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Ameliorative effects of vanillin on potassium bromate induces bone and blood disorders *in vivo*

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Abstract

The objective of this study was to investigate the propensity of potassium bromate ($KBrO_3$) to induce oxidative stress in blood and bone of adult mice and its possible attenuation by vanillin. Our results demonstrated, after $KBrO_3$ treatment, a decrease of red blood cells and hemoglobin and a significant increase of white blood cell. A decrease in plasma levels of folic acid, vitamin B_{12} and iron was also noted. Interestingly, an increase of lipid peroxidation, hydroperoxides, hydrogen peroxide, advanced oxidation protein products and protein carbonyl levels in erythrocytes and bone was observed, while superoxide dismutase, catalase and glutathione peroxidase activities and glutathione, non-protein thiol and vitamin C levels were decreased. $KBrO_3$ treatment resulted in blood and bone DNA fragmentation, a hallmark of genotoxicity-$KBrO_3$-induced, with reduction of DNA levels. Calcium and phosphorus levels showed a decrease in the bone and an increase in the plasma after $KBrO_3$ treatment. These biochemical alterations were accompanied by histological changes in the blood smear and bone tissue. Treatment with vanillin improved the histopathological, hematotoxic and genotoxic effects induced by $KBrO_3$. The results showed, for the first time, that the vanillin possesses a potent protective effect against the oxidative stress and genotoxicity in bone and blood of $KBrO_3$-treated mice.

Key words: Potassium bromate, vanillin, bone, erythrocyte, genotoxicty, antioxidant.

Introduction

Bromate is a major by-product formed during the ozonation process used for the disinfection of drinking water. As a consequence, bromate has been detected within both surface water and more recently within a United Kingdom chalk aquifer. The increasing use of ozonation for treatment of drinking water increases the health risks associated with exposure of humans to bromate (1). Moreover, significant bromate contamination, nobly from industrial origin, has led to formation of a plume within this aquifer and is currently affecting potable water abstraction in the area. Furthermore, $KBrO_3$ is used in the bread making process and is also added to flour, fish paste, beer and cheese (2). It is a constituent of cold wave hair solutions, used in cleaning boilers and oxidation of sulphur and dyes. This xenobiotic has been reported to have toxic and carcinogenic effects in rodents. It is classified as a complete renal carcinogen in animals and a probable human carcinogen (Group 2B carcinogen) by the International Agency for Research on Cancer (3). Indeed, exposures to $KBrO_3$ can cause renal cell tumours in rats, hamsters and mice, and thyroid and testicular mesothelial tumours in rats as well as causing DNA strand breaks and poly (ADP) ribosylation in the kidney with proliferative responses (4). It has been also demonstrated that $KBrO_3$-exposure can cause mutations, rearrangements and transcriptional errors that impair important cellular functions, in many cases necrosis and/or apoptosis (5).

The toxic effects of $KBrO_3$ are attributed probably to its ability to induce oxidative stress leading to enhanced production of reactive oxygen species (ROS) which are important mediators of tissue injury (6). The ROS are widely thought to be generated in the cell due to reduction of $KBrO_3$ to bromide by intracellular reductants. $KBrO_3$ directly or indirectly via ROS production, has been shown to induce oxidative modification of lipids and proteins in several animal tissues (4).

Supporting the involvement of ROS in bromate action, several antioxidants such as oligonol and catechin have been shown to ameliorate the bromate-induced toxicity (7). Vanillin (4-hydroxy-3-methoxybenzaldehyde) (figure 1), a phenolic compound isolated from the bean and pod of tropical vanilla orchid, is widely used in the food and beverage industry and is responsible for the characteristic vanilla flavor. Currently, it is added in a wide range of products such as pastry products, ice cream, soft drinks and baked products (biscuits, cereals), it used as a sleep prevention agent and an aphrodisiac (8). Discovered functional uses of vanillin indicate that it exhibits chemopreventive effects in multiorgan carcinogenesis models in rats and prevents the invasion and migration of cancer cells (9). Vanillin has been also shown to inhibit lipopolysaccharide-stimulated NF-KB activation and cyclooxygenase-2 gene expression in

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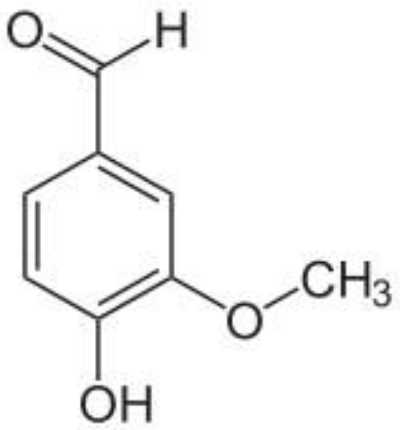

Figure 1. Chemical structure of vanillin.

marine macrophages (10). Besides its flavoring properties, vanillin possesses several bioactive properties such as antioxidant, and antimicrobial activities against yeasts, moulds and bacteria (10). It can also be used to treat sickle cell anemia (11). Bone and red blood cells have a close connection in some diseases such as sickle cell anemia which is characterized by an abnormality in the oxygen-carrying haemoglobin molecule in red blood cells.

To our knowledge there are no reports on oxidative stress induced by $KBrO_3$ in erythrocytes and bone. Thus, the purpose of this study was to investigate the effects of $KBrO_{3-}$ exposure on hematological parameters, oxidative stress, genotoxicity and histophatological changes and the ability of vanillin to improve $KBrO_3$-induced cyto and genotoxicity.

Materials and methods

Reagents

Potassium bromate ($KBrO_3$) and vanillin was purchased from Sigma–Aldrich (Germany: purity ≥99.8%). Other products such as glutathione (oxidized and reduced), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), vanillin and thiobarbituric acid (TBA) were also purchased from Sigma. Chemicals of analytical grade, used in our study, were purchased from standard commercial suppliers.

