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Content

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Effects of Boiling and Steaming Time on Physicochemical Properties of Unripe Banana Pulp and Flour

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Abstract

The effects of boiling and steaming times on physicochemical properties of unripe banana (*Musa* ABB cv. Kluai Namwa) pulp and unripe banana flour were investigated. Unripe bananas were boiled or steamed by using boiling water for 15, 30 and 45 min and compared unheated (raw) banana acted as a control. The hardness of unripe banana pulp significantly decreased with boiling and steaming times from 36.73 N to 24.29 and 22.97 N, respectively ($p< 0.05$). However, pulp brightness (L*) from both processes decreased with heating time ($p< 0.05$). Ash and soluble dietary fiber of unripe banana flour showed markedly increases with heating time. Whereas fat, protein, total dietary fiber, insoluble dietary fiber content and resistant starch content of the pulp decreased inversely with the boiling and steaming time. The boiling and steaming process caused the decreased pasting temperature (from about 87.23 to 67.70°C). While, breakdown and setback viscosity of unripe banana flour increased to 37.46 and 56.13 RVU, respectively. Total phenolic content (TPC) and antioxidant activity (2,2-diphenyl-1-picrylhydrazyl, DPPH; and Ferric reducing antioxidant power, FRAP) of the flour increased directly with boiling and steaming time. Results indicated that the increase in antioxidant activity shown by DPPH and FRAP values of heated banana flour corresponded to an increase in phenolic compounds. Finally, boiled banana flour had higher TPC, DPPH and FRAP values than steamed banana flour. The findings can promote the application of boiled and steamed banana flour as a beneficial ingredient in the food industry.

Introduction

Banana (*Musa* sp.) is an important food crop that is widely cultivated in Southeast Asia, South Asia and West Africa, alongside rice, wheat, maize and tapioca (Aurore et al., 2009; Chaipai et al., 2018). In Thailand, common banana varieties include Kluai Hom, Kluai Namwa, Kluai Hakmuk and Kluai Khai. Kluai Namwa

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(*Musa* ABB) is the most extensively grown, accounting for 70% of the total production (Suvittawat et al., 2014). Bananas are rich sources of important nutrients including carbohydrates, vitamins and minerals, as well as functional components, such as dietary fibers and polyphenols, which all play important roles in promoting health and well-being (Singh et al., 2016). The 100 g of Kluai Namwa contains 91.71-96.01% carbohydrate, 0.05-5.56% protein, 0.00-0.79% fat and 7.98-10.39% total dietary fiber (Vatanasuchart et al., 2015). Ripe bananas have a high amount of sugar and are eaten raw as a sweet fruit or used as a dessert ingredient. On the other hand, unripe bananas are a good source of starch, accounting for 70-80% of the total composition (Chaipai et al., 2018). Bananas are processed into starch and flour, then used as culinary ingredients or as a drug with beneficial properties, such as the treatment of peptic ulcers (Jirukkakul & Rakshit, 2011). Unripe banana flour is rich in resistant starch (RS) about 49-65% (Menezes et al., 2011; Rodríguez-Damian et al., 2013) and has been evaluated as a functional ingredient that plays an important role in decreasing the glycemic index (Detchewa et al., 2021). Several studies indicated that the polyphenol content of bananas had high potential as a food source with preventive health benefits. Total phenolic content (TPC) in unripe bananas ranges from 0.90% to 3.0% (dry weight), mainly as tannin components, polymeric catechins and flavonoids (Jannoey et al., 2021; Sulaiman et al., 2011).

Unripe bananas are generally cooked (e.g., boiling, steaming, frying, or roasting) before consumption. However, when the unripe are fruit is cooked, the physicochemical qualities changed and natural RS becomes digestible (Muyonga et al., 2001; Rodríguez-Damian et al., 2013; Chaipai et al., 2018; Tsamo et al., 2015). The way different varieties and ripening stages impact banana properties have been recently reported in the literature, but to the best of our knowledge changes in the physicochemical properties, rheology and health-promoting benefits of unripe bananas after heat processing, such as boiling and steaming, have yet to be clearly defined. Better use of bananas might be obtained by examining how various types of processing and process conditions influence the quality of heated bananas. Therefore, the effects of boiling and steaming duration time on the properties of the pulp and flour from unripe bananas were investigated.

Materials and methods

1. Materials

Unripe or green bananas (*Musa* ABB cv. Kluai Namwa) with 90 days of age, counted from the time the banana blossom produced. The bananas were obtained from a farm in Khlong Luang District, Pathum Thani, Thailand.

2. Boiling and steaming process

Banana fingers were separated from the hand, washed in cleaned water at room temperature and then heated by boiling and steaming. For the boiling process, unripe bananas (22 fruits) were submerged in 3,000 mL of boiling water (100°C) for 15 min (BB15), 30 min (BB30) and 45 min (BB45). Steaming (~110 °C) was carried out by placing unripe bananas in a steamer and steamed with boiling water for 15 min (SB15), 30 min (SB30) and 45 min (SB45). The temperature at the center of boiled and steamed bananas varied from 88°C to 96°C and from 83°C to 90°C, respectively. The heated unripe bananas were immediately soaked in cold water at 4°C for 30 min to reduce the temperature.

3. Banana flour preparation

The raw, steamed and boiled banana fruits were peeled, cut into 0.5 cm thick slices and then freeze-dried (Home freeze dryer, FD4L model, Epsilon Co. Ltd., Thailand). The dried samples were grounded by using a grinder (Fritsch, Pulverisette16, Germany), sifted through a 177 μm (80 mesh) filtered sieve (Fritsch, Analysette 3 Spartan, Germany), packaged in plastic bags and stored in a desiccant jar for further analyses. Consequently, the flours were obtained from raw bananas that had been boiled and steamed at 15, 30 and 45 min resulting as BBF15, BBF30, BBF45, SBF15, SBF30 and SBF45, respectively.

4. Determination of pulp properties

4.1 The moisture content of banana pulp

The moisture content of banana pulp was determined by gravimetric heating at 105°C using a 3-5 g sample according to the AOAC (2004) method in triplicate.

4.2 Hardness analysis of banana pulp

Analysis of hardness was performed by using a Texture Analyzer (TA.XT plus Texture Analyzer, Stable Micro Systems Ltd., UK). The measurement was performed using a penetration probe (6 mm diameter). The texture analyzer was set in return-to-start mode with a pre-test speed of 1.0 mm/s, test speed of 2.0 mm/s and post-test speed of 10 mm/s. Banana pulp samples were positioned in the middle of the texture analyzer platform

and penetrated 50% of the sample height.

4.3 Color analysis of banana pulp

Color scales were determined by using a colorimeter (Color Flex 4510, VA, USA). The scales L*, a* and b* indicating in terms of lightness, redness/greenness and yellowness/blueness of banana pulp, respectively. The parameters were L* (L* = 0 (black) and L* = 100 (white)), a* (–a= greenness and +a = redness) and b* (–b = blueness and +b = yellowness).

5. Determination of flour properties

5.1 Protein, fat and ash content of banana flour

The banana flour was analyzed for chemical composition (protein, fat and ash content) according to the AOAC (2004) method in triplicate. Protein content was determined by the Kjeldahl method. Soxhlet extraction was used for total crude fat content. Ash content was estimated after incinerating 1 g of the sample in a muffle furnace at 525°C and calculated as follows:

$$\text{Crude ash } (\%) = \left(\frac{W_1 - W_2}{\text{Weight of sample}}\right) \times 100$$

where: W_1 is the weight of the crucible with ash and W_2 is the weight of the empty crucible.

5.2 Dietary fiber

The total, insoluble and soluble dietary fiber contents were measured using a Megazyme test kit (Megazyme International Ltd., Ireland) following AACC (2012) Method 32-05.01: total dietary fiber (TDF) and method 32-21.01: soluble/insoluble dietary fiber. To remove starch and protein, 1 g of flour was exposed to sequential enzymatic digestion by heat-stable α-amylase, protease and amyloglucosidase. The sample solution was filtered to remove insoluble dietary fiber (IDF) and the filtrate was treated with 95% (w/v) ethanol to precipitate the soluble dietary fiber (SDF). IDF and SDF residues were dried for the final estimation of dietary fiber content.

5.3 Resistant starch

Resistant starch (RS) was determined by using the Megazyme assay kit (Megazyme International Ltd.) following AOAC (2011) Method 2002.02: resistant starch. Flour samples were digested overnight with α-amylase and amyloglucosidase. To remove soluble starch, the flour suspension was consecutively washed with 95% and 50% ethanol. The pellet was then dissolved in potassium hydroxide (KOH) solution, hydrolyzed with amyloglucosidase and spectrophotometrically quantified using a glucose oxidase-peroxidase (GOPOD) reagent.

5.4 Pasting viscosity

The pasting properties of banana flour were determined by using a Rapid Visco Analyzer (RVA-Super3, Warriewood, Australia). Briefly, 2.5 g of flour, corrected to dry weight basis, was dispersed in 25 mL of water in an aluminum can. The suspension was then heated at 50°C for 1 min with a rotation of 160 rpm. The temperature was raised to 95°C at a constant rate (14°C/min) and held at 95°C for 4 min. Finally, the samples were cooled to 50°C (14°C/min) and held at 50°C for 2 min. Variations in the viscosity of the flour suspension including peak viscosity (PV), minimum viscosity (MV), breakdown (BD), final viscosity (FV), setback (SB) and pasting temperature (PT) were recorded.

5.5 Total phenolic content and antioxidant activity

Banana flour extraction was performed following the procedure of Moongngarm et al. (2014) with some modifications. Two grams of banana flour were placed in 25 mL of 80% ethanol and extracted in an ultrasonic bath (2600D model, CREST, Malaysia) at 35-37°C for 1 hr. After one hour of extraction, the extract was filtered through Whatman No. 1 paper. A rotary evaporator (Heidolph,Vakuumbox Hei-VAP,Germany) was used to concentrate the filtrate at 40°C and the weight of each extract was recorded. The extract was then re-dissolved in 80% ethanol to a final volume of 25 mL and used for phenolic content and antioxidant analyses.

Total phenolic content (TPC) of banana flour were determined according to the method described by Fatemeh et al. (2012) with some modifications. The sample extract (100 µL) was mixed with 750 µL of Folin-Ciocalteu's phenol reagent and 500 µL of distilled water and allowed to stand for 5 min. Then, 750 µL of 6% (w/v) sodium carbonate (Na_2CO_3) solution was added to the mixture. The absorbance was measured after 90 min of reaction at 765 nm using a spectrophotometer (Gene quant 1300, Kista, Sweden). Total phenolic content (TPC) was calculated using the gallic acid calibration curve within the dilutions of 0-0.1 mg/mL and TPC values were expressed as mg gallic acid equivalent (GAE) per 100 g of dry matter. All experiments were performed in triplicate.

The radical scavenging ability of the flour samples was evaluated using a DPPH free radical scavenging assay, following the modified modification method of Fatemeh et al. (2012). Briefly, 100 µL of the diluted extract was mixed with 750 µL of 0.025 g/L DPPH methanolic solution and allowed to stand in darkness

for 15 min. The absorbance of the resulting solution was measured at 517 nm using a UV-visible spectrophotometer. Dilutions of different concentrations (0.01-0.05 mg/L) of ascorbic acid were used to plot a calibration curve, with results expressed as milligrams of ascorbic acid equivalent (AAE) per g of sample dry basis.

The FRAP assay was performed following the method of Benzie & Strain (1999). In brief, FRAP reagent was prepared using a volume ratio of 10:1:1 of 300 mmol/L sodium acetate buffer (pH 3.6), 10 mmol/L 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution (40 mmol/L hydrochloric acid (HCl) as solvent) and 20 mmol/L iron (III) chloride ($FeCl_3$) solution. The FRAP reagent was warmed in a water bath to 37°C before use. A 30 µL aliquot of banana flour extract was added to 2.7 mL of the FRAP reagent and allowed to stand for 20 min. The absorbance was then measured at 615 nm using a spectrophotometer. Ferrous sulfate ($FeSO_4$) solution (100–500 µmol) was used to construct the standard curve, with results expressed as mmol $FeSO_4$ per g of sample dry basis.

6. Statistical analysis

All measurements were performed in triplicate, with results presented as mean values and standard deviation (SD). Data were subjected to analysis of variance (ANOVA), with comparison among means determined according to Duncan's new multiple range test using IBM SPSS Statistics v.20 (International Business Machines (IBM) Corporation, Armonk, NY, USA). The significance between means was determined by the least significant difference values at the 5% level.

Results and discussion

1. Physicochemical properties of boiling and steaming banana pulp

The effects of boiling and steaming on changes in moisture content, hardness and color of the banana pulp are shown in Table 1. The results showed that the heating process significantly ($p<0.05$) affected the pulp moisture content that increased directly with boiling and steaming time but no significant difference in moisture content was recorded for samples heated for 30 and 45 min. This might be due to the boiling and steaming process providing large amounts of water and vapor, causing the transfer of water into the banana pulp throughout the process (Tsamo et al., 2015). However, water diffuses into banana pulp continuously with boiling and steaming duration until maximum, resulting in no further water diffusion. As a result, no difference in moisture content was found between boiled or steamed samples heated for 30 and 45 min. At the same boiling and steaming duration, the steamed samples had less moisture than the boiled samples. This occurred because boiling delivered more water to bananas than steaming, consequently, boiling bananas in plenty of water increased their water content due to osmotic exchange (Gafuma et al., 2018).

Table 1 Physical properties of raw, boiled and steamed unripe banana pulp

Processing	Moisture content (%)	Hardness (N)	Color		
			*L**	*a**	*b**
Raw banana	66.26±0.25^{d}	36.73±1.66^{a}	86.26±0.29^{a}	1.17±0.18^{bc}	16.71±0.49^{a}
BB15	68.22±0.21^{b}	28.32±0.30^{b}	71.72±1.77^{b}	0.85±0.53^{bc}	16.64±2.75^{a}
BB30	68.66±0.21^{a}	26.42±0.06^{c}	67.95±0.66^{cd}	1.48±0.47^{bc}	12.22±1.37^{b}
BB45	68.92±0.30^{a}	24.29±0.32^{d}	65.01±0.44^{d}	2.69±0.80^{a}	12.27±0.32^{b}
SB15	67.04±0.09^{c}	27.05±0.65^{bc}	69.17±2.27^{bc}	0.56±0.03^{c}	13.43±0.28^{b}
SB30	68.20±0.17^{b}	26.22±0.87^{c}	68.16±2.91^{c}	1.63±0.79^{bc}	11.92±1.65^{b}
SB45	68.30±0.10^{b}	22.97±0.11^{d}	67.22±1.02^{cd}	3.52±0.29^{a}	12.19±0.32^{b}

Remark: a – d = Means ± SD (n = 3) with different lowercase superscript letters within a column are significantly different ($p < 0.05$)
BB = Unripe bananas heated by boiling
SB = Unripe bananas heated by steaming
15,30,45 = boiling and steaming time of unripe bananas (min)

The boiling and steaming process had a significant ($p<0.05$) effect on the textural characteristic of unripe banana pulp, resulting in a decrease in hardness. Independent of the boiling and steaming process, the textural hardness of all heated banana pulp decreased with increasing boiling and steaming time from 15 to 45 min. Boiling and steaming reduced hardness rapidly in the first 15 min and slightly decreased thereafter. The initial hardness of raw unripe bananas was 36.73±1.66 N. After heat processing, hardness decreased by 22.90, 28.07, and 33.87% for BB15, BB30 and BB45, respectively and by 26.35, 28.61 and 37.46% for SB15, SB30 and SB45, respectively. This phenomenon was also observed by Gafuma et al. (2018) with boiled bananas and occurred due to an increase in the water content of unripe bananas after boiling and steaming. Excess water promotes starch swelling and gelatinization while also promoting the breakdown and solubilization of other intercellular components such as pectin, resulting in structural separation.

Boiling and steaming time significantly ($p<0.05$) affected the color of unripe bananas by decreasing the lightness (L*) and yellowness (b*) of banana pulp. BB45 had the least lightness (65.01±0.44), followed by SB45 (67.22±1.02). The L* and b* values of boiled and steamed

unripe bananas decreased because the banana pulp absorbed pigment from the banana peel while being heated. Moreover, the redness (a*) of banana also increased from 1.2 to 2.7 and 3.5 from boiling and steaming, respectively. There was no significant change in the redness (a*) of the boiled and steamed samples compared to a raw banana, except for BB45 and SB45.

2. Physicochemical properties of boiling and steaming banana flour

2.1 Chemical compositions

The heated banana flour compositions (ash, fat and protein) compared to unheated banana flour are presented in Table 2. Raw banana contained 2.70±0.02% (db) ash, 0.51±0.02% (db) fat and 2.61±0.02% (db) protein. Boiling and steaming processes significantly ($p<0.05$) affected the ash content of unripe banana flour. Boiling and steaming unripe bananas for longer periods (45 min) markedly increased the ash content of the banana flour up to 3.02 and 3.37% (db), respectively. These results concurred with Tsamo et al. (2015) who found that the total ash content of plantain banana (*Musa* sp.) pulp decreased after boiling without peel, whereas it remained stable or increased after boiling with peel. An increase in ash content induced by boiling and steaming whole bananas (with peel) was attributable to the migration of minerals such as potassium (K), sodium (Na), calcium (Ca), magnesium (Mg) and phosphorus (P) which are contained in the banana peel and transferred to the pulp. At the same boiling and steaming time, the ash content of steamed banana flour was higher than boiled banana flour. This might be due to some minerals in banana peel being leached into the boiling water, resulting in reducing the portion that migrated to the banana pulp. Conversely, the boiling and steaming processes decreased both the fat and protein contents of unripe banana flour. The fat content of raw unripe banana flour was 0.51±0.02% (db), while fat contents of boiled and steamed banana flours ranged from 0.11-0.23% and 0.13-0.18%, respectively. Low levels of fat in boiled and steamed banana flour were attributed to the formation of starch-lipid complexes during the heat process (Mohammed et al., 2009). As a result, the longer boiling and steaming time caused the lower fat content of the flour. Raw unripe banana flour had 2.61±0.02% (db) protein content, while boiled and steamed banana flour had protein content ranging from 2.47-2.53% and 2.45-2.56%, respectively. The protein content of heated unripe banana flour was slightly lower than raw banana flour because of protein denaturation by heat treatment. Likewise, Vongsumran et al. (2014) observed a decrease in the protein of 1-3% in cooked legume flour.

2.2 Dietary fiber

The effects of heat processing by boiling and steaming on SDF, IDF and TDF contents of unripe banana flour are shown in Table 2. Results revealed that heat processing noticeably modified the soluble and insoluble dietary fiber ratio. As a result, the IDF content of BBF and SBF decreased inversely with boiling and steaming times from 10.43 to 7.48 ($p<0.05$). Conversely, the SDF content of unripe banana flour markedly increased directly with the heating time. Boiling and steaming process increased the SDF content by 0.93, 1.01, 1.24, 1.52, 1.68 and 1.73% for BBF15, SBF15, SBF30, BBF45, BBF30 and SBF45, respectively. Results were consistent with Bader Ul Ain et al. (2019) who suggested that thermal processing including boiling, pressure cooking and roasting changed the SDF/IDF ratio of barley dietary fiber. Interestingly, the boiling process increased SDF by 51.2-53.9%, while decreasing IDF by 8.79-24.4% in barley varieties Haider-93 and Jau-87. The high temperature impacted change in SDF by destroying the glycosidic bonds of polysaccharides, allowing the release

Table 2 Chemical properties of raw, boiled and steamed unripe banana flour

Samples	Ash (% db)	Fat (% db)	Protein (% db)	TDF (% db)	IDF (% db)	SDF (% db)	RS (% db)
Raw banana flour	2.70±0.02^{d}	0.51±0.02^{a}	2.61±0.02^{a}	12.31±0.20^{a}	10.43±0.89^{a}	1.88±0.13^{d}	53.17±3.46^{a}
BBF15	2.85±0.13^{cd}	0.23±0.11^{b}	2.47±0.02^{c}	12.28±0.48^{a}	9.47±0.31^{ab}	2.81±0.24^{c}	18.93±0.18^{c}
BBF30	2.90±0.04^{bc}	0.14±0.04^{bc}	2.53±0.01^{bc}	11.75±0.42^{b}	8.19±0.47^{b}	3.56±0.17^{a}	22.23±0.02^{b}
BBF45	3.02±0.01^{b}	0.11±0.01^{c}	2.51±0.01^{bc}	10.88±0.70^{c}	7.48±0.08^{d}	3.40±0.06^{a}	23.69±1.31^{b}
SBF15	2.70±0.09^{d}	0.18±0.07^{bc}	2.45±0.02^{c}	12.44±0.33^{a}	9.55±0.25^{ab}	2.89±0.14^{bc}	18.42±1.03^{c}
SBF30	3.34±0.15^{a}	0.13±0.02^{bc}	2.53±0.01^{bc}	12.27±0.29^{a}	9.15±0.28^{b}	3.12±0.07^{b}	20.88±0.80^{bc}
SBF45	3.37±0.06^{a}	0.13±0.04^{bc}	2.56±0.01^{ab}	11.94±0.16^{b}	8.33±0.29^{c}	3.61±0.09^{a}	22.56±1.14^{b}

Remark: a – d = Means ± SD (n = 3) with different lowercase superscript letters within a column are significantly different ($p < 0.05$)
TDF = Total dietary fiber, IDF = Insoluble dietary fiber, SDF = Soluble dietary fiber, RS = Resistant starch, BBF = Unripe banana flour heated by boiling, SBF = Unripe bananas flour heated by steaming, 15, 30, 45 = boiling and steaming time of unripe bananas (min)

of oligosaccharides and thus, increasing the amount of SDF (Bader Ul Ain et al., 2019). Meanwhile, the TDF content of the flour was also decreased with heating time especially for the boiling process from 12.31 to 10.88% db. These findings indicated that heat treatment improved the functional properties of dietary fiber by increasing SDF. Boiling and steaming banana caused the decreasing of IDF. This might be due to IDF was hydrolysis by heating to short chain molecule. This caused IDF to become more soluble as a result of SDF increasing. Therefore, heated banana flour should be considered a promising source of SDF for use in therapeutic and health-promoting food products.

