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Original Article

Enhancing carotenoid contents, antioxidant properties and cytotoxicity against human colon adenocarcinoma (HT-29) of gac aril juice (*Momordica cochinchinensis* Spreng) through kefir grain fermentation and hydrolytic enzyme treatment

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Abstract

A tropical fruit, Gac (*Momordica cochinchinensis* Spreng), is valued for its high carotenoid content and antioxidant potential. This study investigated the physicochemical and biofunctional changes in Gac aril juice (GAJ) subjected to fermentation with 2% (w/v) kefir grain and enzymatic treatment using (2% v/v) food-grade pectinase or cellulase for 48 h under control conditions. Parameters assessed included pH, color, total dissolved solid, lycopene, β-carotene content (via HPLC), total flavonoid content, phenolics, antioxidant activity (DPPH, FRAP), volatile organic compounds (GC-MS), and cytotoxicity against HT-29 human colon cancer cells (MTT assay). Results revealed that both treatments significantly improved carotenoid content and antioxidant activity. Pectinase-treated juice showed the highest β-carotene and antioxidant levels, while kefir-fermented juice notably increased phenolic content and exhibited cytotoxic effects with an IC50 of 401.00 ± 1.76 µg/mL. Additionally, the volatile compound profile exhibited treatment-dependent changes in aroma. Morphological changes in HT-29 cells confirmed the cytotoxic effect of the fermented GAJ. This is the first report to demonstrate the cytotoxic potential of kefir-fermented GAJ against HT-29 cells, indicating its promise as a functional ingredient for value-added product development in the food and beverage sector.

Keywords: Cellulose, Pectinase, Lycopene, Flavonoids, β-carotene, Probiotic

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Introduction

Gac *(Momordica cochinchinensis Spreng)* is a tropical fruit known for its rich carotenoid content, including lycopene, β-carotene, fatty acids, phenolic compounds, flavonoids, and Vitamin C (Chuyen et al., 2015). Especially in the aril part, β-carotene and lycopene were significantly higher than in the other fruits (Müller-Maatsch et al., 2017). These compounds have been associated with antioxidant, anticancer, also provitamin A activity (Chuyen et al., 2015; Huynh and Nguyen, 2020). Gac fruit aril was found to have anticancer potential, with extracts from different samples inducing significant apoptosis and necrosis in breast cancer and melanoma cells (Wimalasiri et al., 2020). Gac is typically ovoid or round, and cultivar is present in Thailand, Vietnam, China, India, Indonesia and Malaysia. The fruit consists of two main parts: 1. The mesocarp, which includes orange/yellow spines and a thick, spongy orange layer called the pulp. 2. The endocarp contains red, soft, and sticky arils that cover the blank seeds. The weight distribution of the fruit is as follows: yellow pulp (50%), aril (10-25%), skin (17%), and seeds (16%). The size and weight of the fruit are correlated with aril production (Thavamany et al., 2020). Gac fruit has traditionally been used in Asia for its red color and vision-enhancing properties (Yu et al., 2017). In addition to its traditional use, gac fruit has been processed into products like gac powder and gac oil, which are used as natural colorants and medicinal supplements (Le et al., 2018). In Thailand, traditional consumers like consuming young gac fruit with dipping chilli paste (Chuyen et al., 2015; Kubola and Siriamornpun, 2011*)* and gac fruit aril juice.

Over the last decade, non-dairy fermented juices made from fruits and vegetables have become more attractive for several reasons, such as the health benefits of fermented foods, the rising number of people who cannot digest lactose the growing popularity of vegan diets. As a result, researchers have been more interested in developing and studying lactic acid fermented juices and their functional properties (Szutowska, 2020)**.** Fermentation with lactic acid bacteria (LAB) or LAB in kefir grains known as probiotics, can enhance the nutritional and functional properties of vegetable and fruit products (Crespo et al., 2021; Iruene et al., 2021; Chen, 2021). Several studies have demonstrated that *Lactobacillus plantarum, L. acidophilus, L. casei,* and *L. halveticus* can increase the phenolic compound contents including caffeic acid and rutin contents, and antioxidant capacities of jujube during fermentation (Li et al., 2021)**.** LAB fermentation could change the carotenoid content of vegetables and fruits (Morifuji et al., 2020; Marnpae et al., 2022). Some LAB strains, such as *L. fermentum* and *L. plantarum,* could also produce carotenoid-like compounds during fermentation (Turpin et al., 2016). Similarly, kefir fermentation could increase carotenoid content in vegetable and fruit products (Paredes et al., 2022). Moreover, Marnpae et al. (2022) reported that fermenting fruit and vegetables with composite microbial solutions could increase antioxidant compounds, potentially benefiting health. This implies that consuming fermented fruit and vegetables with LAB or kefir grains may lead to higher carotenoid levels, increased antioxidant activity and potential anticancer effects. However, no studies on the impact of fermenting gac fruit aril juice with kefir grains on carotenoid content and cytotoxicity against the human colon adenocarcinoma (HT-29) were found in the previous study. This research aims to compare kefir grain with the commercial pure food-grade hydrolysis enzyme treated in GAJ with respect to physicochemical properties (colour, pH, total dissolved solids, carotenoid content, and volatile compound profile) and bioactive properties (antioxidant activity and cytotoxic activity against HT-29).

Material and Methods

Kefir grain and commercial enzyme concentration preparation

Kefir grain from the Natural Product Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University was activated by using 0.5 g dry weight with 200 mL of pasteurized fresh milk covering the jar with four layers of cheesecloth at room temperature (25-28 °C) for the fermentation period of 24 h before use. After filtration, the kefir grain was rinsed through the sterilised cheesecloth, washed with sterilised distilled water and inoculated with 2% (w/v) for fermentation. The commercial, pure, food-grade hydrolytic enzymes, such as pectinase and cellulase, were purchased from Reach Biotechnology Co., Ltd. (Thailand), and 2% (v/v) of each enzyme was used in the treatment of gac aril juice.

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Gac aril juice preparation

Fully ripened Gac fruits were procured from local farms in Nakhon Ratchasima Province. The arils were carefully separated from the fruit's rind and seeds. The aril was thoroughly washed to remove impurities. After washing, dry out the moisture. Extracted juice (Gac aril 1 kg extracted with drinking water 1 L) was blended and filtered through a fine mesh to remove residual solids and particles. The juice was pasteurized at 80 ˚C for 30 s. The pasteurized juice was then rapidly cooled and stored in sterilized glass bottles for further fermentation.

