

# Enhancing Antioxidant Potential via Fermentation of Red Violet Pigments Extracted from Red Spinach (*Amaranthus tricolor*)

Putri Elia Natasha Shahrin<sup>1</sup>, Shakila Abdullah<sup>1\*</sup>

<sup>1</sup> Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia (Pagoh Campus), 84600 Pagoh, Muar, Johor, MALAYSIA

\*Corresponding Author: shakilaa@uthm.edu.my

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## Abstract

*Amaranthus tricolor* (*A. tricolor*), commonly known as red spinach, is a widely available and affordable leafy vegetable in Malaysia. It is rich in minerals, natural antioxidants, and bioactive compounds suitable for nutraceuticals which makes it an ideal source of natural food colouring where it can replace synthetic colorants which is lack of benefits such as antioxidants and also harmful to human health. This research aims to develop a natural red food colouring alternative from red spinach that offers high antioxidants in response to the growing concerns about synthetic food colourants. The research encompasses the extraction of red-violet pigments through maceration with acidified ethanol, followed by inoculating lactic acid bacteria (LAB) that were isolated from cultured milk for fermentation purposes using various parameters such as pH, temperature, carbon source concentration, and time until obtained the optimal fermentation for achieving the highest level of antioxidant activity. The optimized fermentation conditions include pH 6, 37°C, 0.5 g of glucose, and 7 days of fermentation, yielding the highest antioxidant activity. The fermented red spinach extract demonstrates superior DPPH and ABTS radical scavenging activity at 83.20% and 45.12% respectively compared to the unfermented extract which are 57.98% and 25.06% respectively. This shown an increase of antioxidant activity which are 25.22% in DPPH assay and 20.06% in ABTS assay The total phenolic content for the fermented extract is 27.56 µg GAE/mL, while the unfermented extract is 17.62 µg GAE/mL. The fermented red spinach extract reveals a darker colour compared to the unfermented red spinach extract. No significant antimicrobial activity is observed against *B. cereus* and *E. coli* in both fermented and unfermented extracts. The findings contribute to the exploration of sustainable and health-promoting alternatives to synthetic food colourants in the food industry. The objective of this study was to create natural colourants that are rich in antioxidants as an alternative to artificial colouring, which most of them have negative effects on people.

## 1. Introduction

*Amaranthus tricolor* which also known as red spinach is one of common species that can be obtained in Malaysia. Despite being accessible and affordable, *A. tricolor* is a great source of minerals, a natural antioxidant, and it also

contains other bioactive chemicals that belong in a balanced diet and are beneficial in nutraceuticals [1]. *A. tricolor* has rich in betalains, comprising two categories of pigments which are betacyanins and betaxanthins [2]. In addition, according to a previous research study by Khanam and Oba [3], *A. tricolor* leaves have an appealing colour and high levels of betacyanins and betalains. As the pigments in *A. tricolor* are abundant and have enticing shade it is perfect to be extracted as a food colourant.

Majority of synthetic colourant were banned because of their adverse effects, signals of toxicity over both the short and long term as well as potentially harming human health such as cancer [4]. Additionally, artificial food colouring does not increase the nutritional value of food [5]. In fact, they are commonly used in food items to hide low-quality ingredients. Moreover, the manufacturing practices harm aquatic life as it uses non-renewable chemical sources [6]. Some sectors continue to rely on synthetic colourants due to their affordability and durability [7]. Insufficient knowledge about the optimal conditions for fermentation might result in less efficient processes, as seen by lower yields and slower production rates. Therefore, determining the ideal fermentation parameters is essential to the production of fermented products.

Lactic acid fermentation is a reliable biotechnology method for preserving food and changing its characteristics to enhance flavour and add additional healthy elements [8]. This study was conducted to enhance antioxidants activity by phenomenon of synergetic effects that will be produced by the fermentation of the extracted of red pigments in *A. tricolor* with LAB. Therefore, LAB strain was selected for red spinach fermentation to enhance antioxidant activity of the natural pigment extract. Next, this study focused on determining the optimal fermentation parameters for achieving the highest level of antioxidant activity. Additionally, studies were conducted on the antioxidant activity, colour, and antimicrobial activity before and after fermentation. The fermented red spinach extract was expected to have high antioxidant activity, colour and antimicrobial resistance compared to the unfermented red spinach extract.

