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In Vitro Formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in Calf Thymus DNA upon Treatment of 2'-deoxyguanosine with Propyl Gallate and 2,6-di-tert-butyl-p-benzoquinone

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Cover Page Footnote

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Abstract

Oxidative DNA damage caused by propyl gallate (PG) and 2,6-di-*tert*-butyl-*p*-benzoquinone (BHT-quinone, a metabolite of butylated hydroxytoluene (BHT)) was analyzed from the 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation in calf thymus DNA and DNA base, 2′-deoxyguanosine (dG). PG in the presence of $CuCl_2$ increased the 8-OHdG formation in calf thymus DNA by around 9.17 times as compared to the control (untreated DNA). In the presence of $CuCl_2$ at 1.28×10^{-5} M, the 8-OHdG per dG ratio resulting from the reaction of dG with PG at various concentrations (20–150 ppm) ranged from 75.50 to 312.06 8-OHdG per 10^5 dG. The 8-OHdG formation increased when the PG concentration was increased from 20 ppm to 80 ppm, and then, it began to plateau around 80 ppm. On the other hand, BHT-quinone increased the formation of 8-OHdG in the presence of $CuCl_2$ by 0.05 times as compared to the control (untreated DNA). LC-MS/MS analysis was used to identify the molecular structure of 8-OHdG, which had a base peak ($M^{+\cdot} + 1$) at m/z = 284 and two main fragments at m/z = 167.9 and m/z = 139.9.

Abstrak

Pembentukan 8-Hidroksi-2′-Deoksiguanosin (8-OHdG) dalam *Calf thymus* DNA yang Disebabkan dari Reaksi 2′-Deoksiguanosin dengan Senyawa Propil Galat dan 2,6-Di-*Tert*-Butil-*p*-Benzoquinon secara *In Vitro*. Kerusakan oksidatif DNA yang disebabkan oleh propil galat (PG) dan 2,6-di-*tert*-butil-*p*-benzoquinon (BHT-quinon, metabolit BHT), dianalisis dari pembentukan DNA *adduct*, 8-hidroksi-2'-deoksiguanosin (8-OHdG), terhadap *calf thymus* DNA dan basa tunggal DNA, 2′-deoksiguanosin (dG) secara *in vitro*. PG dengan dimediasi oleh $CuCl_2$ menyebabkan peningkatan 8-OHdG terhadap *calf thymus* DNA sebesar 9,17 kali lebih besar dibandingkan terhadap kontrol (DNA tanpa perlakuan). Dengan adanya $CuCl_2$ pada konsentrasi 1,28 $x10^{-5}$ M, rasio pembentukan 8-OHdG dari hasil interaksi antara dG dengan PG pada berbagai variasi konsentrasi (20-150 ppm) berkisar antara 75,50-312,06 8-OHdG terhadap 10^5 dG. Pembentukan 8-OHdG tersebut, meningkat dengan bertambahnya konsentrasi PG dari 20-80 ppm, kemudian mulai stabil dengan bertambahnya konsentrasi PG diatas 80 ppm. Sementara itu, BHT-quinon dengan adanya $CuCl_2$ menyebabkan peningkatan 8-OHdG terhadap *Calf thymus* DNA sebesar 0,05 kali dibandingkan kontrol (DNA tanpa perlakuan). Analisis menggunakan LC-MS/MS dilakukan untuk mengidentifikasi 8-OHdG, dengan puncak induk ($M^{+\cdot} + 1$) 284 dan memiliki dua fragmen utama m/z 167,9 dan m/z 139,9.

Keywords: 2,6-di-tert-butil-p-benzoquinone, DNA adduct, 8-hydroxy-2´-deoxyguanosine, propyl gallate

Introduction

Propyl gallate (PG) and butylated hydroxytoluene (BHT) are synthetic antioxidants that are widely used as food additives to prevent food deterioration due to oxidation. PG and BHT have been investigated as chemopreventive agents in several animal experiments [1-3]. Although their use has been approved as food additives in many countries, the safety of these antioxidants is a controversial issue because several research reports have indicated that they have some adverse health effects [4-10].

In contrast to these protective effects, PG was shown to induce apoptosis due to DNA fragmentation in hepatocytes [4]. Jacobi *et al.* [5] reported that PG

induced *single strand breaks* in the presence of Cu (II). Gallic acid and its alkylesters caused strand scission in the presence of Cu (II) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) was the best-characterized base adduct formed via the action of the reactive oxygen species (ROS) [6].

Ito *et al.* reported that BHT can act as tumor promoter in rat urinary bladder and thyroid [7]. BHT is known to be extensively metabolized in its target tissues, and the toxic as well as tumor-promoting activities of BHT are thought to be mediated by metabolites of the parent compound [8]. One of the BHT metabolites, 2,6-di-*tert*-butil-*p*-benzoquinone (BHT-quinone), has been reported to cause cleavage of supercoiled DNA [9]. Oikawa *et al.* [10] reported that BHT metabolites, *i.e.*, BHT-quinone and 2,6-di-*tert*-butyl-4-hydroperoxyl-4-methyl-2,5-cyclohexa-dienone (BHT-OOH), induced oxidative DNA damage.

Because of the widespread use of PG and BHT and the adverse effect of these antioxidants on DNA, it is necessary to investigate their interaction with DNA.

In this study, the binding or interaction of PG and BHT-quinone with calf thymus DNA was observed using a UV-Vis spectrophotometer. Furthermore, to determine the interaction of PG and BHT-quinone with DNA bases, we examined the covalent addition products, known as "DNA adducts" (*i.e.*, 8-OHdG), which served as biomarkers for oxidative DNA damage in calf thymus DNA at the DNA base, 2′-deoxyguanosine (dG). This adduct formation was mediated by cupric chloride and was detected with HPLC equipped with UV-Vis detectors. Finally, the molecular structures of these adducts were identified using LC-MS/MS.