2.3 Resistant starch (RS)

Resistant starch (RS) can be neither hydrolyzed by human digestive enzymes nor absorbed in the small intestine, passing onto the colon (Englyst et al., 1992). RS can be classified into five different types RS1, RS2, RS3, RS4 and RS5. Unripe banana flour is rich in RS2 which is characterized by native granular starch present in uncooked starchy foods (Detchewa et al., 2021; Vatanasuchart et al., 2009). RS2 is slowly hydrolyzed by α-amylase because of its high β-type granular structure (Jaiturong et al., 2020).

Table 2 shows RS content in raw and heated unripe banana flour. RS content of raw banana flour (53.17±3.46 g/100 g db) was consistent with previous reports, ranging from 32.26-58.10% (db) (Moongngarm et al., 2014; Vatanasuchart et al., 2012). Results revealed that heat processing significantly ($p<0.05$) reduced the RS content of unripe banana flour. After heating for 15 min, the RS content of boiled and steamed banana flour decreased to 18.93±0.18% (db) and 18.42±1.03% (db), respectively. These results concurred with Rodríguez-Damian et al. (2013) who found that boiling unripe banana flour decreased RS content from 65.6% (db) to 23.3% (db). The decline in RS2 was attributed to gelatinization of the starch during the autoclaved heating process, resulting in loss of crystallinity and other unreversible changes in starch characteristics (Aparicio-Saguilán et al., 2005). However, the RS content of boiled and steamed banana flour was increased to 22.23 and 20.88% after heating for 30 min. This might be due to the time of the boiling process which provided moist heat that enabled starch gelatinization and consequently, amylose was leached from the starch granules of banana (Chaipai et al., 2018; De la Rosa-Millan et al., 2014). Then, the leached amylose chains are associated to form hydrogen bonds and recrystallized to form RS3 (retrograded starch). Therefore, conditions that promoted gelatinization allowed the greater formation of RS3 in banana starch (Chaipai et al., 2018).

2.4 Pasting properties

The pasting properties of unripe banana flour were determined by gradually raising the temperature of moist starch to 95°C, maintaining this temperature for a period of time and then steadily reducing the temperature to 50°C. When the unripe banana flour was heated, amylose leached out of the swollen starch molecules, resulting in a rapid increase in viscosity with increasing temperature until PV was reached. Amylose leached out of the starch granules because the high temperature (95°C) and mechanical shear stress reduced the viscosity. The MV and BD were recorded. As the temperature of the mixture reduced, the starch molecules retrograded and formed a viscous gel network, resulting in an increase in FV (Amini Khoozani et al., 2020; Wang et al., 2017).

The pasting properties of raw and heated unripe banana flour are presented in Table 3. PT is the lowest temperature required to cook the sample. The highest PT was found in raw unripe banana flour (87.23±0.04°C). The boiling and steaming process

Table 3 Pasting properties of raw, boiled and steamed unripe banana flour

Samples	PT (°C)	PV (RVU)	MV (RVU)	BD (RVU)	FV (RVU)	SB (RVU)
Raw banana flour	87.23±0.04^{a}	188.77±0.56^{e}	178.82±0.15^{c}	9.96±0.71^{d}	219.92±1.18^{d}	41.11±1.33^{d}
BBF15	67.70±2.26^{d}	180.32±1.92^{f}	145.13±1.17^{e}	9.21±1.12^{d}	186.15±3.15^{e}	41.03±1.97^{d}
BBF30	69.01±0.04^{cd}	228.83±3.31^{c}	171.38±7.14^{d}	50.33±2.48^{b}	216.21±4.65^{d}	44.83±2.48^{c}
BBF45	69.88±0.67^{c}	251.21±1.12^{a}	200.88±1.35^{a}	57.46±3.83^{a}	257.00±0.35^{a}	56.13±1.00^{a}
SBF15	70.35±0.01^{c}	191.42±2.83^{e}	182.08±3.54^{bc}	9.33±0.71^{d}	227.38±3.60^{c}	38.34±2.24^{d}
SBF30	70.33±0.04^{c}	219.46±0.18^{d}	187.00±2.36^{bc}	32.46±2.18^{c}	225.33±0.12^{c}	45.29±0.06^{c}
SBF45	82.07±0.26^{b}	235.00±2.83^{b}	199.42±0.94^{a}	35.59±1.89^{c}	248.00±2.12^{b}	48.59±1.18^{b}

Remark: a – d = Means ± SD (n = 3) with different lowercase superscript letters within a column are significantly different ($p < 0.05$)
PT = Pasting temperature, PV = Peak viscosity, MV = Minimum viscosity, BD = Breakdown, FV = Final viscosity, SB = Setback viscosity, RVU= Rapid Visco Analyzer units, BBF = Unripe banana flour heated by boiling, SBF = Unripe banana flour heated by steaming, 15, 30, 45 = boiling and steaming time of unripe bananas (min)

affected the PT of the flour. For example, the PT of the BBF15 and SBF15 was decreased to 67.70 and 70.35°C, respectively. This behavior was caused by the type and amount of RS contained in the flour. Raw unripe banana flour is rich in RS2 which has strong resistance to gelatinization and high temperatures are necessary to gelatinize high-amylose starches. However, heated unripe banana flour contains RS3, a retrograded starch that easily gelatinizes in the presence of water at 60°C (Champ, 2004). As a result, a longer heating duration could cause the re-increase PT of the flour, especially the flour treated by steaming process such as SBF45 showed 82.07°C of PT. Other viscosity values including PV, MV and FV gave comparable results; as boiling and steaming time increased, the viscosity significantly ($p<0.05$) increased. This occurred because the longer heating time increased the quantity of RS (RS3) in the boiled and steamed banana flour, making the flour more resistant to high temperature and shear stress, resulting in an increase in viscosity. BD is defined as the difference between peak and hold viscosity, explaining hydration, starch swelling power and shear resistance of starch paste during boiling and steaming. Low BD is often related to poor hydration and swelling power and strong shear resistance (Shafie et al., 2016). BD values of BBF15 and SBF15 were not different from raw banana flour; however, there were notable increases in BD of boiled and steamed samples heated for 30- and 45-min. BD values of steamed unripe banana flour were lower than the boiled samples for the same heating time.

SB viscosity is the difference between the final and lowest viscosity, indicating starch retrogradation tendency after gelatinization and cooling. Viscosity changes while cooling was mainly due to amylose molecular reassociation, resulting in the formation of new structures (Shafie et al., 2016). SB values of the BBF30 and SBF45 were higher than for BBF15 and SBF15 because with longer boiling and steaming time, more banana pulp granules were gelatinized, and more amylose was destroyed. When the gelatinized starch cooled, the structure rapidly rearranged with the formation of intermolecular hydrogen bonds, resulting in high starch retrogradation.

2.5 TPC and antioxidant activity

The TPC values of unripe banana flour and antioxidant activity determined by FRAP and DPPH assays are presented in Table 4. Results indicated that the boiling and steaming processes impacted antioxidant activity and TPC values of unripe banana flour. The TPC values of boiled flour samples ranged from 8.25 to 9.42 mgGAE/g (db) and those of steamed flour samples ranged from 7.53 to 8.46 mgGAE/g (db). While, the lowest TPC value of 1.80 mgGAE/g (db) was obtained from raw unripe banana flour. The TPC values significantly ($p < 0.05$) increased with increasing the boiling and steaming time. TPC values of boiled banana flour were higher than steamed banana flour at the same heating time. These results concurred with Ebun & Santosh (2011), who found that boiling impacted the phenolic compounds in plantain banana (*Musa paradisiaca*). Plantain boiled with skin had higher TPC values than raw plantain banana. Similarly, Tsamo et al. (2015) observed an increase in TPC in the pulp of boiled plantain banana (*Musa* sp.) with peel. These phenomena can be explained because heat processing weakens the cell wall and enhances the release of bound phenolic compounds by breaking down the cellular constituents (Dewanto et al., 2002). An increase in the bound phenolic content is statistically significantly associated with both time and temperature of retorted heating (Dewanto et al., 2002). A result of a preliminary experiment indicated that the temperature at the center of boiled bananas was 88°C and 96°C for BB15 and BB45, respectively, compared with 83°C and 90°C for SBF15 and SBF45, respectively. In addition, BBF45 contained the highest TPC values.

The antioxidant activity of boiled and steamed banana flour increased markedly with increasing the heating duration, especially in samples heated by boiling. As a result, DPPH and FRAP values of raw banana flour were 3.99±0.04 mg AAE/g (db) and 9.25±0.12 mmol $FeSO_4$/g (db), respectively. While DPPH values of BBF15, BBF30 and BBF45 samples increased by 28.3, 48.6 and 58.9%, respectively. Comparing, SBF15, SBF30 and SBF45 exhibited increases in DPPH values by 5.5, 24.3 and 35.8%, respectively. Similarly, FRAP of heated samples increased as boiling and steaming time increased. For the same treatment time, steamed banana flour had lower FRAP values than boiled samples. Results indicated that the increase in antioxidant activity shown by DPPH and FRAP values of heated banana flour corresponded to an increase in phenolic compounds. Previous studies reported that the antioxidant activity of food is linearly and positively correlated with the phenolic content (Başyiğit et al., 2018; Ruengdech et al., 2019). The Pearson correlation coefficients were calculated between the means of TPC, DPPH and FRAP (Table 5). Results showed high correlation between TPC and DPPH ($r=0.762$, $p<0.01$) and TPC and FRAP ($r=0.890$, $p<0.01$); DPPH and FRAP ($r=0.792$, $p<0.01$) were also observed.

Table 4 Phenolic content and antioxidant activity of raw, boiled and steamed unripe banana flour

Samples	TPC (mg GAE/g db)	DPPH (mg AAE/g db)	FRAP (mmol FeSO4/g db)
Raw banana flour	1.80±0.02^{g}	3.99±0.04^{g}	9.25±0.12^{g}
BBF15	8.25±0.01^{d}	5.12±0.02^{d}	17.38±0.18^{f}
BBF30	9.14±0.03^{b}	5.93±0.03^{b}	26.42±0.13^{b}
BBF45	9.42±0.03^{a}	6.34±0.05^{a}	30.39±0.31^{a}
SBF15	7.53±0.04^{f}	4.21±0.03^{f}	20.80±0.18^{e}
SBF30	8.12±0.03^{e}	4.96±0.01^{e}	24.46±0.22^{d}
SBF45	8.46±0.03^{c}	5.42±0.03^{c}	25.71±0.07^{c}

Remark: a – g = Means ± SD (n = 3) with different lowercase superscript letters within a column are significantly different ($p < 0.05$)
TPC = Total phenolic content, DPPH = 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity, FRAP = Ferric reducing antioxidant power, BBF = Unripe banana flour heated by boiling, SBF = Unripe banana flour heated by steaming, 15,30,45 = boiling and steaming time of unripe bananas (min)

Table 5 Pearson correlation coefficients of phenolic content and antioxidant activity of raw, boiled and steamed unripe banana flour

	TPC	DPPH	FRAP
TPC	1	0.762**	0.890**
DPPH	0.762**	1	0.792**
FRAP	0.890**	0.792**	1

Remark: ** Correlation was significant at $p < 0.01$, TPC = Total phenolic content, DPPH = 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity, FRAP = Ferric reducing antioxidant power

Conclusion

Results demonstrated that boiling and steaming unripe bananas for 15 to 45 min significantly caused the physicochemical properties alternation of pulp and flour banana. Boiling and steaming caused the soft texture of the unripe banana pulp because of starch gelatinization so it was simpler to eat and increased SDF and antioxidant levels. Results revealed that boiling had a stronger impact on the qualities of banana flour than steaming, particularly, pasting properties and antioxidant activity. Meanwhile, the resistant starch content of unripe banana flour was reduced about 30% by steaming and boiling process. Boiled banana flour showed higher TPC, DPPH and FRAP than steamed banana flour. These findings can assist researchers to determine the optimal nutritional advantages and disadvantages of consuming cooked bananas and to promote the application of boiled and steamed banana flour as a beneficial ingredient in the food industry.

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Effects of Emblica Extract (*Phyllanthus emblica*) on Color and Antimicrobial Quality of Avocado Puree During Freezing Storage

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Abstract

Emblica (*Phyllanthus emblica*) fruits have both antioxidant and antimicrobial activities as well as enzyme-inhibiting properties. They contain high levels of ascorbic acid and citric acid, preventing the browning reaction in minimally proceeded fruits and vegetables. This research investigated the effect of emblica fruit extract on the qualities of avocado puree during freezing storage. Emblica fruits were ethanolic extracted and used as anti-browning and anti-microbial agents. Ripe avocados were selected and blanched in carbonate buffer (pH 10.6). Acidulants, including ascorbic acid and emblica fruit extract, were then added prior to blending to become homogenous. The puree was packed and stored at -20°C for 4 weeks. During storage, the puree was determined for their color, pH and total bacterial plate counts. The result showed that the emblica fruit extract had antioxidants activity and could inhibit *Staphylococcus pasteuri,* spoilage bacteria isolated from avocado puree. The emblica fruit extract could delay the browning reaction during storage. However, the extract concentration had an influence on the puree color, causing the puree lightness to decrease. Blanching helped destroyed some contaminated bacteria in the avocado. After storage at -20°C for 4 weeks, less than 1 log CFU/g were detected in all avocado puree treatments, which were blanched. This study indicates that the emblica fruit extract could be used as an acidulant to prevent the blanched avocado from the browning reaction.

Introduction

Avocados are considered as potential economic fruits, gaining huge global attention. They are relatively high in nutrition, including good fat, low sugar and high antioxidants. They are well-known for their health benefits, mainly associated with hypoglycemic, antihypertensive, anti-obesity and hepatic-protective effects. Although avocados originated from Mexico, they began to be cultured in Thailand in 1978 and have been continuously grown in the North of Thailand for more than 40 years, producing approximately 1,200 tons/year.

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Unfortunately, the Thai-grown avocados usually fail to meet the food industry requirements and are rejected or cut-priced, as their characteristics have been changed from the originated avocado breeder. Yearly, more than 25% of the Thai avocados were disposed, causing huge economic losses to Thai farmers. To overcome the problem, avocados could be minimally processed to avocado-based products such as puree, frozen pieces, or powder. However, due to their high perishability and susceptibility, they tend spoil rapidly and become brown during processing and storage (López-Ramírez & Duarte-Sierra, 2020). Polyphenol oxidase, known as PPO, plays an important role in catalyzing browning reaction in avocados. Several technologies have been successfully introduced to reduce or halt those reactions such as high hydrostatic pressure, gamma radiation, flash vacuum-expansion and microwave; however, they require advance equipment, technicians and a high cost of investment (Stephen & Radhakrishnan, 2022).

It has been well-documented that heat causes PPO to be denatured until it cannot catalyze (Lv et al., 2017) and inhibited when the pH is lower than 4. The addition of acidulants such as ascorbic acid, citric acid (Ali et al., 2014) or juice such as lime and onion juice (Bustos et al., 2015) were reported to be able to halt the activity of PPO. Emblica, a fruit originated from India, has the potential to preserve avocados from browning reaction. It contains high levels of ascorbic acid, citric acid and tannin (Liu et al., 2008). Emblica fruits have both antioxidant and antimicrobial activities as well as enzyme-inhibiting properties (Priya et al., 2012; Majeed et al., 2020). It was reported that emblica extract (1%) was more effective in reducing oxidation in biscuit than BHA (200 ppm), synthetic antioxidant, during storage for 6 weeks at ambient temperature and had no influence on the surface color and texture of the biscuit (Reddy et al., 2005). Similarly, less than 7% of emblica juice added into enriched vitamin C aloe vera-apricot beverage was accepted from panelists in terms of appearance, flavor and overall acceptability (Sharma et al., 2022). In addition, emblica extract was successfully used as a natural preservative in raw ground pork during refrigerated storage at 4°C. It helped effectively decrease the number of total viable counts and total Pseudomonas in raw ground pork after 12-day storage as well as lowering lipid oxidation (Nanasombat et al., 2012). Therefore, emblica could be used to not only preserve the avocado puree from the browning reaction, but also protect the puree from microbial spoilage. However, inappropriate amount of heating time, temperatures and concentrations of acidulants may result in unusual and unacceptable color, odor and taste of the avocado.

Moreover, it was also found that heating and acids could influence the structure of chlorophylls and the pigment retention. Heating converted chlorophylls to pheophytins and pyropheophytins, causing color change from light green to an unpleasant olive green, while in acidic conditions, the color could change from bright green to olive brown due to loss of Mg in the porphyrin ring which was replaced by hydrogen ion (Koca et al., 2007). On the other hand, it was found that alkaline conditions did not affect the chlorophyll structure by inducing oxidation of the isocyclic ring and de-esterification of phytol in chlorophylls. This helped retain the basic structure of the chromophore group with Mg linked to the porphyrin ring (Koca et al., 2007). Therefore, to apply simple techniques as heating or the addition of acids to avocado products, such as avocado puree, it is necessary to ensure that the structure of chlorophylls is stabilized, while simultaneously, the puree is prevented from a browning reaction. This research investigated the effects of emblica fruit extract combined with alkaline water boiling on quality changes of avocado puree during freezing storage. The study could contribute to the avocado farmers and the food industry by developing a stable avocado puree using a simple method.

Materials and methods

1. Isolation of spoilage bacteria from avocado puree

The fresh avocado was cleaned and halved lengthwise. The seed was then pitted after cleaning and sanitation. The pulp was cut into small pieces and ground for 2 min in a blender (MX-AC400, Panasonic, China). The puree was immediately packaged in polypropylene zip-lock bags after blending and kept at 4°C until it spoiled. Serial dilution from 10^{-1} to 10^{-6} dilutions was performed to isolate bacteria from the spoiled avocado puree. A 100 µL of each of diluted suspension was spread on nutrient agar medium. The plates were incubated at 37°C for 48 h. Morphological analysis of the isolated bacteria was observed and identified by microscopic examination. A 16S ribosomal DNA sequencing method was also used to confirm the identity of these bacteria (Ananchaipattana et al., 2012). The bacterial isolate was sub-cultured and stored on nutrient agar slants at 4°C for the next experiment.

2. Preparation of Emblica fruit extract

Dried emblica fruits were purchased from Popaya Natural Products Co. Ltd. in Pathumthani, Thailand. The dried fruit was pulverized until becoming a powder. The powder (100 g) was soaked in 95% of ethanol (1,000 mL) for 24 h. The extract was filtered through Whatman filter paper No. 1 and evaporated to remove ethanol under a vacuum using a rotary evaporator (R-300, Buchi, Germany) (Patel et al., 2009). The crude extract was then freeze dried and stored at 4°C in storage vials for experimental use. The extract was used to determine the total phenolic contents, scavenging activity and antimicrobial activity.

3. Determinations of Emblica fruit extract

3.1 Total phenolic contents

Total phenolic contents of emblica extract powder were determined by a modified method as described by Hatami et al. (2014). The solution consisted of 0.5 mL of emblica extract solution (0.1 mg of emblica extract powder dissolved in 1 mL of methanol) mixed with 2.5 mL of 10%v/v diluted Folin–Ciocalteu phenol reagent and 2 mL of 7%w/v sodium carbonate. The samples were incubated in the dark for 2 h at room temperature. One hundred microliter of the solution was then added into 96 wells plates and absorbance at 760 nm was determined using a Microplate Reader (EPOCH 2, Bio Tek, USA). Total phenolic content was expressed as mg gallic acid/g using the equation obtained from a calibration curve of gallic acid at the concentrations of 0-120 mg/L. All samples were measured in triplicate.

3.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical- scavenging activity

DPPH scavenging activity was analyzed using a spectrophotometric method described by Jan et al. (2013). A solution of DPPH in methanol was prepared freshly. To measure the scavenging capacity of a single antioxidant, a 2.9 mL aliquot of DPPH solution was mixed with 0.1 mL of sample solutions (100 mg/mL), shaken well and incubated in the dark for 30 min at room temperature. The decrease in absorbance was measured at 517 nm. The percentage inhibition of the radicals due to the antioxidant property was calculated using the equation 1 shown below.

$$\% \text{ inhibition} = [(Abs_{blank} - Abs_{sample})/Abs_{blank}] * 100 \ldots\ldots (1)$$

where, Abs_{sample} = absorbance of 1 mmol DPPH with sample in methanol and Abs_{blank} = absorbance of methanol solvent in absence of DPPH and sample.

3.3 Antimicrobial activity

The bacteria isolated from avocado puree were then used to determine the antimicrobial activity of emblica fruit extract by agar well diffusion method. The isolate was grown in Muller Hinton broth. The turbidity of the isolate was adjusted to 0.5 McFarland standards. An 0.1 mL of the isolate was then inoculated on Muller-Hinton agar. The plates were dried for 15 min prior to being punched by using sterile cork borers to form wells. A 100 µL of the extracts, prepared by dissolving the extract powder in distilled water to obtain the solution at concentration of 100-500 ppm, was added into the wells and distilled water was used as control. The plates were then incubated for 24 h at 37°C. The diameters of the clear zone of inhibition were measured in millimeters. An agar well showing no clear zone was determined as having no antimicrobial activity. All experiments were done in triplicate.

