Antioxidant Activity of Natural Products against Aluminium Fluoride Induced Oxidative Stress

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Abstract

Evaluation of antioxidant potential of natural products against Aluminium fluoride (AlF₃) induced oxidative stress in albino mice were represented in the present study. Gossypin, Quercetin dehydrate, (-)-Epicatechin gallate, Gallic acid and Suramin sulphate (G-protein inhibitor) were evaluated for antioxidant activity by measuring various biochemical / enzymatic markers such as lipid peroxidation, glutathione, total thiols, catalase and superoxide dismutase. Exposure to Aluminium fluoride resulted in the oxidative stress and free radical mediated damage. The flavonoids like Gossypin, Quercetin, (-) -Epicatechin gallate, Gallic acid and Suramin sulphate are significantly reduced lipid peroxidation, reversed the reduced protective enzymes SOD,CAT and non-enzymatic like glutathione and total thiols levels were approached to normal levels in Aluminium fluoride exposed mice. All the test compounds were showed significant protection against fluoride toxicity. Results of the present study reveals that the flavonoids and Suramin sulphate showed significant antioxidant activity on Aluminium fluoride induced oxidative stress. Further research warranted to study their exact mechanism of action.

INTRODUCTION

Over the last several years, numerous reports from China, India and elsewhere indicate that fluoride in varying concentrations induces free radical toxicity in both animals and in people living in areas of endemic fluorosis. There is much evidence that superoxide free radical and lipid peroxidation play an important role in fluorosis (Chinoy, 2003). Laboratory investigations have often used fluoride activation for stimulation of guanine nucleotide binding proteins (G-Proteins) and activation of adenylate cyclase depends on traces of aluminium (Gilman, 1987; Sternweis & Gilman, 1982). These metallofluoride complexes may thus mimic or potenates the action of numerous extracellular signals and significantly affects many cellular responses.

Fluoride in the presence of trace amounts of Aluminium affects blood elements, cells of the
immune system, protein phosphorylation and organization of cytoskeleton proteins, processes of calcium homeostasis, ion transport and energy metabolism (Ann strunecka & Tiri palocka, 1999). Also include abnormal behavior patters, altered neuronal and cerebrovascular integrity (Mullenix et al., 1995). In blood, brain and liver tissues of animals, various changes occur after chronic administration of fluoride are generation of free radicals, lipid peroxidation and altered antioxidant defense systems are considered to play an important role in the toxic effects of fluoride (Rzeuski et al., 1998; Sharm & Chinoy, 1998).

Defense mechanism against free radical induced oxidative stress involved preventative mechanisms, mainly by enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), non-enzymatic antioxidants are ascorbic acid (Vitamin C), alph-tocopherol (Vitamin E), glutathione (GSH), plant derived polyphenolic and flavonoids are showed antioxidant properties. This balance is essential for the survival of organism and their health.

The protection offered by dietary flavonoids and phenolic compounds have generally been considered as non-nutrients and their possible beneficial effect on human health has only recently been recognized. These compounds are known to possess anti-inflammatory, neuro-protective, radioprotection and anticarcinogenic activities (Rajnaryana et al., 2001; Ganapaty, 2007). On this above basis, in our study we evaluated antioxidant properties of different flavonoids and Suramin sulphate against Aluminium fluoride (G-protein activator) induced oxidative stress in mice.

MATERIALS AND METHODS

Materials Required

Quercetin dehydrate, (-)-Epicatechin gallatee, Suramin sulphate, Nitro blue tetrazolium (NBT), Thiobarbituric acid (TBA), 1,2-dithio-bis-nitrobenzoic acid (DTNB) and Aluminium fluoride were obtained from the Sigma chemical Co (St. Louis, MO,USA) and Trichloroacetic acid (TCA), Hydroxylamine HCL were obtained from Merk, KGOA, Germany. All other chemicals and reagents used were of analytical grade.

Refrigerated centrifuge (Model MPW-350R) from MPW Med. Instrument, Warszawa, Poland and UV-Spectrophotometer (UV-1601) Shimadzu Corporation, Kyoto, Japan were used. The flavonoid gossypin was isolated from Hibiscus vitifolius in Prof. S. Ganapaty’s laboratory and was used in this study.

