before sequencing, identical results were obtained when it was sequenced directly. This method has great potential to give conclusive results for the diverse samples being tested in forensic laboratories.

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ACKNOWLEDGEMENTS. We thank Dr Padma Shastry for a critical reading of the manuscript and Prof. S. K. Brahmachari, Director, CBT, for permission to use the sequencing facility.

Received 11 January 2000; revised accepted 25 February 2000

Fusarium – A new threat to fish population in reservoirs of Kumaun, India

Deepa Bisht*, G. S. Bisht,† and R. D. Khulbe**

Botany Department, Kumaun University, Nainital 263 002, India
*Present address: GBPIHED, Kosi-Katarmal, Almora 263 643, India
**Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora 263 601, India

***Fusarium moniliforme* and *F. udum* (Moniliales: Deuteromycetes) were found to be natural pathogens of freshwater fish in reservoirs, causing mycosis and high mortality in *Barbus rana*, *Channa punctatus*, *Labeo rohita*, *Mastaceamblus armatus*, *Mystus tengra*, *Puntius sophore* and *Wallago attu*. Both the species produced clinical symptoms similar to natural infection in *C. punctatus* and *P. sophore* and caused 40–80% mortality under artificial inoculation. Though 9 other species of extra aquatic fungi belonging to 8 genera of hyphomycetes were also associated with the diseased fish in the reservoir, they were unable to infect the test fish. *Fusarium* species parasitize fish more commonly during summer through rainy season. A temperature above 25°C, coupled with relatively low pH (7.1–7.7) and DO (8.3–9.5 mg l^{-1}) encouraged association and infection of these fungi, whereas low temperature during winter (< 20°C) adversely affected their colonization on the fish. Notably mycosis due to watermolds is prevalent during winter–spring, while extra-aquatic fungi dominate during summer through the rainy season, thus posing a continual threat to fish in the reservoirs. This necessitates an integrated approach to combat mycosis in reservoirs. Besides, prophylatic measures to protect fingerlings before being introduced into the reservoirs, intensive research on biological control of fish diseases caused by *Fusarium* and other fungal species is warranted, not only to increase production in reservoirs but also to conserve several rare fish species.**

RESERVOIR fishery is an important component of inland fisheries in south and south-east Asia. Indian reservoirs exceeding 3 million ha in area are recognized as an invaluable resource for inland fish production[1]. Owing to the existence of both lentic and lotic components, reservoirs not only harbour a wide diversity of fish but also diverse micro-organisms, including fungi pathogenic to fish[2]. Watermolds are a constant and ubiquitous component of aquatic environments and a continuous source of challenge to fish[3,5]. However, geofungi, the extra-aquatic fungi, constitute an integral component of mycoflora in the freshwater ecosystem.

A high prevalence of mycosis in fish species, characterized by descaling, dermal necroses and haemorrhage, which resulted in mass mortality was observed during 1991 in Nanaksagar, a huge man-made reservoir and a well-recognized fish production centre of the Govt. of UP in India. The grievous situation has been implicated due to the infection of watermolds; several species of *Achlya*, *Aphanomyces* and *Saprolegnia* were detected from the symptomatic fish during 1991–1994 and their pathogenicity has been demonstrated[6–8]. Nevertheless, in spite of rigorous isolation, associated pathogens could not be detected from a large number of fish exhibiting mycotic lesions, particularly during the warm period. Since the studies were concentrated on watermolds pathogenic to fish, specific baiting techniques were employed for the isolation of associated fungi and involvement of other fungi was overlooked. We presumed that extra-aquatic fungi (hyphomycetes), which are known to cause diseases in marine as well as freshwater fish in different parts of the world[9–14], are also involved. Consequently, the involvement of these fungi in fish mycosis was investigated during 1994–1996 at Nanaksagar.

A large number of individuals of major contributing fish species was randomly inspected at the fish catching site at Nanaksagar (29°55′N and 79°40′E; 200 m amsl), an artificial reservoir spread over 4662 ha in Kumaun division, India, which harbours a wide variety of fish fauna[15]. The reservoir was built in 1962 primarily to facilitate irrigation in the nearly 40,000 ha downstream areas, however, it has also made a substantial contribution to the state economy through fish production. Im-

†For correspondence.

portant physico-chemical characteristics were studied by sampling of surface water each month for two successive years, following the standard methods of the American Public Health Association[16].

