

Study of DNA adduct 8 hydroxy-2'-deoxyguanosine (8-OHdG) formation through fenton reaction with tert-butylhydroquinone (TBHQ) and butyl hydroxy toluene (BHT)

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Abstract. The research of DNA adduct formation 8-hydroxy-2'-Deoxyguanosine (8- OHdG) as a biomarker of DNA damage due to oxidative stress was carried out by reacting the DNA base 2'-deoxyguanosine-5'-monophosphate with TBHQ and BHT. The formation of 8-OHdG was carried out in various conditions, at temperature of 37° C and 60° C, pH 7.4 and pH 8.4, within 5 hours of incubation time and in the addition of FeSO₄. The formation of DNA adducts profile were analyzed using reversed phase HPLC with UV detector at a wavelength of 254 nm. The results of the study showed that TBHQ and BHT can trigger the formation of 8-OHdG from the reaction of 2'-hydroxy Deoxyguanosine-5'-monophosphate in the presence of Fe (II). Meanwhile, in the addition of hydrogen peroxide, the formation of DNA adducts only occur in the test substance TBHQ. The results showed that the condition of higher temperature at 60°C and pH 8,4 affects the higher formation of DNA adducts.

1. Introduction

Food is a basic needed which is essential for human life and the most important or necessary things in order to survive. Generally in food processing, treatment are given by various ways such as adding additional materials in order to extend the shelf life, improving the texture, or appearance of delicacy[1]. A safe food is a food that does not contain (1) biological hazard or microbiology hazard, (2) chemical hazards, and (3) physical hazard [2].

Antioxidants are natural antioxidants that have been produced synthetically for commercial purposes [3]. Synthetic antioxidants are commonly used for food additives to prevent rancidity caused by fat oil oxidation in food. Butyl hydroxyanisole (BHA), butyl hydroxy toluene (BHT), propyl gallate (PG), Tert-Butyl Hidoksi Quinon (TBHQ) and tocopherols are allowed to be used in foods. Antioxidant BHT in the form of white crystalline solid is widely used because its relatively inexpensive [3]. Antioxidant TBHQ is known as the most effective antioxidant for fats and oils, particularly oil plant because it has antioxidant abilities both in the frying process, but low in the combustion process. Antioxidant TBHQ known shaped white to light brown powder, has a sufficient solubility in fats and oils, not forming complexes with Fe and Cu colors but can turn to be pink in the presence of a base [3]. Acceptable daily intake for TBHQ is 0.70 mg/kg body weight [4-5].

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In abnormal biological conditions, there is always the potential for an antioxidant to become a pro-oxidant if a suitable receptor molecule presents to accept the electron and promote the autoxidation. Mineral ions are particularly important as pro-oxidants [6]. Okubo, et.al 1997, reported that TBHQ can cause the breakdown of DNA in vitro and form 8-hydroksi-2'-deoksiguanosine as a result of oxidative DNA damage in Calf Thymus DNA [7]. BHT-quinone (2,6-di-tert-butyl-p-benzoquinone), has been reported to cause cleavage of supercoiled DNA [8]. Oikawa et al. 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 [9].

Furthermore, this study was conducted to determine the formation of 8-hydroxy-2'-deoksiguanosin (8-OHdG), which is an indicator of DNA damage due to chemical carcinogens exposure. In this study 2'-deoxyguanosine-5'-monophosphate is used to be reacted with BHT and TBHQ in the various pH and temperature and the addition of Fe (II) to see the profile of 8-OHdG formation and prove that carcinogen compounds exposure can cause DNA damage.

2. Experimental and Method

In this research, the study conducted were in vitro study of DNA base 2'-deoxyguanosine and antioxidants trigger to DNA Adduct formation by using HPLC, the analytical method has been validated in the previous research.

