RESEARCH ARTICLE
Ethoxyquin and Butylated Hydroxyl Toluene Induced Hepatotoxic Effect Via Apoptosis, Oxidative Stress in Rats: Tissue Injury-Related CYP1A1 Gene Expression
Amany T. Mohammed, Mohamed M.M. Metwally, Dina Y. Hegab*, Mervat H. Ghoneim, and Ali H. Abou-Hadeed
Forensic Medicine and Toxicology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt.
Pathology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt.
*Corresponding author e-mail: dinahegab23@gmail.com

Abstract
Animal feed may contain different phenolic antioxidants, such as butylated hydroxyanisole, butylated hydroxy toluene (BHT), and ethoxyquin (EQ). EQ and BHT at high concentrations can have a pro-oxidant effect and can cause adverse health effects in animals. This study was designed to evaluate the hepatotoxic effect of EQ and/or BHT in rats. Fifty male Sprague–Dawley rats were assigned to five groups of 10 rats each as following: the first group served as control and did not receive any treatments; the second group served as vehicle control and was orally given corn oil. The third group was orally administered EQ day after day in a dose of 1/5 of LD50, the fourth group was orally received BHT day after day in a dose of 1/5 LD50. The fifth group was orally administered both EQ and BHT at the same doses and durations described above. The duration of this study was 45 and 90 days. The results revealed that EQ, BHT and their co-exposure caused a significant decrease in levels of antioxidant enzymes (superoxide dismutase (SOD), catalase enzyme (CAT) and reduced glutathione (GSH)) in rats. Also a significant increase in malondialdehyde (MDA) and 8-hydroxyguanosine (8-OH-dG) levels was recorded in serum of rats. Co-exposure group had a significant increase in DNA damage variables (% tail DNA, tail length, and Olive Tail Moment (OTM)), strong immuno positive reactivity of caspase3 and up regulation of CYP1A1 gene expression in the liver cells of exposed rats. In conclusion, we can state that EQ and BHT are potentially hepatotoxic compounds where the oxidative stress could be accepted as a possible mechanism of their toxicity. Therefore, the utilization of such compounds as feed additives should be more controlled and limited.

Keywords: Ethoxyquin, Butylated hydroxyl toluene, DNA damage, CYP1A1.

Introduction
Antioxidants are the most suitable additives used to prolong the shelf-life of feeds by preventing oxidation deteriorations which might result in fat rancidity and color changes [1]. Antioxidants that are used in animal feeds can be generally divided into natural and synthetic ones. The most common natural antioxidants used for protecting feed against peroxidation are vitamin E and vitamin C [2]. While the commonest synthetic ones are butylated hydroxy anisole (BHA), tert-butyl hydroquinone (TBHQ), butylated hydroxy toluene (BHT), propyl gallate, and ethoxyquin (EQ) [3].

Ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, EQ) is a lipid-soluble, quinolone-based synthetic antioxidant that accumulates in human and animal tissues. In addition, it is used as a colour preservative in spices and as an anti-degradation agent in rubber [4]. It is widely used as a technological additive in food for pets and livestock animals and farmed fish feed to protect it from lipid peroxidation and preserve the stability of fat-soluble vitamins [5]. It affect the animal’s well-being and
food safety together [6-10]. Additionally, it can contaminate food products of an animal’s source that are prepared for humans, including cultivated fish, meat, and eggs [11]. Many side effects such as apoptosis and cytotoxicity have been observed in animals that fed on feeds containing EQ [12-14]. Experimental studies observed that it resulted in mortality, weight loss, lethargy, changes in the liver, kidney, alimentary duct and urinary bladder and immunosuppression in fish, rodents and dogs [4,15,16]. EQ could effectively induce DNA damage in human lymphocytes. It significantly increased all the evaluated parameters of the comet assay (% tail DNA, tail length, and Olive Tail Moment (OTM)) at concentrations starting from 1µM EQ [17].

Butylated hydroxyl toluene (BHT) (2,6-di-tert-butyl-4-methyl phenol 2,6-di-tert-butylp cresol) is a lipophilic organic compound [18]. It is the most commonly used synthetic antioxidant in foods containing fats like meat, fats, and oils in addition to food containers, packaging materials, milk products and cosmetics [19-21]. The Food and Drug Administration (FDA) organization authorized that BHT can be added separately or in combination with other antioxidants at concentrations up to 0.02% according to the ratio of fat or oil content in the food products [22]. It is used not only as an antioxidant in dog food, but also as an aflatoxin inhibitor at levels as high as 0.015% [21,23].

