The study of DNA adduct 8-hydroxy-2’deoxyguanosine (8-OHdG) formation of butylated hydroxyanisole (BHA) and its metabolite ter-butyl hydroquinone (TBHQ) through in vitro reaction with Calf Thymus DNA and 2’deoxyguanosine

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Abstract. Butylated Hydroxyanisole (BHA) and its metabolite Tert-Butyl Hydroquinone (TBHQ) are synthetic antioxidants, commonly used as food and beverage preservatives. Although WHO declared their safety, the use of these preservatives are still controversial because some studies showed that BHA induced proliferative effects in animal testing and TBHQ is considered as carcinogenic and causes DNA cleavage. This study is aimed to analyze the interaction between Calf Thymus DNA with BHA and TBHQ which are mediated with Copper (II) Chloride. The result of the study in spectrophotometric showed there was bathochromic shift as much as 2-3 nm in DNA treated with TBHQ. The next analysis used HPLC method in stationary phase of ODS, mobile phase of 10mM Natrium Hydrogen Phosphate Buffer and Methanol (85 : 15) for DNA adduct formation, 8-Hydroxy-2-Deoxyguanosine (8-OHDG) as biomarker of risk cancer. The result of the study showed the formation of DNA adduct 8-OHdG in the interaction between DNA and 20-500 ppm of TBHQ. The 8-OHdG formation was greatly increased by the higher concentration of TBHQ. The relative amount of 8 OHDG which formed was reached 946/105 deoxyguanosine in DNA bases. Confirmation test by LCMS/MS was characterized with the detection of mother ion peak (m/z 284); fragment ion peaks at m/z 167.9, and 139.9; at retention time 3.52 min. Meanwhile the interaction between DNA and 50-250 ppm BHA did not induce 8-OHDG.

Keyword: Butylated hydroxyanisole (BHA), tert-butyl hydroquinone (TBHQ), 8-hydroxy-2-deoxyguanosine (8-OHDG), DNA adduct, spectrophotometric, HPLC, LCMS/MS

1. Introduction
Butylated Hydroxyanisole (BHA) and Tert - Butyl Hydroquinone (TBHQ) are synthetic antioxidants in phenolic groups which commonly used as a preservative in food and beverage industry. They have a highly solubility in animals fat and stable in various conditions during food processing. BHA is expected to prevent free radicals formation and prevent lipid oxidation [1]. This substance is able to significantly obstruct or inhibit the oxidation of a substance that is easily oxidized even in low concentrations, not more than 0.02% of oil or fat contained in food [2]. TBHQ is formed by BHA through an O-methylated process [3]. TBHQ is a preservative in products of fats and oils; animal fat; margarine and similar products; snacks made from potatoes, cereals, flour and starch; and nut products [3].
Although the use of preservatives such as BHA and TBHQ is permitted by the WHO with BHA acceptable daily intake (ADI) of 0.5 mg/kg body weight [3], and TBHQ at 0.70 mg/kg body weight [4-6] until now, BHA and TBHQ acceptable consumption still makes controversy because several studies reported that BHA and TBHQ could damage DNA, leading to initiate and promote tumors in several animal tissues. TBHQ can cause the breakdown of DNA in vitro [7] and form 8-hydroxy-2'-deoxyguanosine on Calf Thymus DNA [8].

Study on xenobiotics (chemical compounds) interaction with DNA became a hot topic in this recent years. The covalent interaction between the DNA bases with chemical compounds can produce a form of adducts known as DNA adduct and expected as one of the important stages in the process of cancer initiation [9]. DNA adducts can cause either the coding (miscoding) during transcription or sometimes cause mutations and cancer [10]. DNA adduct can be useful as a molecular biomarker that provides the best approach to early detection of cancer risk [11]. DNA adduct formation by BHA and TBHQ interaction with DNA can be proved through in vitro and in vivo study. This research is aimed to confirm if the interaction between DNA, BHA and TBHQ could lead to DNA adducts formation as biomarkers of cancer risk.

2. Materials and methods

In this research, the study conducted was DNA binding formation in spectrophotometry and study of DNA adducts formation by using HPLC and LC MS/MS. The analytical method has been validated in the previous research.

Study of DNA binding interaction was observed by incubating 100 µL Calf Thymus DNA 10 mg/mL and various concentrations (20, 25, 30, 35 dan 40 ppm) of BHA and TBHQ compounds in 10 mM Tris HCl solvent, pH 7.4; the various incubation times were held in 1, 2 and 3 hours at room temperature 25°C. The interaction between DNA with BHA and TBHQ was analyzed using spectrophotometry.

