

Original Research Article

Minimizing bioavailability of fluoride through addition of calcium-magnesium citrate or a calcium and magnesium-containing vegetable to the diets of growing rats

Abstract

Introduction: Fluorosis is a public health problem in Ethiopia. Fluoride absorption may be decreased by dietary divalent cations which form insoluble complexes with the fluoride ion.

Aim: This study aimed to assess the effect of dietary calcium-magnesium citrate or *Moringa Stenopetala* dry leaf on apparent absorption of fluoride in animals.

Samples: Animals (14 weeks) were on fluoridated/non-fluoridated water and calcium and magnesium supplemented diet.

Study Design: Rats received fluoridated or non-fluoridated (control) water (10mg/L) with or without Calcium magnesium citrate (0.5mg) or moringa stenopetala dry leaf powder(0.1g) blended with daily ration.

Place and Duration of Study: Department of food science and nutrition research, Ethiopian Public Health Institute, 2014

Methods: Twelve female Wistar rats of similar age (14 weeks) and weight (186.7±5.4 g) were placed in metabolic cages for 42 consecutive days and given one of 4 treatments: corn-soya blend (CSB control); CSB and fluoridated water (10 mg/L F); CSB, fluoridated water (10mg F/L) and calcium-magnesium citrate (0.5mg); CSB, fluoridated water (10mgF/L) and *Moringa Stenopetala* (0.1g). Urine and faeces were collected weekly.

Result: Supplementation of calcium-magnesium or *Moringa Stenopetala* leaf significantly ($p < 0.05$) reduced urinary fluoride and increased fecal fluoride level, indicating less absorption of fluoride.

Conclusion: This study provides evidence for a dietary approach in reducing fluorosis.

Keywords: *fluorosis, divalent cations, moringa, fluoride bioavailability*

30

31 **Introduction**

32 Fluorosis has no treatment but can be prevented through use of non-fluoride contaminated water
 33 and appropriate dietary intervention. Data obtained from dietary supplementation studies have
 34 suggested that inadequate levels of ascorbic acid and calcium are related to the manifestation and
 35 severity of fluorosis (Srirangareddy and Srikantia 1971). According to Susheela and Bhatnagar
 36 (2002) toxic effects of fluoride can be reversed not only by withdrawing the fluoride source but
 37 also by providing a diet adequate in protein, calcium and vitamins C, E, and D. Researchers have
 38 proposed that antioxidants play a protective role in fluorosis (Sridharan et al 1999; Chinoy
 39 1978). Superoxide dismutase (SOD) and β -carotene were also implicated in effectively
 40 mitigating impaired growth due to fluoride toxicity in rats (Wang et al., 1994). The divalent
 41 cations calcium and magnesium can form insoluble complexes with fluoride and this is assumed
 42 to reduce fluoride bioavailability through minimizing absorption when supplements or foods rich
 43 in either of these cations are consumed (Cerklewsk 1997; WHO, 1994). Calcium intake in
 44 Ethiopia is reported to be low (Ethiopian Public Health Institute, 2013; Malde et al, 2004).
 45 Moringa is a calcium-magnesium and antioxidant rich food (Babu 2000). *Moringa Stenopetala*
 46 leaves (Moringa) are commonly consumed as a vegetable in some communities of south Omo in
 47 Ethiopia. However, the consumption of Moringa leaf as vegetable is not widely practiced in
 48 known fluorosis endemic areas of Ethiopia. Hence, the possibility of using Moringa leaf powder
 49 for minimization of the bioavailability of ingested fluoride has not yet been attempted, to our
 50 knowledge.

51

52 The objective of this animal trial was to assess the potential migration effect of a common bone
 53 health supplement (calcium-magnesium citrate) and a locally available food (Moringa). The
 54 hypothesis was that when calcium-magnesium combines with fluoride it forms insoluble salt
 55 (calcium-magnesium fluoride) in the gut and thereby reduces the bioavailability of fluoride in
 56 plasma and consequently in urine (Buzalaf and Whitford, 2011). A further benefit may be seen
 57 with Moringa due to its antioxidant content.