Experimental procedures

The experimental procedures were carried out according to the general guidelines on the use of living animals in scientific investigations (12) and approved by the Ethical Committee of the Sciences Faculty of Sfax. Adult mice (aged 2-3 months; weighing 25–30 g) were obtained from the Central Pharmacy (SIPHAT, Tunis, Tunisia). The animals were housed at a 22±2 °C temperature, 45±5% humidity and a 12-h light–dark cycle. 48 mice were housed individually in polyethylene cages and provided daily with standard pellet diet and water ad libitum. They were then randomly divided into four groups of twelve each: The first group of mice served as the control group, received ad libitum distillate water and standard diet. The second group ($KBrO_3$) received through drinking water 2g/L of $KBrO_3$. While the third group ($KBrO_3$+vanillin) was given a single intraperitoneal injection of vanillin, 100 mg/kg b.w per day, and 2g/L of $KBrO_3$ added to their drinking water. Animals in the fourth group (vanillin) were given daily a single intraperitoneal (i.p.) dose of vanillin in (100 mg/kg bw). The treatments were carried out for a period of 15 days. The present study was designed to investigate the toxicity of $KBrO_3$ administrated to mice via oral route as 2 g/L. This dose was selected on the basis of previous studies (13) and checked before the setting of the experiment. In fact, in a pre-study, we have tested several doses of $KBrO_3$ and an oxidative stress without lethal effects was observed in mice treated with $KBrO_3$ at doses under 2 g/L (0.5 g/L) only in peripheral blood (14). At dose corresponding to 2 g/L, few clinical signs of toxicity, oxidative stress in blood as well as in the bone were observed without mortality in adult mice. But with doses over 2 g/L, $KBrO_3$ provoked severe signs of toxicity and mortality. The vanillin dose was tested in our previous study and was proven to be effective against toxicity (14). Lower doses of vanillin gave less protection, while higher doses were not much more effective, as demonstrated by us and by others (14, 15).

During the experimental period (15 days), food and water intakes of the animals were monitored daily. At the end of the experimental period, the animals of different groups were killed by cervical decapitation to avoid stress.

- Some blood samples were collected in heparin tubes, some others were collected in EDTA. Other blood samples were immediately used for determination of hematological parameters:

* Heparined tubes were centrifuged at 2,200×g for 15 min. Plasma samples were then removed and served for determination of vitamins, iron, calcium and phosphorus levels. The sediment-containing erythrocytes were suspended in phosphate-buffered saline solution (0.9 % NaCl in 0.01 M phosphate buffer, pH 7.4) and centrifuged as reported by Sinha et al. (16).

* Blood samples collected with EDTA were served for determination of white blood cells (WBCs), Red blood cells (RBCs), hematocrit (Ht), Hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), platelet number, and MCH concentration (MCHC) by electronic automate Coulter MAXM (Beckman Coulter, Inc, Fullerton, CA).

- Femurs were dissected out and the surrounding muscles and connective tissues were removed. All samples were weighed. Some of them were intended for histological examination, others were mineralized to serve for calcium and phosphorus determination and others were homogenized with 2 mL of 0.1 M Tris–HCl buffer (pH 7.2) using a mortar and pestle according to Ramajayam et al. (17). The homogenates were centrifuged at 10.000 xg for 30 min at 4 °C and supernatants were used for biochemical assays.

Biochemical estimations

Hematopoietic factors

Some plasma samples were used for iron determinations by colorimetric method (BIOMERIEUX kit, France, Ref: 61075). Folate and vitamin B_{12} measurements were performed using immuno-electro-chemilu-

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minescence analysis (Elecsys Folate Immunoassay and Elecsys B_{12} Immunoassay for Elecsys 2010 System; Roche Diagnostics, USA).

Preparation of blood smear

A drop of fresh blood was spread on a slide, fixed with May-Gruinwald for 2 minutes, and rinsed with water. Then, Giemsa was used for staining. Different blood cells and platelets were visualized using an optical microscope at magnification (100x).

Calcium and phosphorus levels in plasma and femurs

Calcium and phosphorus levels were determined in femurs after nitric acid mineralization and in plasma using commercial reagent kits (Biocon, Ref. 2004 and 1904, respectively).

Protein quantification

Hemolysate and bone protein contents were measured according to the method of Lowry et al. (18) using bovine serum albumin.

Measurement of malondialdehyde (MDA) and lipid hydroperoxides (LOOHs)

The erythrocyte and bone MDA levels, index of LPO, were determined spectrophotometrically according to the Draper and Hadley method (19). The MDA values were calculated using 1,1,3,3-tetraethoxypropane as standard and expressed as nmoles of MDA/mg protein.

LOOHs were estimated using the ferrous oxidation in xylenol orange assay (FOX assay) as described by Jiang et al. (20). The amount of hydroperoxide produced was calculated using the molar extinction coefficient of 4.59 X 104 M 1 cm-1 and the results were expressed as nanomoles per milligram of protein.

Determination of advanced oxidation protein products (AOPP) levels in erythrocyte and bone

AOPP levels were determined according to the method of Witko's (21) and were cited by Kayali et al. (22). The concentration of AOPP for each sample was calculated using the extinction coefficient of 261 cm^{-1} mM^{-1} and the results were expressed as µmoles/mg protein.

Measurement of hydrogen peroxide (H_2O_2)

Measurement of H_2O_2 was carried out by the ferrous ion oxidation xylenol orange (FOX1) method (23). The amount of H_2O_2 in the supernatant was determined using a spectrophotometer at 560 nm.

Determination of protein carbonyl (PCO) levels in erythrocyte and bone

PCO contents were measured using the method of Reznick and Packer (24). The absorbance of the samples was measured at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($e = 2.2 \times 10^4$ cm^{-1} M^{-1}) and expressed as µmoles/mg of protein.

Determination of antioxidant enzyme activities in erythrocyte and bone

Catalase (CAT) activity was assayed by the method of Aebi (25). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of µmoles H_2O_2 consumed/min/mg of protein.

Superoxyde dismutase (SOD) activity was estimated according to Beauchamp and Fridovich (26). Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as units/mg of protein.

Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler (27). The decrease in absorbance at 340 nm was measured. The enzyme activity was expressed as nmoles of GSH oxidized/min/mg of protein.

Non-protein thiol (NPSH) levels

Erythrocyte and bone NPSH levels were determined by the method of Ellman (28). Absorbance of colorimetric reaction was measured at 412 nm. NPSH content was expressed as µmoles/mg of protein.

