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Research Article

Protection of Radiation-induced DNA Damage in Albino Rats by *Oroxylum indicum* (L.) Vent.

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ABSTRACT

Development of radioprotective agents has been a subject of intense research to ameliorate the aftermath of exposure to radiation exposure. The aim of the study was to determine the antioxidant and radioprotective potentials of the fruit extract of *Oroxylum indicum* (L.) Vent. Free radical scavenging activity of *O. indicum* fruit extracts was determined through DPPH and superoxide assays. Ascorbic acid, total flavonoid and total phenolic contents were determined spectrophotometrically. In vitro experiments on protection of DNA from radiation-induced (5 and 10 Gy) damages were done using pBR322 plasmid DNA. Albino rats injected intra-peritoneal with 1-2 g/kg of 60% ethanol extract and exposed to 1, 3 and 5 Gy were screened for radio- protective effects using comet assay. 60% methanol extract exhibited highest antioxidant potentials followed by 60% ethanol extract. Treatment with 60% ethanol extract significantly ($P < 0.05$) reduced radiation-induced DNA damage in pBR322. Significant reduction of comet tail DNA and length ($P < 0.05$) in rat bone marrow cells was also observed. The results demonstrate that *O. indicum* fruit has tremendous antioxidant potentials and can protect DNA from radiation-induced damages in both in vitro and in vivo models without recognizable toxic effects.

Keywords: Antioxidant, Comet assay, *Oroxylum indicum,* pBR322, Radioprotection

INTRODUCTION

Free radicals are chemical entities characterized by high reactivity and known to be less stable than non-radicals due to the presence of one or more unpaired electrons.[1] Free radicals are involved in several normal biological processes *in vivo.* Superoxide radicals and other active oxygen species are products of the action of oxidases such as xanthine oxidase, NADPH oxidase, etc., and are generated by a variety of cells to perform useful functions in the body.[2] Normally different forms of free radical are generated at minimal level in cells to help in the harmonization of various physiological functions and are quenched by an integrated antioxidant system in the body. However, destructive effects leading to various disorders occur due to excess production of these free radicals.[3] Reactive oxygen species which are toxic and/or mutagenic can also be produced when visible light excites cellular photosensitizers apart from that done by ionizing radiations.[4] Exposure to ionizing radiations such as x-rays and γ-rays, cosmic rays, ultra sound, natural background radiations knowingly or inadvertently can generate free radicals and lead to associated adverse effects. Harmful intermediates called reactive oxygen species (ROS) are produced in cellular respiration which on accumulation in the body can lead to cumulative damage of biomolecules such as proteins, lipids and DNA, resulting in oxidative stress. Although almost all organisms possess antioxidant defense and repair systems to protect them from oxidative damage, in some cases these systems are insufficient to entirely prevent such damage.[5] In the recent past, there has been growing interest in exploiting the biological activities of different medicinal herbs, owing to their natural origin, cost effectiveness and lesser side effects.[6] Many phytotherapies have been used traditionally to combat diseases for thousands of years.[7] Plants are known to possess various chemopreventive agents like phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites which are rich in antioxidant activity.[8,9,10]. A number of chemopreventive phytochemicals have been identified in plants[11] and these

Fig. 1 Oroxylum indicum (inset: seeds)

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Table 1: Free radical scavenging capacities of fruit extract of *Oroxylum indicum* in different solvent systems

Free radical scavenging capacity (IC_{50})*	Samples		
	Aqueous (Mean ± SE)	60% ethanol (Mean ± SE)	60% methanol (Mean ± SE)
DPPH scavenging assay (µg/mL)	333.49 ± 0.33	178.69 ± 0.43	133.74 ± 0.49
Superoxide scavenging assay (µg/mL)	103.72 ± 0.44	91.07 ± 0.66	70.25 ± 0.76

Values of IC_{50} are mean ± SE (n = 3). Values are statistically significant at P≤0.05. ANOVA does not apply between rows.

Table 2: Antioxidant components in fruit extract of *Oroxylum indicum* in different solvent systems

Antioxidant components*	Samples		
	Aqueous (Mean ± SE)	60% ethanol (Mean ± SE)	60% methanol (Mean ± SE)
Total phenolics (mg GAE/100 g)	783.84 ± 0.62	1735.36 ± 0.21	2339.89 ± 0.08
Total flavonoids (mg QE/100 g)	655.09 ± 0.12	2677.58 ± 0.75	2873.19 ± 0.83
Ascorbic acid (mg/100 g)	585.43 ± 0.66	537.21 ± 0.16	486.77 ± 0.34