Materials and Methods

Chemicals. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) (Sigma-Aldrich); 2′-deoxyguanosine (dG) (Sigma-Aldrich); *calf thymus* DNA (Invitrogen); *micrococcal nuclease* from *Staphylococcus aureus* (Sigma-Aldrich); phosphodiesterase II from bovine spleen (Sigma-Aldrich); propyl gallate (PG) (Merck); 2,6-di-*tert*-butyl-*p*-benzoquinone (BHT-quinone) (Sigma-Aldrich); tris (hydroxymethyl) aminomethane (Merck); cupric chloride dihydrate ($CuCl_2.2H_2O$) (Merck); sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) (Merck); formic acid (Merck); methanol (Merck); hydrogen chloride (HCl)(Sigma-Aldrich); disodium succinate hexahydrate ($NaOOCH_2OHCOONa.6H_2O$) (Merck); calcium chloride-2-hydrate ($CaCl_2.2H_2O$) (Merck).

Analysis of interaction between DNA and PG using UV-Vis spectrophotometer. The reaction mixture of 5 mL contained 20 ppm calf thymus DNA and various concentrations of PG (0, 5, 10, 15, and 20 ppm) in 50 mM Tris HCl buffer (pH 7.4). The reaction mixture was incubated at room temperature for 1 h and then analyzed using a UV-Vis spectrometer (UV-1800, Shimadzu).

Analysis of formation of 8-OHdG in calf thymus DNA by PG and BHT-quinone in the presence of $CuCl_2$. Calf thymus DNA was treated with PG in the presence of $CuCl_2$ in 50 mM Tris HCl (pH 7.4). The reaction mixture of 100 µL was incubated at 37 °C for 1 h. Then, the reaction mixture containing 2.67 mg/mL DNA, 4.27×10^{-5} M $CuCl_2$, and 40 ppm PG was hydrolyzed to deoxyribonuleosides with a mixture of *micrococcal nuclease* (2 mU/µg DNA) and phosphodiesterase II (0.04 mU/µg DNA) in 0.1 M sodium succinate and 0.05 M $CaCl_2$ buffer (pH 6). The mixture was incubated at 37 °C for 3 h. The same procedure was applied to 200 ppm BHT-quinone. Then, the hydrolyzed DNA was analyzed by HPLC equipped with UV-Vis detectors using an octadecylsilane column (XBridge C18, 4.6 x 250 mm, 10 µm (Waters)) with 10 mM $NaH_2PO_4.H_2O$/15% methanol at a flow rate of 0.1 ml/min. The hydrolyzed DNA was also analyzed by LC-MS/MS with a TQ *Detector* (Waters); the column used was a reversed-phase column (Atlantis T3, 4.6 x 50 mm, 5 µm (Waters)). Gradient elution was performed using 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). The flow rate was set to 0.5 mL/min. The gradient elution applied for this analysis is summarized in Table 1.

Mass spectrometric detection was performed on a triple quadrupole tandem mass spectrometer coupled with an electrospray ionization source. The optimized ESI source parameters are listed in Table 2. The determination of 8-OHdG formation was performed under the multiple reaction monitoring (MRM) mode.

Table 1. Gradient Elution

Time (min)	Eluent A (%)	Eluent B (%)
0	50	50
3	5	95
5	5	95
5.5	50	50
10	50	50

Table 2. Working Parameters for Mass Spectrometry

Parameter	Value
Scan type	MRM
Ionization mode	Positive
Collision cell pressure (mbar)	1.33×10^{-5}
Desolvation gas flow (L/h)	1000
Collision gas flow (mL/min)	0.4
Source temperature (°C)	150
Desolvation temperature (°C)	350
Dwell time (s)	0.15
Capillary voltage (kV)	3.5
Cone (V)	24
Collision energy (eV)	20 (167.9) and 40 (139.9)
Ion transition (m/z)	284 > 167.9 and 139.9

The 8-OHdG had a retention time of 3.5 min and a base peak ($M^{+.} + 1$) at m/z = 284 with two main fragments at m/z = 167.9 and m/z = 139.9.

Analysis of 8-OHdG formation resulting from the reaction of DNA base dG with PG and BHT-quinone in the presence of metal ion. The reaction mixture of 500 µL contained 200 ppm dG, 1.28×10^{-5} M $CuCl_2.2H_2O$, and PG at various concentrations (20, 30, 40, 50, 60, 80, 100, and 150 ppm) in 50 mM Tris HCl buffer (pH 7.4). The reaction mixture was incubated at room temperature for 1 h and then measured with HPLC equipped with UV-Vis detectors. The same procedure was applied to BHT-quinone at various concentrations (160, 200, 240, 320, and 360 ppm). The amount of 8-OHdG was measured by calculating the ratio of the peak area of 8-OHdG to that of the initial dG and it was expressed as $8\text{-OHdG}/10^5$ dG. Furthermore, the molecular structure of the formed adduct was identified using LC-MS/MS.

Results and Discussion

UV-Vis spectrophotometer analysis of interaction of PG with DNA. Binding or interaction of a compound with DNA causes adsorption spectra changes [11]. Figure 1 shows the spectra changes of DNA when it is incubated with various concentrations of PG. The control (untreated DNA) exhibited maximum absorption at 259 nm, while PG exhibited maximum absorption 273 nm. With increasing amounts of PG added to the DNA solution, the absorption intensity of DNA centered at 259 nm increased (hyperchromism) gradually, accompanied by red shift (bathochromic). The observed hyperchromism and bathochromic shift might be attributed to the interaction between PG and DNA.

