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Determination of the Phytochemical Components, Nutritional Content, Biological Activities, and Cytotoxicity of Ripening Karanda (*Carissa carandas*) Fruit Extract for Functional Food Development

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ABSTRACT: Ripening karanda fruits are a natural source of phytochemicals, which exhibit various biological properties. The present study aimed to determine the types of phytochemicals, biological properties, and cytotoxic and hemolytic effects of ripening karanda fruits. Two mechanical tools were used to collect the phytochemicals under low temperatures during the extraction process. The extracts were investigated for antioxidants using a 2,2-diphenyl-1-picrylhydrazyl assay. The total phenolic contents were studied using the Folin-Ciocalteu method. The phytochemicals of the total extract were analyzed by gas column chromatography-mass spectrometry. The saccharide types, including the total sugar content, were determined using thin-layer chromatography and the Lane-Eynon method. The total ascorbic acid was analyzed in accordance with the AOAC 967.21 method. The cytotoxic and hemolytic effects of phytochemicals were investigated using human peripheral blood mononuclear cells (hPBMCs) and human red blood cells (hRBCs). The results showed that the appropriation for repeated ultrasonic extraction is four times. The fresh ripening karanda fruit (Fresh-RKF) and freeze-dried powder of ripening karanda fruit extracts exhibited antioxidant activity *in vitro* and exerted a noncytotoxic effect on hPBMCs at a concentration of $\leq$2.5 mg/mL and a hemolytic effect on hRBCs at a concentration of >5.0 mg/mL. The Fresh-RKF extract comprised 0.27% of total sugar and 0.01% of ascorbic acid. These data could support the development of supplemental foods using ripening karanda fruits as the primary ingredient.

Keywords: carissa carandas, cytotoxicity, functional food, nutrition content, phytochemicals

INTRODUCTION

Global population aging has a significant impact on the economy, society, and public health, leading to an increase in noncommunicable diseases (NCDs), which are responsible for over 70% of global deaths (Abegunde et al., 2007; Terzic and Waldman, 2011; GBD 2015 Risk Factors Collaborators, 2016). Aging and certain risk factors, including smoking, pollution, and unhealthy diets, cause oxidative stress, which leads to the breakdown of biological molecules (Abegunde et al., 2007; Dudonné et al., 2009; Terzic and Waldman, 2011). Oxidative stress is associated with NCDs, especially in the elderly. Free radicals, particularly reactive oxygen species, can impair immune and red blood cells, increasing the risk of diseases (Delesderrier et al., 2020). Individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency and sickle cell disease are susceptible to oxidative stress, which can cause hemolysis (Delesderrier et al., 2020; Lee et al., 2022). Thus, they should avoid foods rich in free radicals, including fava beans (Luzzatto et al., 2020; Garcia et al., 2021).

Antioxidants from natural and synthetic sources help counteract oxidative stress and maintain redox balance via enzymatic and nonenzymatic pathways (Cimen, 2008). Plant-based extracts, including those from *Centaurea ammocyanus*, exert antioxidant effects that protect red blood cells, including those from individuals with G6PD defi-

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ciency (Joujeh et al., 2020). Similarly, extracts from mangroves and *Diospyros lotus* fruits also exhibit antioxidant properties, preventing oxidative damage and hemolysis (Azadbakht et al., 2011; Karim et al., 2020). These findings demonstrate the potential of phytochemicals in functional foods to reduce oxidative stress and the risk of NCDs.

Carissa carandas, commonly known as karanda, is a plant from the Apocynaceae family that is found in Southeast Asia, particularly Thailand. It is widely used in traditional medicine and has gained recognition for its diverse bioactive compounds, including antioxidants with significant health benefits (Sahreen et al., 2010; Kumar et al., 2013; Patel, 2013; Bhosale et al., 2020). Karanda fruits, particularly in the ripening stage, are rich in proteins, fiber, carbohydrates, calcium, and bioactive substances with antioxidant and anticancer properties (Kumar et al., 2013; Srithongkerd et al., 2023). According to *in vitro* studies, karanda fruit extract exhibits inhibitory effects on the growth of human colorectal adenocarcinoma (Caco-2) cells and human liver hepatocellular carcinoma (HepG2) cells. These attributes make karanda a promising candidate for the development of functional foods aimed at promoting health.

In the food and pharmaceutical industries, it is important to optimize extraction methods in order to ensure the safety and efficacy of plant-based products. There are various extraction methods, ranging from basic methods such as steam distillation, maceration, and mechanical processes (Bandara et al., 2014) to more advanced approaches such as supercritical CO_2 extraction (Kao et al., 2007) and microwave-assisted extraction (Chemat and Cravotto, 2013). Among the many solvents used for extraction, water is the most preferred because of its safety, cost-effectiveness, and accessibility (Awe et al., 2013; Nam et al., 2018; Villalobos-Vega et al., 2023). To further improve the extract quality, enzymes (e.g., pectinase) are often used to improve the clarity of, reduce the turbidity of, and preserve bioactive compounds (Luzzatto et al., 2020; Garcia et al., 2021; Arya et al., 2022; Patel et al., 2022; Roman-Benn et al., 2023). These optimized extraction processes are crucial for developing functional foods that are effective and safe for consumers.

However, no studies have explored the extraction of ripening karanda fruits using water as a solvent combined with pectinase treatment and its effects on human peripheral blood mononuclear cells (hPBMCs) and human red blood cells (hRBCs). Therefore, the present study aimed to investigate the phytochemical components, nutritional content, and biological activities of karanda fruit extracts. Moreover, the antioxidant activity, cytotoxicity, and hemolytic effects of pectinase-treated and untreated extracts were compared. In addition, the study conducted extraction using two methods (i.e., homogenization and ultrasonic extraction) under cold temperatures. These methods were chosen for their ability to preserve bioactive compounds and improve the quality of the final extract.

The results of this study will provide valuable insights into the potential of ripening karanda fruit extracts as a raw material for developing functional foods with low toxicity and high health benefits. Moreover, these findings will contribute to the growing body of research on functional food development and pave the way for future studies on the integration of karanda-based products into dietary regimens to improve health outcomes.

MATERIALS AND METHODS

Collection and extraction and ripening karanda fruits

The growth of karanda fruits has three stages: (1) raw fruit stage (white, pink color), (2) semi-ripening fruit stage (red color), and (3) ripening fruit stage (purple color) (Kumar et al., 2013). In this study, freshly ripening karanda fruits were collected in the morning (6 – 8 a.m.) in Chlongsong Village, Chlonglaung District, Pathum Thani Province, Thailand. Ripening karanda fruits were washed and soaked in deionized water (DI) for 10 min and then frozen at −20°C overnight. Next, the fruits were cut seeds were removed. The fruits were then soaked in ice-cold sterile 0.01% w/v NaCl (4°C) for 30 min. Subsequently, they were washed thrice with ice-cold DI water. After washing, the fruits were placed in a 20-nm mesh to remove water, weight balanced, and then frozen at −20°C. The frozen ripening karanda fruits were separated into two groups (A and B). The fruits in group A were homogenized at 125 *g* for 5 min using a blender and then frozen dry. Next, 400 mL of ice-cold DI water was added to the extracted chamber containing 20.0 g of frozen dry karanda fruit powder. The extracted chambers were placed in an ultrasonic machine, and the extraction system was performed four times at 45 kHz and 4°C for 1 h. The supernatant in each time program (SET 1 to SET 4) was collected, filtered, and incubated at 60°C for 10 min. The clear supernatant (SET 1 to SET 4) was gathered in a small volume to determine the antioxidant potential and total phenolic contents of the extract. Afterward, the supernatants in all sets were mixed and lyophilized. The extracted powder of group A was called the freeze-dried powder of ripening karanda fruit (FD-RKF) extract.