Study of DNA binding interaction was observed by incubating 350 μ L of 2'-deoxyguanosine 5'-monophosphate (500 ppb) in potassium phosphate buffer 0,1 M (pH 7.4 and 8.4) with 350 μ L of TBHQ (5000 ppb) and 350 μ L of aquabide st for 5 hours at variation of temperature 37°C and 60°C. The variation condition undergo in the addition of 350 μ L of FeSO₄ (5000 ppb), using BHT (5000 ppb) and under Fenton reaction using 175 μ L of FeSO₄ (10000 ppb), and 175 μ L of H₂O₂ (10000 ppb). All variation were shown in Table 1. The incubation products were analyzed by using HPLC with analysis condition shown in Table 2.

Table 1 Variation mixture and condition of dGMP incubation

Variation mixtures			
In the presence of H ₂ O ₂		without H ₂ O ₂	
Temperature incubation 37°C	Temperature incubation 60°C	Temperature incubation 37°C	Temperature incubation 60°C
dGMP pH 7,4 + TBHQ + H ₂ O ₂		dGMP pH 7,4 + TBHQ	
dGMP pH 8,4 + TBHQ + H ₂ O ₂		dGMP pH 8,4 + TBHQ	
dGMP pH 7,4 + BHT + H ₂ O ₂		dGMP pH 7,4 + BHT	
dGMP pH 8,4 + BHT + H ₂ O ₂		dGMP pH 8,4 + BHT	
dGMP pH 7,4 + FeSO ₄ + H ₂ O ₂		dGMP pH 7,4 + FeSO ₄	
dGMP pH 8,4 + FeSO ₄ + H ₂ O ₂		dGMP pH 8,4 + FeSO ₄	
dGMP pH 7,4 + TBHQ + FeSO ₄ + H ₂ O ₂		dGMP pH 7,4 + TBHQ + FeSO ₄	
dGMP pH 8,4 + TBHQ + FeSO ₄ + H ₂ O ₂		dGMP pH 8,4 + TBHQ + FeSO ₄	
dGMP pH 7,4 + BHT + FeSO ₄ + H ₂ O ₂		dGMP pH 7,4 + BHT + FeSO ₄	
dGMP pH 8,4 + BHT + FeSO ₄ + H ₂ O ₂		dGMP pH 8,4 + BHT + FeSO ₄	

Table 2 Analysis condition using HPLC

Column	X Bridge C 18 Waters (4,6 X 250 mm, particle size 5 μ m)
Column temperature	31°C
Eluent	Sodium hydrogen phosphate buffer 10 mM and Methanol (85 : 15)
Flow rate	1,0 mL/minute
Volume Injection	20 μ L
Detector	UV wavelength 254 nm

3. Result and Discussion

3.1 Result of Adducts

This study was conducted by incubated the nucleoside of deoxyguanosine with TBHQ and BHT. This reaction was assumed to produce adducts 8-OHdG. The 8-OHdG compounds are formed from a radical hydroxyl radical ($\bullet\text{OH}$) attack on basic groups of guanine in the C8 atom [10]. The changing of deoxyguanosine structure becomes 8-OHdG due to oxidative stress can be seen in Figure 1.

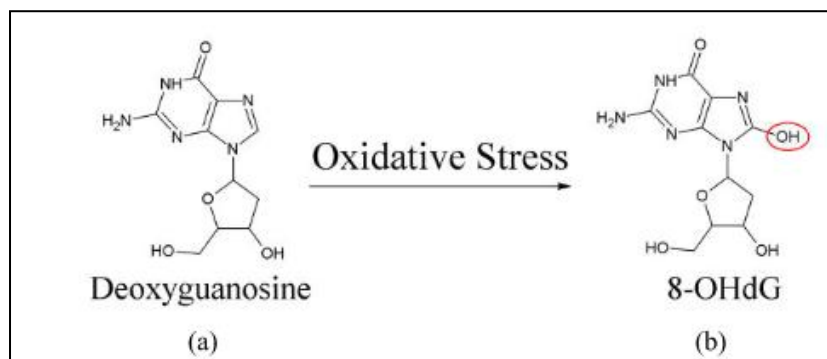


Figure 1 (a) Deoxyguanosine structure ; (b) 8-OHdG structure, deoxyguanosine modification [10]

3.2 The Effect of Xenobiotics

The reaction between DGMP with TBHQ and BHT which are xenobiotic trigger to radical formation, was undergo, to see the result of 8-OHdG formation which indicate the presence of DNA damage when the human body has been exposed to xenobiotics contained in the antioxidant artificial in food such as TBHQ and BHT.

