated in Table 3. Organ somatic index for Liver, Kidney and pancreas was calculated as weight of organ in mg and final body weight in gram given in table 4. Organ somatic index of the liver increases in mice treated with fluoride only and but not both fluoride and moringa stenopetala, and there is slight reduction in indexes of kidney and pancreas in group treated with NaF without Moringa but similar with control in groups co-treated with NaF with Moringa Stenopetala.

As shown in Table 3, relative weights of livers were larger when treated with 60ppm and 100ppm NaF added without moringa than the control. As a very active site of metabolism, the liver is especially susceptible to fluoride intoxication. In our experiment, abnormalities in the liver including degenerative and inflammatory changes and hepatic hyperplasia were observed when treated high dose of fluoride in drinking water. Compared to the control, relative weights of kidneys and pancreas were smaller in all groups treated with fluoride alone or fluoride with moringa, this decrease can be attributed to weight loss, degeneration of structure of kidneys and pancreas. The results showed that accumulation of excessive fluoride induced abnormalities of liver, kidneys and pancreas in mice and groups treated with moringa the effect is attenuated.

Gross examination of kidney and pancreas of treated mice showed no visible sign of toxicity as compared to control group. The mean absolute weights of liver, kidney and pancreas showed

reduction as compared to control mice, but the decrease in organs weight were statistically insignificant. There was no significant difference in organ weight (visceral weight gain) recorded between the control and treatment groups (table 3). No significant change in the OSI of liver, kidney and pancreas of any treatment group was observed as compared with the control group (Table 4).

Similar finding was reported by Zhan X, et al administration of 100 ppm of fluoride for 6 weeks starting in 3 month-old rats also did not cause significant changes in body weight compared to controls(75).

In a contras to our finding the study by Hiaohong Wang et al showed significant decrease in the OSI of organs was found in fluoride alone treated rats (86).

Our results show no significant differences in relative organ weights among the groups was in agreement with those of previous studies. In the study of Collins et al.(74), male and female rats exposed to fluoride for 10 weeks at levels up to 175 ppm in drinking water show significant differences in body weight gain among groups. Mice in exposed to 100 ppm fluoride in drinking water without Moringa showed a decrease in body weight gain compared to the control. Relative organ weights were also not affected in this study.

6.2 LIVER FUNCTION TESTS

In this study, the serum levels of AST, ALT and ALP in the control mice were in the normal range of healthy mice, mean and standard deviation were given in the table 4.

After checking the assumptions (skewness < |2| kurtosis <|9|, P=0.656 after Levene's Test of Equality of Variances) independent students t test, paired t test and one way ANOVA followed by *post hoc* Benferroni test were performed for liver functions parameters.There was statistically significance difference in liver function tests between the treated and the control groups. Compared with the controls, weight of 100 ppm fluoride without Moringa treated group had signicant increase in plasma ALP level with F(5 17)= 5.466, P=0.008, η=0.695 and power of 0.917 independent t test yields no significance difference between fluorotic without *Moringa stenopetala* and fluorotic with *Moringa stenopetala* groups(P =0.168).

The level of ALP were increased significantly in 100 ppm fluoride treated group compared to the control group (M_3=529.67±441.29 versus M_1= 86.00±29.72). There was gradual reduction in serum AST and ALT in the control and treatment groups, Co-administration of Moringa stenopetala crude extract has shown ameliorative effect, in group taking NaF + Moringa.

As a very active site of metabolism and an organ of vital importance, the liver is especially sensitive to fluoride intoxication(87). Fluoride-induced changes of some biochemical parameters. Along with biochemical changes in mice induced by NaF treatment, significant decrease in serum total protein as well as in serum albumin levels. The increased levels of these ALP in blood serum can be related to cytotoxic effects fluoride on the liver(88).

The reduction in liver enzymes content in mice induced by NaF observed here might be due to either increased proteolysis or decreased protein synthesis or fluoride induced osmotic imbalance caused by lipid peroxidation(89). The reduction in protein content of NaF-treated animals supports the view that fluoride inhibits oxidative decarboxylation of branched chain amino acids and simultaneously promotes protein breakdown.

The finding by Anamika JHA et,al. reported, Oral administration of NaF (10 mg/kg body weight/day) for 30 days caused significant reduction in the content of protein in the liver of mice.(90)

Many investigators have reported protein degradation in liver of rabbits during experimental fluorosis. Also, fluoride is known to affect the rate of cellular protein synthesis, which is mainly due to impairment of peptide chain initiation(90).