The fruits in group B were placed in the extraction chamber of a blender containing ice-cold sterile DI water at a 1:1 ratio (w/v). The fruits were homogenized at 503 *g* for 2 min, and the process was repeated five times for a total of 10 min. The homogenate was centrifuged or separated using an 80- to 120-nm mesh, and then the

obtained supernatant was called SET 1. The precipitant (SET 1) was balanced and placed in an ice-cold water chamber. The compounds were extracted using an ultrasonic machine at 45 kHz and 4°C for 1 h. Afterward, the supernatant was collected. The extraction process was repeated according to the schematic in Fig. 1. The extract of each set was maintained at a few milliliters to determine the antioxidant activity and total phenolic contents. The major volume of extracts (SET 1 to SET 4) was mixed and called the complete set. This extract was pasteurized in a 60°C water bath for 10 min and dried using a freeze-dryer. The extracted powder of the whole set was named the fresh ripening karanda fruit (Fresh-RKF) extract.

Analysis of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The antioxidant activity of the FD-RKF and Fresh-RKF extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The reactions were prepared in a small volume using a 96-well plate as a container. In the initial step, 190 μL of 0.101 mM DPPH solution (0.101 mM DPPH in ethanol) was added to the wells. Next, 10 μL of each extracted set was placed into the wells that contained DPPH solution. The plate was shaken to mix the solution and then incubated at 37°C for 1 h in the dark. DI water was used as the negative control, whereas L-ascorbic acid and butylated hydroxytoluene (BHT) were used as the positive controls. The absorbance was measured using a microplate reader (EZ Read 2000, Bio-

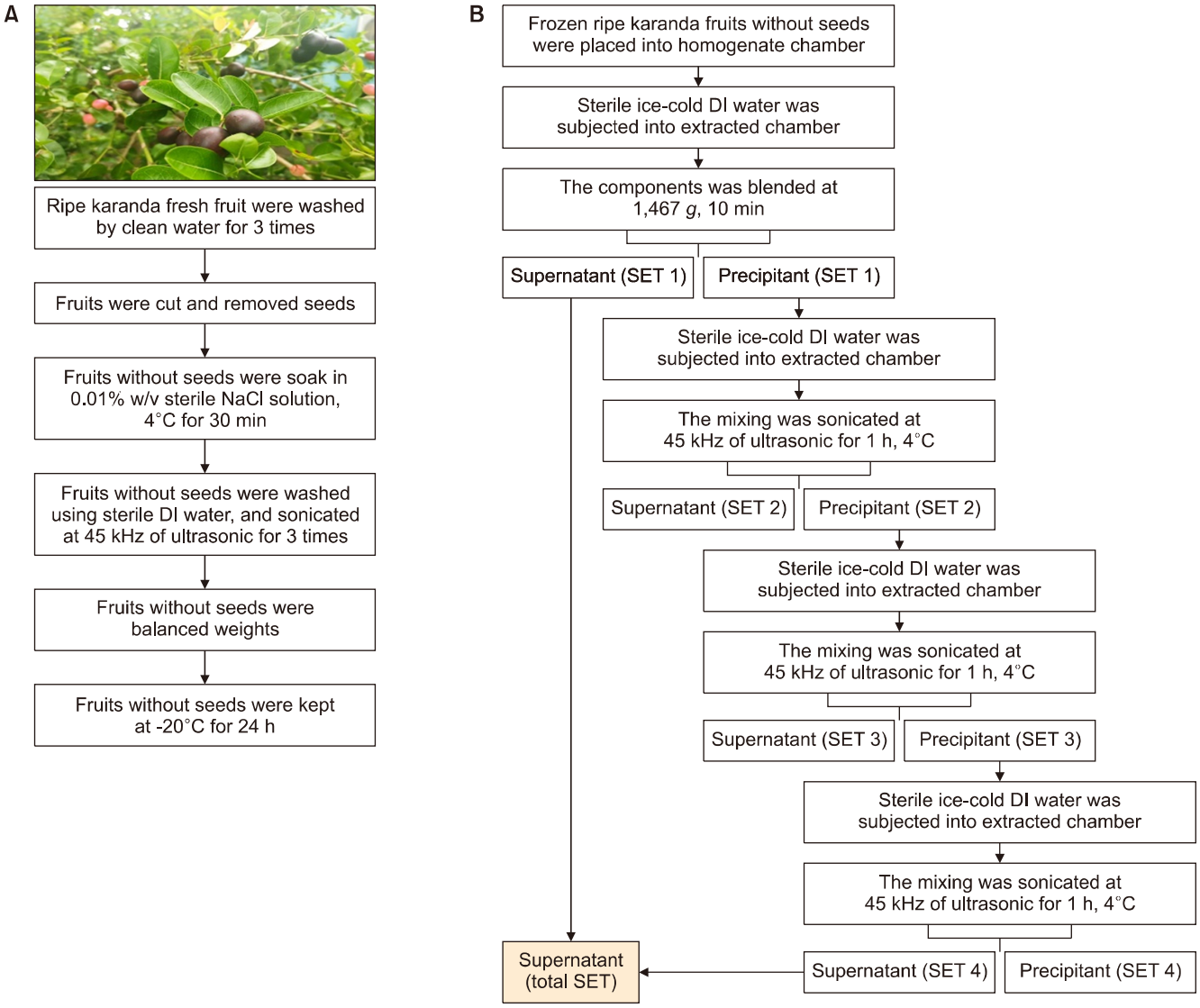

Fig. 1. Schematic of the extraction process of karanda fruit. (A) Preparation of fresh, ripening karanda fruits. In the preparation process of group 1, the fresh ripening karanda fruits were homogenized and then frozen dry. The dried powder was extracted at 45 kHz and 4°C for 1 h, and the process was repeated four times. The supernatant of the extract was named the FD-RKF extract (SET 1 to SET 4). (B) Fresh-RKFs were extracted using two methods: (1) homogenization (SET 1) and (2) ultrasonication at 45 kHz and 4°C (SET 2 to SET 4). The supernatant of the extract was called the Fresh-RKF extract. DI, deionized water; Fresh-RKF, fresh ripening karanda fruit; FD-RKF, freeze-dried powder of ripening karanda fruit.

chrome) at 515 nm. The percentage of antioxidant activity was calculated using a previously described equation (Dudonné et al., 2009).