Study of DNA Adduct formation (8-OHdG) from BHA and TBHQ interaction with Calf Thymus DNA was observed by incubating 40 µL Calf Thymus DNA 10 mg/mL with each compound of 100 ppm BHA and 80 ppm TBHQ, in 50 mM Tris HCl solvent, pH 7.4; the reaction mixture was incubated for 1 hour at temperature 37°C. After incubation, the reaction mixture was hydrolyzed by adding 20 µL volume of natrium succinate mixture 0.1 M, and calcium chloride 0.05 M in ratio 1:1, and 30 µL volume of microccocal nuclease mixture (2 mU/µg DNA) and phosphodiesterase II (0.04 mU/µg DNA). The mixture was incubated at temperature 37°C for 4 hours. The hydrolysis products were analyzed by using HPLC and LC MS/MS with analysis condition shown in table 1 and 2.

Study of DNA adduct formation was performed using DNA base 2’ deoxyguanosine with BHA and TBHQ. Around 100 ppm of 2’ deoxyguanosine was incubated with BHA in various concentrations, 50, 100, 150, 200, and 250 ppm in 50 mM Tris HCl solvent, pH 7.4; the reaction mixture was incubated for 1 hour at temperature 37°C. Meanwhile, incubation with TBHQ was conducted in various concentrations, 20, 40, 60, 80, 100, 120, 160, 200, 300, 400, and 500 ppm in 50 mM Tris HCl solvent,

| Table 1. Analysis condition using HPLC |
|---------------------------------------|
| Column | X Bridge C 18 Waters |
|        | (4.6 X 250 mm, particle size 5 μm) |
| Column temperature | 31°C |
| Eluent | Sodium hydrogen phosphate buffer 10 mM and Methanol (85 : 15) |
| Flow rate | 1.0 mL/minute |
| Injection Volume | 20 µL |
| Detector | UV wavelength 254 nm |
Table 2. Analysis Condition using LCMS/MS LC Condition

| Column          | Atlantis T3 diameter 5 µm, size 4.6 x 150 mm (Waters) |
|-----------------|-------------------------------------------------------|
| Column temperature | 35°C                                                   |
| Eluent          | Eluent A : Eluent B = Formic acid 0.1 % :            |
|                 | Formic acid 0.1 % in Methanol                         |
| Flow rate       | 0.5 mL/minute                                         |
| Injection Volume | 10 µL                                                  |
| Detector        | MS/MS, Triple Quadrupole                              |
| Analysis Time   | 10 min.                                               |
| Analysis Technique | Binary Gradient Programme                         |

Table 3. Binary Gradient Programme (with modification) [12]

| Time (minute) | Eluent A (%) | Eluent B (%) | MS/MS Condition | Ionization | Mode | Parent ion | Product ion |
|---------------|--------------|--------------|------------------|------------|------|------------|-------------|
| 1.0           | 50           | 50           | MS/MS Condition  | positive   | ESI  | 284        | 167.9 ; 139.9 |
| 3             | 5            | 95           | Ionization : positive | ESI      | Mode | 284        | 167.9 ; 139.9 |
| 5             | 5            | 95           | Ionization : positive | ESI      | Mode | 284        | 167.9 ; 139.9 |
| 5.5           | 50           | 50           | Mode : ESI       |            |      | 284        | 167.9 ; 139.9 |
| 10            | 50           | 50           | Parent ion : 284 | Product ion : 167.9 | 139.9 |      |             |
| 10            | Stop         |              |                  |            |      |            |             |

pH 7.4; the reaction mixture was incubated for 1 hour at temperature 37°C. The formation of DNA adduct was analyzed by using HPLC, and 8 – OHDG was measured towards 2’ deoxyguanosine (table 3 [12]).

3. Results and discussion

3.1. DNA binding formation of BHA and TBHQ using spectrophotometry

Binding or interaction of the compounds such as BHA and TBHQ causes spectra changes. Calf thymus DNA was incubated with BHA in various concentrations 20, 25, 30, 35 and 40 ppm in 0.01 M Tris HCl solvent, pH 7.4 at temperature 25°C in three various incubation times (1 hour, 2 hours, and 3 hours). The spectra profile shows that in each incubation time, DNA control has a maximum wavelength absorbance at around 258 nm, meanwhile BHA control has a maximum wavelength absorbance at 286.6 (figure 1). Interaction between DNA and BHA in various concentrations provides maximum wavelength absorbance at around 282-283.4 nm. There is no specific difference in spectra of DNA and BHA in each incubation time. There is only an increasing of absorbance in the higher BHA concentrations. The result stated that there is no binding interaction between DNA and BHA. Calf thymus DNA incubation with TBHQ in various concentrations, 0, 20, 25, 30, 35, and 40 ppm, in 0.01 M Tris HCl pH 7.4, at temperature 37°C for 30 minutes showed that the formed spectra have a maximum wavelength absorbance at around 282.4-284.8 nm. The increasing amount of spectra absorbance was equal with the increasing of TBHQ concentration. Meanwhile, the DNA control has a maximum wavelength absorbance at around 258-258.4 nm after incubation for 1, 2, and 3 hours and the TBHQ control has a maximum wavelength absorbance at around 288 nm. The spectra showed maximum wavelength absorbance at around 259.6-261 nm indicated the bathochromic shift, the shift of peak absorbance to the higher wavelength around 2-3 nm from maximum wavelength absorbance of DNA. In addition,
there was a hyperchromic shift, the increasing of maximum wavelength absorbance intensity in the interaction of DNA and TBHQ which was in line with the increasing of TBHQ (figure 2). The spectra produced in the treatment of DNA with TBHQ in various concentrations show that there is binding interaction between DNA and TBHQ. According to the previous research, this bonding interaction is