## 2. Materials and Methods

### 2.1 Materials

The red spinach which is *Amaranthus tricolor* was used. Analytical grade chemicals which are ethanol absolute (HmbG chemicals, Malaysia), acetic acid (CH<sub>3</sub>COOH) (HmbG chemicals, Malaysia), Sodium Hydroxide (NaOH) (Bendosen, Spain), acid hydrochloric (HCl) (Bendosen, Spain), glucose (HiMedia, India), DPPH powder (Emory, Spain), ascorbic acid (Emory, Spain), methanol (HmbG chemicals, Malaysia), ABTS powder (Emory, Spain), potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>) (HmbG chemicals, Malaysia), Folin-Ciocalteu reagent (HmbG chemicals, Malaysia), sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) (Bendosen, Spain) and gallic acid (Bendosen, Spain) were utilized in the study. Three media were used in this study such as De Man, Rogosa and Sharpe (MRS) Broth (Emory, Spain), The De Man, Rogosa and Sharpe (MRS) Agar (HiMedia, India) and Mueller-Hinton Agar (MHA) (HiMedia, India).

### 2.2 Methods

#### 2.2.1 Red spinach preparation

The red spinach which is *Amaranthus tricolor* was purchased from the supermarket. Then, any damaged or discoloured parts of red spinach were removed and washed thoroughly with water to remove any foreign substances [1]. The red spinach was pat dry using a towel and grinded to increase surface contact between samples and extraction solvents and to create a homogenous mixture.

#### 2.2.2 Extraction of the red spinach

1 M of acetic acid (HmbG chemicals, Malaysia) was added to ethanol absolute (HmbG chemicals, Malaysia) to create an acidified ethanol until pH 4.5 was obtained. 200 g of ground red spinach was extracted for 3 days at room temperature with the acidified ethanol at the ratio of 1:5 (w/v) and frequently stirred [9,10]. The extracted material was centrifuged at 8000 rpm at 4°C for 15 minutes and filtered via Whatman No. 1 filter paper. The extracted sample was placed in McCartney bottles and kept at 4°C in the refrigerator.

#### 2.2.3 Isolation of lactic acid bacteria

The techniques were referred from previous studies [11,12,13]. MRS agar (HiMedia, India) and MRS broth (Emory, Spain) were used as selective media for LAB for the purpose of the isolation of the bacteria from cultured milk. Cultured milk was diluted with distilled water until a dilution factor of 10<sup>5</sup> was achieved. Then, the resulting dilution factor was pipetted into MRS agar plates, incubated upside down in an anaerobic jar for 72 hours with anaerogen at 37°C for 3 days. After 3 days, one single isolated colony from the previous agar plate was picked and inoculated on another MRS agar to grow the lactic acid bacteria by using a sterilised flamed loop and incubated. After 3 days, one single isolated colony was selected and inoculated in MRS broth and incubated.

### 2.2.4 Fermentation

The extract of the red spinach was fermented through submerged fermentation. The extract of red spinach was fermented with variable parameters such as pH, temperature, carbon sources and time. The initial pH was monitored within the desired range which are 5, 6, and 7 using 0.1 mol NaOH (Bendosen, Spain) and 0.1 mol HCl (Bendosen, Spain). The incubation temperature varies between 27°C, 30°C and 37°C. Different concentrations of carbon sources were used such as 0.5 g, 1 g and 1.5 g of glucose (HiMedia, India) [14]. The extract and the conical flask were sterilised by using autoclave (Hirayama HV-85, Japan) at 121°C prior fermentation. The cultured LAB that has been isolated and growth was inoculated into the extract. The conical flask that contains the extract and the LAB was fermented for 3 days in the incubator (Mettler, Germany) with changes of each variable. Then, the highest antioxidant activity was determined for each of the variables until optimal fermentation parameters were obtained.

### 2.2.5 2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity assay

The DPPH radical scavenging activity assay was used to assess the antioxidant potential of both fermented and unfermented red spinach [15]. Fermented spinach with varying pH levels such as pH 5, pH 6 and pH 7 and unfermented spinach as a control were prepared in test tubes. The brewed sample was combined with methanol (HmbG chemicals, Malaysia) and DPPH solution and allowed to sit in the dark for half an hour. Other variables, including temperature, carbon source concentration, and duration, were added to the assay, and triplicated. The DPPH solution without any sample was used as the control assay, and methanol was utilised as a blank. An UV-Vis Spectrophotometer (PG Instrument Limited, UK) was used to measure absorbance at 517 nm, and ascorbic acid (Emory, Spain) was utilised to create a standard curve. The percentage of scavenging activity will be calculated using (1):

$$\text{Percentage of radical scavenging activity} = ((A_{\text{DPPH}} - A_s) / A_{\text{DPPH}}) \times 100 \quad (1)$$

Where  $A_{\text{DPPH}}$  is absorbance of DPPH solution and  $A_s$  is absorbance of the solution added with red spinach extract before or after fermentation.

