Quinalphos-induced chromosomal instability, DNA Damage, and Bax/Bcl-2 dysregulation in mouse bone marrow: Protective effects of Piperine

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Abstract

Quinalphos (QNP), an organophosphate insecticide, is known to induce genotoxic and cytotoxic effects in non-target organisms. This study evaluated the impact of subacute QNP exposure on chromosomal stability, DNA integrity, and apoptosis-related gene expression in bone marrow cells of male Swiss albino mice, and assessed the genoprotective potential of piperine (PIP), a bioactive compound from Piper nigrum. Mice were allocated into six groups: control (2% gum acacia), QNP I (0.375 mg/kg b.w.), QNP II (0.75 mg/kg b.w.), PIP alone (10 mg/kg b.w.), QNP I + PIP, and QNP II + PIP. All treatments were administered orally for 28 days. Genotoxicity was evaluated using micronucleus, chromosomal aberration, and alkaline comet assays. QNP caused a significant, dose-dependent increase in micronucleated erythrocytes, chromosomal aberrations, and DNA strand breaks, along with suppression of erythropoiesis. It also upregulated pro-apoptotic Bax and downregulated anti-apoptotic *Bcl-2* mRNA expression, indicating activation of the mitochondrial apoptotic pathway. Co-administration of piperine significantly reduced genotoxic alterations, though it did not fully restore Bax/*Bcl-2* balance. These findings suggest that piperine exerts protective effects against QNP-induced genetic damage and apoptotic dysregulation.

1. Introduction

Pesticides are recognized as one of the major contributors to environmental contamination in the modern world (Mostafalou and Abdollahi 2013; Kim et al., 2017). The continuous development of newer and more potent pesticide formulations has become necessary due to increasing pest resistance, the need for improved hygienic control, and the growing global demand for food driven by rapid population expansion (Mostafalou and Abdollahi, 2013) where in, only 1% of the pesticide applied hits the target pest, while the remaining 99% of the pesticide drifts into the environment contaminating soil, water and biota (Pimentel and Levitan, 1986; Ramakrishnan et al., 2010). Prolonged or repeated exposure to pesticides has been shown to adversely affect multiple organ systems, including the nervous, endocrine, immune, reproductive, renal, cardiovascular, and respiratory systems (De Souza et al., 2011; Mostafalou and Abdollahi, 2013; Costa, 2018). Among the different types of pesticides, organophosphates (OPs) class of insecticides are widely used and currently makes up 27% of the total sales of pesticides (Ngoula et al., 2007, Amin and Hashem, 2012).

Quinalphos (QNP) is a synthetic OP, non-systemic, broad spectrum hard insecticide and acaricide, acting as a cholinesterase (ChE) inhibitor after dermal contact, ingestion, and inhalation. QNP has wide applicability to control pests in farming of oilseeds, grams, rice, fruits and vegetables such as okra, carrot, brinjal, cauliflower, groundnut, rice, paddy, sugarcane, wheat, potato, corn, cotton etc. due to its good penetrative properties. Its long residual action in animals and aquatic organisms put it as a matter of concern (Muttappa et al., 2014). Despite use of atropine and pralidoxime (therapeutic tools) and improvements in intensive care and related disciplines, the mortality associated with OP insecticide poisoning has not decreased.

Genotoxicity of pesticides for non-target organisms and their influence on ecosystems are of worldwide concern (Bolognesi and Cirillo, 2014). Pesticides have been considered as potential chemical mutagens and tend to be very reactive compounds that can form covalent bonds with various nucleophilic centres of cellular biomolecules, including DNA. For instance, the induction of DNA damage can potentially lead to adverse reproductive outcomes, the induction of cancer and many other chronic diseases (Dimitrov et al., 2006). Wide varieties of mutagenic assays covering gene mutation, chromosomal alterations, DNA damage and micronucleus (MN) frequency have been tested in a number of pesticides (Bolognesi 2003). OP pesticides induce DNA damage (genotoxic, alkylating and clastogenic properties) in *in vivo* as well as *in vitro* systems (Sarabia et al., 2009; Wu et al., 2011).

The growing recognition of medicinal plants and their bioactive constituents in animal nutrition has led to intensified research on various phytogenic compounds incorporated into animal diets. These phytogenic feed additives are administered in different forms, including dried plant material, aqueous extracts, and essential oils, and are increasingly being evaluated for their beneficial effects in livestock species. PIP (1-Piperoylpiperidine) is a dietary, natural secondary metabolite isolated from the oleoresin of fruits and roots of *Piper nigrum L*. (black pepper) and *Piper longum L*. (long pepper) species of Piperaceae family (Zahin et al., 2010; Zheng et al., 2016; Gorgani et al., 2017). It is used widely in various systems of traditional medicine, accounts chemopreventive and antioxidant activities (Selvendiran et al., 2005; Srinivasan, 2007). It has immunomodulatory, anticarcinogenic, stimulatory, hepatoprotective, radioprotective (Darshan and Doreswamy, 2004; Mona et al., 2009), antimicrobial and antiulcer activities (Khamis et al., 2018; Tedasen et al., 2019).

Despite of the significant toxicity, quinalphos is widely used all over the world and systematic studies on the quinalphos toxicity are lacking. Reports indicated that QNP produces genotoxicity (Wu et al., 2011) and PIP was found to be genoprotective (Selvendiran et al., 2005). But the ameliorative potential of PIP has not been studied in QNP-induced subacute toxicity. Hence, the This study was designed to estimate the dose-dependent effect of QNP on DNA fragmentation, chromosomal aberration, micronuclei formation and apoptosis in toxicity model of Swiss Albino mice. Further the study was done to if piperine co-treatment could counteract these alterations, similar to how it has shown potential to modulate effects in other toxicity models.

2. Materials and methods

2.1. Chemicals and reagents

The formulation product of organophosphate (OP) insecticide QNP (VAZARA 25® Cheminova India Ltd.) used in this experiment. May-Grunwald’s stain, Giemsa stainand low melting agarose (LMA) were purchased from Himedia Laboratories Pvt. Ltd., Mumbai.Piperine, Dulbecco’s phosphate buffer saline (devoid of calcium and magnesium),2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) dye, normal melting agarose (NMA), ethidium bromide (EtBr) was purchased from Sigma Chemical Co., USA. Sodium hydroxide (NaOH), tris base,

glacial acetic acid and sodium chloride (NaCl) were procured from Sisco Research Laboratories, Mumbai.

