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Research Article

Physical and chemical properties and antioxidant characteristics of gac aril juice fermented with *Aspergillus niger*

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Abstract - Gac (*Momordica cochinchinensis* (Lour.) Spreng) is a medicinal herb that contains carotenoid substances, phenolic compounds, and flavonoids, and is well-known for reducing the risks of coronary heart disease and prostate cancer. This study investigated the carotenoid and antioxidant properties of Gac aril juice fermented for a 48-hours fermentation process with 2% (v/v) *Aspergillus niger.* The fermented juice exhibited an orange hue with L*, a*, and b* values of 51.47±4.50, 28.37±1.16, and 43.19±0.97, respectively. Total dissolved solids (TDS) decreased to 8.20±0.69 °Brix, and pH also decreased during fermentation. High-performance liquid chromatography (HPLC) revealed higher lycopene (8349.48 μg/100 mL) and β-carotene (57.09±0.64 μg/100 mL) contents than the control. Antioxidant activity, assessed using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and ferric ion-reducing antioxidant power (FRAP) assays, increased with fermentation time. At 48 hours, DPPH activity reached 1.76±0.01 μg of TE/mL, and FRAP activity reached 108.92±0.78 μg Fe (II)/mL. Total phenolic content (TPC) and total flavonoid content (TFC) were 267.30±1.53 μg GAE/mL and 742.10±2.65 μg RE/mL, respectively. Fermented Gac aril juice with *A. niger* contained CO_2 and carbamic acid, which were not found in the non-fermented juice. Our findings created value-added products, such as health-promoting beverages and cosmetic ingredients.

Keywords: *Aspergillus niger*, β-carotene, carotenoids, gac aril juice, lycopene

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1. Introduction

Modern consumers are increasingly prioritizing health by incorporating fruits, vegetables, and herbs into their diets. Nutrient-rich foods such as carrots, pumpkins, and tomatoes play a vital role in promoting overall well-being. Carrots and pumpkins are excellent sources of vitamin A, which supports eye health, while tomatoes provide vitamins C, E and lycopene, which are beneficial compounds for skin nourishment found in many fruits and vegetables. Lycopene has been associated with reduced risks of various cancers, improved skin health, and enhanced immunity (Maoka, 2020). Gac (*Momordica cochinchinensis*), a medicinal herb native to Southeast Asia, is particularly noteworthy for its high carotenoid content and associated health benefits. Epidemiological studies indicate that lycopene, abundant in Gac, may help prevent coronary heart disease and cancers, particularly prostate cancer (Suwanlert et al., 2005). Additionally, Gac's antioxidant properties support skin health and may delay aging (Vuong et al., 2006; Ishida & Chapman, 2009).

Fermentation has emerged as a powerful tool to enhance the bioavailability of phytochemicals and antioxidants in plants. Certain molds and bacteria produce enzymes during fermentation that release beneficial compounds. For instance, apple peel fermented with *Aspergillus oryzae* demonstrated increased levels of free phenolics and antioxidant activity (Li et al., 2024). Similarly, Jambolan fruit pulp has been shown to serve as an effective substrate for antioxidant production under solid-state fermentation with *A. flavus* (Rajan et al., 2023). Enzymes such as cellulase and pectinase, produced by microorganisms like *Aspergillus niger*, can further enhance the release of plant bioactives. For example, fermented soymilk treated with *A. niger* exhibited significantly higher antioxidant activity compared to untreated samples (Elshafei et al., 2022). Building on these findings, this study investigates the phytochemical composition and antioxidant activity of fermented Gac aril juice using *A. niger.* The fermentation process promotes the production of cellulase and pectinase enzymes, which facilitate the release of bioactive compounds. The results highlight the potential applications of fermented Gac aril juice is used in various industries, including food, health, and cosmetics, paving the way for innovative and sustainable solutions.

