EFFECTS OF HEATING AND HYDROGEN PEROXIDE ON THE STABILITY AND ANTIOXIDANT ACTIVITY OF TERMINALIA NIGROVENULOSA EXTRACT AT DIFFERENT pH VALUES

ẢNH HƯỞNG CỦA NHIỆT ĐỘ, HYDROGEN PEROXIDE ĐẾN KHẢ NĂNG ỔN ĐỊNH HOẠT TÍNH KHÁNG OXY HÓA CỦA CAO CHIẾT *TERMINALIA NIGROVENULOSA* Ở CÁC GIÁ TRỊ pH KHÁC NHAU

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ABSTRACT

This study aimed to investigate the stability of Terminalia nigrovenulosa (T. nigrovenulosa) extract in response to heating treatment and hydrogen peroxide at pH 4 and pH 7. The changes of total polyphenol content, DPPH radical scavenging and reducing power were examined. The obtained results showed that the investigated extract was relatively stable under heating and sterilization condition (pH 4). However, it was unstable at pH 7. In addition, hydrogen peroxide also caused diverse effects on the total content of polyphenol compounds, DPPH radical scavenging activity and reducing power at pH 4 and pH 7. The extract reported a better stability at pH 4 compared to that of pH 7. Moreover, the extract solutions which contained higher total amount of polyphenol substances exhibited stronger DPPH radical scavenging and reducing activities. Thus, polyphenol compounds were responsible for the antioxidant activity of extracts. This study highlight the potential of the Terminalia nigrovenulosa extract as a powerful antioxidant additive in food preservation, particularly at acidic condition.

Keywords: *Terminalia nigrovenulosa; hydrogen peroxide; heating; stability; DPPH.*

TÓM TẮT

Mục tiêu của nghiên cứu này là khảo sát sự ổn định của cao chiết Terminalia nigrovenulosa đối với chế độ nhiệt độ, chất oxy hóa hydrogen peroxide ở giá trị pH 4 và pH 7. Sự thay đổi hàm lượng polyphenol tổng số, khả năng dập tắt gốc tự do và năng lực khử của cao chiết được khảo sát. Kết quả cho thấy, cao chiết Terminalia nigrovenulosa tương đối ổn định với điều kiện xử lý nhiệt ở pH 4 nhưng không ổn định ở pH 7. Đồng thời, ở pH 4 thì khả năng kháng oxy hóa và hàm lượng polyphenol tổng số của cao chiết cao hơn so với ở pH 7. Vì vậy, cao chiết Terminalia nigrovenulosa có tiềm năng ứng dụng làm chất chống oxy hóa trong thực phẩm đặc biệt thực phẩm có giá trị pH 4.

Từ khóa: *Terminalia nigrovenulosa; hydrogen peroxide; gia nhiệt; độ bền; DPPH.*

1. INTRODUCTION

Phenolic constitute is the most popular compounds presented in plants including medicinal and edible plants. In recent years, there is an increase in publications reported on the chemistry of phenolic compounds that is because these compounds possess biological properties such as antimicrobial, antiviral, antioxidant, anti-inflammatory activities and ability in the diet to protect against or retard the development of cancer [1], [2], [3], oil protection [4].

However, polyphenolic compounds are not completely stable [5]. These compounds are readily oxidized due to their antioxidant properties, and are thus prone to degradation leading to the content of phenolic compounds and their biological activity may be changed

by storage time and storage conditions as well as processing condition [6]. Therefore, evaluation of the changes of phenolic compounds and their biological activities of materials as the storage and processing conditions are relatively important work. The significant degradation of phenolic compounds during storage have been reported in highbush blueberries juice [7], [8], in *Myrtus communis* L berries extracts [2], and in extracts of basil, savory, lovage, lemon, balm, peppermint, parsley, oregano and sage [9]. In addition, there are some reports indicated that hydrogen peroxide, pH, light and various storage conditions have significant effects on anthyocyanin, phenolic compounds stability and antioxidant capacity in lowbush blueberry products [[10]], the stability of red radish extract [11]; *raphanus sativus* extracts [11]; drumstick leaves, mint leaves and carrot tube extracts [1].

The present study concentrates on the utilization of *Terminalia nigrovenulosa* bark extract which was considered as sources of natural antioxidants [14]. The aims of this research were to investigate the stability of *Terminalia nigrovenulosa* bark extract to pH, hydrogen peroxide, heating treatment.

