

## *Tamarindus indica* L. and *Moringa oleifera* M. extract administration ameliorates fluoride toxicity in rabbits

R Ranjan<sup>1\*</sup>, D Swarup<sup>1</sup>, R C Patra<sup>1</sup> & Vikas Chandra<sup>2</sup>

Division of Medicine<sup>1</sup> and Physiology and Climatology<sup>2</sup> Indian Veterinary Research Institute, Izatnagar 243 122, India

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Aqueous extracts of *T. indica* fruit pulp (100 mg/ kg body weight) and *M. oleifera* seeds (50 mg/ kg body weight) orally once daily for 90 days lowered plasma fluoride concentrations in rabbits receiving fluorinated drinking water (200 mg NaF/ Liter water). Cortical indices and metaphysial width in animals receiving extracts also revealed beneficial effects of plant extracts. Changes in plasma biochemistry suggested less hepatic and renal damages in animals receiving plant extracts along with fluorinated water in comparison to that receiving fluorinated water alone. Preliminary results revealed these plant extracts have some potential to mitigate fluoride toxicity.

**Keywords:** Amelioration, Fluoride-toxicity, *Moringa oleifera*, Rabbit, *Tamarindus indica*

Fluoride is a cumulative poison. Chronic fluoride toxicity leads to bony and dental lesions that develop over a period of time. Since drinking water is the major source of excess fluoride, different techniques were developed for fluoride removal from water. Unfortunately, currently available water defluoridation techniques could not gain much public acceptability due to several social, financial, environmental and technical constraints<sup>1</sup>. Aluminum, boron and calcium salts, hormones and other agents were tested for amelioration of fluorosis with limited success<sup>2,3</sup>. They are assumed to act either by preventing fluoride absorption from gut or by enhancing absorbed fluoride from the body. However, most of them are unfit for clinical use because of chronic nature of the problem and associated side effects after their prolonged uses. Some are reported to be toxic even at effective doses, while others may even enhance the toxic potential of the fluoride<sup>4,5</sup>. Therefore, quest for alternative effective antidote with little or no side effects continue.

Due to rich biodiversity of India, a large number of plant species are available for treatment of various

toxicities and musculoskeletal disorders<sup>6</sup>. Research reports indicate that herbal and plant products may be used for mitigation of fluoride toxicity<sup>7-9</sup>. *Tamarindus indica* L. (Leguminosae; English name: Tamarind, Hindi: Imli or Ambli) is a tree native to Africa, but also widely cultivated throughout India, Sudan, Indonesia, Pakistan, Philippines, Java, Spain and Mexico<sup>10</sup>. Its fresh and dried fruits are used as sour flavoring agent in various Indian cuisines. Anti-inflammatory and antioxidant activity of Tamarind fruit pulp have been reported<sup>11,12</sup>. *Moringa oleifera* M. (Moringaceae; English name: Horseradish-tree, Drumstick-tree, Hindi: Sajjan) is generally considered as vegetable and also used in Indian folk medicine for the treatment of various illnesses. Extracts of *T. indica* fruit pulp and *M. oleifera* seeds have been found to enhance the urinary fluoride excretion in short term trials<sup>7,13</sup>. The present study therefore, aims to investigate their potential to alleviate chronic fluoride toxicity in rabbits.

### Materials and Methods

Plant materials— Seeds of *Moringa oleifera* and fruit pulp of *Tamarindus indica* were procured from a herbal drug trader of Bareilly and authenticated from the Department of Botany, Rohilkhand University, Bareilly, U.P., India, where voucher specimens (08-09/07) were preserved. *T. indica* fruit pulp (100 g) was mixed with 200 mL distilled water in a round bottom flask and left overnight at room temperature. The mixture was shaken vigorously

\*Present address of correspondent author:

Department of Clinical Veterinary Medicine Ethics and Jurisprudence, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141 004, India

Telephone: +91-9417643441

Fax: +91-161-2400822

E-mail: rakesh\_ranjan3@rediffmail.com

several times in between. Next day, the mixture was filtered with Whatman filter paper no. 1. The filtrate was evaporated *in vacuo* using rotary film evaporator. About 20 g of semi-solid material was obtained. The aqueous extract was prepared in a similar way as above by mixing 50 g of dried *M. oleifera* seed powder with 200 mL distilled water. About 15 g of semi-solid material was obtained. Both extracts were stored at  $-20^{\circ}\text{C}$  and diluted to a desired concentration with distilled water just before use.