58

59 **Materials and Methods**

60 *Study Design*

This animal experiment was conducted at Food Science and Nutrition Laboratory of Ethiopian Public Health Institute (EPHI). Female Albino Wistar rats (14 weeks, mean weight (186.7±5.4 g) were randomly assigned to experimental diets (Table 1). The rats were obtained from EPHI zoonosis Research Section. The rats were kept for 3 weeks in separate cages and fed a control diet prior to start the experiment to provide a washout period and stabilize them to the new environment. The rats were then placed in metabolic cages that enabled collection of faeces and urine for 6 consecutive weeks. Faeces and urine were collected at every 24 h interval. After collecting zero-time faeces and urine, rats were fed the experimental diets (Table 1). Each morning leftover food and water was measured during cage cleaning. Rats were housed in a room providing a mean daily temperature of 20 ± 1.2°C and 12 hour day-light cycle.

Table 1: Nutrient content of corn soya blend (CSB) used for animal ration

Nutrient	CSB/100g blend
Carbohydrate, g	68.0
Protein, g	18.3
Moisture, g	9.6
Fat, g	11.2
Ash, g	3.0
Energy, kcal	425.8
Fiber, mg	9.7
Calcium, mg	133.7
Magnesium, mg	258.3
Iron, mg	3.2
Phosphorus, mg	359.6
Zinc, mg	2.9
Potassium, mg	966.9
Sodium, mg	7.8
Thiamin, mg	0.3*
Riboflavin, mg	0.5*
Niacin, mg	3.1*
Pantothenic acid, mg	0.9*
Vitamin b6, mg	0.5*
Tocopherol, mg	0.9*
Vitamin A (IU)	205.0
Folate (µg)	0.1*

*Calculated using food composition table, the rest were analyzed at EPHI laboratory

75

76 *Treatment groups*

77 The four groups of rats were; (i) control diet (corn-soya blend (CSB), called FF (fluoride-free);
 78 (ii) CSB plus fluoridated water, called FC (fluoride control); (iii) CSB + F + calcium-magnesium
 79 citrate, called CA (added calcium-magnesium); (iv) CSB + F + moringa called M (moringa dry
 80 leaf powder added). For treatment (iii), calcium magnesium citrate was added (1000 mg tablet
 81 containing 200 mg calcium and 100 mg magnesium, Solgar LTD, USA). For treatment (iv)
 82 *Moringa Stenopetala* dry leaf powder, collected from Konso in the southwest of Ethiopia was
 83 blended with the food. For treatments (ii), (iii), and (iv), water provided to rats was fluoridated
 84 at 10 mg F/L. The amount of calcium-magnesium citrate, and moringa added to diets was
 85 determined as follows. Based on first week of feeding CSB (washout period), average food
 86 consumed per day per rat was 15 g and average water consumed per day per rat was 18 mL. The
 87 expected F ingested per day per rat from water was ~ 0.2 mg (0.01 mmol F). Thus calcium-
 88 magnesium needed to bind all the ingested fluoride to form CaF_2 and MgF_2 was calculated to be
 89 0.005 mmol total from Ca Mg, based on stoichiometric equivalent. To mix the CA and M diets,
 90 0.5 mg tablet and 0.1 g moringa dry leaf powder were used to blend with 15 g of the CSB,
 91 respectively. The moringa dry leaf powder contained 2000 mg Ca and 450 mg Mg per 100 g of
 92 the leaf powder, and the amount calculated to be consumed per rat was 0.1g per day to provide
 93 0.2 mg Ca (0.005 mmol Ca) and 0.4 mg Mg (0.02 mmol Mg). Therefore, the combined mineral
 94 used to bind the ingested fluoride for the Moringa group (M) was 0.025 mmol. In the study 0.5
 95 mg of the calcium-magnesium citrate tablet, blended with 15g CSB provided 0.1mg Ca (0.0025
 96 mmol Ca) and 0.05 mg Magnesium (0.0021 mmol Mg) which together provided 0.0046 mmol $\approx$
 97 0.005mmol.

