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Research Article

INVESTIGATION ON THE ANTI-GENOTOXIC EFFECT OF OCIMUM SANCTUM IN FLUORIDE INDUCED GENOTOXICITY

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ABSTRACT

The present study was designed to investigate the anti-genotoxic effect of *Ocimum sanctum* on fluoride induced genotoxicity and its impact on oxidative stress. Exposure to fluoride can mainly occur through drinking water when the levels far exceed the permissible limit. Fluorosis is a serious problem the world over resulting in damage to DNA. Micronuclei assessment from bone marrow and peripheral blood was used in the present study to assess the damage to DNA. Sodium fluoride in a single dose (30 mg/kg, i.p.) was used to induce micronuclei in albino mice. Treatment with the aqueous extract of *Ocimum sanctum* was initiated in the single dose study (100, 400 & 800 mg/kg) and as a time course for 1 day, 3 days and 7 days (100 mg/kg). 24 h after injecting sodium fluoride, the animals were sacrificed and micronuclei were determined from smears prepared from bone marrow and peripheral blood. The antioxidant impact of the extract was determined using ferric ion reducing capacity of plasma and thiobarbituric acid reactive substances as a measure of lipid peroxidation. All doses were capable of preventing the formation of micronuclei but 100 mg/kg of the aqueous extract was most efficacious as a single dose and in the time course study. The beneficial effect of *Ocimum sanctum* is possibly due to the synergistic action of constituents like polyphenols, triterpenoids, and flavonoids.

Key words: Fluoride, Ocimum sanctum, Micronuclei.

INTRODUCTION

Fluoride (F) is the thirteenth most abundant element (0.065% by weight) in the earth's crust and is widely distributed in our environment¹. F has been used along with calcium to stimulate osteoblasts and has therefore found a place in the treatment of osteoporosis. Fluoridation of water was found to decrease the prevalence of dental caries. Long term exposure to F has been reported to induce mottling of teeth, skeletal fluorosis, birth defects and cancer². In certain areas around the world, the level of F in ground water might surpass the permissible limit of 1.0 mg/L³ creating distress to osseous tissue, liver, kidney and brain⁴. Nalgonda, a district in Andhra Pradesh in India is deeply afflicted with fluoride related diseases. The fluoride levels in this district range from 2 to 7 mg/L in ground water³. Very large number of people is affected with fluorosis in this district alone.

DNA damage is one of the important consequences of exposure to some genotoxic agents^{4, 5}. Stability of the genome relies heavily on an efficient DNA repair mechanism. DNA repair can be elicited through enzymatic processes which require a few hours or non enzymatic modalities which take only a few seconds. If the DNA repair mechanisms fail to operate efficiently, then nuclear material and cellular components are susceptible to damage by oxidative stress. Exposure to mutagens and genotoxic agents can cause anomalies in the nuclear material characterized by micronuclei (MN) and disruption of chromosomal structure. Micronuclei are small spherical chromatin elements which lie outside the nucleus in a cell or in erythrocytes. They appear identical in structure, shape and size to the main nucleus in a cell.

Previous studies indicate that fluoride induces chromosome aberrations, induction of sister chromatid exchange (SCE) and DNA damage⁶. Damage caused by fluoride intoxication is predominantly mediated through lipid peroxidation^{4, 5}. Very few treatment strategies are available to combat the effects of fluoride.

Ocimum sanctum (family- Labiatae), commonly known as Sacred basil, is a fragrant bushy plant containing volatile oil comprising of 70% eugenol, methyl eugenol and caryophyllene. Triterpenoids like ursolic acid, rosmarinic acid, alkaloids, saponins, flavonoids, phenylpropane glucosides and tannins exist in this plant^{7, 8}. *Ocimum sanctum* have a plethora of reported effects which include growth promoting, anti-hypertensive, cardiac depressant, smooth muscle relaxant, antistress, hepatoprotective, anticancer, antiinflammatory, analgesic and radio protective properties^{8,9}.