**Values are mean ± SE (n = 3). Values are statistically significant at P≤0.05. ANOVA does not apply between rows*

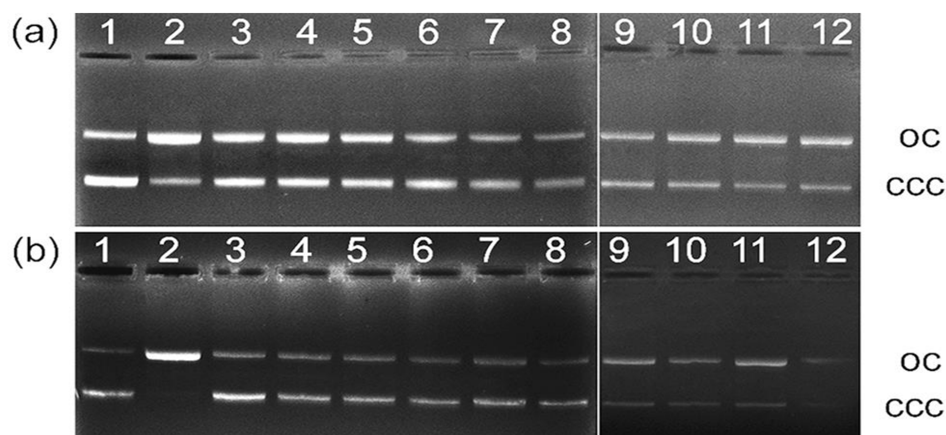

Fig. 2 Agarose gel electrophoresis pattern of pBR322 DNA exposed to different doses of γ-radiation in the absence and presence of fruit extract (60% ethanol) of Oroxylum indicum (a) Lane 2 to 12 were DNA exposed to 5 Gy, Lane1: control, Lane 2: DNA exposed to 5 Gy, Lane 3: DNA with 20 µg/mL, Lane 4: DNA with 40 µg/mL extract, Lane 5: DNA with 80 µg/mL extract, Lane 6: DNA with 120 µg/mL extract, Lane 7: DNA with 160 µg/mL extract, Lane 8: DNA with 200 µg/mL extract, Lane 9: DNA with 300 µg/mL extract, Lane 10: DNA with 400 µg/mL extract, Lane 11: DNA with 500 µg/mL extract, Lane 12: DNA with 600 µg/mL extract and (b) Lane 2 to 12 were DNA exposed to 10 Gy, Lane1: control, Lane 2: DNA exposed to 5 Gy, Lane 3: DNA with 20 µg/mL, Lane 4: DNA with 40 µg/mL extract, Lane 5: DNA with 80 µg/mL extract, Lane 6: DNA with 120 µg/mL extract, Lane 7: DNA with 160 µg/mL extract, Lane 8: DNA with 200 µg/mL extract, Lane 9: DNA with 300 µg/mL extract, Lane 10: DNA with 400 µg/mL extract, Lane 11: DNA with 500 µg/mL extract, Lane 12: DNA with 600 µg/mL extract. Abbreviations: 'oc' denotes open circular or linearised form of DNA and 'ccc' denotes covalently closed circular form or supercoiled form of DNA

phytochemicals react with pro-oxidants and prevent damages to DNA thereby preventing the onset of various diseases.

Oroxylum indicum (L.) Vent. (English: Broken bone plant, Tree of Damocles, Midnight horror; Manipuri: Shamba) belonging to the family Bigoniaceae is a deciduous, small to medium-sized tree with light grayish-brown, soft and spongy bark. The tree is often grown as an ornamental plant for its strange appearance. The flowers are nocturnal, white, hermaphroditic, bell-shaped and bat-pollinated. The long, podded fruits hang down from the bare branches looking like dangling sickles or swords in the night (**Fig. 1**). The seeds are round with papery wings (**Fig. 1**). Leaves are 2-4 inches long, broad, leaflets are 5 inches long and 3-4 inches broad having sharp edges. The stalk of the flower is 1 feet long. The flowers are purple in colour. Fruits are 1-3 foot long, 2-4 inches broad. Seeds are flat, 3 inches in length and 2 inches in width. The flowers are borne in the rainy season and the fruits appear in December to March.[12]

O. indicum is distributed in the Eastern and the Western Ghats, and North-East India up to an altitude of 1200 m and found mainly in ravine and moist places in the forests.[13] It is also sparsely distributed in Manipur (23°50'-25°42' N; 92°58' E to 94°45' E) which is within the Indo-Burmese mega-biodiversity hotspot.[14] Its existence in natural population is highly threatened and has been

Table 3: Protection of pBR322 DNA from radiation-induced damages

Sample	oc form (%)		Protection (%)	
	5 Gy	0 Gy	5 Gy	10 Gy
DNA	85.56	98.62	-	-
DNA + 20 µg/mL extract	34.62	39.54	65.38	60.46
DNA + 40 µg/mL extract	30.45	38.63	69.55	61.37
DNA + 80 µg/mL extract	28.89	36.37	71.11	63.63
DNA + 120 µg/mL extract	26.68	32.17	73.32	67.83
DNA + 160 µg/mL extract	23.76	31.12	76.24	68.88
DNA + 200 µg/mL extract	20.28	28.16	79.72	71.84
DNA + 300 µg/mL extract	47.41	57.9	52.09	42.63
DNA + 400 µg/mL extract	56.24	60.78	43.76	39.22
DNA + 500 µg/mL extract	64.84	64.05	35.16	35.95
DNA + 600 µg/mL extract	66.55	67.94	33.45	32.06

categorized as vulnerable medicinal plant.[12,13] The plant contains flavonoids like chrysin, oroxylin and baicalein as bioactive principles.[15] It is widely used by the Indians for the treatment of various ailments. In India, roots are used in Ayurvedic preparation called *Dasamoola* used as an astringent, anti-inflammatory, anti-helminthic, anti-bronchitic, anti-leucodermatic, anti-rheumatic, anti-anorexic and for treatment of leprosy and tuberculosis.[16] Leaves are emollient, that contain anthraquinone and aloe-emodin. The fruits are acrid, sweet, stomachic and are used in treating diseases of heart and throat, piles, bronchitis, leucoderma and helminthosis. They are used as expectorant and for improving the appetite.[17] The extracts of the bark exhibit anti-microbial, analgesic, anti-tussive and anti-inflammatory properties.[18] In Manipur, the fruits are consumed as non-conventional food and also used as anti-asthmatic.