Symptomatic fry and adult fish of various species were collected in sterile polythene bags, placed in ice in aseptic glass pots and immediately brought to the laboratory. Isolation of associated fungal species was made within 24 h. Portions of infected tissues were washed individually with sterile tap water to remove superficially adhering fungal propagules, and treated with 0.1% potassium tellurite solution to avoid bacterial contamination. The isolation of fungal pathogen(s) was achieved by plating small pieces of invaded host tissue on the surface of C'zapeks agar plates, supplemented with streptopenicillin 0.1 mg l^{-1}. The plates then were incubated at 25 ± 1°C and the outgrowing hyphae were cut and transferred to fresh plates, until a pure culture was obtained. Fungal species thus isolated were identified following Ellis[17] and Booth[18].

Channa punctatus and *Puntius sophore* were used to test the pathogenicity of the isolated fungal species, because of small size and long survival under laboratory conditions. The surface of several individuals of *C. punctatus* and *P. sophore* was sterilized with malachite green (0.05%) to inactivate fungal pathogen(s) including watermolds which might be present. They were washed thoroughly with sterilized tap water to remove the fungicide and placed in a sterilized water-filled aquarium with aerators for continuous supply of oxygen. After 15 days of acclimatization, 5 healthy individuals were artificially injured and transferred to separate smaller aquaria. Each aquarium was filled with sterilized lake water and supplemented with an equal amount of mycelial/conidial suspension (inoculum), prepared using 4 mm agar plugs incubated in PDA broth at 28 ± 1°C for 120 h. The concentration of the inoculum of test isolates was maintained in the range of 4×10^4 to 5×10^4 CFU l^{-1}. Five replicates were prepared for each species. A continuous supply of oxygen was provided through an aerator and readymade mineral food was supplied. An aquarium without inoculum served as the control. The fish were inspected regularly after inoculation for 25 days.

Table 1. Physico-chemical features of surface water of Nanaksagar

Characteristics	Nanaksagar		Other Indian reservoirs[1]
	Range	Mean ± SE	
Temperature (°C)	15–32	23.91 ± 0.94	12.0–31.0
pH	7.1–8.9	8.16 ± 0.11	6.5–9.2
DO (mg/l)	8.3–17.5	13.05 ± 0.58	3.0–12.0
CO_2 (mg/l)	8.8–33.3	18.42 ± 1.62	
BOD (mg/l)	22.6–170.0	75.96 ± 5.67	
COD (mg/l)	125.4–750.0	380.48 ± 35.49	
Conductivity (uscm^{-1})	150.0–450.0	245.00 ± 13.83	76.0–474
Chloride (mg/l)	7.0–10.0	8.45 ± 0.16	
Phosphate (mg/l)	0.07–0.29	0.21 ± 0.01	Trace–0.36
Nitrate (mg/l)	0.16–0.89	0.46 ± 0.041	Trace–0.93

Table 2. Fungal species isolated from diseased fish Nanaksagar reservoir

Fungal species	Fish species*						
	A	B	C	D	E	F	G
Alternaria alternata	+	+	–	+	–	+	+
Aspergillus niger	+	+	–	–	–	+	+
Cladosporium cladosporioides	–	+	+	–	+	+	+
Curvularia lunata	+	–	–	+	–	–	+
Drecshlera australiensis	–	+	–	+	–	+	–
Fusarium moniliforme	–	+	+	+	+	+	+
F. oxysporum	–	+	–	–	+	–	–
F. udum	+	–	+	+	+	–	+
Penicillium chrysogenum	–	+	+	–	–	+	–
P. expansum	+	–	+	–	+	+	–
Trichoderma harzianum	+	–	+	–	–	+	+

*A, *Barbus rana*; B, *Channa punctatus*; C, *Labeo rohita*; D, *Mastaceamblus armatus*; E, *Mystus tengra*; F, *Puntius sophore*; G, *Wallago attu*.

The reservoir water is slightly alkaline, with pH varying between 7.1 and 8.9. The important characteristics of the Nanaksagar reservoir water (Table 1) are comparable with several other Indian reservoirs[1]. The physico-chemical variables reveal monthly/seasonal fluctuations. Water temperature (around 10 am) ranged between 15 and 32°C, with a minimum during winter; it begins to rise during spring and attains a maximum at the summer–monsoon interphase. Fluctuation in concentration of free CO_2, nitrate, BOD, COD, and conductivity was closely associated with temperature, while a decline in pH, concentration of dissolved oxygen and phosphate was evident with increasing temperature.

