

# Evaluation of Biological Properties, Health Benefits and Protective Effects of Kefir against Cytotoxicity and Genotoxicity Induced by Butylated Hydroxytoluene (BHT) *In Vivo*

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## Abstract

Kefir is a beverage made from milk and considered as a probiotic due to its antioxidant and anti-inflammatory properties. The present study aimed to explore the ability of fermented kefir to improve the adverse effects induced by Butylatedhydroxytoluene (BHT) in the blood, liver and renal tissues of rats. Adult Rats were exposed during 21 days either to BHT, Kefir and BHT. BHT treatment resulted in the disruption of hematological parameters. In fact, a genotoxic effect on white blood cells was evidenced by a significant increase in the micronuclei frequency and a decrease in cells viability as well as a significant change in plasma biochemical parameters. Our results also demonstrated important alterations in the liver and kidney expression levels of pro-inflammatory cytokines and oxidative stress. These modifications were substantiated by histopathological data. Interestingly, fermented kefir restored these parameters to near control values.

**Keywords:** Kefir; Butylated hydroxytoluene; Tumor necrosis factor- $\alpha$ ; Antioxidant activity; Histological study

**Abbreviations:** ABTS: Diammonium Salt; BHT: Butylated Hydroxytoluene; Ca: Calcium; Cd: Cadmium; Cr: Chrome; DPPH: 1,1-Diphenyl-Picrylhydrazyl; Fe: Iron; Gpx: Glutathione Peroxidase; GSH: Glutathione; H<sub>2</sub>O<sub>2</sub>: Hydrogen Peroxide; Hb: Hemoglobin; Ht: Hematocrit; IL-6: Interleukin-6; IP: Injected Intraperitoneally; LAB: Lactic Acid Bacteria; MCH: Mean Corpuscular Hemoglobin; MCHC: MCH Concentration; MCV: Mean Corpuscular Volume; MDA: Malondialdehyde; Mg: Magnesium; MN: Micronucleus; Na: Sodium; NADPH: Nicotinamide Adenine Dinucleotide Phosphate Reduced Form; NF-Kb: Nuclear Factor Kappa B; Ni: Nickel; OD: Optical Density; Pb: Lead; PBS: Phosphate Buffered Saline; RBCs: Red Blood Cells; ROS: Reactive Oxygen Species; RT-PCR: Reverse Transcript Polymerase Chain Reaction; SOD: Superoxide Dismutase; TB: Total Bilirubin; TBA: Thiobarbituricacid; TEAC: Trolox Equivalent Antioxidant Capacity; TLC: Thin-Layer Chromatography; TNF- $\alpha$ : Tumor Necrosis Factor Alpha; TVC: Total Viable Counts; UHT: Ultra Heat Treated; WBC: White Blood Cells; Zn: Zinc

## Introduction

Butylated Hydroxytoluene (BHT) is an alkylphenol widely used as a synthetic antioxidant and food preservative [1]. Despite

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its antioxidant activity, its administration in high doses may exert pro-oxidant effects on the target organism. Indeed, its tolerable daily intake is frequently exceeded due to overfeeding, mainly in individuals with extremely high caloric intake, thus leading to its accumulation [2]. In addition, the decline of residual BHT in the tissues after continual feeding takes a long time. Therefore, these residues accumulate, particularly, in adipose tissues and detoxifying organs [3]. Details on blood, liver and kidney antioxidant capacity when using BHT during food poisoning are not known. It is probably exerted through Reactive Oxygen Species (ROS) linked to high levels of Malondialdehyde (MDA), which causes lipid peroxidation [4]. ROS are also often responsible for Nuclear Factor kappa B (NF- $\kappa$ B) activation, thus increasing the expression of pro-inflammatory biomarkers such as Tumor Necrosis Factor-alpha (TNF- $\alpha$ ), and Interleukin-6 (IL-6).

Researches on both animals and humans have revealed that the consumption of some dietary components, such as fibers, phytosterols, polyphenols, bioactive peptides, and probiotics, can modulate metabolism disruption and contribute to the reduction of oxidative stress-related diseases [5]. The nutritional sources of probiotics are fermented yogurts, cheese, pickles, raw sausages, bread, beer, wine, kumis and kefir using *Lactobacilli*, *Bifidobacteria*, *Enterococcus*, and *Streptococcus* [6]. In fact, Kefir is reported to be a probiotic thanks to its health benefits and disease prevention properties. Being a traditional drink, it is obtained via milk fermentation by “kefir grains”, which are complex mixtures of bacteria, yeast and polysaccharides produced by this microflora. Thanks to their health-promoting properties, kefir grains are widely used in China and around the world [5]. The main gradients in kefir are *Lactobacillus kefirianofaciens*, *Lactobacillus buchneri*, and *Lactobacillus helveticus* [6]. Yet, its microbiological composition may change due to growing conditions and the substrate utilized for grains and grains origin proliferation. Besides, the beverage comprises a mixture of lactic acid, carbon dioxide, acetaldehyde, ethanol and vitamin B [6]. Even though studies remain scarce and contradictory, a wide range of biological activities have been reported in kefir, including anti-inflammatory, antifungal and antibiotic activities against yeast and acetic acid bacteria in intestinal microflora [6].

This investigation aimed to validate the *in vitro* and *in vivo* effects of kefir: firstly, it was tested for its mineral and bacterial composition as well as its antiradical activities *in vitro* via 1,1-diphenyl-picrylhydrazyl (DPPH) and diammonium salt ABTS•+-radical-scavenging assays. Secondly, its protective effects against BHT-induced oxidative damage were examined *in vivo* by evaluating the gene expression of pro-inflammatory biomarkers, antioxidant status, plasma biochemical liver and kidney markers and histopathological changes. Also, and in order to test its protection ability against BHT-induced genotoxicity in the peripheral blood, micronucleus and viability tests were carried out to quantify genetic damage. Animal models were used in this study for handling ease purposes, with the intention of exploring kefir’s action mechanisms.

## Materials and Methods

### Chemicals and reagents

The selected alkylphenol Butylated Hydroxytoluene (BHT) required for biochemical assays was obtained from Sigma Chemicals Co. (St. Louis, France). Compounds such as 1,1- diphenyl-picrylhydrazyl (DPPH), Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), glutathione (oxidized and reduced), Nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB) and thiobarbituric acid (TBA) were purchased from Sigma (St. Louis, MO, USA). Other compounds were obtained from various other suppliers.