2. MATERIALS AND METHOD

2.1. Chemical

The following chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA): 2,2-diphenyl-2-picrylhydrazyl hydrate, gallic acid, folin-ciocalteu, potassium ferricyanide, sodium nitrite, and aluminum chloride. Other chemicals and reagents were of analytical grade.

2.2. Preparation of plant extracts

Bark of *Terminalia nigrovenulosa* Pierre ex Laness was subjected to extraction for 24 hours with methanol at a ratio of 1:10 (dry sample/methanol) a glass conical flask on a shaker at room temperature, followed by filtration through filter paper (No. 1, Whatman International LTD, Maidstone, England). The residue was then extracted twice more with methanol as described above. The combined extract were concentrated using a rotary evaporator (Heidolph VV 2011-Antrieb, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 40^0C under a vacuum to obtain dry extract. The extract was stored at -20^0C until use.

2.3. Heat, pH and storage temperature stability

The *Terminalia nigrovenulosa* extract was dissolved in small amount of ethanol and then diluted in Mcllvaine buffer (pH 4 and 7) solution to get the concentration of 2.5 mg/l. the samples were placed into a glass tube with screw cap, wrapped in aluminum foil and then immersed in water bath at 95 – 100 ^{0}C for 20 min or sterilized at 121 ^{0}C for 20 min. afterward, each sample was cooled in water, residual phenolic content and antioxidant activity was determined.

2.4. Stability test to hydrogen peroxide at different pH values

Hydrogen peroxide was added to plant extracts (2.5 mg/mL) dissolved in buffer (pH 4 and 7) solution at final concentrations of 30, 70 and 210 µg/mL. The samples were stored in dark place at 25 ^{0}C for different time periods. The residual phenolic content and antioxidant activity of solutions were determined as 2, 4 and 8 h afterward.

2.5. DPPH radical scavenging activity

Radical scavenging activity of the plant extract against stable DPPH radical (2,2-diphenyl-2-picrylhydrazyl hydrate) was determined by spectrophotometry. When DPPH radical reacts with an antioxidant capable of donating hydrogen, it is reduced. Changes in colour (from deep-violet to light-yellow) were measured at 515 nm using an UV/visible light spectrophotometer.

The free radical scavenging activities of the extracts were measured by a slightly modified method of Nguyen and Eun (2011) [14], as described below. Extract solutions were prepared by dissolving 25 mg of dry extract in 10 mL of methanol. The solution of DPPH radical in methanol (6×10^{-5} mol/L)

was prepared daily before the absorbance measurements. Three milliliters of this solution was then mixed with 77 μL (38 or 19 μL in additional assays) of extract solution (final mass ratios of extracts to DPPH radical were approximately 3:1, 1.5:1, and 0.75:1). The samples were kept in the dark for 15 min at room temperature, after which the decrease in absorption was measured. Absorption of a blank sample containing the same amount of methanol and DPPH radical solution was measured daily. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100,$$

Where A_B and A_A stand for absorption of the blank sample (t=0 min) and absorption of the tested extract solution (t=15 min), respectively.

2.6. Reducing power

The reducing power of the plant extracts was determined by a slightly modified method of Nguyen et al. (2011) [14]. One milliliter of each plant extract concentration (0.1, 0.5, and 1 mg mL^{-1}) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1 %). The mixtures were then incubated at 50°C for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10 %) were added to each mixture, which were then centrifuged for 10 min at 1036×g. The upper layer of the solutions (2.5 mL) were mixed separately with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %), and the absorbance levels were measured at 700 nm using a spectrophotometer. Increased absorbance indicates increased reducing power, and the IC_{50} value is the concentration at which the absorbance is 0.5. BHT was used as positive control.

2.7. Total phenolic content

Total phenolic content of the plant methanol extracts was determined by the Folin–Ciocalteu method (1927). Total content of phenolic compounds in the plant extracts was expressed as mg of gallic acid equivalents (GAE) per g dry weight of plant extracts.

2.8. Data analysis

All experiments were performed in triplicate (n=3), and an ANOVA test (using STATGRAPHICS Centurion XV statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by LSD test ($p<0.05$). The results are presented as mean ± standard deviation (STD) of three replicated determinations.