Fermentation conditions

The fermentation conditions are as follows: the first treatment (T1), the control sample involved GAJ 100 mL was kept at room temperature (25-28 ˚C) for 48 h without agitation. Similarly, the second treatment (T2) combined 98 mL of GAJ with 2 mL of kefir grain (2% v/v), following the same conditions as the control. The third (T3) and fourth (T4) treatments, involving pectinase and cellulase enzymes (2% v/v), respectively, in 98 mL of GAJ, were subject to a temperature at 30 ˚C with rotation at 150 rpm for 48 h. The experiment was conducted in triplicate. Samples were collected at 0, 24, and 48 h, then centrifuged at 10,000 rpm for 10 min before evaluation of physicochemical properties, carotenoid components, volatile organic compounds, and antioxidant capacity.

Physical and chemical property evaluation

pH, color and total dissolved solids

pH determination was conducted using pH meter (Metter Total FiveEASYTMPlus/FEP20), color analysis using a colorimeter (HUNTER Lab Model Ultra Scan PRO/USA) and total dissolved solids (TDS) using a hand refractometer (Master-93H ATAGO/Japan).

Lycopene determination

The samples were sent to the Central Laboratory (Thailand Co. Ltd.), which is accredited to ISO 17025. The procedure was followed by Cucuet et al. (2012). Briefly, homogenize the sample and extract the lycopene using a mixture solvent; n-Hexane 95%: ethanol: Acetone (2:1:1) and centrifuge at 5,000 g at 25 ˚C for 10 min. The supernatant (20 µL) was injected into reversed-phase high-performance liquid chromatography, with isocratic elution and UV detection at 472 nm (Waters, Zellik, Belgium). The stationary phase consisted of a carotenoid C30 reversed-phase column (250×4.6 id, 3 µm, Waters, Zellik, Belgium). The mobile phase consisted of MeOH/isopropyl alcohol/THF (30:30:35) with a flow rate of 1 mL/min and the column temperature was 35 °C. Analytical-grade Lycopene (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard. The experiment was done in duplicate, and the lycopene concentration was expressed as µg/100 mL.

β-carotene determination

The samples were determined using a modified procedure followed by Speek et al. (1986). Briefly, the saponification step used 5.0 g sample adding 10% ascorbic acid (10 mL) and 2M KOH (50 mL). The solution was refluxed for 30 min, and then mixed with 70 mL of hexane. Following separation, the upper fraction was tranfered to a separating funnel containing 50 mL of 5% (w/v) KOH and subjected to two extractions with hexane (35 mL). The mixed hexane extract was washed with 10% (w/v) NaCl (100 mL) and water (100 mL) until alkali-free. A portion of the sample was dried by evaporation at 37 ˚C and then redissolved in chloroform (1 mL) and methanol (1 mL). Analysis of β-carotene was carried out with an LC-201TP, C18 4.6 × 250 mm, 5 µm column, Grace division, USA) and a guard column (Vydac 201TP, C18 4.6 × 12.5 mm, 5 µm, Grace division, USA) at a flow rate of 1.0 mL/min, monitored at 450 nm, with a mobile phase consisting of methanol: tetrahydrofuran: acetonitrile at a ratio of 6:14:80 using standard β-carotene (9750, Sigma, USA). β-carotene content in each sample was determined in duplicate and reported in µg/100 mL All agents used were of analytical grade.

Total phenolic content (TPC) and total flavonoid content (TFC)

The analysed methods were followed in the previous work (Karirat et al., 2023) with a slight modification. For TPC analysis, each sample (20 µL) was added with 10% (v/v) Folin-Ciocalteu solution (100 µL) and then mixed well for 5 min, filled up with 7.5% (w/v) sodium carbonate (80 µL). After 30 minutes in the dark at room temperature (25-28 °C), a measurement at 750 nm was recorded using a microplate reader. Gallic acid (Sigma-Aldrich, St. Louis, MO, USA) at 0, 12.5, 25, 50, 100, 200, and 400 µg/mL was used as the standard. TPC was reported as µg GAE/mL. For TFC analysis, each sample (20 µL) was added with deionized water (60 µL) and 5% $NaNO_3$ (10 µL) in a

96-well microplate. After mixing and incubating at room temperature for 6 min, the mixture was filled with 10% (w/v) $AICl_3 \cdot 6H_2O$ (10 µL) and 1 M NaOH (100 µL) and reacted for 12 min. A510 nm was recorded under a microplate reader (M965+Metertech, Taipei, Taiwan). Rutin (Sigma-Aldrich, St. Louis, MO, USA) at 0, 12.5, 25, 50, 100, 200 and 400 µg/mL was used as a standard, and TFC was reported in µg RE/mL. All experiments were conducted in triplicate.

Characterization of volatile organic compounds (VOC)

Gas chromatography-mass spectrometry (GCMS- Gas chromatography-mass spectrometry (GC-MS-QP2010/Shimadzu/Japan) techniques were used to evaluate the VOC profile in fermented GAJ, as described by Monajemi et al. (2005). The samples (1 µL) were directly transferred to an Agilent HP-5MS column (30 m × 0.25 mm × 0.25 µm) for analysis. The oven temperature program was initiated at 50 °C for 5 min, then 150 to 250 °C at 4 °C/min. Helium was used as the carrier gas at a flow rate of 2 mL/min at a pressure of 7.56 KPa. While the injector temperature was held at 240 °C. The analysis was performed using the pulsed splitless mode. Identification of compounds was analysed based on comparison to the National Institute of Standards and Technology (NIST) Mass Spectral Search Program, NIST Standard Reference Database Number 69 14 (https://webbook.nist.gov/chemistry/) and ChemStation Wiley Spectral Library with % a quality match of > 86%. The chemical structure was searched by National Library of Medicine (Pubchem; https://pubchem.ncbi.nlm.nih.gov/ (National Library of Medicine, 2023).

Determination of antioxidant activity

4,4,1 Dipheny1-1-picrydrazy1 (DPPH) assay

The antioxidant capacity of the sample was followed by Tantaisong et al. (2023) with some modifications. Briefly, fermented gas juice (20 µL) was mixed with 180 µL of 0.2 mM DPPH (Sigma-Aldrich, St. Louis, MO, USA in methanol) in a 96-well microplate, then mixed and left in a dark room for 30 min. The 520 nm absorbance was read by a microplate reader (M965+Metertech, Taipei, Taiwan). Distilled water and Trolox (Sigma, St. Louis, MO, USA) at 0, 12.5, 25, 50, 100, 200 and 400 µg/mL were used as blank and standard antioxidants, respectively. The antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (µg TE/mL). All experiments were conducted in triplicate and all chemical agents were analytical grade.

Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was followed by Tantaisong et al. (2023). FRAP reagent (0.02 M $FeCl_3$, 0.01 M 2,4,6-Tri (2-pyridyl) s-triazine, and 0.3 M acetate buffer at pH 3.6 in a ratio (v/v) 1:1:10) 180 µL were combined with 20 µL of sample. After 30-minute incubation, the absorbance at 593 nm was measured. The deionized water was used as a blank and ferrous (II) sulphate (Sigma, St. Louis, MO, USA) at 0, 12.5, 25, 50, 100, 200 and 400 µg/mL concentrations were used as antioxidant standards. The relative antioxidant activity was reported in µg Fe^{2+}/mL**.** All experiments were conducted in triplicate and all chemical agents were analytical grade.