98

99 *Homogenization of Corn Soya Blend (CSB) with calcium sources*

100 To prepare the two treatment diets (CA and M), each added constituent was first blended in a
 101 small amount of diet before being diluted into one kilo gram used for two weeks. For M, 6.7g of
 102 Moringa dry leaf powder (MDLP) was first homogenized with 100g CSB. The blended 100g
 103 CSB-MDLP was back homogenized thoroughly with 900g CSB in a blender so that MDLP
 104 homogenously distributed in the final 1kg blend. The homogenization was repeatedly conducted
 105 for 15 minutes by reducing the portions of the blended powder and back adding for maximum

homogenization. The calcium magnesium citrate was similarly homogenized by first adding 33.3mg tablet to 100 g CSB and back making the final 1 kg of CA diet. These blends of Moringa and calcium magnesium citrate were made every two weeks.

Animal care

The rats were placed in plastic (made of polyethylene) metabolic cages equipped with secondary cups that collected any spilled food and water. The rats had free access to food and water (Table 2). All procedures were carried out according to the guidelines for animal research. Chemical reagents used were of analytical grade and of the best possible quality. The research protocol was approved by research ethical clearance committee of Ethiopian Public Health Research Institute (EPHI). After completing the experiment, the animals were back handed to animal house, zoonosis for proper removal as described/explained by rules and regulations for experimental animals

Table 2: Food and water consumed by rats over study weeks (mean,(SD)) and room temperature housing metabolic cages

	Group	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6
Food Intake (g)	FC	15.0 (0.0)	16.8 (0.7)	18.8 (0.7)	20.9 (0.8)	21.9 (0.8)	22.8 (0.6)
	CA	15.0 (0.0)	17.3 (1.1)	18.2 (1.0)	19.4 (0.9)	20.7 (0.1)	21.2 (0.2)
	M	15.0 (0.0)	16.1 (0.5)	17.1 (0.5)	18.9 (0.4)	20.5 (0.4)	21.0 (0.7)
	FF	15.0 (0.0)	17.1 (0.7)	18.4 (1.2)	19.6 (1.2)	21.4 (1.8)	22.4 (1.0)
Water Intake (ml)	FC	18.7 (2.0)	21.0 (0.0)	25.0 (3.6)	20.0 (1.7)	26.7 (1.2)	24.7 (2.3)
	CA	19.0 (3.5)	22.7 (0.6)	27.7 (7.6)	26.0 (0.0)	27.7 (0.6)	27.0 (1.7)
	M	36.7 (1.2)	26.7 (1.2)	27.0 (1.70)	26.3 (1.5)	22.3 (5.5)	19.3 (2.3)
	FF	19.8 (4.0)	16.7 (1.2)	20.3 (4.0)	20.3 (6.7)	27.7 (2.9)	25.7 (9.0)
Room Temperature	noon	20.2	19.3	22.3	22.1	21.8	20.9

FF= fluoride free; FC = fluoride control (+ F in water); Ca =added calcium-magnesium citrate + F; M = moringa dry leaf +F

Analysis of fluoride in urine and feces

Fluoride in urine was analyzed by the method of Orion (2001) using combination fluoride electrode (perfect ion) and pH/ion meter (Jenway 3345). The rat feces were dried and moisture content analyzed prior to analysis for fluoride in faeces using the method adapted for food by

Malde et al. (2001). The validation of method was conducted using spiked samples and analysis of reference materials. As a quality control 5µg fluoride was spiked onto feces and urine and analyzed along with the samples. Standard reference materials, plant material (Timothy high - 2695, NIST) and fish tissue (Fish powder, 0268, in house material, National Institute of Nutrition and Seafood Research (NIFES), Norway) were also analyzed along with the samples to validate the method. The recoveries of spiked samples show that the method had percent error < 5%. The analysis from reference material showed 104.4 % recovery from feces and 98.6 % from urine. Coefficient of variation (CV) was found as 6% for plant material.

Statistical analyses

Data are presented as mean ± standard deviation (SD). Comparisons among groups were carried out using one-way ANOVA.

Results

Figure 1 shows the weight gain of animals throughout the six week study. The percent weight increment of Moringa or calcium supplemented rats are higher than the controls (40%). The weight gain of animals given fluoride (FC) was greater than that of rats not given fluoride (FF). By week 7, significant differences ($p > 0.05$) were observed in percent increment of body weight among the treatment groups.

