Podophyllum hexandrum ameliorates endosulfan-induced genotoxicity and mutagenicity in freshwater cyprinid fish crucian carp

Sabzar Ahmad Dar¹, Abdul Rehman Yousuf², Masood-ul-Hassan Balkhi³, Bashir Ahmad Ganai⁴, Mudasir Tantry⁵ and Farooz Ahmad Bhat⁶

¹Linmonology and Fisheries Laboratory, Centre of Research for Development (CORD), University of Kashmir, Srinagar, Jammu & Kashmir, India; ²Division of Fisheries, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K), Jammu & Kashmir, India; ³Phytochemistry Laboratories, Center of Research for Development (CORD), University of Kashmir, Srinagar, Jammu & Kashmir, India

ABSTRACT

Context: Medicinal plants continue to act as a repository for novel drug leads with novel mechanisms of action. Podophyllum hexandrum Royale (Berberidaceae) treats diverse conditions in folk medicine. Objective: The antimutagenic potential of P. hexandrum was evaluated against endosulfan-induced clastogenicity in a piscine model by cytogenetic endpoints. Materials and methods: Podophyllum hexandrum rhizomes were subjected to successive solvent extraction. Fish were exposed to hexane, chloroform, ethyl acetate, methanol and aqueous extracts (15 mg/L each) of plant and endosulfan (0.05 mg/L) alone followed by their combination for antimutagenicity estimates. Chromosomal aberrations (CA) were made from kidney cells and micronuclei (MN) slides from peripheral blood erythrocytes at 48, 72 and 96 h. Antioxidant activity was analyzed by the DPPH assay. Phytochemical analyses were carried out using chromatographic and spectroscopic techniques. Results: Endosulfan induced significant (p < 0.05) MN, authenticated by scanning electron microscopy, and CA in a time-dependent manner. However, methanol and ethyl acetate extracts revealed ameliorating effects. The column eluted methanolic fraction-2 (ME-F2) showed highest reduction profile of 83 and 84% in CA and MN, followed in its extent (73 and 72%) by ethyl acetate fraction-4 (EE-F4). ME-F2 and EE-F4 showed three and six major peaks when analyzed by GC-MS. To explore possible mechanism of action, ME-F2 showed potent antioxidant potential and strong correlation (R² = .900) with antimutagenic activity, whereas EE-F4 seemed to act through a different mechanism. Discussion and conclusion: This study confirms the antimutagenic potential of the subject plant with the identification of some novel compounds, justifying their use in folk medicine, and their corresponding benefit to mankind.

Introduction

The medicinal use of Podophyllum hexandrum Royale syn. P. emodi Wall (Berberidaceae), a high altitude perennial herb native to the alpine and subalpine areas of Himalayas, dates back to ancient times. The plant has been described as ‘Aindri’ – a divine drug in the traditional Indian System of Medicine – the Ayurveda used for the treatment of several ailments including taenia capitis, moncytoid leukaemia, genitai warts, constipation, cold, biliary fever, septic wounds, inflammation, burning sensation, mental disorder, Hodgkin’s and non-Hodgkin’s lymphoma (Singh & Shah 1994). Podophyllotoxin is the most abundant cyclolignan isolated from podophyllin, a resin produced by species of the genera Podophyllum, has cathartic, antirheumatic, antiviral, pesticidal and antimitotic activities (Xu et al. 2011; He et al. 2013).

Several xenobiotics generally defined as environmental mutagens are of great health concern to the modern man as they induce mutational events, thus posing significant toxicological risks to a myriad of genetic processes (Anand et al. 2008). Therefore, the discovery and exploration of compounds possessing antimutagenic and anticarcinogenic properties are gaining credence. Nowadays, the significance of novel bioactive phyto-compounds in counteracting the promutagenic and carcinogenic effects are gaining credence. Antimutagenic activity consists of the suppression of clastogenic processes and can occur by different mechanisms either inside or outside the cell. A variety of genetic tests such as Ames, micronucleus, and chromosomal aberration, have been used to evaluate qualitatively and quantitatively the clastogenic activity of mutagenic compounds (Farah et al. 2006; Dar et al. 2016). Furthermore, for testing chemicals of human health concern, vertebrate assays have advantage as they are metabolically and physiologically more closely related to the human reactions. Fish act as sentinel organism for indicating the potential for exposure of human population to genotoxic chemicals and subsequently can be used to screen natural products to evaluate their pharmacological activities (Dar et al. 2014a, 2015). Fish are frequently used as bioindicators since they are sensitive to changes in their}

CONTACT Sabzar Ahmad Dar sabzar.cord@gmail.com Limnology and Fisheries Laboratory, Centre of Research for Development (CORD), University of Kashmir, Srinagar 190006, Jammu & Kashmir, India

Supplemental data for this article can be accessed here.

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environment and play significant roles in assessing potential risks associated with contamination. Some characteristics of *Carassius carassius* L. (Cyprinidae) such as its wide distribution and availability throughout the year, cost-effectiveness, easy handling and acclimatizing in the laboratory make it an excellent ecotoxicological model. In piscine model, antimutagenic studies are still in infancy and few reports are available. One such report concerns the antimutagenic and anticarcinogenic activity of chlorophyllin towards aflatoxin in rainbow trout (Guha & Khuda-Bukhsh 2002). The ameliorating effect of vitamin C, β-carotene and azadirachtin (principle compound of neem) against genotoxicity of ethyl methanesulfonate and cadmium chloride has been demonstrated in a fish, *Oreochromis mossambicus* Peters (Cichlidae) (Ferguson 1994). Recently, the antimutagenic effect of neem leaves extract in freshwater fish, *Channa punctatus* Bloch (Channidae) has been evaluated by cyto genetic tests (Farah et al. 2006).