4. Avocado puree production

Avocados were kindly provided by Boriboon Farm located in Nakhon Ratchasima Province, Thailand. Avocados in stage 5 were selected to be processed by measuring the avocado firmness by a texture analyzer (TA.XT plus C, Stable micro system, United Kingdom) which had to be between 200-300 N. The selected avocados were cleaned, halved lengthwise and pitted to separate the pulp. They were then cut into pieces and scalded in sodium bicarbonate buffer (pH 10.6) at 85°C for 3 min After that, the pulp was ground with a blender for 2 min. Emblica extract prepared by dissolving emblica extract powder in distilled water to obtain the concentrations of 300 ppm and 500 ppm (AAP/E300 and AAP/E500), was then added. Ascorbic acid was used as the positive control (AAP/AA). Blanched avocado puree in alkaline solution (AAP) was used as the blank. The puree was mixed until homogenous, packaged in polypropylene zip-lock bags and stored at -20°C for 4 weeks. During storage, the frozen avocado puree was separately determined for their color and pH, while total bacteria plate count was conducted after storage.

5. Determination of avocado puree

5.1 Color and pH

The colors of avocado puree were monitored using a colorimeter with LED white light (D65) and CIE 10° standard observer (NR 200, 3nh, China). Each sample were measured in the CIE Lab scale for triplicates. Three replicates were carried out. All data were averaged. Data collected included lightness (L*), redness (a*), yellowness (b*) and the total color difference (ΔE) was

calculated according to the equation 2 shown below. The pH of avocado puree was determined by using a pH meter (pH700, Eutech, Singapore) with triplicates.

$$\Delta E = \sqrt{(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2} \quad \text{..... (2)}$$

where L_0^*, a_0^* and b_0^* were the values of the sample at day 0, while L^*, a^* and b^* were the values of the sample during storage.

5.2 Total bacterial plate counts

Twenty-five grams of chilled avocado puree was added into a stomacher bag containing 225 mL of saline solution. The sample was then homogenized with a stomacher for 2 min. The serial dilution from 10^{-1} to 10^{-6} was performed prior to transferring 1 mL of suspension of each dilution to 3M Petrifilm Aerobic Count Plate. The plates were then incubated at 37°C for 24-48 h. The number of colonies was counted and expressed as log CFU/g. Fresh avocado puree (FAP) was used as the positive control.

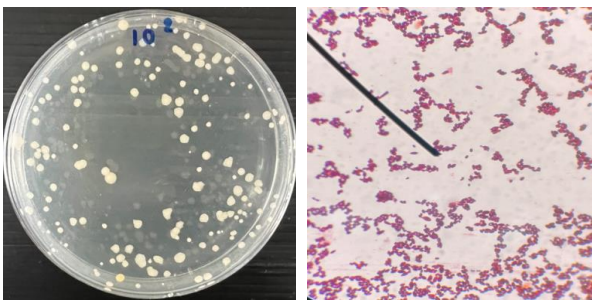
6. Statistical analysis

Each experiment was repeated three times. The influences of the various parameters were assessed by analysis of variance (ANOVA) and Duncan's new multiple range test (DMRT) for mean discrimination or mean comparison, depending on the data. Differences were considered significant at a confidence level superior to 95%. The SPSS statistical program version 16.0 (SPSS Inc., Armonk, NY, USA) was used for the analyses.

Results and discussion

1. Isolation of spoilage bacteria from avocado puree

Spoiled avocado puree was isolated for its microorganisms. The result showed that only one isolate was observed. As shown in Fig. 1, the isolate had yellow, glistening and smooth colonies with regular edge shapes. When observed under light microscope, the isolate was gram-positive bacteria, round shape, nonmotile and nonsporulating. After 16S ribosomal DNA sequencing, the phylogenetic trees of the isolate are shown in Fig. 2. The isolate belonged to the genus of *Staphylococcus* which was identified as *Staphylococcus pasteuri* with the sequence identity of 99%. The bacteria was coagulase-negative which could be found in food products including goat milk, Italian sausage, sea fish and retail beef (Wainwright et al., 2003) or on the surface of drinking water, as well as naturally occurring in the air (Faria et al., 2009). *S. pasteuri* could grow at 15 to 45°C on agar with or without 5 to 15% sodium chloride supplementation. It was able to utilize D-glucose, glycerol, D-fructose and sucrose and produced catalase and urease (Chesneau et al., 1993).



(a) (b)

Fig. 1 Morphology of *Staphylococcus pasteuri* on a nutrient agar plate (a) and under microscope (b), isolated from spoilage avocado puree

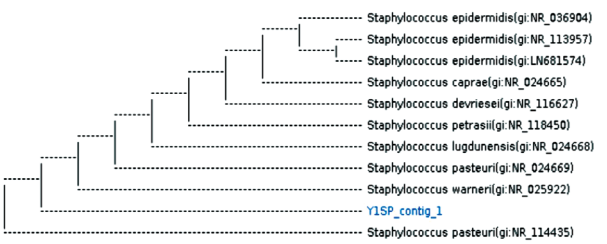


Fig. 2 Phylogenetic tree of bacterial isolate obtained from spoiled avocado puree by 16S rRNA gene sequence method.

2. Antioxidant and antimicrobial activities of Emblica fruit extract

Emblica fruit has been well-known for their rich phenolic compounds and antioxidant activity. In this study, the result showed that the extract had total phenolic content at 167.22 mg GAE/g of emblica fruit extract and its free radical scavenging activity was 37.45%. Sirichai et al. (2022) found that freeze dried emblica fruit extract contained 193.51 mg GAE/g dry weight and had 0.064 µmol Trolox equivalent/g dry weight of radical-scavenging activity investigated by DPPH assay. The presence of quercetin, kaempferol, naringenin were found in the emblica extract, as well as gallic acid which was four-times higher than *Careya arborea Roxb* (Sirichai et al., 2022). Phenolic compounds contributed to the overall antioxidant activities by inactivating lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Sirichai et al., 2022). Alsahli et al. (2021) reported that the ethanolic *P. emblica* fruit extract from Thailand

exhibited strong antioxidant activities. Addition of emblica extract in food products could help enrich vitamin C and increase antioxidant activities of the products (Reddy et al., 2005; Nanasombat et al., 2012: Sharma et al., 2022). However, the activity could be different, depending on the plant variety, extraction methods and drying methods. Besides, phenolic compounds tend to have an important role in antimicrobial activities of plant extracts. *Salmonella* sp. and *Staphylococcus* sp. were inhibited by phenolics and organic acids in cranberry extracts (Puupponen-Pimiä et al., 2005).

As shown in Fig. 3, emblica fruit extract had antimicrobial activity against the spoilage bacteria isolated for avocado puree. The diameters of the clear zone expressed the inhibition activity. They were significantly increased according to the extract concentrations, which was 0.45±0.07 mm, 0.24±0.07 mm, 0.20±0.00 mm, 0.16±0.07 mm and 0.05±0.07 mm, for the emblica fruit extract at concentrations of 500, 400, 300, 200 and 100 ppm, respectively. Gandhi et al. (2020) reported that an ethanolic extract from *P. emblica* showed higher inhibition against gram-positive bacteria (*Staphylococcus aureus*) than gram-negative bacteria (*Escherichia coli*) and fungal (*Candida albicans*). Antimicrobial activity of the emblica fruit extract contributed to Emblicanin A and B, two major tannins which could inactivate enzyme activity and cells envelop transport proteins as well as blocking microbial adhesions (Wang et al., 2017).

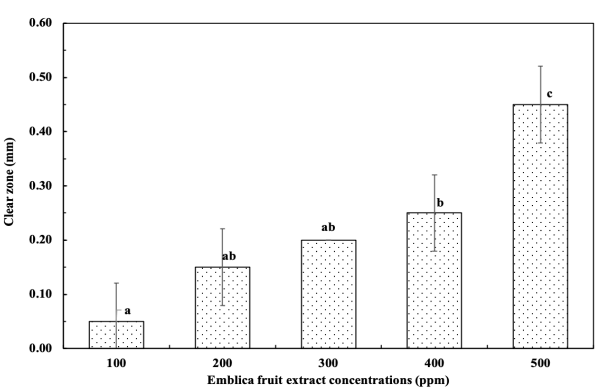


Fig. 3 The clear zone inhibition of emblica fruit extract against *S. pasteuri*
Remark: $^{a\text{-}c}$Mean±S.D. with different letters showing significant difference within the same treatment (p<0.05)

3. Effects of emblica fruit extract on quality of avocado puree during freezing storage

3.1 Color

Emblica fruit extract was added into the avocado puree prior to storage at -20°C for 4 weeks. The extract at the concentration of 300 ppm and 500 ppm were selected to be added into the puree to compare its ability with 300 ppm of ascorbic acid, which was generally used to prevent browning in fruits (Singh et.al, 2017; Singh & Mirza, 2018). Regardless of the extract concentration, after mixing AAP/E300 and AAP/E500 had no significant difference in lightness or L* when compared with AAP/AA but lower than AAP. During storage, L* of all treatments trended to decrease. After 4-week storage, ascorbic acid and the emblica fruit extract could help delay the effects of browning reaction in the puree. The L* of AAP/AA was 39.83±0.06, which was significantly greater than AAP/E300 (39.60±0.05) and AAP/E500 (39.43±0.06) (Fig. 4). In regards to the greenness, as shown in Fig. 5, the greater a* was found in AAP/AA (-1.97±0.02), followed by AAP (-1.55±0.04), AAP/E300 (-1.22±0.01) and AAP/E500 (-0.96±0.06). This could be due to the brown color of the emblica fruit extract, lowering greenness of the puree, while increasing the redness. For the yellowness, AAP/AA showed significantly higher b* than AAP/E300, AAP/E500 and AAP, which were 17.02±0.01, 16.45±0.01, 16.23±0.01 and 16.23±0.06, respectively (Fig. 6). Regarding the color change (ΔE), as shown in Fig. 7, after storage for 4 weeks the puree without an acidulant (AAP) had higher color changes than the addition of acidulants, which was 3.31, 0.92, 0.93 and 2.12 for AAP, AAP/AA, AAP/E300 and AAP/E500, respectively. However, the extract could negatively affect the puree color if its concentration was too high. The more extract was added, the more changes were detected. Ospina et al. (2019) reported that the average human eyes could not distinguished any color when the ΔE was less than 3. The results indicating that ascorbic acid and emblica fruit extract could help maintain avocado color during storage.

Emblica fruit extract contained high levels of phenolic compounds and antioxidants as well as ascorbic acid, citric acid and tannin (Koca et al., 2007). It has been well-documented that ascorbic acid behaved where it could reduce instantly the formed color and acted as quinone reducer (Altunkaya & Gokmen, 2008; Dincer et al., 2002; Neves et al., 2009). Ascorbic acid reduced the conversion of O-quinones to diphenols, leading to the formation of colorless compounds (Ding et al., 2002).

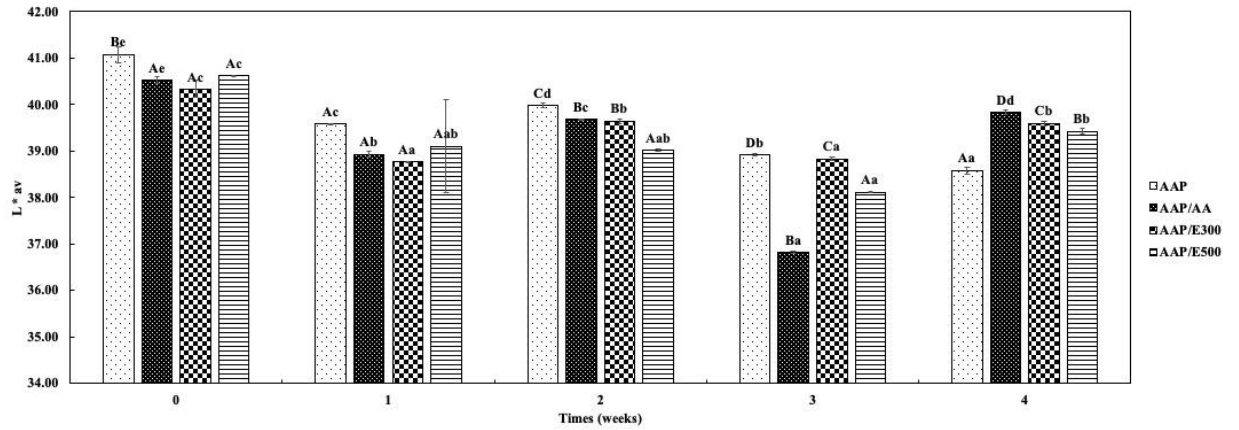


Fig. 4 Average lightness (L*) of avocado puree during storage at -20°C for 4 weeks. (AAP = alkaline blanched avocado puree, AAP/AA = alkaline blanched avoca do puree added with ascorbic acid, AAP/E300 and AAP/500 = alkaline blanched avocado puree added with the emblica fruit extract at the concentration of 300 and 500 ppm, respectively.)

Remark: [a-c] Mean±S.D. with different letters showing significant difference within the same treatment (p<0.05)
[A-D] Mean±S.D. with different letters showing significant difference within the same time (p<0.05)

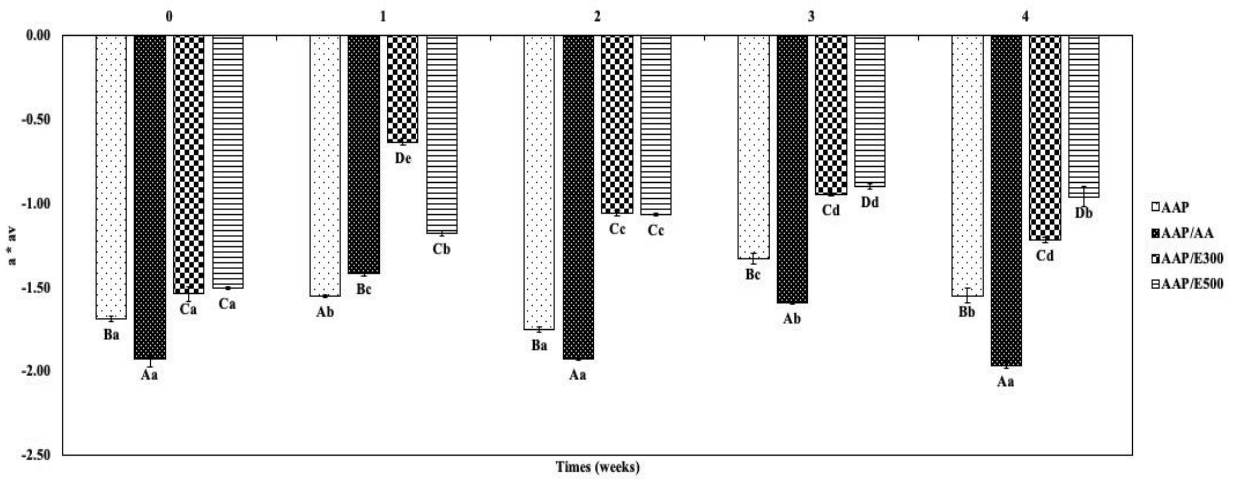


Fig. 5 Average greenness (a*) of avocado puree during storage at -20°C for 4 weeks. (AAP = alkaline blanched avocado puree, AAP/AA = alkaline blanched avo cado puree added with ascorbic acid, AAP/E300 and AAP/500 = alkaline blanched avocado puree added with the Emblica fruit extract at the concentration of 300 and 500 ppm, respectively.)

Remark: [a-c] Mean±S.D. with different letters showing significant difference within the same treatment (p<0.05)
[A-D] Mean±S.D. with different letters showing significant difference within the same time (p<0.05)

However, their effectiveness depends on environmental factors such as pH, water activity, temperature, light and composition of the atmosphere (Lindley, 1998). In addition, citric acid contributed to pH reduction or chelation of the copper at PPO active site, resulting in lowering the PPO activity (Sedaghat & Zahedi, 2012). However, it was found that although emblica fruits contained high antioxidants as vitamin C, they could turn to be brown easily even if they were at low temperature as 25°C or 35°C (Scartezzini et al., 2006). Li et al. (2022) reported that gallic acid, tannic acid, 1,2,3,4,6-penta-O-galloyl-β-D-glucose (β-PGG) and the substrates of *P. emblica* could promote enzymatic and nonenzymatic browning reactions (Li et al., 2022). They also found that the change of PPO activity in *P. emblica* was related to titratable acid during browning. Therefore, appropriate concentration of the emblica extract was needed. The result also indicated that combination of blanching and

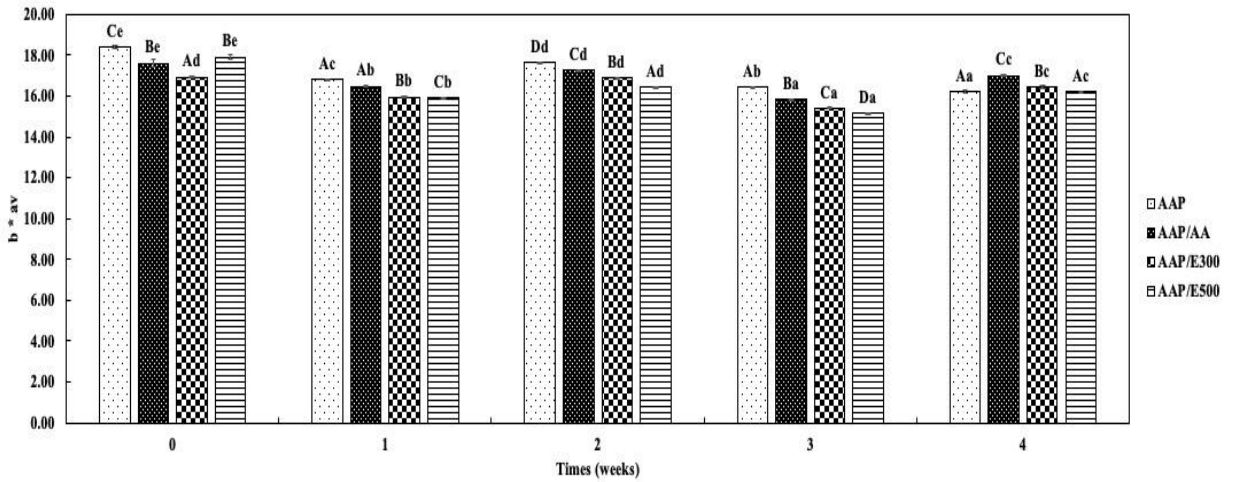


Fig. 6 Average yellowness (b*) of avocado puree during storage at -20°C for 4 weeks. (AAP = alkaline blanched avocado puree, AAP/AA = alkaline blanched av ocado puree added with ascorbic acid, AAP/E300 and AAP/500 = alkaline blanched avocado puree added with the emblica fruit extract at the concentration of 300 and 500 ppm, respectively.)

Remark: $^{a\text{-}c}$ Mean±S.D. with different letters showing significant difference within the same treatment (p<0.05)
$^{A\text{-}D}$ Mean±S.D. with different letters showing significant difference within the same time (p<0.05)

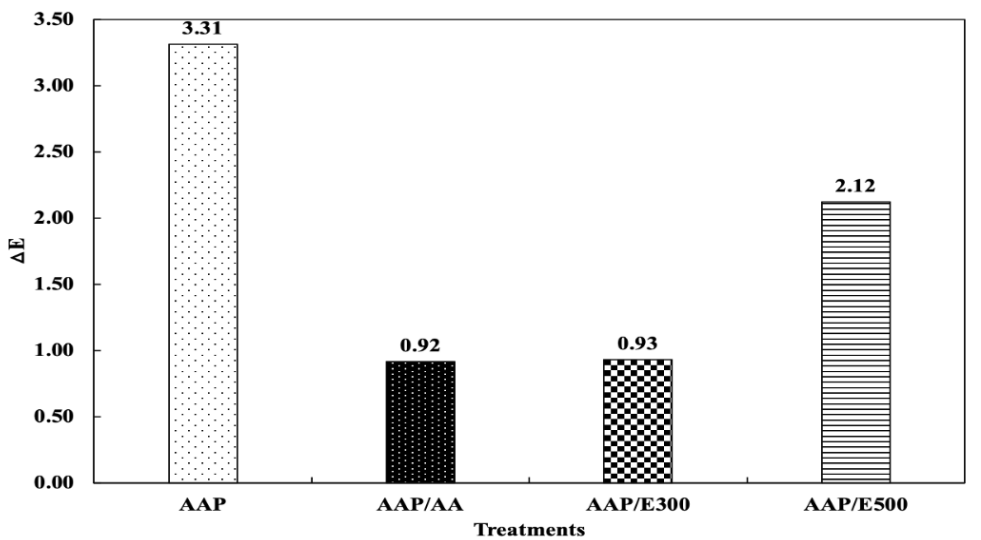


Fig. 7 ΔE of avocado puree during storage at -20°C for 4 weeks (AAP = alkaline blanched avocado puree, AAP/AA = alkaline blanched avocado puree added with ascorbic acid; AAP/E300 and AAP/500 = alkaline blanched avocado puree added with the emblica fruit extract at the concentration of 300 and 500 ppm, respectively.)

adding of ascorbic acid or emblica extract helped improve the puree color. Hasan et al. 2017 treated fresh slices of apple with 1% ascorbic acid solution for one min and hot water with 50°C for 2 min. The results showed that both heat and ascorbic acid treatments could significantly reduce cut surface browning. The combination between heating and addition of ascorbic acid showed inhibitory effects on PPOs peroxidase (PODs), which was one of the factors mainly causing enzymatic browning in fresh cut browning. Hot water treatments played a more important role in suppressing both monophenolase and diphenolase activity of PPOs and POD than ascorbic acid.

