



EVALUATION OF ANTIOXIDANT PROPERTIES OF *TAMARINDUS INDICA* FRUIT PULP EXTRACT ON FLUORIDE-INDUCED OXIDATIVE STRESS IN RAT ERYTHROCYTES

^{a*}Amit Raj Gupta, ^bSahadeb Dey, ^bMohini Saini, ^bDevendra Swarup

^aTeaching Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar-751003, Odisha, India.

^bEnvironmental Medicine Laboratory, Division of Medicine, Indian Veterinary Research Institute, Izatnagar - 243122, India

*Corresponding author email: amitraj24@rediffmail.com

ABSTRACT

This study examined the toxic effects of fluoride in the rat erythrocytes antioxidant system and evaluated the ameliorative effect of hydro-methanolic extract of *Tamarindus indica* fruit pulp on the oxidative damage induced by fluoride. Eighteen rats were divided into three equal groups. The first group served as control and received only tap water. The second group exposed with 200 ppm of sodium fluoride through drinking water. The third group received *T. indica* fruit pulp extract (200 mg/kg body weight) along with fluorinated drinking water, daily by gavage for a period of 6 weeks. At the end of the experiment, significant increase in malondialdehyde (MDA) levels and decrease in superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities were recorded in fluoride exposed rats erythrocytes as compared to control group. Furthermore, higher plasma nitric oxide concentration was observed in fluoride exposed rats as compared to healthy rats. In the tamarind supplemented group, the level of MDA along with the activities of SOD, catalase, GSH and plasma nitric oxide concentration were comparable with the healthy control rats. Thus, it appears that extract of *Tamarindus indica* fruit pulp ameliorate fluoride-induced oxidative stress in rat erythrocytes.

KEYWORDS: Tamarind, Sodium fluoride, Oxidative stress, Erythrocytes.

INTRODUCTION

Plant derived preparations with potential health benefits and active ingredients are components of herbal medicines with definite therapeutic or prophylactic activities. World Health Organization reported that more than 80% of world's population depends on plants and their products to meet the primary health care needs. Rich biodiversity of our country has gifted more than 1000 plant species for ethno-veterinary use (Jain, 2000). *Tamarindus indica* L. (tamarind) of the family Caesalpinaceae is found in both tropical and subtropical regions of the world. In India, it is colloquially known as ambli or imli or Indian date. Different parts of tamarind like fruits, fruit pulp, seeds, leaves, flowers and barks have been used for various medicinal purposes (Williamson, 2002). The fruit is the richest source of protein (2-3 g/100 g) and carbohydrate (41.1–61.4 g/ 100 g) of any fruit, and serve a good source of potassium, phosphorus, calcium, thiamin and niacin (Feungchan, 1996). High antioxidant activities with high phenolics content in the tamarind fruits have already been reported (Martinello *et al.*, 2006). Fluorine, the 13th most abundant element of the earth, represents about 0.3g / kg of earth's crust. In nature no free fluorine is found and it combines with other elements to form fluorides. Fluoride is having both beneficial and harmful effects on living beings. Drinking water having fluoride concentration between 0.5 to 1.5 mg/L is beneficial to health but higher concentration causes serious health hazards. Prolonged exposure to fluoride in high albeit sub-lethal doses usually

causes fluorosis. The major clinical signs of chronic fluorosis include dental, skeletal and non-skeletal fluorosis (Shanthakumari *et al.*, 2004; Ozsvath, 2009). Mechanism of fluoride induced pathotoxicity at cellular and macromolecular levels is still unclear and merits detailed investigation. Previous studies have demonstrated toxic effects of fluoride on various organs and structures in the body. Cytotoxic, genotoxic, immunotoxic, teratogenic, hepatotoxic, thyrotoxic, neurotoxic and carcinogenic effects of fluoride has been reported in various in vitro and in vivo studies (Shashi, 2003; Ge *et al.*, 2005; Zhang *et al.*, 2008). Interactions between fluoride and free radicals have been reported in various biological systems in experimental as well as natural cases of fluorosis (Akdogan *et al.*, 2004; Wang *et al.*, 2004) and increased production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) with resultant peroxidation of lipids, proteins and carbohydrates have been considered to play pivotal role in the patho-toxicity of fluoride (Chauhan and Flora, 2008). The susceptibility of erythrocytes to oxidative damage is due to presence of polyunsaturated fatty acid, haem iron and oxygen, which may produce oxidative changes in erythrocytes (Aguirre *et al.*, 1998). Earlier studies reported fluoride induced increased lipid peroxidation and increased or decreased enzyme activity associated with free radical metabolism in erythrocytes of experimental animals (Shivarajashankara *et al.*, 2001; Shivarajashankara *et al.*, 2003; Ranjan *et al.*, 2009). Beneficial effect of tamarind in fluoride toxicity by