Determination of cytotoxic activity (*in vitro*) against human colon adenocarcinoma (HT-29)

The human colon cancer cell line HT-29, obtained from American Type Culture Collections (ATCC, Manassas, VA, USA), was subjected to cytotoxic analysis. This evaluation was conducted utilizing the 3, 4, 5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) assay followed by Saengha et al. (2023). HT-29 cancer cells (5×10^3 cells/well) were maintained in flasks using Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc.) and antibiotic consisting of 100 U/mL of penicillin and 100 µg/mL of streptomycin. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced every 2-3 days until the cells reached approximately 80% confluence. Before detachment, cells were rinsed with 10% phosphate-buffered saline (PBS, pH 7.2), followed by treatment with 0.25% Trypsin-EDTA solution for cell harvesting. A culture of cancer cells (5×10^3 cells/well) was prepared in 96-well plates overnight in DMEM medium. Next, the juice extracts at 20 mg/mL in DMEM stock solution was added into the cells in the microplates to make final concentrations of 0-800 µg/mL. The cytotoxic effect was evaluated in triplicate throughout 24 h. Substituting MTT reagent for the medium, the cells were allowed to undergo a 4-h reaction period.

Following that, 200 µL of dimethylsulfoxide (DMSO) was employed to dissolve the formazan crystals, followed by the measurement of A590nm. Each chemical was obtained from Sigma- Aldrich (St. Louis, MO, USA). The IC50 values were calculated from three independent replicates using nonlinear regression analysis (inhibitor concentration versus normalized response, variable slope model) with GraphPad Prism version 8.0.1 (Windows platform).

% Cytotoxicity equals $[(A_o - A_e)/A_o] \times 100$

where A_o indicates the absorbance measured without the sample, whereas A_e denotes the absorbance recorded when the sample is present.

To examine morphological alterations in cancer cells, HT-29 cells were seeded at 5×10^3 cells/mL under the same conditions as previously described. Cells were then treated with either 48-h Gac aril juice or 48-h kefir-fermented Gac aril juice at concentrations of 0, 50, 100 and 200 µg/mL, followed by incubation for 24 h. Morphology changes were assessed using an inverted microscope (model NIB-9000; Xenon, Nanjing, China) at 400× magnification.

Statistical analysis

The experimental data were analysed using a Completely Randomized Design (CRD), and one-way Analysis of Variance (ANOVA) was performed at a 95% confidence level with SPSS (version 15.0 for Windows). Each experiment was carried out in triplicate. For the cytotoxicity test, statistical differences among samples were assessed using One-way ANOVA in a CRD and the Duncan Multiple Range Test (DMRT) ($p < 0.05$).

Results

Physicochemical properties of native and treated Gac aril juice

The GAJ mixed with commercial enzymes (T3, T4) and the control (T1) were lighter than the fermented juice with kefir grain as indicated by L*. For a* and b* values, all treated juices contained red and yellow components (Fig.1 and Table 1). TDS in all treated GAJ decreased during fermentation, with Gac aril juice mixed with pectinase showing the lowest TDS (Table 1).

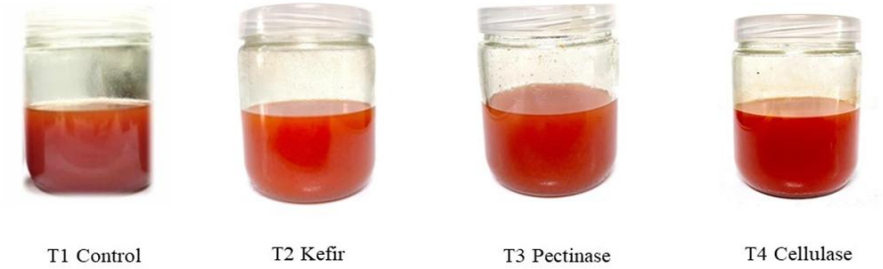

Figure-1. Characteristics of fermented Gac aril juice: T1 Gac aril juice, T2 Gac aril juice fermented with 2% kefir grain, T3 Gac aril juice mixed with 2% pectinase, and T4 Gac aril juice mixed with 2% cellulose.

Table-1. Physical properties of treated GAJ during 48 h.

Sample	Color at 48 h			pH			Total dissolved solid; TDS (°Brix)		
	L*	a*	b*	0 h	24 h	48 h	0 h	24 h	48 h
T1	44.32 ±3.01[a]	33.08±2 .24[a]	51.83 ±2.83[a]	6.52 ±0.02	6.44 ±0.06[a]	6.30 ±0.21[a]	10.50 ±0.87	10.13 ±0.32[a]	9.97 ±0.21[a]
T2	26.17 ±3.79[b]	34.11±3 .09[a]	40.81 ±2.14[b]	6.52 ±0.02	4.52 ±0.02[c]	3.16 ±0.04[c]	10.00 ±0.00	9.87 ±0.06[b]	8.47 ±0.25[b]
T3	48.39 ±1.59[a]	27.72±7 .21[b]	45.59 ±6.89[a]	6.58 ±0.01	5.78 ±0.04[b]	5.58 ±0.01[b]	10.30 ±0.17	9.67 ±0.29[b]	7.53 ±046[d]
T4	51.53 ±7.33[a]	32.77±7 .33[a]	40.83 ±7.69[b]	6.56 ±0.02	5.76 ±0.03[b]	5.52 ±0.06[b]	10.17 ±0.15	9.13 ±0.15[b]	8.17 ±0.58[c]

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Data were presented as mean ± SD (n=3). Within each column, varying superscript letters (a,b,c,d) represent statistically significant differences at $P < 0.05$.
T1 = Non-fermented GAJ; T2 = GAJ fermented with 2% kefir grain; T3 = GAJ mixed with 2% pectinase; T4 = GAJ mixed with 2% cellulose.

Carotenoid, TFC and TPC in native and treated Gac aril juice

Fermented juice and treated juice with hydrolytic enzyme showed higher levels of bioactive phytochemicals, such as lycopene, β-carotene, TFC and TPC compared to non-fermented juice during 48 h (Table 2). The fermented juice and the juice mixed with pectinase enzyme showed significantly higher levels of lycopene and β-carotene than untreated juice, with increase ranging from 9-50 fold increase. However, the juice mixed with cellulase had lower lycopene content but the highest β-carotene content. The kefir-fermented juice increased TPC and TFC than other treatments.

Table-2. Carotenoid, TFC and TPC in treated GAJ during 48 h.