Determination of the total phenolic content

The total phenolic contents of the FD-RKF and Fresh-RKF extracts were determined in accordance with the method of Patathananone et al. (2019). In this method, gallic acid was used as the standard phenolic compound. In the initiation step, different concentrations of gallic acid and 10-fold diluted Folin-Ciocalteu reagent were prepared. Next, 3.5 mL of 2.0% w/v Na_2CO_3 solution was pipetted and placed into test tubes. Then, gallic acid and sample extracts were added into the test tubes and mixed. Subsequent, 400 μL of 10-fold diluted Folin-Ciocalteu reagent was added into the test tubes, mixed, and incubated for 30 min in the dark. The absorbance of all reactions in each tube was measured using an ultraviolet-visible spectrophotometer (Genesys 20, Thermo Scientific) at a wavelength of 750 nm. The standard curve ($y=0.0078x+0.0061$, $R^2=0.9978$) was calculated as the quantity of the total phenolic content of the extract.

Determination of the cytotoxic effect of karanda fruit extracts on human peripheral blood mononuclear cells

In this study, buffy coat bags were prepared from blood donor bags. The preparation process of buffy coat bags was supported by the Central Blood Bank, Faculty of Medicine, Khon Kaen University, and performed in accordance with biological safety limitations. After preparation, the bags were cleaned using 70% v/v ethanol and placed into a class II biosafety cabinet (BSL class II). Twenty milliliters of the buffy coat were transferred into sterile centrifuged tubes that contained 20 mL of the separated reagents (Hi-Sep, Invitrogen). The tubes were inverted gently. The mixture in the tubes was centrifuged at 1,467 *g* at 4°C for 30 min. hPBMCs located at the interphase layer were collected into new tubes containing 10.0 mL of 1X phosphate-buffered saline (PBS, pH 7.4) for washing cells. The tubes were gently shaken and then centrifuged at 1,467 *g* at 4°C for 5 min. The supernatant was removed and then subjected to 10.0 mL of red blood cell lysis buffer (Invitrogen). Subsequently, it was mixed and incubated at room temperature for 30 min. hPBMCs were collected at 1,467 *g* at 4°C for 5 min. Then, the cells were washed thrice using 1X PBS (pH 7.4). Afterward, hPBMCs were resuspended in Roswell Park Memorial Institute (RPMI) medium. Approximately 10 μL of resuspended cells were stained with 10 μL of 1X trypan blue exclusion (Invitrogen). The stained cells were loaded into a counter slide and placed into an automatic cell counter (Bio-Rad). The cell survival was calculated and prepared to the number 1×10^6 to 2×10^6 cells/mL. This resuspended survival hPBMCs was pipetted of 100 μL, subjected to wells of a 96-well plate, and then mixed plates to distribute cells. The plates were incubated at 37°C in a 95% air/5% CO_2 incubator for 2 h. The cells were treated with different concentrations of samples, 20% dimethyl sulfoxide (DMSO, positive control), and 1X PBS (negative control). The treatment plates were incubated under the same conditions for 24 h. Next, the cells were collected by microplate centrifugation at 366 to 1,467 *g*. The supernatant was removed, and the cells were resuspended in each well with 100 μL of RPMI medium. Surviving cells were stained using 100 μL of 1X Presto blue (Invitrogen) in 1X PBS and incubated for 2 h. This dye can stain only viable cells and represent fluorescence values. Thus, the fluorescence intensity of reactions was measured using a microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The percentage of cell viability was calculated using the following formula:

$$\text{Cell viability}(\%) = \frac{\text{fluorescence values}_{\text{test}}}{\text{fluorescence values}_{\text{negative control}}} \times 100$$

Determination of human red blood cell hemolysis

In this section, the effect of treatment on the stability of erythrocytes (hemolysis effect) was investigated. The plasma membrane of hRBCs was broken by some compounds, and then hemoglobin was released into the solution. The porphyrin structure can absorb a maximum wavelength of 525 nm. Thus, the absorbance values are related to the direct variation of hRBC lysis. hRBCs were collected, and 2% w/v of hRBCs were resuspended in 1X PBS (pH 7.4) and allowed to stand at 4°C before use. Subsequently, 100 μL of resuspended hRBCs were placed into a 96-well plate; added with 100 μL of samples, 1.0% w/v Triton X-100 (positive control), and 1X PBS (pH 7.4, negative control); and mixed gently. The reactions took place for 30 min at room temperature. The plates were centrifuged at 1,467 *g* for 10 min. A clear supernatant in each condition was transferred into new wells, and the absorbance was measured using a microplate reader at 525 nm. The percentage of hRBC hemolysis was calculated using the following equation:

$$\text{Hemolysis } (\%) = \frac{\text{Ab}_{525}\text{ nm of test}}{\text{Ab}_{525}\text{ nm of negative control}} \times 100$$

Separation of bioactive agents and carbohydrate types using thin-layer chromatography and analysis of ascorbic acid and total sugar content

Thin-layer chromatography (TLC) was used to separate the components of the FD-RKF and Fresh-RKF extracts (total set). Carbohydrates, sugar, and glycoconjugate molecules were separated, and the relative retention factor (R_f) was compared with the standard saccharides. A silica-

coated aluminum sheet was used as a stationary phase. The standard solution of 1% w/v glucose, fructose, galactose, xylose, maltose, lactose, and sucrose was spotted on the starting separation line using capillary tubes. The FD-RKF and Fresh-RKF extracts (total set) were dropped at 1 and 2, respectively. The stationary phase was placed into a chamber that contained the mobile phase (1-butanol:acetone:H_2O at a ratio of 7:2:1), and then the chamber was covered. Subsequently, the mobile phase was moved to the ending line, and the stationary phase was transferred to a hot air oven at 100°C for 10 min. Next, 10% v/v of sulfuric acid in methanol was sprayed onto the stationary phase, which was then transferred to an oven at 100°C for 10 min. The brown spots were represented, and then the R_f of each spot was analyzed using the following formula:

$$R_f = A/B$$

where A is the distance traveled by the substances and B is the distance traveled by the solvent (solvent front).

Determination of total sugars and ascorbic acid of the Fresh-RKF extract

Total sugars are categorized in two subgroups: reducing and nonreducing sugars. The total sugars of the Fresh-RKF extract were determined in accordance with the method of Lane and Eynon (AOAC 923.09) (Sewwandi et al., 2020).

Meanwhile, the total ascorbic acid in the Fresh-RKF extract was analyzed in accordance with the standard AOAC 967.21 method (Nielsen, 2010).