scavenging reactive oxygen species (Ekambaram *et al.*, 2010) with increased urinary fluoride excretion along with decreased fluoride retention in bone has been reported by various authors (Khandare *et al.*, 2010; Ranjan *et al.*, 2009; Dey *et al.*, 2011; Gupta *et al.*, 2013a). In the present study we aimed to evaluate the anti-oxidant effect of hydro-methanolic extract of *T. indica* fruit pulp on erythrocytes of rats, continually exposed to 200 ppm of sodium fluoride in drinking water.

MATERIALS AND METHODS

Chemicals and reagents

Cacodylic acid, sodium turgate dehydrate, diethylene triamine penta acetic acid (DTPA), 5,5'-dithiobis (2-nitrobenzoic acid) [DTNB, (Ellman's reagent)], nitro blue tetrazolium (NBT), pyrogallol, sulfanilamide and N-naphthylethylenediamine and triton X-100 were purchased from Sigma Chemicals, USA. Thiobarbituric acid (TBA), tris buffer, potassium chloride, disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), ethylene diamine tetraacetic acid (EDTA), perchloric acid, sulphuric acid, hydrogen peroxide (H_2O_2), pyridine and 1-butanol were procured from Sisco Research Laboratories, India.

Animals

Healthy female wistar albino rats (n = 18) weighing about 110-120g, were obtained from Laboratory Animal Resource Section of Indian Veterinary Research Institute (IVRI), Bareilly, India, after obtaining approval from Institute Animal Ethics Committee (IAEC, IVRI). Animals were housed in polypropylene cages in the laboratory animal house ($23 \pm 2^\circ\text{C}$) on standard pellet diet and tap water *ad-libitum* and in daily dark/light cycle. Rats were acclimatized for 15 days prior to the start of experiments.

Experimental design

Rats were randomly assigned into three groups of six each. Rats of group I served as control and received only tap water., group II received sodium fluoride (NaF, MW 41.99, 99% pure, Qualigens Chemicals, Mumbai, India) @ 200 mg/L of drinking water., group III received fluorinated drinking water (200 mg/L added NaF) along with hydro-methanolic extract of *T. indica* fruits (200 mg/kg body weight), daily by gavage for a period of 6 weeks. The dose of sodium fluoride to induce toxicity was selected based on published literature and earlier studies conducted in our laboratory (Yan *et al.*, 2007; Gupta *et al.*, 2013b). The dose of *T. indica* (200 mg/kg body weight) was selected based on previous studies in our laboratory (Dey *et al.*, 2011; Gupta *et al.*, 2015) in which a dose-dependent effect of *T. indica* on reducing serum and bone fluoride concentration with concomitant increase in excretion of fluoride in urine was obtained.

Animals in different groups were sacrificed by decapitation at the end of experiment. Blood samples were collected by cardiac puncture in a centrifuge tube containing acid-citrate-dextrose solution (1 ml per 4 ml of blood). Blood samples were immediately centrifuged at 3000 rpm for 10 min to separate plasma which was used for the estimation of nitric oxide. Packed RBCs were washed three times with buffered saline (0.9% saline in 0.01 M phosphate buffer, pH 7.4), and chilled distilled

water was used to prepare 10% RBC haemolysate for determination of oxidative stress indices. Cyanomethaemoglobin method was used to estimate the concentration of haemoglobin in the haemolysate (Vankampen and Zinglstra, 1961).

Assay of antioxidant indices

Lipid peroxide in erythrocytes

Lipid peroxides (LPOs) level in 10% RBC hemolysate was assessed spectrophotometrically as reported by Placer *et al.* (1966). Plasma and buffy coat was removed from blood samples after centrifugation at 2000 rpm for 10 min. The sedimented cells were washed with chilled 0.85% sodium chloride solution three times. Washed erythrocytes were hemolysed with nine-fold volume of distilled water to make 10% RBC hemolysate. Lipid peroxidation was calculated using 1.56×10^5 as extinction coefficient and finally LPO level was expressed as nmol MDA/ mg of haemoglobin.