Eleven species of geofungi, belonging to 8 genera of dematiaceous hyphomycetes were isolated from different individuals of seven fish species (Table 2). These species were more commonly associated during the warm period. *F. moniliforme* Sheldon and *F. udum* Butler (Figures 1 and 2) were more consistantly isolated. *F. moniliforme* was the most common, detected from a large number of individuals from *C. puctatus*, *Labeo rohita*, *Mastaceamblus armatus*, *Mystus tengra*, *P. sophore* and *Wallago attu*; *F. udum* was detected from *Barbus rana*, *L. rohita*, *M. armatus*, *M. tengra* and *W. attu*. Among other prominent species, *Alternaria alternata* (Fr.) Keissler and *Cladosporium cladosporioides* (Fresen.) de Vries were associated with individuals of five fish species. *F. oxysporum* Schl. ex Fries was associated with *C. punctatus* and *M. tengra*. Although association of these fungi with fish is being reported for the first time from the central Himalayan waterbodies, it was not unusual, and several hyphomycetes have previously been demonstrated as fish pathogen[9,14]. Of the fungi isolated in the present study, species of *Aspergillus*, *Cladosporium*, and *Curvularia* have been isolated from fish and prawn showing mycotic lesions[10,11]. Several other members of this group, including *Exophiala psychrophila*[12], and *Ochroconis humicola*[13,14] have been described as fish pathogens. The results of our study, therefore, provide further evidence that extra-aquatic fungi (hyphomycetes) also possess great potential to parasitize fish.

Species of pathogens and hosts demonstrated differential pathogenicity and immunity, respectively. *F. moniliforme* and *F. udum* appeared virulent, while scaly fish, namely *B. rana*, *C. punctatus*, *L. rohita*, *M. tengra* and *P. sophore* more vulnerable to fungal attack than the non-scaly fish. This is because, when scales get lost naturally or mechanically, the resulting wounds on the body surface remain partly covered by loose skin and the scales lying nearby. As a result, fungal propagules adhering to the wounds are not easily washed away by the water current and get sufficient time to infect the fish. In non-scaly fish, the wounds are continuously washed by the water current. Consequently the conidia fail to adhere to the wounds for a long time to establish infection. Besides, differences in secretion of mucus, which provides a protective cover against invasion by