### 2.2.6 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid Radical Scavenging Activity assay

The method involved preparing a  $7 \times 10^{-3}$  M ABTS solution by dissolving 0.9006 g of ABTS (Emory, Spain) in 250 mL distilled water. Next, 0.1656 g of potassium persulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ) (HmbG chemicals, Malaysia) with 250 mL of distilled water to create  $2.45 \times 10^{-3}$  M potassium persulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ) (HmbG chemicals, Malaysia). 2 mL of  $7 \times 10^{-3}$  M ABTS solution was then added with 2 mL of  $2.45 \times 10^{-3}$  M potassium persulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ) (HmbG chemicals, Malaysia) and stored at room temperature for 12 to 16 hours. The experiment was repeated with different parameters like temperature, carbon source concentration, and time. The absorbance was determined at the 734 nm wavelength using a T60 UV – Vis Spectrophotometer, (PG Instrument Limited, UK) with ascorbic acid (Emory, Spain) as a standard curve [16]. The ABTS scavenging activity was calculated as follows (2).

$$\text{Percentage of radical scavenging activity} = ((A_{\text{ABTS}} - A_s) / A_{\text{ABTS}}) \times 100 \quad (2)$$

where  $A_{\text{ABTS}}$  is ABTS $\cdot^+$  solution without samples meanwhile  $A_s$  is sample with ABTS $\cdot^+$  solution.

### 2.2.7 Total phenolic content

The study used Folin–Ciocalteu reagent (HmbG chemicals, Malaysia) to determine the total phenolic compounds in fermented and unfermented red spinach. In brief, 0.5 mL of each sample of fermented red spinach with different pH and unfermented red spinach were mixed with 2.5 mL of Folin–Ciocalteu reagent (HmbG chemicals, Malaysia) allow to react in room temperature for 3 to 8 minutes. Then, 2 mL of 707 mmol/L sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) (Bendosen, Spain) was added to the mixture at room temperature for 30 minutes [17]. The experiment was performed three times, and then repeated with variable time, temperature, and carbon source concentration. T60 UV – Vis Spectrophotometer (PG Instrument Limited, UK) was used to determine the absorbance at 765 nm. Gallic acid (Bendosen, Spain) was utilised as a standard and the total phenolic content was expressed as  $\mu\text{g/g}$  of gallic acid equivalent (GAE).

### 2.2.8 Measurement of colour

Using a colour spectrophotometer (MiniScan EZ Hunter Lab 4500, US),  $L^*$ ,  $a^*$ , and  $b^*$  readings were measured to determine the colour. Both unfermented and fermented red spinach were put into individual sample cups, each

painted an opaque colour. The colour was represented by the letters L\*, which stands for lightness, and the values a\*, which stands for red (+a) or green (-a), and b\*, which stands for yellow (+b) or blue (-b) [18].

### 2.2.9 Antimicrobial activity

Briefly, 20  $\mu\text{L}$  of fermented and unfermented red spinach and sterile distilled water were beaded onto sterile filter sheets with a 6 mm diameter. Sterile distilled water serves as the negative control, and penicillin discs act as the positive control. Every disc was given some time to dry at room temperature. The MHA plates were injected with *B. cereus* and *E. coli*. The surface of the inoculated plates was placed with each sample of the dried disc. After being left upside down, the plates were incubated for 24 hours at 37°C. The antibacterial activity was evaluated by measuring the inhibitory zone formation's diameter in millimetres (mm) after an overnight incubation period [11,19].