It has been reported from several studies that *Ocimum sanctum* is capable of protecting DNA from damage when exposed to xenobiotics like cyclophosphamide, cyproterone acetate¹⁰ and heavy metals like Cr and Hg¹¹. However no study was undertaken to ascertain the impact of *Ocimum sanctum* on fluoride induced genotoxicity. *Ocimum sanctum* is widely accessible, easily recognized medicinal herb and is found in every household. If it is found effective in reducing the deleterious effect of fluoride, it can be a boon to the population in this area. Keeping this in mind the present investigation was undertaken to evaluate the effect of aqueous extract of *Ocimum sanctum* on fluoride induced genotoxicity and oxidative stress in experimental animals.

MATERIALS AND METHODS

Drugs and chemicals

Sodium fluoride (Batch No.D024290179) was purchased from Universal Laboratories Pvt. Ltd, Mumbai. Bovine albumin serum and MayGrunwald-stain were purchased from Rolex Chemical Industries, Mumbai. Giemsa stain was purchased from Thermo Fischer Scientific India Pvt Ltd, Mumbai. Thiobarbituric acid was purchased from LOBA Chemie Pvt Ltd, Mumbai. All other reagents and chemicals used were of analytical grade.

Animals

Healthy albino mice weighing 25-30 g were procured from National Institute of Nutrition, Hyderabad and acclimatized in our own animal house for one week prior to the experiment. The animals were maintained at 22 ± 3 ⁰C under natural light dark conditions and fed on standard diet with free access to deflourinated water (RO water). This experimental protocol has been approved by the institutional animal ethical committee and the experiments have been conducted as per guidelines laid down by Committee for the Purpose and Control of Supervision of Experiments on Animals (CPCSEA), Chennai, India.

Plant Material

Leaves of *O. sanctum* were collected from the local areas of Nalgonda district, Andhrapradesh, India, and were authenticated by Professor A. Laxma Reddy, Department of Botany, Nagarjuna Government Degree College, Nalgonda. A voucher specimen (No. 005) has been deposited in our herbarium.

Preparation of Ocimum sanctum leaf extract

The leaves were dried at room temperature and powdered. The aqueous extract of *Ocimum sanctum* (AEOS) was obtained by refluxing the powder with distilled water. 75 g of powder is refluxed with 500 ml distilled water for 6 h at 60 °C. The extract obtained was filtered, concentrated (yield, 10% w/w) and stored in refrigerator for further use.

Dose selection

It has been reported that *Ocimum sanctum* is safe up to a dose of 4 g/kg¹⁰. 100, 400 and 800 mg/kg of aqueous extract were selected for the study based on maximum tolerated dose of *Ocimum sanctum*. The dose of sodium fluoride selected was 30 mg/kg^{12} .

Experimental design

The animals were divided into 7 groups of 6 mice each for the dose dependent and time dependent studies for peripheral and bone marrow micronucleus assay.

Dose dependent study

Group I: Animals were administered with 0.2 ml of 1% w/v acacia by oral route.

Group II: Animals were administered with in a single dose of 30 mg/kg of sodium fluoride by the i.p. route to induce micronuclei.

Group III: Animals were treated with 100 mg/kg of AEOS, after 1 h they were injected with sodium fluoride by the i.p. route.

Group IV: Animals were treated with 400 mg/kg of AEOS, after 1 h they were injected with sodium fluoride by the i.p. route.

Group V: Animals were treated with 800 mg/kg of AEOS, after 1 h they were injected with sodium fluoride by the i.p route.

Time dependent study

Group VI: Animals were treated with 100 mg/kg of AEOS once daily for 3 days, 1 h after the last dose, sodium fluoride was injected i.p.

Group VII: Animals were treated with 100 mg/kg of AEOS once daily for 7 days, 1 h after the last dose, sodium fluoride was injected i.p.

The aqueous extract was suspended in 1 % w/v acacia and administered to animals by oral route. Blood was withdrawn from same animals for estimation of oxidative parameters before sacrificing the animals.