Separation of the Fresh-RKF extract

Phenolic and polyphenolic compounds are the major types of phytochemicals that are present in plants. Thus, the Fresh-RKF extract was separated and analyzed using C18 reverse-phase high-performance liquid column chromatography (NEX QC 1600 HPLC). The separation condition was achieved according to the mobile phase gradient program shown in Table 1. Approximately 1,000 μg/mL of sample was prepared, to 1,000 μg/mL and then 10 μL of sample was injected into the C18 column (Agilent Eclipse Plus C18, 3.5 μm, 4.6×100 mm, Agilent). The system was run with a flow rate of 1.0 mL/min and detected using an ultraviolet detector at 272 nm. The standard phenolic compounds, including gallic acid, chlorogenic acid, vanillin, epicatechin, *p*-coumaric acid, vanillic acid, epigallocatechin-3-gallate, epigallocatechin, and quercetin, were separated under the same purified conditions of the sample. The retention times of each sample peak were compared to those of standard compounds to determine the types of substances in the sample.

Table 1. Mobile phase gradient program for the purification of the Fresh-RKF extract using C18-reverse-phase HPLC

Time (min)	Mobile phase	
	A: 0.1% acetic acid (%)	B: acetonitrile (%)
0.00	93	7
5.00	85	15
10.00	75	25
15.00	60	40
15.01	93	7
20.00	93	7

Fresh-RKF, fresh ripening karanda fruit; HPLC, high-performance liquid column chromatography.

Moreover, the small phytochemical molecules in the Fresh-RKF extract were analyzed using gas column chromatography-mass spectrometry (GC-MS; Agilent 7890B, Agilent), as previously described by Kunu et al. (2025). GC was used to determine the biological agents in the Fresh-RKF extract. An HP-5ms column (25 m×250 μm×0.25 μm) was used to sort the samples, with a flow rate of 1.2 mL/min and helium as the carrier gas. The initial and transfer line temperature of the mass selective detector was conducted at 250°C; the splitless mode was used for 0.75 min. The time program was conducted as follows: The oven was set to 60°C for the first step, held for 3 min, and then ran for 3 min. The oven on Rapid1 ran for 23 min at 80°C. The temperature of the oven was then increased to 120°C and held for 36.33 min. Thereafter, the temperature was increased to 240°C, and the timer stopped after 66.33 min. The electron energy of 70 eV was set for the electron ionization of the Agilent 5977B mass analyzer. The scan mode began from a mass of 45.00 to 500.00, with the ion source set at 230°C and the quadrupole set at 150°C.

Statistical analysis

In this study, all data were analyzed using SPSS Statistics ver. 29.0 (IBM Corp.). The statistical differences between groups were analyzed using one-way ANOVA. The values presented are the mean±SD of three independent experiments. Statistical significance was considered at $P \leq 0.05$.

Human ethics

The study was approved by the Institutional Review Board of the Rajamangala University of Technology Thanyaburi (RMUTT_REC No. Exp 17/65).

RESULTS

Effects of the extraction technique on the DPPH radical scavenging activity and total phenolic contents

The antioxidant properties of phytochemicals in each extracted set (SET 1 to SET 4) of the FD-RKF and Fresh-

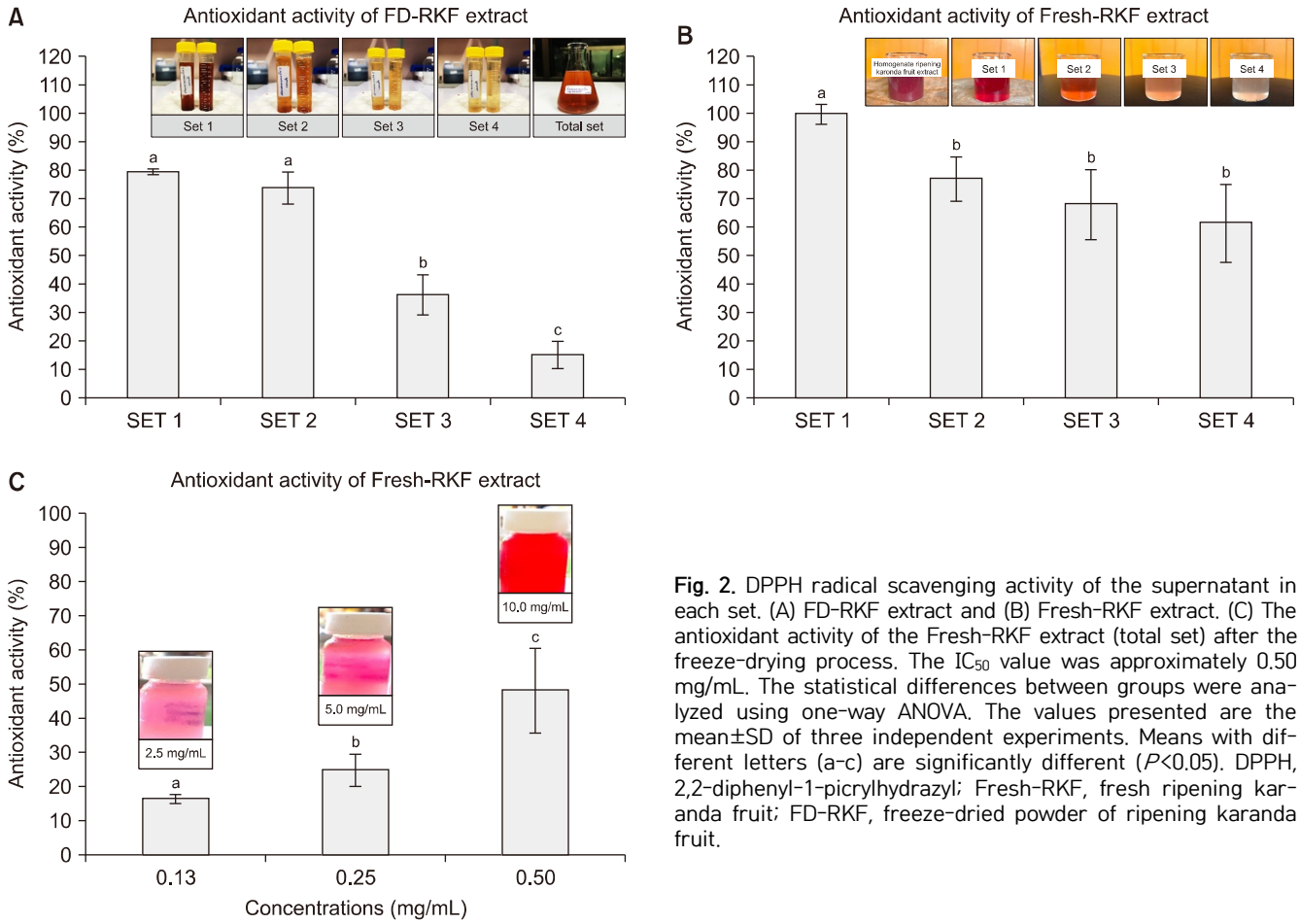

Fig. 2. DPPH radical scavenging activity of the supernatant in each set. (A) FD-RKF extract and (B) Fresh-RKF extract. (C) The antioxidant activity of the Fresh-RKF extract (total set) after the freeze-drying process. The IC_{50} value was approximately 0.50 mg/mL. The statistical differences between groups were analyzed using one-way ANOVA. The values presented are the mean±SD of three independent experiments. Means with different letters (a-c) are significantly different (P<0.05). DPPH, 2,2-diphenyl-1-picrylhydrazyl; Fresh-RKF, fresh ripening karanda fruit; FD-RKF, freeze-dried powder of ripening karanda fruit.

