

***In-vitro* STUDY OF ACRYLAMIDE AND TBHQ COMPOUNDS ON THE GENERATION OF DNA ADDUCT 8-HYDROXY-2'-DEOXYGUANOSINE AS BIOMARKER OF OXIDATIVE DNA DAMAGE**

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ABSTRACT

The research on DNA adduct 8-OHdG formation is performed by reacting 2'-deoxyguanosine and H₂O₂ with acrylamide (AA) and TBHQ compounds that were exposed to UVA light. The 8-OHdG forming test was conducted at various incubation times (5 h and 7 h) and pH variations (7.4 and 8.4). The 8-OHdG formation was analyzed using a reversed-phase HPLC instrument using a buffer solution of sodium phosphate pH 6.7: methanol (85:15) as eluent. The results showed that samples containing TBHQ and acrylamide (AA) produced 8-OHdG and shows synergistic and antagonistic effects. The 8-OHdG level of mixing acrylamide (AA) and TBHQ compounds increased significantly when exposed to UVA light. Samples incubated with UVA exposure had higher levels of 8-OHdG than samples without UVA exposure. The longer the incubation time, the greater the 8-OHdG content was produced. At pH 8.4, most of the samples resulted in greater 8-OHdG formation when compared to samples with the same mixture at pH 7.4. Samples with the greatest 8-OHdG formation results were found in samples containing 2'-deoxyguanosine, acrylamide (AA), TBHQ, and H₂O₂ at pH 8.4 with UVA light irradiation for 7h.

Keywords: Acrylamide (AA), TBHQ, 8-OHdG, Synergistic Effect, UVA Light.

RASĀYAN J. Chem., Vol. 15, No.4, 2022

INTRODUCTION

The heating process of carbohydrate-rich foods at high temperatures (> 120 °C) such as baking, frying, and roasting will form AA in a moderate concentration.¹ In plant-derived foods including coffees, cereals, olives, almonds, and potatoes, the heat-induced reactions between the carbonyl groups of fructose, glucose, and amino acid asparagine.² It was reported that the reaction of AA with guanine resulted in the formation of adduct 2-formamidoethyl through the Michael addition reaction.³ From observation, it was also found that AA and glycine could serve as biological alkylating agents that can induce the substitution of base mutations in DNA, which can bring about the beginning of carcinogenic processes.² TBHQ is an example of the most important antioxidants commonly used in food.⁴ Under irregular biological circumstances, the likelihood of the antioxidants growing into pro-oxidants can always be expected when a compatible receptor molecule is present to receive electrons and trigger auto-oxidation.⁵

Despite the well-known chemoprotective effects of TBHQ as an antioxidant, the literature also suggests that high TBHQ exposure may have a possible carcinogenic effect in mice due to the contribution of semiquinone anion radicals and the generation of ROS from TBQ, which is the oxidized form of TBHQ. In 1997, Okubo notified that TBHQ could lead to DNA breakdown in vitro and generate 8-OHdG as a product of oxidative damage to ct-DNA.⁵ Mutations and cancer can result from many DNA modifications. In their daily activities, humans are often exposed to direct sunlight, the exposure time of which can reach 7 h / day. Ambient sunlight is dominated by UVA (90 %–95 %) and UVB (5 %–10 %). UV rays can penetrate the skin according to the wavelength it has. UVA with longer wavelengths can penetrate deep into the dermis. DNA base is oxidatively modified by free radicals due to UVA exposure, both in the in vitro and in vivo studies.⁶ Reactive oxygen species (ROS) is a product of regular metabolism and xenobiotic exposures.

Excess ROS can result in oxidative modification of cellular macromolecules, inhibiting protein function, and promoting cell death.⁷ The excess level of ROS can damage proteins, lipids, and DNA, thus causing a change in structural and functional cellular.⁸ 8-OHdG has been studied in many types of research as a biological marker of oxidative stress, which can cause DNA damage, leading to carcinogenic effects.⁸⁻¹¹ In this study, research was performed to see the forming of 8-OHdG in vitro from 2'-deoxyguanosine due to exposure to AA, TBHQ, H₂O₂, and exposure to UVA rays for 5 and 7 h and carried out at 37 °C at pH 7.4 and 8.4. The generation of 8-OHdG was analyzed by using HPLC.