In Ayurveda, the traditional Indian system of medicine, several plants have been used to treat ailments and, therefore, it is logical to expect that such plants may also render some protection against radiation damage. Plant extracts eliciting radioprotective efficacy contain a plethora of compounds including antioxidants, immunostimulants, cell proliferation stimulators, anti-inflammatory and anti-microbial agents, some of which may act in isolation as well as in combination with other constituents from the same plant. They may also augment the efficacy of compounds present in other plant species, to provide protection against radiation-induced damage. In traditional Ayurveda, Chinese, Japanese, Korean, Siddha, European, Tibetan and Unani systems of medicine, it is a common practice to use a multi-plant formulation for treating diseases. Synergistic effects may be present, and some of the toxic effects generated by active constituents of one plant may be countered by other constituents present.

Most of the studies using natural plant products have focused on evaluation of radioprotective efficacy of whole extracts or polyherbal formulations, and in some cases fractionated extracts and isolated constituents. Plants with radioprotective properties have been shown almost invariably to possess antioxidant biomolecules. The development of radioprotective agents in the form of pure compounds or as combination of antioxidant rich phyto formulations prepared in the line of traditional medicine systems has been subject of intense research with a view to ameliorate or abstain the aftermath of exposure to a radiation environment . However, no ideal, safe synthetic radioprotectors are available till date except amifostine.[19] Hence, the search for alternative sources, including plants, has been ongoing for several decades. The fact remains that till date there is no single radioprotective agent available which meets all the pre-requisites of an ideal radioprotector, i.e., produces no cumulative or irreversible toxicity, offers effective long-term protection, possesses a shelf-life of 2–5 years, and can be easily administered.[20,21] In view of this, the search for newer, less toxic and more effective radioprotector drugs continues. Evidently, the use of plants and their bioactive constituents with antioxidant activity is highly relevant in mitigation of radiation-induced oxidative damage.

The alkaline single gel electrophoresis is a rapid and sensitive procedure for quantifying DNA damage in individual cells induced by a variety of chemicals and ionizing radiation.[22,23] The assay is based on the embedding of cells in agarose, then analysis in alkaline buffer and finally subjection to an electric current. The resulting images which appear like comets are measured to determine the extent of DNA damage. In the present study, an attempt has been made to evaluate the antioxidant capacities of various extracts of *O. indicum* extracted in different solvent systems and to establish its radioprotective properties *in vitro* in pBR322 DNA and *in vivo* using alkaline single cell gel electrophoresis (comet assay).

MATERIALS AND METHODS

Reagents and chemicals: Chemicals and reagents, viz., ascorbic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), glutathione, hypoxanthine, xanthine oxidase, nitroblue tetrazolium (NBT), Folin-Ciocalteu reagent, gallic acid, ethidium bromide, agarose and quercetin were purchased from Sigma-Aldrich, St. Louis, USA, Dulbecco's Phosphate Buffered Saline (without Ca and Mg), DMSO (Dimethyl sulfoxide), TritonX-100, HBSS (Hank's Balance Salt Solution) and bovine calf serum was purchased from HiMedia (Mumbai, India). Ethidium bromide and agarose was purchased from Merck

Table 4: Acute toxicity of *Oroxylum indicum* fruit extract (60% ethanol) based on mortality of *Rattus norvegicus albus* at 72 h

Extract dose (g/ kg)	Log_{10} dose	72- hours mortality		30 days mortality	
		Death (%)	Probit value	Death (%)	Probit value
2.5	0.40	0	3.04	0	3.04
3.0	0.48	0	3.04	0	3.04
3.5	0.54	20	4.16	30	4.48
4.0	0.60	50	5.00	60	5.25
4.5	0.65	70	5.52	90	6.28
5.0	0.70	100	6.96	100	6.96

[a] *Corrected formula for 0% mortality: 100 x (0.25/n); for 100% mortality: 100 x [(n – 0.25)/ n], n = 5. LD_{50} (72 h): 4.02 g/ kg, log_{10} LD_{50} (72 h): 0.60 and LD_{10} (72 h): 2.47 g/ kg. $LD_{50/30}$: 3.83 g/ kg, log_{10} $LD_{50/30}$: 0.58 and $LD_{10/30}$: 2.33 g/ kg.*