RKF extracts were determined using DPPH assay. The results are shown in Fig. 2A and 2B. The extract in SET1 had higher antioxidant activity than those in SET 2, SET 3, and SET 4 (Fig. 2). The whole set of Fresh-RKF extracts exhibited the ability to scavenge DPPH radicals. The results are shown in Fig. 2C. The IC_{50} value of the Fresh-RKF extract was 0.5 mg/mL, whereas the IC_{50} values of L-ascorbic acid and BHT were 0.004 and 0.003 mg/mL, respectively. Recently, Rumjuankiat et al. (2018) reported that the antioxidant activity of karanda juice presented IC_{50} values of 0.84% (or 8.4 mg/mL). The data indicated that extraction using an ultrasonic instrument influenced the collection of antioxidant components in ripening karanda fruits.

However, the entire set of the FD-RKF extract represented the percent transmittance at 600 nm expressed at 48.50±0.59, indicating turbidity. This problem was solved. The whole set of the FD-RKF extract was incubated with pectinase enzyme (iKnowZyme Pectinase-P2111005, Reach Biotechnology Co., Ltd., specific activity 30,000 units/mL) using an extract: pectinase ratio of 8:2 and 9:1 v/v. The reaction chambers were incubated at 37°C for 2, 4, and 6 h and then placed in a water bath at 100°C for 5 to 10 min to stop pectinase activity. These extracts were called mini-modified FD-RKF extracts. The data showed that the percentage transmittance at 600 nm in all conditions increased to more than 80%. These results suggested that the turbidity in the FD-RKF extract was decreased. Interestingly, the percentage of antioxidation was greater than 95% for all pectinase treatments (data not shown). The results indicated that reducing the turbidity in the extract with pectinase did not affect the antioxidant efficacy. Therefore, a mini-modified FD-RKF extract with an extract:pectinase ratio of 9:1 v/v incubated for 6 h was collected and frozen dry. The powder of this extract was used to study the hemolytic effect of hRBCs in the next step.

Moreover, this technique is also associated with the total phenolic contents, which are presented in Table 2. The data showed that SET 1 had the highest total phenolic content, whereas SET 2, SET 3, and SET 4 had significantly decreased total phenolic contents (all P<0.05). Thus, the program time of extraction using ultrasonication at low temperatures could collect phytochemicals and be suitable to repeat four times.

Cytotoxic effect of ripening karanda fruit extract on human peripheral blood mononuclear cells

Fruit extracts or fruit juice are used to develop concentrated drinks or syrup-based beverages. Food safety

Table 2. Total phenolic contents of karanda fruit extracts

Extraction set	Total phenolic contents (mg of gallic acid/mL supernatant)	
	FD-RKF extract	Fresh-RKF extract
SET 1	0.497±0.029[a]	0.7247±0.0115[a]
SET 2	0.215±0.012[b]	0.2471±0.0134[b]
SET 3	0.095±0.005[c]	0.0539±0.0030[c]
SET 4	0.049±0.005[d]	0.0374±0.0031[d]

Values are showed as mean±SD.
The superscript letters (a-d) show statistically significant differences within the same extract at $P<0.05$. The statistical information was analyzed using one-way analysis of variance. FD-RKF extract, freeze-dried powder of ripening karanda fruit extract; Fresh-RKF extract, fresh ripening karanda fruit extract.

is the foremost priority in the development of food products. Thus, the extract samples should be studied for toxicity and side effects in many cell systems of the human body. In the present study, hPBMCs were used to investigate the cytotoxic effect of the FD-RKF and Fresh-RKF extracts (whole set). The results are shown in Fig. 3. The percent survival of hPBMCs decreased to <10% after treatment with 20% v/v DMSO (positive control).

Interestingly, the percent survival of hPBMCs was more significant than 100% for both extracts at a concentration of 0.63 to 2.50 mg/mL. However, the percent survival of hPBMCs was reduced to <50% at treatment concentrations of 5.0, 10.0, or 20.0 mg/mL. The EC_{50} was approximately 3.75 mg/mL.

Hemolysis effect

hRBCs are cells that carry oxygen to target cells through the blood circulation system. Oxidizing nutrients and biomolecules, which produce energy, depend critically on oxygen. However, the components in food sources may have a hemolytic effect on hRBCs. Thus, the safety and side effects of food components on hRBCs need to be determined, especially for patients with hemolytic anemia and G6PD deficiency (Garcia et al., 2021; Ryan and Tekwani, 2021). In the present study, the hemolytic effect of the FD-RKF and Fresh-RKF extracts (whole set) on hRBCs was investigated. The results are shown in Table 3. The data indicated that 1.0% w/v Triton X-100 (positive control) resulted in 100% hRBC hemolysis, whereas 1X PBS (negative control) resulted in <1% hRBC hemolysis. The final concentration of the FD-RKF and Fresh-RKF extracts (whole set) was less than 10.0 mg/mL, which resulted in a percent hemolysis of <1.0%. However, the percent hemolysis was higher than 50.0% after treatment with 10.0 mg/mL of extracts.

Thin-layer chromatography data

The types of saccharides were investigated using TLC. The results are shown in Fig. 4. Both extracts (Lanes 1 and 2) showed a smear brown spot. The R_f of fructose was similar to the spot in Lane 1. However, the major spots of both extracts showed a characteristic conjugation with other compounds, such as glycosides. Therefore, the reducing and nonreducing sugars in karanda fruit extracts might be related to saccharide conjugated with phytochemicals.

Reducing and nonreducing sugars and ascorbic acid in karanda fruit extracts

The Fresh-RKF extract was analyzed for reducing and nonreducing sugars using the Lane-Eynon method. The total sugar of the Fresh-RKF extract was 0.27%, with reducing and nonreducing sugars comprising 0.25% and

