Australian Journal of Basic and Applied Sciences

2020 November; 14(11): pages 86-93
DOI: 10.22587/ajbas.2020.14.11.9

Original paper AENSI Publications

Estimation of antioxidants activity and capacity to capture free radical using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

Khalid A. Alsaleem [1, 2] *, Mohamed Salem Elfaruk [1, 3] and Ahmed R. A. Hammam [1, 4]

[1]Dairy and Food Science Department, South Dakota State University, Brookings, South Dakota 57007, USA
[2]Department of Food Science and Human Nutrition, College of Agriculture and Veterinary Medicine, Qassim University, Buraydah 51452, Saudi Arabia
[3]Medical Technology College, Nalut University, Nalut 00218, Libya
[4]Dairy Science Department, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt

Correspondence Author: Khalid A. Alsaleem, Dairy and Food Science Department, South Dakota State University, Brookings, South Dakota 57007, USA ; khalid.alsaleem@sdstate.edu

Received date: 22 September 2020, Accepted date: 20 November 2020, Online date: 19 January 2021

Abstract

Background: Antioxidants are food additives with various capacities commonly utilized to reduce free radical and delay food oxidation. Antioxidants within the food materials provide a degree of protection against oxidation and the presence of free radicals. It can emerge in the food natural, added during formulations, or created during food processing.
Objective: This study's objective was to evaluate the capacity of various concentrations of ascorbic acid, gallic acid, and butylated hydroxytoluene (BHT) using The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay and a spectrophotometric at an absorbance of 510 nm.
Results: The Effective concentration (EC50) of ascorbic acid, gallic acid, and BHT was 136.46 ± 15.22, 41.27 ± 1.27, and 0.239 ± 0.08 µM, respectively. The remained of DPPH value was decreased by increasing the concentration of antioxidants. The remains of DPPH for the highest concentrations of ascorbic acid, gallic acid, and BHT were 9.5, 8.7, and 21.09 %, respectively. The antioxidants significantly decreased ($p < 0.05$) the free radical, with gallic acid having higher efficiency followed by ascorbic acid and BHT.
Conclusion: Antioxidants' capacity is a crucial factor in avoiding per-oxidant actions that resulted from a high concentration of antioxidants. The DPPH assay can be used to estimate antioxidants' activity by measuring the remained of DPPH assay using a spectrophotometric measurement. This work's outcome can be applied as reference standards to match and estimate the properties of novel antioxidants.

Keywords: DPPH assay; Free radicals; Antioxidants activity assessment; Ascorbic acid; Gallic acid; BHT

INTRODUCTION

Antioxidants are food additives that decrease free radicals and delay the oxidation of lipids, which in turn, increase the shelf life of foods during storage (Mcclements and Decker, 2000). Antioxidants within the food materials increase the degree of protection against free radicals. The main role of an antioxidant is its capacity to catch free radicals. Thus, food processors formulate food products with antioxidants routinely to decrease free radicals. Nowadays, the consumption of antioxidants has been associated with many health benefits. According to the European Commission Concerted Action on Functional Food, it proves a relationship between the human's disuses and free radicals; thus, the antioxidants have been added to reduce free radicals' harmful effects (Diplock et al., 1998). The estimation of the antioxidant capacity should take into account to decrease the additive antioxidant and avoid the per-oxidant that resulted from using a high concentration. Antioxidants can decrease free radicals in many ways, based on their structure and mechanisms (Quinchia et al., 2011). The antioxidants can primary or secondary antioxidants. The primary antioxidant interacts with peroxy radicals produced from the initiation stage to inhibit the oxidation reaction (Wanasundara and Shahidi, 2005). On the other hand, secondary antioxidant plays an essential role in catching pro-oxidant and catalyst metal ions, which inhibit the oxidation reaction (Wanasundara and Shahidi, 2005).