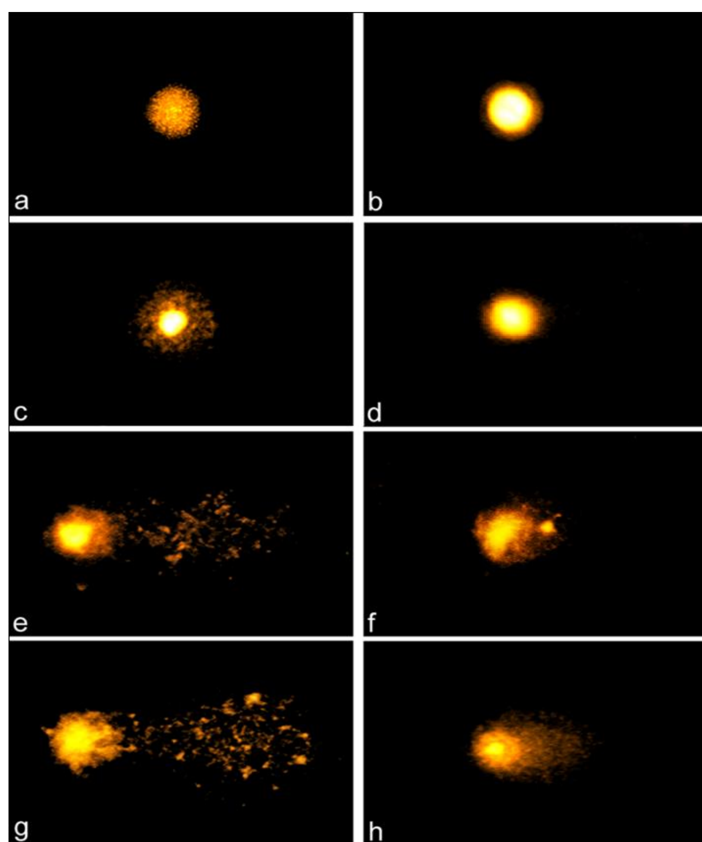

Fig. 3: Images of comets (bone marrow cells of rat) stained with ethidium bromide. (a) Cell from control group, (b) 60 % ethanol group, (c) 60 % ethanol group exposed to 1 Gy (d) 1g/kg extract group exposed to 1 Gy (e) 60 % ethanol group exposed to 3 Gy (f) 2g/kg extract group exposed to 3 Gy (g) 60 % ethanol group exposed to 5 Gy (h) 2g/kg extract group exposed to 5 Gy

(Darmstadt, Germany). Plasmid pBR322 DNA was procured from Merck Biosciences, India. Low melting Agarose was purchased from Promega, USA and all other chemicals and reagents were of analytical grade obtained from Merck, India.

Plant materials and preparation of extracts: Fresh fruits of *O. indicum* were collected from the wild natural habitat of Manipur and herbarium specimens were deposited to the Centre of Advanced study in Life Sciences, Manipur University, Imphal, India and its voucher number was assigned (Herbarium Voucher No. 003403). Fresh matured fruits with seeds were used in the experiments. Moisture content was determined by drying the fruits in an oven at 55 °C for 24 hours. Freshly collected fruits of *O. indicum* were washed thoroughly in tap water and excised with a sharp scalpel. One gram dry weight equivalent of fresh fruit including seeds relative to moisture content (88.39 ± 0.23%) were ground using mortar and pestle, and the paste was treated with distilled water, 60% ethanol or 60% methanol and homogenized separately in each solvent to get a final concentration of 20 mg/mL. The homogenate was collected and centrifuged at 3000g for 10 min to get a clear supernatant. Finally, the clear supernatant was decanted and filtered through Whatman No. 1 filter paper

and stored at 4 °C for various analyses.

Antioxidant screening by DPPH assay: Antioxidant activity of the different extracts was studied by DPPH assay. Solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was used as a reagent for the spectrophotometric assay.[24,25] The reaction mixture in triplicates consisted of 100 µM DPPH with different concentrations of the fruit extract. Comparative DPPH decoloration was assessed from a solution of DPPH supplemented with different concentrations of aqueous, 60% ethanol and 60% methanol extracts of *O. indicum* fruit. The same DPPH concentration in methanol without sample was used as a control. Absorbance was read against a blank at 515 nm after incubation of the reaction mixtures for 30 min in dark at 25 °C. Ascorbic acid was used as positive control. Percentage decoloration of DPPH was determined by comparison with methanol treated control as: DPPH Inhibition (%) = ($A_{control}$ - A_{sample} / $A_{control}$) x 100. Where, $A_{control}$ is the absorbance of control reaction and A_{sample} is the absorbance of the reaction supplemented with fruit extract.

Superoxide scavenging assay: Superoxide radicals were generated *in vitro* by hypoxanthine/ xanthine oxidase system in this experiment. Superoxide scavenging activity of the fruit extracts was assessed through reduction of NBT by hypoxanthine/ xanthine oxidase activity as described earlier[26] with minor modifications. Different concentrations of aqueous, 60% ethanol and 60% methanol extract of the fruit were added to a reaction solution containing 500 µM EDTA (pH 7.4), 500 µM hypoxanthine in 50 mM NaOH, and 1.5 mM NBT. After a three min reaction 0.015 U/mL xanthine oxidase was added to the mixture and the final volume was adjusted to 3 mL with 50 mM phosphate buffer (pH 7.4) and incubated at 25 °C for 30 min. Absorbance was measured at 560 nm against a blank. Quercetin was used as positive control. Superoxide scavenging activity was calculated as: Inhibition (%) = ($A_{control}$ - A_{sample} / $A_{control}$) x 100. Where, $A_{control}$ is the absorbance of control reaction and A_{sample} is the absorbance of the reaction with fruit extract. The concentration of different extracts providing 50% inhibition (IC_{50}) was determined from the plot of inhibition percentage against extract concentration.