Erythrocyte and bone homogenate glutathione (GSH) levels

GSH was determined by the method of Ellman (28) modified by Jollow et al. (29). The method is based on the development of a yellow color when DTNB (5,5-dithiobtis-2 nitro benzoic acid) is added to compounds containing sulphydryl groups. The absorbance was measured at 412 nm after 10 min. Total GSH content was expressed as µg/mg of protein.

Hydrophilic antioxidants in erythrocyte and bone

Vitamin C determination was performed as described by Jacques-Silva et al. (30). The reaction product was determined using a color reagent containing dinitrophenyl hydrazine (4.5 mg/ml) and $CuSO_4$ (0.075 mg/mL). The data were expressed as µg of ascorbic acid/mg of protein.

Histological studies

Femurs, intended for histological examination, were taken and immediately demineralised for 72 h in acetic acid (1.7 mol L^{-1}) according to Talbot et al. (31). Then they were fixed for 48 h in 10% of formalin solution, embedded in paraffin, serially sectioned at 5 µm, and stained with hematoxylin–eosine for light microscopy examination (32).

DNA extraction and quantification

Total DNA and mRNA were isolated from 100 mg of bone tissue, and 100 ml of blood tissue according to the method of Chamczynski and Sacchi (33). Each sample was measured at a wavelength of 260 nm and total DNA content were expressed in µg/µl (Sambrook and Russell) (34). All determinations were performed in triplicate.

DNA fragmentation analysis

DNA fragmentation analysis of normal and experimental mice was isolated from the erythrocyte and bone tissue by the method described previously by Kanno et al. (35). The DNA fragmentation assay was performed by electrophoresis on genomic DNA samples using agarose/EtBr gel following the procedure described by Sellins and Cohen (36). All determinations were performed in triplicate

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Table 1. Effects of $KBrO_3$, vanillin and their combination on some hematologic variables in adult mice.

Erythrocyte parameters	Controls	$KBrO_3$	$KBrO_3$+ vanillin	Vanillin
RBC (10^6/µl)	8.11±0.26	6.54±1.05 **++	8.70±1.89	8.66±0.63
Hb (g/100ml)	12.58±0.34	11.63±0.11*	12.41±0.46	12.72±0.34
Ht (%)	42.76±1.52	42.9±5.09	41.9±8.91	43.32±2.42
VGM (mm^3/GR)	50.06±1.7	50.58±6.04	48.15±1.58	50.04±1.16
MCH (pg/GR)	15.08±0.6	14.66±2.38	14.35±0.96	14.72±0.80
MCHC (g/100ml)	30.12±0.3	28.88±1.11	29.75±1.70	29.8±0.55

Values are mean±S.D. for twelve mice in each group. $KBrO_3$ and vanillin treated groups vs control group: *p < 0.05; **p<0.01. $KBrO_3$-treated group vs ($KBrO_3$+ vanillin)-treated group: ++p < 0.01.

Statistical analysis

The data were analyzed using the statistical package program Stat view 5 Software for Windows (SAS Institute, Berkley, CA). Statistical analysis was performed using one-way analysis of variance followed by Fisher's protected least significant difference test as a post hoc test for comparison between groups. Student unpaired t-test was also used when comparison between two groups was required. All values were expressed as means ±SD. The 0.05 level was selected as the point of minimal statistical significance.

Results

Effects of $KBrO_3$ on general health

Death was not observed in any experimental groups during the treatment period (15 days). However, in $KBrO_3$ treated group, few clinical signs such as reduced activity, increasing weakness.

General characteristics

Our results show that $KBrO_3$ treatment induced a decrease in drinking water and food consumptions (-12% and -21% respectively). Co-treatment with vanillin improved food and water consumption which reached near normal values.

The femur weight and length decreased by 27% and 14% respectively when compared to the controls. The supplementation of vanillin increased the femur weight and length, reaching normal values.

Erythrocyte parameters

Compared with the controls, the $KBrO_3$-treated group had a significant decrease in RBC (-24%) (P<0.01) and in hemoglobin concentrations (-8%) (P<0.05). Other erythrocyte parameters such as Ht, MCV, MCH and MCHC did not change significantly after $KBrO_3$ treatment. Vanillin supplemented to the $KBrO_3$-treated group improved hematological parameters which reached normal values (table 1).

Hematopoietic factors

Exposure of adult mice to 2g/L of $KBrO_3$ altered also haematopoietic factors, especially folic acid and vitamin B_{12} in plasma which decreased by 34 and 48% respectively (P<0.001). Plasma levels of iron were also signantly decreased in $KBrO_3$-treated mice by 52% (P<0.001) were compared to controls. Supplementation of vanillin of $KBrO_3$ group ameliorated folic acid, vitamin B_{12} and iron levels, when compared to $KBrO_3$-treated group (figure 2).

WBC counts and platelet rates

A significant increase in WBC counts (+38%, P<0.001) and a significant decrease in platelets (-56%, P<0.001) of $KBrO_3$-treated mice were observed when compared with controls. The changes induced by the ad-