2. Materials and methods

2.1 *A. niger* preparation

A. niger was purchased from the National Science and Technology Development Agency cultured on potato dextrose agar plate and incubated at 30 °C. After 7 days, a 5-mm diameter mycelial plug of the fungus was transferred to potato dextrose broth (PDB) at pH 5.5 in a 100 mL Erlenmeyer flask and incubated at 30 °C for 3 days in a shaking incubator (LSI-1005R, Lab Tech, Korea) at 150 rpm. A spore suspension of *A. niger* with a concentration of 10^9 conidia/L was then prepared and used at 2% (v/v) for the fermentation Gac aril juice at 30 °C for 48 hours.

2.2 Preparation of Gac aril juice

One kilogram of frozen Gac aril from the region of Nakhon Ratchasima was blended well with 1000 mL of drinking water. After 30 min of pasteurization at 80 °C, the liquid was cooled to room temperature.

2.3 Gac aril juice fermentation conditions

Gac aril juice was mixed with 2% (v/v) *A. niger* in a total volume of 100 mL and incubated in a shaker at 150 rpm and 30 °C for 24 and 48 hours. The samples were collected at 0, 24, and 48 hours. Then, the sample was centrifuged at 10,000 rpm for 10 min before further analysis. Non-

fermented Gac aril juice was used as the control.

2.4 Physical and chemical characteristics of Gac aril juice

For color analysis, the L*, a*, and b* values were measured using a ColorFlex spectrophotometer (model CM-600d, Konica Minolta, Japan). The brightness levels ranged from 0 to 100. Red values were denoted by (+a*) values, green values by (-a*) values, and blue values by (+b*) values. Yellow values corresponded to (-b*) values. A cuvette was filled with a sample of Gac aril juice solution and the color properties of all treatments were observed as bright orange tones. The pH analysis was conducted using a pH meter (Mettler Toledo FiveEasy™ Plus, FEP20 model) to determine the acidity-alkalinity values of the samples. Total dissolved solids (TDS) were analyzed using a hand refractometer (Master-93H, Atago, Japan) with all experiments were conducted in triplicate.

2.5 Carotenoid content analysis

2.5.1 Lycopene content analysis

The procedure was followed by Cucu et al. (2012). Briefly, the sample was homogenized and lycopene was extracted using a solvent mixture of n-hexane 95%, Ethanol, and Acetone (2:1:1), and centrifuged (Universal 320R, Germany) at 3000 rpm at 25 °C for 10 min. The supernatant was injected into a Reverse-phase HPLC using isocratic elution and UV detection at 472 nm (Waters, Zellik, Belgium). The stationary phase was a carotenoid C30 reverse-phase column (250x4.6 mm id, 3 μm, Waters, Zellik, Belgium). The mobile phase consisted of MeOH/isopropyl alcohol/ THF (30:30:35) containing 250 ppm BHT and 0.05% TEA, with a flow rate of 1 mL/ min, column temperature at 35 °C, and injection volume of 20 μL (mg/100 mL of lycopene). Then, 0.1 g of Gac aril sample was ground in a mortar, and incorporated with solvent mixture (1.5 mL, 0.75 mL, 0.75 mL) was added. Furthermore, 5 mL of deionized water was added and the mixture was centrifuged for 10 min at 3000 rpm, 25 °C. Then, 5 mL of the supernatant was diluted with 5 mL of n-hexane 95% and an HPLC analysis was conducted.