3.2.1. The Effect of Compounds Trigger to Free Radicals Fe^{2+} , and H_2O_2 . The reaction of the DGMP with BHT or TBHQ in the presence of Fe^{2+} have different results. At the temperature of 37°C and at pH 7.4 for TBHQ and BHT reaction, the formation of adducts were not detected. However, in another variation on the test xenobiotics substance TBHQ and BHT at pH 8.4 at temperature of 37°C (Figure 2), the adducts were detected as well as the variation reaction at the temperature of 60°C with pH 7.4 and 8.4.

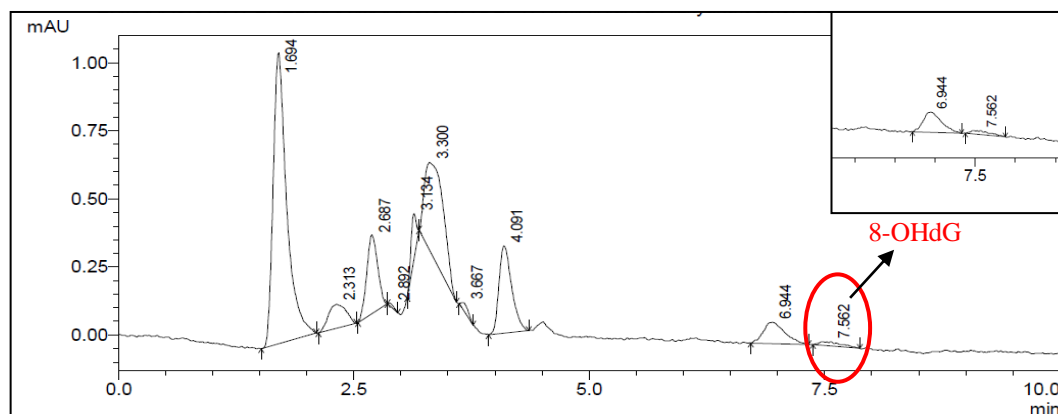
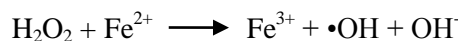


Figure 2. Chromatogram of dGMP and BHT in the presence of Fe^{2+} in the temperature 60°C and pH 8,4

The addition of Fe^{2+} is according to its contained in the body particularly containing as the active site of CYP450 enzymes. The Fe^{2+} can trigger to the formation of radicals in its oxidized form, Fe (III), through Fenton reaction:



The differences of 8-OHdG levels shown in the variation of the test substances. The formation of adducts was not detected from TBHQ and BHT reaction in the addition of Fe^{2+} at pH 7.4 and at temperature 37°C . Meanwhile, at pH 8.4, the adducts were detected as much as 6.90 ppb for TBHQ reaction and 6.90 ppb for BHT reaction. The graphic of DNA adduct formation at temperature 37°C as the effect of H_2O_2 presence has shown in Figure 3.3. At the temperature of 60°C with the same conditions, the level of 8-OHdG were measured as much as 6.74 ppb at pH 7.4 and 7.86 ppb at pH 8.4 with the test substance TBHQ and in the addition of Fe^{2+} . On the test substance of BHT, the DNA Adduct 8-OHdG at pH 8.4 was measured as much as 10.22 ppb, but at pH 7.4 the DNA adduct formation was not detected (Figure 3).