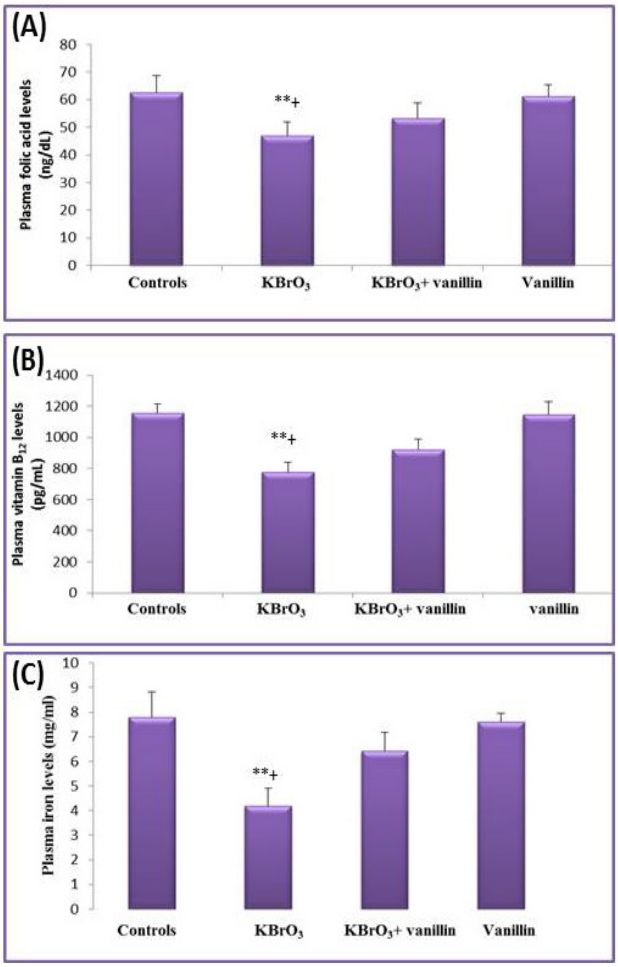

Figure 2. Folic acid (A), vitamin B_{12} (B) and iron (C) levels of adult mice: Controls and treated with $KBrO_3$ (2g/L), $KBrO_3$+vanillin or vanillin (100 mg/kg). The number of determinations for each parameter is n=12. The values are expressed as the means±S.D. Treated groups vs controls: **p<0.01. $KBrO_3$-treated group vs ($KBrO_3$+ vanillin) -treated group: +p < 0.05.

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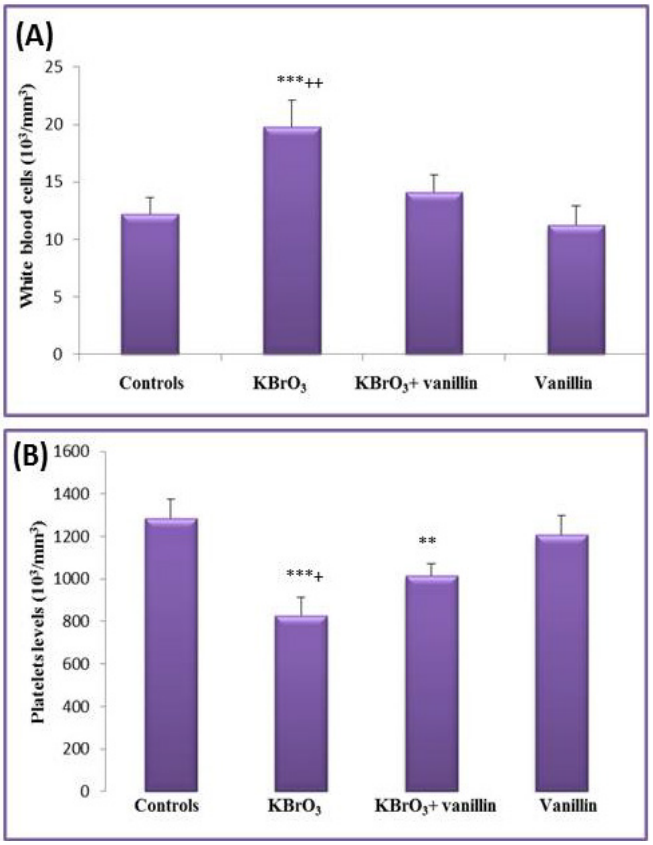

Figure 3. White blood cell counts (WBC) (A) and platelet (B) levels of adult mice: Controls or treated with $KBrO_3$ (2g/L), $KBrO_3$+vanillin or vanillin (100 mg/kg). The number of determinations for each parameter is n=12. The values are expressed as the means±S.D. Treated groups vs controls: **p<0.01; *** p<0.001. $KBrO_3$-treated group vs ($KBrO_3$+ vanillin)-treated group: +p < 0.05; ++ P<0.001.

ministration of $KBrO_3$ were significantly reversed by co-treatment with vanillin as observed in vanillin+$KBrO_3$ mice, while no significant changes were observed in WBC and platelet contents in vanillin treated mice when compared to the controls (figure 3).

Blood smears

In the mice treated with 2g/L of $KBrO_3$, an increase of lymphocyte number (figure 4 B) was observed. Blood smears of mice belonging to this group showed the presence of some necrotic cells (figure 4 B). The association of vanillin with $KBrO_3$ improved histopathological aspect of blood smears (figure 4).

Calcium and phosphorus levels in bone and plasma

$KBrO_3$ altered the bones mineral composition of adult mice, especially calcium and phosphorus contents which decreased by 39% and 16%, respectively. Calcium levels increased by 22% in plasma, while phosphorus levels decreased by 9% in plasma (table 2).

Histological studies

The disorders in bone formation seen in the histological sections of the femur growth plate of the control group, reflected in changes in its mineralization and consequently, the rate of bone turnover were correlated with histological studies. In fact, the growth plate in $KBrO_3$ treated mice was grossly disorganized compared to that of controls. Proliferating chondrocytes failed to form discreet columns and the hypertrophic zone was markedly diminished and morphologically indistinct. In addition, bone trabeculae in the primary spongium of control mice were organized parallel to the columns of proliferating chondrocytes, reflecting the functional continuity between maturing chondrocytes and mineralizing osteoblasts which were required for normal endochondral ossification. While in femur sections of $KBrO_3$

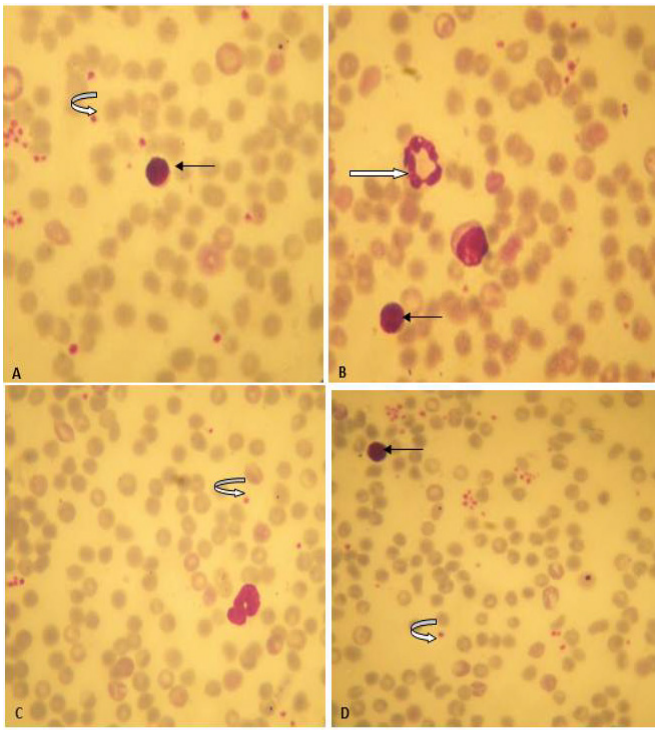

Figure 4: Blood smear of adult mice, controls (A) and treated for 15 days with $KBrO_3$ (B_1 and B_2), KBrO3+ vanillin (C) or vanillin (D). **Arrows indicated:** ⟶ : lymphocytes; ⟲: platelets; ⟹: necrosis.