Animal Care

Swiss albino mice weighing in between 20-24 g were used for the study. They were kept for one week in our laboratories before the experiments for acclimatization to the laboratory conditions and fed with low-fluoride food and water Ad libitum. Animal care and protocols were in accordance with and approved by the Institutional Animal Ethics Committee, Hanagal Shri Kumarseshwara college of Pharmacy, B.V.V.S. Campus, Bagalkot-587101, Karnataka, India (IAEC/05/01-09). All mice were housed in polypropylene cages in a temperature (25±2 °C) and humidity (60±10%) controlled room submitted to a 12-dark/light cycle (artificial lights, 7 a.m – 7 p.m.) and air exhaustion cycle (15 min/h). All procedures were carried out in accordance with the conventional guidelines for experimentation with animals. Prior to experimental treatments, animals were fasted overnight but were allowed free access to water. Six animals were used for each group of study.

Experimental Procedure

The activity was carried out with slightly modification of method described by Sinha et al. (2007). Mice were divided into seven groups of six animals each. Group I, receives normal saline serves as control and group II, receives Aluminium fluoride treated group. Group III, IV, V, VI and VII treated with Gossypin (10 mg / Kg body wt) i.p, Quercetin dehydrate (10 mg / Kg body wt) i.p,(-)-Epicatechin gallate (10 mg / Kg body wt) i.p ,Gallic acid (10 mg / Kg body wt) i.p and Suramin sulphate (5 mg / Kg body wt) i.p respectively for the period of 14 days and from 7th day onwards Aluminium fluoride (600 ppm) treatment through their drinking water. After 24 hrs the last dose of Aluminium fluoride administration, all the animals were sacrificed by cervical decapitation, brain and liver was removed and washed in cold 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized in cold phosphate buffer (0.1 M, pH 7.4) using a potter Elvehjem Teflon homogenizer as possible as under standard condition and homogenate was kept on ice until assayed.
Biochemical Estimations

Total Protein

The protein contents of 10% liver homogenates were determined by using the modified Lowry’s method (Lowry et al., 1951).

Lipid Peroxidation (LPO)

A Thiobarbituric acid reactive substance (TBARS) in the homogenate was estimated by using standard protocol (Prabhakar et al., 2006). Briefly, the 0.5 ml of 10% homogenate was incubated with 15% TCA, 0.375% TBA and 5N HCl at 95 °C for 15 min, the mixture was cooled, centrifuged and absorbance of the supernatant measured at 512 nm against appropriate blank. The amount of lipid peroxidation was determined by using $\varepsilon = 1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ and expressed as TBARS nmoles/mg of protein (Braughler et al., 1987).

Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined based on the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH (Misra and Fridovich, 1972). Briefly, 25 μl of the supernatant obtained from the centrifuged liver homogenate was added to a mixture of 0.1 mM adrenaline in carbonate buffer (10.2) in a total volume of 1ml and the formation of adrenochrome was measured at 295 nm. The SOD activity (U/mg of protein) was calculated by using the standard plot.

Catalase (CAT)

Catalase activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M), and 0.05 ml homogenate (10%, w/v) in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of units/mg protein.

Total Thiols

This assay is based on the principle of formation of relatively stable yellow color by sulphydryl groups with DTNB (Moron et al., 1979). Briefly, 0.2 ml of liver homogenate was mixed with phosphate buffer (pH 8.0), 40 μl of 10 mM DTNB and 3.16 ml of methanol. This mixture was incubated for 10 min and the absorbance was measured at 412 nm against appropriate blanks. The Total thiol content was calculated by using $\varepsilon = 13.6 \times 10^3 \text{cm}^{-1} \text{M}^{-1}$ (Sedlak & Lindsy, 1978).

Glutathione (GSH)

Briefly, proteins were precipitated using 10% TCA, centrifuged and 0.5 ml of the supernatant was mixed with 0.2 M phosphate buffer (pH 8.0) and 0.006 mM DTNB. This mixture was incubated for 10 min and the absorbance was measured at 412 nm against appropriate blanks. The glutathione content was calculated by using the standard plot under same experimental conditions (Prabhakar et al., 2007).

Histopathology

The mice were sacrificed under light ether anesthesia, liver samples of all group were preserved in 10% neutral buffered formalin as described by Luna (1968). Liver 5-6 μm thickness were cut and stained with hematoxylin and eosin.

Statistical Analysis

All the data were expressed as mean ± SEM. The significance of difference in means between control and treated animals for different parameters was determined by using one-way ANOVA followed by Dennett’s test. Significance of differences between groups was evaluated with Students t-test. A p-value of <0.05 was considered statistically significant.