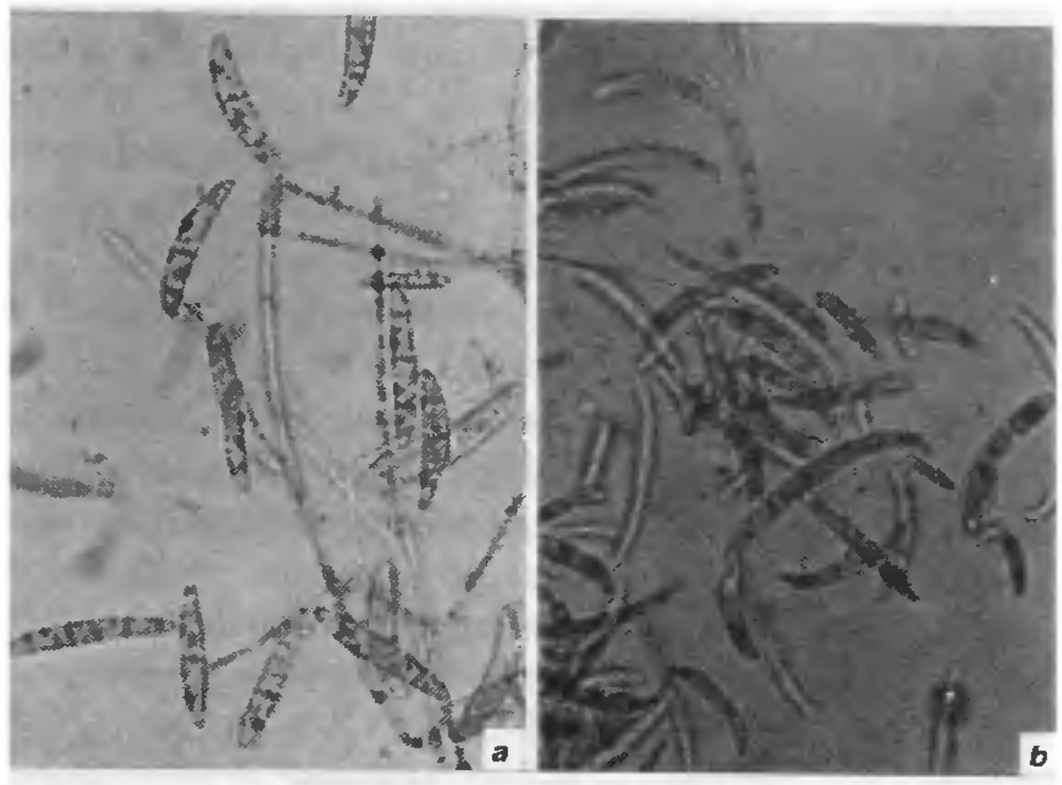

Figure 1. Mycelium, conidiophore and conidia; ***a***, *Fusarium moniliforme* and ***b***, *F. udum*.