**Experimental animals**— New Zealand White male rabbits (24) of 4 to 6 weeks of age, weighing in between 600-800 g were procured from laboratory animal resource section of the Institute. They were maintained in individual rabbit cages and acclimatized for 15 days before starting the experiment. Rabbit feed was given *ad libitum* and a photoperiod of 12 h was maintained. The experiment was conducted as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India, after approval from Animal Ethics Committee of the Institute.

Rabbits were randomly divided into 4 equal groups (Gr I, II, III and IV) of 6 animals each. Gr I received drinking water with fluoride concentration less than 0.28 ppm. Gr II received drinking water with 200 mg/L added sodium fluoride (NaF). Group III and IV were given fluorinated drinking water (200 mg/L added NaF) with aqueous extracts of *T. indica* fruit pulp @ 100 mg/ kg and *M. oleifera* seed @ 50 mg/ kg body weight orally once daily for 90 days. The dose of sodium fluoride to induce toxicity was fixed after perusing the available literature<sup>14,15</sup>. Doses of *T indica*<sup>16</sup> and *M oleifera*<sup>17,18</sup> were fixed on the basis doses used in earlier studies by different workers. Blood samples were collected by cardiac puncture on day 0, 45 and 90 using heparin as anticoagulant. All rabbits were sacrificed on day 90 just after blood sample collection and radiography. Bone and blood samples were immediately brought to the laboratory; plasma was separated and stored at  $-20^{\circ}\text{C}$  till analysis.

**Radiography**— Dorso-ventral and lateral radiographs of all rabbits were taken on day 90. The radiographs were observed for density, metaphysial width of tibia and femur and thickness of bone cortex. The cortical index (C.I.) was estimated as the ratio of combined cortical thickness to bone diameter at mid-diaphysis, i.e.  $\text{C.I.} = 2 \times \text{CWT} / \text{D}$ , where CWT

= cortical wall thickness (mm) and D = mid-diameter (mm) of the bone<sup>19</sup>.

**Fluoride estimation**— Fluoride concentration in plasma was measured by digital Ion-analyzer (Orion Research Model 701A) equipped with a fluoride specific electrode<sup>20</sup>. The quality control criterion was met using repeated standard solutions and slope determination<sup>21</sup>. Electrode operation (slope) was checked and the instrument was calibrated every time before estimation.

Fluoride concentration in bone was estimated after ashing and using the same digital ion-analyzer<sup>22</sup>.

**Plasma biochemistry** — Activities of alkaline phosphatase (ALP), aspartate transaminase (AST) and alanine transaminase (ALT) were estimated as per the standard procedures<sup>23,24</sup>. Creatinine level was estimated by alkaline picrate method<sup>25</sup> and urea concentration was measured by di-acetylene monoxime method<sup>26</sup>.

**Statistical analysis**— The data obtained were compared by Analysis of Variance (ANOVA) and Student's *t* test<sup>27</sup>.  $P < 0.05$  was considered significant.

## Results

On day 90, cortical index of both tibia and femur was significantly ( $P < 0.05$ ) lower in Gr II in comparison to healthy control (Gr I). This revealed thinning of the cortices of long bones in fluoride intoxicated rabbits. Metaphysial width of tibia was significantly ( $P < 0.05$ ) higher in Gr II in comparison to Gr I, indicating broadening of metaphysis. However, in femur, values did not differ significantly in between Gr I and II (Table 1). In animals treated with plant extracts (Gr III and IV), cortical indices were numerically higher than Gr II and values were close to Gr I value. Changes in metaphysial width of femur did not differ significantly among different treatment groups. However, metaphysial width of tibia was significantly ( $P < 0.05$ ) higher in Gr II and IV in comparison to both Gr I and III.