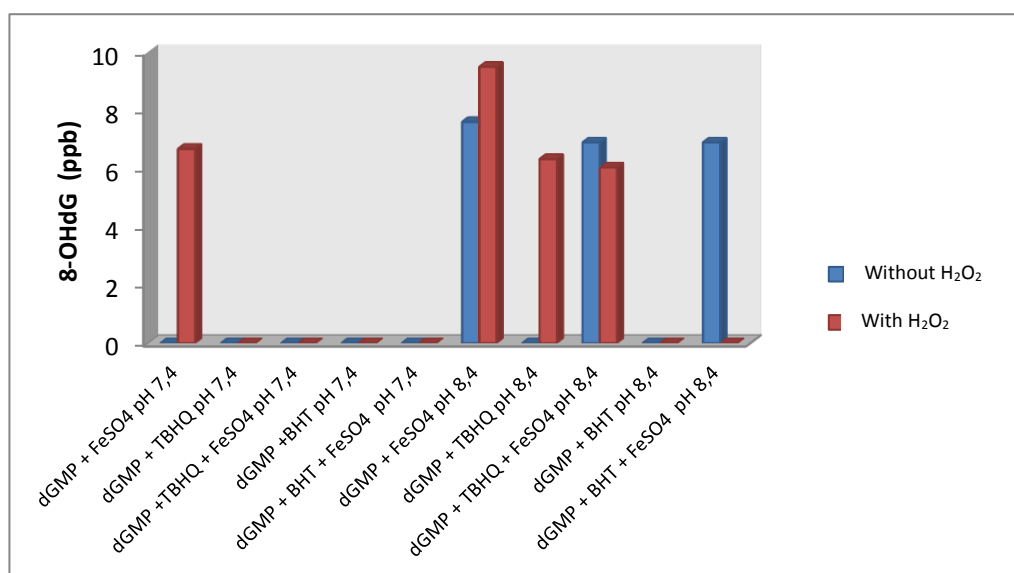


Figure 3. The effect of Fe^{2+} and H_2O_2 in DNA Adduct 8-OHdG formation in the temperature of 37°C

The result of the reaction by the addition of H_2O_2 to the test substance TBHQ without the presence of Fe^{2+} showed the formation of DNA Adducts 8-OHdG adduct which only detected at temperature of 37°C and pH 8.4 as much as 6.32 ppb. Meanwhile, at temperature of 60°C the DNA Adduct 8-OHDG was detected as much as 7.09 ppb at pH 7.4 and 8.21 ppb at pH 8.4. It was different from BHT reaction, which has produced the DNA adduct formation only at temperature of 60°C at both pH as much as 7.38 ppb at pH 7.4 and 7.34 ppb at pH 8.4. The graphic of DNA Adduct level in the temperature of 60°C in the presence and without a presence of H_2O_2 has shown in Figure 4.

3.2.2 The Effect of Fenton Reaction. The addition of H_2O_2 is an important factor in the reaction of this study. The results of the test substance incubation of DGMP and TBHQ or BHT in the addition of Fe^{2+} and in the variation of pH 7.4 and 8.4 and temperature of 37°C and 60°C , showed the variation results of adducts. The results of analysis of 8-OHdG levels in the reactions using the test substance TBHQ and Fe^{2+} at pH 8.4 and temperature 37°C was 6.03 ppb, meanwhile at pH of 7.4 the 8-OHdG was not detected. At the same condition of incubation but in the temperature variations of 60°C , showed different results. The 8-OHdG levels at pH 7.4 was 6.01 ppb and at pH 8.4 was 6.81 ppb. The graphic

of DNA adduct formation from the effect of Fenton reaction in the presence of TBHQ has shown in Figure 5.