EXPERIMENTAL

This research was conducted at UI-Chem Laboratory and the Biochemistry / Organic Chemistry Laboratory in the Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Indonesia from February to July 2020.

Material and Methods

The equipment used, namely: HPLC instrument (Thermo Scientific Ultimate 3000), UVA lamp, vortex, pH meter, measuring flask, measuring pipette, micropipette, volumetric pipette, incubator shakers, analytical balances, Millipore (PTFE and Nylon), and sonicator. While the materials used, namely: acrylamide (AA) (Sigma Aldrich), TBHQ (Sigma Aldrich), 2'-deoxyguanosine (Sigma Aldrich), H₂O₂ (Merck), 8-OHdG (Sigma Aldrich), HCl (Merck), NaOH (Merck), DMSO (Merck), sodium acetate (Merck), methanol grade for HPLC (Merck), Na₂HPO₄ (Merck), NaH₂PO₄ (Merck), KH₂PO₄ (Merck), K₂HPO₄ (Merck), and distilled water (Merck).

General Procedure

Validation of 8-OHdG Analysis Method Using HPLC

HPLC specifications used for 8-OHdG analysis were reversed-phase HPLC with a mobile phase buffer mixture of sodium phosphate: methanol (85: 15) and a stationary phase of column C18. Sample analysis was performed at 254 nm with an injection volume of 20 µL / injection. Determination of the optimum conditions was carried out to find the right mobile phase ratio in order to produce a good separation chromatogram between 2'-deoxyguanosine, and 8-OHdG. Determination of the optimum conditions was carried out by injecting 20 µL of a standard solution of 2'-deoxyguanosine pH 7.4, standard 8-OHdG, and the mixture using the HPLC instrument at conditions of 254 nm wavelength, 1.2 mL/min flow rate, and variations in mobile phase ratios. Variations in the mobile phase ratio used were sodium phosphate: methanol buffer solutions of (1) 80: 10, (2) 85: 15, and (3) 90: 10.

The linearity curve was made using the optimum conditions of the HPLC, 20 µL standard solution of 8-OHdG 0 ppb – 100 ppb was injected into the HPLC column with an eluent ratio of 10 mM sodium phosphate pH 6.7: methanol (85: 15). Then, a linearity curve was made between the areas of the peak obtained against the concentration of the standard solution. Testing of the limit of detection (LOD) and the limit of quantification (LOQ) was carried out with a linear regression equation obtained from a linearity curve. The repeatability (precision) test was measured by using the repetition of injection of 20 ppb 8-OHdG standard about 6 times under the same HPLC conditions as in the linearity test.

Repeatability was calculated by comparing the peak area between the measurements as much as 6 times and the peak area of the linearity curve to produce a standard deviation (SD) value and the percentage coefficient of variation.

In-vitro Study of 8-OHdG Formation

The samples were incubated at 37°C (representing human body temperature) by mixing 2'-deoxyguanosine, xenobiotic substances, and H₂O₂ which were varied at incubation times of 5 and 7 h and a pH of 7.4 and 8.4. 2'-deoxyguanosine was used as a nucleoside compound which is one part of DNA, AA, and TBHQ were used as xenobiotic compounds, while H₂O₂ was used as a source of hydroxyl radicals which are described as a representation of reactive oxygen species formed in the human body. The choice of pH variation of 8.4 is because there are several small parts in the human body that have a pH that can reach pH 8, such as in the intestines.