The mutagenic and carcinogenic action of various genotoxic substances, like endosulfan, also involves the generation of DNA reactive free radicals, which overcharges the endogenous antioxidant defense systems, characterizing oxidative stress. Thus, in general, some antioxidant agents are capable of retaining the mutagenesis and carcinogenesis (Dar et al. 2014b). Various methods are used to determine the antioxidant activity; one of the most widely used is the scavenging activity of the stable free radical 2,2-di-phenyl-1-picryl-hidrazila (DPPH), since it is a rapid, reliable and cost effective test (Huang et al. 2005).

A large group of mutagens comprises of pesticides and constitute a major risk that give rise to concerns at local, regional, national and global scales (Dar et al. 2015). One such compound is endosulfan, a persistent organic pollutant, commercially comprising of two isomers (α- and β-endosulfan) at a ratio of 70:30, belong to the group of chlorinated cyclodienes. Endosulfan is widely used in agriculture around the world to control insect pests and noted for its strong genotoxic effects in various organisms. In our previous studies, we have demonstrated the genotoxicity, clastogenicity and oxidative stress of endosulfan in freshwater fish *C. carassius* (Dar et al. 2014a, 2015). Although there are some preliminary reports regarding the antimitotic activity (He et al. 2013), there is scanty data regarding the antimutagenic potential of the plant. Therefore, the present study will evaluate the antimutagenic potential of *P. hexandrum*, using piscine model, along with its mechanism of action and identification of bioactive compound(s) and their corresponding benefit to humans.

### Materials and methods

#### Experimental fish and chemicals

Healthy fish specimen of *C. carassius*, having chromosome number 100 (2n), were procured in the month of January, 2013 with the help of a local fisherman from the Dal Lake (34°07′N 74°52′E) Srinagar, India. These specimens were identified by Prof. A. R. Yousuf and were transported live in plastic jars to the limnology and fisheries laboratory, University of Kashmir, where they were subjected to a prophylactic treatment by bathing in a 0.05% aqueous solution of potassium permanganate for 2 min to avoid dermal infection. Their average length and wet weight (± SD) were recorded as 12.5 ± 1.6 cm and 33 ± 5 g, respectively. The fish stock was then acclimatized for at least 3 weeks to 1:1 diurnal photo-period in artificially aerated 60 L glass aquaria (10 fish in each) with aged dechlorinated tap water (pH 7.6–8.4), and fed *ad libitum* daily with commercially available fish food (Feed Royal®, Maa Agro Foods, Andhra Pradesh, India). Every effort as suggested by Bennett and Dooley (1982) was taken to maintain optimal conditions during acclimatization: no fish died during this period. The acclimatized fish were used for the experiments, conducted in accordance with the principles of the Institutional Ethical Committee (IEC) for the protection of research animals at the University of Kashmir. Endosulfan, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and cyclophosphamide were purchased from the Sigma Aldrich, Bengaluru, India. All other chemicals and organic solvents used in the present study were of analytical grade.

#### Plant material and extraction

The fresh rhizomes of *P. hexandrum* were collected from the shady and hilly slopes of Daraw, Gurai (34°38′N 74°50′E), Jammu and Kashmir, India in the month of July, 2012. The plant material was authenticated by curator of the Centre of Biodiversity & Taxonomy (CBT), University of Kashmir, India and a voucher specimen (KASH-1752) has been deposited. The rhizomes of *P. hexandrum* were shade dried for 15 days. After being macerated to fine powder, 1 kg rhizome powder was extracted successively with hexane, chloroform, ethyl acetate (EtOAc) and methanol for 16 h using Soxhlet apparatus (Dar et al. 2013). The extracts were filtered through a Buchner funnel using Whatman no. 1 filter paper and were concentrated to dryness under vacuum using Heidolph rotary evaporator, yielding 3.4, 71.73, 16.53 and 97.77 g of hexane, chloroform, EtOAc and methanol extracts, respectively. However, 500 g of the residue left after methanol extract was soaked overnight in 500 mL of distilled water at room temperature with constant stirring. Next morning the extract was filtered over muslin cloth and the filtrate was centrifuged at 5000 rpm for 10 min at room temperature. The supernatant was further lyophilized in lyophilizer (Mac-Flow, India) for complete dryness to obtain powder (21.33 g). All the extracts were stored at 4°C in air tight glass bottles before use.

#### Fractionation of active extracts

The active EtOAc and methanol extract of *P. hexandrum* were fractionated using silica gel 60G (0.063–0.200 mm) column chromatography, as per standard procedures (Dar et al. 2012a, b). Solvents were distilled prior to use. In case of ethyl acetate extract (15 g), column was successively eluted with hexane (2000 mL), hexane:EtOAc [19:1 (1600 mL), 4:1 (1500 mL), 7:3 (1000 mL), 3:2 (1000 mL), 1:1 (1100 mL) and 3:7 (1300 mL)] mixtures, EtOAc (3000 mL), EtOAc:methanol [19:1 (1400 mL), 17:3 (2000 mL) and 7:3 (1100 mL)] mixtures and methanol (3000 mL). Forty fractions of 500 mL each were collected and combined on the basis of their thin-layer chromatography (TLC) profiles to afford five main fractions. Fractions 1–9, 10–17, 18–24, 25–32 and 33–40 were referred to as EE-F1, F2, F3, F4 and F5, respectively. Similarly, the methanol extract (15 g) loaded column was eluted with the solvent systems of gradually increasing polarity using hexane, chloroform, EtOAc and methanol. The following ratios of solvent combinations were sequentially used in the elution process; pure hexane (2500 mL); hexane:chloroform 95:5, 80:20, 60:40, 40:60 and 20:80 (total solvent 4500 mL); chloroform:EtOAc 95:5, 80:20, 60:40, 40:60 and 20:80 (5500 mL); EtOAc:methanol 95:5, 80:20, 60:40, 40:60, 20:80 and 0:100 (7000 mL). Thirty-nine fractions of 500 mL each were collected and combined on the basis of their TLC profile into five major fractions: fractions 1–7, 8–15, 16–23, 24–30, and 31–39 were referred to as ME-F1, F2, F3, F4 and F5, respectively.
**In vivo exposure experiment**