On the other hand, the interaction between BHT-quinone and DNA was not analyzed using the UV-Vis spectrophotometer, because BHT-quinone had maximum absorption at around 260.40 nm, which was close to the absorption peak of calf thymus DNA at 259 nm (not shown); thus, the spectra were not selected for analyzing the interaction between BHT-quinone and DNA.

Formation of 8-OHdG in *calf thymus* DNA. The HPLC chromatogram of the control (untreated DNA) shown in Figure 2 indicates that the DNA was hydrolyzed into its four nucleosides. The nucleosides were separated from one another. The retention time for dG was 6.938 min. The HPLC analysis revealed approximately 49.27% and 52.87% decrease in the peak area of dG following treatment with PG and BHT-quinone, respectively, in the presence of $CuCl_2$.

It is suggested that the treatment of DNA with PG and BHT-quinone in the presence of $CuCl_2$ destroyed dG. Beach and Gupta [12] had shown that guanine bases in DNA are the predominant sites to be attacked by chemical
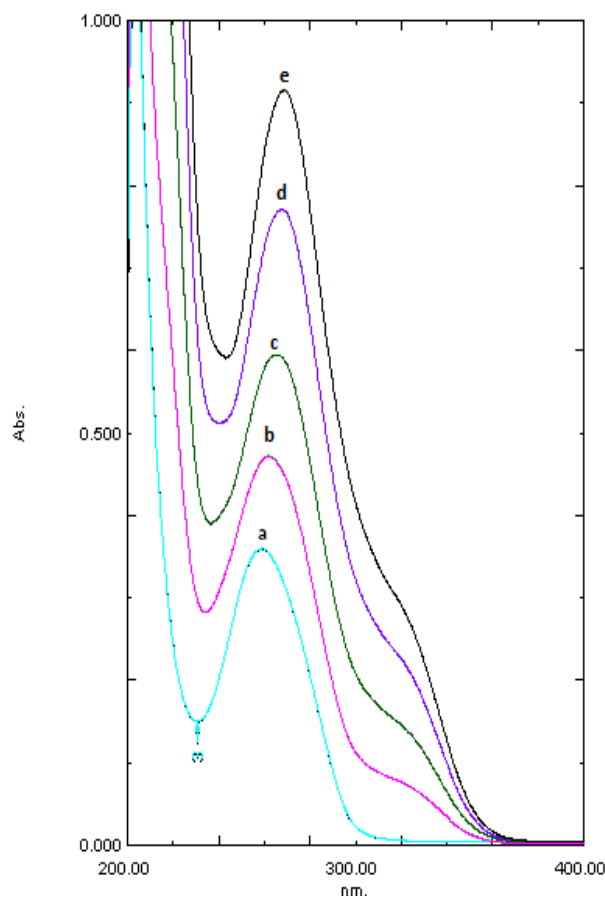

Figure 1. UV–Vis Absorption Spectra of DNA with Varying PG Concentration. [PG]/[DNA] = 0, 0.25, 0.50, 0.75, and 1 Correspond to the Curves a–e, Respectively

carcinogens. However, within the limits of UV detection, we found no evidence of the formation of 8-OHdG either in the control (untreated DNA) or in the DNA treated with PG and BHT-quinone in the presence of $CuCl_2$.

On the other hand, LC-MS/MS analysis of the control (untreated DNA) and the DNA treated with PG and BHT-quinone in the presence of $CuCl_2$ detected 8-OHdG, which had a base peak ($M^{+.} + 1$) at m/z = 284 and two main fragments at m/z = 167.9 and m/z = 139.9 (Figures 3, 4, and 5, respectively). This is due to the lower limit of detection of LC-MS/MS as compared to that of HPLC.

The formation of 8-OHdG in calf thymus DNA in PG mediated with $CuCl_2$ was 9.17 times higher than that in the control (untreated DNA). On the other hand, the formation of 8-OHdG in calf thymus DNA in BHT-quinone mediated with $CuCl_2$ was 0.05 times higher than that in the control (untreated DNA).

Formation of 8-OHdG resulting from the reaction of dG with PG and BHT-quinone in the presence of

CuCl$_2$. The formation of 8-OHdG was observed as a result of the reaction between dG and PG at various concentrations (20–150 ppm) mediated by CuCl$_2$. The

8-OHdG formation was induced by neither PG (in the absence of CuCl$_2$) nor CuCl$_2$ (in the absence of PG), as detected by HPLC equipped with UV detectors. The
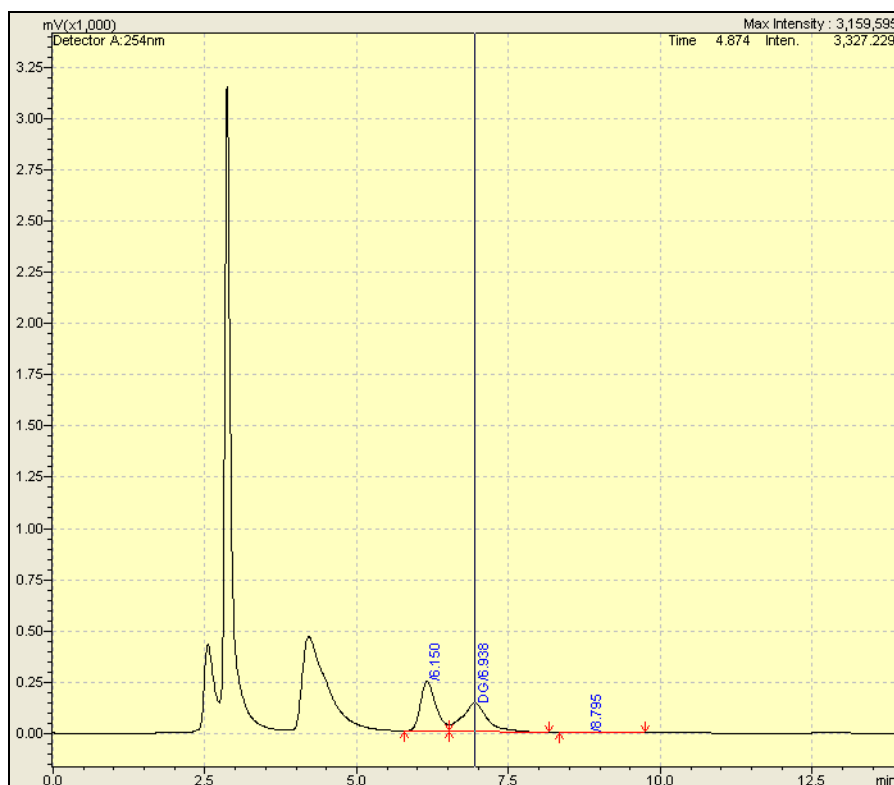