2.2. Experimental animals

Male Swiss albino mice weighing between 20–24 g were procured from Disease Free Small Animal House (DFSAH), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana. They were acclimatized for a period of one week in the Departmental Animal House. Animal house temperature varied between 22 ºC to 27 ºC throughout the study. The study was carried out after the approval from the Institutional Animal Ethics Committee (IAEC). (Approval No. VCC/IAEC/1630-58 dated 26-07.2018).

2.3. Dose selection and preparation of QNP and PIP

Dose for toxicity study was selected according to maximum tolerated dose (MTD). The solutions of QNP (liquid form) were prepared in distilled water to be given orally at 1 ml/100 g b.wt. Two different oral doses of QNP, namely 2.5% and 5% of MTD (15 mg/kg b.wt.) i.e. 0.375 and 0.75 mg/kg b.wt. were selected in mice for subacute toxicity study. Dose of PIP (10 mg/kg b.wt.) given orally was prepared in 2% aqueous gum acacia and was selected according to previous studies performed by other researchers (Mona et al., 2009, Sehgal et al., 2012). 2% aqueous gum acacia suspension was also used in control animals. Doses of QNP and PIP were administered orally in mice through a metal gavage needle.

2.4. Experimental design

In this study mice were divided in six groups comprise of 5 mice each for 28 days treatment schedule. Mice in group I were kept as control and received 2% gum acacia. Mice in groups II, III, IV, V and VI were administered with lower dose of QNP i.e. QNP I (0.375 mg/kg), higher dose of QNP i.e. QNP II (0.75 mg/kg), PIP (10 mg/kg), lower dose of QNP i.e. QNP I (0.375 mg/kg) + PIP (10 mg/kg) and higher dose of QNP i.e. QNP II (0.75 mg/kg) + PIP (10 mg/kg), respectively. All groups of mice were orally administrated with solutions as explained above as per their body weight and a gap of 12 h was maintained between the dosing of QNP and PIP. Group 2 and 3 mice were also administered 2% gum acacia at a gap of 12 h of QNP administration. Animals were observed critically at the time of dosing and body weights were recorded weekly during 28 days of exposure period. After 28 days of treatment, animals were sacrificed on 29th day of first dosing and bone marrow was collected.

2.5. Micronucleus test

The technique to asses micronucleated erythrocytes (MNEs) was described by earlier researchers (Boller and Schmid, 1970; Heddle and Salamone, 1973) and was used for screening of bone marrow cells. To determine the frequency (%) of PCE to total erythrocytes (PCEs + NCEs) and PCE/NCE ratio, at least 500 erythrocytes (i.e., PCEs + NCEs) from each animal were scored randomly (Borroto et al., 2003). From each mouse, a minimum of 2000 erythrocytes i.e. a minimum of 1000 PCEs along with 1000 NCEs were scored to calculate the micronuclei frequency in both PCEs and NCEs. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic

erythrocytes (MNNCEs) were calculated by using the following equation and expressed in percentage (%) (Haynes et al., 1996):

$$\%MNE = \frac{\text{Number of micronucleated cells}}{\text{Total count of cells}} \times 100$$

Evaluation of the activity of PIP to reduce micronuclei induced by QNP in PCEs and NCEs was carried out according to following formula (Koura et al., 2017):

Reduction in micronucleated cells (%) = [(micronucleated cells in QNP-treated mice) − (micronucleated cells in QNP + PIP-treated mice)/ (micronucleated cells in QNP-treated mice− micronucleated cells in control)] × 100

2.6. Chromosomal aberration assay

Before 2 h prior to sacrifice, animals of each group were injected with colchicine. Bone marrow was collected in an aqueous potassium chloride (0.075 M) kept at 37°C for 20 min. After this treatment was done with fixative containing methanol and glacial acetic acid (3:1). Repeated washing was performed with chilled fixative. The fixed cells were dropped onto slides, flame dried, and then Giemsa staining was done, as described by earlier researchers (Bagri and Jain, 2019). Slides prepared from each animal were screened, and 100 well-spread metaphases were counted. For each mouse, total of 100 metaphase cells was counted randomly for various types of chromosomal aberrations. The total number of aberrant metaphases is represented as percent total aberrant metaphases and the different type of aberrations are expressed as aberrations per 100 metaphase cells in each mouse. The percent total aberrant metaphases (TAM) were calculated according to following formula (Prasad et al., 2009):

$$\text{TAM (\%)} = \frac{\text{Total number of aberrant metaphases}}{\text{Total number of metaphases counted}} \times 100$$

Evaluation of the activity of PIP to reduce chromosomal aberrations induced by QNP was carried out according to following formula (Koura et al., 2017):

$$\text{Reduction of CA (\%)} = \frac{\text{Frequency of CA in A- Frequency of CA in B}}{\text{Frequency of CA in A- Frequency of CA in C}} \times 100$$

A= Group treated with QNP, B= Group treated with QNP and PIP, C= Control group

The end points analyzed were the presence of structural chromosomal aberrations, which were recorded as chromatid-type (including breaks or fragments, gaps, and deletion) or chromosome-type (including

centromeric gaps, breaks or fragments and centric fusion), and in numerical chromosomal aberrations polyploidy and endoreduplication were recorded. Data of total aberrant metaphases were evaluated including and excluding gaps.

2.7. Comet assay

The comet assay was performed in mouse bone marrow cells following the methods of Patel et al. (2006) and Dhawan et al. (2009) with minor modifications. Slides were pre-coated with 1% normal melting agarose (NMA). Bone marrow cells were isolated in Dulbecco's phosphate-buffered saline (DPBS), washed thrice, and adjusted to 1×10^6 cells/ml after assessing viability with 0.4% Trypan blue. A mixture of cell suspension and 1% low melting agarose (LMA) was layered over the base slide, covered with a coverslip, and allowed to solidify at 4°C. A third layer of 0.5% LMA was added and solidified similarly.

Slides were immersed overnight at 4°C in chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO; pH 10). DNA unwinding was carried out for 25 min in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA; pH > 13), followed by electrophoresis at 24 V (0.7 V/cm, 300 mA) for 20 min under dark, chilled conditions. Slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with ethidium bromide, and examined under a fluorescent microscope at 200× magnification. A total of 100 cells per treatment (50 per replicate slide) were analyzed using TriTek CometScore™ software. Parameters recorded included tail length, tail DNA (%), tail moment, Olive tail moment (OTM), and comet length.