Determination of total phenolics: The total phenolic content in the extracts were determined using Folin-Ciocalteu phenol reagent based on the procedure described by Singleton and Rossi with slight modifications[27]. Equal quantities (0.4 mL) of the fruit extracts (10 x dilutions) were mixed with 0.3 mL of Folin-Ciocalteu reagent (10x dilution). After 3 min, 0.25 mL sodium carbonate (7.5 % w/v) was added and total volume was adjusted to 4 mL with distilled water. The solution was mixed and allowed to stand in dark at 25 ºC for 30 min. Absorbance was measured at 765 nm. The amount of total phenolics was calculated from the standard curve equation and expressed as gallic acid equivalent (GAE) in mg/100 g. The concentration range of gallic acid used for standard curve was 2-10 µg/mL and the equation of standard gallic acid curve was $y = 0.0858x + 0.0043$ ($R^2 = 0.998$)

Total flavonoid analysis: The total flavonoid content of the fruit extracts was determined by colorimetric method [28]. In brief, 0.5 mL of sample solution was mixed with 2mL of distilled water and subsequently with 0.15 mL of 5% $NaNO_2$ solution. After 6 min of incubation, 0.15 mL of 10% $AlCl_3$ solution was added and then allowed to stand for 6 min, followed by adding 2mL of 4% NaOH solution to the mixture. Immediately after water was added to the sample to bring the final volume to 5 mL, the mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance of the samples was determined at a wavelength of 510 nm. The total flavonoid content was finally expressed in mg/100g quercetin equivalents (QE) by using the expression: $y = 0.0098x - 0.008$ ($R^2 = 0.9984$)

Ascorbic acid content: Ascorbic acid content was estimated according to the method of Klein and Perry[29] with minor modifications. Fruit extract in different solvents containing 100 mg were treated with 2 mL of 1% metaphosphoric acid and filtered. The filtrates were mixed with 9 mL of 2, 6-dichloroindophenol (15 mg/mL), and the absorbance was measured at 515 nm. Content of ascorbic acid was calculated on the basis of the calibration curve and was expressed in mg/100 g. Concentration range of ascorbic acid used for construction of calibration curve was 20-200 µg/mL and equation of the calibration curve was $y = - 0.0006x + 0.2631$ ($R^2 = 0.88$)

Evaluation of radiation-induced DNA damage and protection: DNA from pBR322 plasmid was used to assess protection of DNA damage induced by exposure to gamma radiation using a GC-4000A ^{60}Co-Gamma Chamber (Bhabha Atomic Research Center, Mumbai) as described earlier.[30] DNA (300 ng) was exposed to 5 and 10 Gy doses of γ-radiation at a dose rate of 0.062 Gy/sec in presence or absence of 60% ethanol extract of *O. indicum* fruit in different concentrations (20-600 µg/ mL). DNA sample without the extract which was not irradiated was taken as control. After irradiation, DNA was loaded on 1% agarose gel and electrophoresed in Tris acetic acid EDTA buffer (TAE, pH 8) under constant voltage.[31] The DNA bands in the gel was stained with ethidium bromide and documented using a Vilber Lourmat gel documentation unit. DNA bands were analysed by PerkinElmer-GeneTool software, version-3.07. The experiments were conducted in three replicates. Data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's test. Values are presented as means ± standard error (SE). P values ≤ 0.05 were considered significant.

Single-cell gel electrophoresis analysis (comet assay): Animal preparation: Albino rat (*Rattus norvegicus*, 2n = 42) was obtained from the Regional Institute of Medical Sciences, Imphal, India. Experiments were carried out as described earlier[32] with the approval of the Institutional Animal Ethics Committee (No. MU/8-199/06/UGC dated 03-09-2008) and maintained as per ethics guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), a statutory body under the Prevention of Cruelty to Animals Act, 1960. 2-3 month-old male rats were housed in sanitized rice-husk bedded polypropylene metabolic cages and maintained at 25 ± 2 ºC in the animal house under 12 hours light and 12