The method and procedure recommended by OECD (1997) was followed. The first experiments were semi-static assays consisting of 13 treatments each with 3 replicates, containing 60 L dechlorinated and well-aerated tap water with 10 fish specimens in each aquarium (n = 390). Fish were exposed through aqueous medium to each of single sublethal concentration of endosulfan (0.05 mg/L), hexane, chloroform, ethyl acetate, methanol and aqueous extract (15 mg/L each) of *P. hexandrum*, followed by their combination for 96 h. These concentrations were selected on the basis of LC_{50} (0.07 mg/L) value of endosulfan (Dar et al. 2014a) in *C. carassius*, and optimally high concentrations of *P. hexandrum* extracts were used to ascertain if they had any genotoxic effect. Since endosulfan was an emulsifiable concentrate, it was directly added to the semi-static system, whereas plant extracts were dissolved in 0.5% methanol before adding to the system. The specimens maintained in dechlorinated tap water and those exposed to 0.5% methanol were considered as the negative and solvent control. The specimen maintained in the sublethal concentration of endosulfan served as positive control. The samples were collected at the time intervals of 48, 72 and 96 h and on each sampling interval, 10 fish specimen were sacrificed; 5 fish were processed for the chromosomal aberration (CA) test (0.05% colchicine treatment was given prior to 3 h of autopsy) and the micronucleus assay was carried out from the blood erythrocytes of the rest 5 fish as per standard protocols. In the second set of experiments (n = 90), three concentrations of the active extract(s) of plant (5, 10 and 15 mg/L) were used simultaneously with endosulfan (0.05 mg/L) in order to find out the most effective concentration. In the third and final set of experiments (n = 300), the column eluted fractions of the most effective concentration of the active extract(s) were used simultaneously with endosulfan, so that the fraction(s) with maximal activity can be identified by various chromatographic and spectroscopic techniques. Furthermore, the mean concentration of endosulfan in the water samples during the experiment was always within 5% of the intended concentration, when analyzed by dispersive liquid-liquid micro-extraction (DLLME) followed by GC-MS (Supplementary Figure 1).

**Chromosomal aberration test**

Chromosome preparations were made from the highly hemopoietic and mitotically active head kidney cells, following the standard techniques (Dar et al. 2014a). The fish of all groups (5 fish/group/exposure) were injected with 0.05% colchicine intramuscularly at 1 mL/100 g body weight 3 h prior to dissection, to arrest the metaphase stage. The head kidney was dissected out, macerated and homogenized in 2 mL of 0.56% KCl, in glass tissue homogenizer, to prepare cell suspension. The cell suspension was poured into Eppendorf tubes and incubated for 20–30 min at room temperature for hypotonic treatment. The cell suspension was fixed in chilled Carnoy’s fixative (methanol:glacial acetic acid, 3:1 v/v), mixed gently with Pasteur pipette, centrifuged at 1500 rpm for 10 min and supernatant was discarded. The pellet was resuspended in chilled Carnoy’s fixative and the above process was repeated 3–4 times until the whitish pellet was obtained. Chromosome slides were prepared by dropping one or two drops of cell suspension onto pre-cold slides in 70% alcohol. The slides were then air dried and stained with 5% Giemsa prepared in Sorensen’s buffer (pH-6.8) for 20 min. Finally, the slides were cleared in xylene and permanently mounted in DPX.

The slides having brightly stained well-spread metaphase chromosomes were independently coded and observed at 100× under oil immersion with light microscope for chromosomal aberrations. Replicate slides were selected per fish and a minimum of 25 metaphases were scored from each slide in each group including control. Since the number of fish processed per group was five on every exposure time, a total of 250 metaphasic complements were studied. The CA was recorded under two broad categories, i.e., classical aberrations and non-classical aberrations. In the classical aberrations, both chromosome and chromatid type breaks, including acentric fragments, sister chromatid union and multiple aberrations (polyploidy, aneuploidy, rings etc) were counted and non-classical aberration comprised of stickiness, pulverization and c-metaphases.

**Micronucleus test**

Slides were prepared using the standard fish micronucleated erythrocytes method (Al-Sabti & Metcalfe 1995). Blood samples were withdrawn by caudal puncture with heparinized syringes and peripheral blood smears were immediately made by applying two drops of blood on precleaned and grease-free slides. The smeared slides were left to air dry at room temperature for overnight in a dust and moisture-free environment. The next day slides were fixed by dipping in cold absolute methanol (4 °C) for 15 min and again left to air dry at room temperature for 1 h. Finally, the slides were stained in May-Grunwald stain for 10 min followed with 6% Giemsa in phosphate buffer for 30 min. The slides were then washed thoroughly in double-distilled water, dried and made permanent with DPX-mounting.

For every sampling event, 5 fish were used and replicate slides per fish were prepared. About 1000–1200 cells were examined from each slide, i.e., a minimum of 10,000 erythrocytes were scored, under oil immersion at 100× using Olympus BX 50 microscope (Tokyo, Japan), in each treatment group for the presence of MN. Coded and randomized slides were scored using blind review by a single observer to avoid any technical variation. Only the cells clearly isolated from the surrounding cells were scored.

**Scanning electron microscopy (SEM)**

The SEM was carried out by standard procedures (Dar et al. 2015). Briefly, the aforementioned micronucleated slides were reshaped, sputter-coated with a gold and platinum to a layer of 3–5 nm and were exclusively examined in the secondary electron mode, at an accelerating voltage of 10 kV, with a scanning electron microscope (JSM6510LV, JEOL, Japan). The images were recorded simultaneously with Digiscan™ hardware and processed with Digital Micrograph 3.4.4 software (Gatan, Inc., Pleasanton, CA).