Plasma fluoride concentration was significantly ( $P < 0.05$ ) higher in Gr II on both day 45 and 90 in comparison to corresponding values in Gr I (Table 2). At end of the experiment, fluoride concentration in both plasma as well as bone were significantly ( $P < 0.05$ ) lower in Gr III and IV in comparison to Gr II. Among different fluoride exposed groups, lowest mean fluoride concentration was observed in Gr III on both day 45 and 90. F concentration in Gr III and IV did not differ significantly from each other on

Table 1—Cortical index, metaphyseal width and bone fluoride concentration in rabbits

[Values are mean  $\pm$ SE from 6 animals in each group]

Parameter	Group	Group			
		I	II	III	IV
Cortical index	Tibia	0.42 $\pm$ 0.03 <sup>b</sup>	0.29 $\pm$ 0.01 <sup>a</sup>	0.42 $\pm$ 0.03 <sup>b</sup>	0.42 $\pm$ 0.03 <sup>b</sup>
	Femur	0.37 $\pm$ 0.01 <sup>b</sup>	0.29 $\pm$ 0.03 <sup>a</sup>	0.33 $\pm$ 0.02 <sup>ab</sup>	0.34 $\pm$ 0.02 <sup>ab</sup>
Metaphyseal width (cm)	Tibia	13.10 $\pm$ 0.33 <sup>a</sup>	14.45 $\pm$ 0.34 <sup>b</sup>	13.13 $\pm$ 0.32 <sup>a</sup>	14.75 $\pm$ 0.32 <sup>b</sup>
	Femur	13.92 $\pm$ 0.31 <sup>a</sup>	14.58 $\pm$ 0.36 <sup>a</sup>	13.83 $\pm$ 0.31 <sup>a</sup>	14.38 $\pm$ 0.55 <sup>a</sup>
Fluoride concentration ( $\mu$ g/ g)		475.07 $\pm$ 20.11 <sup>a</sup>	8365.70 $\pm$ 384.30 <sup>c</sup>	6019.20 $\pm$ 531 <sup>b</sup>	7144.30 $\pm$ 190.90 <sup>bc</sup>

Gr I: Control; Gr II: F; Gr III: F+Aq Tamarind; Gr IV: F+ Aq Moringa Figures bearing different superscript in small letters in a row differ significantly ( $P<0.05$ )

Table 2—Alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) activities and creatinine, urea nitrogen and fluoride concentrations in plasma of rabbits

[Values are mean $\pm$  SE from 6 animals in each group]