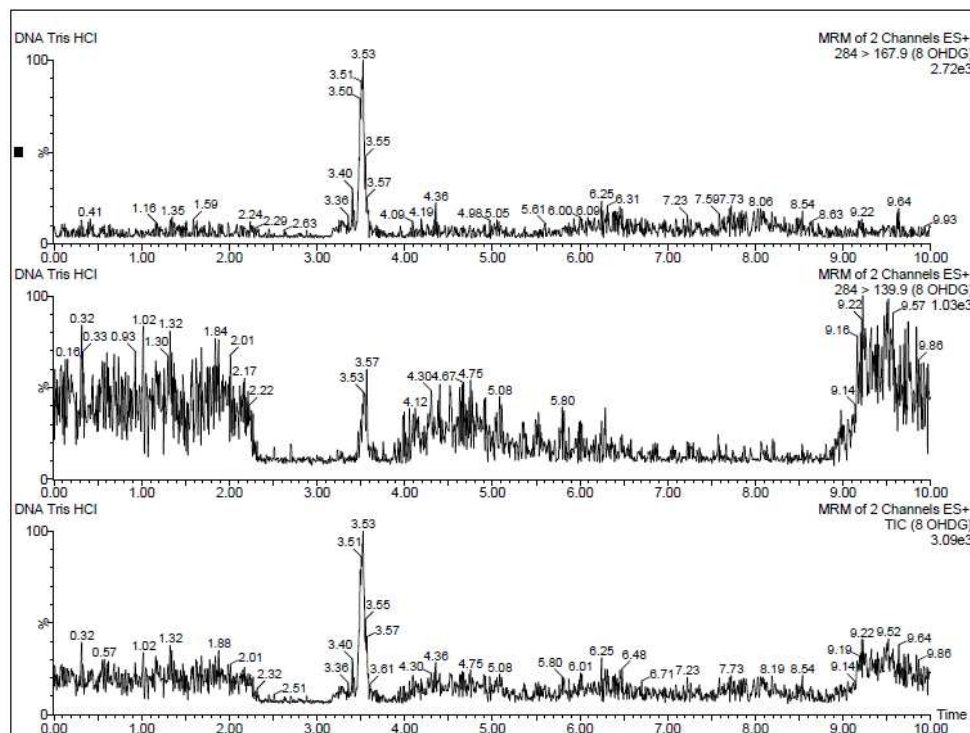**Figure 2. HPLC Chromatogram of Hydrolyzed Control (untreated DNA)**

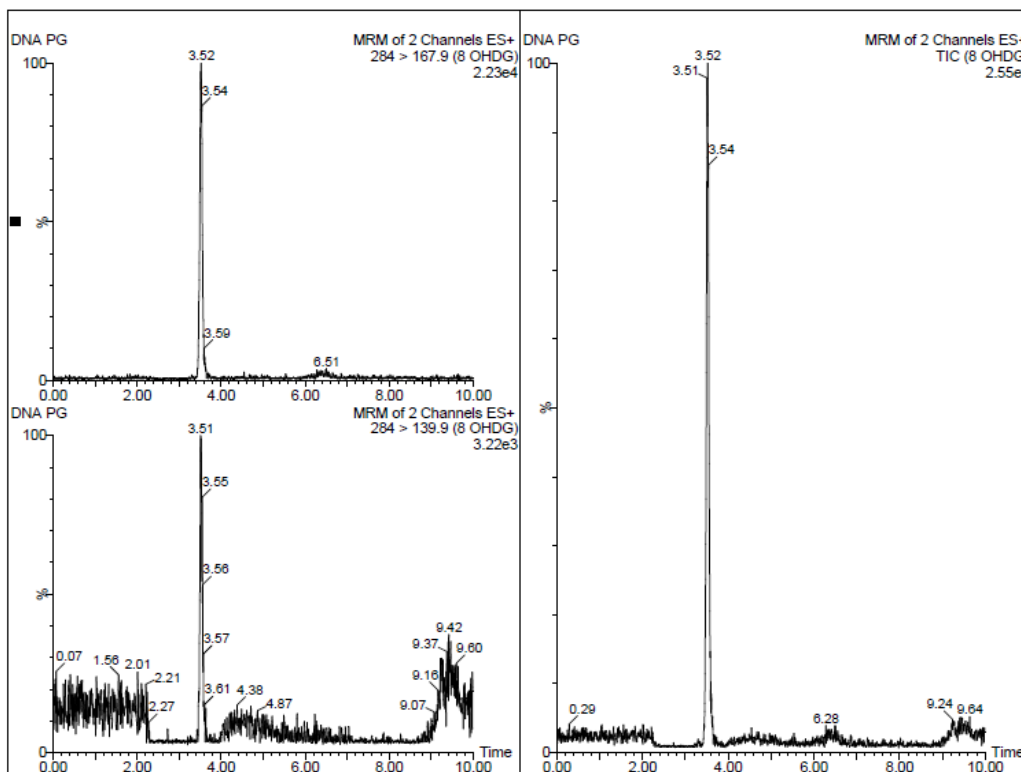**Figure 3. LC-MS/MS Chromatogram of Hydrolyzed Control (untreated DNA)**