**Evaluation of antioxidant activity by the DPPH method**

The antioxidant activity of the active plant fractions and the standard was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical activity by modified method (Braca et al. 2002). The diluted working solutions of the active fractions were prepared in methanol. Rutin was used as standard in 1–100 µg/mL solution. DPPH (0.002%) was prepared in methanol and 1 mL of this solution was mixed with 1 mL of sample solution and standard solution separately. These solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm using UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan). Methanol (1 mL) with DPPH solution (0.002%, 1 mL) was used as blank.
The optical density was recorded and % inhibition was calculated using the formula given below (Noipa et al. 2011):

\[
\text{% Inhibition of DPPH} = \left(\frac{Abs_{0} - Abs_{1}}{Abs_{0}}\right) \times 100
\]

Where, Abs<sub>0</sub> = absorbance of control; and Abs<sub>1</sub> = absorbance of the sample. The experiment was performed in triplicate for each concentration tested.

**GC-MS analysis**

GS-MS analysis was carried out with GCMS-QP2010 Plus, Shimadzu, Japan fitted with programmable head space auto sampler and auto injector. The capillary column used was DB-1/RTX-MS (30 m) with helium as a carrier gas, at a flow rate of 3 mL/min with 1 μL injection volume. Samples were analyzed with the column held initially at 100 °C for 2 min after injection, then increased to 170° C with 10 °C/min heating ramp without hold and increased to 215 °C with 5 °C/min heating ramp for 8 min. Then, the final temperature was increased to 240 °C with 10 °C/min heating ramp for 15 min. The injections were performed in split mode (30:1) at 250 °C. Detector and injector temperatures were 260 °C and 250 °C, respectively. Pressure was established as 76.2 kPa. Run time was 55 min. Temperature and nominal initial flow for flame ionization detector (FID) were set as 230 °C and 3.1 mL/min, correspondingly. MS parameters were as follows: scan range (m/z): 40–650 atomic mass units (AMU) under electron impact (EI) ionization (70 eV). The constituent compounds were determined by comparing their retention times of authentic samples obtained by GC and as well as the mass spectra from the Wiley and Nist database.

**Data evaluation and statistical analysis**

The reduction percentage in number of chromosome aberration and micronuclei in the treatments with the *P. hexandrum*.
Table 2. Frequency profile of CA induced alone by endosulfan and in combination with the variable concentrations of the active extracts of P. hexandrum for different time intervals to evaluate the concentration-dependent antimutagenic response in C. carassius.

| Exp. | Treatment | TMS | Csb | Ctb | Frg | Scu | Dic | Mla | Stp | Cmt | Total aberrations |
|------|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------------------|
| 48   | Cont. 1   | 105 | 1   | 1   | –   | –   | –   | –   | 1   | –   | 2.85 ± 0.212     |
|      | Cont. 2   | 102 | 1   | 1   | –   | –   | –   | –   | –   | 1   | 2.94 ± 0.162     |
|      | Endosulfan| 117 | 2   | 2   | 1   | 1   | 1   | 1   | 2   | –   | 7.69 ± 0.371Bb   |
|      | EE-T1    | 111 | 2   | 1   | 2   | 1   | 1   | 1   | –   | –   | 6.30 ± 0.30Bb    |
|      | EE-T2    | 104 | 1   | 2   | 1   | 1   | 1   | 1   | –   | –   | 5.76 ± 0.27Bb    |
|      | EE-T3    | 109 | 1   | 2   | 1   | 1   | 1   | 1   | –   | 1   | 5.50 ± 0.26Bb    |
|      | ME-T1    | 110 | –   | 1   | 2   | 1   | 1   | 1   | –   | –   | 5.55 ± 0.26Bb    |
|      | ME-T2    | 108 | 1   | 1   | 1   | –   | –   | –   | 1   | –   | 4.62 ± 0.21A2B2  |
|      | ME-T3    | 103 | 1   | 2   | 1   | –   | –   | –   | 1   | –   | 4.85 ± 0.25Bb    |
| 72   | Cont. 1   | 104 | 1   | 1   | –   | –   | –   | –   | 1   | –   | 2.88 ± 0.162     |
|      | Cont. 2   | 101 | 1   | 1   | –   | 1   | –   | 1   | 1   | –   | 2.97 ± 0.172     |
|      | Endosulfan| 110 | 2   | 2   | 1   | 1   | 1   | 1   | 2   | 1   | 9.09 ± 0.37Bb    |
|      | EE-T1    | 116 | 1   | 2   | 1   | 1   | 1   | 2   | –   | –   | 6.89 ± 0.31Bb    |
|      | EE-T2    | 111 | 1   | 1   | 1   | 1   | 1   | 1   | –   | –   | 6.30 ± 0.27Bb    |
|      | EE-T3    | 112 | 1   | 2   | 2   | 1   | –   | –   | 1   | –   | 5.98 ± 0.30Bb    |
|      | ME-T1    | 113 | 1   | 2   | 1   | 1   | 1   | 1   | –   | –   | 6.21 ± 0.27Bb    |
|      | ME-T2    | 119 | 2   | 1   | 1   | 1   | 1   | 1   | –   | –   | 5.04 ± 0.25Bb    |
|      | ME-T3    | 115 | 2   | 1   | 1   | –   | –   | 1   | 1   | –   | 5.21 ± 0.25Bb    |
| 96   | Cont. 1   | 119 | 2   | 1   | –   | –   | –   | –   | 1   | –   | 3.36 ± 0.222     |
|      | Cont. 2   | 108 | –   | –   | 2   | 1   | –   | –   | 1   | 1   | 3.70 ± 0.232     |
|      | Endosulfan| 107 | 3   | 2   | 1   | 1   | 2   | 2   | 1   | 1   | 12.14 ± 0.47Bb   |
|      | EE-T1    | 117 | 2   | 2   | 2   | 1   | 1   | 2   | –   | –   | 8.54 ± 0.38Bb    |
|      | EE-T2    | 101 | 1   | 1   | 2   | 1   | 1   | 1   | 1   | –   | 7.92 ± 0.30Bb    |
|      | EE-T3    | 116 | 1   | 2   | 2   | 1   | 1   | 1   | –   | –   | 6.89 ± 0.31Bb    |
|      | ME-T1    | 107 | 1   | 1   | 2   | 1   | 1   | 2   | –   | –   | 7.47 ± 0.32Bb    |
|      | ME-T2    | 118 | 1   | 1   | 1   | 1   | 1   | 1   | 1   | –   | 5.92 ± 0.23Bb    |
|      | ME-T3    | 113 | 1   | 1   | 1   | 1   | 1   | 1   | 1   | –   | 6.19 ± 0.24Bb    |