2.5.2 β-carotene content analysis

The analysis of β-carotene was performed using an LC-20AD Shimadzu pump connected with a Jasco UV-975 detector. Separation of β-carotene in each sample was performed using a C18 column (Vydac 201TP, C18 4.6 x 250 mm, 5 μm column, Grace Division, USA) with a guard column (Vydac 201TP, cartridge C18 4.6 x 12.5 mm, 5μm, Grace Division, USA) at a flow rate of 1.0 mL/min and monitored at 450 nm. The mobile phase consisted of methanol: tetrahydrofuran: acetonitrile at a ratio of 6:14:80 (Speek et al., 1986). The β-carotene contents in all samples were analyzed in duplicate and expressed as micrograms per 100 g of fresh weight (μg/100g). The calibration graph was created using the standard β-carotene (C9750, Sigma, USA). Then, 3.0-5.0 g of homogenous β-carotene, 10 mL of 10% ascorbic acid solution and 50 mL of 2 M ethanol potassium hydroxide (KOH) solution were mixed in a brown round-bottom flask and boiled in a water bath for 30 min. After allowing the sample to reach room temperature, 70 mL of hexane was added, and the mixture was stirred for 2 min. Two layers were formed. The top layer was transferred to a brown glass separating funnel containing 50 mL of 5% (w/v) KOH solution. The samples were extracted twice with 35 mL of hexane. The combined hexane extract was washed with 100 mL of 10% (w/v) sodium chloride and 100 mL of water until alkali-free. An aliquot was collected and evaporated in a rotary evaporator under vacuum in a 37 °C water bath. The residues were dissolved in 1 mL of chloroform and 1 mL of methanol.

2.6 Antioxidant activity.

2.6.1 Antioxidant activity was analyzed using the DPPH method adapted from Zhang et al. (2016).

The antioxidant capacities were analyzed according to Zhang et al. (2016), with modifications. Each sample of 20 μL was added with 180 μL of 0.2 mM DPPH solution. The mixture was incubated for 30 min at room temperature in the dark. The absorbance was determined using a microplate reader spectrophotometer (M965, master tech, Taiwan) at 520 nm. All experiments were conducted in triplicate. Trolox at a concentration of 0.83-84.58 μg/mL used as standard. The antioxidant capacities were given in μg TE/mL.

2.6.2 Antioxidant activity analyzed using the FRAP method.

The FRAP reagent solution was created by combining 300 mmol of acetate buffer (pH 3.6) with 2,4,6-Tris (2-pyridyl) solution, 20 mmol/L of ferric chloride solution, and Perhydro-1,3,5-Trinitro-1,3,5-Triazine (TPTZ) concentration, following the procedure described by Xu et al. (2009). The mixture was shielded from light during the preparation. Then, 20 μL of the clear top part of Gac aril solution obtained after centrifugation was thoroughly mixed with 180 μL of FRAP reagent. The absorbance was measured at a wavelength of 593 nm. The experiments were conducted in triplicate with a standard solution of ferrous sulfate (12.5-400 μg/mL) used as standard. The antioxidant capacities were given in μg Fe(II)/mL.

2.7 Analysis of total flavonoid content

The procedure was carried out following the method of Tian et al. (2016) with minor adjustments. Specifically, 20 μL of the clear top part of Gac aril solution obtained after centrifugation of the sample and 60 μL of distilled water were pipetted into a 96-well plate (Cole-Parmer, USA), followed by adding 10 μL of 5% sodium nitrate ($NaNO_3$) solution, mixing thoroughly, and incubating for 6 min at room temperature. The solution was then combined with 10 mL of 10% aluminum chloride ($AlCl_3 \bullet 6H_2O$), mixed thoroughly, and allowed to stand for 5 min. Then, 100 μL of 1 M sodium hydroxide (NaOH) was added, thoroughly mixed, and the reaction was allowed to proceed at room temperature for 12 min. The absorbance was determined by a microplate reader at a wavelength of 510 nm. All experiments were conducted in triplicate with rutin (25-400 μg/mL) used as standard. Results were expressed in μg RE/mL.

2.8 Analysis of total phenolic content

The experiment was carried out following the method of Radošević et al. (2017) with minor adjustments. Then, 20 μL of the clear top layer of the Gac aril solution, obtained after centrifugation of the sample, was pipetted into a 96-well plate, mixed thoroughly with 100 μL of 10% Folin Ciocalteu solution, and allowed to sit for 1 min. Next, a volume of 80 μL of 7.5% sodium bicarbonate ($NaHCO_3$) was added, thoroughly mixed, and allowed to react for 90 min in the dark at room temperature. A microplate reader (M965, mastertech, Taiwan) was used to measure the absorbance at a wavelength of 750 nm. The total phenolic content was determined using a gallic acid standard curve. All experiments were conducted in triplicate, with gallic acid (12.5-400 μg/mL) used as standard. The results were expressed in μg GAE/mL.