RESULTS

Lipid Peroxidation

Normal mice showed basal TBARS levels of about 35.25±1.521 and 31.91±3.023 nmol/mg of protein of brain and liver homogenate respectively. Different flavonoids (drug control) did not show a significant difference in lipid peroxidation activity as compared to control. Mice treated with Aluminium fluoride showed significant (p<0.001) decreases in the TBARS levels to about 133.7±9.703 and 79.31±7.810 nmoles/mg of protein of brain and liver tissues homogenate in comparison to the normal control. Different flavonoids like Gossypin (10 mg/Kg), Quercetin dehydrate (10 mg/Kg), (-) Epicatechin gallate (10 mg/kg), Gallic acid (10 mg/Kg) and Suramin sulphate (5mg/Kg) a G-protein inhibitor in AlF₃ exposed mice showed reduced levels of TBARS significantly to about 55.38±4.302, 54.05±8.099, 40.56±4.619, 74.01±12.51 and 69.86±4.619 nmoles/mg of protein of brain tissue and 40.59±1.53 (p<0.001), 57.92±5.302 (p<0.05), 45.41±6.326 (p<0.01), 37.83±2.006 (p<0.001) and 67.89±7.587 nmoles/mg of protein in comparison to Aluminium fluoride treated groups (Figure 1 & 2).
Figure 1. Estimation of TBARS in the brain homogenates of mice which received Aluminium fluoride in drinking water and drug treatment. Statistical significance: \(^{a}p<0.05;^{b}p<0.01;^{c}p<0.001\) Vs Normal control; \(^{d}p<0.05;^{e}p<0.01;^{f}p<0.001\) Vs Aluminium fluoride treated group.

Figure 2. Estimation of TBARS in the liver in homogenates of mice which received Aluminium fluoride in drinking water and drug treatment. Statistical significance: \(^{a}p<0.05;^{b}p<0.01;^{c}p<0.001\) Vs Normal control; \(^{d}p<0.05;^{e}p<0.01;^{f}p<0.001\) Vs Aluminium fluoride treated group.

**Total Thiols**

Normal basal total thiol was found to be 14.29 ±0.726 and 15.82±1.683 µmol /mg of protein of brain and liver tissue of homogenate. Different flavonoids solutions (drug control) did not show a significant difference activity comparison to control. Mice treated with Aluminium fluoride treated decreased the levels 6.014±0.9705 and 4.516±0.335 µmol /mg of protein of brain and liver tissue homogenate respectively as compression to control. The total thiols were should significant \((p<0.001)\) increased and reversed to normal level. AlF\(_4\) exposed mice with different flavonoids like, Gossypin (10 mg/Kg), Quercetin dehydrate (10 mg/Kg), (-)-Epicatechin gallate (10 mg/kg), Gallic acid (10 mg/Kg) and Suramin sulphate (5mg/Kg) of about in brain homogenate 14.71±0.956, 17.41±1.884, 12.93±1.124, 10.25±0.879 \((p<0.01)\), 16.72±1.237 µmol/mg of protein respectively and liver homogenate of about 21.19±2.992, 19.22±2.252, 20.52±1.820, 9.250±0.771 and 17.50±2.084 µmol/mg of protein respectively as comparison with the Aluminium fluoride treated group (Figure 3 & 4).
Figure 3. Estimation of total thiols in the brain homogenates of mice which received Aluminium fluoride in drinking water and drug treatment. Statistical significance: \( a \) \( p < 0.05; \) \( b \) \( p < 0.01; \) \( c \) \( p < 0.001 \) Vs Normal control; \( d \) \( p < 0.05; \) \( e \) \( p < 0.01; \) \( f \) \( p < 0.001 \) Vs Aluminium fluoride treated group.

Superoxide Dismutase

Normal basal level of SOD activity was found to be \( 17.60 \pm 2.811 \) and \( 16.24 \pm 1.073 \) U/mg of protein of brain and liver homogenate. Different flavonoids (drug control) did not show a significant difference in SOD activity compared to control. Mice treated with Aluminium fluoride decreased the levels of about \( 5.114 \pm 0.291 \) and \( 3.213 \pm 0.423 \) in and liver tissue homogenate respectively as comparison to normal control. Different flavonoids in Aluminium fluoride treated groups reversed significantly \( (p < 0.001) \) the SOD level to normal.