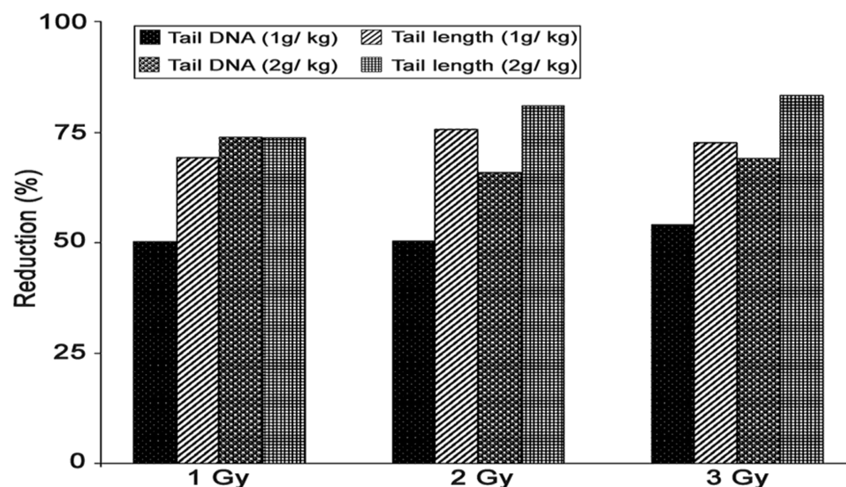

Fig. 4: Percentage of radiation-induced DNA damage reduction by ethanol extract of Oroxylum indicum. The parameters shown are tail DNA and tail length reduction values as a result of the treatment with 1g/kg and 2g/kg body weight of the extract

hours dark cycle. They were fed with standard feed and water was provided *ad libitum*. The crude extract (60% ethanol) was centrifuged and filtered through 0.22 µ filter.

Acute toxicity testing procedure: extract of *O. indicum* (60% ethanol) was injected intra-peritoneally to rats weighing 100-125 g. One control and another four groups with 2.5, 3, 3.5, 4 and 4.5 g/kg body weight doses of the extract were maintained. The animals were kept under constant observation for 30 days. Mortality was recorded every 24 hrs, if any. LD_{50} was calculated based on probit analysis[33] and maximum tolerated dose (MTD) was determined from the observations.

Dose administration of crude extract and radiation exposure: The extract of *O. indicum* at a dose of 1g/kg and 2g/kg body weight were injected intra-peritoneally to experimental animals before 24 hrs of radiation exposure. The rats were divided into 16 different groups of 3 animals each. Four control groups with extract (1g/kg and 2g/kg) and without extract and another 4 group of animals were exposed to 1 Gy, 3 Gy and 5 Gy doses of γ-radiation at a dose rate of 0.058 Gy/s using a GC-4000A ^{60}Cobalt Gamma Irradiator (Bhabha Atomic Research Center, Mumbai) in the Radiation Centre of Manipur University.

Cell sample collection: Animals were euthanized by cervical dislocation at 72 hours post-irradiation. Bone marrow cells were collected by perfusing the femur with one mL of cold mincing HBSS solution (with 20 mM EDTA, 10 % DMSO) into a microcentrifuge tube and used immediately for comet analysis.

Comet analysis: Experiment was performed using the alkaline comet assay based on the protocol by Singh *et al.*[22] Conventional microscopic glass slides were dipped into melted regular agarose (2% in distilled water) and allowed to oven dry at 60°C overnight so as to make a tight coating. 100 µl of bone marrow cell suspension was mixed with 100 µl of 1% low-melting agarose at 37 °C and spread on the precoated slide. After covering with a glass coverslip the slides were allowed to set by keeping in the fridge for 5 min. The coverslips were removed and the slides were then placed in cold (4 °C) lysis buffer overnight (2.5M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, pH 10, 10% DMSO and 1% Triton X-100 freshly added before use). After lysis the slides were placed in fresh, chilled alkaline solution (300 mM NaOH and 1 mM Na_2EDTA in distilled water, pH 13.5), and left for 30 min to allow the DNA to unwind completely. Electrophoresis was conducted for 30 min at 25 V and 300 mA. Slides were then transferred to neutralization buffer (0.4 M Tris in distilled water, pH 7.5) for 15 min in order to remove alkali and detergents. After drying for 10 min the slides were stained with 50 µl of 20µg/ml ethidium bromide and covered with coverslips for immediate analysis. Analysis was done using a Leica DM 2500 fluorescence microscope fitted with COHU High performance CCD Camera and using the Komet 5.5 (Kinetic Imaging Ltd, Liverpool) image-analysis system. Observation was made at 40X magnification. Two slides were used per radiation dose for 3 replicates, and a total of 100 cells were scored for each dose (50 cells from each slide). Tail DNA and tail length was used as criteria for determining DNA damage to cells. The results are expressed as mean ± SE of the comet scores. Statistical significance was evaluated with SPSS 16.0 for Windows using ANOVA followed by Fisher's PLSD test. Reduction of radiation-induced DNA damage expressed as percentage reduction in tail DNA and tail length by treatment with *O. indicum* extract was calculated according to Manoharan and Banerjee[34] and Waters *et al.*[35] using the formula: Reduction (%) = {(mean score in A – mean score in B) / (mean score in A – mean score in C) X 100. where A is the group of cells exposed to γ-radiation; B is the group of cells treated with ethanolic extract of *O. indicum* and exposed to γ-radiation and C is the negative control without any radiation exposure.