Table-1: Data Sample (without UVA exposure)

Incubation Time (hours)	Concentration			
7 h & 5 h	dG	AA	TBHQ	H ₂ O ₂
dG + H ₂ O ₂	150 μ L (12 ppm)	-	-	150 μ L (120 ppm)
dG + AA + H ₂ O ₂	100 μ L (18 ppm)	100 μ L (180 ppm)	-	100 μ L (180 ppm)
dG + TBHQ + H ₂ O ₂	100 μ L (18 ppm)	-	100 μ L (180 ppm)	100 μ L (180 ppm)
dG + AA + TBHQ + H ₂ O ₂	75 μ L (24 ppm)	75 μ L (240 ppm)	75 μ L (240 ppm)	75 μ L (240 ppm)

Table-2: Data Sample (with UVA exposure)

UVA Exposure Time (hours)	Concentration			
7 h & 5	dG	AA	TBHQ	H ₂ O ₂
dG + H ₂ O ₂ + UVA	150 μ L (12 ppm)	-	-	150 μ L (120 ppm)
dG + AA + H ₂ O ₂ + UVA	100 μ L (18 ppm)	100 μ L (180 ppm)	-	100 μ L (180 ppm)
dG + TBHQ + H ₂ O ₂ + UVA	100 μ L (18 ppm)	-	100 μ L (180 ppm)	100 μ L (180 ppm)
dG + AA + TBHQ + H ₂ O ₂ + UVA	75 μ L (24 ppm)	75 μ L (240 ppm)	75 μ L (240 ppm)	75 μ L (240 ppm)

RESULTS AND DISCUSSION

Validation of 8-OHdG Analysis Method Using HPLC

Determination of the optimum condition serves to obtain the most optimum conditions for performing the analysis which can be seen from the relatively fast retention time and has a chromatogram profile that shows good separation (peaks that are not too close together or overlap one another). Determination of the optimum conditions was carried out on three variations of the eluent composition, namely buffer sodium phosphate pH 6.7 and methanol with a concentration ratio of 80:10, 85:15, and 90:10 at 1.2 mL/minute. The three variations of the eluent composition will be assessed for their optimality from the results of the separation profile between a mixture of 2'-deoxyguanosine pH 7.4 and 8-OHdG with a concentration of 100 ppb. From the optimization of condition results, it can be concluded that the most optimum conditions for carrying out the 8-OHdG analysis were at 1.2 mL/min and the optimum mobile phase composition was 85:15. Under these conditions, there is a good separation between the peaks of the 8-OHdG and 2'-deoxyguanosine compounds. It can be seen in Fig.-1, that the peaks of the two compounds do not overlap, have a sharp-pointed shape, and have a retention time that is not too long and also not too fast when compared to the results of separation using the eluent ratio (80: 20) and (90: 10).

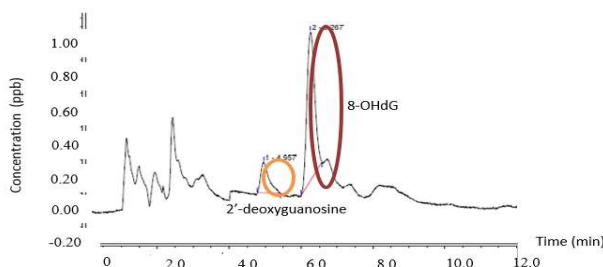


Fig.-1: Chromatogram Profile of 2'-deoxyguanosine pH 7 and 8-OHdG at 1.2 mL/min with eluent composition (85: 15)