3.2 pH

The anti-browning agents of both ascorbic acid and emblica fruit extract had an effect on the pH of the avocado puree. It was evident that after adding anti-browning agents, all treatment had a lower pH compared to the control. In regards to the chilled avocado puree, the emblica fruit extract affected the pH of the avocado puree (Fig. 8). APP/E100 showed higher pH compared to other treatments significantly. The pH of all treatments was reduced during storage. After 4 weeks of storage, AAP/E500 was 6.47±0.01, followed by AAP/E300 (6.60±0.02), AAP/AA (6.64±0.02) and AAP (6.75±0.02). Martinez & Whitaker (1995) reported that adding of acidulants such as ascorbic acid, citric acid and acetic acid could control the browning of fruit juices, causing the pH of a system to be lower than 4. Most of the chemical products used to inhibit darkening enzyme has acidifiers in their composition (Mattos et al., 2007). Guerrero-Beltrán et al. (2006) found that the addition of ascorbic acid (500 ppm) to mango puree adjusted pH to 3.5 showing a reduction in the browning rate during storage at 3°C (Guerrero-Beltrán et al., 2006).

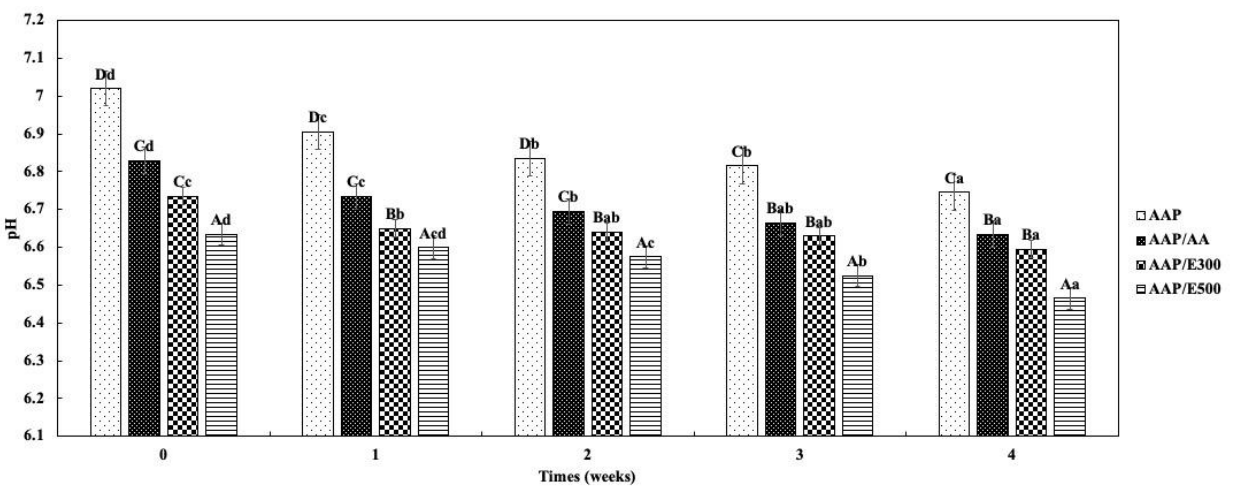


Fig. 8 Changes in the pH of the avocado puree storage at -20°C for 4 weeks (AAP = alkaline blanched avocado puree, AAP/AA = alkaline blanched avocado puree added with ascorbic acid, AAP/E300 and AAP/500 = alkaline blanched avocado puree added with the emblica fruit extract at the concentration of 300 and 500 ppm, respectively.)

Remark: [a-d] Mean±S.D. with different letters showing significant difference within the same treatment (p<0.05)
[A-D] MeanS.D. with different letters showing significant difference within the same time (p<0.05)

3.3 Total bacterial counts (TPC)

During storage at -20 °C, less than 1 log CFU/g were found in all AAP treatments at day 0. However, they were detected in FAP, although the avocado was cleaned before processing. For FAP, after processing (at day 0), it was found that FAP had 4.05±0.10 log CFU/g for frozen avocado. After 4-week storage at -20 °C, TPC of FAP was slightly increased, to 4.18±0.01 log CFU/g. These results probably contributed to avocado pretreatment, boiling of the avocado at 85°C for 3 min, which helped destroy some bacteria as well as addition of certain browning agents. Ukuku et al. (2004) demonstrated that immersion of inoculated cantaloupe in hot water at 70°C for 1 min, resulted in up to a 3.8 log CFU/cm^2 reduction in Salmonella. McCann et al. (2006) reported that surface pasteurization with hot water at 76°C for 3 min, resulted in more than 5 log CFU/cm^2 reduction in *Salmonella enterica* and *E. coli.* In addition, the acidulant such as ascorbic acids, citric acid and emblica fruit extract has an acidic pH, making it unsuitable for microbial growth. Ascorbic acid could function as a sanitizer agent inhibiting *C. albicans, S. aureus* and *E. coli* (EliUz, 2020). Emblica fruit extract acted on the cell membrane of microorganisms, resulting in the inability to grow. The minimum inhibitory concentration of emblica fruit extract at 13.97 mg/mL and the minimum biocidal concentration at 13.97 mg/mL could inhibit and destroy *Staphylococcus* aureus, respectively (Mayachiew & Devahastin, 2008).

Conclusion

Avocado puree could be stabilized by blanching and addition of acidulants during storage. Addition of ascorbic acid into avocado puree helped prevent the puree from color changes, while the emblica fruit extract

could cause the puree to become darker, depending on the extract concentrations. The pH of all treatments tended to decrease during storage. Less than 1 log CFU/g of total bacteria plate counts were detected in all samples, while more than 4 log CFU/g was found in the control, fresh avocado, which was unacceptable. This study indicated that stabilizing avocado puree by blanching in alkaline solutions combined with addition of ascorbic acid or emblica fruit extract could help maintain the puree quality during storage at freezing conditions.

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Influence of Exogenous Sucrose on Total Phenolic, Vitamin C and Antioxidant Enzymes of Soybean (*Glycine max* L.) Sprouts

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Abstract

This research explained the effect of exogenous sucrose on the levels of DPPH free radical scavenging ability, total phenolics and vitamin C as well as the L-galactono-1,4-lactone dehydrogenase (GalLDH), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities of soybean (*Glycine max* L.) sprouts. Soybean seeds were soaked in the various solutions prepared with 0, 1, 10 and 100 mM sucrose at 25°C for 12 hr and then sprayed with these solutions every 12 hr during the germination for 5 days. The higher concentration of exogenous sucrose decreased the L* value of soybean sprouts; whereas, the levels of DPPH free radical scavenging ability, total phenolic, vitamin C and GalLDH, SOD, CAT and APX activities increased. The soybean sprouts from 100 mM sucrose-treated seeds showed the maximum levels of DPPH free radical scavenging ability, total phenolics, vitamin C and GalLDH, SOD, CAT and APX activities, i.e. 85%, 164.95 mg GAE/g FW, 120.65 mg/100g FW, 25.32 U/g FW, 12.00 U/g FW, 25.36 U/g FW and 924 U/g FW, respectively. Thus, it suggested that the sucrose treatment helped to promote the soybean sprouts in containing high-levels of antioxidant activities.

Introduction

Soybean (*Glycine max* L.) sprouts are used for basic ingredients contained in Korean foods and popularly consumed in Southeast Asian countries (Ebert et al., 2017). They are composed of the significantly high contents of ascorbic acid, phenolic compounds, flavonoids, organic acids, amino acids and antioxidative properties (Guo et al., 2012). Tang et al. (2014) reported that soybean sprouts have the important functional ingredients used for human diets.

Sucrose extremely affects plant growth and

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metabolisms found in the cellular and organism levels (Couée et al., 2006). It is one of the important disaccharides available in most plants, regulates the photosynthesis and respiration, serves as a storage compound and maintains osmotic pressure in the cytosol (Eastmond, 2006; Eltayeb et al., 2007; Nishikawa et al., 2005). Thus, its main functions are the promotion of germination and seedling development (Nishikawa et al., 2005).

During germination or sprouting of soybean sprouts, a high number of monosaccharides and disaccharides is generated in soybean sprouts, which activates the sugar metabolism to stimulate the production of secondary metabolites, i.e. phenolic compounds (Chen et al., 2019). The application of exogenous sucrose tended to accumulate the ascorbic acid in harvested broccoli flowers due to regulate gene expression associated with vitamin C metabolism (Nishikawa et al., 2005). It not only played a role in improvement of the nutritional properties and antioxidative properties of broccoli flowers (Xu et al., 2016), but also greatly increased the contents of vitamin C, anthocyanins and phenolic compounds in broccoli sprouts (Guo et al., 2011). Wei et al. (2019) reported that the exogenous sucrose treatment of 0.5 g/L enhanced the levels of ascorbic acid, glucose, L-galactono-1,4-lactone dehydrogenase (GalLDH) activity and total phenolics as well as promoted the activities of antioxidant enzymes, e.g. superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) in mung bean sprouts. Recently, Yu et al. (2023) further suggested that the exogenous sucrose treatment at 30 g/L enhanced the higher levels of phenolic compounds, flavonoids and gamma-aminobutyric acid (GABA) in the treated mung bean sprouts as well as its extract inhibited alcohol-induced oxidative injury in HepG2 cells.

Consequently, the development of immerging and/or soaking solutions with the optimal sucrose concentration for soybean seeds to produce soybean sprouts is expected to lead to worthy scientific data based on the antioxidant enzymes and physicochemical characteristics. Besides, this may be a reference in helping to achieve a more comprehensive understanding for changes in the antioxidants as well as the antioxidant activities and enzymes of other cereal sprouts (treated with the soaking sucrose solution) during sprouting process.

The formation of reactive oxygen species (ROS) in plants exposes to destroy firstly the cellular organelles and properties of cell membrane caused by the lipid peroxidation of membrane, then degradation of the biological macromolecules and lastly cell death (Alscher et al., 1997). There are several antioxidant enzymes (such as SOD, APX and CAT) presented during growing plants and simultaneously eliminating ROS in plants (Ebert et al., 2017). Besides these enzymes remaining in the various substance of cells from the damage of ROS, they help to regulate not only plant growth, cell elongation, senescence and cell death, but also the cell differentiation, cell growth/division and detoxification of xenobiotics. Hence, these antioxidant enzymes, protecting the systems of plants and humans (Dumont & Rivoal 2019) have been of interest to study in different *in vitro* systems.

However, until now the effect of sucrose on color and the biological effects, based on GalLDH, SOD, APX and CAT of soybean sprouts has not been studied. Therefore, this study focused on the different concentrations at 0, 1, 10 and 100 mM sucrose affecting the color, total phenolic content, vitamin C content and bioactive activities in terms of the DPPH free radical scavenging ability, GalLDH, SOD, APX and CAT of soybean sprouts.

Materials and methods

1. Seed sprouting conditions

Soybean (*Glycine max* L.) seeds were obtained from a local market in Bangkok, Thailand. They were cleaned and separated into 4 treatments. They were soaked in 0, 1, 10 and 100 mM of sucrose solution (pH 7.0) at 25°C for 12 h. After the incubation, the seeds were placed into sterile plastic cups (cotton wool thickness of 2.5 cm placed on the bottom) containing many holes to drain water and stored in darkness at 25°C for 5 days. The treated seeds were sprayed by an electric sprayer with a delivery volume of 260 mL/min and the previous soaking solutions were used every 12 hr for 5 days of germination (Qiu et al., 2015.). Every treatment comprised of 60 seeds of soybean and carried out in triplicate. The soybean hypocotyls were harvested on day 5 and then rapidly frozen in liquid nitrogen. The frozen hypocotyls were kept at -100°C prior to analysis (Fig. 1).

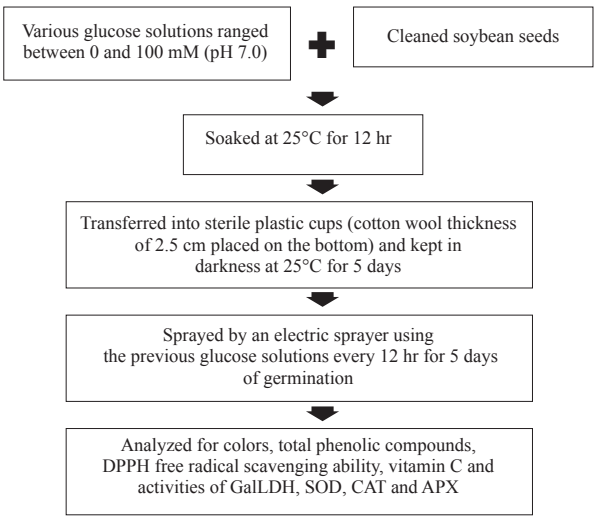

Fig. 1 Overview diagram of this experiment: preparation of soybean sprouts treated with exogenous sucrose solutions at 0-100 mM and their property analysis

2. Color analysis

The color analysis was performed using a colorimeter (Minolta CR-410, Minolta, Japan). The calibration was done by a standard plate. The means of L*, a* and b* were obtained from five points on the sample surface.

3. Total phenolic content

For the total phenolic content, a 2 g of the sample was transferred into 10 mL of 85% ethyl alcohol and then centrifuged at 4,500 g for 20 min. The supernatant was analyzed to find total phenolic content following the Folin-Ciocalteu method, described by Wang et al. (2017). Gallic acid was used as a standard. The phenolic level was calculated as milligrams gallic acid equivalent per gram of fresh weight (mg GAE/g FW).

4. DPPH free radical scavenging ability

To analyze the DPPH free radical scavenging ability, 2.0 g of the sample was put into 15 mL of 85% ethyl alcohol and then centrifuged at 4,000 g for 20 min. The supernatant was used to analyze the DPPH radical scavenging ability, explained by the method of Kraboun (2019) with a slight modification. Two mL of the supernatant was mixed with 5 mL of 0.5 mM DPPH and then kept in darkness for 45 min at ambient temperature. The ability was obtained the absorbance at 515 nm and calculated following the below formula.

$$\text{DPPH radical scavenging ability (\%)} = (100 - (\frac{\text{Abs sample}}{\text{Abs control}})) \times 100.$$

5. Vitamin C content

The extraction of vitamin C from the sample was obtained from 2.0 g of the sprouts transferred into 10 mL of 4.5% phosphoric acid. Then the mixture was centrifuged at 10,000 g for 12 min. The supernatant was read at 525 nm (Kampfenkel et al., 1995). Vitamin C was used as a standard. Vitamin C content is expressed as mg 100 g^{-1} FW.

6. L-galactono-1, 4-lactone dehydrogenase (GalLDH, EC 1.3.2.3) activity

To examine the GalLDH activity, 1.0 g of the sample was extracted with 5 mL of 200 mM potassium phosphate buffer (pH 7.0) and then centrifuged at 6,000 g for 15 min. The supernatant was again centrifuged at 10,000 g for 15 min. The collected precipitate was transferred into 5 mL of the potassium phosphate buffer. GalLDH activity was described by Tabata et al. (2001) with some modifications. 0.50 mL of the extract was put into 5.0 mL of 1.50 mg mL^{-1} cytochrome C and then incubated at ambient temperature for 5 min. To react t = 0, 0.5 mL of 60 mM L-galactono-1, 4-lactone (GalL) was added to the mixture. GalLDH activity was read at 560 nm. 1 unit of the activity is the enzyme content oxidizing 3 nmol of GalL (3 nmol of reduced Cytochrome C) per min.

7. Antioxidant enzyme activities

The activities of SOD, CAT and APX were described according to He et al. (2001) with some modifications. 500 mg of the sample was mixed with 5 mL of 200 mM potassium phosphate buffer (pH 7.5) and then centrifuged at 5,000 g for 25 min. The supernatant was obtained to measure the enzyme activities.

SOD (EC1.15.1.1) activity was explained by the procedure of Giannopolitis and Ries (1977) with a slight modification. The reaction reagent (5 mL) contained 60 mM phosphate buffer (pH 7.0), 70 mM riboflavin (7,8-dimethyl-10-ribitylisoalloxazine), 200 mM methionine [2-amino-4-(methyl-thio)-butyric acid], 5 mM EDTA and 2.0 mM nitro blue tetrazolium [NBT; 2,2′-di-p-nitrophenyl-5,5′-diphenyl-(3,3′-dimethoxy-4,4′- diphenylene) ditetrazolium chloride] and was mixed with 200 mL of the supernatant. The mixture without the enzyme solution was defined as the control. The mixture was exposured to fluorescent lights at 200 mM m^2 s for 10 min and then kept in darkness for 15 min. Subsequently, it was read at 560 nm, where 1 unit of the enzyme activity in which the enzyme content could destroy 50% of NBT photoreduction.

CAT (CAT, EC1.11.1.6) activity was defined as the study on H2O2 oxidation, described by Shao et al. (2013)

with some modifications. The reaction reagent (5 mL) was the mixture composing of 100 mM phosphate buffer (pH 7.5) and 50 mM H_2O_2 and mixed with 200 mL of the supernatant. The absorbance of sample was read at 240 nm in every 10 sec. intervals for 70 sec. 1 unit of CAT activity was the absorbance change of 0.02 per min.

APX (APX, EC1.11.1.11) activity was the study on the ascorbate oxidation, explained according to the method of Nakano & Assada (1981) with a slight modification. The reaction reagent (5 mL) was as the following: 200 mM sodium acetate buffer (pH 6.0), 5 mM EDTA and 6 mM H_2O_2 and transferred into 200 mL of the supernatant. The absorbance of sample was read at 290 nm in every 10 sec intervals for 70 sec. 1 unit of APX activity was the absorbance change of 0.02 per min.

8. Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) for Windows. The difference of data was separated by Duncan's multiple range tests (DMRT). Values are defined as mean± standard deviation (SD). Differences were investigated at a significant level of 0.05.

Results and discussion

1. Impact of exogenous sucrose on L*, a* and b* of soybean sprouts

As shown in Table 1, the higher concentration of sucrose significantly exhibited a decrease in the L* values of soybean sprouts (p<0.05). The L* values of soybean sprouts obtained from 1 mM sucrose-treated seeds and the control (untreated) were highest, which ranged from 72.88 to 75.78 (p>0.05). On the other hand, all the soaking sucrose solutions did not affect the a* and b* values of soybean sprouts (p>0.05). This may be a high concentration of sucrose causing osmotic stress affecting the moisture removal from the plant cells, which resulted in retarding the plant growth and then affecting a less amount of chlorophyll accumulation (Sharma et al., 2019). Thus, the chlorophyll was formed slowly during the growth of the soybean sprouts, affecting higher L* value as well (Price et al., 2003). Moreover, this germination period of soybean sprouts is very short (5 days), indicating the small amount of accumulated chlorophyll. This result was in agreement with Pertiwi et al. (2013), who noted that a short germination period of the legume seeds showed the higher L* values of the sprouts. While, this finding was in disagreement with Murugkar (2014), who claimed that the germination period did not impact on the L* value of soybean sprouts.

Table 1 L*, a* and b* of soybean sprouts from 0-100 mM sucrose-treated seeds

Sucrose (mM)	L*	a*^{ns}	b*^{ns}
0	75.78±0.08^a	-2.54±0.03	-1.46±0.02
1	72.88±0.01^a	-1.60±0.03	-2.47±0.08
10	50.61±0.05^b	-1.96±0.09	-2.61±0.07
100	45.61±0.06^c	-2.61±0.01	-1.32±0.08

Remark: Different letters behind means within a column are significantly different (p<0.05). ns is not significantly different (p>0.05).

2. Impact of exogenous sucrose on DPPH free radical scavenging ability and total phenolic content of soybean sprouts

DPPH free radical method is an antioxidant assay based on both electron transfer (SET) and hydrogen atom transfer (HAT) reactions. This free radical, stable at room temperature, is reduced when appearing as an antioxidant molecule, giving colorless ethanol solution (Guo et al., 2012; Liang & Kitts, 2014). The DPPH free radical scavenging ability of soybean sprouts is displayed in Fig. 2a. The higher sucrose concentrations improved the DPPH radical scavenging ability of soybean sprouts. The soybean sprouts from 100 mM sucrose-treated seeds indicated the highest DPPH radical scavenging ability by 85%. However, the DPPH radical scavenging abilities of soybean sprouts from 1 mM sucrose-treated seeds and the control (untreated) were lowest and showing not different significantly (p>0.05). This was in agreement with Xu et al. (2016), who reported that the sucrose-treated broccoli had higher DPPH radical scavenging activity and total phenolic content as compared with the control (untreated).

Phenolic compounds are the products of secondary metabolism in plants and promote health benefits due to reducing the risk of chronic diseases (Li et al., 2019). The total phenolic content of soybean sprouts is illustrated in Fig. 2b. The patterns of total phenolic content and DPPH free radical scavenging ability were the same. The highest total phenolic content obtained from the soybean sprouts from 100 mM sucrose-treated seeds was 164.95 mg GAE/g FW. The solution of soaking sucrose may play the significant role in accumulating phenolic compounds during growing sprouts due to stimulate gene expression related with phenolic metabolism (Guo et al., 2011; Nishikawa et al., 2005; Xu et al., 2016).

Moreover, Wei et al. (2019) suggested that the sucrose treatment increased the levels of ascorbic acid, phenolic compounds and antioxidant activities of mung bean sprouts. Furthermore, López-Amorós et al. (2006) noted that sucrose could affect a decrease in anti-nutrient phenomenon, but an increase in contents of amino acids,

sugars, dietary fibers and antioxidants during the germination period of legumes. The stimulation of synthesis of polyphenols found in plants, e.g. phenolic compounds and flavonoids was from the abiotic stress, which promotes the plants to handle with the unsuitable environmental conditions (Li et al., 2016). Therefore, phenylpropanoid biosynthetic pathway is generated from the abiotic stress (drought, heavy metals, high content of salt, inappropriate temperatures, high content of carbohydrates and UV radiation) leading to increase phenolic compounds (Aghdam et al., 2020). Winarsi et al. (2020), who confirmed that an increase in phenolic content during the germination is present due to the induction of phenylalanine ammonia-lyase (PAL) activity synthesized biogenetically through a shikimate/phenylpropanoid pathway (Koodkaew, 2019).