Figure 1. Spectra profile of DNA Binding with BHA in 0.01 M Tris HCl pH 7.4; DNA concentration was 20 ppm; BHA in various concentrations 0, 20, 25, 30, 35 and 40 ppm with 3 hours incubation time

Figure 2. Spectra profile of DNA Binding with TBHQ in 0.01 M Tris HCl pH 7.4; DNA concentration was 20 ppm; TBHQ in various concentrations 0, 20, 25, 30, 35 and 40 ppm with incubation time of (a) 30 minutes and (b) 3 hours

Figure 3. Chromatogram profile of enzimatically hydrolyzed DNA using HPLC
Figure 4. Chromatogram profile of DNA hydrolysis using LCMS/MS

called as intercalation, in which the whole or the half of molecules enter the gap between two base-pairs in double helix DNA which can affect the conformation structure and the structure of DNA chain. The responses observed in the spectrum of the intercalation are ‘hyperchromic’ (the increasing of maximum wavelength absorbance) or ‘hypochromic’ (the decreasing of maximum wavelength absorbance).

3.2. DNA hydrolysis
Hydrolysis of DNA was conducted enzymatically by using Micrococcal Nuclease enzymes from Staphylococcus aureus and Phosphodiesterase II from Spleen bovine. Both enzymes are functionate to hydrolyze DNA into single nucleoside. The result of hydrolysis of the mixture compounds was then analyzed using HPLC. The chromatogram results seemed to form 4 chromatogram peaks, one of them is 2’Deoxyguanosine at retention time 6.842 (figure 3). Nevertheless, the DNA adduct 8-OHdG can not be detected using HPLC. Further analysis was performed using LCMS/MS, and the result showed that 8OHdG was formed in very low concentration 7.356 ppb with area of 169.8 (figure 4). The concentrations of 8OHDG are below limit of detection (8 ppb).

3.3. Incubation of 2’ Deoxyguanosine with BHA and TBHQ
Incubation of 2’Deoxyguanosine with BHA in various concentrations 50, 100, 150, 200 and 250 ppm at pH 7.4 mediated with Cu\(^{2+}\) did not trigger the formation of adduct 8-OHDG (figure 5). The formation of 8-OHDG from incubation with TBHQ was analyzed by incubating 2’Deoxyguanosine with TBHQ in various concentrations 20, 40, 60, 80, 100, 120, 140, 200, 300, 400 and 500 ppm, pH 7.4, mediated with Cu\(^{2+}\) at temperature 37°C for 1 hour. TBHQ triggered the formation of 8-OHDG in the increasing of TBHQ concentrations. In TBHQ concentration of 500 ppm, 8-OHDG has formed approximately at 946/105 DG (figure 6).

3.4. DNA incubation with BHA and TBHQ
Interaction between TBHQ and DNA was studied by incubating 80 ppm TBHQ and DNA in the presence of Cu\(^{2+}\) at temperature 37°C, pH 7.4 for 1 hour, then hydrolyzed enzymatically for 3 hours. The reaction mixture was analyzed using LCMS/MS. DNA Adduct 8-OHdG formed was about 10 ppb (figure 7). Nevertheless, the interaction between DNA and 100 ppm BHA by using LCMS/MS was not detected.
Figure 5. Chromatogram profile of HPLC after 2’deoxyguanosine incubation with 250 ppm BHA at pH 7.4 for 1 hour.

Figure 6. Chromatogram profile of 2’deoxyguanosine incubation with 500 ppm TBHQ at pH 7.4 for 1 hour.