3. RESULTS AND DISCUSSION

3.1. Effect of heat treatment and pH on total polyphenol content and antioxidant capacity of plant extracts

The total polyphenol content, DPPH radical scavenging activity and reducing power of the extract solution were found to vary with pH and temperature treatment (Fig. 1). Generally, higher reduction of total polyphenol content, DPPH radical scavenging activity and reducing power were found in extract solution of pH 7.

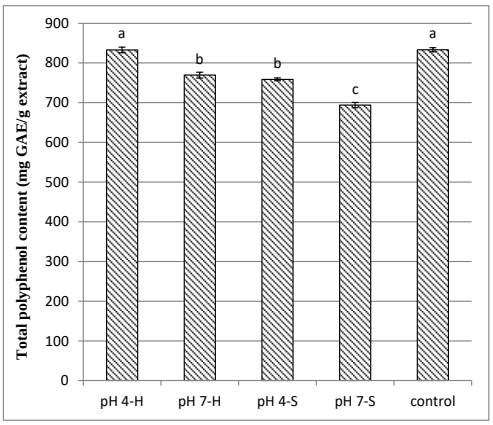

Figure 1. *Effect of heating and sterilization conditions on total polyphenol content of extract at pH 4 and pH 7*

- pH 4-H: Extract solution at pH 4 heated at 100°C for 20 min
- pH 7-H: Extract solution at pH 7 heated at 100°C for 20 min
- pH 4-S: Extract solution at pH 4 sterilized at 121°C for 20 min
- pH 7-S: Extract solution at pH sterilized at 121°C for 20 min

Results are means ± SD of triplicate measurements. Different labels (a-c) above the bars for the same extract indicate a significant difference at P<0.05.

Heating of extract at 100°C for 20 min have no significant effect on total polyphenol content, DPPH radical scavenging activity and reducing power of extract solution (pH 4). However, It induced an decrease in total polyphenol content by 6.65% (from 833.15 mg GAE/g to 769.45 mg GAE/g), reductions of 10.38% DPPH scavenging capacity and 0.04 OD unit of reducing power in extract solution of pH 7.

In the sterilization condition of 121^{0}C for 20 min, the changes in total polyphenol content, DPPH radical scavenging activity and reducing power of extract solutions of pH 4 and pH 7 were totally different. The decrease percentage of polyphenol contents in extract solution of pH 7 was nearly twice higher than that of extract solution (pH 4) (shown in Fig. 1) with the decrease percentage of 8.93% and 16.72%, respectively. The effect of sterilized condition on DPPH radical scavenging activity and reducing power of extract solutions of pH 4 and pH 7 was found in Fig 2. & Fig 3. It showed that higher effects on extract solutions were found in pH 7 with the decrease percentage of DPPH scavenging activity (0,25mg/mL) of 36,20 % and 0,28 OD unit of reducing power compared to 15,41% and 0,18 OD unit in pH 4 extract solution, respectively.

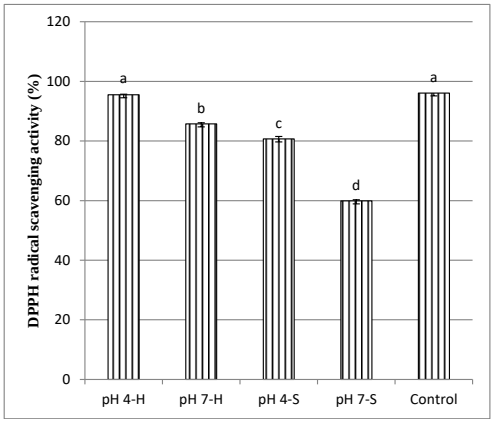

***Figure 2**. Effect of heating and sterilization conditions on DPPH radical scavenging activity of extract at pH 4 and pH 7*

- pH 4-H: Extract solution at pH 4 heated at 100°C for 20 min
- pH 7-H: Extract solution at pH 7 heated at 100°C for 20 min
- pH 4-S: Extract solution at pH 4 sterilized at 121°C for 20 min
- pH 7-S: Extract solution at pH sterilized at 121°C for 20 min

Results are means ± SD of triplicate measurements. Different labels (a-d) above the bars for the same extract indicate a significant difference at P<0.05.