Figure 4. **LC-MS/MS Chromatogram of 8-OHdG Formation in 2.67 mg/mL *calf thymus* DNA Induced by 40 ppm PG in the Presence of $4.27{\times}10^{-5}$ M CuCl$_2$**
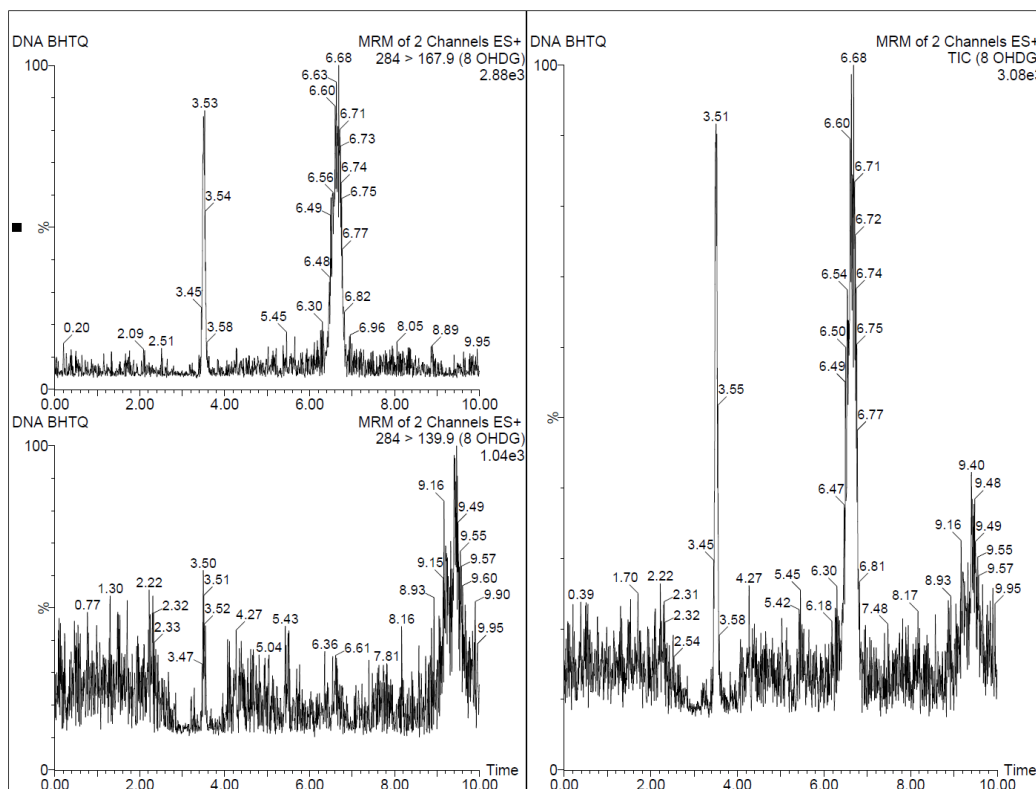

Figure 5. **LC-MS/MS Chromatogram of 8-OHdG Formation in 2.67 mg/mL *calf thymus* DNA Induced by 200 ppm BHT-Quinone in the Presence of $4.27{\times}10^{-5}$ M CuCl$_2$**
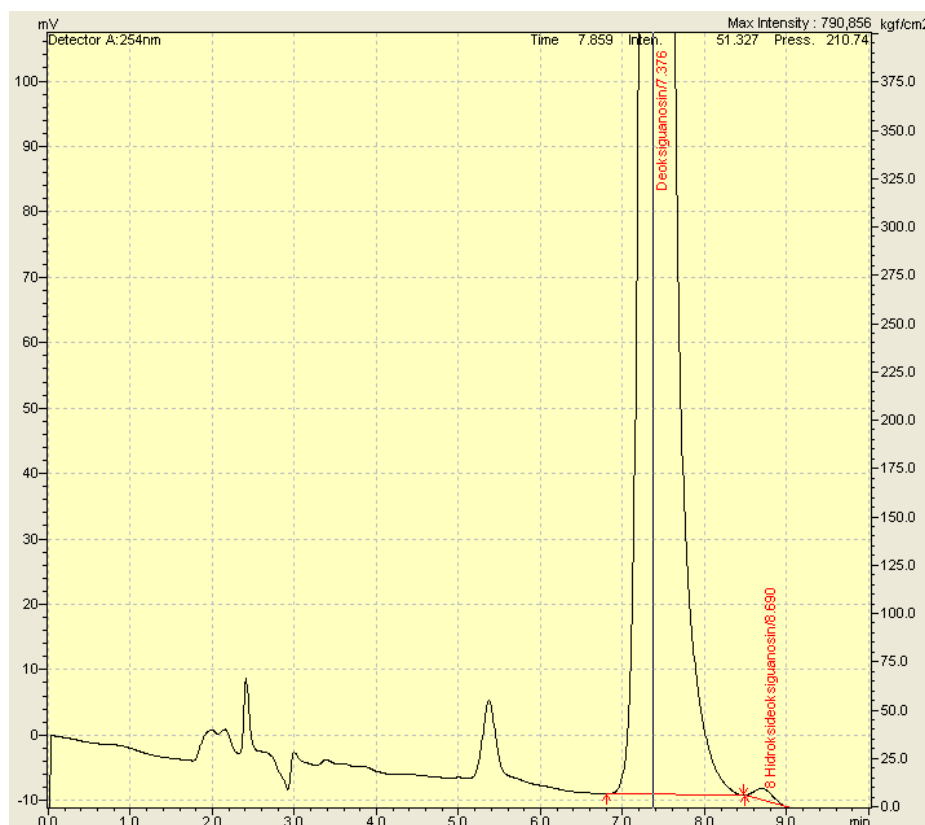
Figure 6. **HPLC Chromatogram of 8-OHdG Formation Resulting from the Reaction between 200 ppm dG and 40 ppm PG in the Presence of 1.28×10^{-5} M CuCl$_2$**