Repeated short-term exposure to BHT caused hepatic toxicity in male and female rats. Several studies have shown that BHT can induce tumors development in the liver of animals when BHT orally ingested [24]. Additionally, it caused the development of hepatocellular tumors in rats and mice, DNA damage in monkeys and endocrine disruption in Zebra fish [25].

EQ and other antioxidant such as BHT and BHA can be used in animal feeds alone or in combination with a maximum concentration of 150 mg/kg for all animal species [26]. As a result, this study examines the effects of EQ and BHT exposure for 45 or 90 days on rat hepatic tissue. For this purpose, serum antioxidant enzymes including superoxide dismutase (SOD), catalase enzyme (CAT) and reduced glutathione (GSH) in addition to oxidative stress biomarkers including malondialdehyde (MDA) and 8-hydroxyguanosine (8-OH-dG) were estimated. DNA damage (comet assay), CYP1A1 gene expression, and immunohistochemistry analysis of Caspase3 and Bcl2 were assessed in the liver cells.

Materials and Methods

Tested compounds

Ethoxyquin (C14H19NO) was bought from Sigma-Aldrich Chemical Company, Saint Louis, MO, USA (≥75% purity). Butylatedhydroxytoluene (C15H24O) was obtained from Alpha Chemica; India (99% pure). The dose of EQ and BHT was selected to be 1/5 LD50 = 500 mg/kg b.wt [27, 28]. Both substances were dissolved in corn oil (Afia Company, Egypt)

Animals and experimental design

Fifty Sprague–Dawley male rats weighing 240-300g were obtained from Laboratory Animal Farm, Faculty of Veterinary Medicine, Zagazig University. Rats were kept with water and feed were accessible ad libitum throughout the acclimatization period (two weeks). The protocol of this study follows the general rules of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals in scientific investigations and was approved by the ethics committee of Institutional Animal Care and Use, Zagazig University, Zagazig, Egypt (ethical approval No. ZU-IACUC/2/F/59/2021).

Rats were divided into five groups of 10 rats each as follows: The rats of the first group were placed as control and did not receive any treatments; the second group served as vehicle control and was orally given 1 mL of corn oil day after day. The third group (EQ) was orally administered EQ dissolved in 1 mL corn oil day after day in a dose of 1/5 LD50 , the fourth group (BHT) was orally administered BHT dissolved in 1 mL of corn
oil day after day for consecutive 45 and 90 days in a dose of 1/5 LD50. The fifth group (combination group) was orally administered both EQ and BHT at the same doses and durations as described above. The duration of the experiment was 45 and 90 days and rats orally administrated all treatments by using a gastric tube.

**Blood and tissue sampling**

On the 45th and 90th days of the experiment, the blood samples were collected from the median canthus of the eyes of all rats (orbital vessels) after anesthesia of rats by 0.05–0.10 ml/100 g Ketamine – Xylazine (Warburg, Germany) intraperitoneally. The blood samples were collected in clean test tubes and centrifuged at 3000 rpm for 15 min to separate sera for the completion of the antioxidants and oxidative stress biomarkers evaluation.

Then, rats were euthanized by cervical dislocation. The liver was directly excised, weighed, and divided into two sets; one set was quickly kept in liquid nitrogen at −80 °C for gene expression analysis and comet assay. The second set was divided horizontally and fixed in neutral buffered formalin at 10% for immunohistochemical staining.

**Estimation of antioxidant enzymes and oxidative stress biomarkers levels in serum**

Superoxide dismutase (SOD), catalase enzyme (CAT), and reduced glutathione (GSH) levels were elevated in serum by using the commercial kits (Spectrum, Diagnostics, Egypt, Co for biotechnology) following the manufacturer instructions. malondialdehyde (MDA) was measured in serum as previously described [29]. The 8-hydroxyguanosine (8-OH-dG) was measured using rat ELISA kits (Cat. No. MBS 267513) obtained from CUSABIO BIOTECH CO., Ltd., China.

**Estimation of cytotoxic effect on liver cells (comet assay: single cell gel electrophoresis)**

The oxidative DNA damage level in hepatic cells was estimated in hepatic cells as described by Singh et al., [30]. Fifty cells/slide were examined and imaged by a CCD camera (Olympus, Tokyo, Japan) connected with the fluorescence microscope (Zeiss Axiovert Inc., Jena, Germany). Tail DNA% and tail length were determined for each cell. Moreover, Comet assay project software was used for estimation of the scores of tail moment from the comet image of each cell.