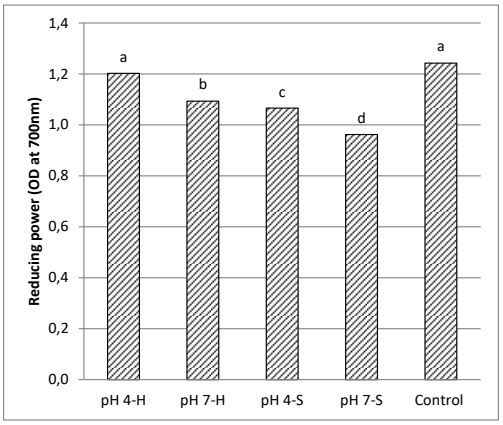

***Figure 3**. Effect of heating and sterilization conditions on reducing power of extract at pH 4 and pH 7*

- pH 4-H: Extract solution at pH 4 heated at 100°C for 20 min
- pH 7-H: Extract solution at pH 7 heated at 100°C for 20 min
- pH 4-S: Extract solution at pH 4 sterilized at 121°C for 20 min
- pH 7-S: Extract solution at pH sterilized at 121°C for 20 min

Results are means ± SD of triplicate measurements. Different labels (a-d) above the bars for the same extract indicate a significant difference at P<0.05.

Sterilization at 121°C for 20 min significantly decreased all parameters of extract solutions. Higher decreased values were also found in extract dissolved in buffer (pH 7) solution. The decrease in DPPH radical scavenging activity and reducing

power of all samples were associated with decreased total polyphenol contents indicated that polyphenolic compounds are responsible for antioxidant activity of the extract.

The loss of polyphenolic compounds by heat treatment is not well understood. There are some researches on the reduction of polyphenolic compound by thermal processing have reported. Cooking both tomatoes and onions resulted in lowered quercetin content [15]. Yu-Wei and Wang (2015) [16] indicated that cooking induced a decrease in total phenolic content by 12-51% and antioxidant activity by 16-67% in the control and germinated pulses. Abhay et al. (2016) [1] represented that the total polyphenols content in cacao reached a maximum at 70°C drying temperature and reduced as the heating time and increasing drying temperature. The different effects between pH 4 and pH 7 on the functional stability of the extract might be due to the difference in structure of compounds being extracted in extract. According to the research of Mansour and Khali (2000) [17] that reported that antioxidant activity of fenugreek seeds and ginger rhizome extracts decreased by increasing the pH of media; whereas the antioxidant activity of various extracts from cocoa by-product increased in alkaline pH [18]; Friedman and Hella (2000) [19] showed that caffeic, chlorogenic and gallic acids are not stable to high pH; chlorognic acid is added to apple juice being stable to acid pH, to heat and to storage.

3.2. Effect of hydrogen peroxide on functional stability of plant extracts

3 The effects of hydrogen peroxide on total polyphenol content, DPPH radical scavenging activity of extract were shown in Table 1. The degradation of total phenolic content, DPPH radical scavenging and reducing power of the extract to H_2O_2 was nearly similar to those of heat treatment, depending on pH of the solution. It was more stable to hydrogen peroxide at pH 4 than at pH 7. Moreover, the stability of investigated parameters also depended on the hydrogen peroxide concentration and incubation time. In general, higher effects were at the first 4 h after treatment and increase hydrogen peroxide concentration led to increase the degradation of total polyphenol content and decrease DPPH radical scavenging activity and reducing power.

Table 1. *Effect of hydrogen peroxide at different concentration on total polyphenol contents of extract solution at pH 4 and pH 7 as time treatment.*

Extract solution	Total polyphenol at different time of H_2O_2 (mg GAE/g extract)			
	Control	4h	8h	12h
pH 4-35 µg/mL	832.86 ± 1.43^{a}	800.25 ± 0.54^{bA}	791.21 ± 1.08^{bA}	798.12 ± 0.54^{bA}
pH 4-70 µg/mL	832.86 ± 1.43^{a}	725.36 ± 0.94^{bC}	719.98 ± 0.94^{bC}	715.51 ± 0.94^{cC}
pH 4-210 µg/mL	832.86 ± 1.43^{a}	705.39 ± 1.43^{bE}	693.02 ± 1.43^{cD}	689.13 ± 1.43^{cC}
pH 7-35µg/mL	832.86 ± 1.43^{a}	746.63 ± 0.94^{bB}	739.25 ± 1.43^{bB}	729.85 ± 0.94^{cB}
pH 7-70µg/mL	832.86 ± 1.43^{a}	715.24 ± 0.94^{bD}	705.42 ± 3.01^{cD}	706.12 ± 3.25^{cD}
pH 7-210 µg/mL	832.86 ± 1.43^{a}	609.12 ± 2.81^{bF}	601.12 ± 0.94^{bE}	598.71 ± 3.38^{bF}

Results are means ± SD of triplicate measurements). In the same raw different labels (a-c) and in the same column different labels (A-F) indicate a significant difference at $p<0.05$.