chromatogram of 8-OHdG induced by 40 ppm PG in the presence of Cu (II) metal ions is shown in Figure 6. In the presence of 1.28×10^{-5} M CuCl$_2$, the 8-OHdG per dG ratio resulting from the reaction between dG and PG at various concentration (20–150 ppm) ranged from 75.50–312.06 8-OHdG per 10^5 dG. The formation of 8-OHdG increased as the PG concentration was increased from 20 ppm to 80 ppm, and it began to plateau above 80 ppm (Figure 7).

LC-MS/MS analysis of the reaction mixture of dG and PG in the presence of CuCl$_2$ also identified 8-OHdG, which had a retention time of 3.5 min and a base peak ($M^{+} + 1$) at m/z = 284 with two main fragments at m/z = 167.9 and m/z = 139.9 (Figure 8).

On the other hand, HPLC analysis of the reaction mixture of dG and BHT-quinone at various concentrations (160–360 ppm) in the presence of 1.28×10^{-5} M CuCl$_2$ did not detect 8-OHdG formation (data not shown), but LC-MS/MS analysis identified 8-OHdG formation (Figure 9). This is due to the lower limit of detection of LC-MS/MS as compared to that of HPLC.

The present study showed that PG and BHT-quinone in the presence of CuCl$_2$ increased the formation of 8-OHdG in calf thymus DNA and dG. The formation of 8-
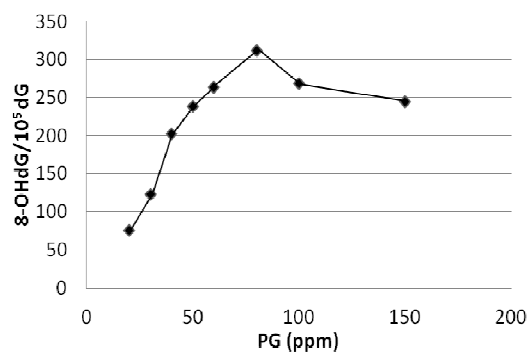

Figure 7. **Formation of 8-OHdG (8-OHdG/10^5 dG) Resulting from the Reaction between 200 ppm dG and PG at Various Concentration (20–150 ppm) in the Presence of 1.28×10^{-5} M CuCl$_2$. The Values are Means of Duplicate Measurements**

OHdG is an indicator of oxidative stress and is known to be mutagenic in mammalian cells [13]. Thus, 8-OHdG is known to cause DNA misreplication, leading to mutation or cancer [14].

The results of the experiments showed that the formation of 8-OHdG induced by PG and BHT-quinone was stimulated by CuCl$_2$. Copper is found in the body, and the normal level of copper in various organs is
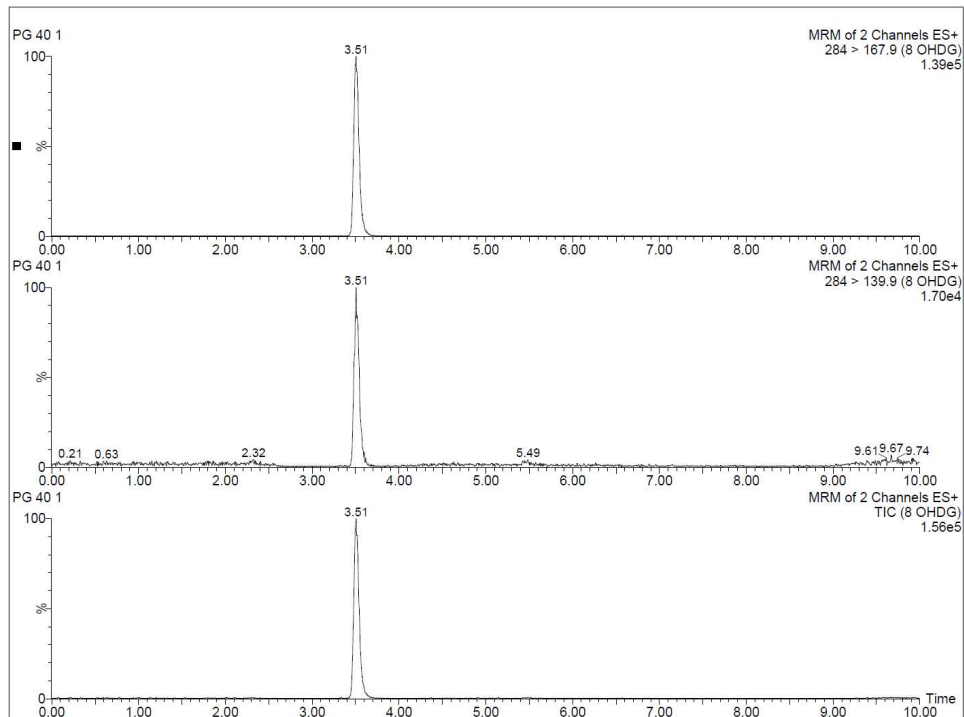
Figure 8. LC-MS/MS Chromatogram of 8-OHdG Formation Resulting from the Reaction between 200 ppm dG and 40 ppm PG in the Presence of 1.28×10^{-5} M $CuCl_2$