Sample	Carotenoid content 48 h (µg/100 mL)		Total flavonoid content (TFC) (µg RE/mL)			Total phenolic content (TPC) (µg GAE/mL)		
	Lycopene	B-carotene	0 h	24 h	48 h	0 h	24 h	48 h
T1	7,345.23 ±0.03^{b}	2.97 ±0.03^{d}	607.77 ±1.15^{a}	607.77 ±1.15^{d}	607.77 ±1.15^{c}	128.08 ±1.76^{b}	128.08 ±1.76^{c}	128.08 ±1.76^{c}
T2	8,490.60 ±0.58^{a}	27.24 ±078^{c}	592.77 ±1.53^{b}	701.77 ±1.53^{c}	733.10 ±2.65^{b}	123.38 ±1.39^{c}	266.95 ±1.70^{a}	273.32 ±1.05^{a}
T3	8,250.00 ±0.71^{a}	55.32 ±1.01^{b}	569.43 ±3.21^{c}	813.43 ±3.06^{a}	913.43 ±3.21^{a}	132.30 ±1.70^{a}	250.64 ±2.22^{b}	258.20 ±1.99^{b}
T4	6,912.19 ±0.53^{c}	149.67 ±3.03^{a}	595.43 ±2.52^{b}	795.10 ±3.61^{b}	916.10 ±2.00^{a}	131.23 ±2.36^{a}	249.68 ±1.93^{b}	255.88 ±1.42^{b}

Data were presented as mean ± SD (n=3). Within each column, varying superscript letters (a,b,c,d) represent statistically significant differences at $P < 0.05$.
T1 = Non fermented GAJ; T2 = GAJ fermented with 2% kefir grain; T3 = GAJ mixed with 2% pectinase; T4 = GAJ mixed with 2% cellulose.

Volatile organic compounds (VOCs) in treated GAJ

Fermented Gac juice was found to have an altered profile of volatile organic compounds compared to non-fermented juice (Table 3). GAJ contained 2-Aziridinylethyl amine and acetic acid as major compounds while fermented juice with kefir consisted of CO_2, aminocarboxylic acid and increased acetic acid content. Fermentation also increased lactic acid, acetic acid, and pyruvate, and decreased pH. GAJ treated with 2% pectinase consisted of acetic acid, dimethylamine, 6-methyl-2-heptanol and sorbitol as major VOCs.

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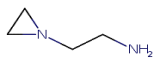
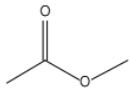
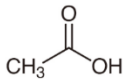
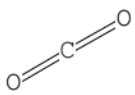
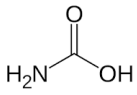
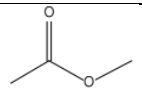
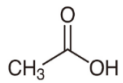
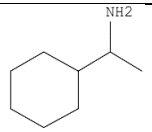
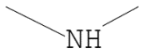
Table-3. Volatile organic compounds in GAJ, fermented GAJ with kefir and enzymatic treated GAJ.

Compound name	Boiling point (°C)	Molecular Weight	Molecular Formula	Structure	Retention time/min	Area %	Similarity %	Benefit	References
GAJ									
2-Aziridinylethyl amine	146	86	$C_4H_{10}N_2$		1.476	73.70	94	Synthesis of many pharmaceutical compounds	National Library of Medicine, 2023 Ikpa et al., 2021
Acetic acid ethenyl ester	72.2	86.09	$C_4H_6O_2$		1.924	0.99	89	Precursor to synthesis industrial polymers	National Institute of Standards and Technology, 2023; Bienewald et al., 2000
Acetic acid	118	60.05	$C_2H_4O_2$		2.117	25.31	96	Food additive in food and drink, solvent	National Institute of Standards and Technology, 2023; Lynch et al., 2019; Sanchez-Sala et al., 2019
Fermented GAJ with 2% kefir grain									
Carbon dioxide	-78.46	44.01	CO_2		1.458	11.17	100	Food additives in food and drink, solvent	National Library of Medicine, 2023; National Institute of Standards and Technology, 2023
Aminocarboxylic acid (Carbamic acid)	251	61.04	CH_3NO_2		1.459	23.64	95	Used in metabolic reactions, catabolic reactions or	National Library of Medicine, 2023; National Institute of Standards and

								waste generation.	Technology, 2023
Acetic acid methyl ester (methyl acetate)	57.1	74.08	$C_3H_6O_2$		1.584	1.17	92	Flavoring agents, solvent	National Library of Medicine, 2023; National Institute of Standards and Technology, 2023
Acetic acid	118	60.05	$C_2H_4O_2$		2.076	64.02	99	Food additive in food and drink, solvent	National Institute of Standards and Technology, 2023 Lynch et al., 2019 Sanchez-Sala et al., 2019
GAJ mixed with 2% pectinase									
(S)-(+)-1-Cyclohexylethylamine	174.6	127.23	$C_8H_{17}N$		1.358	0.93	90	Chemicals and drug development	National Library of Medicine, 2023; National Institute of Standards and Technology, 2023; Royal Society of Chemistry, 2023 Pařík and Chlupatý (2014)
Dimethylamine	7	45.08	C_2H_7N		1.452	25.72	93	food additives, color additives, accelerator in rubber, detergents, and pesticides;	National Library of Medicine, 2023; National Institute of Standards and Technology, 2023

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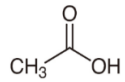
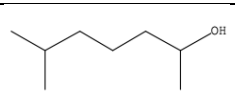
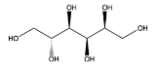
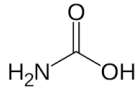
Acetic acid	118	60.05	$C_2H_4O_2$		2.074	30.89	99	Food additive in food and drink, solvent	National Institute of Standards and Technology, 2023; Lynch et al., 2019; Sanchez-Sala et al., 2019
6-Methyl-2-heptanol	176	130.23	$C_6H_{18}O$		8.244	24.49	86	Aroma-volatile compound	National Library of Medicine, 2023; National Institute of Standards and Technology, 2023 Royal Society of Chemistry, 2023
Sorbitol	296	182.17	$C_6H_{14}O_6$		46.09	17.98	88	Sweetening agents	National Library of Medicine, 2023; National Institute of Standards and Technology, 2023; Royal Society of Chemistry, 2023
GAJ mixed with 2% cellulase									
Amino carboxylic acid (Carbamic acid)	251	61.04	CH_3NO_2		1.329	7.91	100	Used in metabolic reactions, catabolic reactions or waste generation.	National Library of Medicine, 2023; National Institute of Standards and Technology, 2023; Royal Society of Chemistry, 2023