### Experimental procedure

**Production and preparation of Kefir:** The used ingredients were as follows: Commercial Ultraheat treated (UHT) sterilised semi-skimmed milk (containing per 100 g: proteins=3.2 g; carbohydrates=4.5 g; lipids  $\approx$  1.5 g {butyric acid=0.0611 g, caproic acid=0.0376 g, caprylic acid=0.0188 g, capric acid=0.047 g, lauric acid=0.065 g, myristic acid=0.17 g, palmitic acid=0.437 g, stearic acid=0.164 g, monounsaturated fatty acid=0.425 g, polyunsaturated fatty acid=0.0558 g, Omega 3=0.00987 g, alpha-linolenic acid=0.00987 g, omega 6=0.033 g, linoleic acid=0.033 g, omega 9=0.326 g, oleic acid=0.326 g} and *Kefir grains microflora* (not genetically modified) originating from Tibet, China, and consisting of gelatinous, irregularly-shaped grains formed by a symbiotic combination of yeasts and acetic and lactic acid bacteria [5,7]. The matrix preparation was carried out in the following way: the activated grains (30 g) were inoculated in commercial UHT skimmed milk (1.000 mL) and statically incubated at 25°C for 24 h. Beverage samples (1 mL) were aseptically taken every 6 h. Once the desired pH of 4.6 was reached, fermentation was stopped by cooling the flasks in an ice bath and storing them under refrigeration at 4°C until use. Kefir was prepared as described above, once a week, thus ensuring freshness.

**DPPH radical-scavenging assay of fermented kefir:** The samples' free radical scavenging activity was determined according to the method of Brand-Williams et al. [8], with minor modifications of Takebayashi et al. [9]. An aliquot of ethanol absolute solution (0.1 mL) containing different sample concentrations (1:2 serial dilutions from the initial sample) was added to 3.9 mL of DPPH solution (0.06 mM in ethanol). The obtained mixture was then vortexed vigorously and incubated at room temperature (25°C) in darkness for 60 min, and absorbance was read at 517 nm against ethanol blank using a spectrophotometer (Uvi Light XT5). The

IC50 values denote the concentration of the tested compounds required to scavenge 50% of DPPH free radicals. The corresponding inhibition percentages were calculated according to the following equation:

$$\text{Radical scavenging activity (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

Where  $A_{\text{blank}}$  is the absorbance of the control (prepared in the same manner without test compound), and  $A_{\text{sample}}$  is the absorbance of the tested compound. Ascorbic acid and BHT were used as a standard control. The values are presented as the means of triplicate analyses.

**Measurement of the Trolox equivalent antioxidant capacity (TEAC):** The radical-scavenging activity was determined according to Re et al. [10] and the radical cation was prepared by reacting an ABTS aqueous solution (7 mM) with potassium persulfate (2.45 mM, final concentration) kept in the dark at 25°C for 12 h-16 h. The obtained solution was diluted in ethanol to an absorbance of 0.70 ( $\pm$  0.020) at 734 nm before use. A volume of 10  $\mu$ L of Trolox or sample in ethanol was mixed with 990  $\mu$ L of this diluted solution and absorbance was determined at 734 nm and 30°C, 6 min after initial mixing. Appropriate solvent blanks were run in each assay, and the decolorization extent was estimated by monitoring the absorbance reduction at 734 nm. The anti-oxidant activity was determined according to compounds and calculated relatively to the equivalent Trolox concentration. Each antioxidant activity was determined at three concentrations, within the range of the dose-response curve of Trolox. The radical scavenging activity was expressed as the TEAC defined as mM of Trolox.

**Mineral Contents of fermented kefir:** The concentrations of Iron (Fe), Magnesium (Mg), Calcium (Ca), Zinc (Zn), Sodium (Na), Lead (Pb), Cadmium (Cd), Chromium (Cr) and Nickel (Ni) in kefir were measured according to the method of Ial [11], following nitro per chloric mineralization (2/1 V/V) by atomic absorption spectroscopy (model Thermo-Scientific ICE 3000, Sherwood Scientific Ltd., Cambridge, UK). The wavelengths used to quantify the analyzed elements were first defined on the device: Mg (285.2 nm), Ca (422.7 nm), Cd (766.5 nm), Na (589 nm), Fe (248.3 nm), Zn (213.9 nm), Cr (324.8 nm), Ni (232 nm) and Pb (283.3 nm).

**Microbiological analysis of fermented kefir:** The growth of the various microflora was monitored through the determination of the Total Viable Counts (TVC), Lactic Acid Bacteria (LAB), *Salmonella*, *Staphylococcus aureus*, and yeast. An amount of 0.1 ml of diluted kefir (1:10, NaCl 0.9%) was spread on the surface of agar plates. TVC were calculated using a Plate Count Agar (PCA; Merck, Darmstadt, Germany) after a 3 days of incubation at 30°C, and LAB were determined on a de Man Rogosa and Sharpe medium (MRS) (Difco, Detroit, MI, USA) after a 48h of anaerobic incubation at 30°C. The required anaerobic conditions were ensured through the use of Anaeropack GENbox Jars combined with Pack-Anaero oxygen absorbers (Mitsubishi Gas Chemical Company, Tokyo, Japan). *Salmonella* enumeration was conducted on a Xylose Lysine Deoxycholate agar medium (XLD agar) (Ciplastraat 3, B-2440 Geel, Belgium) after incubation for 24 h at 37°C. A Baird Parker agar (La Forja, 9-28850 Torrejon de Ardoz-Madrid, Spain) was used for *Staphylococcus aureus* enumeration (24 hr at 37°C), while that of yeast was performed on a Sabouraud agar medium (10 g/l Peptone, 20 g/l Glucose, 15 g/l Agar, pH 6) after incubation at 25°C for 48 h. The 25-250 colony plates were selected and counted, and the CFU ml<sup>-1</sup> average number calculated. All plates were visually examined for typical colony types and morphological characteristics associated with each growth medium. Besides, each medium selectivity was routinely checked by the Gram staining and microscopic examination of smears prepared from colonies randomly selected from the media.

**Thin-Layer Chromatography (TLC) analysis of fermented kefir:** A volume of 10  $\mu$ l of diluted kefir was used for TLC. Migration was performed twice on a silica gel TLC plate (20 cm  $\times$  20 cm) using n-butanol, ethanol, and water (2:1:1, v/v). Visualization of carbohydrates was carried out by heating the TLC plate after spraying with 5% (v/v) sulfuric acid in ethanol. Lactose (1 g/l) was used as standard monosaccharide and commercial milk as reference.

**Animals' preparation:** All applied experimental procedures were carried out according to the general guidelines on the use of living animals in scientific investigations approved by the Ethical Committee of the Sciences Faculty of Sfax. Adult Wistar rats (weighing 180 g-200 g), obtained from the Central Pharmacy (SIPHAT, Tunis, Tunisia), were housed at a 22°C  $\pm$  2°C temperature, 45%  $\pm$  5% humidity and a 12 h light-dark cycle and provided with a daily standard pellet diet and water ad libitum.