SOD assay

Superoxide dismutase (SOD), an important antioxidant defense enzyme, was measured in 10% RBC haemolysate following the method of Marklund and Marklund (1974) with certain modifications reported by Minami and Yoshikawa (1979). Each unit of SOD activity is defined as the amount of enzyme that inhibited pyrogallol auto-oxidation by 50% and the values were expressed as units/ mg of haemoglobin.

CAT assay

Catalase (CAT) activity in 10% RBC hemolysate was recorded spectrophotometrically at wave length of 240 nm after appropriate dilution as reported by Cohen *et al.* (1970) and the values were expressed in units per milligram of haemoglobin.

GSH assay

Glutathione (GSH) level in packed RBC and was measured using DTNB method as per Prins and Loos (1969). The concentration of reduced glutathione was expressed as μmol GSH per ml of packed RBC for erythrocyte.

Nitric oxide estimation

Nitrite concentration was measured in plasma using Griess reagent as per Green *et al.* (1982) to determine plasma concentrations of nitric oxide. Briefly, 100 μl plasma (or nitrite standard) and 100 μl freshly prepared Griess reagent were added in separate wells of a micro-titre ELISA plate and incubated for 30 min at room temperature and then absorbance was taken at 550 nm against DW in ELISA micro-plate reader. A multi-point linear standard curve of nitric oxide, taking a range of concentration from 0 to 200 μM was constructed and the final values were obtained by plotting the absorbance in the curve and expressed as μM NO/ml plasma.

Statistical analysis

The data were analyzed statistically using analysis of variance to compare the means of treatment group with that of healthy control and diseases control groups (Snedecor and Cochran, 1994).

RESULTS

Figure 1 shows the MDA concentration, an end product indicative of the extent of lipid peroxidation, in the

erythrocytes. The lipid peroxide level in the erythrocytes of fluoride exposed rats was significantly ($P < 0.05$) higher than the control values. However, rats treated with

tamarind fruit pulp extract had LPO level comparable to that of healthy control rats.

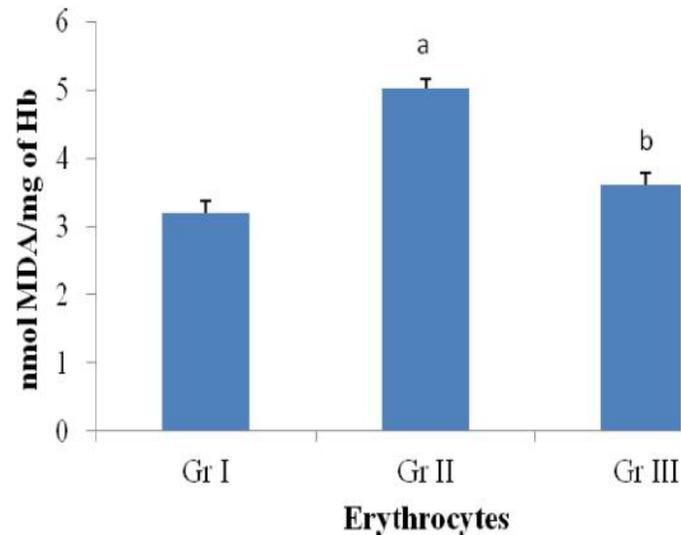


FIGURE 1. Effect of Tamarind fruit pulp extract on the TBARS formation against fluoride induced oxidative damage in the erythrocyte of the experimental rats. Each column represents mean \pm SE, $n=6$; “a” indicates the significant difference between the healthy control and fluoride exposed groups, “b” indicates the significant difference between the fluoride exposed groups and tamarind fruit pulp extract supplemented groups, ($P^a < 0.05$, $P^b < 0.05$).