C (L-ascorbate) biosynthesis in plants (Wheeler et al., 1998). As shown in Fig. 3, the higher sucrose concentrations increased the levels of vitamin C and GalLDH activity of soybean sprouts. The vitamin C content and GalLDH activity of soybean sprouts from 100 mM sucrose-treated seeds increased 1,065.70% and 1,925.60%, respectively as compared with the control (untreated). Obviously, the increased concentrations of sucrose played an important role on the synthesis of vitamin C and GalLDH activity. This was in agreement with Guo et al. (2011), who noted that the treatment of 88 mM sucrose affected the increment of ascorbic acid in broccoli sprouts by 41% versus the control. Moreover, Cao et al. (2015) also found that the application of sucrose solution for soaking cucumber seeds contributed to the increased content of vitamin C in cucumber seedlings.

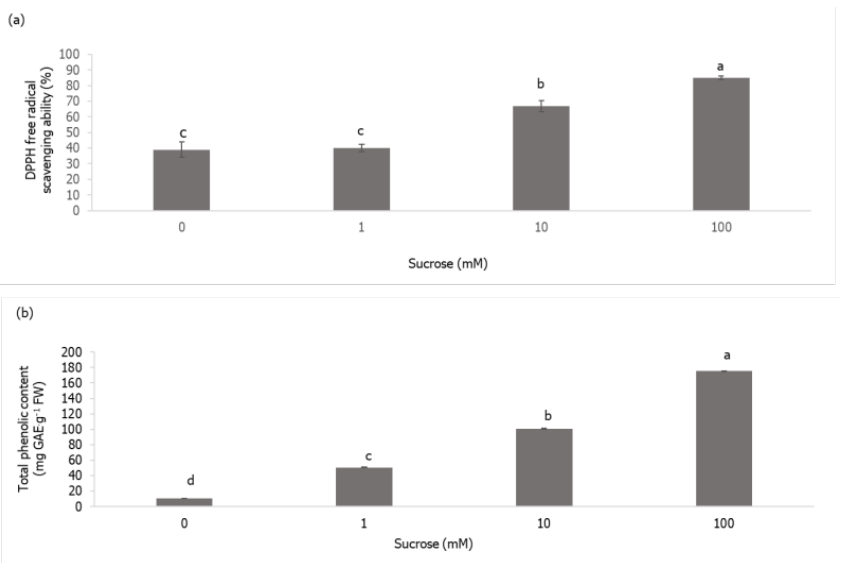

Fig. 2 DPPH free radical scavenging ability (a) and total phenolic content (b) in soybean sprouts on day 5 from 0-100 mM sucrose-treated seeds
Remark: Different letters are significantly different (p<0.05)

3. Impact of exogenous sucrose on vitamin C content and L-galactono-1,4-lactone dehydrogenase (GalLDH) activity of soybean sprouts

Vitamin C or ascorbic acid, possessing a strong antioxidant property, is a cofactor for a lot of the enzymes and neutralizes the effect of reactive oxygen species (ROS) (Aghdam et al., 2022; Smirnoff et al., 2001). L-galactono-1,4-lactone dehydrogenase (GalLDH) is a FAD-containing oxidoreductase that catalyzes the terminal step of Smirnoff–Wheeler pathway of vitamin

Our results indicated the higher GalLDH activity and vitamin C biosynthesis would generate together since our experiment condition may be under the optimal condition for soybean seed germination. This phenomenon was explained because of the appropriate condition for sprouting cereal seeds enhancing the levels of both L-galactose and the enzyme GalLDH in the sprouts; therefore, this enzyme GalLDH can hydrolyze L-galactose to transform ascorbic acid with a high concentration (Guo et al., 2011).

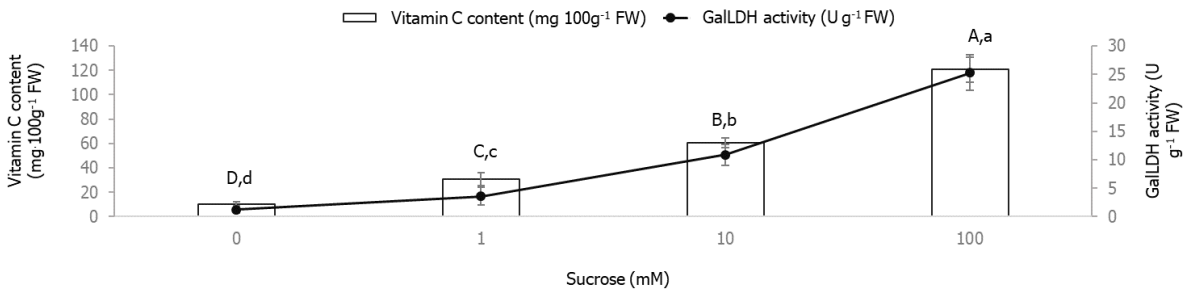

Fig. 3 Vitamin C content and GalLDH activity of soybean sprouts from 0-100 mM sucrose-treated seeds
Remark: Capital and small letters indicate significantly different ($p<0.05$) of vitamin C content and GalLDH, respectively

4. Impact of exogenous sucrose on the activities of antioxidant enzymes of soybean sprouts

SOD catalyses the disproportionation of superoxide ($O_2^{\cdot-}$) radicals to become H_2O_2 and O_2 (McCord & Fridovich, 1969), which plays a significant role in order to protect the biological cells (Xu et al., 2019). Plant SODs are the metalloenzymes consisting of Fe, Mn or Cu/Zn (a prosthetic group). The number, type, and distribution of SOD isoenzymes may be changed due to the species, developmental stage and environmental conditions (Bridges & Salin, 1981). Catalase (CAT) is an enzyme-containing homotetramer ferriheme, with Fe as a cofactor that catalyzing hydrogen peroxide (H_2O_2) into water and oxygen. CAT activity plays an important role in detoxifying H_2O_2, which is increased with age (Lobo et al., 2010). Ascorbate peroxidase (APX) isoenzymes are important in changing H_2O_2 into H_2O, especially found in the chloroplast (Chen et al., 2012).

The antioxidant enzymes, i.e. SOD, CAT and APX are depicted in Fig. 4. The SOD, CAT and APX activities of soybean sprouts of all treatments had the same pattern. The higher SOD, CAT and APX activities were found

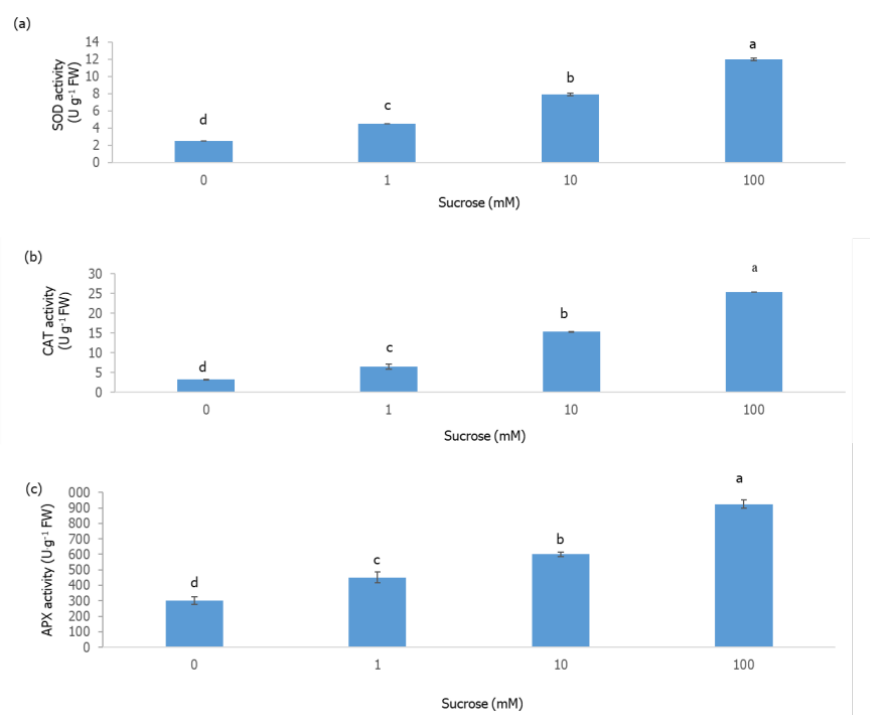

Fig. 4 SOD (a), CAT (b) and APX (c) activties in soybean sprouts from 0-100 mM sucrose-treated seeds
Remark: Different letters are significantly different ($p<0.05$)

in the soybean sprouts produced from the sucrose-treated seeds soakedand/or sprayed using the higher sucrose concentrations. The maximum activities of SOD, CAT and APX obtained from the soybean sprouts produced from 100 mM sucrose-treated seeds were observed, which increased 380, 692.5 and 208%, respectively as compared with the control (untreated). Obviously, the incremental accumulation of secondary methabolites such as the total phenolic content, vitamin C and GalLDH (Fig. 2b and Fig. 3) of soybean sprouts was related to SOD, CAT and APX activities (Fig. 4). These results indicated that the sucrose treatment could serve more effectiveness of SOD, CAT and APX activities of soybean sprouts. This suggested that the osmotic substances, i.e. a high concentration of sucrose could stimulate the development of an anti-oxidative defence system, thus the accumulation of antioxidant enzymes was observed (Xue-Feng et al., 2019). This was in agreement with Cao et al. (2014), who reported that the higher sucrose concentration activated the SOD and APX activities in cucumber seedlings. Wei et al. (2019) and Xu et al. (2016) further reported that the activities of SOD, APX and CAT in sucrose-treated broccoli florets and mung bean sprouts were higher than those in the untreated control. In this study, the results exposed that the changes of DPPH free radical scavenging ability and total phenolic content (Fig. 2) that exactly corresponded to the activities of SOD, CAT and APX (Fig. 4). Thus, exogenous sucrose treatment is an effective way to rise the DPPH free radical ability, total phenolic content and the antioxidant enzymes of soybean sprouts.

Conclusion

Soybean sprouts are an important dietary source containing both antioxidant activities and enzymes. The soybean sprouts from 100 mM sucrose-treated seeds resulted in significantly increased levels of total phenolics, DPPH free radical scavenging ability, vitamin C and GalLDH, SOD, CAT and APX activities. However, the L* values of soybean sprouts were lower when the soybean seeds were treated with the higher sucrose concentration; however, the a* and b* values of all treatments were not significantly different (p>0.05). Therefore, the sucrose treatment at 100 mM should be an alternative way for the production of soybean sprouts composing of good antioxidant properties and physicochemical qualities.

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Development of a Rapid UV-Visible Spectrophotometry Method to Assess of Total Carotenoid Content in a Green Microalgae, *Scenedesmus armatus*

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Abstract

The benefits of microalgae are due to the promising sources of pigments influencing researchers to focus on optimizing the culture conditions for the high-yield pigment of microalgae culture. However, conventional procedures to determine microalgae pigments required a large sample volume and toxic chemicals. Due to the drawback of conventional procedures for determining the pigment of microalgae, this research aims to develop a new UV-visible spectrophotometry method using the Remote Diffuse Reflectance Accessory (RDRA) equipped with a UV-visible spectrophotometer for assessment of the total carotenoid content in green microalgae, *Scenedesmus armatus*. Using an optimal preparation of *S. armatus* on the Whatman GF/C™ glass microfiber (GF/C™) filter and scanning UV-visible spectra using RDRA, the characteristic peak of carotenoid at 480 nm demonstrates good analytical characteristics. It exhibits a strong linear relationship with cell concentrations ranging from 2-20 x 10^7 cells/mL ($R^2 = 0.9884$). The developed method yields a total carotenoid content of 2.16±0.58 ng/10^4 cells for *S. armatus*. A paired t-test at a 95% confidence level indicates no significant difference ($P \geq 0.05$) between total carotenoid content obtained using the developed method and a conventional method (1.96±0.24 ng/10^4 cells). In summary, the developed method shows promise for estimating total carotenoid content in green microalgae. Furthermore, the developed method offers advantages over the conventional method by reducing sample processing time and eliminating the need for hazardous reagents and a large volume of samples.

Introduction

Nowadays, the word "food security" has gained significant importance due to the impacts of climate change. According to food security, there is a global focus on the search for highly nutritious food sources. Microalgae are considered one of the best candidates for high-nutrition food sources. Microalgae are recognized for their ability to provide abundant amounts of various nutrients, including essential vitamins, minerals, proteins

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and fatty acids. Additionally, microalgae are particularly rich in pigments such as chlorophyll, carotenoids and phycobiliproteins. Pigments are widely used in many industries, such as pharmaceuticals, food and functional food and supplements. Pigments serve multiple purposes in the industry as sources of color, antioxidants and bioactive compounds with potential health benefits. *Scenedesmus armatus,* green microalgae, is interested in aquaculture due to its high nutritional value and suitability as a feed source for aquatic organisms due to its high-fat content of 27.4-29.7% of dry weight (Ryckebosch et al., 2011). In addition, *S. armatus* has been an attractive interest because of its potential to produce highly nutritious food and various medical applications due to the high concentration of bioactive compounds such as pigments and carotenoids (Goodwin & Britton, 1988). Carotenoids found in algae are responsible for their colors and possess bioactive properties. Therefore, carotenoids are widely used in many applications, such as food, medicine, cosmetics and aquatic products, due to antioxidant and immune-boosting benefits (Jalal et al., 2013). According to the increasing demands, numerous researchers are conducting studies aimed at optimizing culture conditions for pigments, such as carotenoid production, to maximize pigment yield (Pavel et al., 2016; Razi Parjikolaei et al., 2013; Rise et al., 1994; Seyfabadi et al., 2011). The quantification or determination of pigment content is an essential step in this field of research.

The analysis of each type of pigment in microalgae culture process, particularly carotenoids, involves many techniques, such as flow cytometry (Chen et al., 2017), high performance liquid chromatography tandem mass spectrometry (Soares et al., 2019) and particularly extraction methods using organic solvents (Hyoung, 2001). In 1972, Strickland & Parsons (1972) reported spectrophotometric assays to estimate pigments such as chlorophyll a, b, c and total carotenoid content using acetone as an organic solvent for extraction. These methods have served as standard procedures for estimating pigments in algae, such as carotenoids and chlorophyll. However, these methods are time-consuming and require a large volume of samples and toxic chemical organic solvents for extraction. Therefore, extraction methods do not work well for pigment analysis in microalgae. Due to the large volume of samples and time-consuming pigment estimation in biological samples, spectrophotometric measurement of solid-state samples using diffuse reflectance accessory (DRA) has been considered an effective tool due to rapid and non-invasive sample preparation (Blitz, 1998). DRA and RDRA are widely used in near-infrared (NIR) and UV-visible spectrometers to obtain spectra of various solid-state samples. By means of specific wavelength chemical bond absorption, NIR reflectance spectroscopy is applied for the analysis of plant and animal tissue (Foley et al., 1998) and food quality control applications (Cen et al., 2007; Huang et al., 2008; Nicolai et al., 2007). Diffuse reflectance UV-visible spectroscopy is used in biological research as an in vivo study (Leštan et al., 1993). Besides spectral analysis, DRA is commonly used for color measurement of plant and fruits sample (Kasajima, 2019). For pigment analysis, diffuse reflectance spectroscopy has been applied for carotenoids contents in maize (Brenna et al., 2004), banana and plantain fruit pulp (Davey et al., 2009) and yellow-fleshed watermelon (Davis et al., 2004). In the field of algae research, NIR reflectance spectroscopy has been applied for an estimate and quantitative analysis of protein, carbohydrate and lipid content in microalgae (Dean et al., 2010; James et al., 2011; Larens & Wolfrum, 2013). However, diffuse reflectance UV-visible spectroscopy has rarely been used for studies in pigment analysis for microalgae research (Duppeti et al., 2017). Therefore, this research aims to develop a new method for pigment content estimation, focusing on total carotenoid content in microalgae, *S. armatus*. to create a practical and reliable assay for quantifying total carotenoids in microalgae research. Herein, we describe a protocol for the optimal fabrication of fresh cells on a solid support for absorbance measurements using an RDRA. Moreover, our developed method has been validated by comparing results with the conventional method reported by Strickland and Persons (Strickland & Parsons, 1972).

Materials and methods

1. Apparatus

All UV-visible spectra were recorded on a Cary 60 UV-visible spectrometer (Agilent Technology, Inc. USA) with a xenon flash lamp and a photomultiplier tube (PMT) as a light source and detector, respectively. In all conventional method experiments, sample solutions were carried out with UV-visible spectra measurements using an ultra-microvolume cuvette with an 1 mm path length lid (Hellma GmbH & Co. KG, Germany). UV-visible spectra of solid support samples in developed method

experiments were measured using a VideoBerrelino Remote Diffuse Reflectance Accessory (RDRA) (Harrick Scientific Products, Inc. USA).

2. Reagents and materials

All chemicals in microalgae cultivation were purchased as analytical grade (Sigma Aldrich, USA). Spectroscopic grade acetone (Merck, Germany) was used for the conventional method experiments. Water used for the experiments was purified with a Milli-Q filtration system (Millipore, USA).

3. *S. armatus* cell preparations

The inoculum of *S. armatus* used in the experiment was previously isolated from open-air tilapia tanks by Kamperayanon (2013). According to Chen's modified method, *S. armatus* was cultivated by immobilization techniques using sodium alginate matrices (Chen, 2007). *S. armatus* was immobilized in stable gel beads with a 3-4 mm diameter and an average weight of 0.2 g per bead. The microalgae in gel beads were cultured in Chu's Medium (Chu,1942; Stein, 1973) at 26±2 °C under 3,000 lux light conditions until reaching the stationary growth stage. After removing the algae beads from the media, 3 g of the gel beads were dissolved using 5 mL of 5% $(NaPO_3)_6$ solution (Chen, 2007). The algae suspension was centrifuged at 4,000 rpm for 5 min and the supernatants were discarded. After washing the pellet twice with Milli-Q water and removing the supernatants, the cell pellet was resuspended in 5 mL of Milli-Q water to be used in the subsequent experiments.

4. Determinations of the optimum conditions for preparing samples using RDRA

4.1 Effect of solid supports

Into 1.5 mL Eppendorf tube, 1.0 mL cell solution with the desired number of cells was centrifuged at 4,000 rpm for 5 min and the supernatants were discarded. Then 1.0 mL of Milli-Q water was added to obtain $2x10^7$ cells/mL. To fabricate a solid-state sample for RDRA, 5 mL of cell solution was introduced to three types of solid supports, including the GF/C™ glass microfiber (GF/C™) filter (Whatman), Whatman filter paper No.1 (Whatman) and nylon membrane filter 0.45 μm (ANOW). Then samples were left to dry in the dark at room temperature for 30 min. UV-visible spectra of solid support samples were scanned in the 250-750 nm range using RDRA.

4.2 Effect of volumes of sample

To fabricate a solid-state sample for RDRA, a $2x10^7$ cells/mL cell solution was introduced to the GF/C™ filter with a range of 1-50 mL volume. After drying in the dark at room temperature for 30 min, UV-visible spectra of solid support samples were scanned in the 250-750 nm range using RDRA.

4.3 Effect of temperature

To fabricate a solid-state sample for RDRA, a 5 μL of $2x10^7$ cells/mL cell solution was introduced to the GF/C™ filter. To evaluate the effect of drying temperature, samples were dried in the dark under three conditions at room temperature, 40°C and 60°C for 30 min. Then, UV-visible spectra of solid support samples were scanned in the 250-750 nm range using RDRA.

5. Effect of *S. armatus* concentrations on carotenoids determination under optimum conditions for RDRA

Into a 1.5 mL Eppendorf tube, 1.0 mL of cell solution with the desired number of cells was centrifuged at 4,000 rpm for 5 min and the supernatant was discarded. Milli-Q water (50 – 500 μL) was added to obtain various concentrations as 2 to 20×10^7 cells/mL. To fabricate a solid-state sample for RDRA, 5 μL of the cell solution was introduced onto the GF/C™ filter. Samples were left to dry in the dark at room temperature for 30 min. UV-visible spectra of the solid support samples were scanned in the 250-750 nm range using RDRA.

6. Total carotenoid content determination using developed method

Into a 1.5 mL Eppendorf tube, $1.2x10^7$ cells of *S. armatus* were centrifuged at 4,000 rpm for 5 min and the supernatant was discarded. Then 300 mL Milli-Q water to resuspend cells into a solution. Under optimal conditions, solid-state samples were fabricated using 5 μL of the cell solution onto a GF/C™ filter. Samples were left to dry in the dark at room temperature for 30 min. UV-visible spectra of the solid support samples were scanned in the 250-750 nm range using RDRA.

7. Total carotenoid content determination using conventional method

The conventional method was modified by the Strickland & Parsons (1972) method. Into a 1.5 mL Eppendorf tube, 4.0×10^7 cells of the same bulk sample of *S. armatus* were centrifuged at 4,000 rpm for 5 min. After discarding supernatants, the pellet of *S. armatus* cell was extracted with 400 μL of 90% acetone in water. The suspension was placed on a vortex mixer for 1 min and then centrifuged was allowed for phase separation. The acetone crude extracted solution was separated and transferred into a 2 mL vial. The acetone crude extracted was evaporated under reduced pressure using a rotary evaporator and freeze-drying to remove solvent for dryness. Subsequently, the acetone crude extracted was

redissolved with 400 µL spectroscopic acetone. A 5 µL acetone-crude extract solution was introduced to an ultra-microvolume cuvette with a 1 mm path length lid and then UV-visible spectra in the range of 250-750 nm were recorded.

8. Estimation of total carotenoid content

The total carotenoid content for the developed method and conventional method were estimated using the eq. (1) given by Strickland & Persons calculation (Strickland & Parsons, 1972) as follows:

$$\text{Total carotenoid (ng/104 cells)} = \frac{4xA_{480}xVx1000}{cell\ density} \quad (1)$$

where A_{480} is an equivalent absorbance at 480 nm, V is a volume of cell solution and the cell density is a unit of 10^4 cells. The equivalent absorbance at 480 nm (A_{480}) was calculated using eq. (2) as follows:

$$A_{480} = A^*_{480} \times 10 \quad (2)$$

where A^*_{480} is an absorbance recorded by the ultra-microvolume cuvette with a 1 mm path length lid and RDRA.