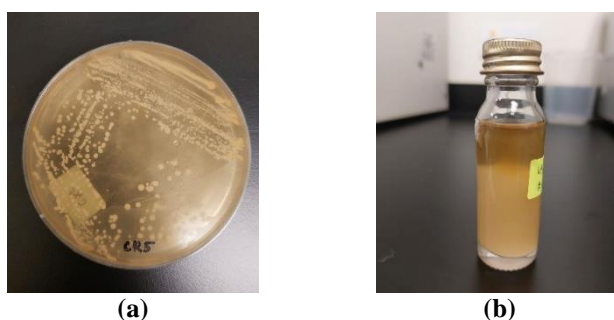
### 2.2.10 Statistical analysis

The collected data of the experiments were expressed using Mean  $\pm$  SD and applied an independent samples t-test by SPSS except for antimicrobial activity as it applied a one-way analysis of variance (ANOVA) by SPSS. Independent samples t-test was used to determine whether the means of fermented and unfermented red spinach are significantly different as it lower or higher than the significance value. Apart from that, one-way ANOVA technique involves the condition of the extract and positive and negative control that were evaluated the reaction on the inhibition zone diameter. The difference between the means of diameter inhibition zone must be considered significant at  $p < 0.05$  using one way ANOVA [20].

## 3. Results and Discussion

### 3.1 Isolation of lactic acid bacteria

The cultured milk underwent serial dilution to reduce the concentration of the bacterial sample in each plate. By using this technique, it was possible to determine that distinct colonies formed of individual bacterial cells as compared to bundles or groups of colonies. Consequently, to obtain well-isolated bacterial colonies on agar plates, serial dilution was essential [21]. The isolated single colony from the agar plate that had a dilution factor of  $10^5$  was inoculated onto another agar plate. It ensured sure the inoculation culture was pure and contained only one type of bacteria by inoculating the isolated colony. Fig. 1 showed that the isolated culture of lactic acid bacteria and stock culture were achieved. The isolated LAB was cultured in MRS broth for further application in food fermentation.



**Fig. 1** Observation of (a) Isolated lactic acid bacteria; (b) Stock culture

### 3.2 Effect of fermentation to antioxidant activity

The study optimized the fermentation conditions for the red spinach extract at pH 6, 37°C, with 0.5g of glucose, and 7 days. The fermented extract showed a higher percentage of inhibition for DPPH and ABTS radical scavenging rates  $83.2024 \pm 1.5903\%$  and  $45.1193 \pm 3.7360\%$ , compared to the unfermented extract  $57.9825 \pm 1.3036\%$  and  $25.0555 \pm 1.8200\%$  respectively as showed in Table 1. Previous study found that fermented samples had significantly higher DPPH and ABTS radical scavenging rates [22]. The total phenolic content of the fermented extract was  $27.5589 \pm 0.5593 \mu\text{g GAE/mL}$ , while the unfermented extract had  $17.6170 \pm 0.2531 \mu\text{g GAE/mL}$ . Fermentation increased the amount of phenolic compounds and flavonoids through microbial hydrolysis that improved antioxidative activity [23]. The results showed there are significant differences in the mean DPPH and ABTS percentage inhibition and the total phenolic content between the two conditions of red spinach.

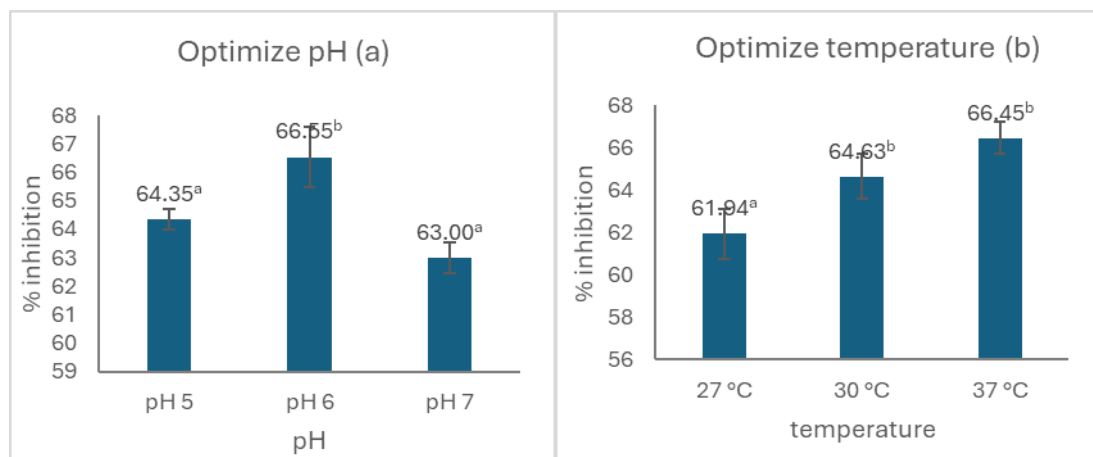
Lactic acid bacteria have an optimal pH range of 5 to 7, which is compatible with the growth and activity of LAB strains isolated from cultured milk [24]. The highest antioxidant activity of the red spinach extract was observed at pH 6, with maximum productivity. The highest yields of lactic acid bacteria were obtained when strains were cultivated at 37°C, which is in line with optimal growth conditions for many lactic acid bacteria strains [25]. Next, 0.5 g of glucose exhibited the greatest antioxidant activity. Lower antioxidant activity was seen when the red spinach extracts were consumed 1 g and 1.5 g of glucose. This finding suggests that a high glucose content may be a contributing factor to substrate inhibition in lactic acid fermentation [26]. The most optimal days for fermentation with high antioxidant activity were 7 days, as it allowed enough time for lactic acid bacteria to produce significant amounts of bioactive compounds. The fermentation environment was stabilized by optimizing pH, temperature, and glucose concentration, allowing antioxidants to reach their peak levels. However, prolonged fermentation can lead to the degradation of certain antioxidants, reducing their effectiveness [27].