Evaluation of the activity of PIP to reduce DNA damage in terms of tail moment induced by QNP [Reduction (%)] was carried out by following formula:

Reduction in tail moment = [(tail moment in QNP-treated mice) − (tail moment in QNP + PIP-treated mice)/ (tail moment in QNP-treated mice − tail moment in control)] × 100

2.8. RNA extraction and quantitative RT-PCR

Total RNA from each mouse bone marrow after removing PBS was isolated with PureLink™ RNA mini kit as per the manufacturer's instructions. Quantification of total RNA was determined at 260 nm by nanodrop spectrophotometry. cDNA was synthesized by using Revertaid® First strand cDNA synthesis kit (Thermo Scientific, USA) using moloney murine leukemia virus reverse transcriptase enzyme by following manufacturer's instructions. For detection of Bax and *Bcl2* mRNA polymerase chain reaction (PCR) primers were designed (Table 4).

2.9. Statistical analysis

Data were analyzed using Graph Pad Prism version 5.03. Results were expressed as mean ± SEM with 'n' equal to number of animals. The differences among the groups were compared by one-way analysis of variance (One-Way ANOVA) with Tukey post-hoc test. In all tests, p values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect on micronucleus test

The effect of subacute oral exposure of QNP, PIP and their combination on micronucleus test in bone marrow cells of mice as well as the percentage of damage reduction by the PIP are presented in Table 1 and Fig. 1. Group treated with QNP II showed significant increase in the frequency of MNPCE in comparison to control and group treated with QNP I. However, there was dose-dependent decreasing trend in frequency of MNPCEs in QNP I and QNP II + PIP-treated group animals as compared to QNP I and QNP II group animals. The values % MNNCE in QNP I and QNP II were non-significantly higher than control animals. There was non-significant decrease in P/N ratio at both doses of QNP in comparison to control in dose dependent manner. The values were higher in QNP I + PIP and QNP II + PIP group animals as compared to QNP I and QNP II treated group animals, respectively. A significant decrease in % PCE of QNP II-treated group was observed in comparison to control. Administration of PIP in QNP-treated groups (QNP I and QNP II) non-significantly increases % PCE as compared to QNP I and QNP II treated group animals, respectively. Administration of PIP in QNP-treated groups (QNP I and QNP II) caused dose dependent reduction in the frequency of MNPCEs up to 26.08 and 30.18% and in the frequency of MNNCEs up to 25 and 45%, respectively. There was no significant difference in the values of control and PIP-treated group animals in all the parameters.

Table 1
Effects of subacute oral exposure of mice to QNP, PIP and their combination on micronucleus test in bone marrow cells

Treatment (mg/kg b.wt.)	MNT parameters				Reduction in MNPCEs (%)	Reduction in MNNCEs (%)
	MNPCEs (%)	MNNCEs (%)	P/N	% PCEs		
Control (200)	0.56 ± 0.09	0.42 ± 0.10	1.09 ± 0.07	52.00 ± 1.75	-	-
QNP I (0.375)	0.79 ± 0.07	0.90 ± 0.15	0.91 ± 0.02	47.28 ± 0.53	-	-
QNP II (0.75)	1.10 ± 0.04^{ab}	0.82 ± 0.06	0.83 ± 0.10	44.60 ± 2.85^{a}	-	-
PIP (10)	0.55 ± 0.07^{c}	0.44 ± 0.14	1.13 ± 0.05^{c}	52.80 ± 1.34^{c}	-	-
QNP I (0.375) + PIP (10)	0.74 ± 0.05^{c}	0.78 ± 0.14	1.11 ± 0.05	53.36 ± 0.98 ^{c}	26.08	25
QNP II (0.75) + PIP (10)	0.94 ± 0.06^{ad}	0.64 ± 0.10	0.89 ± 0.05	46.20 ± 1.17^{e}	30.18	45

Data are presented as mean ± SEM (n = 5 mice/group). QNP: quinalphos; PIP: piperine; MNPCEs: micronucleated polychromatic erythrocytes; MNNCEs: micronucleated normochromatic erythrocytes; P/N: ratio of polychromatic erythrocytes to normochromatic erythrocytes; and PCEs: polychromatic erythrocyte. The values were compared using one-way ANOVA followed by Tukey post-hoc test. Means bearing a, b, c, d, and e superscripts differ significantly ($P < 0.05$) vs. control, QNP I, QNP II, PIP, and QNP I + PIP, respectively.

3.2. Effect on chromosomal aberrations

The effect of subacute oral exposure of QNP, PIP and their combination on % chromosomal aberrations in bone marrow cells of mice as well as the percentage of damage reduction by the PIP are presented in Table 2 and Fig. 2. Groups treated with QNP (QNP I and QNP II) showed a significant increase in the frequency of TAM (both excluding and including gaps) in comparison to control group. QNP II-treated group showed significant increase in the frequency of TAM (excluding gap) in comparison to QNP I-treated group animals. The values were lower in QNP I + PIP and QNP II + PIP group animals as compared to QNP I and QNP II treated group animals, respectively. There was no significant difference in the values of control and PIP-treated group animals. The percentage of reduction of chromosomal damage were observed to be 3.13 and 25.80% respectively, in QNP I + PIP and QNP II + PIP-treated groups. A significant increase was observed in chromatid deletion and centromeric break in QNP I-treated group in comparison to control. QNP II-treated group animals showed significant increase in chromatid deletion, centromeric gap, centromeric break, centric fusion and endoreduplication in comparison to control animals. There was no significant difference in the values of control and PIP-treated group animals in all the parameters.