2.9 Analysis of volatile organic compounds of fermented Gac aril juice using a Gas Chromatograph Mass Spectrometer

The chemical compositions of fermented Gac aril juice were determined using a Gas Chromatograph Mass Spectrometer (GCMS, QP2010, Shimadzu, Japan) following Monajemi et al. (2005) using an Agilent HP-5MS column (5% phenylmethylsiloxane, 30 m × 0.25 mm,

film thickness 0.25 μm) with helium as the carrier gas. The oven temperature was raised from 50 °C for 5 min to 150 °C and 250 °C at a rate of 4 °C per min. Each sample underwent a total practice time of 60 min. The flow rate was maintained at 2 mL/min, with an injection volume of 1 μL per sample. The average speed was recorded at 36 cm/s, and the pressure was measured at 7.56 kilopascals (kPa). The analysis was conducted using a gas chromatography-mass spectrometer, utilizing a Fragment Ion Fingerprint machine's reference database to determine the compound types. Each sample underwent a total practice time of 60 min. The flow rate was maintained at 2 mL/min, with an injection volume of 1 μL per sample. The average speed was recorded at 36 cm/s, and the pressure was measured at 7.56 kilopascals (kPa).

2.10 Statistical analysis

A completely randomized design (CRD) was used to plan the experiment, repeating each step three times. Statistical analysis was performed using SPSS version 15.0 for Windows, with analysis of variance and comparison of mean differences at the 95% confidence level ($P < 0.05$).

3. Results and discussion

All treatments displayed bright orange tones, Gac aril juice with 2% (v/v) *A. niger* provided the filaments (Figure 1).

A: Gac aril juice (Control)

B: Gac aril juice + 2% (v/v) ***A. niger***

Figure 1. Color with no added *A. niger* to Gac aril juice (A) and fermented Gac aril juice with 2% (v/v) *A. niger* (B) at 48 hours of fermentation.

Physical properties of fermented Gac aril juice. During the fermentation process, vivid orange hues were observed in each sample. The L*, a*, and b* values ranged between 44.32 and 51.47, 28.37 and 33.08, and 43.19 and 51.83, respectively. The color difference values (ΔE) of L*, a*, and b* for Gac aril juice with 2% (v/v) *A. niger* and non-fermented Gac aril juice are shown in Figure 2A. pH and total dissolved solids decreased with longer fermentation times (Figure 2B and Figure 2C).