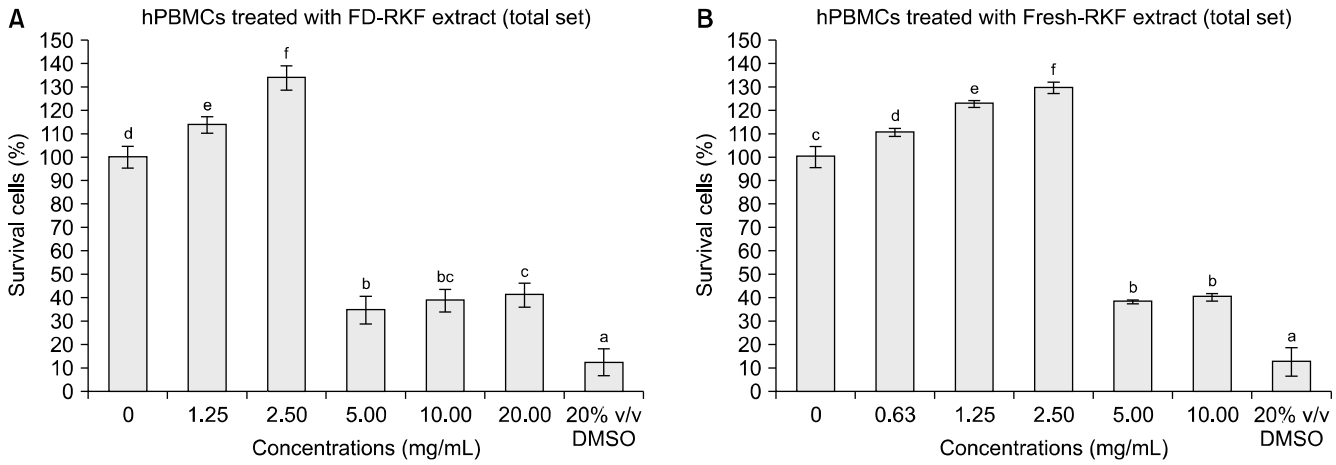

Fig. 3. Cytotoxic effect of the (A) FD-RKF extract (total set) and (B) Fresh-RKF extract (total set) on hPBMCs. RPMI medium without the sample was used as the negative control, whereas 20% v/v DMSO was used as the positive control. The percentage of survival cells is represented as mean±SD. The statistical differences between groups were analyzed using one-way ANOVA. The letters (a-f) showed statistically significant differences at $P<0.05$. FD-RKF extract, freeze-dried powder of ripening karanda fruit extract; Fresh-RKF extract, fresh ripening karanda fruit extract; hPBMCs, human peripheral blood mononuclear cells; RPMI, roswell park memorial institute; DMSO, dimethyl sulfoxide.

Table 3. Hemolysis effect of the FD-RKF extract, Fresh-RKF extract, and FD-RKF extract+pectinase

Samples	Concentration	Hemolysis (%)
Triton X-100	1.0% w/v	100±0.00
PBS control (−)	1X	Less than 1%
FD-RKF extract	10.0 mg/mL	71.84±6.97
	5.0 mg/mL	Less than 1%
	2.5 mg/mL	Less than 1%
Fresh-RKF extract	10.0 mg/mL	62.84±3.05
	5.0 mg/mL	Less than 1%
	2.5 mg/mL	Less than 1%
FD-RKF extract + pectinase (mini-modified FD-RKF extract)	10.0 mg/mL	Less than 1%
	5.0 mg/mL	Less than 1%
	2.5 mg/mL	Less than 1%

Values are presented as mean±SD.
FD-RKF extract, freeze-dried powder of ripening karanda fruit extract; Fresh-RKF extract, fresh ripening karanda fruit extract.

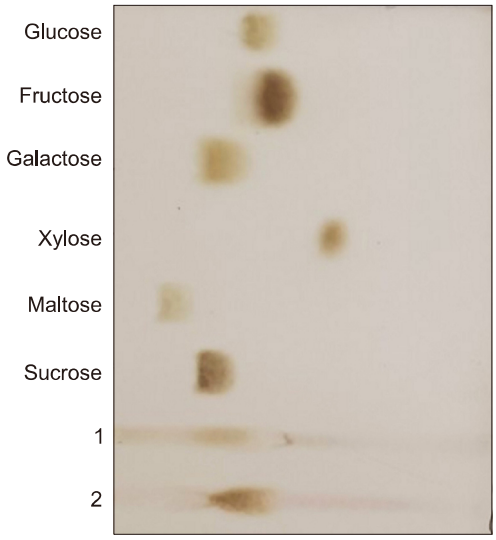

Fig. 4. Thin-layer chromatography profile of carbohydrates and phytochemical agents in ripening karanda fruit extracts. Lane 1, FD-RKF extract; Lane 2, Fresh-RKF extract. FD-RKF extract, freeze-dried powder of ripening karanda fruit extract; Fresh-RKF extract, fresh ripening karanda fruit extract.

0.02%, respectively.

In addition, the percentage of ascorbic acid was 0.01%. Ascorbic acid is displayed effectively in antioxidants (Chambial et al., 2013). Kumar et al. (2013) found that ripening karanda fruits contained more ascorbic acid than semi-ripening and raw karanda fruits. In the present study, the Fresh-RKF extract contained 0.01% w/w ascorbic acid. The data showed that ascorbic acid degraded slightly throughout the extraction process. Thus, the antioxidant activity of the Fresh-RKF extract was associated with the presence of ascorbic acid, which is consistent with previous research (Kumar et al., 2013).

Types of phytochemicals

The results of phytochemical separation by C18 reverse-phase HPLC are shown in Fig. 5. The retention times of nine standard phenolics/flavonoids were used for comparison. The chromatogram profiles of standard compounds and the Fresh-RKF extract are shown in Fig. 5A and 5B, respectively. As shown in the figure, the purified

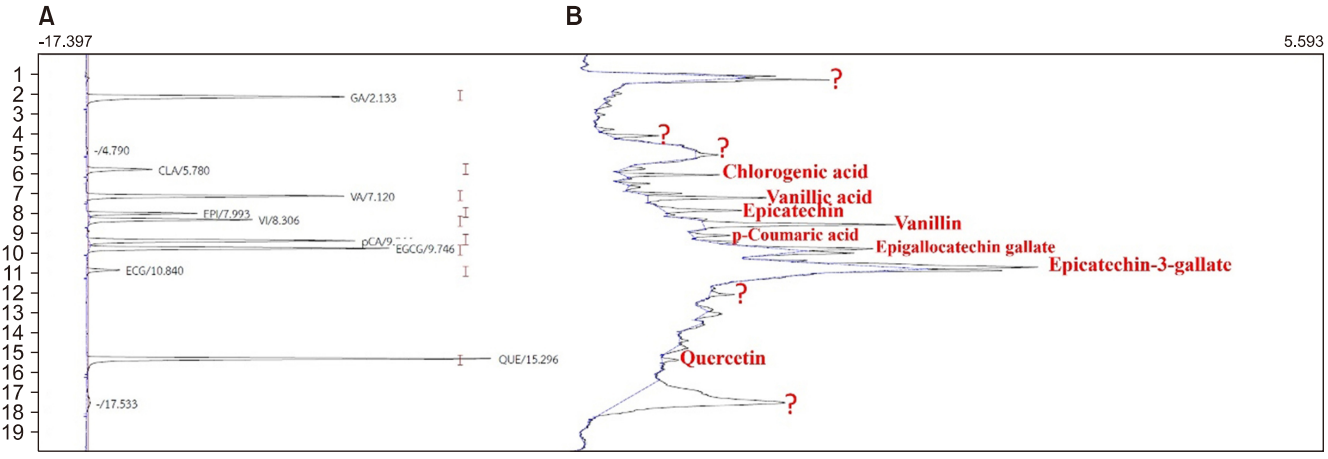

Fig. 5. Chromatography profile of the standard phenolic compounds and karanda fruit extracts separated by C18 reverse-phase HPLC. (A) The profile of standard phenolics and (B) ripening karanda fruit extract. GA, gallic acid; CLA, chlorogenic acid; VA, vanillic acid; EPI, epicatechin; p-CA, p-coumaric acid; EGCG, epigallocatechin gallate; ECG, epicatechin-3-gallate; QUE, quercetin; HPLC, high-performance liquid column chromatography.

peaks presented similar retention times to those of chlorogenic acid, vanillic acid, vanillin, epigallocatechin gallate, and queretin. Therefore, the Fresh-RKF extract might consist of these phenolic compounds. Future studies should perform liquid chromatography-mass spectroscopy to determine the types of phenolic compounds in the Fresh-RKF.