Table 2. Calcium and phosphorus levels in plasma and bone of adult mice for 15 days: controls and treated with 2 g/L of KBrO3 and/or 100 mg/kg of vanillin.

Parameters & treatments	Controls	$KBrO_3$	$KBrO_3$+ vanillin	Vanillin
Plasma levels (mg/L)				
Calcium	72.77±3.46	94.39±5.73**+	88.81±3.29*	72.42±5.11
Phosphorus	43.38±.537	39.97±4.12*	40.79±2.87*	42.76±5.27
Bone levels (mg/g)				
Calcium	74.92±5.79	53.65±3.69**+	62.39±9.85*	76.72±9.85
Phosphorus	110.75±10.97	94.96±17.05**++	100.53±11.27*	115.03±8.90

The number of determinations is: n = 48 for femur weights; n = 12 for phosphorus and calcium contents. $KBrO_3$ and vanillin-treated groups vs. control group: *p<0.05; **p<0.01. $KBrO_3$-treated group vs ($KBrO_3$ + vanillin)-treated group: +p < 0.05; ++p < 0.01.

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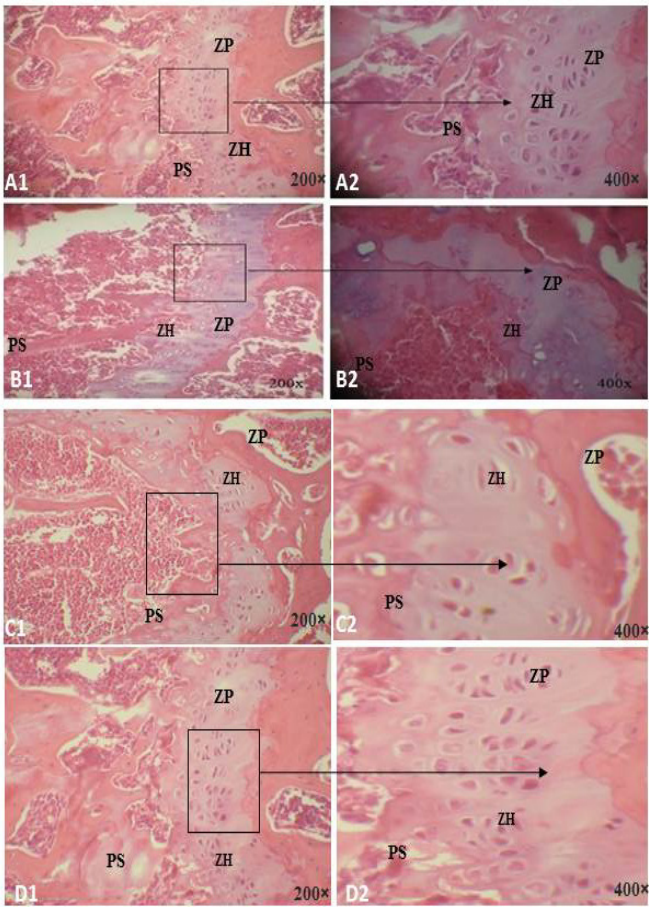

Figure 5. Bone histological sections of adult mice: controls (A1 and A2), treated with 2 g/L of $KBrO_3$ (B1 and B2), $KBrO_3$+ vanillin (C1 and C2) and treated with vanillin (D1 and D2). Optic microscopy; hematoxylin-eosin stain. PZ: proliferative zone HZ: hypertrophic zone PS: primary spongium region showing bone trabeculae.

treated mice, proliferating chondrocytes failed to form discreet columns. Hypertrophic chondrocyte differentiation and neovascularization in this region were greatly reduced. Compared to control group, treated mice displayed few, thin, and fragmented bone trabeculae. Vanillin supplementation improved the histological aspects of the femur (figure 5).

Estimation of lipid peroxidation

Our results revealed an increase of MDA, levels in the erythrocyte and bone of the $KBrO_3$-treated group as evidenced by the enhanced MDA levels in erythrocyte (+31%, P<0.001) and bone extracts (+29%, P<0.001) when compared to the controls. Supplementation of vanillin alleviated LPO and modulated significantly the MDA levels in femur and hemolysate of mice (table 3).

Estimation of LOOHs and H_2O_2 Production

Our results showed that LOOHs was increased in the erythrocyte (+49; P<0.001) and in the bone (+43; P<0.001) of $KBrO_3$-treated group when compared to the controls (Table 3). In addition, the H_2O_2 levels generated in the erythrocytes of adult mice significantly increased by 29%, and 39% in the bone (P<0.001). The administration of vanillin to ($KBrO_3$+vanillin) treated-group decreased significantly LOOHs and H_2O_2 production in both tissues compared to the $KBrO_3$-treated group.

Protein oxidative damage markers

Table 3 shows the levels of AOPP and PCO, markers of protein oxidative damage. In the $KBrO_3$-treated group, a significant increase of AOPP and PCO levels in erythrocyte (+48; +47%) and bone homogenates (+41; +28%) of treated mice was observed, respectively, when compared to the controls. The administration of vanillin to ($KBrO_3$ + vanillin) treated-group decreased significantly AOPP and PCO levels in both tissues compared to the $KBrO_3$-treated group.