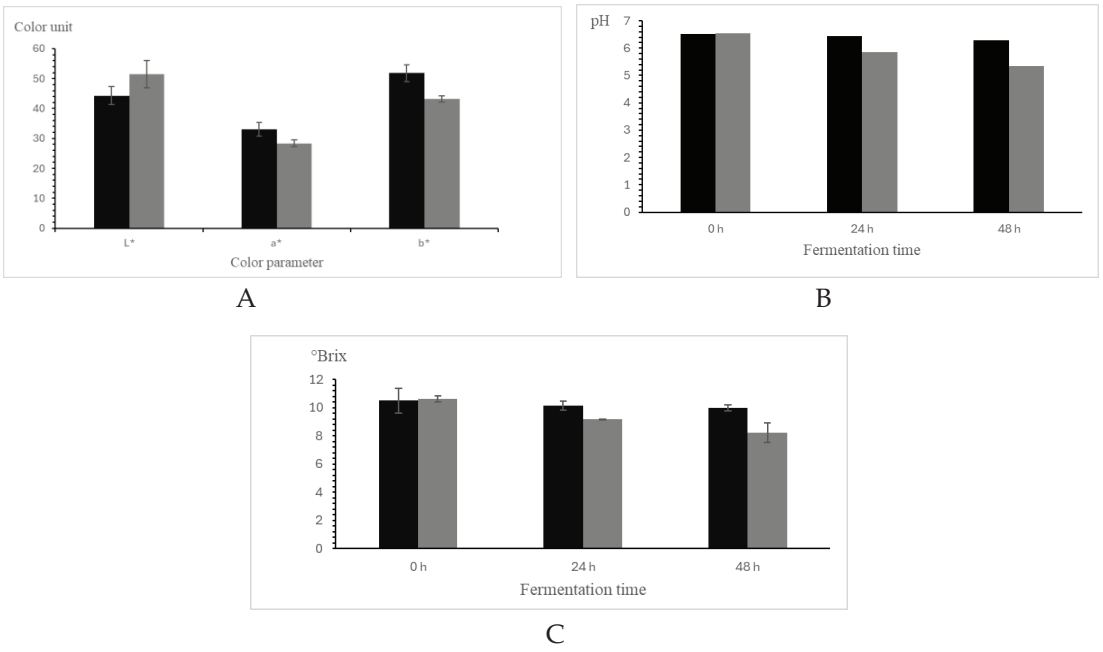


Figure 2. Physical properties of Gac aril juice. A: L*, a*, and b* value, B: pH and C: Total dissolved solids ■ T1 Non-fermented Gac aril juice and ■ T2 Fermented Gac aril juice with 2% (v/v) *A. niger*.

For chemical composition of Gac aril juice after 48 hours, the results showed higher levels of lycopene, β-carotene content (Figure 3A and Figure 3B), total flavonoid content, and total phenolic content in fermented Gac aril juice with *A. niger* than in the control (Figure 4A and Figure 4B). *A. niger* produces a balanced profile of enzymes, which refers to enzymes with optimized activity across a range of conditions or substrates, ensuring well-rounded performance in specific applications such as cellulase, tannase, and pectinase (Meini et al., 2021). These enzymes facilitate the release of phytochemicals during fermentation.

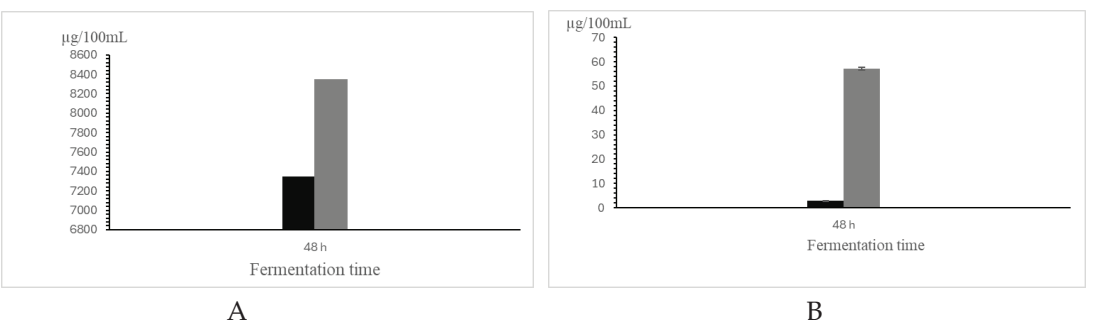
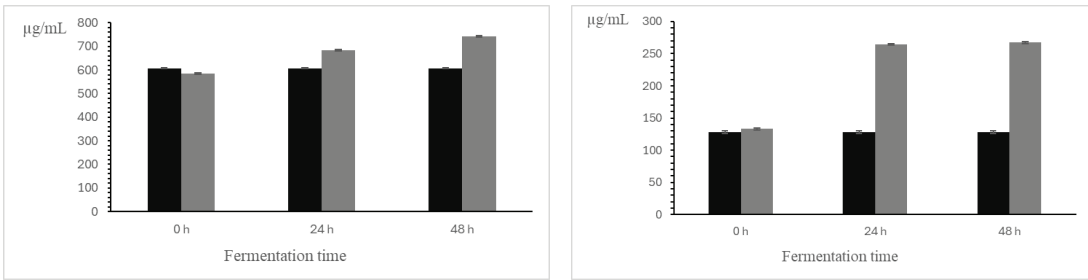


Figure 3. (A) Lycopene content and (B) β-carotene content of non-fermented and fermented Gac aril juice during 48 hours of fermentation. ■ T1 Non-fermented Gac aril juice and ■ T2 Fermented Gac aril juice with 2% (v/v) *A. niger*.