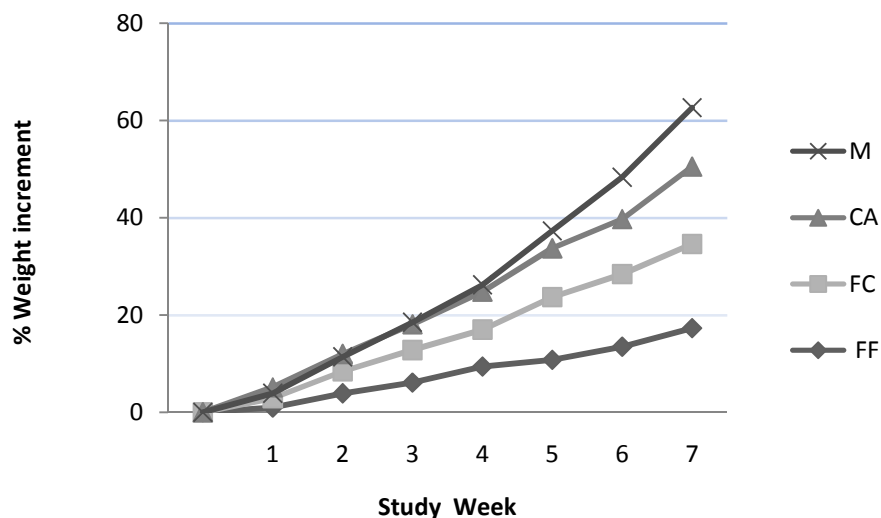


Figure 1. Percent increment of body weight of rats over 7 weeks of study period by treatment group: normal diet, non-fluoridated water (fluoride-free, FF); normal diet, fluoridated water (fluoride control, FC); diet supplemented with Calcium-magnesium, fluoridated water (calcium-magnesium, CA); diet supplemented with moringa, fluoridated water (moringa group, M).

Fecal Fluoride excretion

Fecal excretion of fluoride by treatment group over the 6 weeks of feeding is given in Figure 2. The fluoride-free (FF) control group showed little fecal excretion. However despite being provided with fluoridated water the fluoride control group (FC) also showed little fluoride excretion, indicating almost 100 % absorption of fluoride had occurred. In contrast rats given calcium or Moringa along with fluoridated water (CA and M, respectively) showed higher fluoride fecal excretion which was not different by dietary treatment.

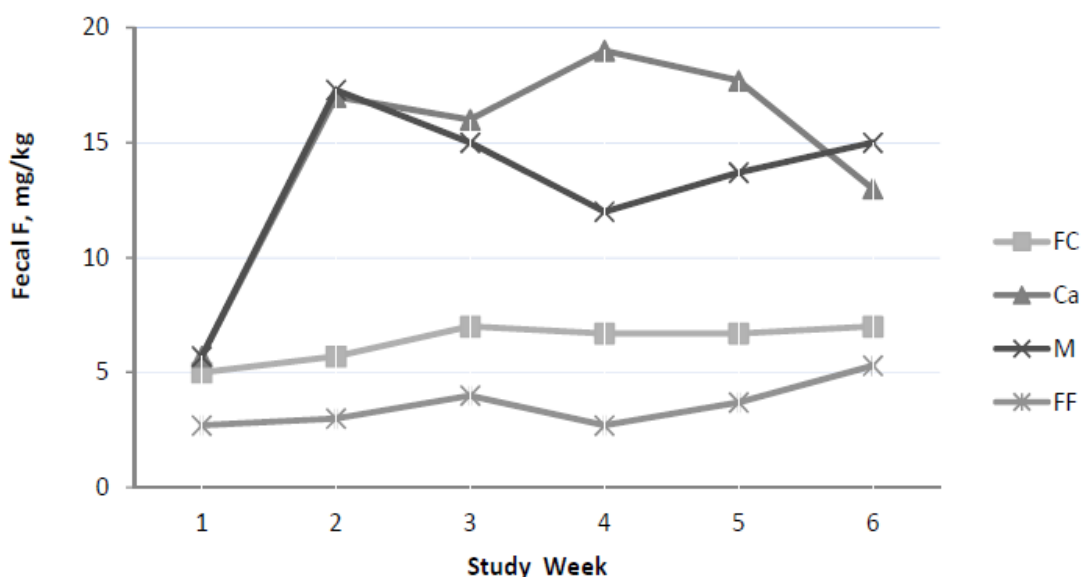


Figure 2: Fluoride in feces of rats (mg/kg of feces) over 6 week study by treatment group: normal diet, non-fluoridated water (fluoride-free, FF); normal diet, fluoridated water (fluoride control, FC); diet supplemented with Calcium-magnesium, fluoridated water (calcium-magnesium, CA); diet supplemented with moringa, fluoridated water (moringa group, M).