Exp: exposure time in hours; TMS: total metaphasic plates studied; Csb: chromosome break; Ctb: chromatid break; Frg: fragment; Scu: sister chromatid union; Dic: dicentric; Mla: multiple aberrations; Stp: stickiness and pulverization; Cmt: c metaphase; Cont: 1: negative control (tap water); Cont: 2: solvent control; EE-T1: ethyl acetate extract-treatment 1(0.05 mg/L endosulfan +5 mg/L EE); EE-T2: ethyl acetate extract-treatment 2 (0.05 mg/L endosulfan +10 mg/L EE); EE-T3: ethyl acetate extract-treatment 3 (0.05 mg/L endosulfan +15 mg/L EE); ME-T1: methanol extract treatment 1(0.05 mg/L endosulfan +5 mg/L ME); ME-T2: methanol extract treatment 2 (0.05 mg/L endosulfan +10 mg/L ME); ME-T3: methanol extract treatment 3 (0.05 mg/L endosulfan +15 mg/L ME).

extract(s) was calculated according to the following formula (Waters et al. 1990):

\[
\text{Reduction\%} = \frac{\text{Frequency of CA or MN in A} - \text{Frequency of CA or MN in B}}{\text{Frequency of CA or MN in A}} \times 100
\]

Where, A = endosulfan alone; B = Plant extract(s) mixed with endosulfan and C = Negative control (tap water).

Data were compared for statistically significant difference between control and treatment groups using one-way analysis of variance (ANOVA). Significant differences in ANOVA were further analyzed by post hoc Bonferroni’s, Newman–Keuls and Dunnett’s multiple comparison tests.

Results

Antimutagenicity of P. hexandrum extracts in chromosome aberration test

The typical diploid metaphase complements of the fish, C. carassius, were found to consist of 100 chromosomes, belonging to four types, namely, submetacentric, metacentric, subtelo-centric and acrocentric. Table 1 summarizes the frequency of CA induced by endosulfan and P. hexandrum extracts separately and by their simultaneous treatment. The frequency of CA was induced significantly \((p < 0.05)\) by endosulfan and reached to \(7.7 \pm 0.37, 9 \pm 0.37\) and \(12 \pm 0.47\%\) after 48, 72 and 96 h, respectively. Endosulfan and its simultaneous treatment with hexane, chloroform and aqueous extract were able to produce CA in a significant \((p < 0.05)\) manner, but the reduction in CA frequency was also observed in case of methanol (71\%) and EtOAc (60\%) extracts at 96 h, which were further studied in a concentration-dependent manner in order to find out the most effective concentration which came to be 10 and 15 mg/L, respectively (Table 2). The reduction profiles by effective concentration of methanol extract (10 mg/L) with endosulfan were estimated as 63, 65 and 71\% for 48, 72 and 96 h, respectively. In the endosulfan group treated with effective concentration of EtOAc extract, the reduction profiles were 45, 50 and 60\% for 48, 72 and 96 h, respectively. The highest reduction of 83\% versus control after 96 h was recorded, in case of column eluted ME-F2. In the EtOAc group with endosulfan, fraction EE-F4 was found to be more effective as the highest reduction of 73\% (0.05 mg/L endosulfan +15 mg/L EE-F4) was recorded after 96 h registering the frequency of 5.71 ± 0.27 compared to 12.14 ± 0.47 of endosulfan (0.05 mg/L) alone (Table 3).

Antimutagenicity of P. hexandrum extracts in micronucleus test

The erythrocytes of C. carassius were generally observed as elliptical with a centrally located oval nucleus and a considerable
amount of cytoplasm, any abnormality could therefore, be seen easily. The size and position of micronucleus in the cytoplasm showed slight variation and normally one MN per cell was observed, though in some instances 2 or 3 MN were also observed at longer duration, when analyzed by SEM, which provides efficient results as compared to simple microscopy.

The frequency of MN induced alone by endosulfan and in combinations with plant extracts is summarized in Table 4. In accordance with the results obtained in CA test, endosulfan induced MN significantly \( (p < 0.05) \) at all durations when used alone, while a clear negative effect on induction of MN by methanol and EtOAc extract was found at all time intervals, with maximum reduction of 69% and 62% at 72 h, respectively. Both these extracts were further studied in a concentration-dependent manner and a concentration of 10 and 15 mg/L came to be effective for methanol and EtOAc extract (Table 5). The reduction profiles in the MN incidence by various column eluted fractions of methanol (ME-1, 2, 3, 4 and 5; 10 mg/L each) with endosulfan were estimated as 54, 74, 67, 50, 26% (48 h); 54, 80, 65, 50, 31% (72 h), and 57, 84, 58, 56, 38% (96 h). Similarly, in the case of endosulfan groups treated with EtOAc fractions (EE-1, 2, 3, 4 and 5; 15 mg/L each), the reduction profiles in the MN incidence were recorded as 46, 54, 58, 61, 42% (48 h); 42, 52, 56, 70, 44% (72 h) and 36, 51, 55, 72, 47% (96 h) (Table 6). Overall, the results revealed that endosulfan was potent genotoxic agent and column eluted fractions ME-F2 and EE-F4 effectively reduced the frequency of CA and MN when used simultaneously with endosulfan.