The data of small molecules in the Fresh-RKF extract analyzed by GC-MS are represented in Table 4. As shown in the table, several phytochemicals were observed. The main components and classes with an peak area percentage >1% were reported, including 1H-imidazole-4-ethanolamine, N,5-dimethyl-; dibutyltin maleate; 5-alpha-aminoethyltetrazole; 1,2-propadiene-1,3-dione; 2-furancarboxaldehyde, 5-methyl-; 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one; 2H-tetrazole, 2-methyl-; propylamine, N,N,2,2-tetramethyl-, N-oxide; sydnone, 3,4-dimethyl-; dimethyl-1,2,3-oxadiazol-3-one; hydrogen isocyanate; delta-2-tetrazaboroline, 5-ethyl-1,4-dimethyl-; (4R,5R)-(+)-2-chloro-4,5-dimethyl-1,3,2-dioxaphospholane-2-oxide; 1-cyclopentanethiol, 2-(diethylboryloxy)-; 2-butenedioic acid (E)-, monomethyl ester; 2-propanamine, N-methyl-N-nitroso-; 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; benzoic acid, silver(1+)-salt; catechol; 1H-tetrazole, 1-methyl-; 5-hydroxymethylfurfural (HMF); malic acid; pyrimidine-5-methyl-1-oxide; 3-phospholene, 3-

Table 4. Biological compounds in the fresh ripening karanda fruit extract investigated by gas column chromatography-mass spectrometry

No.	Biological compound	RT (min)	%Area (higher 1%)	Formula	Score	Mass	CAS
1	1H-Imidazole-4-ethanolamine, N,5-dimethyl-	3.820	2.11	$C_7H_{13}N_3$	47.61	139	53966-46-4
2	Dibutyltin maleate	4.234	100	$C_{12}H_{20}O_4Sn$	79.96	219	1000425-93-2
	Dibutyltin maleate	4.671	7.48		56.35		
3	5-Alpha-aminoethyltetrazole	4.869	3.38	$C_3H_7N_5$	54.28	113	1000227-36-7
	5-Alpha-aminoethyltetrazole	5.837	1.20		67.33		
4	1,2-Propadiene-1,3-dione	6.455	47.06	C_3O_2	83.99	68	504-64-3
5	2-Furancarboxaldehyde, 5-methyl-	7.032	4.91	$C_6H_6O_2$	69.92	110	620-02-0
6	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	7.521	11.34	$C_6H_8O_4$	57.53	144	10230-62-3
7	2H-tetrazole, 2-methyl-	8.757	3.64	$C_2H_4N_4$	60.17	84	16681-78-0
8	Propylamine, N,N,2,2-tetramethyl-, N-oxide	8.996	1.11	$C_7H_{17}NO$	43.65	131	13993-87-8
9	1,2-Propadiene-1,3-dione	9.462	1.65	C_3O_2	53.89	68	504-64-3
10	Sydnone, 3,4-dimethyl-	9.561	1.11	$C_4H_6N_2O_2$	55.10	114	4007-18-5
11	Dimethyl-1,2,3-oxadiazol-3-one	9.701	2.09	$C_4H_6N_2O_2$	44.68	114	1000445-12-4
12	Hydrogen isocyanate	10.010	5.05	CHNO	50.70	44	75-13-8
13	Delta-2-tetrazaboroline, 5-ethyl-1,4-dimethyl-	11.362	38.27	$C_4H_{11}BN_4$	57.26	126	20534-01-4
14	(4R,5R)-(+)-2-Chloro-4,5-dimethyl-1,3,2-dioxaphospholane-2-oxide	11.496	1.58	$C_4H_8ClO_3P$	36.76	182	89104-48-3
15	1-Cyclopentanethiol, 2-(diethylboryloxy)-	12.377	2.47	$C_9H_{19}BOS$	53.14	186	1000161-56-3
16	2-Butenedioic acid (E)-, monomethyl ester	12.884	4.05	$C_5H_6O_4$	68.09	130	2756-87-8
17	2-Propanamine, N-methyl-N-nitroso-	13.531	4.23	$C_4H_{10}N_2O$	66.91	102	30533-08-5
18	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	13.921	35.58	$C_6H_8O_4$	70	144	28564-83-2
19	Benzoic acid, silver(1+)-salt	14.842	1.53	$C_7H_5AgO_2$	56.81	229	532-31-0
20	Catechol	16.352	5.60	$C_6H_6O_2$	65.40	110	120-80-9
21	1H-Tetrazole, 1-methyl-	17.319	1.39	$C_2H_4N_4$	54.97	128	16681-77-9
22	5-Hydroxymethylfurfural	17.681	34.39	$C_6H_6O_3$	67.42	126	67-47-0
23	Malic acid	18.264	1.01	$C_4H_6O_5$	39.99	134	6915-15-7
24	Pyrimidine-5-methyl-1-oxide	19.913	20.07	$C_5H_6N_2O$	64.07	110	17758-50-8
25	3-Phospholene, 3-chloro-1-methyl-, 1-oxide	21.289	4.68	C_5H_8ClOP	42.46	150	22356-34-9
26	Furazanamine, 4-azido-	24.256	1.99	$C_2H_2N_6O$	38.11	127	78350-49-9
27	1,2-Benzenediol, 3-methoxy-	26.558	1.11	$C_7H_8O_3$	51.79	140	934-00-9
28	4-Hydroxy-benzoic acid	28.720	12.76	$C_7H_6O_3$	68.09	138	99-96-7
29	2,6-Dimethyl-4-pyridinamine 1-oxide	29.560	1.41	$C_7H_{10}N_2O$	46.89	138	3512-82-1
30	Lactic acid,	35.237	1.44	$C_7H_{13}BO_3$	62.05	156	24372-01-8
	monoanhydride with 1-butaneboronic acid,	35.318	1.74		62.08		
	cyclic ester	35.522	12.38		61.68		
31	Myo-inositol, 4-C-methyl-	42.167	4.89	$C_7H_{14}O_6$	62.30	194	472-95-7

RT, retention time; CAS, chemical abstract service number.

chloro-1-methyl-, 1-oxide; furazanamine, 4-azido-; 1,2-benzenediol, 3-methoxy-; 4-hydroxy-benzoic acid; 2,6-dimethyl-4-pyridinamine 1-oxide; lactic acid, monoanhydride with 1-butaneboronic acid, cyclic ester, and myo-inositol, 4-C-methyl-.