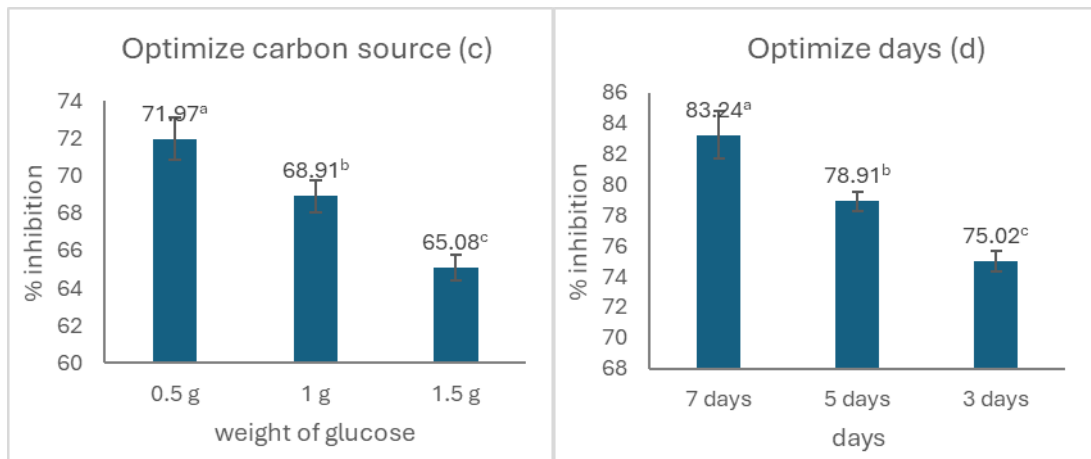
**Table 1** Summary of the result for DPPH, ABTS and TPC assay

Condition	DPPH (% inhibition)	ABTS (% inhibition)	TPC ( $\mu\text{g GAE/mL}$ )
Fermented	83.2024 $\pm$ 1.5903	45.1193 $\pm$ 3.7360	27.5589 $\pm$ 0.5593
Unfermented	57.9825 $\pm$ 1.3036	25.0555 $\pm$ 1.8200	17.6170 $\pm$ 0.2531

### 3.2.1 2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity

The presence of antioxidants in a test sample, such as red spinach extract, lowered DPPH by donating electrons or hydrogen atoms to the unpaired electron of DPPH. This donation led to the reduction of DPPH from its radical form (DPPH•) to a non-radical form (DPPH-H), corresponding to a decrease in absorbance as the reduction of the DPPH radical resulted in a colour change from deep purple to a lighter colour. A greater reduction in absorbance indicated a higher antioxidant activity [28]. Based on Fig. 2, pH 6 and 37°C had the greatest percentage inhibition compared to pH 5 and pH 6, 27°C, and 30°C, respectively. Meanwhile, for the concentration of the carbon source, 0.5 g of glucose showed a higher percentage inhibition compared to other quantities. The fermentation days with the highest percentage inhibition were 7 days. The higher the percentage inhibition, the more effective the sample was at scavenging or neutralizing DPPH free radicals, indicating a greater antioxidant activity [29].