**Chronic BHT treatment to induce inflammation and co-administration of Kefir:** Twenty-four (24) male adult rats were randomly divided into three groups of eight each:

1. The first group received vehicle (corn oil) and constituted the controls
2. The second group was injected Intraperitoneally (IP) with a dose of 150 mg BHT/kg of body weight (b.w.), followed by 3 weekly 200 mg/kg injections. This protocol was adopted because an initial 200 mg/kg dose proved to be lethal; after the initial 150 mg/kg b.w. dose, the rats tolerated succeeding 200 mg/kg injections well. All injections were administered before 10 AM because of circadian influences on BHT pneumotoxic efficacy [12].
3. The third group received Kefir and BHT daily (a dose of 150 mg BHT/kg of b.w., followed by 3 weekly 200 mg/kg injections). The administration of fermented kefir was carried out by gavage at a dose of 1.8 mL/day according to the literature [13].

**Preparation, measurements and analytical procedures:** Throughout the experimental stage, food and water intakes were submitted to daily monitoring. By the end of this period, all the animals were killed by cervical decapitation to avoid stress conditions.

Some blood samples were collected in heparin tubes and some others in EDTA, while additional samples were immediately used for hematological parameters determination.

Heparin tubes were centrifuged at 2200 xg for 15 min and the sediment-containing erythrocytes were suspended in a phosphate-buffered saline solution (0.9% NaCl in 0.01 M phosphate buffer, pH 7.4) and centrifuged as reported by Sinha [14].

EDTA collected blood samples were used for the 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 an electronic automate Coulter MAXM (Beckman Coulter, Inc, Fullerton, CA).

**Micronucleus (MN) assay in peripheral blood:** The preparation of slides was done according to the standard micronucleated erythrocytes method of Hayashi [15]. An amount of peripheral blood was taken directly and then air-dried to make the smear which was subsequently fixed in methanol for 20 min. Fixation was followed by slides staining in acridine orange (Sigma) for 1-3 min. The slides were then photographed under oil immersion at 1000X magnification using the Olympus BX50 (Tokyo, Japan) fluorescence microscope.

**Leucocytes isolation by density gradient centrifugation:** The peripheral WBC fractions were obtained after separation by Ficoll. Fresh blood samples were adjusted to 1 ml with Phosphate Buffered Saline Solution (PBS), pH 7.4 and 2 ml of Ficoll 400 (Sigma ref. F4375-100G), and then centrifuged at 2500 xg for 20 min. The white blood cells were extracted in the middle of the Ficoll gradient.

**WBC viability:** Determination of cell viability was achieved through the trypan blue exclusion technique as described previously [16]. The assay was carried out on purified leucocytes samples. The white blood cells were counted using Malassez cells and viability percentage was scored.

**Biochemical markers estimation in plasma:** To separate plasma, blood was collected in heparinized tubes and centrifuged at 2000 xg for 10 min. Plasma Transaminase Activities (AST, ALT) and Total Bilirubin (TB) levels were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Biomaghreb, Ariana, Tunisia, Ref 20012; 20043; 20047). Urea, uric acid and creatinine levels in plasma were estimated spectrophotometrically using commercial diagnostic kits (References: 20151, 20143 and 20091) purchased from Biomaghreb (Ariana, Tunisia).

**Analysis of antioxidant biomarkers in liver and kidney homogenates:** Kidney and liver Malondialdehyde (MDA) concentrations, index of lipid peroxidation, were determined spectrophotometrically according to the method of Draper and Hadley [17]. The MDA values were calculated using 1, 1, 3, 3-tetraethoxypropane as standard and expressed as nanomoles of malondialdehyde/mg protein.

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was carried out according to the ferrous ion oxidation xylenol orange (FOX1) method described by Ou and Wolff [18]. The amount of H<sub>2</sub>O<sub>2</sub> in the supernatant was determined by a spectrophotometer at 560 nm. Values were expressed as μmoles/mg of protein.

Glutathione (GSH) contents in the kidney and liver were determined by Ellman's method [19], modified by Jollow [20] based on the development of a yellow color when 5,5-dithiobis-2 nitro benzoic acid was added to compounds containing sulfhydryl groups. Absorbance was measured at 412 nm after 10 min. Total reduced glutathione content was expressed as mg/mg of protein.

Superoxide dismutase (SOD) activity was estimated according to Beauchamp and Fridovich [21] and its units were expressed as the amount of enzyme required to inhibit NBT reduction by 50%. The activity was expressed as units/mg protein.

The activity of Glutathione peroxidase (GPx) was measured according to Flohe and Gunzler [22] and the decrease in absorbance at 340 nm determined. The enzyme activity was expressed as nmol of GSH oxidized/min/mg protein.

Vitamin C was determined as described by Jacques-Silva et al. [23]. The data were expressed as mmol of ascorbic acid per milligram of protein.

**Total RNA extraction from liver and kidney:** Several liver and kidney samples were collected from both control and treated rats. Total mRNA was isolated from 100 mg of these organs (stored at -80°C before RNA extraction) using a kit purchased from Invitrogen (Pure Link RNA ref 12183018A) according to the manufacturer's recommendations. The prepared RNA was checked by electrophoresis for its integrity and by optical density (OD) measurement for its purity. All samples had 260/280 OD ratio values varying between 1.7 and 1.9.

**cDNA synthesis and RT-PCR:** Through reverse transcript polymerase chain reaction (RT-PCR) amplification of mRNA,

transcript abundances of TNF  $\alpha$ , IL1 and IL6 genes were determined from several samples. Total cDNA was obtained from total mRNAs by reverse transcription procedure. First-strand cDNAs were prepared using heat-denatured (5 min at 70°C) total mRNAs (10 mg) as a template, 200 U MMLV reverse transcriptase (Invitrogen), 20  $\mu$ mol of each deoxynucleoside triphosphate, and 20  $\mu$ mol of each primer. A total reaction volume of 20  $\mu$ l for 5min at room temperature and 60 min at 42°C was used to carry out reverse transcription. The cDNA/RNA heteroduplex was then denatured at 70°C for 15 min and cooled on ice. A number of primer pairs were designed to produce overlapping fragments.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
$\beta$ -Actin	TCCTCCTGAGCGCAAGTACTCT	GCTCAGTAACAGTCCGCCTAGAA
IL-1	TCTTCGAGGCACAAGGCA	CAGAGGTCCAGGTCCTGGAA
IL-6	CAGCTATGAAGTTTCTCTCCGCA	CAGAATTGCCATTGCACAACCTC
TNF- $\alpha$	GGTGATCGGTCCCAACAAGG	CACGCTGGCTCAGCCACTC

**Histopathological studies:** Portions of liver and kidney tissues were fixed for 48 h in 10% buffered formalin solution, dehydrated in an ascending graded series of ethanol, cleared in toluene and embedded in paraffin. 5-6  $\mu$ m thick sections were made by means of a rotary microtome and then stained with Hematoxylin and Eosin (H and E) for microscopic observation. Six slides were prepared from each kidney and liver. Six slides were prepared from each liver. All sections were semi-quantitatively evaluated for the degree of kidney and liver injuries.