From the results of the separation, the retention times of 2'-deoxyguanosine and 8-OHdG were obtained, respectively, 4.957 min and 6.267 min. Linearity curves are used to estimate the concentration of unknown analytes in the test sample.¹² The linearity curve shows the relationship between the concentrations of the 8-OHdG standard with the area of the chromatogram. Making this linearity curve is done by injecting the

standard 8-OHdG at 7 different concentrations, namely: 0 ppb (distilled water: sodium acetate (1: 1)), 5 ppb, 10 ppb, 20 ppb, 50 ppb, 80 ppb, and 100 ppb. From these calculations, linearity was obtained at 0.9963. The LOD and LOQ values were obtained from the linearity curve. LOD is defined as the lowest detectable concentration of the Analyte. In contrast, LOQ is the lowest concentration of sample that can be determined with an acceptable level of precision and accuracy in the mentioned study conditions.^{13,14} From the linearity data, the LOD and LOQ were 4.5 ppb and 15.1 ppb. It can be concluded, that 8-OHdG can be detected by HPLC if it has a concentration above 4.5 ppb and can be quantified well at a concentration above 15.1 ppb. The precision test in an analytic process is the level of acceptance of the sample test results when the analytical method is applied repeatedly to several homogeneous samples.¹³ The precision test was carried out by injecting 20 ppb of 8-OHdG standard 6 times. This test aims to see the diversity of the resulting peak area. The results of the analysis of the peak area of 6 times the injection of the standard 8-OHdG were calculated as the coefficient of variation. The coefficient of variation obtained was 0.96 %. As the results of these calculations, the value obtained was smaller than % CV Horwitz (% RSDR)¹⁵, which is 28.82 %. It can be concluded that the measurement has a fairly good precision value so that the analysis results can be accepted.

Analysis Results of the 8-OHdG Formation by *In-vitro*

In Fig.-2, all variations of samples exposed to AA compounds caused an increase in 8-OHdG levels compared to samples that were not exposed to AA compounds. The increase in 8-OHdG occurred in all conditions of incubation, such as at 5 h and 7 h incubation time, 5 h incubation with UVA exposure, and 7 h incubation with UVA exposure. It is not certain if the mechanism of AA influences the formation of 8-OHdG. Jiang *et al.* in 2007 conducted a study to see the genotoxicity of AA in hepatoma G2 (HepG2) cells in humans. From these results, it was found that AA caused the breakdown of DNA strands in HepG2 cells by a possible underlying mechanism, namely due to the increased levels of ROS and GSH depletion. This research also resulted in the formation of 8-OHdG. From these studies, it was concluded that AA exerts a genotoxic effect in HepG2 cells, with a mechanism made possible using oxidative DNA damage caused by intracellular ROS and GSH depletion.¹⁶

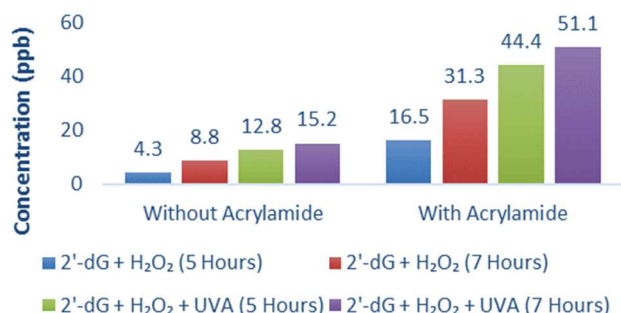


Fig.-2: Graph of acrylamide (AA) addition on the formation of 8-OHdG at 5 and 7 h incubation time

Strengthening the results of research from Jiang *et al.* in 2007, Wajda *et al.* explained that the decrease in the level of glutathione in reduced form (GSH), caused by the conjugation of GSH with AA, may be accountable for the effect of cytotoxic of AA, which may be correlated to the accumulation of excessive amounts of ROS.^{17,23} With so many reactive oxygen species accumulated, especially hydroxyl radicals, that can cause the formation of 8-OHdG. From the analysis results that can be seen in Fig.-3, all samples experienced an increase in the formation of 8-OHdG levels after being exposed to TBHQ. In a sample mixture of 2'-deoxyguanosine with H₂O₂ incubated for 5 h, a mixture of 2'-deoxyguanosine with H₂O₂ incubated for 7 h, and a mixture of 2'-deoxyguanosine with H₂O₂ incubated for 5 h with UVA exposure showed the amount of 8-OHdG formation rise with approximately 3-4-fold when compared to the sample without TBHQ exposure. In contrast to the samples previously mentioned, the mixture of 2'-deoxyguanosine and H₂O₂ mixed samples incubated for 7 h with UVA exposure showed a significant increase after being given by TBHQ exposure with an increase of approximately 15 times compared to the samples without TBHQ exposure. From these data, it can be concluded that the presence of TBHQ and UVA rays at a longer incubation time resulted in the greater formation of 8-OHdG. The results of high 8-