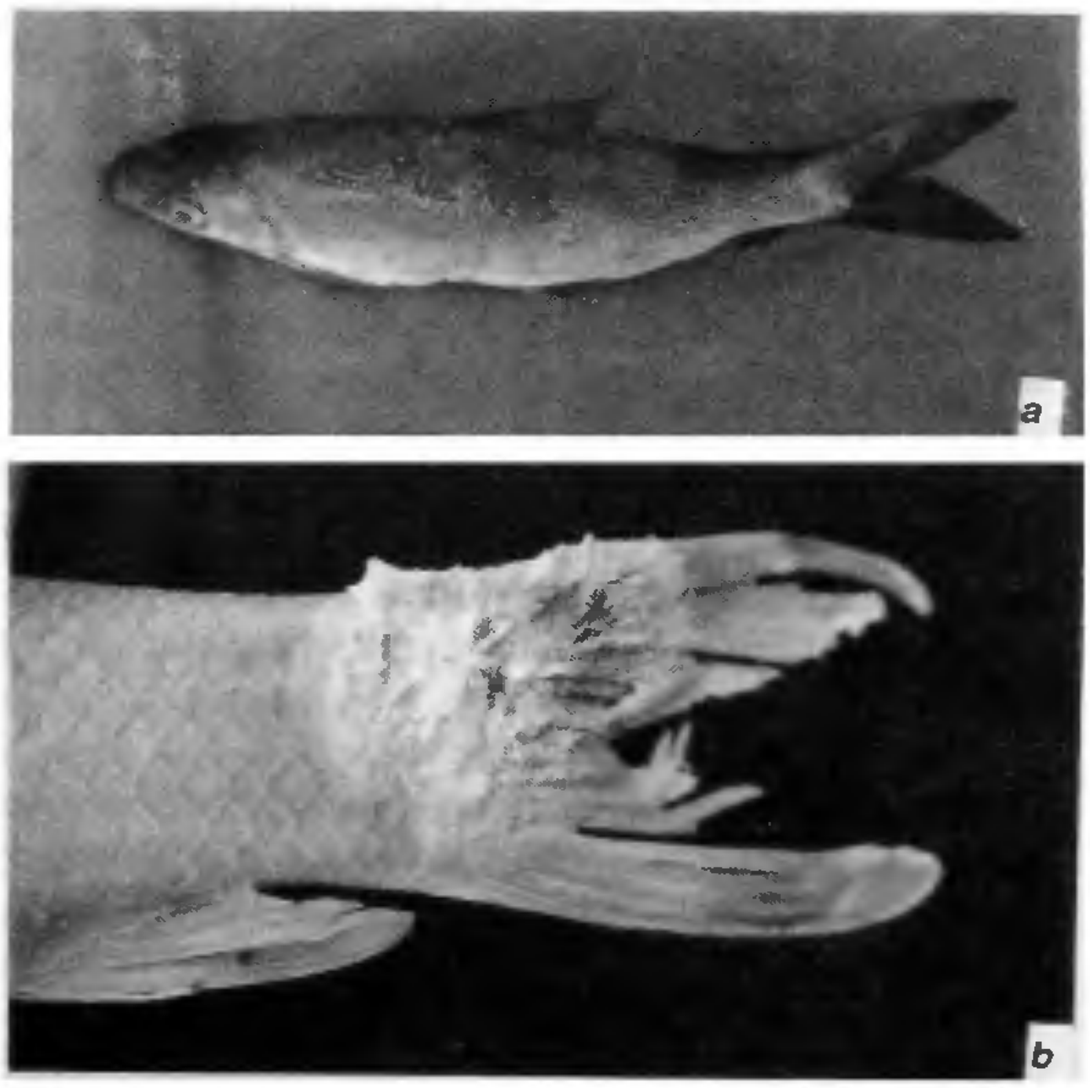

Figure 2. Mycotic symptoms on (***a***) *Barbus rana*, (***b***) *Labeo rohita* and (***c***) *Mastacembelus armatus*.

pathogens, seem to be responsible for differential colonization of species and subsequent development of disease[19,20].

Symptoms produced by these fungi broadly resembled those caused by watermolds and could not be distinguished until mycelial colonization was visible. The primary lesions were characterized by depigmentation of body colour in small patches. As the disease progressed, the lesions grew in size and affected the underlying muscle layer, producing dermal necroses. Descaling, external haemorrhage, eroded tails and dorsal fins were characteristic features of severe infection, which led to sluggishness, surface swimming and ultimately to death. Mycosis spread to the fish population irrespective of age, length and weight. However, injured individuals were more vulnerable to fungal infection.

Though 11 species were detected from the diseased fish, the pathogenicity test clearly showed that only *F. moniliforme* and *F. udum* had the ability to infect fish, causing mortality in 60 and 80% individuals of *C. punctatus* and 40 and 60% in *P. sophore*, respectively (Table 3). Other fungi did not cause gross infection on the test fish. *Fusarium* species were reisolated from the diseased fish previously inoculated. Mortality of fish inoculated with *F. moniliforme* and *F. udum* and lack of lesions in other fungal species and the control (without inoculum) confirmed that *Fusarium* spp. were the primary cause of mycosis. Systemic infection on the test fish, and association of these fungi with a large number of individuals of 6 fish species in the reservoir provided additional evidence that these fungi possess a great potential to parasitize fish. This might be attributed to the production of fusaric acid by these species[21,22]. The pathogenic behaviour of *Fusarium* spp. to fish is striking, because these are ubiquitous plant pathogens present in almost all agriculture soil and diseases caused by these fungi account for enormous losses of major crops throughout the world. Fungal species which failed to establish infection on the test fish under artificial inoculation might be secondary colonizers of tissues, weakened by several abiotic and biotic stresses. These may, thus, be regarded as weak pathogens, which may parasitize fish under stress and congenial conditions for fungal growth.

Table 3. Pathogenicity of *Fusarium* spp. on *C. punctatus* and *P. sophore*

Species	*Channa punctatus*		*Puntius sophore*	
	Fish used	Mortality (%)	Fish used	Mortality (%)
Fusarium moniliforme	5	60	5	40
Fusarium udum	5	80	5	60
Control (without inoculum)	5	Nil	5	Nil

Diseases result from the interaction of a pathogen with its host, but the intensity and extent of interaction is markedly affected by the environmental factors. Unlike infection of watermolds, the common fish pathogens, which were prevalent during spring in the reservoir[7,8], the extra-aquatic fungi more commonly parasitized fish during the warm period (late spring–summer) when infection due to watermolds decreased. The maximum number of species was associated during May–June.

Association of extra-aquatic fungi, and the subsequent infection of fish in the reservoir were governed by fluctuations in water characteristics. Water temperature between 24 and 32°C coupled with relatively low pH (7.1–7.5) and dissolved oxygen (8.3–9.5 mg l^{-1}), and high concentration of free CO_2 (19.5–33.0 mg l^{-1}) were conducive for growth, sporulation and their subsequent infectivity. A maximum association of these fungi with mycotic fish in May–June coincided with water temperature 28–32°C, pH 7.1–7.5 and DO 8.3–9.5 mg l^{-1}. Low temperature during December–January (< 20°C) suppressed the growth, sporulation and pathogenic association of these fungi. This is because, considerable decrease in pH and DO and increase in CO_2 and nitrate levels of water during high-temperature months not only encouraged growth and sporulation in geofungi[23] but also caused stress to fish and predisposed them for infection[24,25]. Moreover, such conditions are not conducive for watermolds[7,8], thus minimizing the competition between these two fungal groups. Conversely, growth and sporulation of these fungi are suppressed by low water temperature, which favours development of watermolds. Besides low temperature, least association of extra-aquatic fungi during winter may be attributed to competition between these two groups. Moreover, low temperature may also adversely affect enzyme secretion by these fungi[26], thereby influencing their colonization on fish tissues.