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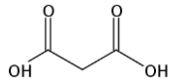
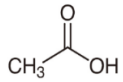
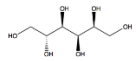
Malonic acid	220	104.06	$C_3H_4O_4$		1.447	36.62	88	Flavoring agents	National Library of Medicine, 2023
Acetic acid	118	60.05	$C_2H_4O_2$		2.072	34.61	99	Food additives in food and drink, solvent	National Institute of Standards and Technology, 2023; Lynch et al., 2019; Sanchez-Sala et al., 2019
Sorbitol	296	182.17	$C_6H_{14}O_6$		45.23	20.85	87	Sweetening agents	National Library of Medicine, 2023

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Antioxidant activity and cytotoxicity activity of native and treated GAJ

The fermented GAJ and the juice treated with 2% of each hydrolytic enzyme (pectinase and cellulase) showed increased antioxidant activity during 24-48 h compared to the non-fermented juice. The juice treated with 2% pectinase at 48 h exhibited the strongest DPPH radical scavenging activity and ferric-reducing antioxidant capacity, followed by the juice treated with cellulase and the kefir-fermented juice. Increasing phenolic and flavonoid content (Table 2) in this study enhanced antioxidant activity at 24 and 48 h (Table 4). This finding suggests that Gac fermented juice, as well as fermented apple juice, have enhanced antioxidant activity due to the fermentation process. This bidirectional association between probiotic bacteria and polyphenols remains poorly understood. Further investigations are required to elucidate the underlying mechanisms and explore its potential applications within the food industry.

Table-4. Antioxidant activity of treated GAJ using the DPPH and FRAP method during 48 h.

Sample	DPPH (µg TE/mL)			FRAP (µg Fe^{2+}/ mL)		
	0 h	24 h	48 h	0 h	24 h	48 h
T1	0.85±0.02[c]	0.85±0.02[c]	0.85±0.02[d]	59.51±1.27[c]	59.51±1.27[c]	59.51±1.27[c]
T2	1.21±0.04[b]	1.28±0.02[b]	1.35±0.02[c]	84.25±0.78[b]	91.67±0.83[b]	104.76±1.08[b]
T3	1.52±0.02[a]	1.60±0.01[a]	1.80±0.02[a]	96.80±1.52[a]	106.22±0.69[a]	152.06±0.66[a]
T4	1.58±0.07[a]	1.61±0.03[a]	1.72±0.02[b]	97.27±0.48[a]	107.82±0.93[a]	150.45±0.85[a]

Data were presented as mean ± SD (n=3). Within each column, varying superscript letters (a,b,c,d) represent statistically significant differences at $P < 0.05$.
T1 = GAJ; T2 = GAJ fermented with 2% kefir grain; T3 = GAJ mixed with 2% pectinase; T4 = GAJ mixed with 2% cellulose.

As for the cytotoxic effects of GAJ, the native juice had the lowest *E*max value of 47.98±0.18% at 800 µg/mL (<50% to be considered as not toxic to cancer cells). However, fermented GAJ with kefir 2% had the highest *E*max value of 59.08±0.59% (Table 5). Similarly, the IC_{50} value of GAJ with kefir 2% was lower indicating higher cytotoxicity against HT-29 cells (401.00±1.76 µg/mL) (Fig. 2). A dose-dependent cytotoxicity trend was observed in both native juice and fermented juice with kefir 2% (Table 5); however, the cytotoxic effect was more significant in fermented juice than native juice at the same concentrations indicating that fermentation of GAJ by kefir grain enhanced cytotoxicity towards colon cancer cell line HT-29. Overall, two treatments of juice had IC_{50} values > 200 µg/mL corresponding to a very weak cytotoxic effect on colon cancer cells. However, in this work, the limitation is that only an *in vitro* cytotoxic experiment using MTT assay was conducted. An *in vivo* experiment was required to confirm the *in vitro* cytotoxic results in a longer term of continuous administration of gac aril extracts in a rat model with induced colon cancer cells.

Table-5. Cytotoxicity (Emax, IC_{50}) of crude extracts from GAJ and fermented GAJ with 2% kefir on colon cancer cells HT-29 at 24 h.

Treatment	HT-29 colon cancer cells	
	***E*max (%)**	**IC_{50} (µg/ml)**
GAJ at 0 h	51.40±0.11[c]	882.03±1.50[c]
GAJ at 48 h	52.46±0.18[b]	826.07±0.84[b]
Fermented GAJ with 2% kefir at 48 h	61.00±0.86[a]	362.70±4.39[a]

Data were presented as mean ± SD (n=3). Within each column, varying superscript letters (a,b,c,d) represent statistically significant differences at $P < 0.05$.

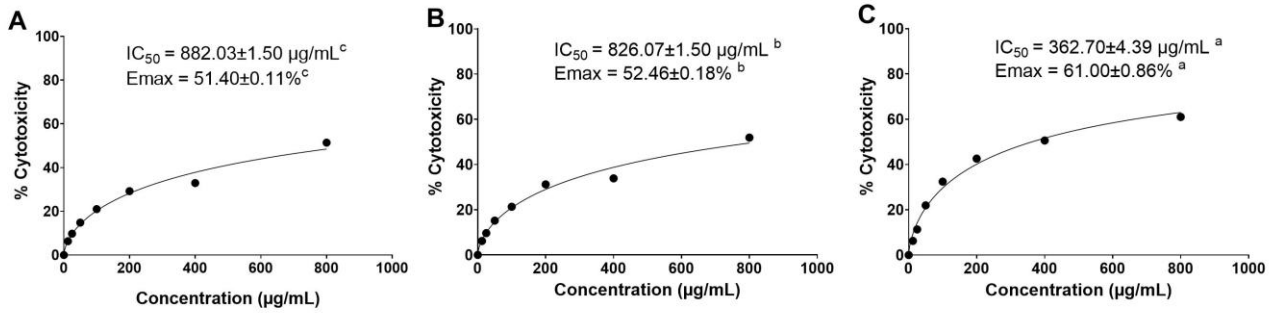

Figure-2. Cytotoxic activity of GAJ at 0 h (A), GAJ at 48 h (B) and fermented GAJ with kefir 2% at 48 h (C) on colon cancer cells HT-29.

As for the morphological results of HT-29 colon cancer cells (Fig. 3), the untreated control group exhibited the highest cell density, whereas increasing concentrations of GAJ treatment led to a gradual reduction in cell density. Moreover, HT-29 cells displayed notable morphological alterations, becoming smaller and more spherical in shape, resembling apoptotic bodies. These morphological changes were indicative but not conclusive of apoptosis, and this was the experimental limitation in the present study. To strengthen future investigations, flow cytometric analysis using Annexin V/PI staining should be employed to confirm apoptotic pathways.