The disturbance of protein synthesizing systems in fluorosis has been attributed to a decrease in activity of a group of enzymes catalyzing the key processes of cellular metabolism. This reduction or loss of enzymatic activity could be due to fluoride generated free radicals ultimately causing inactivation of enzymes(87). Kathpalia and Susheela (18) have observed that administration of large doses of NaF to rabbits caused a 10 to 46% reduction in protein content in most body tissues(90). The higher level of exposure to fluoride in the present study may affect metabolism and increase the fluoride level in the liver.

6.3 RENAL FUNCTION TESTS

In this study, the serum levels of urea, BUN and Cre in the control mice were in the normal range of healthy mice, mean and standard deviation were given in the table 5.

After checking the assumptions (skewness < |2| kurtosis <|9|, P=0.656 after Levene's Test of Equality of Variances) independent students t test, paired t test and one way ANOVA followed by *post hoc* Bonferroni test were performed for liver functions parameters. There was statistically significance difference in RFT(plasma urea) between the treated and the control groups. Compared with the control, renal function of 100 ppm fluoride without moringa treated group had signicant increase in plasma urea level with F(5 17)= 3.966, P=0.023, η=0.625 and power of 0.801 independent t test yields no significance difference between fluorotic without *Moringa stenopetala* and fluorotic with *moringa_stenopetala* groups (P=0.318).

The plasma urea level were increased in 100 ppm fluoride only group compared to the control group (M_3=529.67±441.29 versus M_1= 86.00±29.72). There was gradual decrease in serum BUN and Cre in both the control and treatment groups. Co-administration of *Moringa_stenopetala* crude extract has no ameliorative effect, in group taking NaF and Moringa. There is no significant difference between control group and the other treatment groups.

The serum urea level and BUN were increased in 100 ppm fluoride only group compared to the control group (M_3=0.233±.021). Co-administration of *Moringa stenopetala* with NaF has decreasing effect of fluoride in group 5 and 6 (M_4=0.120±.000 and M_5=0.120±.017). There is no significant difference in G2 and G3, G4 and G5, as well as G1 and G6. Serum BUN level of treatment group increased as well for fluoride and moringa treated groups *but* there is no significant difference between control group and the treatment groups.

Urea in serum is one of the sensitive indices of renal function. In group III, the serum nitrogen level was increased significantly when compared to control group (group I).

During the present investigation, increase in the urea level suggests impairment in glomerular function induced by NaF. The elevated serum levels of UN indicated reduced ability of the kidney to eliminate the toxic metabolic substances(42,91). F is excreted mainly from the kidney, and harmful effects of F retention are directly related to renal function. Fluoride disturbed the

kidney filtration and renal function by significantly increased the level of urea in plasma(92–94).

This study was in agreement previous studies which have demonstrated that NaF treatment of rats causes alterations in the serum biochemical parameters and similar finding was reported indicating an altered renal function(86).

Because the concentration of Cre in blood correlates inversely with the volume of glomerular filtration, the former is a useful marker of the filtration function of kidneys, the more so in that Cre is excreted only via the kidneys(95).

Impaired renal function would also explain the drastic increase of the urea in the blood serum of the treated rats. A lower rate of secretion of urea into urine resulting from renal insufficiency would cause its concentration in serum to increase. Appleton et al. after injection of high doses of sodium fluoride into rats found increases in the concentration of glucose, urea, and Cre in the plasma. The increase of urea and Cre concentration in the serum was interpreted by this author as reflecting impaired renal function(96). Renal lesions caused by the presence of fluorides have already been reported by numerous authors(89,97).

Fluoride at levels of 100 ppm and over in drinking water induced renal damage. For longer periods of administration, 5 ppm fluoride in drinking water induced renal damage. In endemic fluorosis areas, people may be exposed to fluoride at a level comparable to more than 25 ppm, considering intake from water and food. (74).