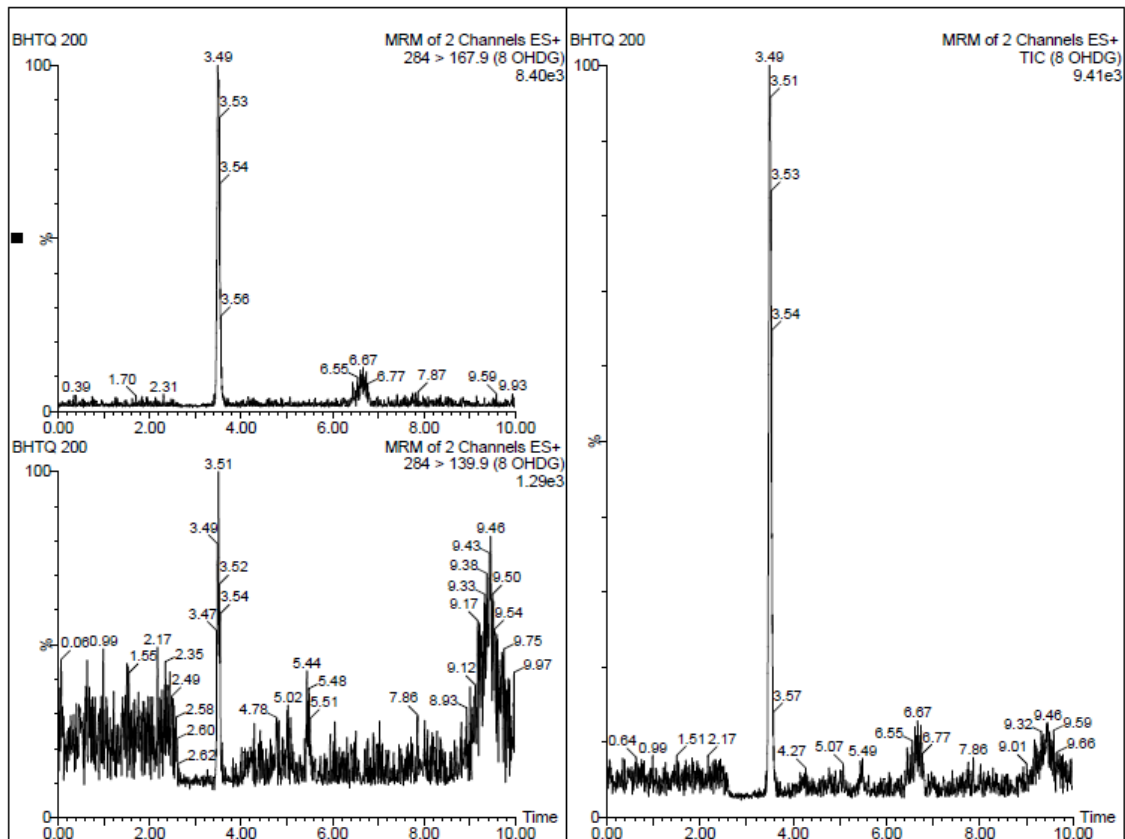**Figure 9. LC-MS/MS Chromatogram of 8-OHdG Formation Resulting from the Reaction between 200 ppm dG and 200 ppm BHT-quinone in the Presence of 1.28×10^{-5} M $CuCl_2$**

approximately 0.85–3.60 µg/mL [15]. Free Cu(II) ions are found in the nucleus, and they have a high affinity for DNA [16]. Copper ions are known to mediate oxidative damage to DNA, which is detected as 8-OHdG formation [17].

An oxidative DNA lesion is assumed to be caused by the hydroxyl radical generated from hydrogen peroxide (H_2O_2) and copper through the Fenton reaction [18]. The formation of 8-OHdG induced by gallic acid may be due to the hydroxyl radical ($^{\cdot}OH$) produced depending on the transition metals [6]. The possible mechanisms of metal-mediated hydroxyl radical formation induced by PG are shown in Figure 10.

Metal-mediated auto-oxidation of PG generates the semiquinone radical. The Cu (II) ion is reduced by gallate to form Cu(I), which reduces molecular O_2 to the superoxide anion ($O_2^{\cdot-}$) [6]. Then, $O_2^{\cdot-}$ may undergo a dismutation reaction, represented by Equation (1), in which H_2O_2, a nonradical, is formed [19].

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{1}$$
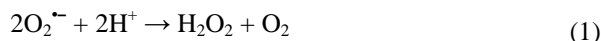
H_2O_2 can combine with Cu^+ to generate $^{\cdot}OH$ through the Fenton reaction (Equation 2) [21]:

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + {}^{\cdot}OH \tag{2}$$
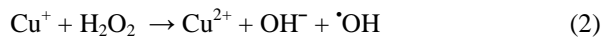
The hydroxyl radical ($^{\cdot}OH$) is a highly reactive ROS that can react with DNA adducts, such as 8-OHdG, as an oxidative DNA damage marker [22]. Although all DNA bases are susceptible to damage, guanine is the most prone to oxidative modification [23]. The mechanism of 8-OHdG formation results from the reaction of 2'-deoxyguanosine (dG) with hydroxyl radicals ($^{\cdot}OH$), as shown in Figure 11. The results showed increasing formation of 8-OHdG induced by PG mediated with $CuCl_2$, which is suggested to occur because of the increase in the hydroxyl radicals.