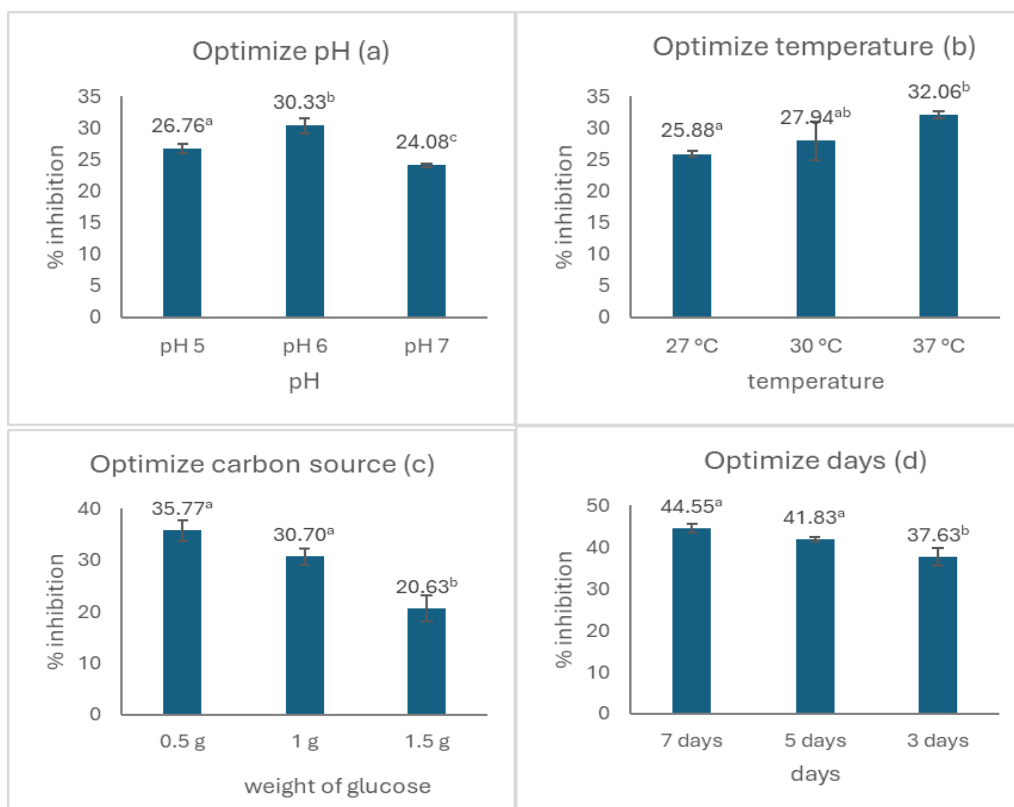




**Fig. 2** Graph bar with standard deviation of DPPH antioxidant assay for (a) pH, (b) temperature, (c) carbon source and (d) days expressed in percentage inhibition.