OHdG formation due to TBHQ exposure may occur due to the contribution of the radicals formed during the photo-oxidation process of TBHQ. Radicals formed during the TBHQ oxidation process due to UV exposure can initiate the breakdown of hydrogen peroxide (H_2O_2) into hydroxyl radicals so that more hydroxyl radicals can be produced. The effect of more hydroxyl radicals will have an impact on 8-OHdG formation.

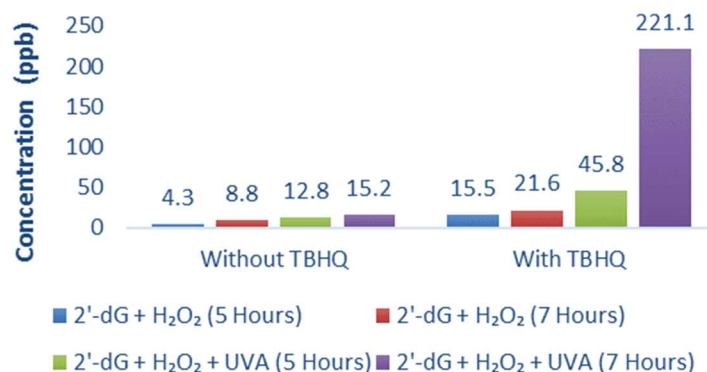


Fig.-3: Graph of TBHQ addition on the formation of 8-OHdG at 5 and 7 h incubation time

From the analysis, the formation of 8-OHdG was increased in a mixture of 2'-dG with AA and TBHQ exposed to UVA light (Fig.-4). From the outcome of the analysis of all samples, it can be observed a decreasing effect and also the effect of an increase in 8-OHdG levels due to the presence of AA and TBHQ compounds simultaneously in the sample. It can also be seen that the samples exposed to UVA light increase the level of 8-OHdG by producing a synergistic effect. Synergistic effects can occur due to the influence of UVA rays which have a major influence on H_2O_2 and TBHQ in initiating the formation of hydroxyl radicals. However, for samples that did not expose to UVA light, it only produced an antagonistic effect on the mixture. It is known that the antagonism properties between antioxidants in the oxidation of food components can occur with less effective antioxidant regeneration by more effective antioxidants, more effective antioxidant oxidation by radicals from less effective antioxidants, competition between the formation of antioxidant radical adducts, and antioxidant regeneration, and environmental changes of antioxidants¹⁸. In conclusion, the mixing effect of AA and TBHQ compounds is influenced by external conditions such as exposure to UVA rays. An additive effect is an effect that refers to a combination resulting in the sum of the effects of each component; a synergistic effect occurs when the resulting effect is higher compared to the total of the individual components, and the antagonistic effect occurs when the combination produces an effect that is smaller than the sum individually.¹⁹