Table 2

Effects of subacute oral exposure of mice to QNP, PIP and their combination on chromosomal aberrations in bone marrow cells

Aberrations / 100 metaphase cells (%)	Treatment (mg/kg b.wt.)					
	Control (200)	QNP I (0.375)	QNP II (0.75)	PIP (10)	QNP I (0.375) + PIP (10)	QNP II (0.75) + PIP (10)
ICG	3.20 ± 0.49	8.20 ± 0.58	6.00 ± 1.14	1.80 ± 0.58	7.80 ± 0.86	5.80 ± 3.29
CG	0.20 ± 0.20	0.40 ± 0.24	0.80 ± 0.58	0.20 ± 0.20	0.20 ± 0.20	0.40 ± 0.40
CB	0.40 ± 0.40	0.20 ± 0.20	0.40 ± 0.24	0.60 ± 0.24	0.40 ± 0.24	0.60 ± 0.40
CD	2.20 ± 0.37	7.80 ± 0.37[a]	7.80 ± 0.86[a]	1.60 ± 0.24[bc]	6.40 ± 0.40[ad]	11.00 ± 1.95[ade]
Cg	1.20 ± 0.20	5.00 ± 0.63	5.60 ± 1.91[a]	2.00 ± 0.71	5.20 ± 0.66	5.40 ± 0.81
Cb	3.20 ± 0.49	7.20 ± 0.49[a]	8.00 ± 0.45[a]	1.80 ± 0.37[bc]	5.40 ± 0.93[d]	5.60 ± 0.93[d]
CF	2.00 ± 0.45	4.00 ± 0.63	5.60 ± 1.08[a]	1.60 ± 0.60[c]	4.40 ± 0.51	3.80 ± 0.58
R	0.20 ± 0.20	0.20 ± 0.20	0.60 ± 0.40	0.20 ± 0.20	0.00 ± 0.00	0.40 ± 0.40
E	0.80 ± 0.37	1.60 ± 0.68	3.00 ± 0.45[a]	1.60 ± 0.51	1.60 ± 0.51	2.20 ± 0.37
Poly	0.60 ± 0.24	0.80 ± 0.37	1.40 ± 0.51	1.40 ± 0.40	1.20 ± 0.37	2.00 ± 0.32
TAM (including gaps) (%)	9.00 ± 0.71	18.00 ± 1.30[a]	22.40 ± 1.33[a]	8.60 ± 0.68[bc]	16.20 ± 1.59[ad]	19.80 ± 2.40[ad]
TAM (excluding gaps) (%)	6.20 ± 0.37	13.00 ± 0.89[a]	18.40 ± 0.51[ab]	6.80 ± 0 .49[bc]	12.40 ± 1.21[acd]	15.40 ± 1.72[ad]

Data are presented as mean ± SEM (n = 5 mice/group). QNP: quinalphos; PIP: piperine; ICG: isochromatid gap; CG: chromatid gap; CB: chromatid break; CD: chromatid deletion; Cg: centromeric gap; Cb: centromeric break; CF: centric fusion; R: ring; E: endoreduplication; Poly: polyploidy; TAM: Total aberrant metaphases. The values were compared using one-way ANOVA followed by Tukey post-hoc test. Means bearing a, b, c, d, and e superscripts differ significantly ($P < 0.05$) vs. control, QNP I, QNP II, PIP, and QNP I + PIP, respectively. Gaps have been mentioned and calculated both including and excluding in the total aberrations.

Aberrations / 100 metaphase cells (%)	Treatment (mg/kg b.wt.)					
	Control (200)	QNP I (0.375)	QNP II (0.75)	PIP (10)	QNP I (0.375) + PIP (10)	QNP II (0.75) + PIP (10)
Reduction (%) TAM (excluding gaps	-	-	-	-	3.13	25.80

Data are presented as mean ± SEM (n = 5 mice/group). QNP: quinalphos; PIP: piperine; ICG: isochromatid gap; CG: chromatid gap; CB: chromatid break; CD: chromatid deletion; Cg: centromeric gap; Cb: centromeric break; CF: centric fusion; R: ring; E: endoreduplication; Poly: polyploidy; TAM: Total aberrant metaphases. The values were compared using one-way ANOVA followed by Tukey post-hoc test. Means bearing a, b, c, d, and e superscripts differ significantly ($P < 0.05$) vs. control, QNP I, QNP II, PIP, and QNP I + PIP, respectively. Gaps have been mentioned and calculated both including and excluding in the total aberrations.

3.3. Effect on comet assay

The effect of subacute oral exposure of QNP, PIP and their combination on comet assay in bone marrow cells of mice as well as the percentage of damage reduction by the PIP presented in Table 3 and Fig. 3. Results of the present study showed a significant dose-dependent increase in tail length, tail DNA (%), tail moment, olive tail moment and comet length at both doses of QNP (QNP I and QNP II) in comparison to control. The values were lower in QNP I + PIP and QNP II + PIP group animals as compared to QNP I and QNP II treated group animals, respectively. There was no significant difference in the values of control and PIP-treated group animals except that a significant increase in comet length in PIP alone group was found in comparison to control. The comet length was significantly reduced in QNP II + PIP-treated group animals as compared to QNP II group animals. The dose-dependent percentage of reduction in tail moment of comet assay of the group treated with PIP were observed to be 30.34 and 18.03% in QNP I + PIP and QNP II + PIP-treated group animals, respectively.

Table 3
Effects of subacute oral exposure of mice to QNP, PIP and their combination on comet assay parameters in bone marrow cells

Treatment (mg/kg b.wt.)	Comet assay parameters					Reduction in tail moment (%)
	Tail length (µm)	Tail DNA (%)	Tail moment	Olive Tail moment (AU)	Comet length (µm)	
Control (200)	2.29 ± 0.29	9.82 ± 0.97	0.65 ± 0.10	1.60 ± 0.15	58.46 ± 2.30	-
QNP I (0.375)	17.65 ± 1.88[a]	29.42 ± 1.95[a]	11.23 ± 1.21[a]	15.85 ± 0.84[a]	149.4 ± 0.83[a]	-
QNP II (0.75)	20.20 ± 1.28[a]	30.09 ± 2.13[a]	13.46 ± 1.08[a]	18.07 ± 1.62[a]	164.7 ± 7.31[a]	-
PIP (10)	2.00 ± 0.16[bc]	8.16 ± 0.86[bc]	0.67 ± 0.12[bc]	1.85 ± 0.20[bc]	85.80 ± 2.84[abc]	-
QNP I (0.375) + PIP (10)	13.12 ± 2.06[acd]	23.04 ± 1.79[acd]	8.02 ± 1.40[acd]	11.00 ± 1.82[acd]	133.8 ± 6.02[acd]	30.34%
QNP II (0.75) + PIP (10)	16.75 ± 1.12[ad]	28.87 ± 2.28[ad]	11.15 ± 0.77[ad]	13.66 ± 1.15[ad]	132.2 ± 5.53[acd]	18.03%

Data are presented as mean ± SEM (n = 5 mice/group). QNP: quinalphos; PIP: piperine. AU: arbitrary unit.The values were compared usingone-way ANOVA followed by Tukey post-hoc test. Means bearing a, b, c, and d superscripts differ significantly ($P < 0.05$) vs. control, QNP I, QNP II and PIP, respectively.