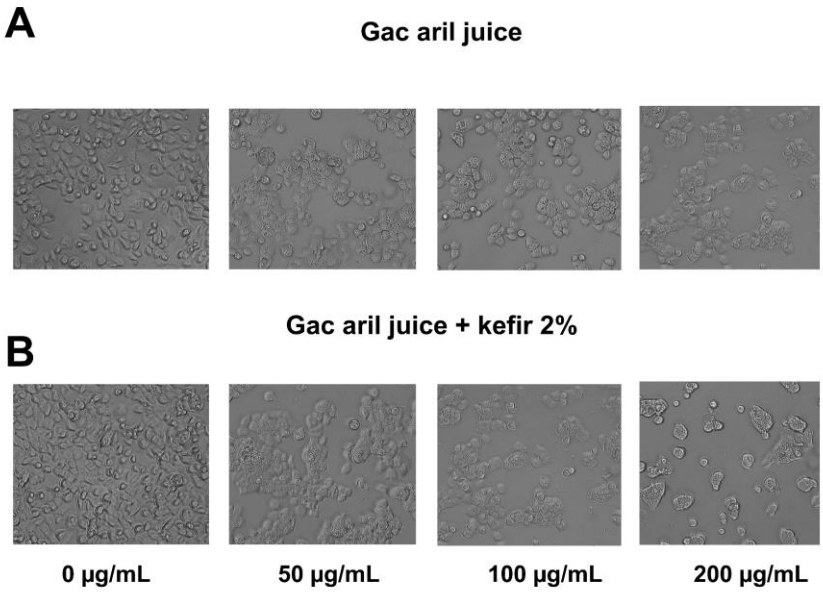

Figure-3. Morphology of HT-29 colon cancer cells upon treatment (A) GAJ at 48 h, (B) GAJ fermented with 2% kefir at 48 h.

Discussion

The physicochemical properties, volatile composition, antioxidant activity, and cytotoxic potential of GAJ were markedly influenced by kefir fermentation and enzymatic treatments. The changes in color parameter (L^*, a^*, b^*), pH and TDS (Table 1) were closely associated with the release and transformation of carotenoids, phenolics and flavonoids during fermentation and enzymatic treatment (Table 2). Kefir fermentation (T2) resulted in the most pronounced acidification (pH decreased from 6.52 to 3.16), which corresponded with a significant darkening of the sample (L*=26.17±3.79). This effect is consistent with

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the high organic acid production typically observed during lactic acid bacteria fermentation, which not only reduces pH but also alters pigment stability through acid-induced structural changes (Wang et al., 2021; Limbad et al., 2023). The relatively high redness value (a*=34.11 a*±3.09) in T2 aligns with the increased lycopene content (8,490.60 µg/100mL), suggesting that acidic condition can enhance lycopene extractability or stability, as reported in fermented gac products by Marnpae et al., 2022. The slight decrease in yellowness (b*=40.81±2.14) corresponds with the modest β-carotene content (27.24 µg/100mL), reflecting the known sensitivity of β-carotene to acidic degradation (Ersṃan and Müller-Maatsch, 2022)

Enzymatic treatments (T3 and T4) produced higher lightness values (L* = 48.39-51.53), indicating greater pigment liberation due to structural breakdown of the cell wall. This aligns with previous findings that pectinase and cellulase disrupt pectin-cellulose matrices, facilitating enhanced carotenoid extraction. Pectinase was more effective than cellulase in enhancing lycopene extraction. This is due to its pectolytic and hemicellulolytic activities, which enable it to degrade pectin in the middle lamella. In contrast, cellulase treated the 1,4-β-d-glycosidic bonds within cellulose of the primary cell wall. Nonetheless, it is important to consider that enzymatic treatment may also contribute to carotenoid degradation when exposed to oxygen (Pinthong et al., 2019). This is supported by the substantial increase in β-carotene content, particularly in T4 (149.67 µg/100mL), and by elevated lycopene levels observed in T3 and T4. The marked increases in TPC and TFC during enzymatic treatment (TPC up to 913.43 µg/ GAE/mL in T3 and 916.10 µg/ GAE/mL in T4; TFC up to 258.20-255.88 µg/ RE/mL) indicate enhanced release of bound phenolic. These results are consistent with evidence that enzyme–mediated hydrolysis increases phenolic accessibility by breaking down cell wall polysaccharides (Li et al., 2021). Furthermore, the moderate redness (a*=27.72-32.77) and relatively high yellowness (b*=40.83-45.59) in T3 and T4 match the observed increase in carotenoid content, consistent with carotenoid color relationship previously documented in plant-based systems (Müller-Maatsch et al., 2017; Ersṃan and Müller-Maatsch, 2022).

The reduction in TDS across all treatments, particularly in T2 (from 10.00 to 8.47 ºBrix) supports the hypothesis that carbohydrates were metabolized by acetic acid and lactic acid bacteria, including yeast producing hydrolytic enzymes capable of breaking down complex phenolic compounds into simpler, more bioavailable forms. Specifically, *L. plantarum* has demonstrated the ability to deglycosylate phenolic glycoside and to liberate both soluble conjugated and insoluble bound phenolics from plant cell walls (Li et al., 2021). The diversity of LAB species and yeast strains in kefir grain enables them to adjust their metabolic pathways to varying environmental conditions. This adaptation is likely influenced by their metabolism and the production of phenolic and flavonoid compounds, which generates organic acid and secondary metabolites (Wang et al., 2021; Patil et al., 2022; Sharma et al., 2022; Limbad et al., 2023). This metabolic activity, together with pigment liberation facilitated by enzymatic treatments, explains why T3 and T4 displayed both the highest TPC/TFC values and brightest color profile.

Collectively, these results demonstrate that kefir fermentation primarily influences pH and pigment stability through microbial acidification, whereas enzymatic treatments enhance carotenoid and polyphenol release through cell wall degradation, resulting in distinct and measurable differences in color, biochemical composition, and functional attributes (Sharma et al., 2022; Patil et al., 2022)

The volatile organic compound profile of GAJ was markedly modified by kefir fermentation and hydrolytic enzyme treatments (Table 3), and these changes can be directly linked to alterations in aroma and flavor perception. Untreated GAJ was characterized by a high proportion of 2-aziridinylethyl amine (73.70%) and acetic acid (25.31%), compounds associated respectively with pungent, ammoniac-like oder and sharp vinegar-like sourness (Doeun et al., 2017). This composition explains the relatively harsh and acidic aroma typical of fresh GAJ before any bioprocessing. Kefir fermentation significantly reshaped the VOC profile, most notably by increasing acetic acid to 64.02% and generateing methyl acetate (1.17%), a fruity ester well known for imparting sweet, pear-like aroma (Zhang et al, 2023) The rise in acetic acid is consistent with heterofermentative LAB metabolism, which converts sugar into organic acid and contributes to the pronounced sour flavor (Wang et al., 2024). Meanwhile, methyl acetate production adds a light fruity aroma that softens the acidity, thereby improving the overall aroma balance of fermented GAJ. Additionally, the detection of CO_2 (11.17%) enhances freshness and mouthfeel, characteristics commonly associated with fermented beverages. In GAJ treated with pectinase, the