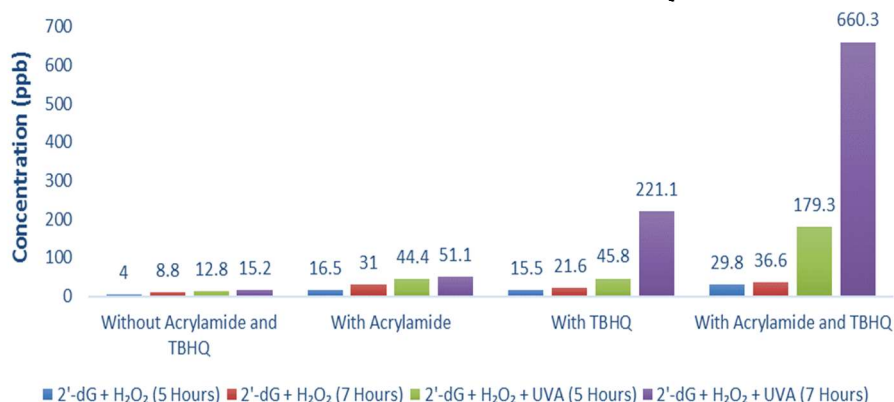


Fig.-4: Graph of the Acrylamide (AA) and TBHQ addition on the formation of 8-OHdG at 5 h and 7 h incubation time

As seen in Fig.-4, the highest value of 8-OHdG levels occurred in samples containing AA and TBHQ which were exposed to UVA light for 7 h. The effect of exposure to UVA rays significantly increases the effect of AA and TBHQ mixture which can be seen from the increase of 8-OHdG level from 29.8 ppb to 179.3

ppb at 5 h incubation time, and 36.6 ppb to 660.2 ppb at 7 h time incubation. In addition, the data from the 5 h and 7 h incubation results showed that TBHQ was quite reactive to UVA rays. From Fig.-4, there was an increase in 8-OHdG levels from sample without UVA light exposure at 5 h to 7 h incubation time, but with an average increase that is not too significant. This can be occurred because there was no UVA exposure which can break down H_2O_2 into hydroxyl radicals. In contrast, sample incubation with UVA irradiation containing TBHQ and a mixture of AA and TBHQ experienced a notable increase in levels of 8-OHdG from 5 h incubation time to 7 h incubation time. This shows that the effect of the incubation time is very influential on the active sample to produce ROS in the system. At a longer incubation time, UVA rays will take longer to shine on the sample and cause an increase in the duration of the H_2O_2 photolysis process. With the longer duration of photolysis of H_2O_2 , more hydroxyl radicals will also be produced. As a result, the level of 8-OHdG increases the length of incubation time increases. UVA rays act as a breaking agent for H_2O_2 (can be seen in Fig.-5) so that hydroxyl radicals can be obtained which will later attack 2'-deoxyguanosine and generate 8-OHdG (can be seen in Fig.-6).

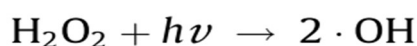


Fig.-5: The scheme of Photolysis Reaction of Hydrogen Peroxide (H_2O_2)²⁰

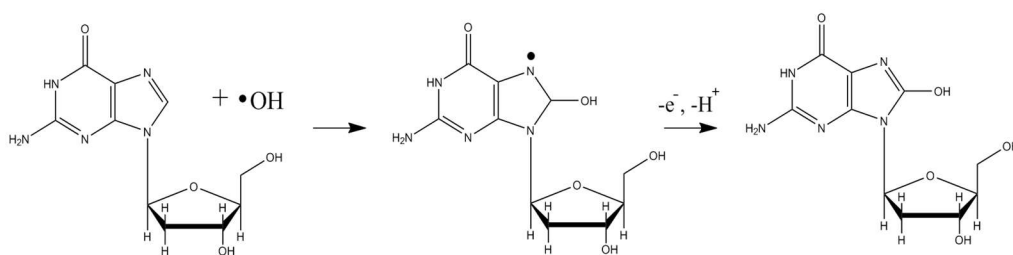


Fig.-6: The scheme of 8-OHdG formation from 2'-deoxyguanosine and hydroxyl radicals