**Expression of CYP1A1 gene**

The mRNA expression levels of CYP1A1 gene in the liver of rats was estimated by real-time PCR using the primer pairs FW 5´ TAACTCTTCCCTGGATGCCTTCAA 3´, and Rv: 5´ GTCCCCGGATGGGCCCTTTCAA 3´ [64]. Total RNA from hepatic cells of different treatment groups was extracted by utilizing the RNeasy Mini Kit (Qiagen, Heidelberg, Germany) according to the manufacturer’s instructions. The total RNA was converted to cDNA using RevertAid Reverse Transcriptase (Thermo Fisher, catalog number: EP0441). RT-qPCR of CYP1A1 gene was done on a Rotor-Gene Q cycler (Qiagen, Germany) using QuantiTect SYBR Green PCR kits (Qiagen, Heidelberg, Germany). Expression of gene was performed on real-time PCR machine (Stratagene MX3005P) at 95°C for 15 min, followed by 94°C for 15s, 56°C for 30s and 72°C for 30s. Expression levels of the target gene were normalized with respect to the reference gene β-actin using the oligonucleotide primer pairs 5´ TCCTCCTGAGCGCAAGTACTCT 3´ and 5´ GCTCAGTAACAGTCCGCCTAGAA 3´ [63]. Relative fold changes in the expression of target gene were calculated using the ΔΔCt method [31].

**Immunohistochemistry examination**

Avidin biotin peroxidase method was performed for immunohistochemistry staining. The paraffin-embedded sections were deparaffinized in new xylene for 4 times, and remoisten in ethanol with graded concentration (100 %, 95 %, and 70 %) then cleaned in phosphate buffered saline (PBS) at pH 7.2 for 5 min. The sections were incubated with 0.3% hydrogen peroxide in water to stop endogenous peroxidase.
activity. Then, distilled water used to wash the sections three times and then washed in PBS. Washed sections were incubated with blocking reagent (10% normal rabbit serum) for 30 min to diminish the non-specific binding of immunoglobulins in a humid chamber. Antisera containing the specific primary antibody (Anti-caspase3 and Bcl2 antibody; abcam company, USA.) were incubated with the sections overnight at room temperature in a humid chamber. Antisera were incubated with biotinylated secondary anti-immunoglobulin for 30 min at room temperature in a humid chamber, and then slides were cleaned with two changes of PBS for 5 min each. The slides were kept for 30 min at room temperature in a moist chamber and then cleaned in two changes of PBS for 5 min to each change. The slides were incubated with diaminobenzidine (DAB; abcam company, USA.) used as chromogen for 4 min at room temperature. Slides were cleaned in distilled water for 5 min. Mayer's hematoxylin was used as a counterstain, so sections were counterstained with it, then slides were dehydrated in alcohol with ascending grades, cleaned in xylene and fixed with Canada balsam. Light microscope was used to visualize the slides [32].

**Statistical analysis**

Data were expressed as mean ± SEM to assess the impact of EQ, BHT, and their combination. Results were analyzed through two-way ANOVA of the SPSS 22.0 computer program. Duncan’s multiple range test was performed to compare mean values between treated groups. A value of \( P < 0.05 \) was considered statistically significant [33].

**Results**

**Effects of EQ, BHT, and their combination on serum antioxidant enzymes**

The effects of EQ, BHT, and their co-exposure on serum antioxidant enzymes of treated rats were summarized in Table (1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD (u/mL)</th>
<th>CAT (ng/mL)</th>
<th>GSH (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 days</td>
<td>22.30 ±2.00</td>
<td>39.90 ±3.00</td>
<td>4.00 ±0.40</td>
</tr>
<tr>
<td>Control</td>
<td>36.00 ±2.00</td>
<td>63.50 ±2.00</td>
<td>8.70 ±0.60</td>
</tr>
<tr>
<td>VH</td>
<td>30.00 ±2.00</td>
<td>62.70 ±2.00</td>
<td>8.10 ±0.30</td>
</tr>
<tr>
<td>EQ</td>
<td>22.50 ±3.00</td>
<td>44.00 ±5.00</td>
<td>5.50 ±1.20</td>
</tr>
<tr>
<td>BHT</td>
<td>10.45 ±0.40</td>
<td>24.00 ±1.00</td>
<td>2.00 ±0.20</td>
</tr>
<tr>
<td>EQ+ BHT</td>
<td>28.00 ±0.43</td>
<td>42.00 ±1.00</td>
<td>5.20 ±0.40</td>
</tr>
</tbody>
</table>