### Evaluation of antioxidant activity by the DPPH method

The analyses of the antioxidant activity showed that the percentage inhibition of 40 \( \mu \) g/mL of fraction ME-F2 was 81%, which was comparable with the standard antioxidant activity of rutin (85%). However, the free radical scavenging activity of fraction EE-F4 was significantly (45%) debased as compared to the standard (Figure 1). The potent antioxidant activity of methanolic fraction ME-F2 was confirmed in the present investigation.  

### Table 3. Frequency profile of CA induced by endosulfan alone and in combination with column eluted fractions of active *P. hexandrum* extracts for different time intervals to evaluate the antimutagenicity in *C. canusius*.

| Exp. | Treatment | TMS | Csb | Ctb | Frg | Scu | Mla | Stp | Cmt | Total aberrations mean (%)± S.D. |
|------|-----------|-----|-----|-----|-----|-----|-----|-----|-----|----------------------------------|
| 48 Cont. 1 | 105 | 1 | 1 | – | – | – | 1 | – | 2.85 ± 0.21 |
| | 102 | 1 | 1 | – | – | – | – | 1 | 2.94 ± 0.16 |
| Endosulfan | 117 | 2 | 2 | 1 | 1 | 1 | – | 2 | 7.69 ± 0.37 |
| EE-F1 | 103 | 1 | 1 | – | 1 | 1 | 2 | – | 6.92 ± 0.27 |
| EE-F2 | 106 | 1 | 1 | – | 1 | 1 | 1 | – | 6.46 ± 0.25 |
| EE-F3 | 113 | 1 | 1 | 2 | 1 | 1 | – | – | 5.30 ± 0.26 |
| EE-F4 | 101 | 1 | – | 1 | – | – | 1 | 1 | 4.95 ± 0.21 |
| EE-F5 | 107 | 1 | 2 | 1 | 1 | 1 | – | – | 5.60 ± 0.26 |
| ME-F1 | 116 | 1 | 1 | 1 | 1 | 1 | 1 | – | 5.17 ± 0.22 |
| ME-F2 | 118 | 1 | 1 | 2 | – | – | 1 | – | 4.23 ± 0.24 |
| ME-F3 | 113 | 2 | 1 | 1 | – | – | 1 | – | 4.42 ± 0.26 |
| ME-F4 | 114 | 1 | – | 1 | 1 | – | 1 | – | 5.26 ± 0.38 |
| ME-F5 | 115 | 1 | 2 | 2 | 1 | 1 | – | – | 6.08 ± 0.30 |
| 72 Cont. 1 | 104 | 1 | 1 | – | – | – | 1 | – | 2.88 ± 0.16 |
| | 101 | 1 | – | 1 | – | – | 1 | – | 2.97 ± 0.17 |
| Endosulfan | 110 | 2 | 2 | 1 | 1 | – | – | 2 | 9.09 ± 0.34 |
| EE-F1 | 120 | 1 | 1 | 1 | 2 | 2 | – | 1 | 6.66 ± 0.33 |
| EE-F2 | 100 | 1 | 1 | 1 | 1 | 1 | – | – | 6.00 ± 0.23 |
| EE-F3 | 119 | 2 | 1 | 2 | – | – | 1 | 1 | 5.88 ± 0.29 |
| EE-F4 | 102 | 1 | – | 1 | – | 1 | 1 | 1 | 4.90 ± 0.21 |
| EE-F5 | 108 | – | 1 | 2 | – | – | 2 | 1 | 6.48 ± 0.31 |
| ME-F1 | 120 | 1 | 1 | 2 | – | – | 2 | 1 | 5.83 ± 0.29 |
| ME-F2 | 114 | 1 | 1 | 1 | – | – | 1 | 1 | 4.38 ± 0.20 |
| ME-F3 | 107 | 2 | 1 | 1 | 1 | 1 | – | – | 5.60 ± 0.26 |
| ME-F4 | 109 | 1 | 1 | 1 | – | – | 1 | 1 | 5.50 ± 0.22 |
| ME-F5 | 103 | 1 | 2 | 1 | 1 | 1 | 1 | – | 6.79 ± 0.28 |
| 96 Cont. 1 | 119 | 2 | 1 | – | – | – | 1 | 1 | 3.36 ± 0.22 |
| | 108 | – | – | 2 | – | – | 1 | 1 | 3.70 ± 0.23 |
| Endosulfan | 107 | 3 | 2 | 1 | 1 | 2 | 2 | 1 | 12.14 ± 0.47 |
| EE-F1 | 109 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 8.25 ± 0.33 |
| EE-F2 | 111 | 2 | 1 | 1 | 1 | 1 | 1 | – | 7.20 ± 0.29 |
| EE-F3 | 112 | 1 | 1 | 2 | 2 | 1 | – | 7.14 ± 0.34 |
| EE-F4 | 105 | 1 | 1 | 2 | – | – | 1 | 1 | 5.71 ± 0.27 |
| EE-F5 | 106 | 1 | 2 | 1 | 1 | 1 | – | 1 | 7.54 ± 0.29 |
| ME-F1 | 113 | 2 | 3 | – | – | 2 | – | 1 | 7.07 ± 0.39 |
| ME-F2 | 103 | – | 1 | 2 | – | – | 1 | 1 | 4.85 ± 0.25 |
| ME-F3 | 104 | 2 | 1 | 1 | – | – | 1 | 1 | 6.73 ± 0.28 |
| ME-F4 | 108 | 2 | 2 | 1 | – | – | 2 | – | 7.40 ± 0.34 |
| ME-F5 | 116 | 1 | 2 | 1 | 2 | 2 | – | 1 | 7.75 ± 0.35 |
the hexane, chloroform, ethyl acetate, methanol and aqueous extract (15 mg/L each) of

Table 4. Frequency profiles of micronuclei induced alone by endosulfan and P. hexandrum extracts followed by their simultaneous exposure for different time intervals to evaluate antimitogenicity in C. carassius.