### Statistical analysis

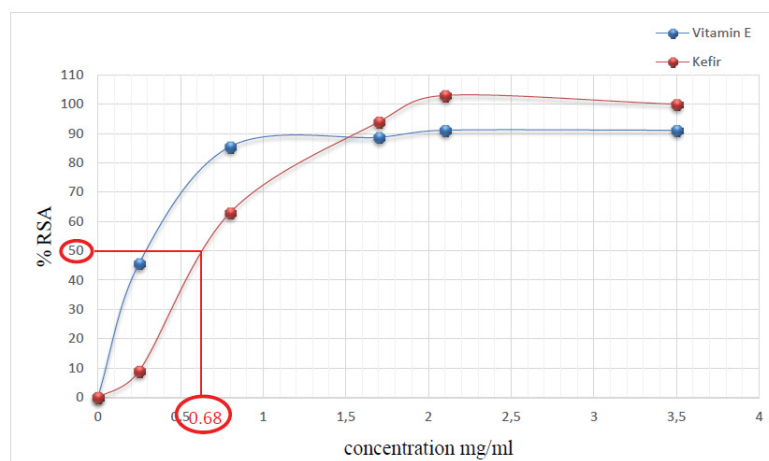
The values of each parameter are expressed as the mean  $\pm$  standard deviation ( $x \pm SD$ ). Duncan's multiple range tests provided mean comparisons with the level of statistical significance set at  $p < 0.05$ . Statistical analyses were performed using SPSS for Windows (Version 17.0).

## Results

### *In vitro* study

**Evaluation of the antiradical activity of kefir using DPPH and ABTS $\bullet+$ -radical scavenging activities:** DPPH and ABTS $\bullet+$  are relatively stable radicals in which their characteristic colors disappear when they are quenched. The DPPH and ABTS $\bullet+$ -radical scavenging activities of Kefir were determined in the present study and compared with those of standard antioxidants. The kefir displayed a DPPH scavenging activity that was lower than that of vitamin E. Its concentration that allows reducing the free radical to half concentration (IC<sub>50</sub>) was 0.68 mg/ml whereas this value was 0.3 mg/ml for Vitamin E. When using the ABTS $\bullet+$ -scavenging test to assess anti-oxidant capacity, Kefir displayed a high scavenging capacity since its concentration needed to reduce the free radical amount to half (IC<sub>50</sub>) was comparable to that of Vitamin E (1,42 mg/ml) and lower than that of vitamin C (73 mg/ml).

**Monosaccharide composition of fermented Kefir:** Monosaccharide composition of kefir was determined by Thin Layer Chromatography (TLC). Results presented in **FIG. 1** show the appearance of one plug of monosaccharide having a retention factor of 0.28. The hydrolysis of the kefir led to the emergence of a plug having a retention factor of 0.28 corresponding to the same retention factor of the lactose (used as standard P3). The hydrolysis of the kefir was not total since part of the plug corresponding to the kefir persisted (RF=0.28). The appearance of a plug corresponding to glucose indicated that the kefir product contains a homo polysaccharide consisting of a chain of lactose molecules (**FIG. 2**).



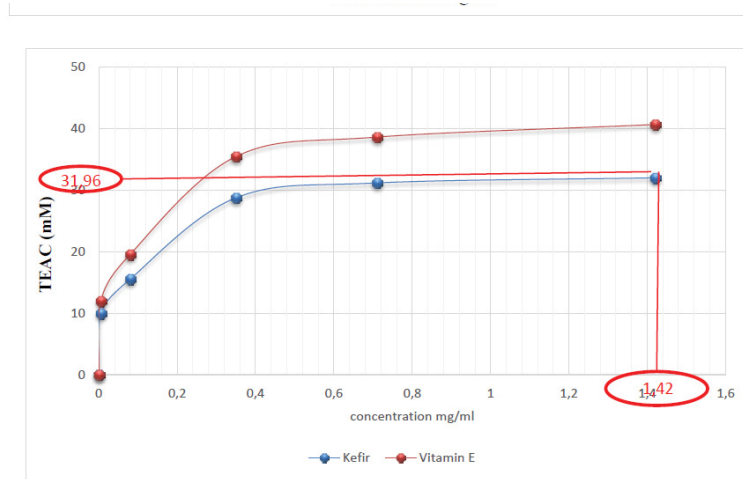


FIG. 1. DPPH and ABTS radical scavenging activities of kefir in comparison with vitamin E used as standard.

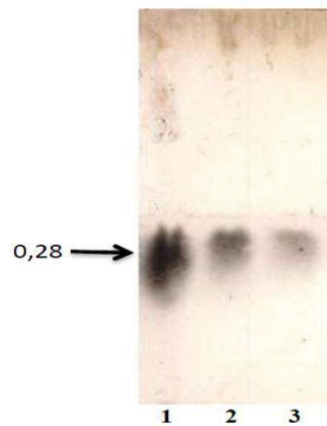


FIG. 2. Monosaccharide composition of fermented kefir. Commercial milk (lane 1); Kefir (lane 2) and Lactose (lane 3).

**Mineral content of fermented kefir:** Our data indicated that fermented kefir was rich in Na ( $162.2 \pm 8.34$  mg/L), Ca ( $18 \pm 3.65$  mg/L) and Mg ( $2.6 \pm 0.023$  mg/L) and contained small amounts of Fe and Zn ( $0.02 \pm 0.001$  and  $0.04 \pm 0.01$ , respectively). On the contrary, there is a complete absence of Cd, Cr, Pb, and Ni (TABLE 1).

TABLE 1. Mineral composition of kefir.

Mineral Contents (mg/L)	Na+	Ca++	Mg++	Zn+	C d	Pb	Cr	Ni
<b>Kefir</b>	162.2	18.08	2.63	0.04	0	0	0	0

**Microbial growth:** Conventional microorganism enumeration was carried out during the 24h milk fermentation (3% kefir grain) at 30°C. Presumptive LAB counts increased significantly at 12 h, reaching maximum values ( $10.46$  log units) at 24 h and TVC ( $4.8$  log units). Yeast counts increased until 12 h, remaining constant at around  $4.36$  log units over the fermentation period. Microorganisms were also enumerated during kefir storage at 4°C for 48h. During this period, yeast (approximately  $4.36$  log units) and LAB group counts (approximately  $10$  log units) remained constant until the end of the storage period (TABLE 2).

TABLE 2. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of kefir on Bacteria.