**The main effect**

<table>
<thead>
<tr>
<th>Duration</th>
<th>SOD (u/mL)</th>
<th>CAT (ng/mL)</th>
<th>GSH (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 days</td>
<td>25.00 ±3.00</td>
<td>42.6 ±4.00</td>
<td>5.60 ±0.70</td>
</tr>
<tr>
<td>90 days</td>
<td>25.00 ±3.00</td>
<td>42.6 ±4.00</td>
<td>5.60 ±0.70</td>
</tr>
<tr>
<td>Sig.</td>
<td></td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Control</td>
<td>39.90 ±2.00</td>
<td>63.50 ±2.00</td>
<td>8.70 ±0.60</td>
</tr>
<tr>
<td>VH</td>
<td>62.70 ±2.00</td>
<td>62.70 ±2.00</td>
<td>8.10 ±0.30</td>
</tr>
<tr>
<td>EQ</td>
<td>44.00 ±5.00</td>
<td>44.00 ±5.00</td>
<td>5.50 ±1.20</td>
</tr>
<tr>
<td>BHT</td>
<td>24.00 ±1.00</td>
<td>24.00 ±1.00</td>
<td>2.00 ±0.20</td>
</tr>
<tr>
<td>EQ+ BHT</td>
<td>42.00 ±1.00</td>
<td>42.00 ±1.00</td>
<td>5.20 ±0.40</td>
</tr>
</tbody>
</table>

**The interaction effect**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD (u/mL)</th>
<th>CAT (ng/mL)</th>
<th>GSH (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33.00 ±0.40</td>
<td>59.00 ±1.20</td>
<td>7.50 ±0.20</td>
</tr>
<tr>
<td>VH</td>
<td>35.00 ±0.20</td>
<td>57.00 ±0.20</td>
<td>7.60 ±0.20</td>
</tr>
<tr>
<td>EQ</td>
<td>14.00 ±0.40</td>
<td>32.00 ±0.40</td>
<td>2.50 ±0.10</td>
</tr>
<tr>
<td>BHT</td>
<td>27.00 ±0.50</td>
<td>27.00 ±0.50</td>
<td>2.6 ±0.10</td>
</tr>
<tr>
<td>EQ+ BHT</td>
<td>36.00 ±0.24</td>
<td>43.00 ±0.50</td>
<td>5.00 ±0.40</td>
</tr>
<tr>
<td>Control</td>
<td>39.00 ±2.00</td>
<td>68.20 ±1.00</td>
<td>8.00 ±0.80</td>
</tr>
<tr>
<td>VH</td>
<td>46.00 ±3.00</td>
<td>68.00 ±1.00</td>
<td>7.60 ±0.60</td>
</tr>
<tr>
<td>EQ</td>
<td>56.00 ±1.00</td>
<td>56.00 ±1.00</td>
<td>8.50 ±0.60</td>
</tr>
<tr>
<td>BHT</td>
<td>71.00 ±1.00</td>
<td>71.00 ±1.00</td>
<td>1.58 ±0.20</td>
</tr>
</tbody>
</table>
Results showed that SOD, CAT, and GSH activities were significantly higher on the 90th day compared to their respective values on the 45th day. Regarding the effect of treatments, the lowest values of all studied antioxidants were observed in the BHT group compared to EQ and EQ+BHT group and both were also significantly decreased compared to the control group. The interaction between duration of exposure and treatment effect showed that on the 45th day of exposure, the lowest value of SOD was observed in BHT treated group relative to the control and other treated groups. EQ also significantly reduce the SOD level than control but still higher than BHT. The combined EQ+BHT exposure was less effective than separate exposure in reducing SOD activity, but its value was also lower than the control. On the 90th day all treated groups had a significant decrease in level of SOD. But EQ group had higher value compared to the BHT and combination groups.

The separate exposure to EQ and BHT for 45 days showed significantly lower values of CAT than combined exposure and BHT was the lowest one. On the other hand, the CAT activity was significantly lower in the BHT and combined group than EQ on the 90th day.

The results also demonstrated a comparable reducing effect of EQ and BHT on the GST content on the 45th day than EQ+BHT. On the 90th day, the GSH content was comparable to control in EQ and significantly higher than EQ+BHT and the lowest value was observed in BHT group.