Parameter	Group	Day of observation		
		0	45	90
ALP (KA units/ ml)	I	6.51 $\pm$ 0.53 <sup>aA</sup>	7.56 $\pm$ 0.92 <sup>aA</sup>	6.01 $\pm$ 0.59 <sup>aA</sup>
	II	6.05 $\pm$ 0.99 <sup>aA</sup>	16.11 $\pm$ 1.66 <sup>bB</sup>	18.05 $\pm$ 4.38 <sup>bB</sup>
	III	6.38 $\pm$ 0.85 <sup>aA</sup>	7.33 $\pm$ 0.93 <sup>aA</sup>	7.27 $\pm$ 0.95 <sup>aA</sup>
	IV	6.54 $\pm$ 1.04 <sup>aA</sup>	14.13 $\pm$ 2.46 <sup>bCB</sup>	8.79 $\pm$ 1.19 <sup>abA</sup>
ALT (RF units/ ml)	I	14.68 $\pm$ 0.80 <sup>aA</sup>	14.27 $\pm$ 0.94 <sup>aA</sup>	16.60 $\pm$ 1.40 <sup>aA</sup>
	II	17.08 $\pm$ 0.81 <sup>aA</sup>	22.82 $\pm$ 3.45 <sup>aB</sup>	70.33 $\pm$ 7.75 <sup>bC</sup>
	III	15.58 $\pm$ 1.31 <sup>aA</sup>	13.00 $\pm$ 0.57 <sup>aA</sup>	28.63 $\pm$ 4.37 <sup>aB</sup>
	IV	16.25 $\pm$ 0.80 <sup>aA</sup>	12.80 $\pm$ 2.38 <sup>aA</sup>	38.63 $\pm$ 3.68 <sup>bB</sup>
AST (RF units/ ml)	I	23.75 $\pm$ 2.02 <sup>aA</sup>	23.50 $\pm$ 1.27 <sup>aA</sup>	27.60 $\pm$ 3.42 <sup>aA</sup>
	II	24.25 $\pm$ 1.93 <sup>aA</sup>	32.50 $\pm$ 2.47 <sup>bB</sup>	45.33 $\pm$ 1.45 <sup>cB</sup>
	III	23.08 $\pm$ 1.45 <sup>aA</sup>	27.50 $\pm$ 0.89 <sup>aAB</sup>	32.38 $\pm$ 2.76 <sup>bA</sup>
	IV	24.08 $\pm$ 1.12 <sup>aA</sup>	23.60 $\pm$ 0.37 <sup>aA</sup>	29.25 $\pm$ 4.05 <sup>aA</sup>
Creatinine (mg/dl)	I	0.87 $\pm$ 0.16 <sup>aA</sup>	0.61 $\pm$ 0.03 <sup>aA</sup>	0.83 $\pm$ 0.09 <sup>aA</sup>
	II	0.68 $\pm$ 0.17 <sup>aA</sup>	0.73 $\pm$ 0.16 <sup>aAB</sup>	2.12 $\pm$ 0.17 <sup>bC</sup>
	III	0.81 $\pm$ 0.16 <sup>aA</sup>	0.87 $\pm$ 0.03 <sup>aB</sup>	1.49 $\pm$ 0.11 <sup>bB</sup>
	IV	0.70 $\pm$ 0.15 <sup>aA</sup>	0.50 $\pm$ 0.02 <sup>aA</sup>	1.29 $\pm$ 0.18 <sup>bB</sup>
Urea nitrogen (mg/dl)	I	8.43 $\pm$ 0.75 <sup>aA</sup>	8.87 $\pm$ 0.41 <sup>aA</sup>	9.56 $\pm$ 0.55 <sup>aA</sup>
	II	8.66 $\pm$ 0.95 <sup>aA</sup>	18.62 $\pm$ 0.44 <sup>bB</sup>	18.18 $\pm$ 1.30 <sup>bB</sup>
	III	8.83 $\pm$ 0.80 <sup>aA</sup>	11.44 $\pm$ 1.25 <sup>aA</sup>	13.81 $\pm$ 1.41 <sup>bAB</sup>
	IV	8.72 $\pm$ 0.84 <sup>aA</sup>	14.48 $\pm$ 2.12 <sup>bA</sup>	11.94 $\pm$ 2.87 <sup>abA</sup>
Fluoride (mg/L)	I	0.07 $\pm$ 0.01 <sup>aA</sup>	0.07 $\pm$ 0.01 <sup>aA</sup>	0.07 $\pm$ 0.01 <sup>aA</sup>
	II	0.06 $\pm$ 0.01 <sup>aA</sup>	2.24 $\pm$ 0.30 <sup>bC</sup>	2.81 $\pm$ 0.44 <sup>bC</sup>
	III	0.07 $\pm$ 0.01 <sup>aA</sup>	1.12 $\pm$ 0.19 <sup>cB</sup>	0.72 $\pm$ 0.06 <sup>bB</sup>
	IV	0.06 $\pm$ 0.01 <sup>aA</sup>	1.63 $\pm$ 0.42 <sup>bB</sup>	0.77 $\pm$ 0.04 <sup>aB</sup>

Gr I: Control; Gr II: F; Gr III: F+Aq Tamarind; Gr IV: F+ Aq Moringa

Figures for a given parameter bearing no common superscript in small letters in a row and capital letters in a column differ significantly ( $P<0.05$ )

both day 45 and 90, but individual values were numerically lower than Gr II values on both observation days.