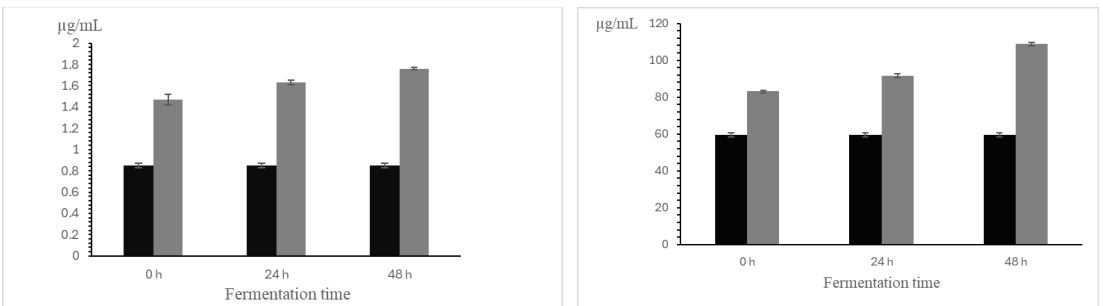


A B

Figure 4. (A) Total Flavonoid Content (TFC) and (B) Total Phenolic Content (TPC) of non-fermented and fermented Gac aril juice mixed with 2% (v/v) *A. niger* during 48 hours of fermentation. ■ T1 Non-fermented Gac aril juice and ■ T2 Fermented Gac aril juice with 2% (v/v) *A. niger.*

Antioxidant activity of Gac aril juice after 48 hours of fermentation, the Gac aril juice supplemented with 2% (v/v) *A. niger* exhibited significant antioxidant activity. A positive correlation was observed between enzyme production and antioxidant activity (Meini et al., 2021), as illustrated in Figure 4.

The analysis of antioxidant activity revealed that the combination of Gac aril juice with 2% (v/v) *A. niger* at 0, 24, and 48 hours, using DPPH and FRAP assays, had significant effects on antioxidant activity. The activity increased over time, reaching its highest level after 48 hours of fermentation (Figure 5A and Figure 5B).

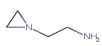
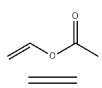
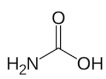


A B

Figure 5. Antioxidant activity (A) DPPH assay and (B) FRAP assay of non-fermented and fermented Gac aril juice for 48 hours. ■ T1 Non-fermented Gac aril juice and ■ T2 Fermented Gac aril juice with 2% (v/v) *A. niger.*

Volatile organic compounds (VOCs) in treated Gac aril juice. Fermented Gac aril juice showed an altered profile of VOCs compared to non-fermented juice (Table 1). Gac aril juice contained 2-aziridinylethyl amine and acetic acid as major compounds while fermented Gac aril juice with 2% (v/v) *A. niger* consisted of CO_2 and carbamic acid. The amounts of volatile chemicals are reduced following the fermentation process.

Table 1. Volatile organic compounds in fermented Gac aril juice using 2% (v/v) *A. niger* and non-fermented Gac aril juice.