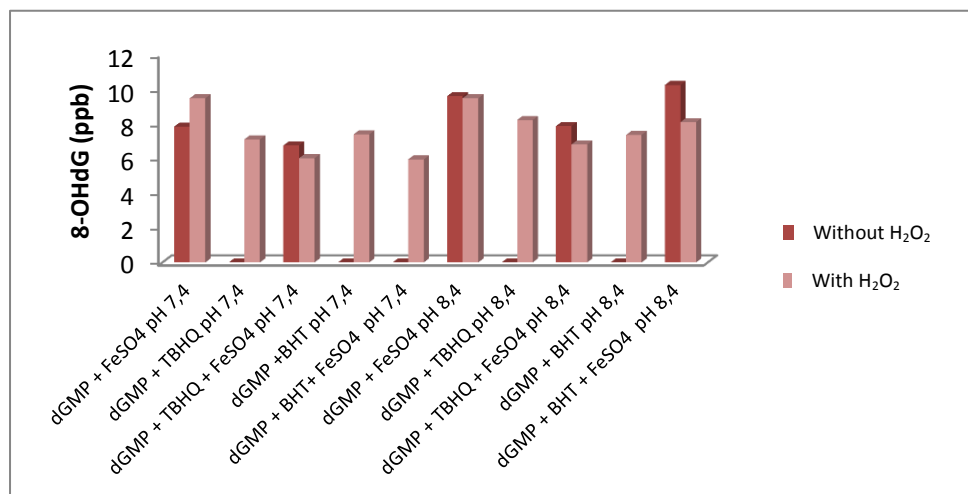


Figure 4. The effect of Fe²⁺ and H₂O₂ towards the formation of DNA Adducts 8-OHdG in the temperature of 60°C

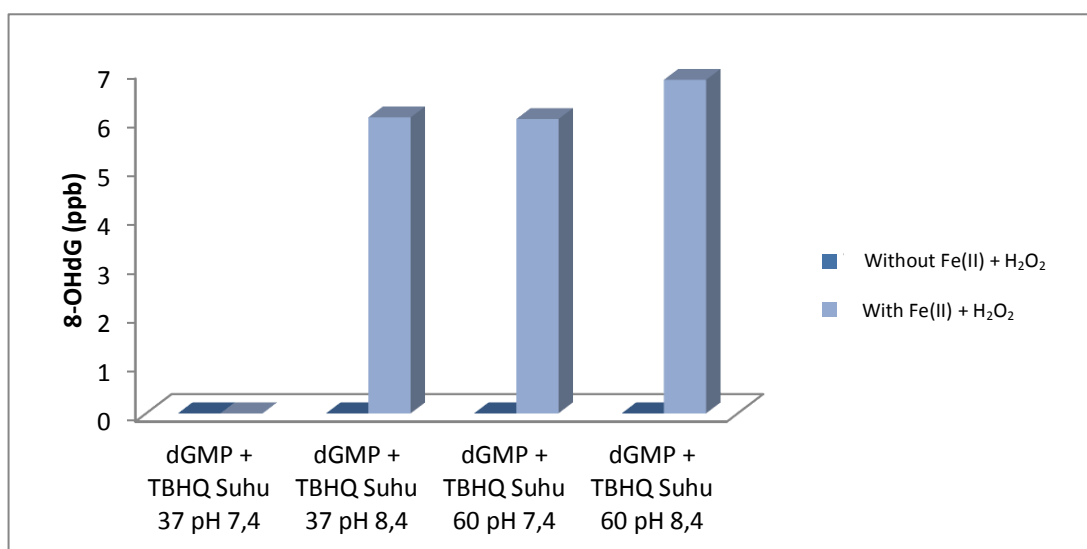


Figure 5. The effect of Fenton reaction towards DNA Adduct 8-OHdG Formation with TBHQ

On the incubation using BHT and Fe²⁺ at temperature 37°C, 8-OHdG level at pH 7.4 and 8.4 was not detected. At a temperature of 60°C both of variation at pH 7.4 and pH 8.4 was detected the level of 8-OHdG as much as 5.94 ppb for pH 7.4 and 8.09 ppb for pH 8.4. The graphic of DNA Adduct formation in the presence of BHT has shown in Figure 6.