DISCUSSION

Studies on natural products offer techniques for extracting valuable components from natural raw materials. Ripening karanda fruits reportedly have high phenolic and flavonoid contents (Kumar et al., 2013). These phytochemicals exhibit antioxidant capabilities, which are essential for protecting biomolecules from free radicals (Silva et al., 2000).

In the present study, ripening karanda fruit extracts were extracted under low-temperature hydrophilic conditions using two methods (i.e., homogenization and ultrasonic extraction). The appropriation of repeating extracted times of ripening karanda fruit using ultrasonic (45 kHz, normal amplitudes) should be performed 3 to 4 times. In this study, extraction process required approximately 4 h, which is shorter than immersion or fermentation methods. The biological substances in ripening karanda fruits can be collected in more than just one extraction cycle. A comparison of the extract preparation from Fresh-RKFs and frozen dry powder of ripening karanda fruits showed that the FD-RKF extract had a lower percentage of transmittance (higher turbidity) than the Fresh-RKF extract. Previous studies have reported that using cold water in the extraction process of ripening karanda fruits may help to stabilize some bioactive compounds (Sahreen et al., 2010; Rumjuankiat et al., 2018).

The turbidity of the FD-RKF extract was improved by incubating with pectinase (Patel et al., 2022) for 6 h, and the reaction was stopped at 80°C for 5 min. The resulting product was called mini-modified FD-RKF extract. This process results in a higher percentage of transmittance (>80%) in the FD-RKF extract. The antioxidant properties of both extracts (with and without pectinase treatment) were analyzed and compared. The results showed that the percentage of antioxidation at the same concentration was higher than 80% (data not shown). These data indicated that the antioxidant properties of both extracts differed significantly. The results of this test showed that the process of removing turbidity with the enzyme pectinase had no effect on the antioxidant efficacy of the extract. However, this process can reduce the extract's turbidity. This is an advantage of developing fruit juice extracts that show functional benefits in the health of beverage and supplement industries in the future.

The results of GC-MS indicated that various types of compounds are present in the Fresh-RKF extract. These substances may be associated with the detected biological properties. For example, tetrazole and its derivatives displayed various biological activities. Kaushik et al. (2018) reported that the synthesized indolyl tetrazolopropanoic acid derivatives and the synthesized 3-substituted-5-(1-phenyl-1H-tetrazole-5-yl) methyl expressed antioxidant properties. In the present study, 5-alpha-aminoethyltetrazole, 1H-tetrazole, 1-methyl-, and 2H-tetrazole, 2-methyl- were detected in the Fresh-RKF extract. HMF is a molecule that is found in many sources of fruit extracts. According to Shapla et al. (2018), HMF exhibits antioxidant properties, which is similar to the findings of Zhao et al. (2013). HMF can downregulate xanthine oxidase, block immune-mediated allergic reactions, and prevent sickle hemoglobin (Shapla et al., 2018) including anticancer effects (Joel and Maharjan, 2021). Therefore, the antioxidant properties of the Fresh-RKF extract may be related to these phytochemicals.

Furthermore, the percentage of hPBMC viability was greater than 95% when the concentration of the extract was ≤2.5 mg/mL. This increased hPBMC viability may be related to the amount of sugar, types of sugar, and some biological agents detected in the extract (Fig. 4 and Table 4). The Fresh-RKF extract had a total sugar level of 0.27%. The TLC profile showed the sample's R_f values, which were similar to the standard fructose that is classified as a reducing sugar. Reducing sugar can promote cell proliferation by acting as the carbon source (Han et al., 2015; Arya et al., 2022; Roman-Benn et al., 2023). However, the high concentration (>2.5 mg/mL) of the Fresh-RKF and FD-RKF extracts resulted in a survival percentage of <50% of hPBMCs, indicating that the phytochemicals in both extracts had a cytotoxic effect on hPBMCs. This result may be due to certain groups of substances that are constituents in both extracts, as shown in Table 4. For example, dibutyltin maleate, which was detected in the Fresh-RKF extract, exhibits inflammation-inducing behavior in adipocytes and macrophages (Milton et al., 2017). HMF exerts genotoxic, indirect mutagenic, carcinogenic, and nephrotoxic effects. Moreover, it causes DNA damage. It also restrains DNA polymerase γ (Shapla et al., 2018). Thus, the increased concentration of the Fresh-RKF extract exhibited a cytotoxic effect on hPBMCs, which may be associated with the presence of HMF.

Moreover, the phytochemicals of the Fresh-RKF and FD-RKF extracts exhibited a hemolytic effect on hRBCs at concentrations of ≥10.0 mg/mL. The percent hemolysis of hRBCs was greater than 50% after treatment with 10.0 mg/mL of the extract. The same categories of substances may be responsible for the breakdown of blood cells by phytochemicals in the Fresh-RKF and FD-RKF extracts, as shown in Table 4. Interestingly, 10.0 mg/mL of the mini-modified FD-RKF extract exerted a

nonhemolytic effect on hRBCs (Table 3). There are several reasons for this finding. First, the structure of some compounds that induce the hemolysis effect may be altered or degraded by high temperatures. Recent research has indicated that high temperatures affect biological activity and the degeneration of phytochemicals. This factor could promote the denaturation or degeneration of compounds (Senisterra et al., 2012). Thus, high temperatures might cause the mini-modified FD-RKF extract to have a reduced percentage of erythrocyte break. Second, the high number of pectin and its derivatives might exert a hemolytic effect on hRBCs (Arya et al., 2022). Therefore, the mini-modified FD-RKF extract treated with pectinase decreased the number of pectins, thereby decreasing the hemolytic effect.

These data suggest that ripening karanda fruits can be used as raw materials for the development of food supplements. The cytotoxic and hemolysis concentrations of the extract could suggest caution in food safety, including supporting future clinical research. Food supplements should be produced similar to pharmaceuticals to guarantee safety and efficacy, including identification of mechanisms of action, chemical and biological standardization, preclinical testing for toxicity and drug-botanical interactions, metabolism, and clinical testing (van Breemen, 2015).

In conclusion, the fresh and frozen dry powder of ripening karanda fruits could collect the bioactive agents by the extracted condition as the regular amplitude, 35 to 45 kHz of the ultrasonic frequency, and repeated extraction cycle 3 to 4 times. The Fresh-RKF and FD-RKF extracts exerted a cytotoxic effect on hPBMCs at concentrations greater than 2.5 mg/mL. Both extracts improved the percent hemolysis of hRBCs at a concentration of 10.0 mg/mL. The turbidity of the FD-RKF extract could be decreased by pectinase treatment. Moreover, pectinase treatment did not affect the antioxidant activity. This method could reduce the percentage hemolysis of hRBCs *in vitro*. Therefore, the extraction process and biological properties of the Fresh-RKF and FD-RKF extracts could be used to develop the food and beverage industries.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: SP. Analysis and interpretation: AK, AW. Data collection: AK. Writing the article: WK, SP. Critical revision of the article: SP, WK. Final approval of the article: all authors. Statistical analysis: WK. Obtained funding: SP. Overall responsibility: SP.

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