### 3.2.2 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid Radical Scavenging Activity

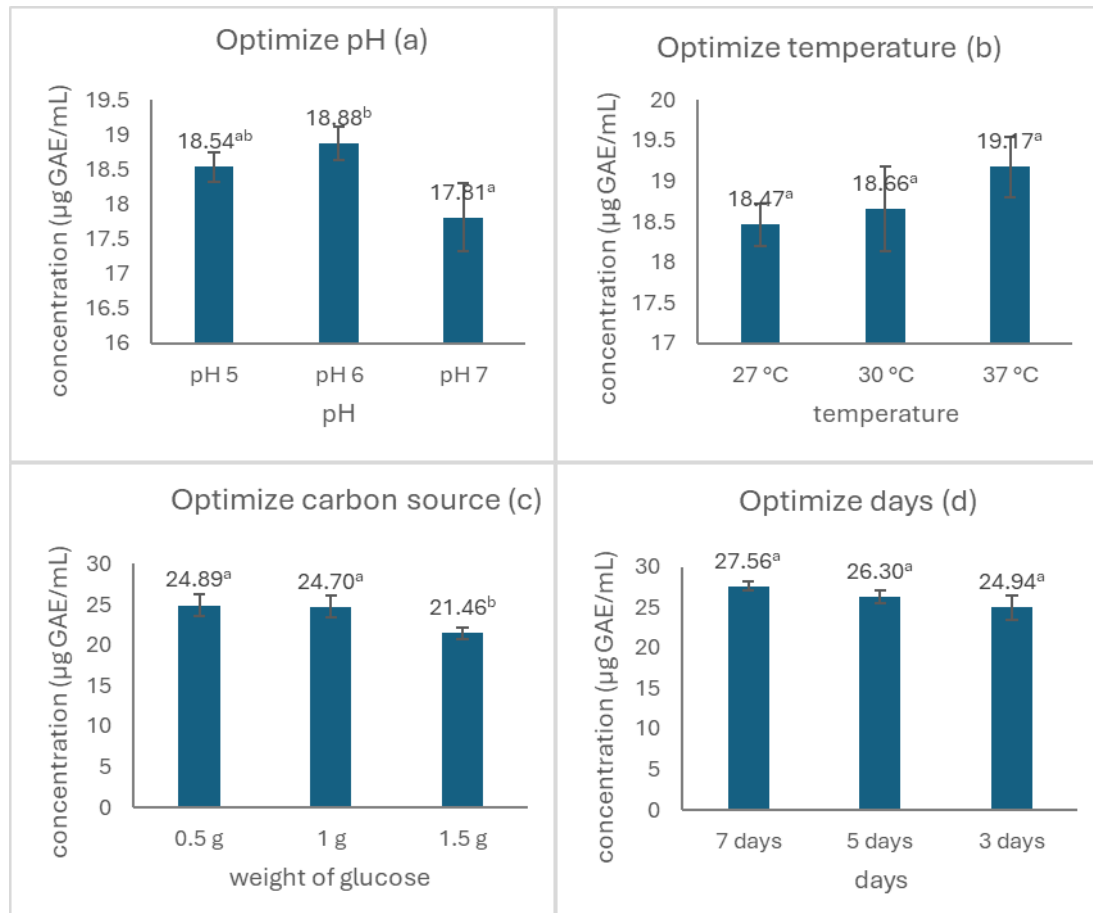
Antioxidants in the red spinach extract reacted with the ABTS radical cation and led to the reduction of the radical cation as the antioxidants donated electrons or hydrogen atoms to the ABTS radical cation. This resulted in a decrease in absorbance, causing a colour change from blue green to a lighter colour. The reduction in absorbance indicates antioxidant capacity of the substance which causing higher the percentage inhibition, indicating a greater antioxidant activity [30]. As shown in Fig 3, pH 6 and 37°C had the greatest percentage inhibition compared to pH 5 and pH 6, 27°C, and 30°C, respectively. Meanwhile, for the concentration of the carbon source and fermentation days, 0.5 g of glucose and 7 days of fermentation showed a higher percentage inhibition compared to other quantities. Therefore, the optimized fermentation for pH, temperature, quantity of carbon source and days were pH 6, 37°C, 0.5g and 7 days respectively.



**Fig. 3** Graph bar with standard deviation of ABTS antioxidant assay for (a) pH, (b) temperature, (c) carbon source and (d) days expressed in percentage inhibition

### 3.2.3 Total phenolic content

Phenolic compounds reduced the Folin-Ciocalteu reagent when mixed with sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) to form a blue-coloured complex. This complex had a peak absorbance at 765 nm, which could be measured spectrophotometrically. The intensity of the colour was directly proportional to the concentration of phenolics in the sample of the red spinach extract. Therefore, the higher the concentration of phenolic compounds in the sample, the greater the intensity of the colour produced, allowing for the quantification of the total phenolic content [31]. In comparison to all their factors, pH 6, 37°C, 0.5 g, and 7 days included the maximum quantity of total phenolic content, as indicated in Fig. 4.



**Fig. 4** Graph bar with standard deviation of TPC for (a) pH, (b) temperature, (c) carbon source and (d) days expressed in ( $\mu\text{g GAE/mL}$ )

### 3.3 Measurement of colour

Table 2 showed the following results for the fermented red spinach which are  $L^*$ ,  $a^*$ , and  $b^*$  values of  $26.8267 \pm 0.5771$ ,  $0.2233 \pm 0.1206$ , and  $-5.5500 \pm 0.2427$ , respectively. The extracts of unfermented red spinach were  $28.1000 \pm 0.8466$ ,  $0.3000 \pm 0.2234$ , and  $-4.6700 \pm 0.5311$ , respectively. The colour of the fermented red spinach extract was darker than the unfermented red spinach extract. Both fermented and unfermented red spinach exhibit a positive value for the  $a^*$  value where the unfermented red spinach appears slightly redder than the fermented red spinach. The fermented and unfermented red spinach were in range blue as the  $b^*$  value shows a negative value which fermented red spinach was bluer than the unfermented red spinach. There was no significant difference of the means of the  $L^*$ ,  $a^*$ , and  $b^*$  values between fermented and unfermented red spinach since the significance values for the  $L^*$ ,  $a^*$ , and  $b^*$  values were greater than 0.05.