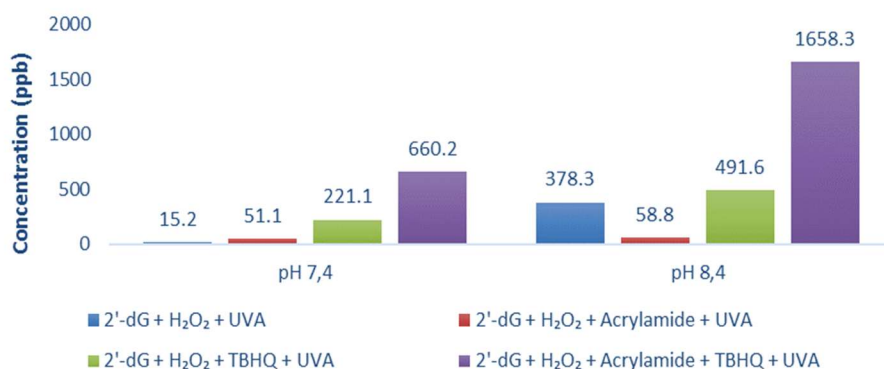


Fig.-7: Graph of pH condition on the formation of 8-OHdG at 7 h incubation time

From the data obtained (Fig.-7), there was an increase in 8-OHdG levels at alkaline condition (pH 8.4). The decomposition rate of hydrogen peroxide at alkaline pH was greater than at pH 7.4, so that formation of hydroxyl radicals were greater too. Several factors that can influence the decomposition activity of hydrogen peroxide are impurities, temperature, pH, and metal ions present in solution.²¹



Fig.-8: The scheme of hydrogen peroxide decomposition reaction²¹

The decomposition rate of H_2O_2 (Fig.-8) will increase significantly at alkaline pH (pH 5-11).^{20,22} According to Brooks and Moore (2000), H_2O_2 will decompose into H^+ and per-hydroxyl anions (HO_2^-) which will then lead to the formation of free radicals.²¹ Research shows that the formation of the per-hydroxyl anion is influenced by pH; The higher the pH, the more per-hydroxyl anions (HO_2^-) are formed, leading to the production of more free radicals (hydroxyl radicals)^{21,23}. The more hydroxyl radicals that are formed will increase the formation of 8-OHdG. It can be concluded, that at a more alkaline pH, a greater level of 8-OHdG formation will be produced

CONCLUSION

From this research, it can be concluded that exposure to AA compounds, TBHQ, and a mixture of AA and TBHQ compounds with 2'-deoxyguanosine in the presence of UVA exposure could lead to the 8-OHdG formation. The highest levels of 8-OHdG were found in a variety of mixtures of 2'-deoxyguanosine, AA, TBHQ, and H_2O_2 exposed to UVA light for 7 hours; pH 8.4; 37 °C with a concentration value of 1658.3 ppb. Exposure to AA and TBHQ compounds to the formation of 8-OHdG produces an antagonistic effect in samples without exposure to UVA light and produces a synergistic effect in samples exposed to UVA light. There is a significant difference in the formation of 8-OHdG due to exposure to a mixture of AA and TBHQ compounds given exposure to UVA light compared to without exposure to UVA light. For further research, it may be possible to do biomonitoring in order to obtain more real data in accordance with the conditions that occur in humans.

ACKNOWLEDGMENT

We are thankful to PUTI Proceedings Grant with contract number NKB-965/UN2.RST/HKP.05.00/2020 for funding this research.