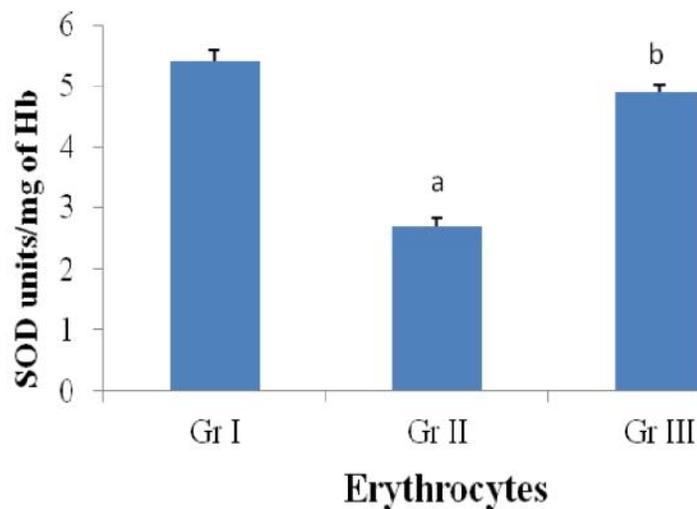


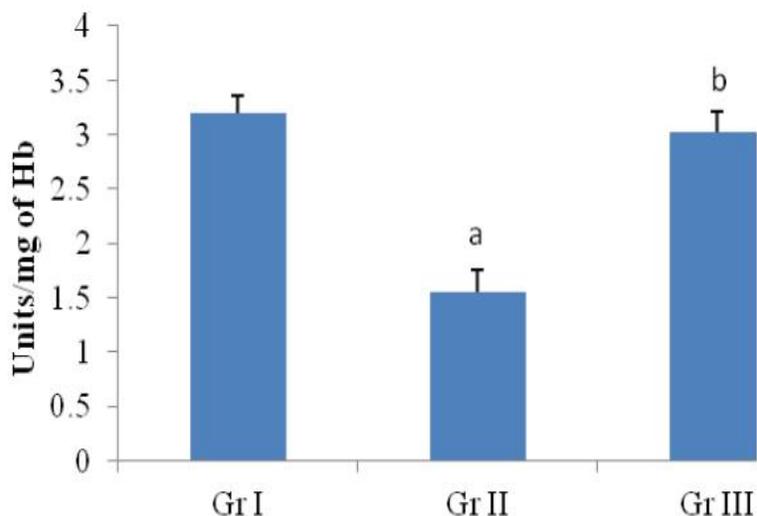
FIGURE 2. Effect of Tamarind fruit pulp extract on the SOD activity against fluoride induced oxidative damage in the erythrocyte of the experimental rats. Each column represents mean \pm SE, $n=6$; “a” indicates the significant difference between the healthy control and fluoride exposed groups, “b” indicates the significant difference between the fluoride exposed groups and tamarind fruit pulp extract supplemented groups, ($P^a < 0.05$, $P^b < 0.05$).

The activity of SOD in the erythrocytes of experimental rats is shown in figure 2. Significant ($P < 0.05$) decrease in the activity of SOD was found in erythrocytes of fluoride exposed rats. However, the supplementation of tamarind fruit pulp extracts significantly ($P < 0.05$) increases the activity of SOD and the mean value was comparable with the healthy control rats.

Significant ($P < 0.05$) suppression in the activity of CAT was found in erythrocytes of rats of Gr II following

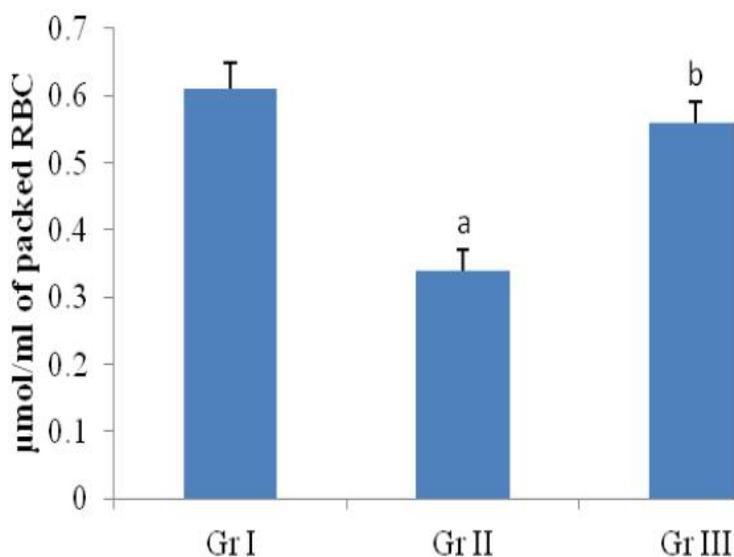
exposure to fluoride (Fig. 3). On the other hand, the activities of CAT in rats of Gr III treated with tamarind fruit pulp extract were comparable to healthy control.