- pH 4-35 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 35 µg/mL
- pH 4-70 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 705 µg/mL
- pH 4-210 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 210 µg/mL

- pH 7-35µg/mL: Extract solution at pH 7 treated with hydrogen peroxide at concentration of 35 µg/mL
- pH 7-70µg/mL: Extract solution at pH 7 treated with hydrogen peroxide at concentration of 70 µg/mL
- pH 7-210 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 210 µg/mL

Table 1 showed the effect of hydrogen peroxide with different concentration on total polyphenol content of extract solutions at pH 4 and pH 7. The results showed that degradation rates of total polyphenol content were gradually increased together with the increase of hydrogen peroxide. The decrease percentages after 4h treatment were of 3.92, 12.91 and 15.31% at pH 4 and 10,35, 14,12, 26,86% at pH 7 with the hydrogen peroxide concentration of 35, 70, 210 µg/mL, respectively. The different effect of hydrogen peroxide on total polyphenol content of the extract depended on pH might be due to the different from the structure of compounds containing in the extracts which were more stable at pH 4 than at pH 7.

The stability (reactivity) of extract to hydrogen peroxide indicates antioxidant ability against reactive oxygen species (ROS). In this study, DPPH radical scavenging activity and reducing power of the extract solutions at pH 4 and 7 was examined. As shown in Table 2 & Table 3, the extract solution at pH 4 showed a significantly stronger antioxidant activity than at pH 7. DPPH radical scavenging activity of extract solution at pH 4 after 4h treatment of 84.49, 81.72 and 75.44 % and at pH 7 of 72.15, 65.00 and 60.12 % with the hydrogen peroxide treatment concentration of 35, 70 and 210 µg/mL, respectively compared to control (without hydrogen peroxide treatment) of 95.52% (Table 2). The changes in reducing power of extract solution to hydrogen peroxide was the same as DPPH radical scavenging activity, higher adverse effect at pH 7 than pH 4 and decrease in reducing power together with the increase in hydrogen peroxide concentration (Table 3). Moreover, increase concentration of hydrogen peroxide increase the degradation rate of total polyphenol content. These results were accordance with results of Elham et al. (2010) [20] that indicated that anthocyanin degradation increased as the concentration of hydrogen peroxide increased. Sapers and Simmons (1998) [22] observed that the decolonization of strawberry, raspberry, and cherry anthocyanins in the presence of hydrogen peroxide and the presence of high concentrations of hydrogen peroxide destruction of anthocyanins was rapid.

Table 2. *Effect of hydrogen peroxide at different concentration on DPPH radical scavenging activity of extract solution at pH 4 and pH 7 as time treatment*

Extract solution	DPPH radical scavenging activity (% inhibition)			
	Control	4h	8h	12h
pH 4-35 µg/mL	95.52 ± 0.16^{a}	84.49 ± 0.21^{bA}	80.78 ± 0.46^{cA}	81.52 ± 0.45^{cA}
pH 4-70 µg/mL	95.52 ± 0.16^{a}	81.72 ± 0.71^{bB}	73.18 ± 0.39^{cB}	72.39 ± 0.39^{cB}
pH 4-210 µg/mL	95.52 ± 0.16^{a}	75.44 ± 0.62^{bC}	73.14 ± 0.52^{bB}	69.98 ± 0.51^{bB}
pH 7-35µg/mL	95.52 ± 0.16^{a}	72.15 ± 0.49^{bD}	69.27 ± 0.48^{bB}	67.45 ± 0.32^{bcB}
pH 7-70µg/mL	95.52 ± 0.16^{a}	65.00 ± 0.71^{bE}	63.50 ± 0.37^{bC}	62.12 ± 0.22^{bC}
pH 7-210 µg/mL	95.52 ± 0.16^{a}	60.12 ± 0.43^{bF}	57.20 ± 0.65^{bD}	54.82 ± 0.11^{bD}

Results are means ± SD of triplicate measurements). In the same raw different labels (a-c) and in the same column different labels (A-F) indicate a significant difference at $p<0.05$.