6.4 HISTOPATHOLOGY

6.4.1 Liver

The liver tissue of mice expose to 60ppm NaF without moringa had moderate increase cell size, variability in number and vacuolated cells, hypertrophic hepatocytes, generally increased nuclear size with nuclear size variation, diffused cellular ballooning with loss of hepatic plate architecture, vacuolar changes with loss of architecture is prominent in preportal areas where observed. The group treated with 100ppm NaF without moringa, were the worst affected. The tissue of this group showed generally increased cell size, variability in number and had

extensive vacuolar degeneration, generally increased nuclear size with nuclear size variation, loss of hepatic plate arrangement, loss of lobular architecture, preportal eosinophiliosis of cytoplasm, hypertrophy, hyperplasia with moderate size variation where observed. In the group expose to 60ppm NaF with moringa had mild increase in cell size, mild vacuolated cells, preserved lobular architecture with mild hypertrophy and hyperplasia, inconspicuous to distinct nucleus, mild vacuolar changes with preserved architecture were observed. In the group expose to 100ppm NaF with moringa mild variation in cell size, mild hyperplastic response, and amphophilic cytoplasm with preserved architecture where observed. In the group expose treated with 100mg/kg moringa uniform cell size, relatively uniform cell shape, and amphophilic cytoplasm with preserved architecture where observed.

Santosh K. Sahu et al. in previous study reported similar finding to this study which was an extensive cell necrosis along with ballooning degeneration of hepatic cells together with the nucleus was observed in rabbits of the highest dose (100ppm of NaF/kg/). The cytoplasm in some hepatic cells exhibited extensive vacuolization.

Shashi A. and Thapar S.(2001) reported similar findings, hepatic cellular hyperplasia showing dilatations of sinusoids with a large number of erythrocytes in them were most pronounced. Some hepatic cells were binucleated indicating hepatocellular adenoma and carcinoma(27).

The study by Lu Y et, al. has been revealed, that cellular degeneration, severe necrosis in hepatocytes, nuclear fragmentation, nuclear degenerative changes, binucleated condition, pushing of nucleus to periphery of hepatocytes, hemorrhage in central vein and pycnotic nucleus is observed in transverse section of liver in rabbit exposed to sodium fluoride for 8 weeks(98).

Chronic fluorosis can severely damage many systems of the human body. ROS and lipid peroxidation have even been considered to play an important role in the pathogenesis of chronic fluoride toxicity and oxidative stress was as one of the important mechanisms of the toxic effects of fluoride. The liver is the main organ responsible for metabolism and detoxification. Fluoride exposure would induce both pathomorphological and metabolic changes in the liver (86).

The histopathological results in the present study was similar to previous finding by Sahu K. et al which indicate that exposure to sodium fluoride from 15 days to 16 weeks in high doses caused necrotic changes in hepatocytes and liver sinusoids.(39)

Ghosh J et al (2009) and Bhattacharyya S et al (2014) reported similar finding, which was necrosis of hepatocytes has been observed in the presence of relatively high fluoride concentrations via increased oxidative stress (99,100).

6.4.2 KIDNEY

In this study, the general morphology of glomeruli (size and shape), basement membrane, medullary and cortical portion cells in the control mice were nearly round, with regular shape and clear cell boundaries, and the nucleus, mostly round or oval, was located in the center of the cells figure 8G.

The mice exposed to sodium fluoride separately and in combination with Moringa for 90 days have shown remarkable kidney histological changes when compared to control (Figures 8G to 9M). The kidney tissue of mice expose to 60ppm NaF without moringa had moderate tubular necrosis, edematous interstitium, destruction of glomeruli moderate granulation Eosinophilic changes observed. The group treated with 100ppm NaF without moringa had extensive necrosis, interstitial hemorrhage and edema, extensive glomeruli destruction and lymphoid proliferation were observed. In the group expose to 60ppm NaF with moringa had diffused eosinophilia, glomerular necrosis, lymphocytic infiltration, mild hemorrhage and edema, and slight glomeruli destruction where observed. In the group expose to 100ppm NaF with moringa slight granulation, few lymphocytic infiltration, few focus of edema and hemorrhage and few focus of necrosis where observed. In the group expose treated with 100mg/kg moringa very few focus of lymphatic infiltration, otherwise normal study where observed.

According the finding by Omireeni N. and Siddiqi A.(2010) higher doses of sodium fluoride 10, 20 and 30 mg of NaF/kg body weight caused a significant decrease in the collagen content of the kidneys when compared to the control rats which was may be either due to increased degradation of collagen by NaF or an inhibition of collagen synthesis by NaF(86).