To conclude, *F. moniliforme* and *F. udum* possess a great potential to parasitize a wide range of fish species. Once established in the reservoir, *Fusarium* species can survive under unfavourable conditions in the form of mycelial fragments, conidia and dormant propagules like chlamydospores, which persist for many years[27]. These fungi are thus virtually impossible to eliminate from the reservoir, where application of fungicides is not feasible. Further, it is of concern that two distinct ecological groups of fungi, viz. aquatic phycomycetes and the extra-aquatic fungi involved in the etiology of mycosis in subtropical reservoir, and their pathogenic activity peaked at different seasons. Thus, fish suffer from fungal infection throughout the year.

In a preliminary study, *Gliocladium roseum* and *Trichoderma harizianum* have shown antagonistic property against *F. moniliforme* and *F. udum*. Species of *Trichoderma* and *Gliocladium* have been used with suc-

cess against diseases of a wide variety of economically important crops including Fusarial rots and wilts[28]. Thus, they have the potential to suppress the fish pathogenic *Fusaria*. Intensive research is needed to exploit their full potential against *Fusarium* spp. pathogenic to fish. Moreover, studies on the integrated use of several micro-organisms will be needed to control the diseases caused by different fungi.

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ACKNOWLEDGEMENTS. We thank ICAR, New Delhi, for financial assistance and Prof. S. P. Singh, Head, Botany Department for providing laboratory facilities.

Received 15 February 2000; revised accepted 22 March 2000

Radioprotective effect of *Phyllanthus niruri* on mouse chromosomes

P. Uma Devi*, Ravindra Kamath and B. S. S. Rao

Department of Radiobiology, Kasturba Medical College, Manipal 576 119, India

Micronuclei (MN) induction in the mouse bone marrow polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) was studied 24 h after whole body exposure to 4 Gy of gamma radiation with or without a single intraperitoneal injection of 25 mg/kg to 150 mg/kg of 50% methanolic extract of *Phyllanthus niruri* (whole aerial part). The extract significantly reduced the radiation-induced MN induction in both PCE and NCE and increased the ratio of PCE to NCE. The effect increased linearly with extract dose from 25 to 125 mg/kg.

PHYLLANTHUS niruri (syn. *P. fraternus* Webster, f. Euphorbiaceae) is a winter weed, growing in the tropical parts of India, and the whole plant, fresh leaves and roots are used as medicine[1]. It is found to be effective in treating infective hepatitis, and is used as an adjuvant with other herbal medicines. Sane *et al.*[2] reported that *P. amarus* (syn. *P. niruri*[3]) protected rats against carbon tetrachloride induced liver toxicity. Some drugs used in liver treatment like Liv. 52 and Thiola (2-mercaptopropionylglycine, MPG) have shown good radioprotective effect against radiation-induced chromosome damage in mouse[4–6]. But there is no study on the radioprotective effect of *Phyllanthus*. Therefore, an experiment was conducted to see if the plant possesses any protective effect against radiation damage *in vivo*.

Fresh *Phyllanthus niruri* plants were collected from the fields near Mangalore, during the months of February to April and were identified. Complete aerial parts were removed, washed, shade dried and powdered. Extract (PNE) was prepared by refluxing with methanol 50% at 60°C and concentrated under vacuum. Extract is not soluble in water. A fine suspension was prepared using 0.5% carboxy methyl cellulose (CMC) in phosphate-buffered saline, freshly before use.

Six- to eight-week-old Swiss albino mice weighing 25 ± 3 g were selected from an inbred colony maintained in our laboratory, under controlled conditions of temperature (23 ± 2°C) and humidity (50 ± 5%). The animals were administered intraperitoneally (i.p.) with 0.2 ml of 0.5% CMC or PNE, at 25 to 150 mg/kg body weight one hour before whole body irradiation with 4 Gy of gamma radiation. Animals were restrained in well-ventilated polypropylene boxes and whole body exposed to cobalt-60 gamma rays from a Gammatron

*For correspondence. (e-mail: info@mahe.ernet.in)