Table 4
Primer sequences used for Real-time RT-PCR analysis

Type of Gene	Primer sequences	Amplicon size	Accession number
Bax	F5'-GCGTGGTTGCCCTCTTCTACTT-3' R5'-CCACAAAGATGGTCACTGTCTGC-3'	256bp	NM_007527
Bcl-2	F5'-TTCGCAGAGATGTCCAGTCAGCT – 3' R5'-TGAAGAGTTCCTCCACCACCGT – 3'	82bp	NM_009741
ß-actin/ Actb	F5'-GGCTGTATTCCCCTCCATCG – 3' R5'-CCAGTTGGTAACAATGCCATGT'3'	154bp	NM_007393

3.4. Effect on Bax and *Bcl-2* gene in bone marrow cells analyzed by RT-PCR

The effect of subacute oral exposure of QNP, PIP and their combination on Bax and *Bcl-2* gene in bone marrow cells are Figs. 4 and 5. A significant upregulation was found in mRNA expression of Bax gene at both doses of QNP in comparison to control. A significant downregulation was found in mRNA expression of *Bcl-2* gene at both doses of QNP in comparison to control. PIP administration did not produce significant effect on QNP induced upregulation and downregulation in mRNA expression of Bax and *Bcl-2* gene, respectively.

4. Discussion

Deliberate use of pesticides during the decades caused serious hazards on human health. QNP is one of the broad-spectrum pesticides, widely used as insecticide and acaricide. Phytotherapy, which involves the use of plant-derived bioactive compounds for therapeutic purposes, has gained increasing attention in toxicology due to its relatively low toxicity and multitargeted mechanisms of action (Yoshitomi et al., 2011, Aziz et al., 2014).

The present study demonstrates that subacute exposure to quinalphos (QNP) induces marked genotoxicity in mouse bone marrow cells, as evidenced by increased micronucleus formation, chromosomal aberrations, DNA strand breaks, and dysregulation of apoptosis-related genes. Importantly, the attenuation of these alterations by piperine (PIP) highlights the relevance of phytotherapy as a complementary strategy in mitigating pesticide-induced genetic damage.

Organophosphate pesticides such as QNP are known to generate oxidative stress through metabolic activation by cytochrome P450 enzymes, leading to excessive production of reactive oxygen species (ROS) (Debnath and Mandal, 2000; Soltaninejad et al., 2009). These ROS interact with nucleic acids, proteins, and membrane lipids, causing DNA strand breaks, chromosomal instability, and impaired cellular proliferation (Chauhan et al., 2005; Kalthur et al., 2017). In the present investigation, the dose-dependent increase in micronucleated polychromatic erythrocytes, total aberrant metaphases, and comet assay parameters confirms that QNP acts as a clastogenic and DNA-damaging agent (De Boeck et al., 2000; Tice et al., 2000). The dose-dependent DNA migration observed in the present study indicates substantial genomic instability. Suppression of erythropoiesis further suggests bone marrow cytotoxicity, indicating that genotoxic insult is accompanied by functional impairment of hematopoietic tissue (Mishra et al., 2015; Sadiqul et al., 2017).

Co-administration of piperine (PIP) attenuated QNP-induced genotoxic alterations, as reflected by reductions in micronuclei frequency, chromosomal aberrations, and comet assay parameters. These findings indicated that PIP causes attenuation particularly at the lower QNP dose, suggesting that phytochemicals may be more effective when oxidative burden is moderate. This aligns with the concept in phytotherapy that natural compounds act primarily as modulators of cellular defence systems rather

than as direct antidotes. By strengthening intrinsic antioxidant mechanisms and possibly modulating xenobiotic-metabolizing enzymes, PIP may limit the formation of reactive QNP metabolites capable of binding to DNA.

Furthermore, the alteration in apoptotic gene expression (increased Bax and decreased Bcl-2) following QNP exposure indicates activation of the intrinsic mitochondrial apoptotic pathway (Zerin et al., 2015). Excessive apoptosis in bone marrow can compromise hematopoietic homeostasis and contribute to long-term toxicity. Although PIP did not significantly normalize Bax and Bcl-2 expression, the overall reduction in DNA damage suggests that phytotherapeutic intervention may prevent the upstream oxidative signals that trigger apoptotic cascades. Thus, the genoprotective effect of PIP may occur primarily at the level of oxidative stress modulation rather than direct transcriptional regulation of apoptotic genes (Selvendiran et al., 2005; Sehgal et al., 2013).

The integration of cytogenetic, molecular, and apoptotic findings underscores a unified mechanism of QNP-induced toxicity driven by oxidative stress and mitochondrial dysfunction. In this context, phytotherapy offers a promising adjunct approach by targeting multiple pathways simultaneously free radical scavenging, enhancement of antioxidant enzymes, stabilization of cellular membranes, and modulation of metabolic activation. Such multitarget action is a hallmark of plant-derived compounds and distinguishes them from single-target synthetic therapeutics.

Importantly, the absence of genotoxic effects in the PIP-alone group confirms its safety at the administered dose, reinforcing its suitability as a dietary phytochemical supplement. These findings support the broader concept that incorporation of bioactive plant constituents into the diet may help reduce environmental and occupational genotoxic risks associated with chronic pesticide exposure.

5. Conclusion

The present findings not only establish the genotoxic potential of quinalphos but also highlights the therapeutic promise of phytochemicals such as piperine in occupational and environmental toxicology. The results strengthen the scientific basis for integrating phytotherapy into preventive toxicology strategies, particularly in populations at risk of prolonged pesticide exposure.

Declarations

Competing Interests

“The authors have no relevant financial or non-financial interests to disclose.”

Ethics approval

“This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of of Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar “(Approval No. VCC/IAEC/1630-58 dated 26-07.2018)”

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Author Contribution

Preeti Singh contributed to the acquisition, analysis, and interpretation of data, drafted the manuscript, and critically revised the article. Vinod Kumar contributed to the conception and design of the study, acquisition, analysis and interpretation of data, drafted the manuscript, critically revised the article, and approved the final version to be published. Preeti Bagri participated sufficiently in the work to take public responsibility for appropriate portions of the content. Deepika Lather assisted in comet assay image acquisition and analysis. Kanisth Batra assisted in molecular experimental work. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work, ensuring the integrity and accuracy of the research.