**Effects of EQ, BHT, and their co-exposure on serum oxidative biomarkers**

The effects of EQ, BHT, and their co-exposure on serum oxidative biomarkers of treated rats were listed in Table (2).

Table (2): The changes in the levels of MDA and 8-OH-G in serum of male Sprague- Dawley rats treated with EQ and /or BHT for 45 and 90 days.

<table>
<thead>
<tr>
<th>The main effect</th>
<th>MDA (nmol/mL)</th>
<th>8OHG (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 days</td>
<td>71.00 b ±4.00</td>
<td>61.80 b ±5.00</td>
</tr>
<tr>
<td>90 days</td>
<td>91.70 c ±7.00</td>
<td>65.00 c ±8.00</td>
</tr>
<tr>
<td>Sig.</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>Control</td>
<td>42.50 c ±10.00</td>
<td>45.50 c ±7.00</td>
</tr>
<tr>
<td>VH</td>
<td>43.00 c ±12.00</td>
<td>42.00 c ±34.00</td>
</tr>
<tr>
<td>EQ</td>
<td>74.00 b ±3.00</td>
<td>57.00 b ±5.00</td>
</tr>
<tr>
<td>BHT</td>
<td>94.00 b ±4.00</td>
<td>40.00 c ±4.00</td>
</tr>
<tr>
<td>EQ+ BHT</td>
<td>93.00 b ±1.00</td>
<td>87.00 a ±1.00</td>
</tr>
<tr>
<td><strong>Treatments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig.</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Control</td>
<td>42.60 c ±1.00</td>
<td>43.00 c ±7.00</td>
</tr>
<tr>
<td>VH</td>
<td>41.00 c ±2.00</td>
<td>43.00 c ±1.00</td>
</tr>
<tr>
<td>EQ</td>
<td>66.00 c ±1.30</td>
<td>44.00 c ±1.30</td>
</tr>
<tr>
<td>BHT</td>
<td>82.90 c ±0.40</td>
<td>40.00 c ±0.40</td>
</tr>
<tr>
<td>EQ+ BHT</td>
<td>92.60 b ±1.00</td>
<td>87.00 a ±1.00</td>
</tr>
<tr>
<td>Control</td>
<td>42.00 c ±1.00</td>
<td>41.00 c ±2.00</td>
</tr>
<tr>
<td>VH</td>
<td>35.00 c ±1.80</td>
<td>40.00 c ±1.00</td>
</tr>
<tr>
<td></td>
<td>EQ</td>
<td>BHT</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>82.00 ±1.30</td>
<td>105.00 ±0.50</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>70.00 ±1.00</td>
<td>70.00 ±0.40</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD. n = 5/group. Means within the same column carrying different superscripts are significantly different * = P < 0.05, *** = P < 0.001. NS = not significant. MDA: malondialdehyde, 8-OH-dG: 8-hydroxyguanosine, VH: vehicle (positive control), EQ: ethoxyquin, BHT: butylated hydroxyl toluene.

Our study showed that lipid peroxidation level as represented by MDA was significantly elevated by increasing the time of exposure. The effect of EQ, BHT, and combination on MDA level revealed that the BHT and EQ+BHT groups had a significantly elevated MDA content compared to the EQ treated group and all were higher than control. The effect of interaction between duration and treatment on MDA level was observed, the level of MDA was significantly increased in EQ+BHT treated group followed by BHT group and EQ group on the 45th day of exposure. On the 90th day of exposure BHT treated group had a significant higher level of MDA followed by EQ+BHT group and finally the EQ group relative to the control group.

Concerning 8-OH-dG level, the effect of duration on 8-OH-dG revealed that level of 8-OH-dG was significantly elevated with increasing the time of exposure. The effect of EQ, BHT, and co-exposure on 8-OH-dG level had a significant (P < 0.001) increase relative to the control group and highest comparing to the EQ and BHT treated groups. The effect of interaction between time and treatment demonstrated a significant elevation in the level of 8-OH-dG in the combination group compared to the BHT and EQ groups on the 45th and 90th days of experiment.