Strain	Gram	CMI (mg/ml)	CMB (mg/ml)
<i>Salmonella Typhimurium</i>	-	15	>15
<i>Klebsiella pneumoniae</i>	-	15	>15
<i>Enterobacter cloacae</i>	-	7.5	>15
<i>Escherchia coli</i>	-	7.5	>15
<i>Staphylococcus epidermidis</i>	+	-	-
<i>Bacillus cereus</i>	+	-	-
<i>Bacillus subtilis</i>	+	-	-

**In vivo study**

**Hematological parameters:** Compared with the control group, RBC, Hb content and platelet counts were reduced by 29% (p=0.05); 23% (p=0.06) and 25% (p=0.09) respectively in rats exposed to BHT (TABLE 3). No changes in MCV, MCH, and MCHC were noted. Interestingly, co-treatment with Kefir improved parameters cited above to reach control values.

TABLE 3. Hematological parameters of controls and BHT treated rats associated with kefir.

Parameters and Treatments	Control	BHT	BHT+kefir	F	P
RBC (10 <sup>6</sup> /μL)	8.33 ± 0.47 <sup>a</sup>	6.46 ± 1.01 <sup>b</sup>	7.72 ± 0.63 <sup>ab</sup>	4.99	0.053
Hb (g/100 mL)	14.24 ± 0.67 <sup>a</sup>	11.55 ± 1.61 <sup>b</sup>	13.71 ± 0.86 <sup>ab</sup>	4.83	0.056
Ht (%)	42.58 ± 2.38 <sup>a</sup>	34.06 ± 5.17 <sup>b</sup>	40.18 ± 2.40 <sup>ab</sup>	4.55	0.063
MCH (pg/RBC)	17.12 ± 0.32 <sup>a</sup>	18.03 ± 4.02 <sup>a</sup>	17.73 ± 1.16 <sup>a</sup>	0.11	0.898
MCHC (g/100 mL)	33.46 ± 0.53 <sup>a</sup>	34.71 ± 7.41 <sup>a</sup>	34.05 ± 1.71 <sup>a</sup>	0.061	0.942
MCV (mm <sup>3</sup> /RBC)	51.12 ± 0.16 <sup>a</sup>	52.71 ± 1.17 <sup>a</sup>	52.05 ± 1.58 <sup>a</sup>	1.47	0.301
Platelets (10 <sup>3</sup> /mm <sup>3</sup> )	728 ± 129.16 <sup>a</sup>	321 ± 70.10 <sup>a</sup>	659.32 ± 312.14 <sup>a</sup>	3.59	0.094

<sup>a</sup> Means not sharing the same letters (a-c) within a column are significantly different (p<0.05). Data are means ± standard deviations values. RBC: Red Blood Cells; WBC: White Blood Cells; Hb: Hemoglobin; Ht: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration

**MN assay in the peripheral blood:** FIG. 3A showed the morphology of the white cell’s nuclei of controls and BHT- treated groups after the treatment period. Nuclei were considered to have the normal phenotype when glowing bright and homogenously. Apoptotic nuclei could be identified by condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. In the control group, cells did not show any nuclear fragmentation. However, there was a characteristic nuclear fragmentation of WBC nuclei in BHT-group (FIG. 3A). The apoptotic cells displayed the characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin. Co-administration of kefir prior to BHT exhibited significant amelioration of the morphology of the white cells, compared to rats that received only BHT.

**White blood cells' number and viability:** The trypan blue exclusion technique showed a cell viability of 100% in the control group (FIG. 3B). Results clearly showed significant DNA damage, evidenced by the decrease (p<0.05) in number and viability WBC, in the BHT group, as compared to those of controls (FIG. 3B). Significant DNA damage was observed after BHT treatment when compared with controls. Supplementation of Kefir to BHT-treated group ameliorated the WBC viability when compared to BHT-group. The assay described was repeated three times.

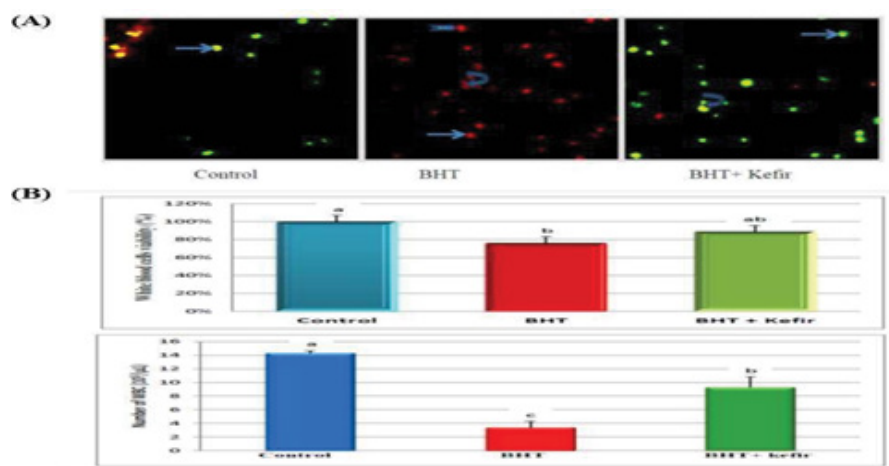


FIG. 3. (A): MN test of control and treated rats with BHT alone or associated to kefir. → Nucleus of WBC; ↻ Apoptotic cells; ↻ Necrotic cells; (B): Effects of BHT alone or associated with kefir on WBC number and viability<sup>a</sup>. <sup>a</sup>Means not sharing the same letters (a-c) within a row are significantly different (p<0.05). Data are means ± standard deviations values.

**Plasma biomarkers levels:** Compared to the controls, bilirubin, AST and ALT levels in the BHT-treated group were increased in plasma of adult rats by 20% (p<0.5), 74% (p=0.05) and 11% (p=0.06) respectively, indicating BHT induced hepatotoxicity (TABLE 4).

Our results also showed a constellation of disorders in the renal function of the BHT- treated group. Creatinine, uric acid and urea levels in the BHT-treated rats were higher by 28% (p=0.2), 89% (p=0.006) and 23% (p=0.01) respectively in plasma than those of controls. Supplementation of Kefir to the BHT-treated group ameliorated all parameters cited above compared with BHT-group (TABLE 4).



TABLE 4. Hematological parameters of controls and BHT treated rats associated with kefir.

Parameters & treatments	Control	BHT	BHT+kefir	F	P
ASAT (U/I)	222.7 ± 69.44 <sup>b</sup>	387.5 ± 92.60 <sup>a</sup>	240.25 ± 38.44 <sup>b</sup>	4.83	0.056
ALAT (U/I)	67.6 ± 12.19 <sup>ab</sup>	75.4 ± 7.66 <sup>a</sup>	54 ± 7.11 <sup>b</sup>	4.09	0.06
Bilirubin (mg/L)	25.8 ± 8.76 <sup>a</sup>	30.87 ± 9.26 <sup>a</sup>	22.53 ± 9.59 <sup>a</sup>	0.625	0.567
Creatinine (mmol/L)	17.71 ± 2.30 <sup>a</sup>	22.75 ± 3.73 <sup>a</sup>	20.26 ± 3.29 <sup>a</sup>	1.903	0.229
Urea (mmol/L)	5.78 ± 0.45 <sup>b</sup>	7.14 ± 0.57 <sup>a</sup>	6.92 ± 0.22 <sup>a</sup>	8.33	0.019
Uric acid (mg/L)	43.66 ± 12.57 <sup>b</sup>	82.51 ± 13.76 <sup>a</sup>	35.88 ± 8.12 <sup>b</sup>	13.59	0.006

a Means not sharing the same letters (a-c) within a column are significantly different (p<0.05). Data are means ± standard deviations values.