**Table 2**  $L^*$ ,  $a^*$ , and  $b^*$  values of the fermented and unfermented red spinach extract

Condition	$L^*$	$a^*$	$b^*$
Fermented	$26.8267 \pm 0.5771$	$0.2233 \pm 0.1206$	$-5.5500 \pm 0.2427$
Unfermented	$28.1000 \pm 0.8466$	$0.3000 \pm 0.2234$	$-4.6700 \pm 0.5311$

### 3.4 Antimicrobial activity

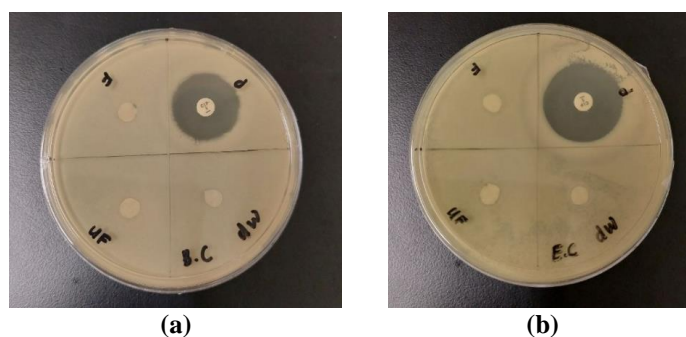
Fig. 5 shows the zone of inhibition of both fermented and unfermented red spinach against *B. cereus* and *E. coli*, measured in millimetres (mm) using the agar disc diffusion method. The results showed no significant difference between the fermented and unfermented red spinach extracts. This could be due to the complex mixture of compounds in fermented plant extracts, the production of a wide range of metabolites [31] and the chemical components affecting multiple target sites against bacterial cells [32]. Technical errors during sample preparation may also contribute to this issue. As shown in Table 3, there is no significant difference between the fermented and unfermented red spinach.

**Table 3** The result of inhibition zone for agar disk diffusion method

Samples	The diameter of inhibition range (mm)							
	<i>E. coli</i>				<i>B. cereus</i>			
	1	2	3	Mean $\pm$ SD	1	2	3	Mean $\pm$ SD
Fermented	0	0	0	0 <sup>a</sup>	0	0	0	0 <sup>a</sup>
Unfermented	0	0	0	0 <sup>a</sup>	0	0	0	0 <sup>a</sup>
Positive control	28	27	28	27.67 $\pm$ 0.58 <sup>b</sup>	24	25	24	24.33 $\pm$ 0.58 <sup>b</sup>
Negative control	0	0	0	0 <sup>a</sup>	0	0	0	0 <sup>a</sup>

Note: Data are presented as mean  $\pm$  standard deviation

Values with different superscript letters within a row are significantly different ( $p < 0.05$ )



**Fig. 5** The observation of inhibition zone by fermented red spinach extract (a) *Bacillus cereus*; (b) *Escherichia coli*

### 4. Conclusion

The study describes the extraction of red violet pigments from *A. tricolor* using maceration techniques. The red spinach extract was then inoculated with lactic acid bacteria to enhance the antioxidant potential of the fermented product. The fermentation process was optimized for pH, temperature, carbon source, and time. The fermented red spinach extract showed superior antioxidant activity and total phenolic content compared to the unfermented extract. The optimal fermentation conditions, including pH 6, 37°C, 0.5 g of glucose, and a 7-day fermentation period, yielded the highest antioxidant activity. The antimicrobial activity assessment showed no inhibition zones for both fermented and unfermented extracts. The study should consider increasing pigment concentrations and optimizing extraction methods to increase the effectiveness of red spinach extracts. Ultrasonicated extraction has been found to enhance antimicrobial activities of spinach leaf extracts, while fermenting red spinach extracts with specific strains may enhance their antimicrobial activity.

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### Conflict of Interest

Authors declare that there is no conflict of interests regarding the publication of the paper.

## Author Contribution

The *study conception and design, data collecting, results analysis* and *interpretation*, and *draft article preparation* are all acknowledged as contributions and responsibilities of the authors to the paper. The final draft of the manuscript was approved by the authors, Putri Elia Natasha Shahrin and Shakila Abdullah after they had evaluated the findings.

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