These authors added that the collagen fibers produced during fluoride toxicity would be defective due to inadequate cross-links. Thus fluoride interferes with the maturation and normal metabolism of tissue collagen. The glomerulus is the functional unit of the kidney. The normal glomerular basement membrane, composed of type IV collagen, plays an important function in

the process of filtration. Therefore any alteration in the kidney collagen content is also likely to affect the renal function.

The study by Zhuo Zhang et al results showed that the ultrastructure of the liver and kidney in fluoride treated rats displayed shrinkage of nuclear and cell volume, swollen mitochondria and ER and vacuoles formation in the liver and kidney cells (86).

Our histopathological findings confirmed that morphological changes of kidney in fluoride-treated mice may be due to oxidative damage induced by the accumulation of fluoride in the kidney. Fluoride-treated mice kidney proximal tubule cells showed swollen, mitochondria, vague or disappeared ridges, and vacuole formation (101).

Similar observations were made by other authors who found cloudy swelling, hypertrophy, and atrophy of glomeruli in kidney of mice treated with fluoride (102,103). Previous studies showed that fluoride-induced apoptosis by oxidative stress and suggested the role of oxidative stress in the apoptotic process (104).

These histological changes could be correlated with oxidative damage. Moringa stenopetala crude extract had significantly attenuated these fluoride-induced pathological changes in liver and kidney tissue of mice since moringa is very rich in antioxidants substance including Rutin. The presence of Moringa alleviated the harmful effects of fluoride, such as mitigating pathomorphological changes of liver and kidney. The antioxidant activities and health benefits of Moringa may be related to its high rutin and phenolic content. Rutin have shown a higher antioxidant activity than vitamins C and E. These compounds are able to capture free radicals by donation of phenolic hydrogen atoms (104).

6.4.3 PANCREAS

In this study, the general morphology of acinar cells (number and size) in the control mice were in normal healthy mice, figure 10M. On gross examination the pancreas of both control and experimental group of animals was normal in appearance. However in the animals of group II and III the color of the pancreas was slightly lighter and discoloration patches were visible.

The mice exposed to sodium fluoride separately and in combination with Moringa for 90 days have shown remarkable pancreatic tissue histological changes when compared to control (Figure

10M to 11R). The pancreatic tissue of mice expose to 60ppm NaF without moringa had mild perivascular chronic inflammatory infiltrate, slight hyperplastic changes observed. The group treated with 100ppm NaF without moringa had decrease in cell size and number, chronic inflammatory infiltrate, fibrosis and necrotic changes were observed. In the group expose to 60ppm NaF with moringa had few focal chronic inflammatory infiltrate where observed. In the group expose to 100ppm NaF with moringa very few focus of chronic inflammatory infiltrate where observed. In the group expose treated with 100mg/kg moringa normal study where observed. The animals of group III were the worst affected. Pancreas of this group showed decrease in cell size and number, chronic inflammatory infiltrate, fibrosis and necrotic changes were observed

Similar finding was reported by Machalinska A et, al. and Bashir Ahmad Shah With increased concentration of fluorides frequent hemorrhages, edema of acini, distortion of cell outline, small areas of parenchymal necrosis and hemorrhages, diffuse areas of parenchymal necrosis and finally fatty infiltration and fibrosis was observed.(105,106).

This study revealed that excessive fluoride causes observable ultrastructural changes. These effects might be an important reason for growth depression induced by fluorosis. Excessive production of free radicals induced by fluoride may damage the structures of digestive enzymes and reduce their activities (107).

The ultrastructural changes in the pancreas observed here are in accordance with previous findings in thyroid, liver, and kidney of fluorotic animals.(54,75) The mitochondrion is an organelle that is the site of aerobic cellular respiration and is responsible for the conversion of food and feed to usable energy.(54) Its morphological changes would disturb the metabolism of substances in the mitochondria and suppress ATP synthesis.

Enlargement of mitochondria in fluoride-treated pigs is believed to a compensatory process due to ATP deficiency. ER is a membrane network within the cytoplasm of cells that is involved in the synthesis, modification, and transport of cellular materials. The dilation of ER in fluoride-treated pigs indicates enhancement of detoxification reactions and its rupture would suppress protein synthesis that might reduce the pancreatic secretions, including digestive enzymes. The destruction of mitochondria and ER can be attributed to oxidative stress induced by fluoride, which can seriously damage the structure of cells and organelles, especially their membranes(54,75).