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appearance of 6-methyl-2-heptanol (24.49%) has also been reported in Black Perigord truffle as a volatile alcohol generated during storage, with its concentration increasing as the truffle progresses through natural aging and degradation processes (Choo et al., 2021). The presence of dimethylamine (25.72%) is a volatile amine with a strong fishy, ammonia-like odor. In seafood and fermented fishery products, this compound is formed mainly via reduction of trimethylamine oxide and contributes to undesirable fishy off-oder (Lu et al., 2022). This treatment also consisted of sorbitol (17.98%) contributes sweetness and viscosity in juice. (Asasta et al., 2024). Cellulase treatment produced a distinct VOC pattern dominated by malonic acid (36.62%), a naturally occurring dicarboxylic acid in higher plants, which can represent a quantitatively important component of the organic acid pool in plant tissues. Classical and more recent plant metabolomics studies have repeatedly identified malonic acid among low molecular weight organic acids in plant extracts (Ma and Qi, 2021). This treatment also consisted of acetic acid (34.61%), including sorbitol (20.85%), contributing to sweetness and smooth mouthfeel. The resulting balance of acids and polyols explains the milder sour-sweet taste compared with kefir-fermented GAL, which exhibited a more intense acidic profile.

Fermentation and enzymatic treatment led to significant alterations in the volatile compound composition of GAJ compared to the untreated sample. A few reports (Wang et al., 2021; Limbad et al., 2023) indicated yeast and LAB in kefir grain could metabolized glucose and sucrose, leading to carbon dioxide through decarboxylation reactions, along with the formation of carboxylic acids, and various amino acids. Biological processes of nitrogen can generate aminocarboxylic acid from the deamination of arginine including water and carbon dioxide through a carbonate dehydratase, resulting in aminocarboxylic acid. Aminocarboxylic acid itself is not highly toxic to humans in small amounts, but its derivatives can be more problematic. One of its derivatives, ethyl carbamate (EC), a human carcinogen, can form during fermentation through a reaction between ethanol and nitrogen-rich precursors such as urea. Urea is a catabolic product of arginine produced by yeast and LAB (Han et al., 2025). However, this finding also raises a critical food safety concern regarding the potential of EC, which is classified as a probable human carcinogen (Group 2A) by IARC (IARC, 2010). formation It's critical to control the process to limit its levels and not pose a risk to sensitive individuals such as: (1) Selecting LAB and yeast strains with high urease activity to degrade urea rapidly (Zhao et al., 2014; Han et al., 2025) and (2) Enzymatic degradation represents an effective approach for the removal of urea or EC generated during the post fermentation phase. Researchers have identified numerous natural acid ureases and EC hydrolases and examined their potential applications (Liang et al., 2023). These steps are critical to ensure the safety of functional beverages, particularly for sensitive consumer groups.

Cellulase and pectinase treatments markedly increased the antioxidant activity of GAJ, (Table 4). At 48 h, the DPPH values in T3 and T4 reached 1.80±0.02 and 1.72±0.02 µg TE/mL, respectively, which were significantly higher ($p< 0.05$) than those of fermented GAJ (T2) (1.35±0.02 µgTE/mL) and the non-treated GAJ T1 (0.85 ±0.02 µgTE/mL). A similar trend was observed for FRAP, where T3 and T4 showed the highest reducing power at 48 h (152.06±0.66 and 150.45±0.85 µ Fe^{2+}/mL, respectively), compared with T2 (104.76±1.08 µ Fe2+/mL) and T1 (59.51±1.27 µ Fe2+/mL). These results clearly demonstrate that enzymatic treatment with pectinase and cellulase is more effective than kefir fermentation alone in enhancing the antioxidant capacity of GAJ. Our findings were consistent with Nadar and Rathod (2019), who reported that a co-immobilized magnetic nanobiocatalyst containing both pectinase and cellulase produced more than two-fold increase in free radical scavenging activity in orange, mango, and banana peel extracts compared with conventional solvent extraction. This improvement is largely because most phenolic and flavonoid compounds are bound within the cell wall matrix, which consists of cellulose, hemicelluloses, lignin, and pectin, through hydrogen bonding and hydrophobic interactions. The enzymatic breakdown of cell walls constituents, particularly pectin and cellulose, can increase cell wall permeability and porosity, thereby promoting the efficient release of intracellular compounds. A similar mechanism is likely occurring in our GAJ system. The higher TPC and TFC observed in enzyme-treated samples correspond well with the superior DPPH and FRAP values in T3 and T4 (Table 2 and 4), suggesting that enzymatic breakdown of pectin and cellulose increased cell wall permeability and facilitated the release of intracellular antioxidants.

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Kefir fermentation (T2) also enhanced the antioxidant activity of GAJ relative to the native juice. DPPH values increased from 0.85±0.02 µgTE/mL at 0 h to 1.35±0.02 µgTE/mL at 48 h, while FARP improved from 59.51±1.27 µFe2+/mL to 104±1.08 µFe2+/mL (Table 4). This pattern aligns with the study of Marnpae et al. (2022), who reported that fermentation of Gac juice with *L. paracasei* CASEI 431 enhanced β-carotene, DPPH, FRAP activity and increased inhibition of lipid peroxidation. In our work, the increase in antioxidant activity in T2 coincides with the elevated carotenoid content, particularly lycopene, and modest increases in TPC and TFC (Table 2), supporting the role of carotenoids as free radical scavengers and metal ion chelators with antioxidant activity comparable to vitamin C (Marnpae et al., 2022).

Moreover, the fermentation process can modify the phenolic profile of GAJ, leading to increased levels of phenolic and flavonoid compounds, similar to observations in fermented apple juice reported by Li et al. (2018) and Li et al. (2021). Polyphenols, a diverse group of secondary metabolites including flavonoids, phenolic acids, stilbenes, and lignans, are known to interact bidirectionally with probiotics. During fermentation, probiotics produced enzymes (e.g. β-glucosidase) that convert complex polyphenolic glycosides into simpler, more soluble aglycones, thereby enhancing the total content and bioavailability of bioactive compounds (de Oliveira et al., 2020; Cheng et al., 2020; Sharma et al., 2022). This mechanism is consistent with an increase in TPC, TFC and the corresponding rise in DPPH and FARP values in our fermented samples.