**Cytotoxic effect of EQ, BHT, and their co-exposure on liver cells (comet assay)**

The effect of duration on comet assay parameters showed no significant differences at 45 and 90 days. Treatment effects on comet parameters demonstrated that the combined exposure to EQ+BHT significantly increased the DNA damage variables (tail length, ratio of DNA tail, and tail moment) than the EQ and BHT groups, but both were significantly higher than the control. The interaction between time and treatment had no significant effect on tail length and %DNA tail on the 45th and 90th days. The BHT treated group showed the highest tail moment on the 45th day relative to other groups. While on the 90th day, the highest value of tail moment was obtained by EQ treatment compared to other groups (Figure1).
Figure 1: The findings of the comet variables in liver tissue of rats from control group after 45 (A) and 90 (B) days showed single cell on micro gel electrophoresis. Liver of rats from EQ group after 45 (C) and 90 (D) days show single cell with tail on micro gel electrophoresis. Liver of rats from BHT group after 45 (E) and 90 (F) days show single cell with tail on micro gel electrophoresis. Liver of rats from EQ+BHT group after 45 (G) and 90 (H) days reveal single cell with tail on micro gel electrophoresis.

Effects of EQ, BHT, and their co-exposure on expression of hepatic CYP1A1 gene

There were a significant ($P < 0.001$) upregulation in transcriptional mRNA expression of $CYP1A1$ in liver tissue with increasing time of exposure. The BHT and combination groups had significantly higher expression of $CYP1A1$ gene than EQ and all were higher than the control group. The effect of interaction between duration and treatment on $CYP1A1$ gene expression revealed that the highest value among different group was observed in the BHT group on the 90th day of exposure followed by the combination group at the same duration, then the combined group and BHT group on the 45th day, respectively. The EQ treatment exhibit a lower upregulation of the studied gene than the BHT and combined groups and its effect was more prominent on the 90th day. On the other hand, the lowest values were recorded in control groups at both durations (Figure 2).
Figure 2: Graphical presentation of real-time quantitative PCR analysis of the expression of CYP1A1 gene in liver of EQ, BHT, and co-exposure treated rats. A: duration effect, B: treatment effect, and C: interaction effect. Means of columns carrying different superscript letters are significantly different. C: control, VH: vehicle, EQ: ethoxyquin, and BHT: butylated hydroxyl toluene.
Figure 3: Immunohistochemistry findings of liver tissue of control rats showed negative to weak caspase-3 (A) and moderate Bcl-2 immunostaining (E). Liver of rats after administration of EQ for 45 and 90 days showed moderate Caspase-3 (B) and mild Bcl-2 (F) immunostaining. Liver tissue of rats that were administered BHT for 45 and 90 days showed moderate caspase-3 (C) and mild Bcl-2 (G) immunostaining. Liver from rats that were administered EQ + BHT for 45 and 90 days showed strong caspase-3 (D) and weak Bcl-2 (H) immunostaining (scale bars = 30 μm).

**Immunohistochemistry findings of apoptosis related proteins in liver tissue**

The effects of EQ and/or BHT on the apoptosis related proteins have been demonstrated in Figure 3. Immunohistochemistry findings of liver tissue of rats from the control group showed negative to weak caspase-3 immunostaining (A) and moderate Bcl-2 immunostaining (E). The hepatic cells of rats that administrated EQ for 45 and 90 days showed moderate Caspase-3 immunostaining (B), and mild Bcl-2 immunostaining (F). While, the hepatic cells of rats which administrated BHT for 45 and 90 days showed moderate caspase-3 immunostaining (C) and mild Bcl-2 immunostaining (G). The hepatic cells of rats administered EQ + BHT for 45 and 90 days showed that strong caspase-3 immunostaining (D) and weak Bcl-2 immunostaining (H).

**Discussion**

Animal feed may contain different phenolic antioxidants such as BHT and EQ that have a pro-oxidant effect and can cause adverse health effects in animals [34]. This study revealed the lowest values of all studied antioxidants (SOD, CAT, and GSH) in the BHT group compared to EQ and EQ+BHT groups and both were also significantly lower than the control group. This result agreed with some earlier studies Ibrahim et al. [35], Chevillard et al. [36], Awah et al. [37], Kubiriza et al. [38], and Li et al. [39]. These may be due to pro-oxidative action of high EQ concentrations which may generate reactive oxygen species (ROS) leading to oxidative stress demonstrated by the reducing levels of serum antioxidants. Pro-oxidative action of high BHT concentrations which may generate ROS leading to oxidative stress demonstrated by the reducing levels of serum antioxidants and some studies
reported that not only BHT but also its metabolites, such as BHT-Q and BHT-QM, can act as prooxidants [65]. GSH is the main body antioxidant, acting as a direct free radical scavenger and as a substrate for GPx and GST. SOD catalyzes the conversion of superoxide radical to H$_2$O$_2$ which is further detoxified by CAT. The decline in liver antioxidants implies that the produced ROS had surpassed the scavenging capacity of antioxidants [28,40-42].