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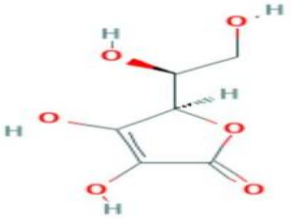
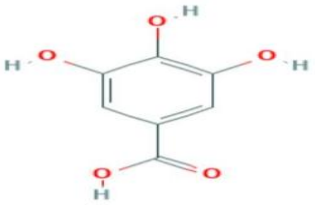
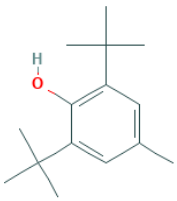
Three ways for the antioxidants' content to emerge in foods: i) present naturally; ii) add during food processing, and iii) produce during food processing (Alsaleem, 2019). Dairy products contain various active antioxidants, such as vitamins and enzymes (Pihlanto, 2006). Milk contains vitamins and bioactive compounds that are present naturally and react as antioxidants (Saxelin et al., 2003). For instance, natural antioxidants in milk are low molecular weight thiols (Niero et al., 2014), ascorbate (Nielsen et al., 2001), tocopherol, retinol, and carotenoids (Jensen and Nielsen, 1996; Nozière et al., 2006). Moreover, a study shows that using some cultures such as pediococcus pentosaxeus during processing yogurt increases antioxidant activities (Balakrishnan and Agrawal, 2014).

The additive antioxidants could be either natural or synthetic antioxidants that can be added during processing foods. The natural antioxidants can be grouped into three groups: First contains glutathione reductase, catalase, superoxide dismutase, and minerals in their structure. The second contains ascorbic acid, glutathione (GSH), albumin, and alpha-tocopherol. In contrast, the last group contains a mixed combination of enzymes that can fix broken DNA, degraded proteins, oxidized lipids, and peroxide as lipase (Sindhi et al., 2013). Moreover, the antioxidants can be divided into enzymatic (primary and secondary) and non-enzymatic antioxidants (Bunaciu et al., 2012). The antioxidants can be synthetic, which have recently tested their capability to reduce free radicals plus investigating their safety to the consumer, such as butylated hydroxytoluene (BHT). The BHT, propyl gallate (PG), and butylated hydroxyanisole (BHA) are chemical compounds that have been formed and examined by scientists to develop their ability to catch free radicals, ensure their safety, as well as their impact on the consumers' health. Using high concentrations of synthetic antioxidants could be affected by the consumers' health (Wanasundara and Shahidi, 2005). The food and dairy processors tend to use fewer synthetic antioxidants to potent effect than natural antioxidants. The synthetic antioxidants should be added at low concentrations to avoid per-oxidant actions (Wanasundara and Shahidi, 2005). Consumers prioritize natural antioxidants rather than synthetic antioxidants due to emotional reasons (Pokorný, 2007). Some antioxidants, such as D-ascorbic acid and alpha-tocopherol, could be synthetic or natural antioxidants that depend on these antioxidants' source.

However, they consider and discuss under natural antioxidants (Wanasundara and Shahidi, 2005). This could be due to the similarity of synthetic antioxidants' structure and capacity to catch free radicals compared with natural antioxidants.

Ascorbic acid is known as vitamin C and a natural antioxidant found naturally or additive in some foods (Table 1).

Table 1. Concentration and chemical characteristics of ascorbic acid, gallic acid, and 3,5-Di-tert-4-butylhydroxytoluene (BHT) used for the DPPH assay.

Name	Concentration (Mole per mole of DPPH)	Empirical formula	Molecular weight	Chemical structure
Ascorbic acid	0, 25, 50, 75, 100, and 125	$C_6H_8O_6$	176.12	
Gallic acid	0, 25, 50, 75, 100, and 125	$C_7H_6O_5$	170.12	
2,6-Di-tert-butyl-4-methylphenol (BHT)	0, 0.25, 0.5, 0.75, 1, and 1.25	$C_{15}H_{24}O$	220.35	

It is a water-soluble widely known for its antiradical properties that exhibit free radical scavenging, contributing to decrease morbidity and mortality from coronary heart disease (Brighenti et al., 2005). Bioactive nanocomposite film (Janani et al., 2020), degradative of various organic pollutants (Hou et al., 2020), and polluted water remediation (Hou et al., 2020) are applications of ascorbic acid. Gallic acid, known as a polyhydroxy phenolic compound, can be a free form or compound (Niemetz and Gross, 2005). It is a water-soluble that can be found in fruit and vegetable (Table 1). Gallic acid has different biological activities that include anti-viral (Kratz et al., 2008), anti-bacterial (Kang et al., 2008) and anti-inflammatory (Kim et al., 2006). BHT is a synthetic phenolic antioxidant and derives from the corresponding alcohol and aldehyde (BHT-CHO) (Fujisawa et al., 2004)