The cytotoxicity results against the colon cancer line HT-29 show that the kefir-fermented GAJ at 48 h achieved an *E*max of 61.00±0.86% and an IC_{50} of 362.70±4.39 µg/mL (Table 5). In contrast, the native juice at 0 h had an *E*max of 51.40±0.11% and IC_{50} 882.03±1.50 µg/mL, indicating a substantially weaker effect. Thus, the enhanced phytochemical profile correlates with enhanced cytotoxicity: the kefir-fermented juice (with higher TPC, TFC and carotenoids) produced higher anticancer activity. However, the overall potency of the kefir-fermented GAJ remains "very weak" (IC_{50} > 200 µg/mL). From a mechanistic perspective, the enhanced cytotoxic effect observed against HT-29 colon cancer cells may be attributed to the elevated levels of multiple bioactive compound classes in the kefir-fermented GAJ, including carotenoids, TPC and TFC (Table 2), together with the significantly increased antioxidant activities, as evidenced by higher DPPH radical scavenging capacity and FRAP values (Table 4).

Lycopene and β-carotene, the predominant carotenoids in Gac fruit, are likely key contributors to the observed cytotoxic and pro-apoptotic effects against HT-29 colon cancer cells in this study. Lycopene has been shown to exert a concentration-dependent inhibitory effect on HT-29 cell viability, markedly suppressing cell proliferation in a dose-dependent manner, with an IC_{50} of 7.89 µM after 24 h of treatment (Ataseven et al., 2023). In the same study, treatment with 7.89 µM lycopene markedly increased the expression of pro-apoptotic proteins, including cleaved caspase-3, Bax, and cleaved PARP, while exhibiting no significant effect on the anti-apoptotic Bcl-2 protein. These findings provide clear evidence that lycopene induces apoptosis in HT-29 colon cancer cells. Similarly, β-carotene (20 and 40 µM) exerts antiproliferative effects towards HT-29 cells (Lee et al., 2022). This study also demonstrated that β-carotene inhibited colon cancer stem cells (CSCs) by suppressing their self-renewal ability and downregulating the expression of key CSC markers, including *CD44*, *CD133*, *ALDH1A1*, *NOTCH1*, and Sox2, as well as components of the Wnt/β-catenin signalling pathway. Collectively, these findings indicate that BC possesses therapeutic potential as an agent targeting colon CSCs (Lee et al., 2022).

The considerable rise in TPC and TFC observed in our kefir-fermented Gac juice (TPC reached 733.10±2.65 µg GAE/mL and TFC 273.32±1.05 µg RE/mL in T2 at 48 h in Table 3) compared to the control GAJ supports the notion that these phytochemical classes may contribute to the induction of apoptosis in HT-29 colon cancer cells. Phenolic compounds have been shown to exert anticancer effects by modulating key survival signals—such as inhibiting PI3K/Akt, ERK/MAPK, and NF-κB pathways, while up-regulating tumor suppressors like p53, PTEN and the Bax/Bcl-2 ratio (Anantharaju et al., 2016; Do et al., 2021). Similarly, flavonoids have been documented to trigger apoptosis in colon cancer lines through mechanisms such as ROS generation, cell cycle arrest (often at G2/M), and caspase activation (Abotaleb et al., 2018; Esmeeta et al., 2022; Dubey et al., 2023).

Our results, showing enhanced cytotoxicity (IC_{50} 362.70 µg/mL for kefir-fermented GAJ) concomitant with elevated phenolic/flavonoid concentrations, are therefore consistent with this mechanistic literature. While we did not measure apoptotic markers directly,

the correlation between increased phenolic/flavonoid content (Table 4) and increased cytotoxicity in HT-29 (Table 5) implies that these classes of compounds may be driving—at least in part—the cytotoxic responses we observed. Future work should isolate specific phenolic and flavonoid molecules from the juice and assess their apoptotic activity (e.g., Annexin V/PI, caspase assays) to validate this mechanistic hypothesis.

This report was the first to show the cytotoxic effects of fermented GAJ on HT-29 colon cancer cells, even though the IC_{50} value was > 200 µg/mL (very weak effect). Our finding that kefir-fermented GAJ achieves higher cytotoxicity aligns with these reports, though our IC_{50} values are higher (weaker effect) than many purified compounds or high-dose extracts in literature: e.g., Wimalasiri et al. (2020) reported GA pulp extract IC_{50} 490-730 µg/mL in breast/melanoma cells, so our IC_{50} of 362 µg/mL is comparable or somewhat better in HT-29 cells. Thus, our data add to the literature by showing that GAJ fermentation enhances cytotoxicity, not only antioxidant activity. In addition, Gac pulp extract was able to inhibit the growth of 26-20 colon adenocarcinoma cell lines in Balb/c mice by up to 23.6% as a result of a bioactive protein weighing 35 kDa with anti-cancer activity (Tien et al., 2005). In another report, it was pointed out that unsaturated fatty acids from Gac pulp extract had the effect of reducing blood lipids, cholesterol, blood pressure and preventing cancer (Xu et al., 2019).

Our results suggest that GAJ, when fermented with kefir grains, may serve as a promising base for functional beverages with enhanced antioxidant and anticancer potential. This aligns with the broader trend in plant-based fermented beverages investigated in the literature: the review by Chong et al. (2023) underscores the potential of fermented beverages (kefir, kombucha) as health-promoting drinks (Chong et al., 2023; Paramithiotis et al., 2024). The fact that we observed measurable increases in antioxidant activity (DPPH of 1.35 µg TE/mL, FRAP of 152.06 µg Fe^{2+}/mL in T2 in Table 4) and cytotoxic activity suggests viable functional claims. Attention to sensory attributes (colour, flavour changes via VOC profile) will be critical, since consumer acceptance may be impacted by fermentation-derived acids and volatiles.

Conclusion

In conclusion, this study provides insights into how different fermentation methods can affect the physicochemical properties of GAJ. Fermentation of GAJ with kefir, along with the application of hydrolytic enzymes like pectinase and cellulase, facilitates the breakdown of plant cell walls, leading to the release of free carotenoids that contribute to the reddish-orange coloration of the fermented GAJ. Microbial fermentation of GAJ with kefir enhances its anti-cancer effects through the degradation of glycosyl-containing phenolic compounds, production of free phenolic compounds, and generation of metabolites especially organic acids with increased acidity (lower pH) by lactic acid bacteria, ultimately contributing to higher biological activity and antioxidant potential. Furthermore, kefir-fermented GAJ provided more acidity during the fermentation period than enzyme-treated juice. These findings could be useful for developing new strategies for producing fermented fruit juices with desirable functional properties.

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Contribution of Authors

Mungkunkoth K: Collected data and wrote methodology.

Luang-In V: Collected data, analysed and interpreted data, performed the analysis, drafted the article in cytotoxicity activity and did critical revision.

Butkhup L, Somboonwatthanakul I, Sungsri-in M, Moongngarm A, Limpongsa E & Ma NL: Analysed and interpreted data, final approval of the version to be published.

Deeseenthum S: Conceptulized and designed the study, collected data, analysed and interpreted the data, executed experiments, drafted the manuscript, critically revised intellectual content, and final approval of the version to be published.

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