The differences of 8-OHdG levels is due to the influence of H₂O₂ which has a function as an oxidator. Oxidative DNA damage caused the formation of hydroxyl radicals from H₂O₂ through the Fenton reaction (Tokoyuni and Sagripanti, 1996). The H₂O₂ presences in the reaction process will form the oxidation reaction of Fe²⁺ to Fe³⁺ and produce hydroxyl radical (•OH) which have a reactivity and will interact with cells to form adducts [12]. The hydroxyl (OH•) radical is a reactive ROS which will react with DNA and forming an adduct 8-OHdG as a marker of oxidative DNA damage [13]. The reaction that occurs without the addition of H₂O₂ will lead to autoxidation process. Autoxidation is a

reaction that is triggered by the air on organic and inorganic systems which are actually the result of directly or indirectly reaction from molecular oxygen in free radical process (14).

The formation of adducts in the presence of fenton reaction have been influenced and have synergistic effect either with TBHQ or BHT especially at pH 8.4 and temperature 60°C.

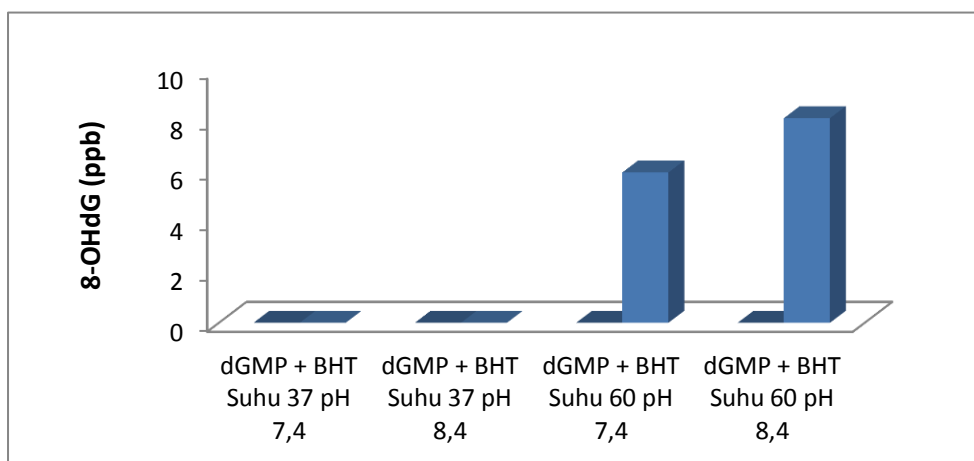


Figure 6. The effect of Fenton Reaction towards 8-OHdG formation with BHT

4. Conclusion

The reaction of Deoxyguanosine monophosphate either with TBHQ or BHT in the presence of Fe^{2+} at temperature 60°C and at pH 8.4 was affected the formation of 8-OHdG. At the temperature of 37°C the formation of adducts either with TBHQ, or BHT in the presence of Fe^{2+} was detected only at pH 8.4. The result of the reaction by the addition of H_2O_2 to the test substance TBHQ without Fe^{2+} produces adduct which only detected at temperature of 37°C and pH 8.4 meanwhile at temperature of 60°C and at both pH 7.4 and 8.4 8-OHdG was formed. The result of the reaction by the addition of H_2O_2 to the test substance BHT without Fe^{2+} produces adduct at temperature of 60°C and at both pH 7.4 and 8.4. The Fenton reaction was influenced the formation of DNA Adduct 8-OHdG by the condition of temperature and pH. At temperature 37°C, the 8-OHdG was detected only in the presence of TBHQ at pH 8.4. At temperature 60°C, the 8-OHdG was detected in the presence of TBHQ or BHT at both pH 7.4 and 8.4. Finally, the formation of adduct at temperature 60°C was higher than at temperature 37°C

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