9. Statistical analysis

All measurements were carried out for replication samples (N = 3), and the results are expressed as mean values ± standard deviation. Linear least squares regression analyses and Paired t-test (N=30) were performed using the Data analysis tool of Microsoft® Excel 2019.

Results and discussion

1. Determinations of the optimum conditions for preparing samples using RDRA

1.1 Effect of solid supports

Three types of materials were examined to study the proper solid support for the preparation of solid-state samples for RDRA measurement, including GF/C™ filter, Whatman filter paper No.1 and nylon membrane filter 0.45 µm for recorded UV-visible spectra. UV-visible spectra and absorbance at 480 nm of carotenoid characteristic peak response are presented in Fig.1. According to the controlled fabrication conditions with a 5 µL of 2×10^7 cells/mL *S. armatus* died out in the dark at room temperature for 30 min, all types of solid supports exhibited absorption bands at 440-480 nm and 670 nm, contributed to carotenoid and chlorophyll, respectively (Fig. 1a). Moreover, UV-visible spectra of solid-state samples compared to the spectrum of acetone crude extract solution of *S. armatus* (8×10^7 cells/mL), all the bands of solid-state sample had longer wavelength than the acetone crude extract solution (Fig. 2). Consideration on the absorbance at 480 nm, which corresponds to the carotenoid characteristic peak, the result revealed that the highest response was observed on the GF/C™ filter, followed by the nylon membrane 0.45 µm. The lowest response was observed on the Whatman filter paper No.1 filtration. According to properties presented on the product website studies as previous studies, retention sizes and hydrophobic properties of GF/C™ filter (Hickel, 1984; Logan et al., 1993; Cytiva Life Sciences, n.d.), Whatman filter paper No.1 (Cytiva Life Sciences, n.d.; Nishat et al., 2021) and nylon membrane filter 0.45 mm (Hangzhou Anow Microfiltration Co., Ltd., n.d.); Zhang et al., 2010) could be considered for this result. As shown in the image of the GF/C™ filter sample in Fig. 1c, the small pore size of 1.2 µm pore diameter and the hydrophilic polarity of the GF/C™ filter allowed for the even dispersion of *S. armatus* cells on the surface, fitting well within the sample spot size of RDRA (1.5 mm) resulting in the highest response. Despite the Whatman filter paper No.1 having a hydrophilic polarity, which can create a uniform distribution of cells, the retention size of the Whatman filter paper No.1 is 11 mm which means the cells did not retain on the surface, resulting in the lowest absorbance. The pore size of the nylon membrane (0.5 µm pore diameter) is smaller than the size of microalgae cells. The cells can retain well on the surface. However, due to its hydrophobicity, a wider distribution of cells exceeded the sample spot size of RDRA, resulting in a lower signal.

1.2 Effect of sample volumes

This experiment aimed to investigate the effect of *S. armatus* sample volumes on the fabricated GF/C™ filter. As shown in Fig. 3, the absorbance of UV-visible spectra of *S. armatus* at a concentration of 2×10^7 cells/mL increased with the increase of sample volume due to increasing cell concentrations. However, the linearity of the signal and concentrations should be considered. Fig. 3b demonstrates that the absorbance response at 480 nm and the concentration of cells exhibit low linearity and low precision with increasing standard deviations. The measurement uncertainty associated with high sample volumes results from larger diameters, leading to different cell distributions within each volume. Therefore, it can be concluded from this experiment that the optimum sample volume is 5 µL which is an appropriate volume for a sample window of RDRA.

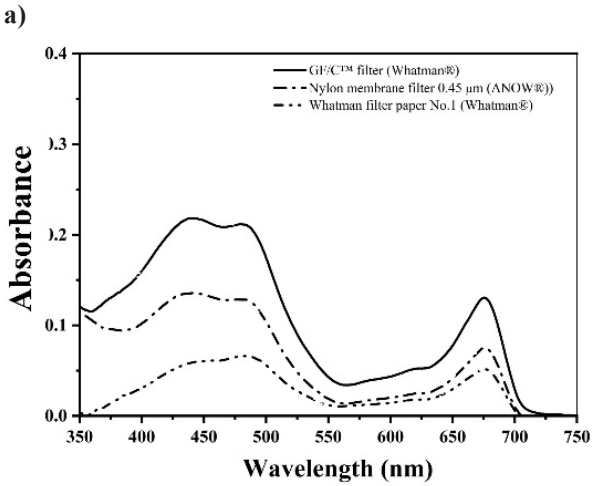

Fig. 1 Effect of solid supports on UV-visible spectra (a) and absorbance at 480 nm (b) of S. armatus 5 µL, 2×107 cells/mL fabricated onto GF/C™ filter (Whatman), Whatman filter paper No.1 (Whatman) and nylon membrane filter 0.45 mm (ANOW); and solid-state samples images of S. armatus fabricated onto solid supports (c).

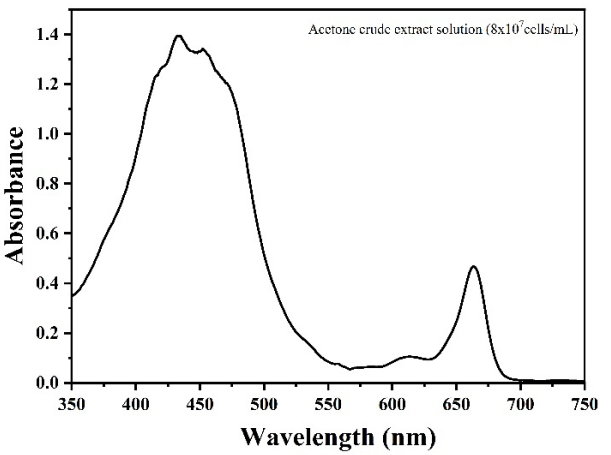

Fig. 2 UV-visible spectrum of acetone-crude extract solution of 8×107 cells/mL *S. armatus*

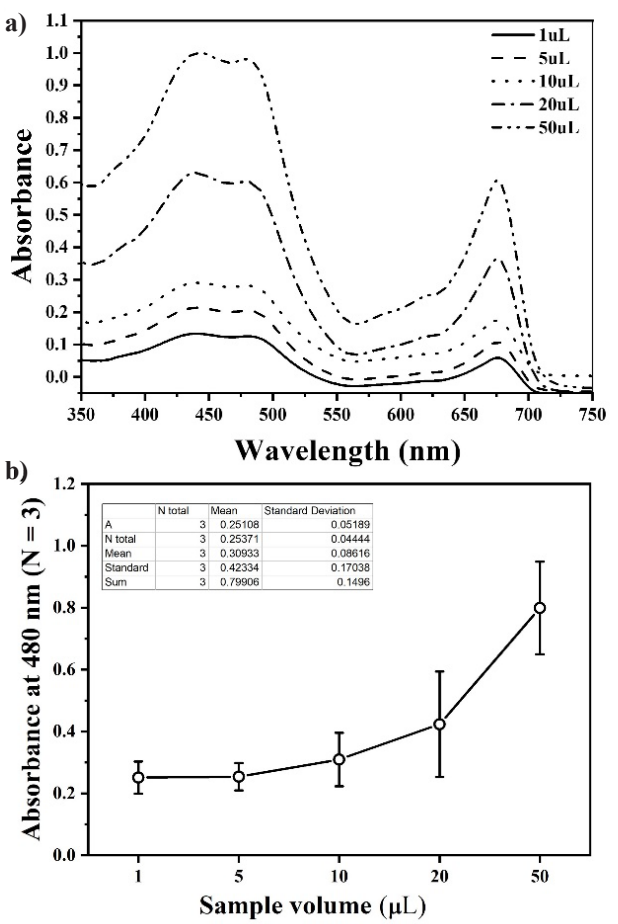

Fig. 3 Effect of sample volumes on UV-visible spectra (a) and absorbance at 480 nm of S. armatus (b) fabricated onto GF/C™ filter (Whatman) with different sample volumes in the range of 1 – 50 mL (cell concentration = 2×107 cells/mL).

1.3 Effect of temperature

In the solid-state sample for RDRA measurement, the sample needs to be dried to minimize the reflection effect of solvent. Therefore, this experiment investigated the effect of temperature on a drying process using an optimal volume of 2 × 10^7 cells/mL introduced on the GF/C™ filter. The samples were dried under a cover at different temperatures, including room temperature, 40°C and 60°C, for 30 min. As shown in Fig. 4, absorbance bands of 40°C and 60°C conditions were lower than room temperature conditions and corresponded to thermal degradation of carotenoids at high temperatures (Sun et al., 2023).

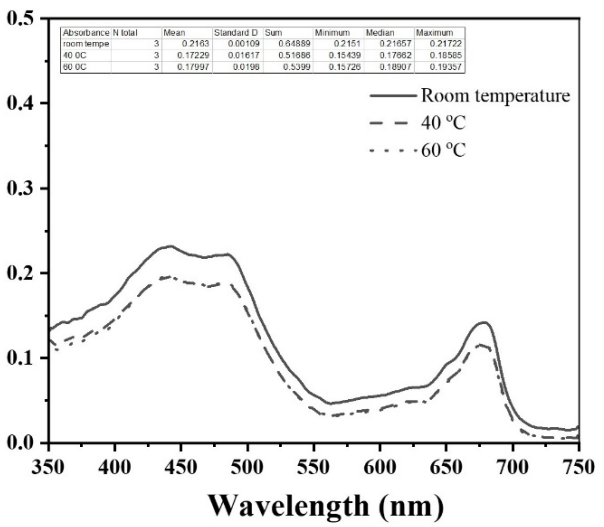

Fig. 4 UV-visible spectra of 5 µL 2×10^7 cells *S. armatus* fabricated onto GF/C™ filter with drying at different temperatures: room temperature, 40°C and 60°C, respectively

2. Possibility to apply the developed method for the analysis of carotenoid content

To apply the method for quantitative analysis, the effect of cell concentrations on the carotenoid response at 480 nm was investigated using the optimum measurement conditions of 5 µL volume sample fabricated on GF/C™ filter and dried in the dark at room temperature for 30 min. The solid-state sample was carried out using optimal conditions as presented in Fig. 5.

Fig. 5 Solid-state sample image of *S. armatus* cells with optimal conditions as the volume of sample 5 µL fabricated onto GF/C™ filter and dried at room temperature for 30 min

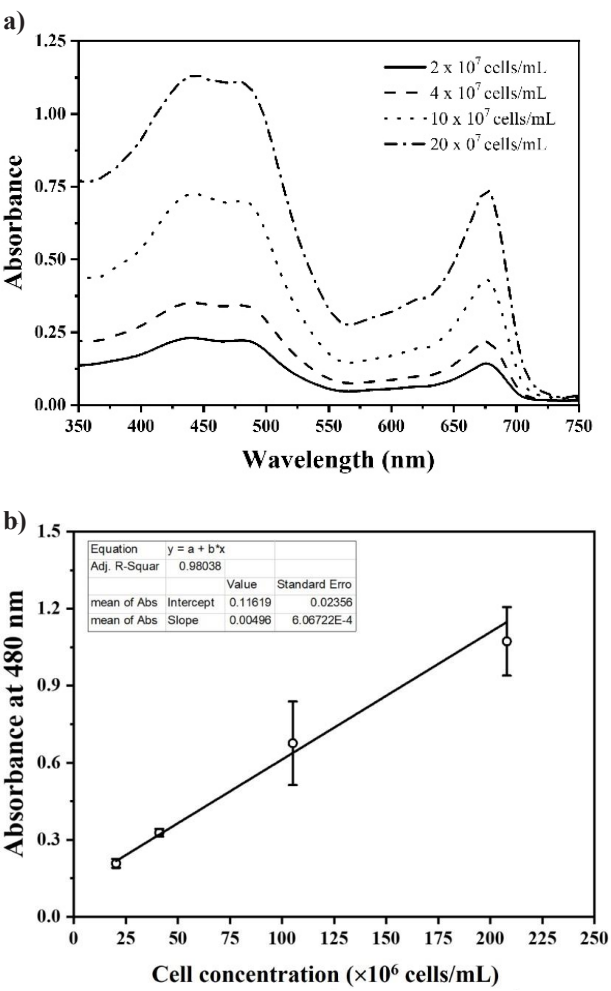

Fig. 6 Effect of cell concentrations on UV-visible spectra (a); and calibration plot of absorbance at 480 nm of S. armatus 2-20×107 cells/mL with optimal conditions as the volume of sample 5 mL fabricated onto GF/C™ filter and dried at room temperature for 30 minutes.

As shown in Fig. 6a, the absorbance band at 480 nm increases with an increase of cell concentrations. The response of the carotenoid characteristic peak at 480 nm in Fig. 6b exhibits a strong linear relationship with cell concentrations ranging from 2-20×10^7 cells/mL (R^2 = 0.9884). These results can be noted that the developed method can be applied to quantitative applications.

3. Comparison of the total carotenoid content of the developed method with the conventional method of Strickland and Parsons

To compare the analytical results of the developed method with the conventional method of Strickland & Parsons (1972), the carotenoid content of both methods were analyzed using the same bulk sample of *S. armatus*. The total carotenoid content of both methods were estimated using the equivalent absorbance at a wavelength of 480 nm (A480) and Eq. (1). The total carotenoid concentration obtained by the developed method under optimum conditions was 2.16±0.58 ng/10^4 cells (n = 30). In comparison, according to the conventional method, the total carotenoid content in *S. armatus* were determined to be 1.96±0.24 ng/10^4 cells (n = 30). A paired t-test at the 95% confidence level was performed to compare the results of total carotenoid analysis by the developed and standard methods. The analysis showed that there was no significant difference ($P \geq 0.05$) between the total carotenoid content obtained by the developed method (2.16±0.58 ng/10^4 cells) and the conventional method (1.96±0.24 ng/10^4 cells). The results of this study demonstrate that the developed method allows for the analysis of equivalent absorbance at 480 nm using a solid-state measured with RDRA to estimate total carotenoid content for microalgae. However, this developed method also provides a low precision as a high standard deviation of the analytical results. The measurement uncertainty may be obtained from the sample in the form of cell suspension. There may be some effect on cells transferred in a variable of each sample on solid support. The high standard deviation of the result also appears in the conventional method. However, both methods offer the same range in the standard deviation (≈25% deviation form means). The high variation of the result of this method may be derived from the loss of sample during the extraction process.

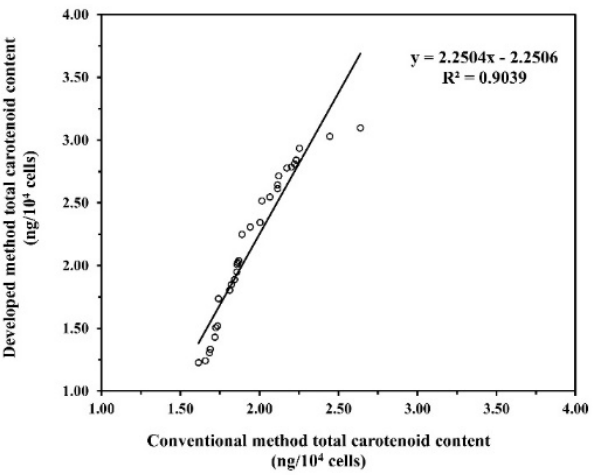

Fig. 7 Total carotenoid content of *S. armatus* (n = 30) using optimal condition of developed method compared to total carotenoid content of the similar bulk sample *S. armatus* (n = 30) using conventional method

Furthermore, the estimated total carotenoid content of *S. armatus* using the developed method was plotted against the total carotenoid content estimated using the conventional method (Fig. 7). A linear relationship was obtained between the estimated two methods with an R^2 of 0.9039. According to the results, the developed method overcomes the drawback of the conventional method for carotenoid estimating for microalgae in terms of large-scale sample requirement, time-consuming and not generating toxic chemicals in the extraction process. It can be concluded that the new development method could replace the conventional method for total carotenoid content estimation for *S. armatus*.

Conclusion

In conclusion, we have successfully developed a new practical method for estimating the total carotenoid content in *S. armatus* using RDRA with a UV-visible spectrometer. The optimal sample preparation on GF/C™ filter as a solid support has demonstrated a characteristic carotenoid peak at 480 nm, indicating promising quantitative application with a well-established linear relationship between the signal and cell concentrations. To assess the suitability of the proposed method for pigment production in microalgae cultivation, we compared the total carotenoid content of *S. armatus* obtained using the developed method with a conventional method using a paired t-test at a 95% confidence level. The results showed that the developed

method exhibits no significant differences in total carotenoid values compared to the conventional method and offers an improvement, including non-hazardous waste generating, saving time and low sample needs. The developed method can serve as a total carotenoid estimating assay in microalgae research due to its simplicity, rapidity and cost-effectiveness. Its advantageous features makes it a practical choice for an efficient and reliable method to routinely estimate carotenoid content in microalgae.

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Antioxidant Activities and Cytotoxicity Effect on Normal Human Dermal Fibroblasts of Roselle (*Hibiscus sabdariffa* L.) Calyces Extracts and W/O/W Emulsion Loaded Extract for Cosmetic Applications

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Abstract

The objective of this study was to evaluate the antioxidant activities and cytotoxicity effect on normal human dermal fibroblasts of roselle (*Hibiscus sabdariffa* L.) calyx extracts and to load with the roselle extract in the inner phase of water in oil in water multiple emulsion for application in facial cosmetic. The extracts of dried roselle calyces were prepared by a maceration technique using ethanol/water at concentrations of 30%, 70%, 95% and 0% (100% DI water). Obtained results showed that the 30% ethanolic extract had a higher amount of total phenolic and anthocyanin content than other extracts. According to the DPPH and ABTS assays, the 30% ethanolic extract exhibited dose-dependent antioxidant activity higher than other extracts with an IC_{50} value of 0.432±0.001 mg/mL and 0.0855±0.01 mg/mL, respectively. The formulation of water in oil in water multiple emulsion containing the roselle calyx extract was prepared by a beaker method with two-step emulsification. The prepared multiple emulsion showed a light pink color with homogeneously formula. The physiochemical stability of the prepared formula was evaluated at accelerated conditions; room temperature (30±5°C), low temperature (4±1°C) and high temperature (40±1°C) for 30 days and 6 cycles of freeze (-25°C, 24 h)-thaw (4°C, 24 h). Results found that the tested formula showed a good physicochemical stability compared to the initial condition. All finding suggests possible application for the 30% ethanolic extract of roselle calyces as a natural active ingredient in skin cosmetic products.

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Introduction

In recent years, the natural substances have traditionally been used in skin care products due to being considered safer than synthetic substances. The term of natural is defined as an ingredient that is produced by nature or found in nature and is directly extracted from plants or animal products. The herbs, fruits, flowers, leaves, minerals, water and land can be used as sources of natural ingredients (Ribeiro et al., 2015). The bioactive compounds in a natural product can enhance skin health as well as protect the skin against various damaging factors, including ultraviolet radiation (UVR) and free radicals. Free radicals are unstable and highly reactive molecules with a neighboring molecule can increase stability. As a chain reaction, the neighboring molecules which lose or accept electron to then becomes a new free radical. These free radicals significantly contribute to skin damage and accelerate ageing especially reactive oxygen species (ROS) (Michalak, 2022). Antioxidant are substances which can donate an electron to reactive species resulting in the prevention of the radical chain reaction. Prior studies reported that the phenolic-containing extracts exhibited the antioxidant properties (Dudonné et al., 2009). Nowadays, the phenolic compounds which derived the plant widely used in cosmetic products and may eventually replace the use of synthetic antioxidants (Przybylska-Balcerek & Stuper-Szablewska, 2019).

Hibiscus sabdariffa L. known as Roselle and it belongs to the Malvaceae family. Roselle is a medicinal plant which grows in subtropical and tropical regions such as Africa, Egypt, Guatemala, India and Thailand (Juhari et al., 2018). The essential part of roselle is calyces. Roselle calyx is rich in bioactive compounds such as vitamins (e.g., ascorbic acid), anthocyanins (e.g., delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside) flavonoids (e.g., quercetin, kaempferol, luteolin and apigenin), phenolic (e.g., chlorogenic acid and protocatechuic acid) and organic acids (e.g., citric acid and hibiscus acid) (Riaz & Chopra, 2018). These compounds in rosella calyx extract are contributed to having biological activities such as antimicrobial (Jung et al., 2013), antioxidation (Villalobos-Vega et al., 2023), anti-Inflammatory (Ariyabukalakorn et al., 2019). In addition, hibiscus acid isolated from roselle calyx showed skin aging potential and no cytotoxicity to human dermal fibroblast (Wang et al., 2022). Roselle extract is one plant extract that has been used in cosmetics, such as skin toner/astringents and anti-aging skin care products (Pinsuwan et al., 2010). For example, roselle calyx extract is used as an active ingredient in anti-aging cream (single emulsion) yet nowadays, cream or lotion as multiple emulsion formation containing roselle extract has not been reported in the market. According to scientific reports, roselle calyx extract contains several polar and acidic compounds, so that, their chemical properties have induced skin irritation and low skin permeation (Hjorth, 1969). The incorporation of roselle extract into a cosmetic dosage form has been improved as well as protection against environmental and to preserve their stable properties over time such as emulsion, nano-emulsion, liposome (Bevan et al., 2023). Among the dosage forms, emulsion technology has attracted much attention because it involves simple processing, low energy cost and easy application; moreover, it is widely used in food, pharmaceutical and cosmetic products.