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(Table 1). It can inhibit cancer induction with various chemical carcinogens (Slaga, 1995). Synthetic antioxidants are known as primary antioxidants (Shahidi, 2015). The BHT number is known as E320 that is under the butylates group (Joint et al., 2004). 158°F (70 °C) and 509 °F (265 °C) are the melting and boiling points of BHT, respectively. It is exceedingly used as a food additive since the 1950s (Humans and Cancer, 1988) to maintain the physical and chemical properties of food (Fries and Püttmann, 2002). The concentration of BHT ranges from 0.01 to 0.02% in most foods regulated by the U.S. Food and Drug Administration (FDA) and the European Union (EU) (Freitas and Fatibello-Filho, 2010). It is mostly insoluble in water and can be dangerous on human health if it takes at a high concentration (Leng and Gries, 2012; Sadowska et al., 2012).

Numerous chemical and volumetric techniques have been developed for quantifying and monitoring the capacity of antioxidants, including thermogravimetrical analysis (TGA) (Alsaleem et al., 2020), differential scanning calorimetry (DSC) (Alsaleem et al., 2019), radical scavenging capacity assay (Torun and Toprak, 2020), oxygen radical absorbance capacity (ORAC) (Paknahad et al., 2020), oxidative stability index (OSI) (Bañares et al., 2019), peroxide values (PV) (Cuomo et al., 2020), derivative thermogravimetry (DTG) (Gray, 1978), and gel permeation chromatography (GPC) (Dudonné et al., 2009). The powers and weaknesses of those techniques for evaluating the capacity of antioxidants have been reviewed (Kamal-Eldin and Pokorny, 2005). Some of those methods are more efficient for liquid products and some for solids. The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay is an easy technique compared with other methods. Blois (Blois, 1958) developed the DPPH assay (considers the steady free radical), which was the first approach to measuring antioxidant activity. Like the many other free radicals, the DPPH molecule does not dimerize and is known as steady free radicals to delocalize the spare electrons through the molecule (Kedare and Singh, 2011). The DPPH assay can be applied for both solid and liquid samples and is not particular to any singular antioxidant component; however, it refers to all the sample's antioxidant capability. It is an easy technique and low cost compared with other methods. Several methodologies have been published to determine the free radical scavenging capacity of antioxidants, and DPPH assay technique is regarded as an easy and accurate technique (Hegedűs et al., 2020). Effective concentration (EC50) is a parameter that has been used for the evaluation of the DPPH result, which is the amount of antioxidant is needed to reduce 50% of DPPH activity (Alexander et al., 1999). This can be applied to determine the antiradical power (ARP), as shown in Equation (1) (Brand-Williams et al., 1995; Bondet et al., 1997).

Equation (1)

$$ARP = \frac{1}{EC_{50}}$$

This work aimed to estimate the ability of different concentrations of ascorbic acid, gallic acid, and BHT using DPPH assay at an absorbance of 510 nm using a spectrophotometric measurement.

2. MATERIALS AND METHODS

2.1. MATERIALS

The DPPH was purchased from Alfa Aesar (Ward Hill, MA, USA). Spectrophotometric grade methanol (900 g/L) was obtained from Fischer Scientific Co. (USA). Ascorbic acid, gallic acid and 3, 5-Di-tert-4-butylhydroxytoluene (BHT) were obtained from Sigma (St. Louis, MO, USA), and 12 Well Cell Culture Cluster was purchased from Costar (NY, USA). The antioxidants were stored at -18 °C until further use. Spectrophotometric data was obtained using VARIAN (Cary Bio 50 UV-Visible Spectrophotometer) attached to a VARIAN Cary 50 MPR Microplate Reader from Agilent Co. (USA).

2.2. Antiradical activity measurement

2.2.1. Preparation of 2, 2-Diphenyl-1-picrylhydrazyl assay

The DPPH assay was prepared using the method reported by Brand-Williams (Bondet et al., 1997) with some changes. A total of 49 mg of DPPH was dissolved into 250 mL of methanol. The DPPH solution was vortexed and prepared on the same day and kept no more than three hours at room temperature.

2.2.2. Antioxidant determinations

Three antioxidant solutions of ascorbic acid, gallic acid, and BHT were prepared by mixing with 250 mL of methanol of each. A total of 44 mg of ascorbic acid, 12.6 mg of BHT, and 4.2 mg of gallic acid were dissolved into 250 mL methanol. The antioxidants' solutions were vortexed and kept for further analysis.