REFERENCES

1. B. De Meulenaer, R. Medeiros, and F. Mestdagh, *Advances in Potato Chemistry and Technology (Second Edition)*, 527(2016), <https://doi.org/10.1016/B978-0-12-800002-1.00018-2>
2. M. Friedman, *Food & Function*, **6**(6), 1752(2015), <https://doi.org/10.1039/C5FO00320B>
3. N. Z. Atay, D. Çalgan, E. Öakat, T. Varnali, *Journal of Molecular Structure: THEOCHEM*, **728**(1-3), 249(2005), <https://doi.org/10.1016/j.theochem.2005.06.039>
4. S. Handayani, I. C. Dani, Budiawan and D. Pakuanisa, *Journal of Physics Conference Series*, **835**, 1 (2017), <https://doi.org/10.1088/1742-6596/835/1/012015>
5. T. L. de Jager, A. E. Cockrell and S. S. Du Plessis, *Advances in Experimental Medicine and Biology*, **996**, 15(2017), https://doi.org/10.1007/978-3-319-56017-5_2
6. M.L.Circu, T.Y.Aw, *Free Radical Biology and Medicine*, **48**(6), 749(2010), <https://doi.org/10.1016/j.freeradbiomed.2009.12.022>
7. M. Graille, P. Wild, J-J Sauvain, M. Hemmendinger, I. Guseva Canu, N.B. Hopf, *Toxicology Letter*, **328**, 19(2020), <https://doi.org/10.1016/j.toxlet.2020.04.006>
8. C. Nishisgori, *Photochemical & Photobiological Sciences*, **14**, 1713(2015), <https://doi.org/10.1039/C5PP00185D>
9. S.K.Urbaniak, K. Boguszewska, M. Szewczuk, J. Kaźmierczak-Barańska and B. T. Karwowski, *Molecules*, **25**(1), 202(2020), <https://doi.org/10.3390/molecules25010202>
10. D. Wu, B. Liu, J. Yin, T. Xu, S. Zhao, Q. Xu, X. Chen, and H. Wang, *Journal of Chromatography B*, **1064**, 1(2017), <https://doi.org/10.1016/j.jchromb.2017.08.033>
11. M. Azadeh, B. Gorovits, J. Kamerud, S. MacMannis, A. Safavi, J. Sailstad and P. Sondag, *The AAPS journal*, **20**(1), 22(2017), <https://doi.org/10.1208/s12248-017-0159-4>
12. P. Araujo, *Journal of Chromatography B*, **877**(23), 2224(2009), <https://doi.org/10.1016/j.jchromb.2008.09.030>
13. Ü. Şengül, *Journal of Food and Drug Analysis*, **24**(1), 56(2016), <https://doi.org/10.1016/j.jfda.2015.04.009>
14. A. Elena, C. Orbeci, C. Lazau, P. Sfirloaga, P. Vlazan, C. Bandas, I. Grozescu, *Intechopen Book Series*, 53(2013), <https://doi.org/10.5772/53755>
15. L. Jiang, J. Cao, Y. An, C. Geng, S. Qu, L. Jiang, and L. Zhong, *Toxicology In Vitro*, **21**, 1486(2007), <https://doi.org/10.1016/j.tiv.2007.06.011>

16. A. Duda-Chodak, Ł. Wajda, T. Tarko, P. Sroka, P. Satora, *Food & Function*, **7(3)**, 1282(2016), <https://doi.org/10.1039/c5fo01294e>
17. E. Choe, D. B. Min, *Comprehensive Reviews in Food Science and Food Safety*, **8**, 345(2009). <https://doi.org/10.1111/j.1541-4337.2009.00085.x>
18. S. Wang, K. A. Meckling, M. F. Marcone, Y. Kakuda, R. Tsao, *Journal Agriculture Food Chemistry*, **59(3)**, 960(2011), <https://doi.org/10.1021/jf1040977>
19. N. Liu, S. Sijak, M. Zheng, L. Tang, G. Xu, M. Wu, *Chemical Engineering Journal*, **260**, 826(2015), <https://doi.org/10.1016/j.cej.2014.09.055>
20. C. R. G. Torres, E. Crastechini, F. A. Feitosa, C. R. Pucci and A. B. Borges, Influence of pH on the effectiveness of hydrogen peroxide whitening, *Operative Dentistry*, **39(6)**, E261(2014), <https://doi.org/10.2341/13-214-l>
21. E. S. Abdel-Halima, S. S. Al-Deyab, *Carbohydrate Polymers*, **92(2)**, 1844(2013), <https://doi.org/10.1016/j.carbpol.2012.11.045>
23. A. Valavanidis, T. Vlachogianni and C. Fiotakis, *Journal of Environmental Science and Health Part C*, **27**, 120(2009), <https://doi.org/10.1080/10590500902885684>

[RJC-6770/2021]