Data in Table 3 indicate that average excretion of fluoride by rats that consumed fluoridated

water (FC) was not significantly different ($p = 0.150$) from non-fluoridated (FF). Average excretion rates by the calcium-magnesium or Moringa-supplemented groups were similar to each other ($p = 0.395$) but significantly greater than that of fluoride controls ($p < 0.05$). In fact, the fecal fluoride of supplemented groups (CA and M) are almost double of the non-supplemented one (FC).

Table 3: Average fecal fluoride (mg/kg) and urine fluoride (mg/L) of rats over 6 week study

Treatment	N	Fecal F mg/kg	Urine F mg/L
FF	3	3.6 ± 1.0^a	$4.6^a \pm 0.6$
FC	3	6.4 ± 0.8^a	$76.0^b \pm 8.5$
CA	3	14.7 ± 4.8^b	$55.9^c \pm 6.9$
M	3	13.1 ± 4.0^b	$55.2^c \pm 7.6$

*Means in columns having the same letter are not significantly different ($p < 0.05$)

FF = fluoride free; FC = fluoride control (+ F in water); CA = added calcium-magnesium citrate + F in water; M = moringa dry leaf + F in water

Urine Fluoride excretion

Rats given no fluoridated water (FC) showed little urinary fluoride excretion (Figure 3) over the six weeks. For all three groups given fluoridated water (FC, CA, M), there was a similar pattern of fluoride excretion in urine until week 5 when urinary fluoride of rats having the control diet rose compared to the other two groups (CA and M).

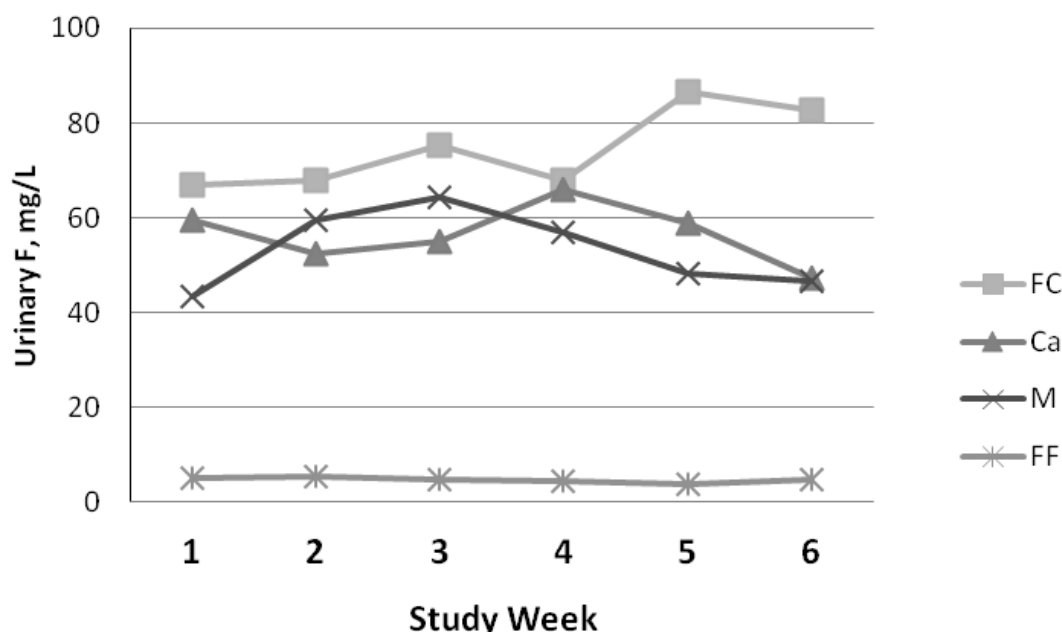


Figure 3: Urinary fluoride (mg F per L of urine) of rats over 6 week study by treatment group: normal diet, non-fluoridated water (fluoride-free, FF); normal diet, fluoridated water (fluoride control, FC); diet supplemented with Calcium-magnesium, fluoridated water (calcium-magnesium, CA); diet supplemented with moringa, fluoridated water (moringa group, M).