Figure 7. Chromatogram profile of DNA hydrolysis after incubated with TBHQ using LCMS/MS.
The formation of 8OHdG induced by TBHQ was stimulated by Cu$^{2+}$ ion which induced the formation of reactive oxygen species. The reaction showed the role of redox cycle of Cu(II)/Cu(I). The ion of Cu$^{2+}$ can promote oxidation process of TBHQ and generate ROS in the form of semiquinone anion radicals; Tert Butyl Quinone (TBQ), H$_2$O$_2$, superoxidant, and hydroxyl radicals (figure 8). The oxidized form of TBHQ generated intermediates of TBQ anion radical, and released one electron and two H$^+$. protons. The missing electron will alter oxygen into superoxide. In the presence of H$^+$, superoxide will generate H$_2$O$_2$. Copper (Cu$^{2+}$) as free ions are present in the nucleus at a concentration of approximately 16 x 10$^{-6}$ M. In the presence of H$_2$O$_2$, Cu$^{2+}$ can produce hydroxyl radicals continuously through the Fenton reaction as follows:

$$\text{Cu}^{2+} + \text{O}_2 \rightarrow \text{Cu}^{+} + \text{O}_2^\bullet$$

The last reaction (O$_2^\bullet$+ H$_2$O$_2$ $\rightarrow$ O$_2^+$ HO$^+$ + HO$^+$) is known as the Haber Weiss reaction.

The interaction between hydroxyl radicals with DNA nucleoside caused the DNA damage, as it can oxidize single base of DNA, 2’deoxyguanosine generating 8-OHDG. The formation of 8-OHDG was occurred through two stages of reactions, the hydroxyl radical attack on carbon C-8 of 2’deoxyguanosine
generating C8-OH- radical adduct, followed by the release of electrons and H⁺, generating 8-OHDG (figure 9).

The conformation test of 8-OHdG formation using LCMS/MS with electro spray ionization (ESI) is aimed to identify molecular mass from DNA adduct 8-OHDG formation and its fragmentations which generated from molecule collided with high-energy electrons. LCMS/MS chromatogram profile and mass spectra in figure 10 show the chromatogram of 8-OHdG at retention time 3.5 min and base peak (M⁺+1) at m/z = 284. This is equal with 8-OHdG molecular mass (C10H13N5O5) = 283.24. Pieta et al. [14] reported base peak of 8-OHdG (M⁺+1) = 284. The fragmentation of 8-OHDG with positive ion generated two main fragments at m/z = 167.9 and m/z = 139.9. The first fragment ion m/z

![Figure 10. Base peak profile of 8-OHDG (M⁺+1 = 284) and its fragmentations generating fragment ions m/z = 167.9 and m/z = 139.9](image)

![Figure 11. Chromatogram profile of 2’Deoxyguanosine and 50 ppm TBHQ mediated with Cu²⁺ incubation at pH 7.4 for 1 hour using LCMS/MS generating base peak (M⁺+1) = 284 and two main fragment ions at m/z = 167.9 and m/z = 139.9](image)
= 169.9 was a result of N-glicoside bond breaking, thus the glycosides (C5H8O3, M\(^+\) 116) were separated. Meanwhile, the fragment ion m/z = 139.9 was formed from the losing of carbon monoxide (CO, M\(^+\) 28). The same result was occurred in conformation study using 2’deoxyguanosine treated with 50 ppm TBHQ (figure 11).

4. Conclusions

From the result of this study, it was concluded that there was no binding interaction between DNA and BHA in various concentrations 20, 25, 30, 35 and 40 ppm in 10 mM Tris HCl solvent for 1-3 hours incubation time, at temperature 25°C. Based on the analysis by spectrophotometry, it can be concluded that there was DNA binding interactions between DNA and TBHQ in various concentrations 20, 25, 30, 35 and 40 ppm in 10 mM Tris HCl solvent for 1-3 hours incubation time, at temperature 25 °C. The interaction has been confirmed by the ocurrence of bathochromic shift as much as 2 – 3 nm while spectrum absorption increased with the increase of TBHQ concentrations. Calf thymus DNA treated with 100 ppm BHA mediated with Copper (II) Chloride and incubated for 4 hours at temperature 37°C did not trigger the DNA Adduct formation 8-OHdG. Calf thymus DNA and 80 ppm TBHQ incubation at 37°C for 4 hours, mediated with Copper (II) Chloride triggered the DNA Adduct 8-OHdG formation. BHA and 2’Deoxyguanosine incubation at 37°C for 1 hour, mediated with Copper (II) Chloride did not trigger the DNA Adduct 8-OHdG formation. Incubation of TBHQ in various concentrations 20-500ppm with 2’Deoxyguanosine at 37°C, for 1 hour mediated with Copper (II) Chloride, triggered the DNA Adduct 8-OHdG formation. There was an increase of DNA Adduct 8-OHdG formation as TBHQ concentrations increased. According to HPLC analysis, relative amount of 8-OHdG formation was 946/10\(^5\) Deoxyguanosine (DG) from DNA. The conformation test of 8-OHdG formation using LCMS/MS showed the peak of 8-OHdG at retention time 3.52 min and base peak of 8-OHdG (M\(^+\)+1) = 284, generating two main fragment ions: m/z = 167.9 and m/z = 139.9.

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