A type of emulsion is double emulsions also called multiple emulsions, which consist of two types: water-in-oil-in-water ($W_1/O/W_2$) emulsions and oil-in-water-in-oil ($O_1/W/O_2$) emulsions, mainly depending on the loaded active agents. (Lamba et al., 2015). In $W_1/O/W_2$, water is first dispersed in oil which is then dispersed in another water phase, similarly for $O_1/W/O_2$, oil is first dispersed in water and then this emulsion is dispersed in a second oil phase. Multiple emulsions provide protective encapsulation to bioactive substances (hydrophilic and hydrophobic) in the inner phase droplets (Wang et al., 2017; Niknam et al., 2020). The $W_1/O/W_2$ emulsions have been investigated for loading the hydrophilic active agents (e.g., polyphenols, phenolics) better than O/W emulsions, because the release of hydrophilic compounds can be prolonged and better controlled (McClements, 2015). However, the emulsifier is very important to obtain emulsion because it can effectively protect the double emulsion droplets against flocculation, creaming and coalescence, thus contributing to the multiple emulsion stability. In addition, emulsifier must be biodegradable and nontoxic.

In this study, the roselle calyx extract was prepared using a maceration technique at room temperature to treat the bioactive compound in the extract. Then, the antioxidant activities and cytotoxicity effect on normal human dermal fibroblasts of roselle calyx extract were investigated. This extract was loaded into the internal phase of $W_1/O/W_2$ emulsion which was formulated by two-step emulsification method. The physiochemical characterization of prepared multiple emulsion and their stability were analyzed.

Materials and methods

1. Raw materials

The dried red calyx of roselle (*Hibiscus sabdariffa* L.) was purchased from Chao Krom Poe dispensary pharmacy, Samphanthawong, Bangkok, Thailand. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Folin-Ciocalteu reagent, Gallic acid (HPLC grade), Quercetin (HPLC grade) and Trolox were purchased from Sigma-Aldrich (USA). Ethanol (99%, AR grade) was purchased from Labscan (Thailand). Ethanol (Commercial grade) and deionized water were purchased from S.N.P. General Trading (Thailand). Sodium carbonate (Na_2CO_3, AR grade), Aluminium chloride ($AlCl_3$, AR grade), Sodium acetate (AR grade), Potassium chloride (KCl, AR grade) and Sodium chloride (NaCl, AR grade) were purchased from Univar Ajax Finechem (New Zealand). Mineral oil, Sorbitane monooleate, Polysorbate 80, Phenoxyethanol and Triethanolamine were supplied by Namsiang (Thailand).

2. Preparation of roselle calyx extracts

The red calyx of roselle is the type most commonly used (green, red and dark red) because of their high content of anthocyanin and acids (Wong et al., 2002; Peredo Pozos et al., 2020). An extract of red calyx of roselle was produced by a maceration technique as previously studied (Kusnadi & Purgiyanti, 2021), with modification. Dried calyx was ground into powder using a grinder and then the fine powder was kept in a sealed container protected from light at 4°C in refrigerator until usage. The extract of roselle calyx was macerated at room temperature (30±5°C) in various concentrations of ethanol including 30%, 70%, 95% and 0% (100% deionized water). The extraction was done in the ratio of dried roselle and solvent 1:10 w/v. Briefly, 10 g of dried calyx powder was macerated in 100 mL of each solvent and then kept at room temperature for 24 h. The resulting solvent was filtered through a Whatman No.1 filter paper. The marc was re-extracted again in the same method and done in triplicates. The pooled filtrate was concentrated using a vacuum rotary evaporator (Buchi, R-205/v, Switzerland) at 45°C. Each crude extract was weighed and kept in a tight container protected from light at 0°C in refrigerator. The yield of each extract was calculated using the following equation:

$$\text{Yield (\%)} = (W_1 \times 100)/(W_2) \quad [1]$$

where W_1 is the weight of roselle calyx extract and W_2 is the weight of the dried roselle calyx powder.

3. The quantitative analysis

3.1 Total phenolic content (TPC)

Total phenolic content of roselle calyx extracts was analyzed by a Folin-Ciocalteu method of previous research (Mohd-Esa et al., 2010) with some modifications. Briefly, 0.2 mL of each extract solution (1.0 mg/mL) was mixed with 1.0 mL of Folin-Ciocalteu reagent (10% v/v) and 0.8 mL of sodium carbonate (Na_2CO_3) (7.5% w/v) was added and vortex. After incubation for 30 min at room temperature, the absorbance of the incubated solution was measured at 765 nm, using a UV-visible spectrophotometer (Shimadzu, UV-2401PC, Japan). The amount of total phenolic was expressed as milligram of gallic acid equivalents per gram of extract (mgGAE/g extract) from the calibration curve. This assay was performed in triplicate.

3.2 Total flavonoid content (TFC)

Total flavonoid content of roselle calyx extracts was determined based on the formation of a complex flavonoid aluminium as previously studied (Meda et al., 2005), with slightly modification. Briefly, 100 µL of aluminium chloride ($AlCl_3$) (2.0 %w/v) was mixed with the same volume of each extract solution (1.0 mg/mL) and allowed to stand at room temperature for 10 min. The absorbance was measured at 415 nm using a microplate reader (Biochrom, EZ Read 2000, England). The total flavonoid content was expressed as milligram of quercetin equivalent per gram of extract (mgQE/g extract) from the calibration curve. This assay was done in triplicate.

3.3 Total anthocyanin content (TAC)

Total anthocyanin content of roselle calyx extracts was determined using the pH differential method of previous research (Wu et al., 2018) with some modifications. This pH differential method is based in the change of color of anthocyanin with pH: at pH 1.0 colored oxonium ions are formed, whereas at pH 4.5 predominates the colorless hemiketal form. The difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. Briefly, two buffer solutions were prepared at pH 1.0 (0.025 M of potassium chloride (KCl)) and pH 4.5 (0.4 M of Sodium acetate (CH_3COONa). The extract solution (1.0 mL) was mixed with each buffer solution (2.0 mL) and then shaken. Absorption measuring at 517 and 700 nm was read using a microplate reader (Biochrom, EZ Read 2000, England) after incubation for 15 min in the dark at room temperature. Total

anthocyanin value was expressed as milligram of cyanidin-3-glucoside equivalents per gram of extract according to the following equations:

$$\text{Absorbance (A)} = (A_{517} - A_{700})\ \text{pH 1.0} - (A_{517} - A_{700})\ \text{pH 4.5} \quad [2]$$

$$\text{TAC (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times l) \quad [3]$$

where MW is molecular mass of cyanidin-3 glucoside (449.2 g/mol), DF is the dilution factor (1.0), 1000 is the g to mg conversion coefficient, ε is the molar absorptivity, calculated as cyanidin-3-glucoside (26,900 L/mol/cm) and l is the cuvette radius (1.0 cm).

4. *In vitro* antioxidant activities

4.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The free radical scavenging activity of roselle calyx extracts was determined by using DPPH assay according to the method described by Yakaew et al. (2016) with slightly modification. Briefly, 150 µL of DPPH ethanolic solution (0.2 mM) was then mixed with 75 µL of various concentrations of each extract solution. The mixture was incubated at room temperature for 30 min in the dark. After incubation, absorbance was measured at 515 nm and measured using a microplate reader (Biochrom, EZ Read 2000, England). This study was compared with the positive control, Trolox. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_S/A_B)] \times 100 \quad [4]$$

where A_S is an absorbance of DPPH with the tested sample and A_B is an absorbance of DPPH without the tested sample. The concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against the sample concentration. The study was run in triplicate.

4.2 $ABTS^+$ [(2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] assay

The free radical scavenging capacity of $ABTS^+$ was assayed as previously studied (Wu et al., 2018), with some modification. Briefly, to prepare an $ABTS^{\bullet+}$ radical, the $ABTS^{\bullet+}$ solution (7.0 mM) was mixed with potassium persulphate (2.45 mM) at a ratio of 1:1 (v/v). After that, the mixture was kept in the dark at room temperature for 12-16 h. The working reagent was prepared by mixing the $ABTS^{\bullet+}$ solution with ethanol to an absorbance of 0.70±0.02 at 734 nm using a microplate reader. Then, to determine the $ABTS^{\bullet+}$ radical scavenging activity, 100 µL of various concentrations of the extract solution was added to 2.0 mL of $ABTS^{\bullet+}$ solution. The mixture was well mixed and left to stand for 10 min in the dark place at room temperature, The absorbance was taken at 734 nm using a microplate reader (Biochrom, EZ Read 2000, England). Trolox was used as a positive compound. The $ABTS^{\bullet+}$ scavenging activity was calculated in the same way as DPPH radical scavenging activity.

5. Cytotoxicity to normal human dermal fibroblasts

Cytotoxicity assay was also done to determine cell viability of the cultured fibroblast cells using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay according to the method described by Ala AA, et al (2018) with modification. Briefly, normal human dermal fibroblast (NHDF) cell was used as a cell model to study the cytotoxicity. The NHDF (ATCC# PCS-201-010) cells was trypsinized and seeded at approximately $8x10^3$ cells/well into 96-well plate and cultured in the complete growth media of DMEM-F12 supplemented with 10%FBS and 1% v/v Penicillin/Streptomycin. NHDF cells were maintained at 37°C with 5%CO_2 for 24 h. Then, the culture media was removed and washed with sterile phosphate buffer saline (PBS). The NHDF cells underwent treatment with different concentration of crude extracts comparison with the control. After 24 h, the media containing the samples was discarded carefully and washed with sterile PBS. Then, NHDF cells were incubated with MTT solution and incubated at 37°C in the dark for 2 h. The medium was removed and the formazan crystals was dissolved with dimethyl sulfoxide (DMSO). The absorbance of each well was read on microplate reader at 570 nm. The % cell viability was calculated by the below equation:

$$\text{Cell viability (\%)} = (A_S/A_C) \times 100 \quad [5]$$

where A_S is an absorbance of the tested sample and A_C is an absorbance of control (cell untreated with the tested sample).

6. Preparation of water in oil in water emulsion

Water in oil in water containing roselle calyx extract was prepared using beaker method with two-step emulsification as previously studied (Mahmood et al., 2014) with modification. The extract of roselle calyx was loaded in the inner phase (primary water in oil emulsion) of water in oil in water multiple emulsion. The concentration of roselle calyx extract was considered from the obtained result of antioxidant activity. The

compositions and their functions of water in oil in water formula is shown in Table 1.

Table 1 The compositions and their functions of water in oil in water formula

Ingredients	Quantity (%w/w)	Functions
First step: Primary W/O emulsion		
Deionized water	qs to 100	Diluent
Sodium chloride (NaCI)	0.3	Inner phase checking
Mineral oil	20.0	Emollient
Sorbitane monooleate (Span 80, HLB=4.3)	4.0	Lipophilic emulsifier
Triethnolamine (TEA)	0.2	pH adjuster to 5.0-5.5
Phenoxyethanol	1.0	Preservative
Second step: W/O/W multiple emulsion		
Primary w/o emulsion	80.0	Inner phase of multiple
Polysorbate 80 (Tween 80. HLB=15)	6.0	emulsion Hydrophilic
Deionized water	14.0	emulsifier Diluent.

Remark: q.s.is quantum satis (as much as is enough), HLB is hydrophile-lipophile balance

Briefly, primary W/O emulsion prepared by emulsifying the oil phase (mineral oil and sorbitan monooleate) with the aqueous phase (deionized water and sodium chloride) in the presence of lipophilic surfactant while heating both phases at 75°C. The oil phase and aqueous phase was mixed by homogenizer (Silverson L5M, England) at 1500 rpm for 30 min and the temperature of this mixture was maintained at 70-75°C. Preservative and pH adjuster and/or roselle extract added to the mixture at 40°C, then stirred to emulsion formation.

For the second stage emulsification, the primary W/O emulsion was added slowly to a secondary aqueous phase containing hydrophilic emulsifier (deionized and tween 80) at stirring speed of 700 rpm for 15 min using a homogenizer (Silverson L5M, England) and the formation of multiple emulsion was confirmed by microscopic analysis.

7. Evaluation of physicochemical characterization

7.1 The appearance, homogeneously formula, phase separation and color were determined by visualization.

7.2 The morphology was investigated using a microscope (MLB 3200, Kruss, Germany). Observations were made at 40X magnification after diluting the multiple emulsions.

7.3 Electrical conductivity value of the multiple emulsion was determined with an electrical conductivity probe of pH/mV/EC/TDS/NaCl/Temp Starter B100 (Ohaus, Ohaus Instruments, Shanghai, China).

7.4 pH value was determined using pH meter (Starter B100, Ohaus Instruments, Shanghai, China) which was calibrated using standard buffer solution.

7.5 The viscosity was determined by Brookfield viscometer (DV-I Prime, Brookfield Ametex, USA) at 50 rpm using a spindle no.63.

7.6 The L* a* b* color value was measured using a Chroma meter (CR-400, Centasia, Konica Minolta, Thailand)

8. Investigation of physiochemical stability

The multiple emulsion loaded roselle calyx extract was kept in air-tight containers under accelerated condition using 6 cycles of alternative freeze-thaw (-25°C for 24 hr followed by 4°C for 24 hr as 1 cycle), low temperature (4±1°C) for 30 days, high temperature (40±1°C) for 30 days and room temperature (30±5°C) for 30 days. The samples were removed from the storage conditions and allowed to achieve room temperature prior to the evaluation of the physicochemical characteristics compared with initial condition.

9. Statistical analysis

Statistical analysis of the data was analyzed by performing a one-way analysis of variance (ANOVA) and Duncan post-hoc tests using SPSS program (SPSS ver. 22.0 for Windows, SPSS Inc., Chicago, IL, USA) and the statistical significance was $p < 0.05$.

Results and discussion

1. Roselle calyx extracts and quantification of antioxidant compounds in the extracts

The effective extraction and proper assessment of antioxidants from herbals are crucial when exploring the potential antioxidant sources and promote the application in the cosmetic field. Extraction is the first step in the isolation of phenolic compounds from plant materials by traditional methods such as maceration and soxhlet extractions, which have been used for many decades. In this present study, obtained extracts from roselle calyx was produced using a maceration method with different concentrations of water and ethanol; aqueous (100% water), 30% ethanol, 70% ethanol and 95% ethanol. The extraction results found that the maximum yield value was obtained extract by maceration with 30% (v/v) ethanol which was 50.67% (w/w). The yield then tended to decrease when the ethanol concentration was 70% and 95%, respectively (Table 1). This might be due to the combination between water and ethanol which serves the extraction of compounds that are soluble in water and ethanol in roselle calyx such as phenolic acid, flavonoid and anthocyanin. Other compounds may have been extracted and contribute to higher yield.

Presented in Fig. 1, the physical appearance of the crude extracts from roselle calyx is visually evaluated and shows that both extracts from 30% ethanol and aqueous gave a red powder and a red-brown powder for 70% ethanolic extract whereas the 95% ethanolic extract shows a dark brown viscous liquid. All crude extracts were kept in tight container at 0°C for further studies.

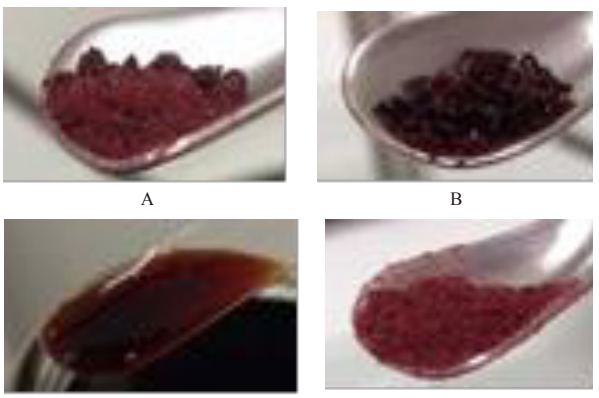

Fig. 1 Visual appearance of the crude extracts from roselle calyx prepared by various concentrations of ethanol; (A) 30% ethanol, (B) 70% ethanol, (C) 95% ethanol and (D) 100% aqueous (0% ethanol)

The content of total phenolic, total flavonoid and total anthocyanin in each extract are shown in Table 2. The content of total phenolic compounds in the roselle calyx extracts were calculated with the calibration curve equation of gallic acid (0.0625-1.0 mg/mL, Y= 1.005X + 0.0272, R^2 = 0.999) based on Folin-Ciocalteu assay. The total phenolic content of 30% ethanolic extract had no significantly difference ($p>0.05$) higher than 70% ethanolic extract and the lowest content was extracted with 95% ethanol; this means that ethanol is not a good solvent for extracting phenolic compounds. This demonstrated that the total phenolic content in roselle calyx extract might contain mainly the protocatechuic acid (PCA) and chlorogenic acid (Riaz & Chopra, 2018). The results were similar to previously research (Villalobos-Vega et al., 2023) which showed that phenolic compounds are soluble in hydroalcoholic mixtures containing equal amounts of ethanol and water.

The amount of total flavonoid compounds of roselle calyx extracts were calculated with the calibration curve equation of quercetin (0.039-0.625 mg/ml, Y=13.925X + 0.1051, R^2 = 0.9995) which found that 95% ethanolic extract had significantly ($p<0.05$) higher amount of total flavonoid and followed by 30% ethanolic extract, 70% ethanolic extract and aqueous extract, respectively. The finding imply that the ethanol-water (5%) solvent could extract the flavonoid compounds from roselle calyx such as quercetin, kaempferol, luteolin and apigenin more than other solvents which contain higher percentage of water.

According to the pH-differential method, the total anthocyanin content of roselle calyx extract showed no significantly differences ($p>0.05$) in the higher content of 30% ethanolic extract which was 321.73±26.72 mg Cyanidin-3-glucoside equivalents/g extract than aqueous extract (274.60±23.79 mg Cyanidin-3-glucoside equivalents/g extract. This study confirms that roselle calyx extract displayed high content in anthocyanins. Prior research reported that anthocyanins had been identified in the calyx of roselle including delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside (Piovesana et al., 2019). The determination showed lower content of both total phenolic and total anthocyanin which were extracted with 95% ethanol and results were similar to prior reports (Villalobos-Vega et al., 2023).

Table 2 Yield value and content of total phenolic, total flavonoid and total anthocyanin in roselle calyx extracts

Roselle calyx extracts	Yield (%w/w)	Total phenolic content (mgGAE/g extract)	Total flavonoid content (mgQE/g extract)	Total anthocyanin content (mg Cyanidin-3-glucoside equivalents/g extract)
30% ethanolic extract	50.67	48.78±0.83^a	6.18±0.91^b	321.73±26.72^a
70% ethanolic extract	29.77	47.45±0.12^a	3.46±0.17^c	66.80±11.05^c
95% ethanolic extract	23.43	32.72±0.76^c	14.98±1.47^a	39.89±10.07^d
Aqueous extract	39.96	44.80±1.10^b	1.67±0.25^d	274.60±23.79^b

Remark: The content of total phenolic, total flavonoid and total anthocyanin are given as mean ± SD (n=3). The different superscript letter in the same column represents significant differences when compared with each extract at $p<0.05$

2. *In vitro* antioxidant activities

The antioxidant activities of roselle calyx extracts were determined by measuring its DPPH and $ABTS^+$ radicals scavenging activities. The concentration providing 50% inhibition (IC_{50}) of roselle calyx extracts and positive control (Trolox) are presented in Table 3.

Table 3 Antioxidant activities of roselle calyx extracts

Samples	IC_{50} (mg/mL)	
	DPPH radical	$ABTS^+$ radical
30% ethanolic extract	0.43±0.001^d	0.08±0.001^d
70% ethanolic extract	0.51±0.014^c	0.10±0.001^c
95% ethanolic extract	1.62±0.037^a	0.11±0.008^c
Aqueous extract	1.04±0.003^b	0.27±0.003^a
Trolox	0.04±0.001^e	0.25±0.001^b

Remark: Values are given as mean ± S.D of triplicate. The different superscript letter in the same column represents significant differences at $p<0.05$

The antioxidant activity was carried out using a radical scavenging assay including DPPH and $ABTS^+$ methods. A DPPH assay was used to assess nitrogen radicals including reactive nitrogen species (RNS) that are well-known to be pro-inflammatory mediators, whereas $ABTS^+$ radical assay was used to target oxygen radicals for estimating neutralization of reactive oxygen species (ROS). The IC_{50} value is a parameter widely used to measure free radical scavenging activity. A smaller IC_{50} value corresponds to a higher antioxidant activity. Our results found that the 30% ethanolic extract showed significantly ($p<0.05$) exhibited DPPH and $ABTS^+$ radical scavenging activity higher than other extracts. This demonstrated that antioxidant activities result of 30% ethanolic extract are related to both total phenolic and total anthocyanin contents. The free radical scavenging activity of the extract may result from high phenolic and anthocyanin contents. Thus, the antioxidant properties of this extract may be attributed to the both phenolic and anthocyanin which may act as an antioxidant. Our results were similar to prior reports (Wu et al., 2018).

3. Cytotoxicity effect on normal human dermal fibroblasts of roselle extracts

Cell viability is one of the criteria for evaluation of cytotoxicity test of crude extracts. Here, the cytotoxic effect of roselle calyx extract on normal human dermal fibroblast (NHDF) cells was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The obtained results are expressed as percentage of cell viability and concentration of extracts are shown in Fig. 2. The percentage of cell viability after exposure to 5-250 ug/mL of all roselle calyx extracts was significantly decreased ($p<0.05$) when compared with control cell (0 ug/mL, untreated the extracts) of each extract. However, the results showed that the percentage of cell viability was more than 70% after treating with all concentration of each extract. It demonstrated that all extracts from roselle calyx was not cytotoxic to NHDF cells. These results were similar to prior reports (Wang et al., 2022) which found that the ethanolic extract of roselle calyx did not show a cytotoxicity effect on human dermal fibroblasts.

4. Physicochemical characterizations of water-in-oil-in-water emulsion

Water in oil in water (W/O/W) emulsion was prepared using beaker method with two-step emulsification. Roselle calyx extract was produced by 30% ethanol and selected for loading in the inner phase of W/O/W multiple emulsion. The physicochemical data of W/O/W emulsion base (no extract) and W/O/W emulsion loaded extract including physical appearance, color value (L*, a*, b*), electrical conductivity, pH value and viscosity value are presented in Table 4.