RESULTS

DPPH free radical scavenging capacity:The fruit extracts of *O. indicum* in different solvent systems showed variable

Table 5: Detection of DNA damage using comet assay in bone marrow of rat exposed to γ-radiation and its protection by treatment with ethanolic extract of *Oroxylum indicum*

Treatments	Comet Score (Mean ± SE)	
	Tail DNA	Tail length
Control	$8.83 \pm 0.73^{a,d}$	1.39 ± 0.12^{a}
60% Ethanol	$8.97 \pm 0.69^{a,d}$	1.41 ± 0.09^{a}
1g/kg Extract	$9.04 \pm 0.50^{a,d}$	1.17 ± 0.06^{a}
2g/kg Extract	$9.02 \pm 0.54^{a,d}$	1.35 ± 0.14^{a}
1 Gy	$16.12 \pm 0.82^{b,h}$	6.91 ± 0.19^{b}
60% Ethanol + 1Gy	16.36 ± 0.95^{b}	7.73 ± 0.46^{b}
1g/kg Extract + 1Gy	$12.65 \pm 0.72^{c,d}$	$3.36 \pm 0.18^{c,e,h}$
2g/kg Extract + 1Gy	$10.75 \pm 0.63^{a,c,d}$	$3.07 \pm 0.17^{c,e}$
3Gy	20.93 ± 0.71^{e}	11.37 ± 0.38^{d}
60% Ethanol + 3Gy	21.44 ± 0.77^{e}	11.80 ± 0.43^{d}
1g/kg Extract + 3Gy	$15.16 \pm 0.60^{b,d,h}$	$3.94 \pm 0.29^{c,e,h}$
2g/kg Extract + 3Gy	$13.24 \pm 0.81^{c,d}$	$3.39 \pm 0.16^{c,e,h}$
5Gy	29.14 ± 0.90^{f}	16.69 ± 0.90^{f}
60% Ethanol + 5Gy	30.13 ± 0.88^{f}	16.99 ± 0.84^{f}
1g/kg Extract + 5Gy	18.67 ± 0.69^{g}	5.68 ± 0.45^{g}
2g/kg Extract + 5Gy	$15.53 \pm 0.72^{b,h}$	$4.01 \pm 0.22^{c,e,h}$

SE: standard error; Values with the same letter (a-h) do not differ significantly (P > 0.05). One way ANOVA followed by Fisher's PLSD test

degree of reactivity against DPPH free radical. When the solution of 100 µM DPPH in methanol was supplemented different concentrations of the fruit extracts (100-400 µg/mL) in different solvent systems, *viz.,* aqueous, 60% ethanol and 60% methanol, a dose dependent decoloration of DPPH solution from purple to yellow colour was observed. The same decoloration pattern of DPPH was also observed when supplemented with 2-10 µg/mL of ascorbic acid with an IC_{50} of 3.83 ± 0.05 µg/ mL. 60% methanol extract of the fruit showed 88.03% decoloration of DPPH at a concentration of 400 µg/mL, whereas aqueous and 60% ethanol extracts showed 63.98% and 84.84% DPPH decoloration at the same concentration. The dose dependent increase in DPPH decoloration was observed to be highest in reaction mixtures supplemented with 60% methanol extract of *O. indicum* fruit by showing the lowest IC_{50} value indicating strong DPPH scavenging capacity (Table 1).

Superoxide scavenging capacity: Fruit extracts of *O. indicum* in different solvent systems, *viz.,* aqueous, 60% ethanol and 60% methanol showed comparatively good response towards scavenging of superoxide radicals with 88.76%, 83.91% and 90.08% respectively at a concentration of 240 µg/mL. IC_{50} of quercetin used as positive control in the experiment was 7.40 ± 0.59. Superoxide scavenging capacity of 60% methanol extract was highest as compared to the extract in other solvents (Table 1). A dose dependent pattern in the increase in superoxide scavenging capacity was observed with the increase in fruit extracts in different solvents, and also with the increase in quercetin concentration in the case of positive control. Fruit extracts of *O. indicum* in different solvents (aqueous, 60% ethanol and 60% methanol) exhibited comparatively strong superoxide scavenging potentials.

Total phenolic, total flavonoid and ascorbic acid content: Total phenolic content in gallic acid equivalent calculated from the calibration curve of gallic acid was highest in 60% methanol extract. The fruit extract in 60% ethanol was found to have an intermediate phenolic content as compared to 60% methanol and aqueous extracts. The aqueous extract of *O. indicum* fruit showed the least total phenolic content. The results indicate that contents of total phenolics depend on the type and polarity of solvent used in the extraction. Total flavonoid content calculated using the standard curve equation of quercetin was highest in 60% methanol extract. Total flavonoid content of the aqueous extract of *O. indicum* fruit was comparatively lower as compared to that of 60% ethanol and 60% methanol extracts. Ascorbic acid content was highest in aqueous extract of *O. indicum.* The results indicate differential association of total phenolics, flavonoids and ascorbic acid in different solvent systems (Table 2).