2.2.3. Determination of Antioxidant Capacity

Different concentrations of ascorbic acid, gallic acid, BHT were prepared by mixing with DPPH using a 12-well microplate. A 0, 25, 50, 75, 100, and 125 moles of ascorbic acid and gallic acid/mole of DPPH were mixed with the DPPH solution to 1000 mL. A 0, 0.25, 0.5, 0.75, 1, and 1.25 mole of BHT / mole of DPPH was mixed with DPPH solution to 1000 mL. Table 1 presents the concentration used, empirical formula, molecular weight, and antioxidants' chemical structure. Each concentration was done in triplicate. The mixtures were vortexed for 5 seconds and left in the dark for 5 min at room temperature. Then, the spectrophotometer was used at 510 nm absorbance. Equation (2) calculated the remaining DPPH % of each antioxidant:

Equation (2)

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$$\text{Remaining DPPH (\%)} = \frac{A_t}{A_{t0}} * 100$$

Where At is the corrected absorbance reading of each antioxidant. At0 is the corrected absorbance of DPPH %.

The DPPH % obtained were plotted against the mole antioxidants/mole DPPH of antioxidants concentrations to obtain the standard curves. The EC50 values for antioxidants were reported. Different models were assigned for each antioxidant. The gallic acid model was logistic as follow in Equation (3):

Equation (3)

$$DPPH = \frac{min + (max - min)}{1 + \frac{x}{EC-50^{-slope}}}$$

On the other hand, the ascorbic acid model was linear as follows in Equation (4):

Equation (4)

$$DPPH = (m * x) + b$$

However, the BHT model was exponential decay as follows in Equation (5):

Equation (5)

$$DPPH = y_0 + a * exp^{-b*x}$$

2.2.4. Statistical Analyses

ANOVA was fulfilled to calculate p-values using R software (R x 64 3.3.3 using R studio). When a significant difference was identified at $p < 0.05$, mean separation was done by the least significant difference (LSD test).

3. RESULTS AND DISCUSSION

The estimation capacity of ascorbic acid, gallic acid, and BHT was evaluated using the DPPH assay at an absorbance of 510 nm using a spectrophotometric measurement. The EC50 of ascorbic acid, gallic acid, and BHT were determined and reported in Table 2. The EC50 value of ascorbic acid, gallic acid, and BHT were 136.46 ± 15.22, 136.46 ± 15.22, and 0.239 ± 0.08 µM, respectively. It was expected that the antioxidants in this study give high antiradical activities with EC50 values lower than 0.239 µM. The results presented that the DPPH of antioxidants significantly reduced ($p < 0.05$) the free radical with gallic acid having higher efficiency followed by ascorbic acid and BHT. The BHT was used with a higher concentration comparing to other antioxidants in this study. Lower BHT (> 25 µM) cannot reach completion even for up to two hours. It was expected that increasing the number of antioxidants elevates the antiradical activity of antioxidants.

Table 2. The EC_{50}, p-value, and R^2 of gallic acid, ascorbic acid, and BHT

Antioxidant	EC_{50} (µM)	P-value	R^2
Gallic acid	41.27 ± 1.27^b	< 0.0001	> 0.99
Ascorbic acid	136.46 ± 15.22^a	< 0.0001	> 0.99
Butylated hydroxytoluene (BHT)	0.239 ± 0.08^c	< 0.0001	> 0.99

Mean (n = 3) of three independent experiments ± standard deviation

The % DPPH values were plotted against the mole antioxidant/mole DPPH of different antioxidants to obtain the standard curves (Figure 1-3). Figure 1 shows the remaining DPPH values against the variable concentrations of gallic acid. The standard curve was obtained using Equation (3). It is a nonlinear correlation in the gallic acid standard curve. The remained of DPPH value was decreased by increasing the concentration of gallic acid, which was expected. The remains of DPPH were 9.5 % at 125 µM of gallic acid. The gallic acid with a < 40% concentration shows a sharp decrease in the remaining free radicals. The EC50 of gallic acid was 41.27 ± 1.27 µM with R2 > 0.99, and p-value < 0.0001. Mamat (Mamat et al., 2020) and Olugbami (Odunola et al., 2015) reported that the EC50 values of gallic acid were 18.23 and 38.0 µM that was lower than the study result caused to sample preparations and solvent for the study.