- pH 4-35 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 35 µg/mL
- pH 4-70 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 705 µg/mL
- pH 4-210 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 210 µg/mL
- pH 7-35µg/mL: Extract solution at pH 7 treated with hydrogen peroxide at concentration of 35 µg/mL
- pH 7-70µg/mL: Extract solution at pH 7 treated with hydrogen peroxide at concentration of 70 µg/mL
- pH 7-210 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 210 µg/mL

Table 3. *Effect of hydrogen peroxide at different concentration on reducing power of extract solution at pH 4 and pH 7 as time treatment*

Extract solution	**Reducing power (OD at 700nm)**			
	Control	**4h**	**8h**	**12h**
pH 4-35 µg/mL	1.25 ± 0.01^{a}	0.95 ± 0.01^{bA}	0.91 ± 0.02^{cA}	0.89 ± 0.01^{cA}
pH 4-70 µg/mL	1.25 ± 0.01^{a}	0.83 ± 0.01^{bC}	0.78 ± 0.01^{cB}	0.75 ± 0.02^{dB}
pH 4-210 µg/mL	1.25 ± 0.01^{a}	0.68 ± 0.02^{bD}	0.62 ± 0.01^{cC}	0.63 ± 0.01^{cC}
pH 7-35µg/mL	1.25 ± 0.01^{a}	0.87 ± 0.01^{bB}	0.8 ± 0.01^{cB}	0.78 ± 0.03^{cB}
pH 7-70µg/mL	1.25 ± 0.01^{a}	0.72 ± 0.02^{bD}	0.64 ± 0.01^{cC}	0.64 ± 0.01^{cC}
pH 7-210 µg/mL	1.25 ± 0.01^{a}	0.61 ± 0.01^{bE}	0.57 ± 0.01^{bD}	0.58 ± 0.01^{bD}

Results are means ± SD of triplicate measurements). In the same raw different labels (a-c) and in the same column different labels (A-E) indicate a significant difference at $p<0.05$.

- pH 4-35 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 35 µg/mL
- pH 4-70 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 705 µg/mL
- pH 4-210 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 210 µg/mL
- pH 7-35µg/mL: Extract solution at pH 7 treated with hydrogen peroxide at concentration of 35 µg/mL
- pH 7-70µg/mL: Extract solution at pH 7 treated with hydrogen peroxide at concentration of 70 µg/mL
- pH 7-210 µg/mL: Extract solution at pH 4 treated with hydrogen peroxide at concentration of 210 µg/mL

The decomposition of hydrogen peroxide in an aqueous solution occurs in two ways, dissociation and homolysis cleavage of O-H or O-O bonds which lead to the formation of highly reactive species including perhydroxyl anion (HOO^-), and perhydroxyl ($^*OOH$) and hydroxyl ($^*OH$) radicals. The decomposition and association products of H_2O_2 have shown to be responsible for the oxidation and subsequence degradation of phenolic compounds[22], [22]. In addition, the decomposition rate of H_2O_2 to reactive

products at pH 7 was higher than that at pH 4 [23]. In this study, the oxidation and degradation rate of polyphenolic compounds at pH 7 was higher than that at pH 4. The results of Kahtan et al. (2015) [24] indicated that Rosella anthocyanin extract at higher pH values showed faster rates of degradation than those with lower pH values. Moreover, the degradation of phenolic compounds associated with the reduction of DPPH radical scavenging activity and reducing power. Thus, polyphenol compounds were responsible for the antioxidant activity of extracts.

4. CONCLUSION

The results of this study indicated that the stability of polyphenol compounds in the extract of *Terminalia nigrovenulosa* bark significantly affected by pH, temperature and hydrogen peroxide. The antioxidant capacity of the extract was associated with total polyphenol content. The antioxidant components in extract were relatively stable under heating and sterilization conditions at pH 4 but were unstable at pH 7. Moreover, the stability of the investigated extract also depended on the H_2O_2 concentration and the solution pH. It is more stable at pH 4 than pH 7 and higher effects were found in the first 4h. It need to be screening of polyphenol based on their chemical structure and apply them effectively in the food system, particularly at acidic pH values.

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