Also, BHT and EQ+BHT exposed groups presented increments in serum MDA and 8-OHdG level which reflects the failure of antioxidant defense system to counteract the ROS-induced damage. This further supports to consider oxidative stress a major mechanism of EQ and BHT-induced toxicity, as the two biomarkers represent lipid peroxidation and oxidative damage to DNA, which eventually results in tissue injuries. These results were proven by previous works obtained by Bernhard et al. [16], Awah et al. [37], and Elatrash [43]. Additionally, BHT has been reported to produce several reactive species during its oxidative metabolism and this can possibly mediate its adverse effects [44, 45].

Single cell gel electrophoresis (SCGE) or comet assay is a useful, simple, and flexible method to evaluate DNA damage and repair at individual cell level. Our results demonstrated that the combined exposure to EQ+BHT significantly increased the variables of DNA damage (length of tail, ratio of DNA in tail and tail moment) than the EQ and BHT groups, but both were significantly higher than the control. These results are in agreement with that given by Błaszczyk [17] and Sasaki et al. [46]. The interaction between time and treatment had no significant effect on tail length and %DNA tail at 45 and 90 days. This may be attributed to the pro-oxidant activity of EQ [47,48] and the intermediate metabolites of BHT such as BHT-quinone and BHT-quinonemethide (BHT-QM) transformed by the cytochrome P450 enzyme system in the liver [49, 50]. These metabolites form peroxides may induce DNA damage which was proved by the elevated 8-OHdG level in treated groups.

Calcium dependent endonuclease may be activated by BHT resulting in DNA fragmentation which is important stage in the process of apoptosis [51]. The results of our study are augmented by immunohistochemistry findings. Where, our study revealed that rat’s hepatic cells that administrated EQ or BHT for 45 and 90 days showed moderate expressions of Caspase-3 immunostaining and mild expressions of Bcl-2 immunostaining. However, the hepatic cells of rats that administrated EQ and BHT in combination for 45 and 90 days showed strong expressions of caspase-3 and weak expressions of Bcl-2. EQ has been reported to induce cytotoxic effects and apoptosis of cultured human lymphocytes [13, 14]. These results were supported by the increased lipid peroxidation in liver of treated rats which can regulate the mitochondrial oxidative stress resulting in reduction of the mitochondrial membrane potential and permeability transition which is the first stage in the process of apoptosis and the release of apoptogenic factors such as cytochrome C and procaspases 2, 3 and 9 [52].

Using of biomarkers is important in toxicology for numerous reasons, such as variabilities of several contaminants, sensitivities of many biomarkers, and the chemical specificity of some biomarkers [53, 54]. In Particular, CYP1A acts as an enzyme modulator of chemical detoxification and transformation of chemotherapeutics, steroids, and xenobiotics that can be used as biomarkers for oxidative stress [55]. Additionally, some xenobiotics cause damage over a lengthy period of time and have difficult or imprecise techniques of analysis. So, biomarkers can supply with early sensitive warning signs about the possible damages [56]. The obtained qPCR data revealed significant upregulation of CYP1A1 gene expression in the liver of BHT and combination groups than the EQ group and all were higher than the control group. These findings agree with those reported by
Bohne et al. [57] and Holaas et al. [58]. Similarly, BHT-induced over expression of hepatic CYP enzyme in rat [59]. Moreover, BHT is a potent inducer of the microsomal monooxygenase system and its major route of degradation is the oxidation catalyzed by cytochrome P450 [60]. EQ had inhibitory action through the formation of a complex with hepatic antioxidant response elements (EQ-ARE) which can interfere with CYP1A1 gene directly or indirectly through its binding with promoters and/or enhancers [61, 62]. The elevated expression of the tissue injury marker CYP1A1 in addition to the increased apoptosis and DNA damage comes in line with the depleted antioxidant activity and increased lipid peroxidation, suggesting oxidative stress as a possible mechanism of EQ and BHT toxic action.

Conclusion
The current findings suggested that EQ and BHT could induce alterations in the serum levels of antioxidants especially in combined exposure. In addition, EQ and BHT increased the level of serum lipid peroxidation, DNA damage, cytotoxicity, apoptosis, and upregulated the expression of the tissue injury marker CYP1A1 gene. These effects suggested that EQ and BHT are potentially hepatotoxic compounds and ROS could be implicated in their mechanism of toxic action. Therefore, the use of such compounds should be more controlled and limited as their excessive and prolonged exposure over time are causing extensive damages of vital body organs and tissues.