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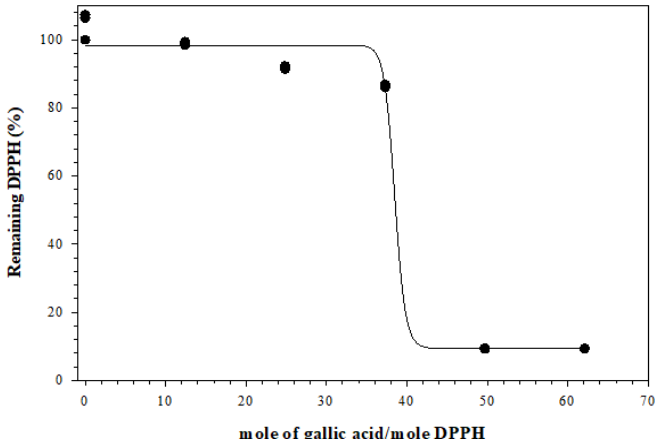

Figure 1. The remaining DPPH (%) evolution with a mole of ascorbic acid

The DPPH radical scavenging profile of ascorbic acid appears in Figure 2. The standard curve was obtained using Equation (4). It is a linear correlation in the ascorbic acid standard curve. The ascorbic acid has quickly reacted with the DPPH solution. The remains of DPPH were 8.7 % at 125 µM of ascorbic acid. The EC50 of ascorbic acid was 136.46 ± 15.22 µM with R2 > 0.99 and p-value < 0.0001. Charrier (Charrier et al., 2006); and Kanimozhi (Kanimozhi and Prasad, 2009) reported that the EC50 of ascorbic acid was 91 and 284 µM, respectively. Sharma & Bhat (Sharma and Bhat, 2009) and Brand-Williams et al. (Brand-Williams et al., 1995) studied the scavenging activity of DPPH by ascorbic acid and reported that EC50 values were 11.8 and 10.2 µM, respectively. These differences could be related to the concentration of antioxidants and solvents that were used in the studies. The experiment environment, such as light, presence of oxygen, and pH of the mixing solution, could affect the absorbance of DPPH (Sharma and Bhat, 2009), which makes it impossible to compare the result with other studies (Kano et al., 2005; Ricci et al., 2005).

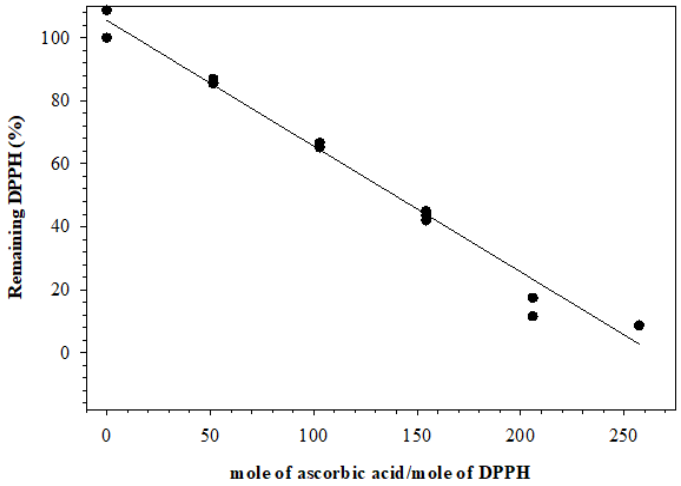

Figure 2. The remaining DPPH (%) evolution with a mole of gallic acid.

Figure 3 shows the remaining DPPH values against the variable concentrations of BHT. The standard curve was obtained using Equation (4). It is a nonlinear correlation in the BHT standard curve. The concentration of BHT was increased to decrease the remaining time. Some antioxidants failed to reach EC50 with low concentrations to increase the antioxidants' concentrations (Chen et al., 2013). A study found some antioxidants at low concentrations act as prooxidants, but at high concentrations behave as antioxidants (Chen et al., 2013). The purple color of the DPPH solution was changed by elevating the antioxidants' concentrations. At the higher BHT concentration, the solution's color changed to yellow, and the remains of DPPH were 21.09 %. The EC50 of BHT was 0.239 ± 0.08 with R2 > 0.99 and p-value < 0.0001. The EC50 of BHT was fairly lower than those reported by earlier studies as 16.14 (Boulebd, 2020) and 18.9 µM (Brand-Williams et al., 1995). The previous studies were used ethanol and lower concentrations of BHT that could decrease the antioxidant activity (Mishra et al., 2012). All antioxidants curves showed the existence of a correlation between the concentration of antioxidants and the residual of free radicals, judging by the shape of the curves.