**Estimation of MDA and H<sub>2</sub>O<sub>2</sub> levels:** Our results revealed an increase of lipid peroxidation and ROS production levels in the liver and kidney of the BHT-treated group as evidenced by the enhanced MDA by 91% and 49% (p<0.001) and H<sub>2</sub>O<sub>2</sub> (p<0.001) levels when compared to controls (TABLE 5). Kefir co-administration significantly improved the MDA and H<sub>2</sub>O<sub>2</sub>, when compared to the BHT-treated rat.

**Enzymatic and non-enzymatic antioxidant status:** GPx and SOD activities increased significantly in the liver (43%; p=0.02 and 69%, p=0.04) and kidney (24%; p<0.1 and 108%; p<0.005, respectively) of BHT treated-group when compared to those of controls (TABLE 5).

A significant decrease in GSH and vitamin C values (24%; p=0.01 and 76%; p<0.001, respectively) in the liver and in kidney (26%; p=0.01 and 93%; p=0.001, respectively) was evident in the BHT-treated group compared to controls (TABLE 2). Co-treatment with Kefir significantly improved the antioxidant status in the kidney and liver tissues compared with the BHT-treated group.

TABLE 5. Liver and kidney oxidative stress parameters of controls and treated groups.

Parameters and treatments	Control	BHT	BHT+Kefir	F	p
MDA (nmoles MDA/g tissue)	115.65 ± 18.81 <sup>c</sup>	221.55 ± 19.04 <sup>a</sup>	153.79 ± 13.10 <sup>b</sup>	29.16	0.001
GPx (nmol/min/mg protein)	4.92 ± 0.50 <sup>b</sup>	7.04 ± 0.50 <sup>a</sup>	5.80 ± 0.87 <sup>ab</sup>	8.12	0.02
GSH (mg/mg protein)	486.91 ± 34.5 <sup>b</sup>	392.03 ± 25.72 <sup>a</sup>	426.99 ± 19.68 <sup>b</sup>	9.25	0.015
Vitamin C (mmol/mg protein)	2.52 ± 0.11 <sup>a</sup>	1.43 ± 0.10 <sup>c</sup>	1.94 ± 0.18 <sup>b</sup>	49.12	<0.001
SOD (units/mg protein)	72.81 ± 7.26 <sup>b</sup>	123.32 ± 25.43 <sup>a</sup>	105.94 ± 18.56 <sup>ab</sup>	5.68	0.041
H <sub>2</sub> O <sub>2</sub> (µmoles/mg of protein)	0.05 ± 0.007 <sup>c</sup>	0.10 ± 0.008 <sup>a</sup>	0.08 ± 0.001 <sup>b</sup>	43.36	<0.001
<b>Kidney</b>					
MDA (nmoles MDA/g tissue)	202.82 ± 14.83 <sup>c</sup>	303.44 ± 15.30 <sup>a</sup>	240.51 ± 17.67 <sup>b</sup>	30.35	0.001
GPx (nmol/min/mg protein)	4.56 ± 0.69 <sup>a</sup>	5.60 ± 0.67 <sup>a</sup>	5.12 ± 0.50 <sup>a</sup>	2.79	0.139
GSH (mg/mg protein)	620.35 ± 46.56 <sup>a</sup>	489.38 ± 27.82 <sup>b</sup>	557.08 ± 27.56 <sup>ab</sup>	10.43	0.011
Vitamin C (mmol/mg protein)	1.97 ± 0.09 <sup>a</sup>	1.02 ± 0.22 <sup>c</sup>	1.56 ± 0.17 <sup>b</sup>	23.97	0.001
SOD (units/mg proein)	58.16 ± 13.89 <sup>b</sup>	120.94 ± 14.68 <sup>a</sup>	93.65 ± 14.68 <sup>ab</sup>	14.31	0.005
H <sub>2</sub> O <sub>2</sub> (µmoles/mg of protein)	0.11 ± 0.009 <sup>a</sup>	0.21 ± 0.03 <sup>a</sup>	0.16 ± 0.01 <sup>a</sup>	3.13	0.117

<sup>a</sup>Means not sharing the same letters (a-c) within a column are significantly different (p<0.05). Data are means ± Standard deviations values

**Effects of BHT and Kefir on the expression of inflammatory mediators:** The effect of Kefir associated with BHT on pro-inflammatory genes expression was examined by measuring IL-1 β, IL-6 and TNFα mRNA accumulation by RT-PCR (FIG. 4). The results show a concomitant significant increase in IL and TNFα mRNA in the liver and kidney of BHT-treated rats. Importantly, Kefir co-treatment reduced inhibited these increases of the pro-inflammatory cytokines.

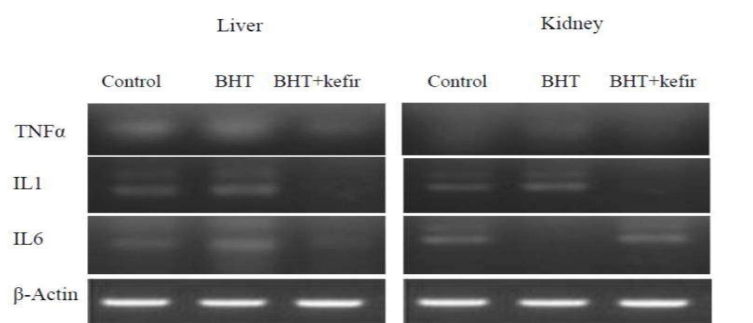


FIG. 4. Kefir down-regulated BHT induced of IL1, IL6, and TNFα in liver and kidney expressions analyzed by RT-PCR.



**Histopathological studies:** In the BHT-treated rats, liver, and kidney histological data showed numerous abnormalities (FIG. 5 and 6). Light microscopic examination indicated a liver normal structure in controls, showing a normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein. While with BHT treatment, severe histopathological changes were observed. BHT caused necrosis, hemorrhages, infiltration of inflammatory leucocyte cells and hepatocyte vacuolization.