Gossypin (10 mg/Kg), Quercetin dehydrate (10 mg/Kg), (-)-Epicatechin gallate (10 mg/kg), Gallic acid (10 mg/Kg) and Suramin sulphate (5mg/Kg) a G-protein inhibitor increases the SOD level significantly of about \( 14.09 \pm 1.303, 14.25 \pm 1.715, 12.27 \pm 0.883, 12.77 \pm 0.795, \) and \( 13.09 \pm 1.409 \) of brain tissue homogenate and 12.76 \pm 1.763, 13.09 \pm 2.394, 11.10 \pm 0.638, 10.60 \pm 0.781 and 13.12 \pm 1.409 of liver tissue homogenate respectively as comparison with the Aluminium fluoride treated group (Figure 5 & 6).
Figure 5. Estimation of superoxide dismutase in the brain homogenates of mice which received Aluminium fluoride in drinking water and drug treatment. Statistical significance: $^a p<0.05$; $^b p<0.01$; $^c p<0.001$ Vs Normal control; $^d p<0.05$; $^e p<0.01$; $^f p<0.001$ Vs Aluminium fluoride treated group.

Figure 6. Estimation of superoxide dismutase in the liver homogenates of mice which received Aluminium fluoride in drinking water and drug treatment. Statistical significance: $^a p<0.05$; $^b p<0.01$; $^c p<0.001$ Vs Normal control; $^d p<0.05$; $^e p<0.01$; $^f p<0.001$ Vs Aluminium fluoride treated group.

Catalase
Normal basal level of catalase activity was found to be 2.263±0.250, 0.987±0.054 U/mg of protein of brain and liver homogenate. Different flavonoids (drug control) did not show a significant difference in catalase activity compared control. Mice treated with Aluminium fluoride decreased the levels of about 0.3597±0.114 and 0.9871±0.054 of brain and liver tissue homogenate respectively as comparison to normal control. Different flavonoids in Aluminium fluoride treated groups reversed the catalase level to normal. Gossypin (10mg/Kg), Quercetin dehydrate (10 mg/Kg), (-)-Epicatechin gallate (10 mg /kg), Gallic acid (10 mg/Kg) and Suramin sulphate (5mg/Kg) a G-protein inhibitor increases the catalase level significantly of about 1.566±0.134 ($p<0.001$), 1.712±0.272 ($p<0.01$), 1.169±0.186 ($p<0.01$), 1.458±0.2200 ($p<0.01$) & 0.948±0.157 ($p<0.05$) of brain tissue homogenate respectively and 1.384±0.294 ($p<0.01$), 1.868±0.0463 ($p<0.001$) and 1.250±0.248 ($p<0.001$) of liver tissue homogenate respectively as comparison with the Aluminium fluoride treated group (Figure 7 & 8).
Figure 7. Estimation of catalase in the brain homogenates of mice which received Aluminium fluoride in drinking water and drug treatment. Statistical significance: *p<0.05; **p<0.01; ***p<0.001 Vs Normal control; *p<0.05; **p<0.01; ***p<0.001 Vs Aluminium fluoride treated group.

Glutathione
Normal basal GSH level was found to be 6.051±0.428 and 5.208±0.335 nmol/mg of protein of brain and liver homogenate. Different flavonoids solutions (drug control) did not show a significant difference activity comparison to normal. Mice treated with Aluminium fluoride treated decreases the levels 2.099±0.2152 and 1.691±0.1187 nmol/mg of protein of brain and liver tissue homogenate respectively as compared to normal control. The GSH levels were significantly (p<0.001) increased to normal levels. AlF₄ exposed mice with different flavonoids like Gossypin (10 mg/Kg), Quercetin dehydrate (10 mg/Kg), (-)-Epicatechin gallate (10 mg /kg), Gallic acid (10 mg/Kg) and Suramin sulphate (5mg/Kg) increases SOD levels of about 7.262±0.375, 7.689±0.9507, 5.290±0.3608, 6.188±0.4281 & 5.727±0.489 of brain tissue homogenate respectively and 5.783±0.8512, 6.779±0.808, 7.255±0.539, 6.397±0.403 and 5.541±0.447 of liver tissue homogenate respectively as comparison with the Aluminium fluoride treated group(Figure 9 & 10).
Histopatological Examinations

Histopatological examinations of all the groups were represented from the figure 11-18.