In comparison to Gr I, ALP activities were significantly ( $P<0.05$ ) higher in Gr II on both day 45 and 90. However, in Gr III, values were significantly ( $P<0.05$ ) lower than Gr II values on both observation days. In Gr IV, values did not differ from Gr II on day

45, but on day 90 activity was significantly ( $P<0.05$ ) lower than corresponding value in Gr II. Activities of liver specific enzymes (ALT and AST) in Gr II were significantly ( $P<0.05$ ) higher in comparison to control (Gr I) on both day 45 and 90. In Gr III and IV co-treated with plant extracts, no consistent pattern in change of activities of these enzymes was observed. However, values were numerically lower in these

groups in comparison to Gr II on both sampling days. Gr IV values were, in general, lower than Gr III values. Creatinine and urea nitrogen concentrations in plasma were also lower in Gr III and IV in comparison to Gr II. However, values varied considerably and did not show any consistent pattern in terms of statistical significance.

### Discussion

Radiographic findings of long bone in chronic fluoride toxicity vary considerably and may include osteosclerosis, osteopenia, intermittent growth lines, metaphyseal widening or soft tissue ossification<sup>28</sup>. Smaller doses of fluoride stimulate osteogenesis and new bone formation, while larger doses result into increased bone resorption and defective matrix formation<sup>29</sup>. Homogenous reduction in bone density and thinning of cortices are most common changes observed in human patients<sup>30</sup>. This is perhaps due to reduction in collagen synthesis at high doses of fluoride exposure<sup>31</sup>. Results of the present study revealed that exposure to 200 mg NaF/ L drinking water for 90 days induce thinning of cortices of long bones. Also, *M. oleifera* and *T. indica* extract treatment had some potential to reduce fluoride accumulation and resultant cortical thinning in long bones.

Fluoride concentration in blood reflects severity of daily exposure, while that in bone reflects both duration and severity of total exposure<sup>32</sup>. In fluoride exposed non-treated rabbits plasma fluoride concentration increased many fold to baseline values on day 45. This reflected excess intake of fluoride through drinking water. Following ingestion, fluoride is rapidly absorbed to reach systemic circulation. In blood, about 75% of fluoride remains free in plasma, 5% bound to plasma proteins and the remaining is found mainly in or on the erythrocytes<sup>33</sup>. Due to high aqueous solubility of NaF, rapid and extensive fluoride absorption occurs through gastrointestinal tract<sup>34</sup>. In present experiment, plasma and bone fluoride concentrations in Gr III and IV, treated with plant extracts were lower than corresponding values in Gr II given only fluoride.

Changes in plasma biochemistry also revealed cytotoxic potential of excess fluoride intake to cells present in bone, liver and kidney. Increased ALP activity following fluoride administration occurs due to lysis of osteoblasts and osteocytes<sup>35</sup>. Increase in ALP activity is almost a consistent finding in natural and experimental fluorosis<sup>36-38</sup>. Elevated liver enzyme