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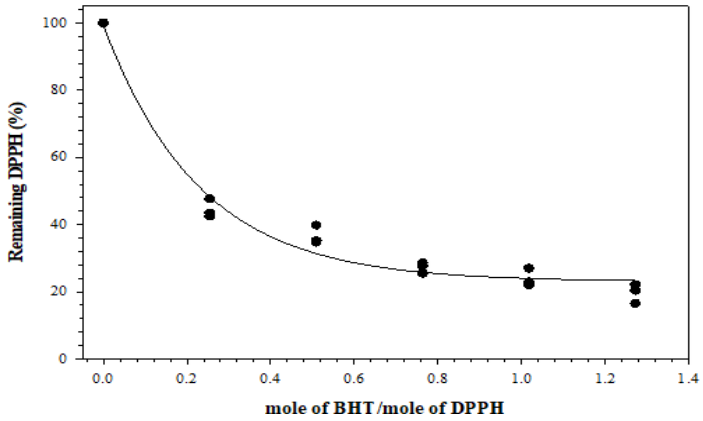

Figure 3. The remaining DPPH (%) evolution with a mole of 3,5-Di-tert-4-butylhydroxytoluene (BHT).

Beside EC50, the antioxidants activities can be ordered as BHT < ascorbic acid < gallic acid. Thus, gallic acid is the highest effective antioxidant and has more ability to trap free radicals, while BHT is the least efficient antioxidant because it was not reaching the EC50 at the same concentration. In general, the EC50 value was affected by the concentration of antioxidants, a study's solvent, the physical and chemical structure of antioxidant itself (Nenadis et al., 2007). The concentration of antioxidants can increase the antioxidants' capacity by decreasing the required time to reach EC50. Moreover, the EC50 could be affected based on the phenolic hydroxyl groups on the antioxidant structure, antioxidant structure itself, and polarity and solubility of antioxidants (Ramirez et al., 2006). The electronegativity, softness, hardness, and electrophilic index are examples of molecular descriptors that could affect the antioxidant activity (Sadasivam and Kumaresan, 2011; Praveena et al., 2014). The hydroxide group's strength in the antioxidants structures plays a role in antioxidant activity (Bendary et al., 2013). The ability of an antioxidant to break the hydroxide group could be considered as antioxidant activity. The strength of the hydroxide bond reduces the activity of the antioxidant (Boulebd, 2020). The broken bond between hydroxide and antioxidants helps the antioxidant to catch free radicals. The amount ratios of antioxidant solution to the DPPH solution play a vital role in the antioxidant activity (Dawidowicz et al., 2012). Therefore, the volume ratios of the antioxidant solution to the DPPH solution should use the same ratio to compare various antioxidants' antioxidant activity. In terms of solvent, the phosphate buffer gives higher values of EC50 for BHT and ascorbic acid, but it gives lower values with gallic acid compared with methanol (Mishra et al., 2012). The EC50 of BHT and ascorbic acid were 89.63 and 110.77 µM, respectively, that was used ethyl alcohol as dissolvent (Ricci et al., 2005). It was less effective for BHT and higher useful for ascorbic acid comparing to our results. The EC50 of BHT, which was dissolved in chloroform, shows better antioxidant activity than ethyl acetate (Dawidowicz et al., 2012). The observation of DPPH could be affected by the light when using methanol as a solvent (Min and Jung Kim, 2008).

4. CONCLUSION

The EC50 values of gallic acid, ascorbic acid, and BHT were 41.27 ± 1.27, 136.46 ± 15.22, and 0.239 ± 0.08 µM, respectively. The low value of EC50 referred to the higher antiradical power. The concentration of antioxidants and the remaining DPPH at higher concentrations should be considered. The remains of DPPH for the highest concentrations of ascorbic acid, gallic acid, and BHT were 9.5, 8.7, and 21.09 %, respectively. As a result, gallic acid has more antiradical power as compared with ascorbic acid and BHT. The EC50 values are affected by the concentration of antioxidants, type of antioxidants, and the solvent. The activity of different antioxidants can be measured precisely, rapidly, and low-cost using DPPH assay.

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