DISCUSSION

It is conventional wisdom that the oxidative stress response is triggered by an imbalance in the production and metabolism of reactive oxygen species (ROS). Common oxidants produced in organisms include reactive oxygen species (ROS), such as hydrogen peroxide, superoxide (\( \cdot \)O\(_2\)), hydroxy radical (OH\(_{-}\)), singlet oxygen and nitrogen species. These reactive oxygen species (ROS) has a role in disease and aging in animals (Singh et al., 2002).
The term redox signaling is used to describe regulatory process in which the signal is delivered through redox reactions. Redox signaling requires that the steady state of redox balance is disturbed either by an increase in ROS formation or a decrease in the activity of antioxidant system. Signal transduction is triggered by intracellular signals such as hormones, growth factors, cytokines, neurotransmitters and fluorides (Thannickal & Fanburg, 2000; Bigay et al., 1987).

Fluoride anions have long been known to influence the activity of a variety of enzymes. Most of the researchers used fluoride activation or stimulation of guanine nucleotide binding protein (G-protein) (Gilman, 1987). Aluminium fluoride complex mimics the action of many neurotransmitters, hormones and growth factors (Sternweis & Gilman, 1982). The treatment of isolated hepatocytes with sodium fluoride produced an efflux of calcium and rise in free cytosolic calcium, alteration in the phosphatidylionositol-4,5-bisphosphate content and increase in the level of inositol -1,4,5-triphosphate observed (Chabre, 1999; Blackmore et al., 1985). Similar effects were observed, G-protein mediated cell responses are of key importance in the process of neurotransmission and intracellular signaling in the brain (Blackmore et al., 1988).

Fluoride enhances lipid peroxidation and decreased activities of antioxidant enzymes have been recorded in soft tissue of fluoride treated mice (Rana and Hokin, 1990) and produced changes in organs the reduced in the concentration of free radical scavenger glutathione as well as reduced ascorbic acid and impairments in the activity of the protective enzymes SOD, CAT and reduced GSH (Vani & Reddy, 2000).

On this basis the present studies with different flavonoids antioxidant properties were evaluated in animals exposed with Aluminium fluoride in drinking water. The results showed the significant antioxidant defense mechanism and protection against oxidative stress. All flavonoids showed significantly decreased the lipid peroxidation, induced by Aluminium fluoride and reveres to the normal level of the protective enzymes catalase, superoxide dismutase, indicating overall fluoride toxicity protective effects.

However, thiols/glutathione content is reported to serve as an index of oxidative stress (Willson, 1983). In our present studies also significant improvement in thiols and glutathione content in both liver and brain. This result indicates that, the selected flavonoids may exert their protection through modulation of signal transduction mechanism via G-protein. Earlier results substantiates that, Suramin sulphate showed significant in G-protein inhibitor (Ref), may be it showed it effects through membrane stabilization and reported to regulates the level of cyclic AMP, which is stimulated by G-protein and inhibiting the intracellular release of calcium, both are critical to the degranulation of mast cells (Sang-Hyun et al., 2005).

The flavonoids rich plant extract Lucopus lucidus, showed antioxidant and potent antiallergic activity in compound 48/80 induced mast cell degranulation through inhibits the release of calcium through membrane stabilizing activity through G-proteins and quercetin also showed potent inhibition of histamine released, which may act by altering intracellular levels of cAMP (Madelein Ennis et al., 1980; Jae-Yong Shin et al., 2005). With the above basis, present selected flavonoids and G-protein inhibitor Suramin sulphate (direct inhibitor of Adenyl cyclase) (Jiri Stohr et al., 2005) may exert their actions as a antioxidant activity and decreased the fluoride toxicity through regulating the cyclic AMP level or modulate adenylylcyclase activity and inhibition of intracellular calcium release, calcium play an important role in increase of intracellular rise of ROS and oxidative stress (Russell, 2004).

In short, ALF$_4$ induced damage produces alteration in the antioxidant status of the tissues, which is manifested as an abnormal histopathology. All flavonoids restored all these changes. The flavonoids have potent antioxidant activity in AlF$_4$ induced oxidative stress.

**CONCLUSION**

The results of the present study indicates that, all the tested flavonoids exerts remarkable antioxidant activity due to its possible multiple effects involving significant protection against the oxidative damage, which may be attributed to its protective action on lipid peroxidation and to the enhancing effect on cellular antioxidant defense contributing to the protection against oxidative damage in Aluminium fluoride induced oxidative stress. Further work is necessary to elucidate the molecular mechanism involved in the antioxidant activity of these compounds.
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