Figure 4 shows the level of GSH in erythrocytes of all experimental rat groups. Fluoride exposure significantly ($P < 0.05$) depleted the level of GSH as compared to healthy control rats. Concomitant use of tamarind fruit pulp extract enhanced the level of GSH compared to fluoride exposed groups.



Erythrocytes

FIGURE 3. Effect of Tamarind fruit pulp extract on the CAT activity against fluoride induced oxidative damage in the erythrocyte of the experimental rats. Each column represents mean \pm SE, n=6; “a” indicates the significant difference between the healthy control and fluoride exposed groups, “b” indicates the significant difference between the fluoride exposed groups and tamarind fruit pulp extract supplemented groups, ($P_a < 0.05$, $P_b < 0.05$).



Erythrocytes

FIGURE 4. Effect of Tamarind fruit pulp extract on the GSH level against fluoride induced oxidative damage in the erythrocyte of the experimental rats. Each column represents mean \pm SE, n=6; “a” indicates the significant difference between the healthy control and fluoride exposed groups, “b” indicates the significant difference between the fluoride exposed groups and tamarind fruit pulp extract supplemented groups, ($P_a < 0.05$, $P_b < 0.05$).

Significant ($P < 0.05$) increase in plasma nitric oxide concentration was recorded in fluoride exposed rats as compared to healthy rats. The concentration of plasma

nitric oxide in rats treated with tamarind fruit pulp extract was found statistically ($P < 0.05$) comparable with those found in healthy rats (Fig. 5).

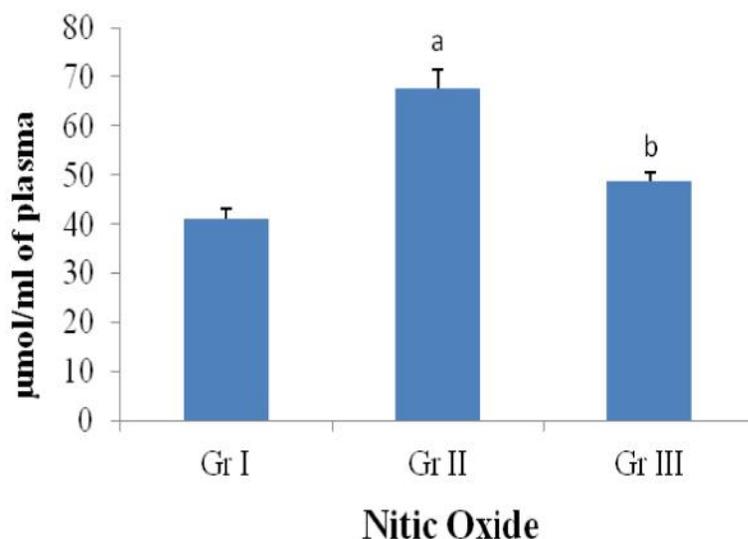


FIGURE 5. Plasma nitric oxide concentration in rats of different experimental groups. Each column represents mean \pm SE, $n=6$; “a” indicates the significant difference between the healthy control and fluoride exposed groups, “b” indicates the significant difference between the fluoride exposed groups and tamarind fruit pulp extract supplemented groups, ($P^a < 0.05$, $P^b < 0.05$).

DISCUSSION

The aim of the present study was to evaluate the anti-oxidant effect of hydro-methanolic extract of *T. indica* fruit pulp on oxidative damage induced by sodium fluoride in erythrocytes of rats. Reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide are implicated as important pathologic mediators in many disorders. Increased concentration of intracellular ROS and enhanced lipid peroxidation play a pivotal role in the toxicity of a wide range of compounds (Cross, 1987). Earlier studies have reported relationships between fluoride and free radical reactions in various biological systems in natural as well as experimental cases of fluorosis (Patel and Chinoy, 1998; Vani and Reddy, 2000). Increased production of free radicals in fluoride toxicity reacts with polyunsaturated fatty acids to yield lipid hydroperoxides which in turn initiates a lipid-radical chain reaction following to oxidative damage to cell membrane (Chinoy, 2003). Erythrocytes, role and their inclination to generate radical species, may be considered as sensitive and intermediate cells in oxidative reactions (Sato *et al.*, 1998). The presence of iron in erythrocytes, a powerful transitional metal catalyst renders erythrocytes highly susceptible to peroxidative damage (Sato *et al.*, 1998). The presence of polyunsaturated fatty acid in membrane of erythrocytes, a primary target for reactions involving free radicals and may allow the erythrocytes vulnerable to oxidative damage (Clemens and Waller, 1987).