| Exp. | Treatment | Total no. of MN | Frequency of MN mean (%)± SD |
|------|-----------|-----------------|-------------------------------|
| 48   | Cont. 1   | 11,800          | 28 ± 1.32                    |
|      | ES        | 12,000          | 30 ± 1.32                    |
|      | HEPH      | 11,490          | 36 ± 1.32                    |
|      | CEPH      | 11,730          | 42 ± 1.32                    |
|      | MEPH      | 11,980          | 48 ± 1.32                    |
|      | AEPH      | 11,560          | 54 ± 1.32                    |
|      | ES + HEPH | 11,330          | 54 ± 1.32                    |
|      | ES + CEPH | 11,840          | 60 ± 1.32                    |
|      | ES + EEPH | 11,648          | 66 ± 1.32                    |
|      | ES + MEPH | 11,594          | 72 ± 1.32                    |
|      | ES + AEPH | 11,210          | 80 ± 1.32                    |

72 Cont. 1 11,550 23 – 23 0.19 ± 0.12
Cont. 2 11,000 30 – 30 0.23 ± 0.12
Endosulfan 11,250 320 35 4 400 0.37 ± 0.12
HEPH 11,990 37 3 – 43 0.35 ± 0.12
CEPH 11,100 38 5 – 48 0.43 ± 0.12
EEPH 11,388 35 1 – 37 0.32 ± 0.12
MEPH 11,715 37 1 – 39 0.33 ± 0.12
AEPH 11,845 34 – – 34 0.29 ± 0.12
ME + HEPH 11,645 337 17 8 395 3.44 ± 0.12
ME + CEPH 11,635 331 23 11 410 3.52 ± 0.12
ME + EEPH 11,585 145 10 2 171 1.47 ± 0.12
ME + MEPH 11,560 120 16 4 164 1.41 ± 0.12
ME + AEPH 11,590 127 15 8 140 2.70 ± 0.12

96 Cont. 1 11,980 34 1 – 36 0.30 ± 0.12
Cont. 2 11,220 36 1 – 38 0.33 ± 0.12
Endosulfan 11,760 413 116 23 714 6.07 ± 0.12
HEPH 11,068 34 5 – 44 0.39 ± 0.12
CEPH 11,609 41 3 – 47 0.40 ± 0.12
EEPH 11,872 38 1 – 40 0.33 ± 0.12
MEPH 11,039 45 – – 45 0.40 ± 0.22
AEPH 11,317 45 – – 49 0.43 ± 0.22
ME + HEPH 11,000 421 89 27 680 6.18 ± 0.22
ME + CEPH 11,427 440 99 19 695 6.08 ± 0.22
ME + EEPH 11,005 178 46 15 315 2.86 ± 0.22
ME + MEPH 11,740 197 33 9 290 2.47 ± 0.22
ME + AEPH 11,200 218 8 3 235 2.07 ± 0.22

Table 5. Frequency profiles of micronuclei induced alone by endosulfan and in combination with the variable concentrations of the active extracts of P. hexandrum for different time intervals to evaluate the concentration-dependent antimitogenic response in C. carassius.

| Exp. | Treatment | Total no. of MN | Frequency of MN mean (%)± SD |
|------|-----------|-----------------|-------------------------------|
| 48   | Cont. 1   | 11,800          | 28 ± 1.32                    |
|      | Cont. 2   | 11,320          | 30 ± 1.32                    |
|      | Endosulfan| 12,000          | 30 ± 1.32                    |
|      | EE-T1     | 11,905          | 23 ± 1.32                    |
|      | EE-T2     | 11,730          | 27 ± 1.32                    |
|      | EE-T3     | 11,648          | 31 ± 1.32                    |
|      | ME-T1     | 11,560          | 23 ± 1.32                    |
|      | ME-T2     | 11,670          | 27 ± 1.32                    |
|      | ME-T3     | 11,594          | 31 ± 1.32                    |
|      | ES + EE-T1| 11,330          | 31 ± 1.32                    |
|      | ES + EE-T2| 11,840          | 36 ± 1.32                    |
|      | ES + EE-T3| 11,648          | 42 ± 1.32                    |
|      | ME + EE-T1| 11,594          | 43 ± 1.32                    |
|      | ME + EE-T2| 11,670          | 48 ± 1.32                    |
|      | ME + EE-T3| 11,594          | 53 ± 1.32                    |

72 Cont. 1 11,550 23 – 23 0.19 ± 0.12
Cont. 2 11,000 30 – 30 0.23 ± 0.12
Endosulfan 11,250 320 35 4 400 0.37 ± 0.12
EE-T1 11,490 200 11 7 243 2.11 ± 0.12
EE-T2 11,730 121 29 5 194 1.65 ± 0.12
EE-T3 11,585 145 10 2 171 1.47 ± 0.12
ME-T1 11,670 144 19 3 191 1.63 ± 0.12
ME-T2 11,160 109 12 2 139 1.24 ± 0.12
ME-T3 11,560 120 16 4 164 1.41 ± 0.12

96 Cont. 1 11,550 34 1 – 36 0.30 ± 0.12
Cont. 2 11,220 36 1 – 38 0.33 ± 0.12
Endosulfan 11,760 413 116 23 714 6.07 ± 0.12
EE-T1 11,490 163 77 16 365 6.07 ± 0.12
EE-T2 11,730 126 91 26 386 3.29 ± 0.12
EE-T3 11,005 183 51 10 315 2.86 ± 0.12
ME-T1 11,670 127 61 21 312 2.67 ± 0.12
ME-T2 11,560 123 49 17 272 2.35 ± 0.12
ME-T3 11,740 197 33 9 290 2.47 ± 0.12
GC-MS analysis