Author Contributions
Amany T. Mohammed, Mohamed M.M. Matwally, Dina Y. Hegab, Mervat H. Ghoneim, and Ali H. Abou-Hadded were equally contributed to all stages of preparing, drafting, and writing the manuscript. All authors read and approved the final version of this manuscript.

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الإيثوكسيكوين وهيدروكسيل التولوين الببتيد يسبب تأثير سام على الكبد عن طريق موت الخلايا والاجهاد التاكسدى في الجرذان و التعبير الجيني CYP1A1.

1. على أبو حديد 1، دينا حجاب 2، محمد مولى أمنى محمد 1، أماني محمد 1، محمد متولي 2، ميرفت غنيم 1، علي أبو حديد 1.

1. قسم الطب الشرعي والسموم، كلية الطب البيطري، جامعة الزقازيق، الزقازيق، 44511، مصر.

2. كلية الطب البيطري، جامعة الزقازيق، الزقازيق.

يحتوي العلف الحيواني على العديد من مضادات الأكسدة الفينولية مثل الإيثوكسيكوين وهيدروكسيل التولوين الببتيد و هيدروكسى انيسول. ووجود الإيثوكسيكوين وهيدروكسيل التولوين الببتيد بتركيزات عالية يؤدي إلى تأثير مكب ويبسبب آثار صحية ضارة على الحيوانات. تم تصميم هذه الدراسة لتقديم تأثير الإيثوكسيكوين و/أو هيدروكسيل تولوين ببتيد على الكبد في الفئران. تم تقسيم عدد 50 من ذكور جرذان سبراغ داولي إلى 5 مجموعات (10 جرذان لكل منها) على النحو التالي: المجموعة الأولى خدمت كمجموعة ضابطة حيث لم تتلق أي علاجات. المجموعة الثانية وتم إعطاؤها زيت الذرة. تم تجريع المجموعة الثالثة EQ عن طريق الفم يومًا بعد يوم لمدة 45 و 90 يومًا متتالية بجرعة 5/1 من الجرعة المميتة، وتم تجريع المجموعة الثالثة BHT يومًا بعد يوم لمدة 45 و 90 يومًا متتالية في جرعة من 1/5 من الجرعة المميتة. كانت المجموعة الخامسة تجريع EQ و BHT بنفس الجرعات والمدة الموصوفة أعلاه. أظهرت النتائج أن EQ و BHT وال تعرض المشترك لهما تسبب في انخفاض معنوي في مستويات إنزيمات مضادات الأكسدة، وال الأسلاك المتطايرة في مصل الفئران. كما تسبب أيضاً في زيادة معنوي في مستوى malondialdehyde MDA، 8-hydroxyguanosin في الجرذان. كا أن مجموعة تعرض المشترك زيادة كبيرة في تلف الحمض النووي، والتفاعل الإيجابي المناعي لـ caspase 3 و/or زيادة التعبير الجيني CYP1A1. أظهرت النتائج أن في خلايا الكبد للجرذان المعرضة EQ و BHT، والعرض المشترك لهما تسبب في انخفاض معنوي في مستويات إنزيمات مضادات الأكسدة في الفئران. تسبب أيضاً في زيادة معنوي في malondialdehyde MDA، 8-hydroxyguanosin في مصل الفئران. كان في مجموعة تعرض المشترك زيادة كبيرة في TGF-β في تلف الحمض النووي، والتفاعل الإيجابي المناعي لـ caspase 3  و/or CYP1A1 mRNA المنتظم في خلايا الكبد للجرذان المعرضة EQ و BHT، والعرض المشترك لهما تسبب في انخفاض معنوي في مستويات إنزيمات مضادات الأكسدة في الفئران. تسبب أيضاً في زيادة معنوي في TGF-β في تلف الحمض النووي، والتفاعل الإيجابي المناعي لـ caspase 3 و/or CYP1A1 mRNA المنتزم في خلايا الكبد للجرذان المعرضة EQ و BHT، والعرض المشترك لهما تسبب في انخفاض معنوي في مستويات إنزيمات مضادات الأكسدة في الفئران. تسبب أيضاً في زيادة معنوي في TGF-β في TGF-β في TGF-β.