Peripheral blood micronucleus assay

Peripheral blood was collected by retro orbital puncture under enflurane anesthesia in test tubes containing 1% w/v EDTA as an anticoagulant. Smear was prepared on slides, air dried, fixed with absolute methanol for 10 min and stained with phosphate buffer diluted Giemsa (1:4) for 12 min. Slides were observed under microscope for the presence of micronuclei¹³.

Determination of antioxidant activity by ferric reducing ability of plasma (FRAP) assay

Plasma antioxidant status was evaluated using ferric reducing antioxidant power (FRAP) assay. The assay was carried out according to F.F.Benzie and J.J.Strain (1996). 100 μ l of plasma was mixed with 3 ml of working FRAP reagent and absorbance was measured at 0 min after vortexing at 593 nm. Thereafter, samples are placed at 37 °C in water bath and absorbance was again measured after 4 min. Ascorbic acid standards (100 μ M-1000 μ M) were processed in the same way¹⁴.

Estimation of Thiobarbituric acid Reactive Substances (TBARS) in plasma

The thiobarbituric acid reactive substances levels were estimated as per the spectrophotometric method described by Ohkawa et al. 0.5 ml of plasma was mixed with 0.5 ml of normal saline, 1 ml of 20 % w/v trichloroacetic acid (TCA) and 0.67 % w/v TBA reagent. The samples were kept for boiling at 95 °C for 1 h. To each of the sample, 3 ml of n-butanol was added, mixed well and centrifuged at 3000 rpm for 10 min. The separated butanol layer was collected and read in a spectrophotometer against reagent blank at 535 nm. TBARS concentration was expressed in terms of μ mol of malondialdehyde per milliliter of plasma¹⁵.

Bone marrow micronucleus assay

The same experimental animals were used for both peripheral blood MN and bone marrow MN assays. Animals in all groups were sacrificed by cervical dislocation 24 h after the intraperitoneal injection of sodium fluoride. Animals were dissected to excise femur. Marrow suspension from femur bone was prepared in 5% bovine serum albumin (BSA) and centrifuged at 1000 rpm. The pellet formed was resuspended in a required quantity of BSA. A drop of this suspension was taken on clean glass slides, smears were prepared and the slides were air-dried. The slides were fixed in methanol, stained with May-Gruenwald-Giemsa and MN were identified in two forms of red blood cells which are Polychromatic Erythrocytes as PCE and Normochromatic Erythrocytes as NCE using binocular microscope under oil immersion objective. About 500 PCE and corresponding NCE per animal were observed for the presence of MN¹³.

Statistical analysis

Data are expressed as mean \pm SEM. Analysis of data was done by One-way ANOVA followed by Dunnett comparison test for FRAP and TBARS. Mann-Whitney U test was carried out for micronuclei in bone marrow and peripheral blood using Graph Pad In Stat version 3.10 for Windows 2009 (Graph Pad Software). The statistical significance was set as 0.01 and 0.05 (P<0.01, P<0.05).

Table 1: Effect of aqueous extract of Ocimum sanctum on the frequency of micronuclei in bone marrow and peripheral blood induced by
sodium fluoride

S.NO	Treatment	Bone marrow micronucleus test		Peripheral blood micronucleus test
		% MNPCE ^a	% MNNCE ^b	% MNNCE ^c
1	Negative control	0.4±0.12	0.2267±0.08	0 ±0.00
2	SF (30 mg/kg)	9.2±0.42	0.993±0.09	3 ±0.26
3	AEOS (100 mg/kg)	$1.4\pm0.05^{**}$	$0.2698 \pm 0.07^{**}$	0.233±0.06**
4	AEOS (400 mg/kg)	1.83±0.21**	0.3675±0.10**	0.266±0.04**
5	AEOS (800 mg/kg)	3.4±0.36**	$0.5617 \pm 0.14^*$	0.33±0.04**
6	AEOS (100 mg/kg) for 3 days	0.933±0.12**	0.142±0.05**	0.133±0.04**
7	AEOS (100 mg/kg) for 7 days	$0.4+0.15^{**}$	0.000+0.0	$0.066\pm0.04^{**}$