activities in fluoride exposed rabbits reflected hepatic injury. Increase in ALT and AST activities in fluoride toxicity has also been reported in goat<sup>37</sup>, cattle<sup>39</sup> and man<sup>40</sup>. Increase in urea nitrogen and creatinine is a common finding in fluorotic subjects both under natural and experimental conditions<sup>37,39</sup>. The biochemical changes, however, were less pronounced in rabbits co-treated with plant extracts (Gr III and IV). This may be either due to hepatoprotective or nephroprotective properties of these plant extracts or indirect effects by reducing the fluoride burden in the body. The second possibility seems to be more important since few studies in the past documented increased fluoride excretion when subjects were treated with these plant products. Significant ( $P < 0.001$ ) increase in urinary fluoride excretion was reported in boys given 10 g Tamarind fruit pulp per day for 18 days<sup>8</sup>. The renal clearance of fluoride is influenced by urine pH and the rate of clearance is higher with alkaline urine<sup>41,42</sup>. Tamarind paste is a rich source of tartaric acid which does not get metabolized and is excreted as such through the urine. Tartarate inhibits carbonic anhydrase leading to production of alkaline urine and thereby promotes urinary fluoride excretion<sup>9</sup>. Increase in pH of urine with tamarind fruit pulp supplementation suggested a possibility for increased urinary fluoride excretion<sup>8</sup>. The same author, however, failed to notice any change in urinary pH of dogs fed tamarind fruit pulp paste @ 10 g/day for 3 months along with fluoride @ 10 mg/kg body weight, despite about 50% decrease in femur fluoride concentration and increase in urine fluoride excretion<sup>13</sup>. The present study differed from earlier studies<sup>8,13</sup> in two ways. Here aqueous extract of tamarind fruit pulp was tested in place of crude tamarind fruit pulp. Also, the study period was longer and effect on long bones in terms of radiographic appearance and fluoride concentration were tested that seems to be better parameters than urinary fluoride excretion in chronic toxicity.

Animals on good nutrition have been reported to suffer less from fluoride toxicity in comparison to that on poor nutrition<sup>32</sup>. High nutritive values of Tamarind fruit pulp may, therefore, also be assumed to have protective effects against fluoride toxicity. It contains high concentration of zinc<sup>43</sup>, fat, carbohydrates, fiber, ash, calcium, phosphorous, iron, magnesium, sodium, thiamine, riboflavin, niacin and vitamin C<sup>44</sup>. Food rich in protein, vitamins, essential amino acids and minerals exhibited protection from fluoride induced

oxidative stress to various organs in rats<sup>45-48</sup>. Crude extract of tamarind fruit pulp contains high levels of antioxidants like polyphenols and flavonoids<sup>10</sup>. Tamarind fruit pulp intake is also reported to conserve nutrients like zinc, magnesium, calcium and copper by reducing their urinary excretion<sup>8,9</sup>.

Binding with fluoride by Tamarind is another possibility that may reduce fluoride absorption from gut<sup>44</sup>. This property can also be attributed to high concentration of minerals, like calcium, phosphorus, zinc and iron which are reported to form insoluble complexes with fluoride in gut. However, the extent and avidity of the fluoride binding with tamarind gel are not strong enough to play significant role in fluoride bioavailability<sup>8</sup>.

Alcoholic extracts of flower<sup>50</sup> and roots<sup>51</sup> of *M. oleifera* have been reported to possess hepatoprotective effects. Daily oral administration of *M. oleifera* seeds @200 mg/ kg body weight to rabbits had no harmful effects on body growth<sup>17</sup>. Beneficial effects of *M. oleifera* seed extract was also observed in rats exposed to fluoride for 15 days<sup>7</sup>. Contrary to Tamarind, prevention of fluoride absorption from gastrointestinal tract seems to be more important mechanism of action. It contains a cationic polyelectrolyte that has proved efficient in water treatment as a substitute to aluminium sulfate or other flocculent<sup>52</sup>. Thus beneficial effects of aqueous seed extracts can be attributed to prevention of fluoride absorption by its flocculent action. However, Tamarind seed is also rich in calcium which may also help mitigating fluoride toxicity.

From the present results, it can be concluded that aqueous extracts of *T. indica* fruit pulp and *M. oleifera* seeds have some potential to mitigate toxic effects of excess fluoride. However, further studies are needed for separation and identification of active components so that maximum benefits can be obtained by supplementing smaller doses of these plant products.

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