Figure 7 shows an interesting feature of 8-OHdG formation induced by PG in the presence of Cu(II), *i.e.*, the 8-OHdG formation began to plateau at high
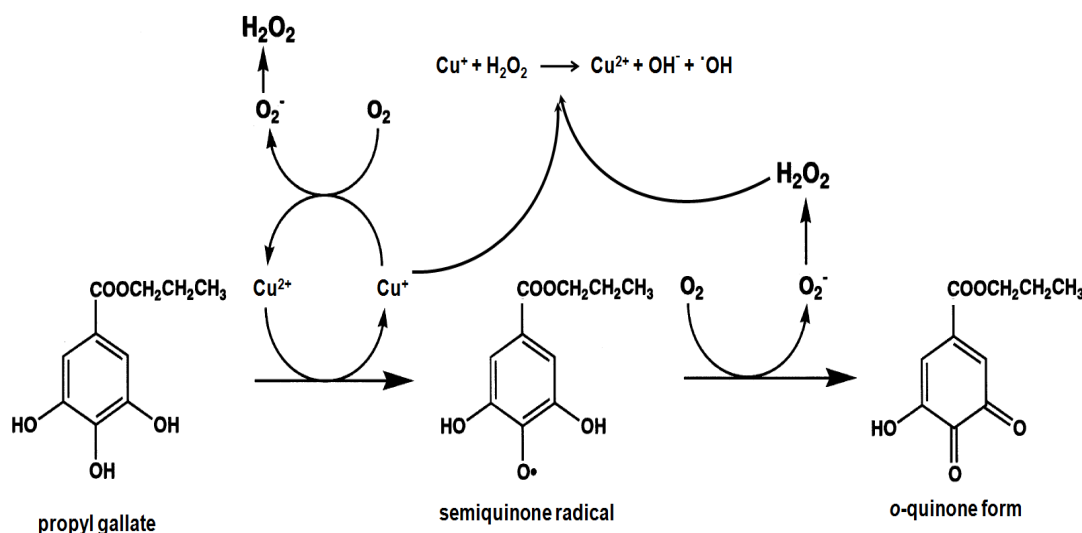

Figure 10. Possible Mechanism of Formation of Hydroxyl Radical Induced by Propyl Gallate (PG) Mediated with Cu(II) (adopted from Kobayashi *et al.* [20] with Modification)
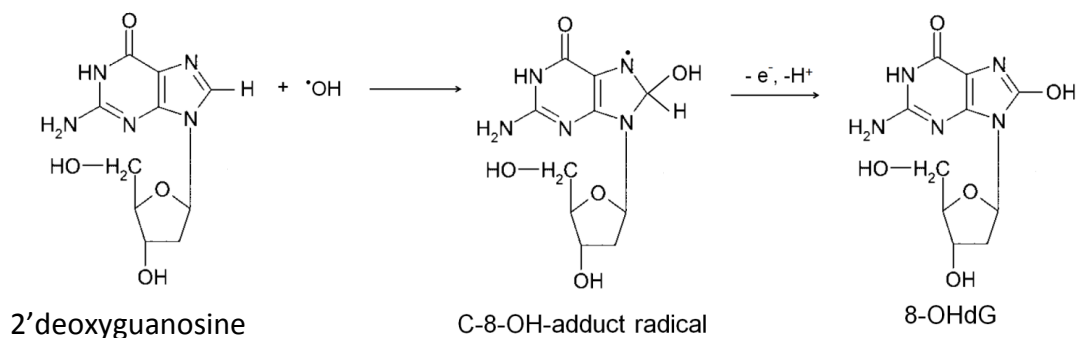

Figure 11. Reaction between 2'-deoxyguanosine and Hydroxyl Radical (adopted from Valavanidis *et al.* [22] with Modification)

concentrations of PG. These phenomena can be attributed to the ability of compounds to participate in more than one reaction. In this situation, a compound could act as a pro-oxidant at low concentrations and stimulate the formation of radicals. However, at higher concentrations, these compounds may scavenge free radicals. This is the case for compounds that are strong reducing agents, such as ascorbic acid and gallic acid [24].

The results of this study showed that PG, a gallic acid derivative, could act as a pro-oxidant at low concentrations in the presence of metal ions. The pro-oxidant effect of such compounds is thought to be responsible for DNA damage, as demonstrated by researchers [4-6].

On the other hand, BHT-quinone is a quinone compound and one of the metabolites of BHT antioxidant. The addition of $CuCl_2$ to 200 ppm BHT-quinone induced a slight increase in 8-OHdG levels (0.05 times as compared to the control). Studies conducted by Oikawa *et al.* [10] showed that BHT-quinone caused DNA damage not in the presence of Cu(II) but in the presence of NADH and Cu(II).

Conclusions

From the results of this study, it was concluded that PG induced the formation of 8-OHdG, as an indicator of oxidative DNA damage, in calf thymus DNA and 2′-deoxyguanosine (dG) in the presence of $CuCl_2$. PG in the presence of $CuCl_2$ increased the 8-OHdG formation in calf thymus DNA by around 9.17 times as compared to the control (untreated DNA). On the other hand, BHT-quinone (BHT metabolite) at high concentration, in the presence of $CuCl_2$, had a slight effect on 8-OHdG formation. Further *in vivo* studies on the formation of 8-OHdG induced by PG and BHT-quinone in the presence of $CuCl_2$ are required to observe additional details as to whether 8-OHdG formation is dose-dependent and to consider the dynamic process of repair and mutagenicity of the adduct target that can ultimately lead to cancer formation.

Acknowledgements

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