The kidney also exhibited several abnormalities such as vascular congestion inside glomeruli and between tubules. The infiltration of lymphocytes and polynuclear cells was observed between tubules while necrosis and vacuolization occurred particularly inside tubules in the BHT-treated rats. In the group co-treated with Kefir associated with BHT, a marked improvement in hepatic and renal histopathological appearance was observed but still with focal areas of less extensive glomerular necrosis and degenerated cells compared to BHT. Particularly, we noted, in (BHT+kefir) fed group, an increase in mitotic cells, indicating the important role of kefir in cell regeneration

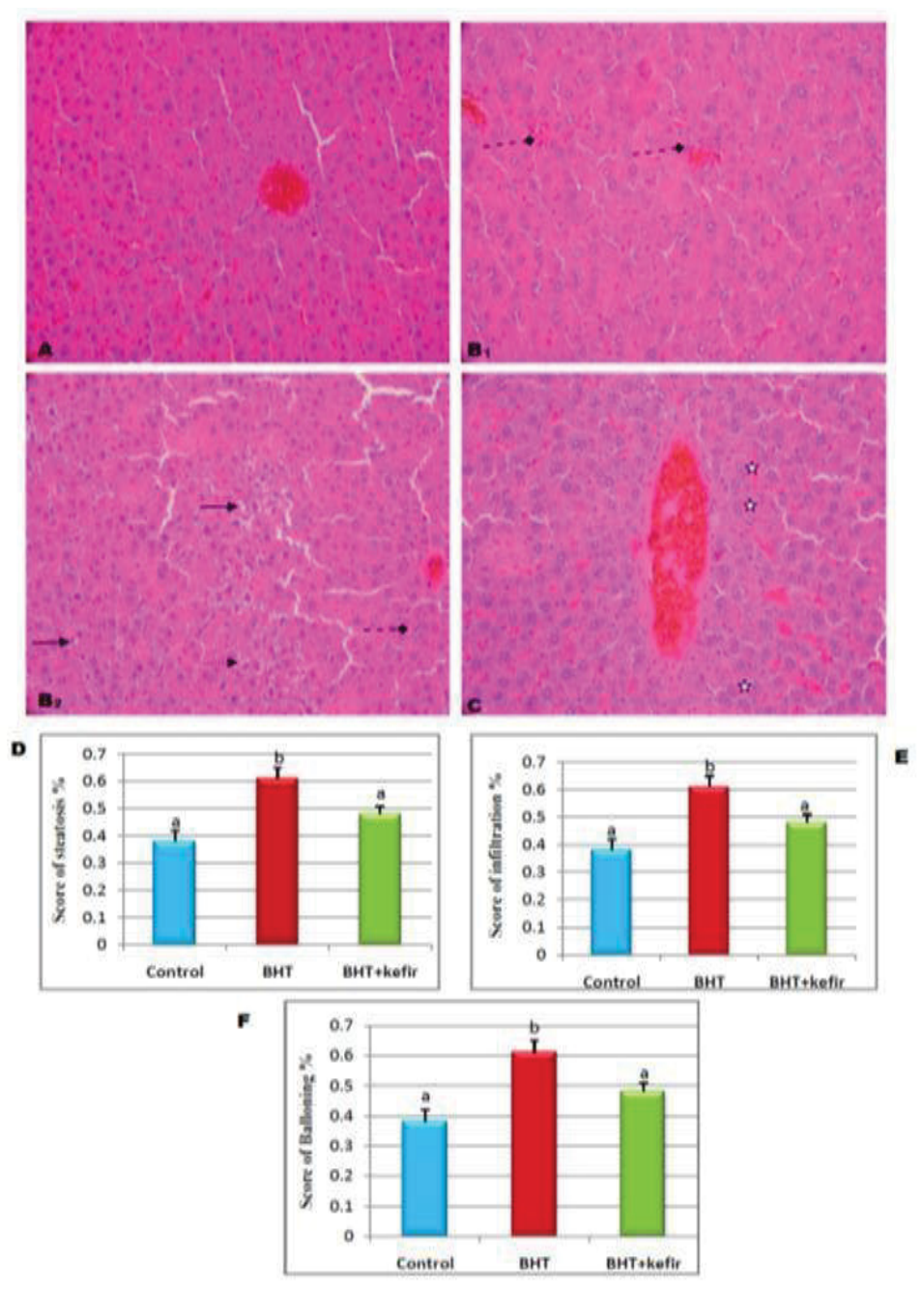


FIG. 5. (A): Liver histological sections of adult rats: Controls; (B1 and B2): BHT-Treated rats; (C): BHT+kefir-Treated rats; (D, E and F): Histological scores of liver tissues. <sup>a</sup>Means not sharing the same letters (a-c) within a row are significantly different (p<0.05). Arrows indicate: ·····◆Leukocytes infiltration, —→Steatosis ·····▶Vacuolization, ☆ Mitotic cell.

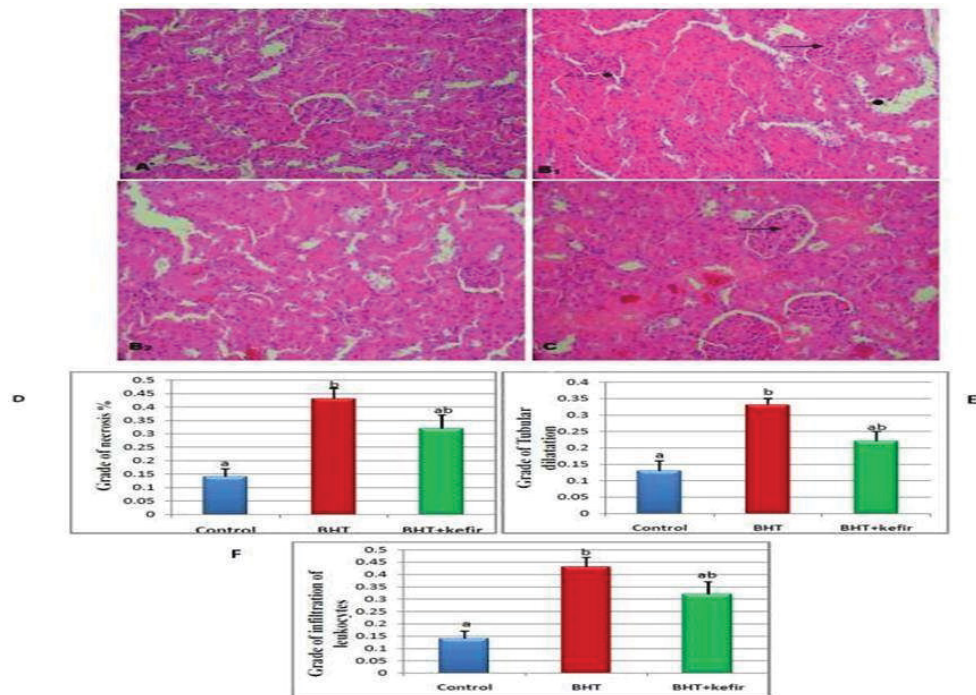


FIG. 6. (A): Kidney histological sections of adult rats: controls; (B1 and B2): BHT; (C): BHT+kefir; (D, E and F): Histological scores of kidney tissues. <sup>a</sup>Means not sharing the same letters (a-c) within a row are significantly different (p<0.05). Arrows indicate: —→Intra-glomeruli hemorrhage, ······•Leukocyte infiltration, ·······•Vacuolization, - - - →Necrosis.