Values are expressed as Mean ± SEM, n=6, Statistics: Mann-Whitney U test, ** P<0.01,* P<0.05 compared with positive control, a) from 3000 PCE and b) corresponding NCE counted. c) from 3000 NCE. MNPCE: micronucleated polychromatic erythrocytes, MNNCE: Micro nucleated normochromatic erythrocytes, SF: Sodium fluoride, AEOS: aqueous extract of *Ocimum sanctum*







Figure 2: Effect of aqueous extract of *O*.sanctum on concentration of TBARS in fluoride induced genotoxicity. Values are expressed as Mean ± SEM, n=6, **P<0.01 compared with positive control. AEOS: aqueous extract of *Ocimum sanctum*, SF: sodium fluoride.



Figure 3: Photographs showing micro nucleated erythrocytes in bone marrow and peripheral blood. Control and MNPCE and MNNCE in bone marrow in sodium fluoride treated group. AEOS treatment 100 mg/kg and AEOS 100 mg/kg for 7 days in bone marrow and peripheral blood respectively.

RESULTS

Peripheral blood micronucleus assay

A single intraperitoneal exposure with sodium fluoride (30 mg/kg) was capable of inducing MN (MNNCE) within 24 h. Treatment with a dose of 100 mg/kg of AEOS exerted a significant reduction in the formation of MNNCE induced by F indicating that low dose of AEOS is more effective in prevention of MN. The number of MNNCE observed with 400 and 800 mg/kg of AEOS also declined but the effect was less pronounced than that produced by 100 mg/kg AEOS (Table I). In time course studies, treatment with a dose of 100 mg/kg of AEOS for 7 days exerted a significant reduction in the formation of MNNCE induced by F compared with the positive control (Table 1).

Determination of antioxidant activity by ferric reducing ability of plasma (FRAP) assay

The antioxidant activity of group of animals treated with 100 mg/kg of AEOS was found to be more than group of animals treated with 400 mg/kg and 800 mg/kg of AEOS. The same dose (100 mg/kg) was found to possess better antioxidant activity when treated for 7 days (Figure 1).

Estimation of Thiobarbituric acid Reactive Substances (TBARS) in plasma

Oxidative stress represented by the concentration of TBARS was found to be less in group-3 treated with 100 mg/kg than animals treated with 400 mg/kg and 800 mg/kg of AEOS. When treatment was continued for 3 and 7 days with 100 mg/kg, a significant reduction in the formation of oxidative stress markers was observed (Figure 2).

Bone marrow micronucleus assay

In dose-dependent study, the number of MNPCE and MNNCE were reduced in groups treated with 400 mg/kg and 800 mg/kg of AEOS. The group treated with 100 mg/kg of AEOS exhibited a fair reduction in MNPCE and MNNCE formed compared with the F treated group (P<0.05). In the time dependent studies, the proportion of MNPCE and MNNCE formed declined as the duration of treatment with AEOS (100 mg/kg) progressed (Table 1).

DISCUSSION

Xenobiotics or chemicals can induce changes in the biological system as they would be recognized by the body as foreign substance. Damage induced by chemicals can be manifested at the cellular or molecular level resulting in organ toxicity or DNA damage. Generally, the physiological system possesses several repair mechanisms to safeguard cell, cell organelles and genetic material from damage. Recurrent exposure for prolonged periods might shift this balance resulting in evident DNA damage.