Significant increase in malondialdehyde level and decreased activities of superoxide dismutase and catalase in erythrocytes suggested that oxidative stress has mediated toxic effect in fluoride intoxicated rats (Guo *et al.*, 2003). Fluoride exposure in rats caused reduction in GSH concentrations in erythrocytes (Akdogan *et al.*, 2004). Being a multifunctional non-enzymatic antioxidant, GSH was consumed by overproduced free radicals in fluoride-induced oxidative stress or it might play role in

the metabolism of fluoride in mammals and depleted in that process (Akdogan *et al.*, 2004; Inkielewicz and Krechniak, 2004). Enhanced lipid peroxidation, decreased GSH levels and lower SOD activity, have been observed in the erythrocytes of fluoride-treated experimental animals (Shivarajasankara *et al.*, 2003). Likewise, enhanced lipid peroxidation and decreased activities of antioxidant enzymes have been recorded in soft tissues of fluoride-treated rats (Shivashankara *et al.*, 2002; Akdogan *et al.*, 2004).

Nitric oxide (NO), a reactive free radical produced from L-arginine by NO synthase, is well recognized as a physiological messenger molecule (Rodeberg *et al.*, 1995). Cells under inflammatory conditions produce increased amount of NO due to induction of nitric oxide synthetase (Noack *et al.*, 2000). In the present study, administration of sodium fluoride found to cause a significant increase in nitric oxide (NO) production that might be responsible for erythrocytic damage and inflammatory changes (Cenesiz *et al.*, 2005). In addition high level of fluorine is reported to cause methaemoglobin excess due to suppression of the activity of SOD, an enzyme that transforms excess NO to nitroxyl ions (Murphy and Sies, 1991). Suppression of SOD activity found in the present study might have contributed to higher levels of plasma NO.

Traditionally, fruits, leaves and seeds of tamarind are used for the treatment of a wide variety of ailments and diseases. Pods (fruits) are rich in ascorbic acid and sugar and have been used in drinks and many food preparations. Medicinally the fruit pulp is used as cathartic, astringent, febrifuge antiseptic and refrigerant (Komutarin *et al.*, 2004). The extract of tamarind fruit is reported to contain high concentration of zinc, a nutritional antioxidant (Glew *et al.*, 2005) and fat, carbohydrates, fibre, ash, calcium, phosphorous, iron, magnesium, sodium, thiamine, riboflavin, niacin, vitamin C and tannin (Feungchan *et al.*, 1996). It also contain pectin, organic acids mainly

potassium–hydrogen tartarates as well as free acids such as malic, tartaric and citric acids, phenolic compounds (+) catechin, (-) epicatechin, taxifolin, apigenin, eriodictyol, luteolin and naringenin (Sudjaroen *et al.*, 2005). These flavonoids participate in the cellular antioxidant network due to their ability to regenerate ascorbyl radicals and to protect endogenous vitamin E and glutathione from oxidative depletion (Rohdewald, 2002). They also exert free radical scavenging activity via inhibition of the enzyme oxido-reductases (Carlo *et al.*, 1999). In vitro tamarind fruit pulp extract (70% ethanolic) exhibited significant radical scavenging activity and decreased lipid peroxidation in serum with improved antioxidant defence in terms of SOD, CAT and glutathione peroxidase activities (Martinello *et al.*, 2006). The efficacy of tamarind fruit pulp extract in alleviating fluoride induced oxidative stress in rats may therefore be associated with the presence of these phyto-constituents.

CONCLUSION

The present study indicated the antioxidant effect of *T. Indica* fruit pulp extract on fluoride induced oxidative damage in rat erythrocytes. The results may be of future therapeutic significance particularly in the fluoride endemic area where man and animals are exposed to fluoride from natural or anthropogenic sources.

ACKNOWLEDGEMENT

Authors are thankful to Mr. Brijesh Tyagi for his technical assistance.

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