Radiation-induced DNA Damage Protection: When pBR322 DNA was exposed to 5 Gy dose of γ-radiation, the covalently closed circular (ccc) form of DNA depleted and when the dose was increased to 10 Gy, the ccc-form almost disappeared and formation of open circular (oc) form of the plasmid DNA increased (Fig. 2). Treatment of plasmid pBR322 DNA with different concentrations (20-200 µg/ mL) of 60% ethanol extract of *O. indicum* fruit before 1 hour of radiation exposure gives a dose dependent protection by checking the depletion of ccc-form of DNA. The extract at a concentration of 20 µg was able to provide 65.38% and 60.46% protection of the DNA from 5 Gy and 10 Gy radiation exposures respectively. Treatment of DNA with 200 µg/ mL of the extract were able to provide 79.72% and 71.84% protection from radiation-induced damages when exposed to 5 Gy and 10 Gy respectively as presented in Table 3. However, decline in the percentage protection of DNA damage was observed when the dose of fruit extract was increased from a concentration of 300 µg/mL.

In vivo toxicity: Mortality records and values of LD_{50} of *O. indicum* extract at 72 h and 30 days are shown in Table 4. The maximum tolerated dose (MTD) of 60% ethanol

extract of *O. indicum* was 2.25 g/kg body weight when injected intra-peritoneal.

Comet assay: No significant changes were observed in bone marrow cells of unirradiated animals (control 60% ethanol treated and extract treated) as determined by scoring comet tail DNA and comet length. In rats exposed to 1, 3 and 5 Gy, no significant differences were observed between the 60 % ethanol treated and non-treated groups. However, significant reduction ($P< 0.05$) of DNA damage caused by γ-radiation was observed in rats treated with *O. indicum* extract before the radiation exposure (Fig. 3). Significant decrease in tail DNA and tail length which was observed in extract treated groups at different radiation doses are presented in Table 5. The percentage reductions in radiation-induced DNA damage revealed that tail DNA was reduced by 50.20-54.15 % and 65.75-73.90% when treated with 1g/kg and 2g/kg body weight of the rat respectively (Fig. 4). Tail length was also reduced by 69.14-75.69% and 73.30-83.31% by the same treatment as mentioned above.

DISCUSSION

In traditional medicine systems, crude extracts of various plant parts are used to control a number of disorders and ailments. Various extraction procedures involve dry sample extractions in a variety of solvents, but in the present study, dry weight equivalent relative to moisture content was taken into account, as drying methods lead to loss in antioxidative quality of the sample.[36] In conformity with the Indian System of Traditional Medicine and also keeping a view towards preventing unwanted loss of heat-labile antioxidant components, crude extract of fresh fruits were used in the experiments. DPPH assay for evaluation of free radical scavenging activity of extracts from plants, food materials and single compounds is based on the capacity of samples in bleaching the purple- coloured methanol solution of DPPH to the corresponding yellow-coloured hydrazine either by H- atom or electron donation.[37,38] Increase in the percentage of decoloration of the reaction solution when substituted with crude extracts of *O. indicum* fruit suggests that they possess antioxidant molecules in the form of polyphenols, flavonoids or pigments and hence, have the capacity to scavenge DPPH free radical.[8,9,10] Superoxide radicals are generally produced within the body by the intracellular organelles of the neutrophils as a part of defense mechanism against infections. Excess generation of superoxide may be triggered by enzymes such as NAD(P)H oxidase and xanthine oxidoreductase leading to oxidative stress.[2,3] Hypoxanthine/xanthine oxidase system employed to generate superoxide free radicals have been reported to be capable of promoting tumourogenesis and inflammation.[26] The decrease in absorbance at 560 nm with the plant extracts and reference compound quercetin indicates their ability to quench superoxide radicals in the reaction mixture suggesting positive antioxidant potentials. Exposure of plasmid pBR322 DNA to doses of 5 and 10 Gy γ-radiation-induced DNA strand breaks and resulted in the depletion of covalently closed circular (ccc) form or supercoiled form. The ccc-form of DNA gets converted to open circular (oc) form or linearised form of DNA as a result of exposure to ionizing γ-radiation.[39] 60% ethanol extract of *O. indicum* fruit reduced the conversion of ccc-form to the oc-form of plasmid DNA. The ability to retain the ccc-form of DNA demonstrates that 60% ethanol extract of *O. indicum* was able to protect the DNA *in vitro* from radiation-induced strand breaks. In comet assay, the ethanolic extract of *O.indicum* showed efficacy in preventing DNA damage in irradiated rats. It reveals that pre-treatment of experimental animals with 1g/kg and 2g/kg extract of the fruit reduced DNA damage which can be observed in terms of reduction of tail DNA and tail length of comets. The results obtained may be attributable to the antioxidant fractions or otherwise bioactive molecules present in the crude extract by countering numerous radiation-induced oxidative damages which otherwise would lead to mutagenesis or clastogenecity.[40] The antioxidant properties of *O. indicum* have been reported to activate DNA repair pathways and act directly on the mutagenic process by increasing the fidelity in DNA replication or by stimulating DNA damage error-free repair.[41,42] Our results clearly justify that fruit extract of *O. indicum* can protect DNA from undergoing strand breakage due to γ-radiation exposure.

CONCLUSION

The results indicate that *O. indicum* fruit has tremendous antioxidant potentials and radioprotective properties and can be incorporated in the dietary regime as a potential source of antioxidants and radioprotective agents. Further, conservational strategies need to be taken up through *in situ* and *ex situ* procedures to make this vulnerable resource available for future generations.

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