Compound name	Boiling point (°C)	Molecular weight	Molecular formula	Structure	Retention time/min	Area %	Benefit	Reference
Gac aril juice								
2-Aziridinylethyl amine	146	86	$C_4H_{10}N_2$		1.476	73.70	synthesis of many pharmaceutical compounds	PubChem Ikpa et. al, 2021
Acetic acid ethenyl ester	72.2	86.09	$C_4H_6O_2$		1.924	0.99	Precursor to synthesis industrial polymers	NIST Chemistry WebBook SRD 69 Bienewald et.al, 2000
Acetic acid	118	60.05	$C_2H_4O_2$		2.117	25.31	Food additive in food and drink, solvent	NIST Chemistry WebBook SRD 69 Lynch et al. (2019) Santos-Sánchez et al. (2019)
Fermented Gac aril juice with 2% (v/v) *A. niger*								
Carbon dioxide	-78.46	44.01	CO_2		1.458	11.17	Food additive in food and drink, solvent	PubChem NIST Chemistry WebBook SRD 69
Carbamic acid	Decomposes below	61.040	NH_2COOH		1.329	7.37	Get rid of pests	NIST Chemistry WebBook SRD 69

Our study utilized 2% (v/v) *A. niger* to ferment Gac aril juice. During the fermentation process, each sample exhibited vibrant color tones. Similarly, Dufossé et al. (2014) observed that filamentous fungi can produce pigments and colorants. Over time, the fermentation darkened the color and reduced the pH as fermentation time increased. The acid production by *A. niger* in our study aligns with findings of Li et al. (2024).

Marnpae et al. (2022) reported a decrease in pH and dissolved solids, accompanied by an increase in organic acids, including lactic and acetic acid. Their study investigated the modification of antioxidant activity and prebiotic potential of apple peel through *A. oryzae* fermentation. After 48 hours of fermentation, a consistent reduction in dissolved solids was observed. Antioxidant activity, as determined by the FRAP and DPPH assay, indicated that fermented Gac aril juice exhibited higher antioxidant activity than the control. Similarly, Rajan et al. (2023) reported increased antioxidant activity in jambolan fruit pulp, as measured by the DPPH method, following solid-state fermentation with *A. niger* at different time interval. The fermented juice exhibited higher total flavonoid contents than the production of α-galactosidase from isolated *A. niger* NRC114 α-galactosidase. Soy milk also had higher total flavonoid content (Elshafei et al., 2022), total phenolic content, lycopene, and β-carotene levels when fermenting Gac aril juice with *A. niger*. Fungal metabolites,

represented by carotenoids such as lycopene (red) and β-carotene (yellow-orange), were produced by *Penicillium oxalicum* (Takahashi et al., 2020). Rajan et al. (2023) also found higher total phenolic contents and total flavonoid contents in jambolan fruit pulp after solid-state fermentation treatment at different times using *A. niger* and *A. flavus*, and the quantity increased compared to unfermented samples. Carotenoids extracted from tomato waste increased from 0.062 mg/L at zero hours of fermentation to 0.164 mg/L of total carotenoids after 36 hours of solid-state fermentation using tannase production *A. niger* GI, a wide-type isolation of tannin-degrading fungal strains from the Mexican desert (Mendez-Carmona et al., 2022). The fermented juice exhibited volatile organic compounds, such as CO_2. The dissolution of CO_2 in water leads to the formation of carbonic acid and it is beneficial to health when present in appropriate amounts. Volatile organic compounds were found in Shanxi-aged vinegar fermented by *A. awamori* (Zhang et al., 2024).

4. Conclusion

Fermented Gac aril juice using 2% (v/v) *A. niger* provided higher antioxidant activity than non-fermented juice after 48 hours with higher total phenolic contents, total flavonoid contents, lycopene, and β-carotene levels. It also exhibited an altered profile of volatile organic compounds compared to non-fermented juice, with 2-aziridinylethyl amine and acetic acid being identified as major compounds. Additionally, fermented Gac aril juice with 2% (v/v) *A. niger* contained CO_2 and carbamic acid. Future research should focus on strategies to preserve these beneficial phytochemicals in fermented Gac aril juice. Fermentation of Gac aril juice with 2% (v/v) *A. niger* after 48 hours significantly increased the contained phytochemicals and antioxidant activity. This study can serve as a foundation for future research, exploring the use of other microbes to monitor trends in phytochemicals or other compounds that could be applied in various contexts as needed.

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