## Discussion

In the present work, GPx, SOD, GSH, vitamin C, H<sub>2</sub>O<sub>2</sub> and MDA were selected as relevant indices to evaluate the effect of kefir on BHT-induced hepatotoxicity and nephrotoxicity *in vivo*. Liver and kidney damage was also assessed at the expression levels of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6). Besides, other indices in plasma samples were evaluated to provide additional evidence supporting our results.

In the current study, BHT treatment brought about a significant increase in MDA and H<sub>2</sub>O<sub>2</sub> levels in the liver and kidney of adult rats. MDA is an indicator of free radical damage induced by membrane lipid peroxidation which can alter cell membrane integrity, induce impairment in membrane transport function and disrupt cellular homeostasis [24]. Hence, BHT-induced cytotoxicity could be partly due to ROS production. The molecular mechanisms by which BHT causes ROS production have not been elucidated, but our data revealed that oxidative stress was responsible, at least in part, for its toxicity. Free radicals may be derived directly from BHT or during its metabolism by the P<sub>450</sub> cytochrome. In fact, oxidative stress leads to the formation of superoxide radicals which, in turn, are converted to hydroxyl radicals via H<sub>2</sub>O<sub>2</sub> or ONOO [25]. These free radicals lead to cell membrane lipid peroxidation, causing phospholipids degradation. In the present study, increased liver and renal MDA levels, the product of lipid peroxidation, along with increased SOD and GPx activities and concomitant decreased vitamin C and GSH levels, the major defense agents against oxidative damage, after BHT injection, suggested its hepato- and nephro-toxicities which may be due to oxidative damage. Damage to liver cells will generally result in plasma transaminase elevation, an indicator of cell necrosis [26]. The increase in plasma AST and ALT enzymes in the BHT groups clearly indicated oxidative damage with hepatocellular necrosis and an increase in the permeability of hepatocyte membrane. In addition, BHT hepatotoxicity was evidenced by an increase in bilirubin levels resulting either from hemolysis or the decreased liver uptake conjugation. On the other hand, the increase of plasma creatinine, uric acid, and urea levels indicates a glomerular filtration rate decline and, consequently, a kidney dysfunction. Indeed, the biochemical results were confirmed by histological data marked leukocyte infiltration in the liver and kidney tissues, thus confirming the inflammatory responses to BHT-treatment. Also, renal lesions were characterized by an enlarged Bowman space and a vascular congestion, while hepatic lesions were marked by vacuolization, indicating the start of a necrosis step. It is worth noting that the supplementation of kefir to the BHT treated rats induced a significant recovery of the histopathological changes. Meanwhile, fermented kefir eliminated lipid peroxidation and improved liver and kidney dysfunction. This suggests that kefir evoked an antioxidant effect and, consequently, protected organs from oxidative damage and dysfunction. Besides, Kefir could eliminate oxides such as lipid peroxides via its antiradical and antioxidant proprieties demonstrated by our *in vitro* study. According to previous findings, the underlying kefir protective effect mechanism is probably due to its bioactive components such as exopolysaccharides, peptides, antioxidants and

also through immunomodulatory properties [27]. The positive effects of kefir on cell regeneration can also probably be through the provision of high-quality nutrition intake which is considered vital to maintain and regenerate body cells [28].

The advanced symptom of excessive ROS targeting of the liver and kidney could be activated by inflammatory cells [29]. Hence, the inflammatory response causes related genes stimulation (IL6, IL-1 $\beta$  and TNF- $\alpha$ ) and further leukocyte infiltration, observed in our results in the BHT-treated group. In addition, pro-inflammatory TNF- $\alpha$  cytokine exerts a considerably increasing effect on tissue inflammatory response and causes severe hepatic and renal oxidative damage [30]. Our investigation has proven that fermented kefir supplementation remarkably affected both the biochemical markers and immune response (cytokines IL1, IL6, TNF $\alpha$ ). In fact, tight regulation of these cytokines appears to be essential to maintain beneficial functions and can offer a new therapeutical strategy against inflammatory diseases. Indeed, kefir seems to be able to activate regulatory T cells (Tregs) whose functions are to maintain the homeostasis of Th1-Th2 responses. Through their cytokine products, these cells have the ability to suppress inflammatory reactions and apoptosis. Thus, our study suggests that kefir action mechanism consists in lowering plasma liver and kidney biomarkers and pro-inflammatory cytokines, and also in decreasing the subsequent effects of free radicals and lipid peroxidation. The reduction of peroxide, molecules affect positively the secretion of pro-inflammation cytokines (IL1, IL6) if the structural damage and function of hepatic and renal cells are inevitable.

Hematological parameters, among other important organism responses, provide a sound basis for judgment regarding the disease, the extent of tissue damage, the response of defense antioxidant mechanism, diagnosis of anemia, and can be an index of health status characterization [31]. Our data revealed abnormalities in some blood cell parameters of BHT-treated rats, evidenced by a significant reduction in RBC number as well as in Ht and Hb concentrations after BHT injection. This could be explained by oxidative stress probably generated by BHT which also disturbed the immune function of treated rats. WBCs decrease occurring with BHT could result from the immune system failure and/or from leukocytes necrosis or apoptosis increase. This hypothesis was confirmed by the micronucleus (MN) and white blood cell viability tests assayed in the present study. These methods are widely used for genetic damage detection, seeing the availability ease of test samples from animals and humans. MN arises from the DNA breaks that lead to acentric chromosome fragments or lagging chromosomes at the interphase, confirming the carcinogenicity and genotoxicity of the tested product [32]. BHT genotoxic effect on white blood cells was evidenced by a significant increase in the micronuclei frequency and a decrease in cell viability, while kefir co-treated rats improved total leukocyte counts, cell viability and micronuclei frequency probably via its antioxidant and anti-inflammatory properties. In fact, fermented kefir possesses health-promoting properties thanks to its complex mixtures of bacteria and polysaccharides, as demonstrated by our results. This corroborates previous findings showing that kefir improves the biological value and digestibility of proteins, and strengthens the immune system through the normalization of the pro-inflammation cytokines [33].

## Conclusion

It can be concluded that co-treatment with kefir provided protection against BHT-induced cytotoxicity and genotoxicity, modulated genes expression and DNA repair. It also exhibits great antioxidant capacity demonstrated by the potentiating of SOD and GPx activities. Furthermore, a key intermediate for its antioxidant activity was identified, *in vitro*, in the reaction with ABTS<sup>•+</sup> and DPPH, proving its ROS scavenging ability.

## Conflicts of Interest

The authors declare that there are no conflicts of interest.

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