The average urinary excretion of F, shown in Table 3, indicates that when given no external source of fluoride (FF), urinary excretion was lower than all other treatment groups ($p = 0.0001$). Those rats given fluoridated water, however, differed in urinary fluoride excretion according to dietary treatment: rats given either calcium-magnesium (CA) or moringa (M) had significantly less urinary fluoride than control rats (FC) ($p = 0.001$), but were not different from each other ($p=0.410$).

Discussion

Fecal and urinary fluoride excretions in rats were compared after dietary supplementation with calcium and magnesium or Moringa in combination with high-fluoride drinking water. Fecal fluoride was measured to reflect the portion of fluoride that was bound and complexed into an insoluble salt. Fecal fluoride was low in rats given fluoridated water (FC) or fluoride-free drinking water (FF), indicating that almost all the fluoride in the water had been absorbed. This

is in accordance with other studies which found reduction in urinary fluoride on addition of calcium and antioxidants (Susheela and Bhatnagar, 2002): In contrast, when adding calcium and magnesium or Moringa to the diet (CA and M groups), the fecal fluoride concentration increased significantly, indicating decreased fluoride absorption. There is no significant difference in fecal fluoride excretion between these two treatments group (Susheela and Bhatnagar, 2002).

Urinary fluoride is also assumed to reflect fluoride absorption, as it shows that fluoride has been absorbed and then is excreted by this route; however, it is less quantitative than fecal fluoride since some absorbed fluoride might be retained in the body such as in bones (Toth *et al.*, 2005). Urine from the FF group had, as expected, low fluoride levels. The FC group had the highest urinary fluoride concentrations, indicating that water-borne fluoride was absorbed ($p < 0.05$). The rats treated with calcium-magnesium or Moringa had significantly lower urinary F compared to the FC-group suggesting less F absorption. The result is similar with the study from Susheela and Bhatnagar (2002). There was no significant difference between CA and M indicates equal amounts of fluoride were absorbed.

The increase of fecal fluoride content and decrease of urinary fluoride in rats supplemented with calcium-magnesium and or calcium-magnesium rich foods (Moringa) strongly suggests that fluoride combines with calcium-magnesium to form insoluble compounds that are not absorbed. However, in the case of Moringa, it is not known whether the binding is solely due to its calcium and magnesium content, or whether other factors such as anti-oxidants contribute to the effect (Susheela and Bhatnagar, 2002). Nevertheless, fluoride-combining elements that prevent F absorption, if consumed in diet/beverages, should prevent or slow the progression of fluorosis. The Rift valley communities of Ethiopia which are living under fluoride burden due to contaminated water with fluoride (Tekle-Haimanot *et al.*, 2006) may benefit from diversifying their diet with calcium-magnesium and anti-oxidant rich foods such as milk, moringa, and kale, all of which are locally available. Moreover since Moringa and vegetables like kale are rich in anti-oxidants (vitamin C and provitamin A), using these foods may also reduce the toxicity that might be caused due to fluoride (Sridharan *et al* 1999; Chinoy 1978).

This study had limitations. The study had a relatively short period (7 weeks) to reflect a condition (fluorosis) that takes a long time to manifest. Calcium, magnesium and antioxidant levels were not directly measured in any of the treatment diets, or in urine or feces. However, the fluoride concentration used for inducing the fluoride load was similar to the drinking water fluoride level in the Ethiopian Rift Valley communities (Tekle-Haimanot et al., 2006).

Conclusions

Adding Moringa or calcium-magnesium citrate to the diet of rats given a high load of fluoride in drinking water increased fecal excretion and reduced urinary fluoride of rats, indicating that diet can prevent fluoride uptake. These experimental trials indicate that fluoride bioavailability can be reduced by complementing the diet with calcium-magnesium rich foods. In areas of the world such as the Rift valley of Ethiopia where fluorosis occurs, mitigation of fluorosis may be possible with calcium-magnesium rich diets. This study provides evidence for a dietary approach may be an effective strategy in reducing endemic fluorosis.

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