Antioxidant enzyme activities in hemolysate and bone

Compared to the controls, a significant decline was noted in the activities of CAT, SOD and GPx in erythrocyte (-73%; -43% and -36% respectively), and bone (-50%; -41% and -32% respectively) extracts of adult mice treated with $KBrO_3$ (table 4). The administration of vanillin ameliorated enzyme activities in the $KBrO_3$+vanillin-treated group.

Table 3. MDA, protein carbonyl (PCO) and advanced oxidation protein products (AOPP) levels in erythrocyte and bone of adult mice: controls and treated with 2 g/L of KBrO3 and/or 100 mg/kg of vanillin for 15 days.

Parameters & treatments		Controls	$KBrO_3$	$KBrO_3$+vanillin	Vanillin
Erythrocyte	**MDA** (nmoles/mg protein)	29.21±3.93	41.92±2.94***+	36.25±3.83*	30.84±5.76
	AOPP (nmoles/mg protein)	0.28 ±0.18	0.53 ± 0.09**+	0.40 ±0.05*	0.31 ±0.03
	PCO (µmoles/mg protein)	2.11±0.46	3.15±0.32***+	2.53±0.57*	2.15±0.51
	H_2O_2 (µmoles/mg of protein)	0.19±0.03	0. 34±0.07***+	0.26±0.08**	0.20±0.04
	LOOH (nmoles/mg protein)	2.48±0.12	3.61±0.44***+	3.12±0.90**	2.61±0.39
Bone					
	MDA (nmoles/mg protein)	48.29±9.40	69.74±9.06***++	58.66±4.23**	46.42±5.10
	AOPP (nmoles/mg protein)	0.48±0.07	0.85±0.18***++	0.69±0.06**	0.50±0.17
	PCO (µmoles/mg protein)	3.21±0.09	6.07±1.20***+	5.47±0.86*	3.56±0.48
	H_2O_2 (µmoles/mg of protein)	0.04±0.006	0.08±0.003**+	0.05±0.002	0.05±0.001
	LOOH (nmoles/mg protein)	1.72±0.21	2.29±0.43***++	1.97±0.65*	1.66±0.82

Values are means ±S.D. for twelve mice in each group. $KBrO_3$ and vanillin-treated groups vs. control group: *p<0.05; **p<0.01;***p<0.001. $KBrO_3$-treated group vs ($KBrO_3$ + vanillin)-treated group: +p < 0.05; ++p < 0.01.

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Table 4. Antioxidant enzyme activities (CAT, SOD and GPx) in erythrocyte and bone of adult mice controls and treated with $KBrO_3$, vanillin or their combination ($KBrO_3$+ vanillin).

Parameters & treatments	Controls	$KBrO_3$	$KBrO_3$+ vanillin	Vanillin
Erythrocyte				
CAT (µmoles H_2O_2 degraded/min/mg protein**)**	13.57±2.69	7.81±3.24**++	10.95±1.47*	12.43±1.38
SOD (units/mg protein)	92.31±4.46	64.27±5.81***+	76.94±4.97**	90.42±6.04
GPx (nmoles of GSH/min/mg protein)	36.17±5.09	27.45±3.97**+	31.93±4.22*	35.44±2.41
Bone				
CAT (µmoles H_2O_2 degraded/min/mg protein**)**	24.28±3.56	16.17±2.97**++	21.19±2.47	26.20±1.39
SOD (units/mg protein)	78.12±3.39	55.46±4.83***+	64.31±4.99**	76.49±6.23
GPx (nmoles of GSH/min/mg protein)	29.38±2.19	21.64±3.54**	25.85±3.61*	28.19±3.22

Values are means ±S.D. for twelve mice in each group. $KBrO_3$ and vanillin-treated groups vs. control group: *p< 0.05; **p<0.01; ***p<0.001. $KBrO_3$-treated group vs ($KBrO_3$ + vanillin)-treated group: +p < 0.05; ++p < 0.01.

Table 5. GSH, NPSH and vitamin C levels in erythrocyte and femur of adult mice controls and treated with $KBrO_3$, vanillin or their combination ($KBrO_3$+ vanillin).

Parameters & treatments	Control	$KBrO_3$	$KBrO_3$+vanillin	Vanillin
Erythrocytes				
GSH (µg/mg protein)	113.47±6.79	94.51±5.42***+	104.27±3.16**	115.29±6.23
NPSH (µmoles GSH/mg protein)	27.42±1.41	19.97±2.03***	21.22±1.90**	28.43±2.66
Vitamin C (µmoles ascorbic acid/mg protein)	193.42±4.67	161.39±6.32***+	177.52±3.42*	196.28±7.55
Bone				
GSH (µg/mg protein)	194.68±10.32	146.30±9.47***++	172.87±8.03**	190.21±7.17
NPSH (µmoles GSH/mg protein)	34.91±3.49	23.18±4.72***+	27.04±2.15*	31.83±3.85
Vitamin C (µmoles ascorbic acid/mg protein)	232.26±7.34	204.30±9.71***+	211.87±10.25**	230.10±4.91

Values are means ±S.D. for twelve mice in each group. $KBrO_3$ and vanillin-treated groups vs. control group: *p< 0.05; **p<0.01; ***p<0.001, $KBrO_3$-treated group vs ($KBrO_3$ + vanillin)-treated group: +p < 0.05; ++p < 0.01.

Non-enzymatic antioxidant levels in erythrocyte and bone

Our results revealed a significant decrease in GSH, NPSH and vitamin C levels in erythrocyte (-20%, -37%, -20%) respectively and femur extracts (-12%, -50%, -13%) respectively of $KBrO_3$-treated mice, when compared to the controls (table 5). Administration of vanillin ameliorated enzyme activities in ($KBrO_3$ + vanillin) treated-group compared to those of the $KBrO_3$-treated group.

Effects of $KBrO_3$ on DNA Fragmentation and DNA quantification

Agarose gel electrophoresis showed undetectable DNA laddering in the blood and bone of the control mice. The DNA intact band appeared to be condensed near the application point with no DNA smearing, suggesting no DNA fragmentation in the control group while a smear (a hallmark of necrosis) without ladder formation on agarose gel was observed in the blood and bone of the $KBrO_3$ treated mice. The co-treatment with vanillin and $KBrO_3$-treated group resulted in the absence of DNA smearing (figure 6).