Exposure to environmental hazards like fluoride can take place mainly through drinking water⁶. Fluoride is a biologically active element which has a tendency to cumulate in the body as only 50% of it undergoes renal clearance¹⁶. F is known to inhibit various enzymes and this might result in its interference in DNA repair mechanism which utilizes several enzymes. F is regarded as a true mutagen as it is capable of forming covalent bonds with DNA inducing significant chromosomal damage⁴. The damage caused by F could be due to its ability to induce oxidative stress. Generation of reactive oxygen species due to oxidative stress occurs when molecular oxygen undergoes reduction and actively accepts single electrons converting into superoxide radicals. The generated reactive oxygen species readily attack the methylene groups of polyunsaturated fatty acids facilitating lipid peroxidation 17 . The end product of lipid peroxidation which is TBARS evidently rises due to oxidative stress. Insult caused by reactive oxygen species is not restricted to the cell membrane alone, but can readily extend to proteins and nucleic acids. Therefore DNA damage is evident due to oxidative stress.

DNA damage can be easily detected by cytogenetic analysis to detect the presence of micronuclei. An important biomarker of DNA damage is detection of MN in bone marrow and peripheral blood. The bone marrow micronucleus assay developed by Schmid relies on the fact that chromosomal fragments or entire chromosomes might fail to incorporate into the nucleus after cell division. Due to this, small chromatin containing element might appear in the cell around the nucleus which are detected as micronuclei. The erythropoeitic lineage comprises various cells during their developmental cascade of which polychromatic erythrocytes and normochromatic erythrocytes are the most prominent. Further nuclear damage can be assessed by counting the number of MN. If the proportion of MN is more than 6%, it implies that the agent is mutagenic¹⁸. Our observations reveal that genomic integrity was disturbed by the administration of sodium fluoride.

Evaluation of the total anti-oxidant capacity of AEOS revealed a significant decline in the FRAP values in F challenged group compared with control. 100 mg/kg of AEOS as a single schedule and for a period of 7 days considerably increased FRAP values. Studies have shown a reduction in the formation of an important marker of oxidative stress which is TBARS following treatment with AEOS in a dose of 100 mg/kg for 7 days. AEOS in lower doses as a single schedule and in the same dose for longer duration was capable of restoring the integrity of DNA by reducing the formation of MN compared to the groups treated with 400 and 800 mg/kg.

Ocimum sanctum is a spiritually revered and popular medicinal plant commonly called as the sacred basil. It contains volatile oils, triterpenes, flavonoids, tannins, alkaloids, phenylpropane glycosides. Analysis of the active constituents reveals the occurrence of polyphenolic compounds. Polyphenolic compounds possess one or more phenolic moieties which can readily interact with free radicals leading to their depletion. TBARS, an oxidative stress marker of lipid peroxidation is known to interact with DNA causing strand breaks leading to genomic instability. The competency of Ocimum sanctum as an anti-oxidant is undoubted based on assessments made from the FRAP and lipid peroxidation studies. Several studies have reported the protective effect of ursolic acid on irradiation induced damage of hematopoietic cells and keratinocytes¹⁹. It has also been documented that ursolic acid has potential antioxidant property. The anti-mutagenic and anti-oxidant profile of O. sanctum extract can be attributed to the presence of triterpenoids like ursolic acid and rosmarinic acid. Reports suggest that flavonoids present in Ocimum sanctum can protect against injury caused by radiation and noise induced oxidative stress. The presence of flavonoids like orientin and vicenin might also contribute to the beneficial effects of O. sanctum against F induced genotoxicity²⁰. Ocimum sanctum contains polyphenols which could exert its antioxidant action by preventing formation of oxidative stress markers or facilitating the detoxification of F or complexing with iron and copper which are minerals functioning as catalyst in free reactions²¹. Appraisal radical generation of lipid peroxidation, bone marrow and peripheral blood micronuclei studies clearly show that AEOS is more effective at a dose of 100 mg/kg in the dose dependent schedule and at the same dose when treated for 7 days. It was difficult to discern the reason for the slight decrease in protection with increase in the dose of the extract. However, we suggest that this might occur due to a complex interaction of several constituents present in O. sanctum such as alkaloids, as alkaloids are reported to possess mutagenic properties²²

In conclusion it can be stated that *O. sanctum* possesses antigenotoxic effect by circumventing F induced oxidative stress and DNA damage.

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