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Figures

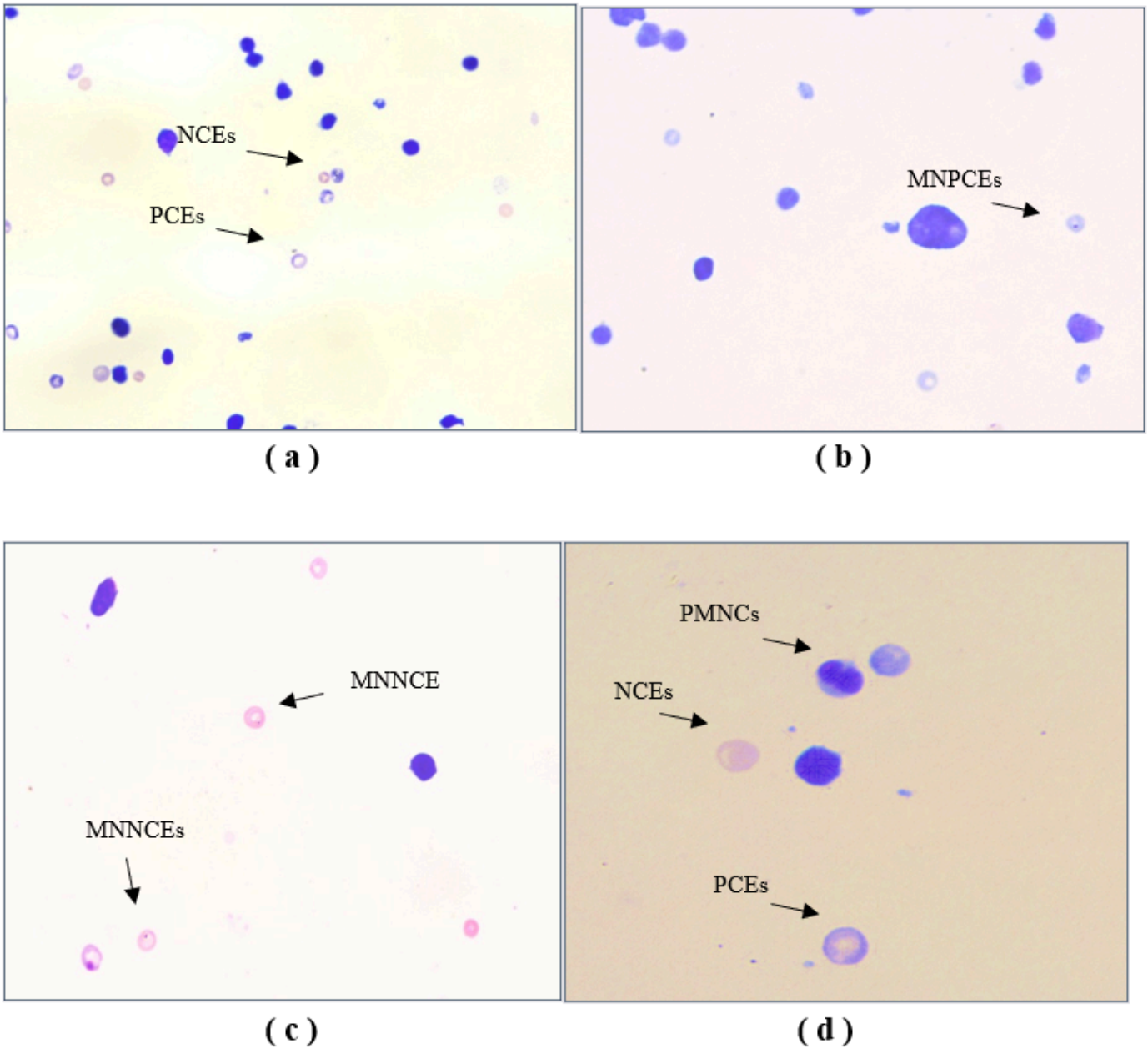

Figure 1

Representative images showing micronuclei in bone marrow cells of mice observed under 40X magnification of light microscope. Plate (a) shows normochromatic erythrocytes (NCEs) and polychromatic erythrocytes (PCEs). Plate (b) shows micronucleated polychromatic erythrocytes

(MNPCEs). Plate (c) shows micronucleated normochromatic erythrocytes (MNNCEs). Plate (d) shows polymorphonuclear cells (PMNCs)