The genotoxicity of $KBrO_3$ was confirmed by a significant decrease in DNA contents in the bone and blood of the $KBrO_3$-treated group. While in mice treated with $KBrO_3$ and vanillin, there was an increase in DNA contents, reaching normal values, when compared with the $KBrO_3$-treated group (figure 6).

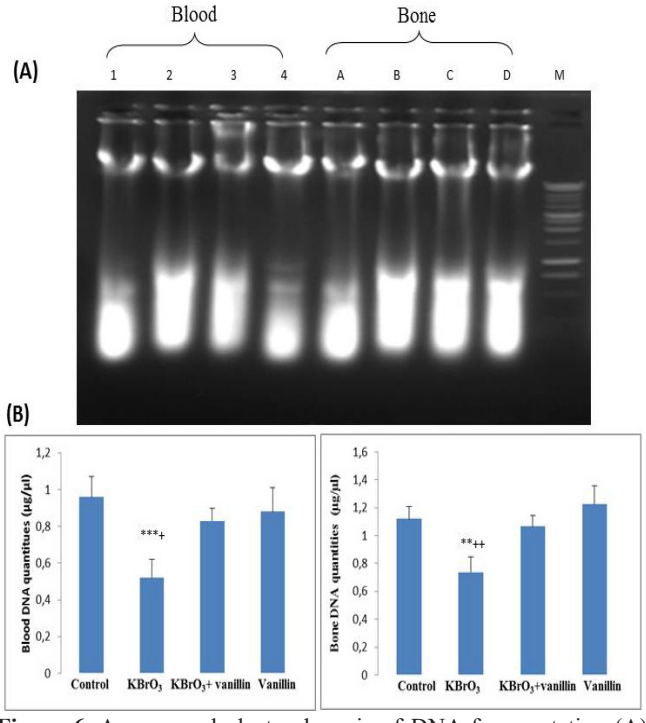

Figure 6. Agarose gel electrophoresis of DNA fragmentation (A) and DNA quantities (B) in the bone and blood. M: marker ; lane 1 control group of blood, lane 2 $KBrO_3$-treated group, lane 3 $KBrO_3$+vanillin-treated group, lane 4 vanillin group, lane A control group of bone, lane B $KBrO_3$-treated group, lane C $KBrO_3$+vanillin-treated group, lane D vanillin group. Treated groups vs controls: **p<0.01; *** p<0.001. $KBrO_3$-treated group vs ($KBrO_3$+ vanillin)-treated group: +p < 0.05; ++ P<0.001.

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Discussion

Our results demonstrated that $KBrO_3$, when administered via drinking water (2g/L) to adult mice, induced abnormalities in some blood cell parameters. We have noted a significant decrease in RBC counts and haemoglobin. Moreover, other erythrocyte parameters such as hematocrit, MCV, MCH and MCHC did not change, as compared to control groups. Thus Hoogstratten et al. (37) reported that anemia could be due to some haematopoietic factors' deficiency. The major ones are iron, folic acid and vitamin B_{12}. Our results showed that mice, after $KBrO_3$ treatment, exhibited a significant decrease of iron, serum folic acid and vitamin B_{12}. These vitamins (B_9 and B_{12}) are necessary for normal haematopoiesis, DNA synthesis and cellular division (38). In fact, folate serves as a coenzyme in single-carbon transfers in the metabolism of nuclei and amino acids. It is required for synthesis of purines and pyrimidines that are needed for DNA production and erythropoiesis. A deficiency of folate causes abnormal cell replication, particularly in the erythropoietic system (38). Anemia also could be obtained after vitamin B_{12} deficiency, since this vitamin is required for normal erythrocytes production. Additionally, the main function of iron in the body is in erythropoiesis. It serves as a functional component of iron-containing proteins including haemoglobin and myoglobin (39). It deficiency also causes an impairments in the erythropoietic system. Vanillin corrected the anomalies induced by $KBrO_3$ in hematological parameters. Previous studies showed that vanillin is useful as anti-sickle cell anemia (11), anti-mutagen and anti-bacteria agent at high concentration of un-oxidized form to be medically effective, as well as antioxidant (40).

Several clinical and experimental studies have indicated that vitamin B deficiency was usually associated with impairment in bone quality (41). In addition to that, it is well known that anemia was occurred if there are any problems at their production site in the bone. The subject of bone and red blood cells arises in regard to the various diseases that lead to anemia. In fact, a number of abnormalities might be seen in red blood cells when there is a problem within the bone. In fact, our results determined a number of abnormalities in bone of $KBrO_3$-treated mice, including lower femur weight and length and reduction of bone mineral composition. These alterations could be attributed to the removal of calcium and phosphorus from bone tissue. As consequence, the net activity of bone-resorbing cells is more accentuated than that of bone-forming cells (41).

Bone mineral loss and anemia, induced by $KBrO_3$-treatment could attribute to oxidative stress generated directly by this xenobiotic or indirectly by its induction to free radicals production. In fact, when administered orally, $KBrO_3$ is rapidly absorbed from the gastrointestinal tract, appears in the blood and then it is distributed to other tissues (42). Inside the cell, $KBrO_3$ is reduced to bromide by intracellular reductants like GSH (42). This reduction process probably generates ROS that are thought to mediate cellular damage. Our data showed an increase in H_2O_2 and LOOHs levels in both erythrocyte and bone of $KBrO_3$-treated mice, suggesting its participation in generating free radicals. These radicals and others could cause deleterious effects on biological molecules (lipids, proteins, DNA, enzymes) in both tissues as well as disorders in bone turnover. Indeed, ROS are involved in bone remodelling by promoting its resorption. During this process, osteoclasts generate large quantities of ROS, which excessive accumulation suppresses bone formation and stimulates further resorption (43). ROS, depending on their concentration, can either have beneficial or deleterious effect on the bone cells. Disorders in bone formation, reflected in changes of mineralization and the rate of bone turnover, are supported by histological studies. In fact, the growth plate in the $KBrO_3$-treated group was grossly disorganized compared to that of the control group. Proliferating chondrocytes failed to form discreet columns and the hypertrophic zone was markedly reduced and morphologically indistinct. In addition, bone trabeculae in the primary spongium of control mice were organized parallel to the columns of proliferating chondrocytes, reflecting the functional continuity between maturing chondrocytes and mineralizing osteoblasts which were required for normal endochondral ossification. Whereas, in treated mice this relationship was disrupted with fragmented and disorganized bone trabeculae relative to controls. These modifications could probably be due to the accumulation of free radicals resulting from the $KBrO_3$-induced bone cytotoxicity. In fact, Garrett et al. (44) demonstrated the relationships between oxygen-derived free radicals and bone mineral and histological disorders. Vanillin supplementation to the $KBrO_3$-treated group improved the histological pictures of the femur evidenced by its return to its normal aspects in which the phenomena of proliferation must have been involved. Vanillin supplementation may probably regulate bone mineral composition. This oligoelement exhibits a protective role against bone impairment via it antioxidant properties. Mitomycin C- and methylmethane sulphonate-induced mutations in mouse bone cells were also reduced by vanillin, as demonstrated by Karathanos et al. (45).