In the present study we have found that excessive fluoride in the water can damage pancreatic tissue, especially acinar cells, which may in turn lead to a series of biochemical and pathological abnormalities. These histological changes could be correlated with oxidative damage. Moringa stenopetala crude extract had significantly attenuated these fluoride-induced pathological changes in pancreatic tissue of mice since moringa is very rich in antioxidants substance including Rutin. The presence of Moringa alleviated the harmful effects of fluoride, such as mitigating pathomorphological changes of acinar (secretory) cells. The antioxidant activities and health benefits of Moringa may be related to its high rutin and phenolic content. rutin have shown a higher antioxidant activity than vitamins C and E. These compounds are able to capture free radicals by donation of phenolic hydrogen atoms (104).

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATION

7.1 Conclusion

In conclusion, this study showed that Fluoride caused general body weight reduction in mice of treatment group while *moringa stenopetala* was found to provide protection to general body weight reduction.

It was found out that fluoride was hepatotoxic chemical caused increased plasma AKP level, crude extract of *moringa stenopetala* provided ameliorative effect on liver function parameter.

From this study fluoride was nephrotoxic chemical caused renal nephron damage and leads to increased plasma urea level, while crude extract of *moringa stenopetala* provided ameliorative effect on renal urea clearance function.

The present findings suggest that high doses of fluoride have potential to cause oxidative stress, which could provoke pathological changes, as well as cell apoptosis in the liver, kidney and pancreas of mice. *Moringa stenopetala* feeding could mitigate the fluoride-induced damage by elevating the antioxidant ability in the organs stated. This study demonstrated the protective role of *moringa stenopetala* against fluoride-induced oxidative stress in liver, kidney and pancreas of mice. *Moringa stenopetala* might be an important antioxidant to apply in ameliorating fluorosis in the future.

The sum-up present study depicted that exposure to F alters the structures and function of organs in question. These are evidenced by necrosis of hepatocytes, glomeruli and pancreatic acinar cells, decreased renal urea clearance and increase in plasma alkaline phosphate level. Moringa has shown a significant protective role against F toxicity probably by its antioxidant role.

7.2 RECOMMENDATIONS

1. Moringa Stenopetala crude extract seems protective against fluoride toxicity; Moringa must be consumed as food additive by the community, special in high water fluoride content areas like East Africa Rift Valley regions.
2. Aqueous and alcohol crude extraction of moringa stenopetala must be repeated in further study since we may have missed essential lipid soluble components.
3. Further chronic and extensive study required on soft tissue toxicity of NaF since the data at hand from sub-chronic study may be inconclusive.

CHAPTER EIGHT: REFERENCES

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ANNEX

Data collection formats

Group	code	Body weight (gm.)	Liver weight (gm.)	Kidney weight (gm.)	Pancreatic weight (gm.)
1	1				
	2				
	3				
	4				
2	5				
	6				
	7				
	8				
3	9				
	18				
	11				
	12				
4	13				
	14				
	15				
	16				
5	17				
	18				
	19				
	20				
6	21				
	22				
	23				

	24				

Liver function test data collection sheet

Group	code	ALT(U/L)	AST(U/L)	ALP(U/L)
1	1			
	2			
	3			
	4			
2	5			
	6			
	7			
	8			
3	9			
	18			
	11			
	12			
4	13			
	14			
	15			
	16			
5	17			
	18			
	19			
	20			
6	21			
	22			

	23			
	24			

Renal function test data collection sheet.

Group	Code	Cre (mg/dl)	Urea (mg/dl)	BUN (mg/dl)
1	1			
	2			
	3			
	4			
2	5			
	6			
	7			
	8			
3	9			
	18			
	11			
	12			
4	13			
	14			
	15			
	16			
5	17			
	18			
	19			
	20			
6	21			
	22			
	23			
	24			

Declaration

ASSURANCE OF PRINCIPAL INVESTIGATOR

The undersigned agrees to accept responsibility for the scientific ethical and technical conduct of the research project and for provision of required progress reports as per terms and conditions of the Faculty of medical sciences in effect at the time of grant is forwarded as the result of this application.

Name of the student: _____

Date._____ Signature _____

APPROVAL OF THE FIRST ADVISOR

Name of the first advisor:_____

Date._____ Signature _____

APPROVAL OF THE SECOND ADVISOR

Name of the second advisor:_____

Date._____ Signature _____

APPROVAL OF INTERNAL EXAMINER

Name of internal examiner:_____

Date._____ Signature _____