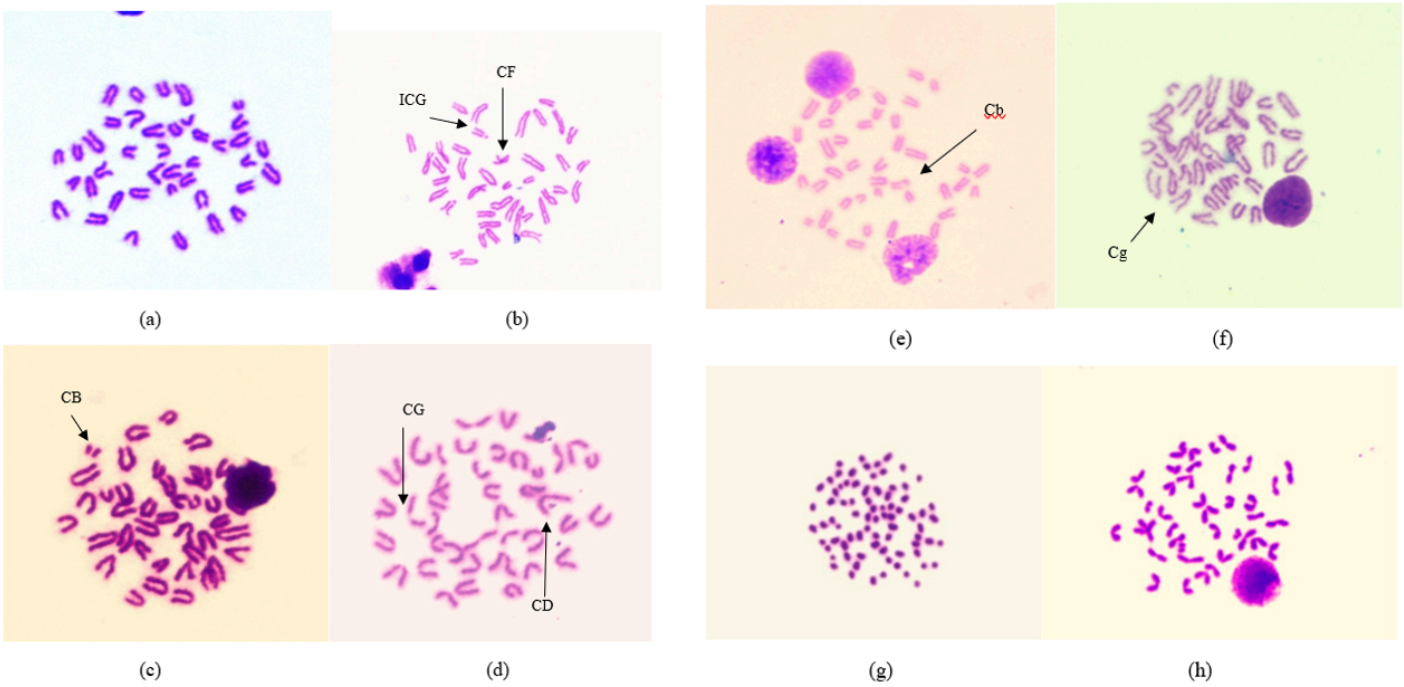

Figure 2

Representative images of different types of chromosomal aberrations induced in bone marrow cells of mice showing effects of subacute oral exposure of mice to QNP, piperine and their combination observed under ×100 objective (oil) in light microscope; a: Normal metaphasic spread; b: centric fusion (CF); isochromatid gap (ICG); c: chromatid break (CB); d: chromatid gap (CG); centromeric deletion (CD); e: centromeric break with fragments (Cb); f: centromeric gap (Cg); g: Polyploidy; h: Endoreduplication

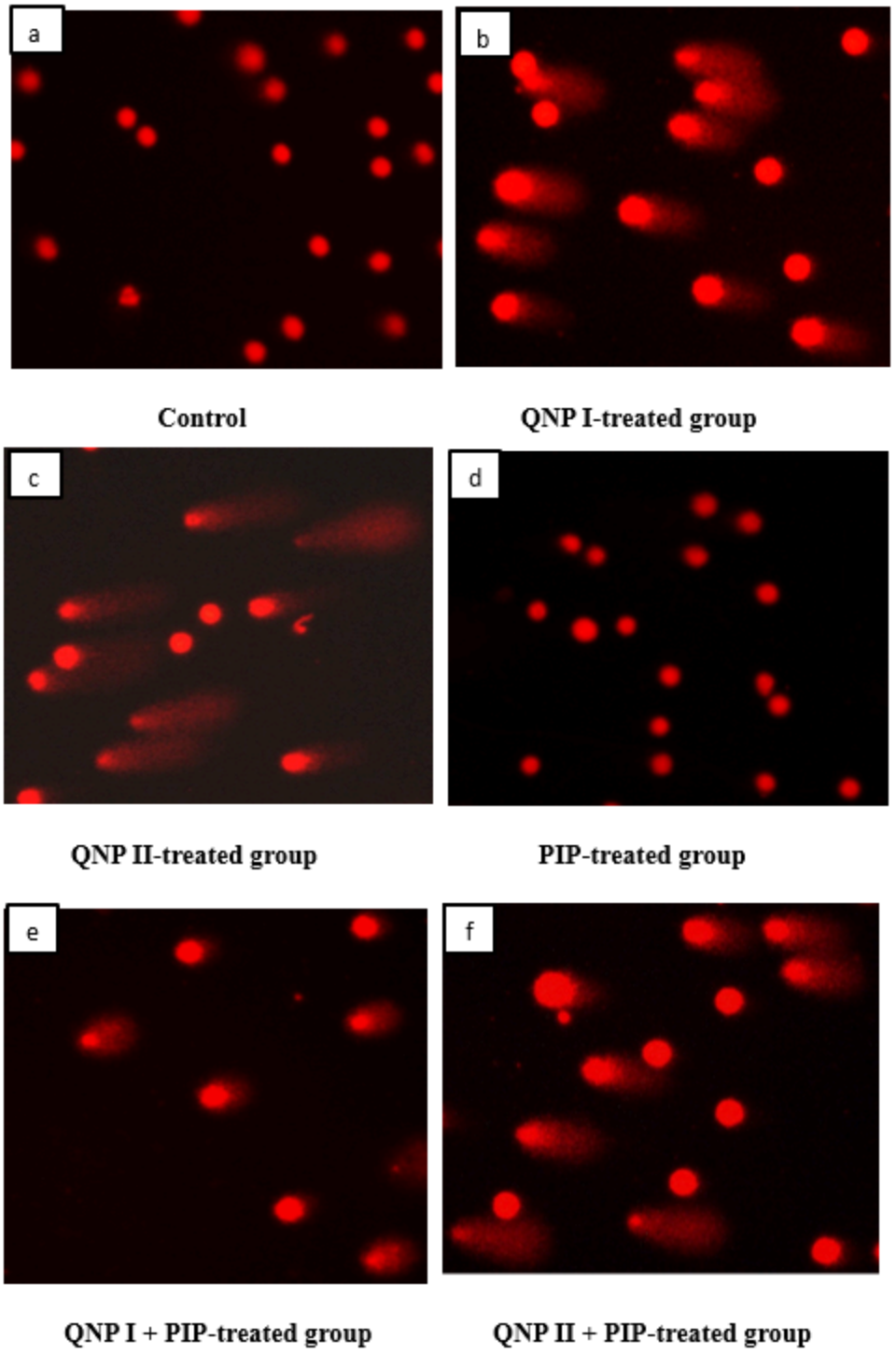

Figure 3

Representative images showing effects of subacute oral exposure of mice to QNP, piperine and their combination on DNA damage (comet assay) in bone marrow cells observed at 200x magnification of fluorescent microscope