The relationship between bone and erythrocyte lies in the fact that red blood cells are produced in the bone marrow which is the soft fatty tissue found within bone cavities. This relationship is so close when blood cells are abnormal. In fact, bone disorganization was accompanied with hematotoxic effects induced by $KBrO_3$. Our data demonstrated that $KBrO_3$ caused a significant increase of lipid peroxidation as shown by the enhancement of MDA production which was more pronounced in the erythrocytes than in the bone of treated mice. In fact, erythrocytes are highly and more susceptible to oxidative damage, than bone tissue, due to their high rate of oxidative metabolic activity and high content of polyunsaturated fatty acids (46). In addition, ROS probably generated by $KBrO_3$, induced a rise of advanced oxidation of protein products (AOPP) and protein carbonyls (PCO) products, markers of protein oxidative injuries in erythrocyte and bone tissues. Free radicals attack also DNA bases in bone and peripheral blood, therefore causing mutagenic lesions. Hematoxicity and bone genotoxicity were confirmed by DNA fragmentation test on agarose gel and a reduction in the DNA levels. As far as smears histopathological observations were marked by a necrosis and apoptotic white blood cells. There are many different kinds of evaluation re-

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ports for the antioxidant activity of vanillin against ROS and other radicals, though the ROS-scavenging activity as mentioned above seems to be one of the factors for the anti-mutagenic property of vanillin. However, it has also been reported that vanillin inhibited protein oxidation and lipid peroxidation induced by photosensitization in rat liver mitochondria and it exhibited hydroxyl radical (47) and 2,2′-azinobis(3- ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•+) (14) scavenging activities. The mechanism by which vanillin prevented against $KBrO_3$ induced cytotoxicity could be explained by a decrease in the metabolic activation of $KBrO_3$ or by its action as a chain-breaking antioxidant for scavenging free radicals, or by a combination of these effects. P*revious* study of Kamat et al. (48) demonstrated that vanillin can react with radicals via adducts formation or self-dimerization mechanism, contributing to a high reaction against ABTS and DPPH-radicals. Furthermore, it has been reported, by previous finding, that vanillin inhibits protein oxidation and lipid peroxidation induced by photosensitization in rat liver mitochondria and exhibits hydroxyl radical and radical cation scavenging activities (48). Part of these biological properties can be attributed to the fact that vanillin is a phenolic compound able to scavenge free radicals.

Although the detailed mechanisms of $KBrO_3$-mediated toxicity is still unknown, our data showed that this compound increase free radicals production, including H_2O_2, which disturbs oxidant/antioxidant balance. The antioxidant system includes different antioxidant enzymes namely SOD, CAT, GPx together with the substances which are able to reduce ROS, like GSH. Besides, different antioxidants like ascorbic acid, vitamin E scavenge ROS (49). In the current study, $KBrO_3$ administered through drinking water at a dose of 2g/L significantly reduced the activities of antioxidant enzymes like SOD, CAT and GPx in erythrocyte and bone. The possible explanation is that superoxide radicals can inhibit CAT activity and CAT inhibition finally reduces SOD activity. This is an indicative of free radicals formation rate (50), mainly that the SOD-CAT system provides the first defense system against oxygen toxicity. For GPx, the decrease in its activity may be the result of O_2 production or a direct action of $KBrO_3$ on the synthesis of the enzyme (51). Likewise, GSH, NPSH and vitamin C, the crucial components of the non-enzymatic antioxidant defense mechanism, function as a direct reactive free-radical scavenger (52). Recent studies have shown that free radicals production such as H_2O_2 or xanthine oxidase generated superoxide anions which are able to inhibit osteoblastic differentiation in mouse and rabbit marrow cells (53). Nevertheless, these super oxide anions are found to stimulate osteoclast differentiation and bone resorption (54). The decrease of GSH and NPSH levels in the hemolysates and in bone homogenate of $KBrO_3$ treated mice might be due to their consumption in the scavenging free radicals probably generated by $KBrO_3$. Since GSH is involved to recycle vitamin C by mediating the reduction of dehydroascorbate, its deficiency would be expected to produce a depletion of vitamin C in the erythrocytes and bone of $KBrO_3$-treated mice. *In vitro* studies indicate that liver and kidney tissues degrade bromate to bromide and that glutathione GSH is probably involved in that degradation (42). The ability of vanillin to modulate the activity of enzyme antioxidants in the erythrocyte and bone tissues has been demonstrated in our study, indicating the antioxidative action of this molecule (55). Vanillin has been reported to inhibit mutagenesis induced by chemical and physical mutagens and to suppress the invasion and migration of cancer cells (56). It also displays chemopreventive effects in multiorgan carcinogenesis and hepatocarcinogenesis models in rats (57). Moreover, vanillin displays antimicrobial and antioxidant properties and is used as a food preservative and for medicinal purposes (10).

In conclusion, co-treatment with vanillin exhibits protective effects against oxidative stress induced by $KBrO_3$ in the erythrocyte and bone. The protection of vanillin includes the improvement of the haematological parameters, the antioxidant enzymes activities, the inhibition of DNA damages and the amelioration of histopathology in blood and bone tissues. The mechanisms through which vanillin lowers oxidative stress can be attributed to its high content of phenolic compounds, which exhibit antioxidant properties**.**

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