In order to find out the bioactive compounds responsible for antimutagenic activity, column eluted fractions ME-F2 and EE-F4 were subjected to GC-MS analysis (Figure 2). ME-F2 showed three major compounds: 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (30.96\%), 2-furancarboxaldehyde, 5-(hydroxy-methyl) (28.65\%) and hexadecanoic acid, methyl ester (27.07\%) constituting 86.68\% of the total peak area (Supplementary Figures 2–4). The minor fractions of ME-F2 include tetradecane (0.45\%), 3-deoxy-D-mannoic lactone (2.08\%), 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z) (2.16\%), deoxy-podophyllotoxin, (2.83\%), podophyllotoxin (2.92\%), epiisopodophyllotoxin-acetate (0.47\%) and 9H-furo[2,3-H]chromene-2,8-dione, 4-methyl-9-(3,4,5-trimethoxybenzylidene) (2.34\%) comprising 13.32\% of the total peak area (Supplementary Table 1). Six major peaks observed in case of EE-F4 were n-hexadecanoic acid (15.47\%), (3β)-stigmast-5-en-3-ol (8.62\%), d (3β)-stigmasta-5,22-dien-3-ol acetate (9.41\%), deoxy-podophyllotoxin (24.22\%), podophyllotoxin (18.91) and epipodophyllotoxin-acetate (21.13) which constitutes 97.76\% of the total peak area.
Discussion

A good strategy for protection against genetic damage caused by xenobiotics is the intake of compounds, natural or synthetic, capable of preventing the formation or repairing an already induced damage (Aydemir et al. 2005). Most of the toxic chemicals that produce genotoxic effects have been known to form reactive oxygen species as well as electrophilic free radical metabolites that interact with DNA to cause disruptive changes (Kim et al. 1991). One of our previous studies demonstrated that genotoxic and mutagenic effects of endosulfan were invariably accompanied and correlated with increased oxidative stress and disturbance of antioxidant enzymes (Dar et al. 2015). The results of the present study clearly showed that methanol and EtOAc rhizome extracts and fractions of P. hexandrum had antimutagenic and anticalcito- genic potential. Several mechanisms have been proposed for antimutagenic activity due to the presence of diverse phytochemical constituents such as tannins, saponin, flavonoids, steroids, terpenoids and glycosides (Berhow et al. 2000).

The GC-MS analysis of ME-F2 showed that it contains three major bioactive constituents, namely, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), 2-furancarboxaldehyde, 5-(hydroxymethyl)-(5HMF) and hexadecanoic acid, methyl ester. All these compounds are likely to possess potent antimutagenic activity. The major constituent DDMP present in the ME-F2 is a Millard reaction product of glucose and glycine, having antimutagenic activity against arylamine (Berhow et al. 2000). This DDMP isolated from onion, in one of the previous studies, have modulated the activity of NF-κB thereby inducing the apoptotic cell death of cancer cells (Ban et al. 2007). 5-HMF, which possesses important biological activities like antioxidant, antymycocar-dial activity, can inhibit mutation and cancer because they can scavenge free radical or induce antioxidative enzyme. Therefore, in order to explore the possible mechanism of action, antioxidant potential of active fractions of P. hexandrum was also carried out. The results confirmed that the potent antimutagenic fraction ME-F2 also possess strong antioxidant potential with a strong correlation (R² = .900) between them. Recently, DDMP, a major compound in ME-F2 fraction, was also isolated and identified as a potent antioxidant from Pyrus pyrifolia (Hwang et al. 2013), supporting this study and some recent studies which showed a strong correlation between the antioxidant and antimutagenic activity.

The six major compounds identified by GC-MS analysis in the second action fraction EE-F4 were deoxypodophyllotoxin, podophyllotoxin, epipodophyllotoxin acetate, palmitic acid, β-sitosterol, and stigmasterol-acetate. Podophyllotoxin and its related derivatives, constituting major percentage of the fraction EE-F4, have been used for a variety of therapeutic purposes including cathartic, antirheumatic and antiviral properties, pesticidal and antimitotic treatments (Xu et al. 2011; He et al. 2013). Because of its inhibitory activity on cell growth, it is often used as a lead compound for drug design in the search for improved antiproliferative agents. Deoxypodophyllotoxin has antiproliferative, anti-inflammatory, antitumour and antiviral activity in diverse cell types (He et al. 2013). The free fatty acids (FFAs) were previously described to possess antitumour effects against...
many different types of human tumour cells, including those from breast, lung and prostate carcinomas, and regression of human gliomas (Reddy et al. 1998). Palmitic acid has been reported to induce apoptosis in tumour cells. Many studies have also shown that supplementing the culture medium with palmitic acid completely rescued prostate and breast cancers cells from fatty acid synthase (FAS) knockdown-induced apoptosis (Kwan et al. 2013). One molecular target of palmitic acid in tumour cells is DNA topoisomerase I. However, it does not affect DNA topoisomerase II; this suggests that palmitic acid may be a lead compound for anticancer drug discovery. Stigmastanol is known to possess many important biological activities like antihypercholesterolemic, antimutagenic, antileishmanial, antimalarial, antitypranosomial, platelet aggregation inhibitor and antiviral (Zhou et al. 2011). β-Sitosterol is known to be effective against a number of cancers like human breast cancer, colon carcinoma and prostatic cancer (Manayi et al. 2013). The significant reduction in the CA and MN in the EE-F4-treated group in our study might be attributed to different mechanism, other than antioxidant potential, of the parent compound podophyllotoxin and needs further study.

Conclusions
In the present work, three novel compounds were identified from the methanol fraction of P. hexandrum; biological evaluation showed that most of these compounds exhibited potent antimutagenic activity, via antioxidant pathway, as compared to the already known lignans from the plant and could therefore, potentially be a repository for pharmacologically active products, suitable for the development of new effective chemotherapeutic agents and their corresponding benefit to mankind. This work states the importance of considering these novel identified compounds other than podophyllotoxin and its derivatives in biological studies when using P. hexandrum rhizome extracts.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding
This work is part of the PhD thesis of the first author who thanks the University Grants Commission (UGC) for his Junior Research Fellowship (Sr. No. 2061330965; Ref. No: 23/06/2013-i) and Director of the Centre of Research for Development (CORD), University of Kashmir, for providing necessary research facilities.

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