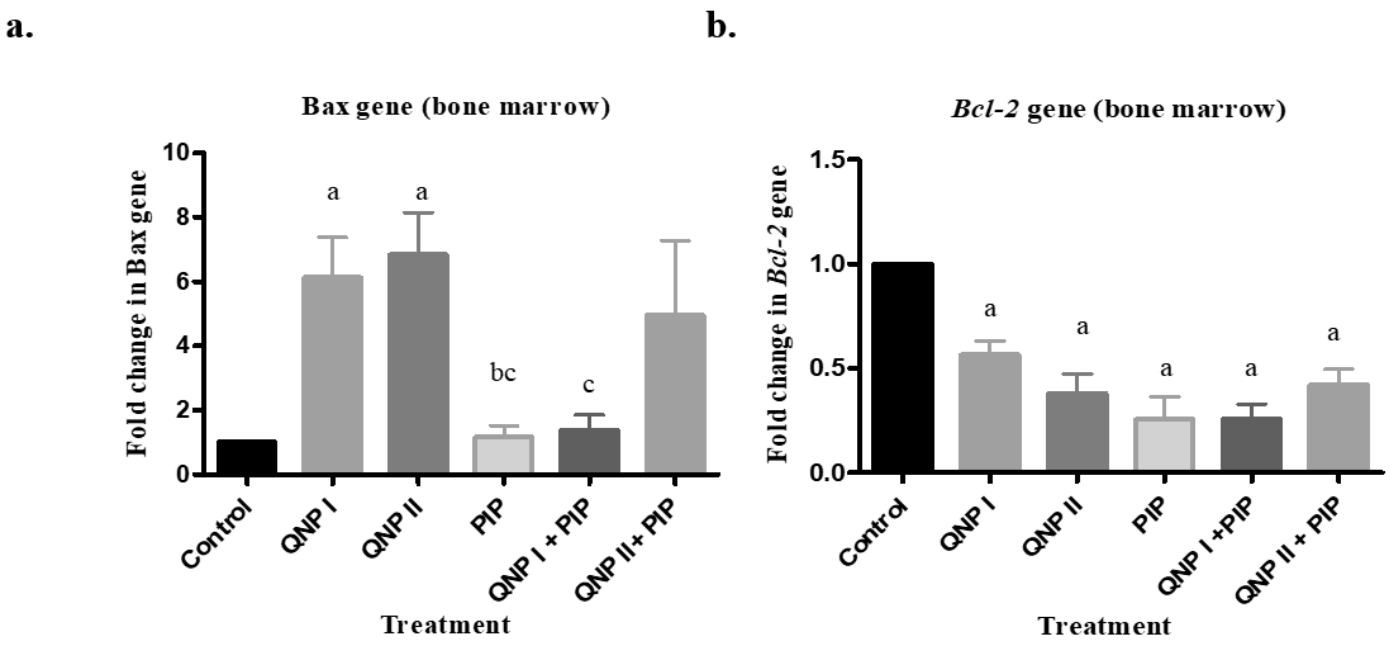

Figure 4

Effects of subacute oral exposure of QNP, piperine and their combination in the mRNA expression of apoptosis related gene Bax and *Bcl-2* of mice. QNP: quinalphos; PIP: piperine (10 mg/kg). Fig. a and b represent fold changes in the mRNA expression of apoptosis related gene Bax and *Bcl-2* of mice. The values were compared using one-way ANOVA followed by Tukey post-hoc test. Means bearing a, b, and c superscripts differ significantly ($P<0.05$) vs. control, QNP I, and QNP II, respectively

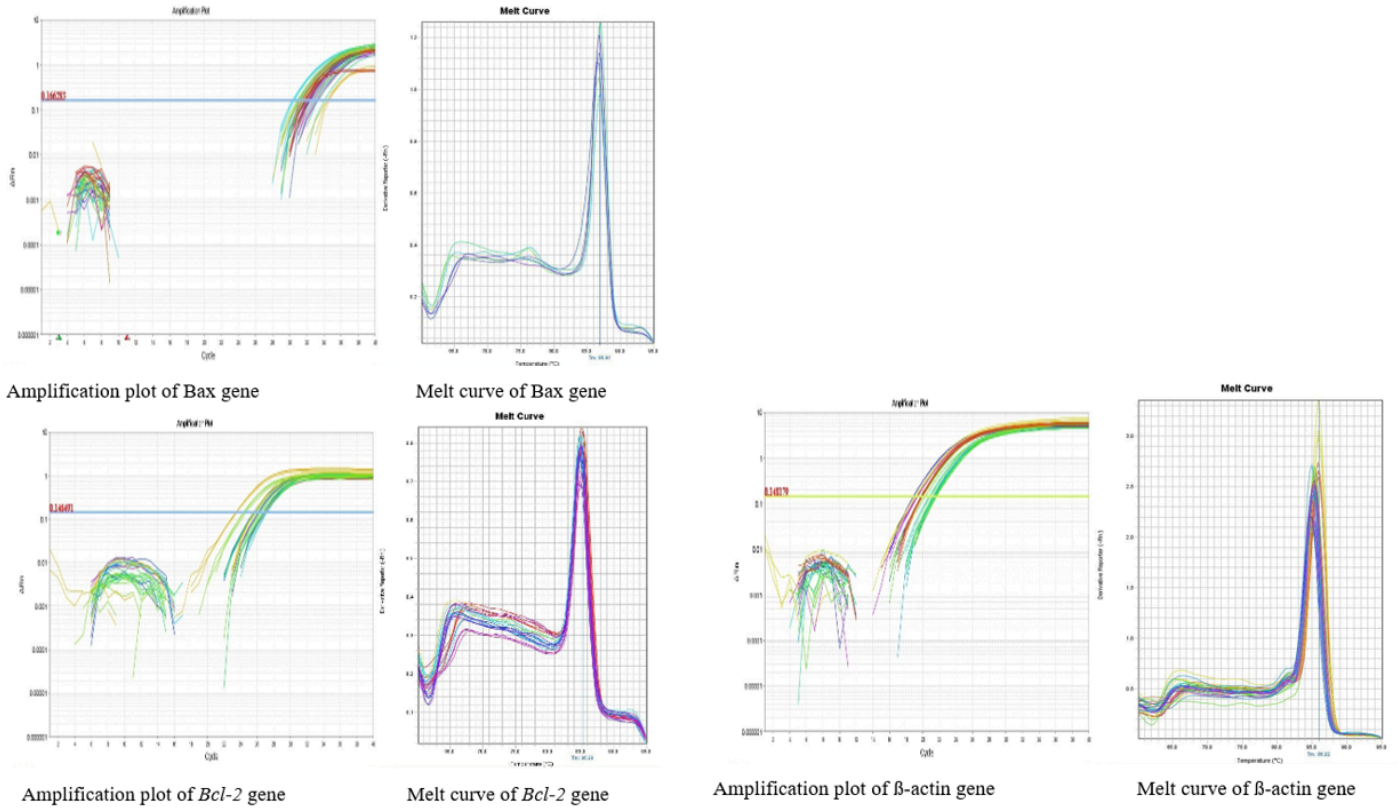

Figure 5

Representative images showing amplification plot and melt curve of Bax, *Bcl-2* and ß-actin gene in bone marrow of mice

Supplementary Files

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