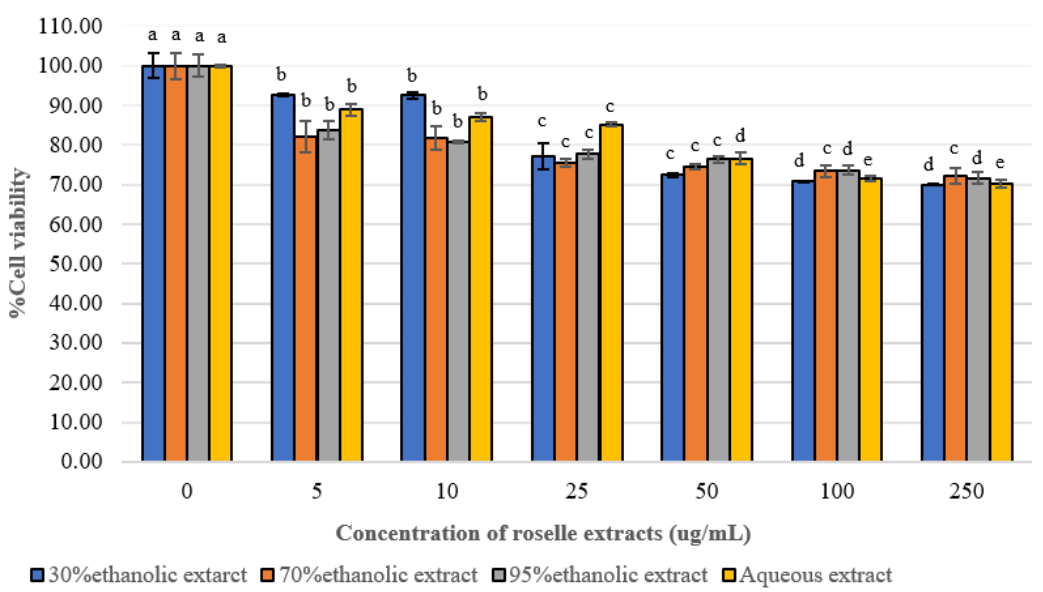

Fig. 2 The percentages of NHDF cell viability after exposure to roselle calyx extracts. Each bar represents mean ± S.D. of triplicate. The different subscript letters on the chart in each extract represents significantly different when compared with each concentration in the same extract at $p<0.05$

Table 4 The physicochemical data of W/O/W emulsion base (no extract) and W/O/W emulsion loaded extract

Formulations	Physical appearance	Electrical conductivity (μS/cm)	pH	Viscosity (cP)	L*, a*, b* value
W/O/W emulsion base (no extract)	Homogeneously Non-separation Non- flocculation White color	+83.23±1.11[a]	6.56±0.03[a]	12.5±0.17[a]	L* = 65.12±1.21[a] a* = -2.64±0.22[a] b* =2.62±0.39[a]
W/O/W emulsion loaded extract	Homogeneously Non-separation Non-flocculation Light pink color	+82.67±1.15[a]	5.59±0.04[b]	11.7±1.08[a]	L* = 53.47±0.22[b] a* = 0.32±0.04[b] b* = 7.24±0.04[b]

Remark: Values are given as mean ± S.D of triplicate. The different superscript letter in the same column represents significant differences at $p<0.05$

The electrical conductivity of multiple emulsion was evaluated. The basic principle of this test is that water is a good conductor of electricity. In this study, the electrical conductivity of multiple emulsion base (no extract) and multiple emulsion loaded extract showed positive charge. This test found to be positive as water which is the continuous phase. And in the formulations added sodium chloride (NaCl) to the aqueous phase used as electrolyte, thus, its conductivity increases greatly. Obtained result showed that the electrical conductivity of multiple emulsion loaded extract and multiple emulsion base (no extract) was not significantly ($p>0.05$) decreased. This implied that the increasing of electrical conductivity values may be due to the transfer of NaCl which was entrapped in the internal aqueous phase of the multiple emulsion to the external aqueous phase. While the decrease may be attributed to the transfer of NaCl lost into external aqueous phase during the process of manufacturing towards the internal aqueous phase (Jiao & Burgess, 2003).

The pH value of multiple emulsion loaded extract was 5.59±0.04 which was significantly ($p<0.05$) decreased compared with multiple emulsion base (no extract). The decreasing in pH value of multiple emulsion loaded extract was probably due to the production of highly acidic of the extract which had a high content of phenolic compound. Both formulations were similar to prior reports that the topical products should be acidified and possess pH in the range of 4 to 6 (Lukić et al. 2021).

The viscosity of multiple emulsion loaded extract and multiple emulsion base (no extract) was not significantly ($p>0.05$) decreased. The viscosity of multiple emulsion may be due to the adding of sorbitane monooleate (Span 80) in the oil phase of water in oil primary emulsion and incorporated NaCl salt in the internal aqueous phase. The decreasing of viscosity in the multiple emulsion loaded extract may be due to a gradual permeation of salt through the oil layer to the external continuous aqueous phase. Our study was similar to prior reports (Jiao & Burgess, 2003).

The color of the prepared multiple emulsion was measured in terms of the L*, a*, b* color space system. In this color space, L* represents the lightness and a and b are color coordinates: where +a is the red direction, -a is the green direction, +b is the yellow direction and -b is the blue direction. The resulting showed L* a* b* color value of multiple emulsion base (no extract) and multiple emulsion loaded extract were significantly ($p<0.05$) different. The color value of multiple emulsion loaded extract was L*=53.47±0.22, a*=0.32±0.04 and b*=7.24±0.04 which showed creamy light pink in color which may be caused by the color of 30% ethanolic extract. This is similar to visual investigation.

The morphology of prepared multiple emulsion was investigated using an optical microscope. The physical appearance and micrograph images of W/O/W emulsion base (no extract) and W/O/W loaded roselle calyx extract are shown in Fig.3. Physical appearance of W/O/W emulsion base (no extract) and W/O/W emulsion loaded extract provided the good physical characteristics including homogeneously, non-separation or non-flocculation occurrence. According to color appearance, multiple emulsion base (no extract) showed a white color and light pink color for multiple emulsion loaded extract, determined by visualization (Fig.3A and 3B). The shape of the W/O/W droplet in emulsion base (no extract) (Fig.3C) and emulsion loaded roselle calyx extract (Fig.3D) appeared to be multiple globules droplets showing inter and external phases of water and oil phase in the middle and both micrographs showed the same in behaviour of W/O/W emulsion structure.

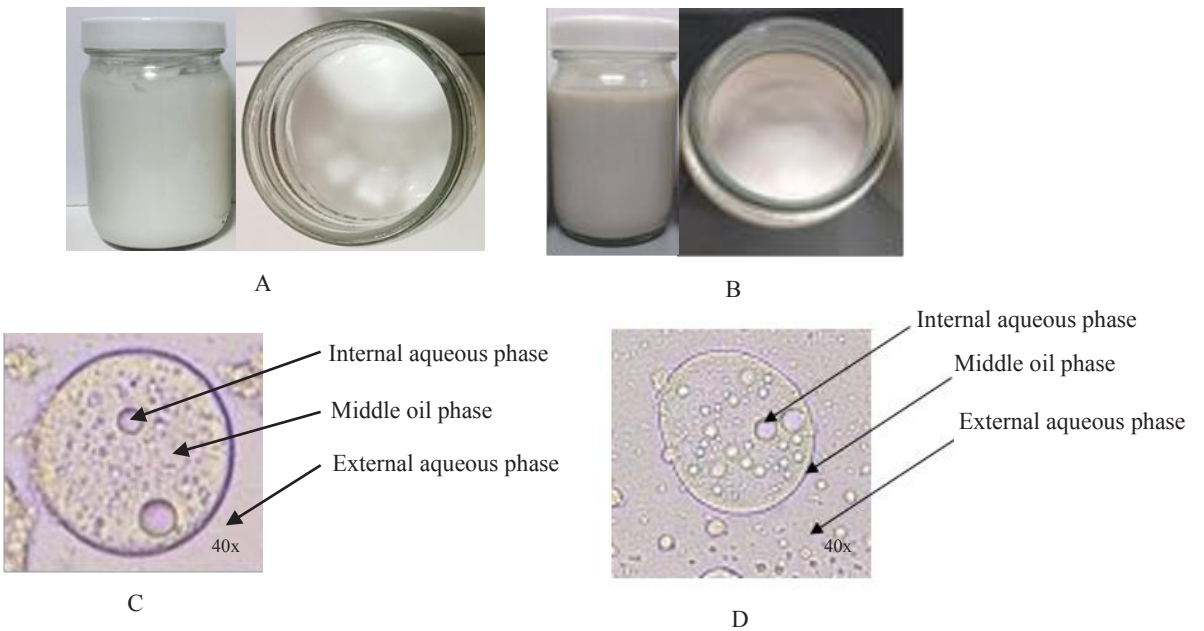

Fig. 3 The physical appearance of (A) water in oil in water emulsion base and (B) water in oil in water emulsion loaded roselle calyx extract and a typical photomicrograph of (C) water in oil in water emulsion base and loaded roselle calyx extract in the internal aqueous phase (D)

5. Physicochemical stability study of W/O/W emulsion loaded roselle calyx extract

The physicochemical stability of prepared multiple emulsion was determined under accelerated conditions including freeze-thaw (-25°C for 24 h followed by 4°C for 24 h as 1 cycle) for 6 cycles, low temperature (LT) (4±1°C) for 30 days, high temperature (HT) (40 ±1°C) for 30 days and room temperature (RT) (30±5°C) for 30 days, results are shown in Table 5. The physical appearance of prepared multiple emulsion was determined in the terms of color, odor and homogeneity, according to visual investigation compared with initial condition. Color had little changes in color of samples kept at high temperature whereas others conditions were not different. Odor did not change at all by conditions tested. The homogeneity of the remaining formation and the phase separation or flocculation did not occur, results are shown in Fig 4. This demonstrates that the concentration of sorbitane monooleate (Span 80) in the oil phase and polysorbate 80 (Tween 80) in the continuous phase had enhanced multiple emulsion stability. In addition, sodium chloride (NaCl) salt has a complex effect on the stability of W/O/W multiple emulsion. It stabilizes the inner droplet (Jiao & Burgess, 2003).

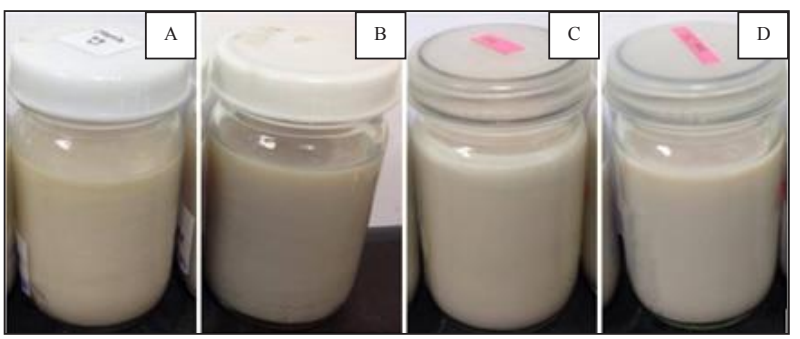

Fig. 4 The physical appearance of W/O/W emulsion loaded extract after storage at (A) room temperature (RT), (B) high temperature (HT) (40 ±1°C), (C) low temperature (LT) (4±1°C) and (D) freeze-thaw cycle

Table 5 Physicochemical stability of W/O/W emulsion loaded roselle calyx extract

Conditions	Electrical conductivity^{ns} (µS/cm)	Viscosity^{ns} (cP)	pH	Color		
				L*	a*	b*
Initial	+82.67±1.15	11.7±1.08	5.59±0.04^{b}	53.47±0.22^{c}	0.32±0.04^{b}	7.24±0.04^{a}
RT	+82.33±2.08	12.7±0.60	5.49±0.06^{b}	50.33±0.44^{d}	0.41±0.07^{b}	5.62±0.24^{c}
HT	+83.33±0.58	11.5±1.05	5.34±0.15^{d}	57.86±2.08^{a}	0.15±0.02^{c}	6.52±0.40^{b}
LT	+83.00±1.00	10.8±0.60	5.40±0.10^{c}	56.38±1.05^{b}	0.39±0.02^{b}	7.46±0.65^{a}
Freeze-thaw cycles	+82.00±2.65	13.5±1.20	5.75±0.07^{a}	55.24±2.54^{c}	0.96±0.03^{a}	5.51±0.17^{a}

Remark: Values are given as mean ± S.D of triplicate. The different superscript letter in the same column represents significant differences when compared with initial condition at $p<0.05$. ns represents not significant difference. RT: room temperature; LT: low temperature; HT: high temperature

The electrical conductivity and viscosity were not significantly ($p>0.05$) changed compared with the initial condition. The electrical conductivity and viscosity values slightly changed indicating a small net water diffusion through the oil layer during storage. Previously revealed pH of skin range of 4 to 6 are considered to be average pH of the skin. Results of pH value analysis found that the multiple emulsion under the room temperature condition did not significantly ($p>0.05$) change compared with the initial condition. This indicated that that primary emulsion which was entrapped in the extract was stable after storage at room temperature. However, other conditions, the pH value did significantly ($p<0.05$) change which was due to the production of highly acidic of the extract which had high content of phenolic compound. For the colorimetry detection, there was little change in L* a* b* color value under all condition tests and these results were similar to evaluating by visual.

Conclusion

The crude extract from roselle calyx which was extracted by 30% ethanol was found to have high content of phenolic and anthocyanin contents which possessed DPPH and $ABTS^{+}$ radical scavenging activities. Additionally, all crude extract from roselle calyx showed no toxicity effect on normal human dermal fibroblast (NHDF) cell. The W/O/W emulsion which loaded roselle calyx extract into the internal aqueous phase of primary water in oil was formulated by beaker method with two-step emulsification. Prepared formular showed a good physicochemical stability after storage under all conditions tested when compared with the initial conditions. All findings indicated that the 30% ethanolic extract of roselle calyx can be used as a natural antioxidant for an active ingredient in skin cosmetic products.

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Development of Healthy Ready-to-Cook Sauce for Thai Food

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Ready-to-cook, Sauce, Thai food, Tea seed oil, Gac fruit

Abstract

Thai cuisine is widely popular both domestic and international. Thai cuisine is characterised by the use of herbs and spices as ingredients to provide unique flavors. However, due to the vast variety of ingredients and the delicacy in preparing or even the difficulty in acquiring the ingredients indicated in the recipes, the flavors of the dish are very likely to be distorted from authenticity. This research therefore developed ready-to-cook sauces for Thai food to increase convenience for consumers and to survey consumers' acceptance of these products. Additionally, tea seed oil and powdered Gac fruit were utilised for their health-wise beneficial values. The developed ready-to-cook sauces included Pad Thai sauce and Ka Phrao (Holy basil) sauce. The results of product acceptance test indicated that consumers rated the liking scores for Pad Thai sauce and Ka Phrao sauce in the range between like moderately and like very much (8.2±1.3 and 7.8±1.2). Over 92% of the consumers accepted both sauce products, and more than 89% decided to purchase the products. Hence, the development of the healthy ready-to-cook sauces as products are viable for commercial distribution.

Introduction

Thai cuisine combines a variety of ingredients to create deliciousness as well as offering health advantages, such as using less oil in cooking especially for vegetables, as the raw foods have great nutritional value. Nowadays, Thai food has gained wide popularity both within Thailand and abroad. The highlight of Thai food is the use of herbs and spices as ingredients to create a unique aroma. As a result of the numerous ingredients and seasonings used in Thai cuisine, it can be challenging to create, and occasionally it may be impossible to locate all the necessary components, which results in the cooked dish tasting different from the original recipe. In addition, Thai cuisine preparation involves complicated processes and steps, and it takes a long time to cook. Therefore, this inconvenience may be solved by getting instant meals. The application of current manufacturing processes and raw ingredients to enable easier Thai cooking is fascinating. Consequently, the research team has created ready-to-cook seasoning sauce formulas for Thai food that are popular with both Thais and foreigners, including Pad Thai sauce and Ka Phrao sauce, which consumers may use to prepare a range of

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foods. The convenience of usage and storage were both considered by the researchers in order for it to be utilized internationally.

The Codex Alimentarius defines sauces as "substances added to food to enhance aroma and taste". They have always been a significant component of culture across the world, and now that they are being used more often in daily life, they are becoming more significant from both a nutritional and financial perspective (García-Casal et al., 2012). Many different factors can be used to categorize sauces, such as their geographical origin (e.g., Italian, Indian, or Japanese sauces), serving temperature (e.g., warm or cold sauces), flavor (e.g., mild or hot sauces), acidity (e.g., low-acid or acid sauces), sweetness (e.g., sweet or salty sauces) and color (e.g., brown, pink, or green sauces). Sauces are an essential part of the human diet and have a significant added value. It's interesting to use healthy ingredients as components of ready-to-cook sauces. Tea seed oil and Gac (*Momordica cochinchinensis*) fruit aril powder were employed as sauce ingredients in this study. Unsaturated fatty acids found in tea seed oil, which include 81–87% oleic (Omega-9), 13–28% linoleic (Omega-6) and alpha-linoleic acid (Omega-3), can lower levels of harmful cholesterol, encourage the production of healthy cholesterol and reduce the risk of developing chronic non-communicable diseases such as cardiovascular disease, diabetes, and obesity (Kim et al., 2008). Tea seed oil also contains a number of antioxidants, which assist in cancer prevention (Liu et al., 2022). In an application test for soy sauce, Choi et al. (2018) determined green tea seed extract as a healthy food preservative and reported antibacterial substances that eliminated all yeast below the minimum inhibitory concentration (MIC) value.

Color is an important factor for enhancing attractive value and consumer acceptability of foods and beverages. For a variety of purposes, including to ensure uniformity, maintain stability throughout the shelf life, and contribute to a desired flavor, color is added to soups, sauces and condiments (Corradini, 2019). Depending on the physical form and end-user usage, different colors may be needed for these applications. Color performance can be considerably affected by processing issues including heat, pressure, pH and interactions with other ingredients (Dey & Nagababu, 2022). For stability in sauces, soups and condiments, a variety of synthetic food colors are provided. However, the scientific community has turned its attention to natural colorants that serve to replace their synthetic harmful counterparts due to the belief in bio-safety measures, health advantages and the nutritional significance of food colors (Manzoor, 2021). The use of natural colorants like the carotenoid pigment in Gac fruit arils may be beneficial for the consumers' health because they have outstanding antioxidant properties (Kha, 2010). Nevertheless, there is still little research on the use of Gac fruit aril as a natural colorant in food and beverages. Thuy et al. (2023) investigated the addition of Gac fruit aril and xanthan gum with functional properties and natural coloring compounds to macaroni. They found the physicochemical components, bioactive compounds, firmness, microstructure and cooking quality of the products highlighted the significance of the ingredients utilized in the outcomes. Kumkong et al. (2020) studied the color quality of whey protein-mixed gelatin with Gac fruit aril powder that changed to a dull color during storage. El Haggar et al. (2023) developed a new tomato-free sauce formula fortified with beneficial oils and contained in nanometer capsules that enhanced the product's nutritional value and antioxidant capacity and may have beneficial effects on general health in relation to biochemical factors including lipid profile and liver function. The ultimate goal is to market this new mixture as a beneficial product that can boost immunity and potentially even enhance health. The investigation of the impacts and advantages of the novel product will involve biological studies. Therefore, in this study, we used tea seed oil and Gac fruit aril powder to develop healthy formulas for ready-to-cook sauces for Thai cuisine, analyzed their properties, including sensory, physical and chemical properties and tested the customer acceptability of these sauces.

Materials and methods

1. Materials

The following ingredients were used to make ready-to-cook sauces: tea seed oil (Patpat, Chiang Rai, Thailand), Gac fruit (Kanchanaburi, Thailand), fish sauce (Tiparos, Bangkok, Thailand), sugar (Mitr Phol, Bangkok, Thailand), coconut sugar (Lin, Bangkok, Thailand), chili sauce (Mabin, Samutsakhon, Thailand), tamarind paste (Aro, Pathumthani, Thailand), red chili (Thewet Market, Bangkok, Thailand), holy basil (Thewet Market, Bangkok, Thailand), and Xanthan gum (Ziboxan® F80, China).

2. Preparation of Gac fruit aril powder

Freeze-dry Gac fruit aril sample has been generated in accordance with the method indicated by Kumkong et al. (2018). The Gac aril sample was processed by filtering and separating the seeds before being mixed with maltodextrin DE10 at a ratio of 5 g/100 g (w/w) and freeze dried at the National Food Institute, Thailand. Condenser temperature was -20 C and pressure was 250 Pa for 48 hr during operation. Before usage, a powder sample of freeze-dried aril was stored at 4°C and packaged individually in aluminum foil.

3. Formulation of healthy ready-to-cook sauce

3.1 Pad Thai Sauce

The prototype Pad Thai sauce consisted of tamarind paste, fish sauce, sugar, coconut sugar, chili sauce, vinegar and chili powder (Onnompun, 2012). The formulation was developed by replacing unhealthy ingredients with healthy ones, such as vegetable oil was substituted with tea seed oil, to prevent the noodles from sticking during the stir-frying process. As well as using Gac fruit aril powder as a coloring agent for the sauce to have even color and antioxidants from Gac fruit. According to research by Kha et al. (2015), Gac fruit aril powder can be used in a variety of foods, including yogurt, cake and pasteurized milk, to maintain color consistency during processing, enhance the amount of beta-carotene and lycopene and lessen the peroxide value or rancid smell. The prototype Pad Thai sauce formulation and the other sauce with gac fruit aril powder and tea seed oil replacement are shown in Table 1.

Table 1 The ingredients of the prototype Pad Thai sauce and the tea seed oil and Gac fruit aril powder replacement formulation

Ingredients (%)	Prototype*	Tea seed oil and Gac fruit aril powder replacement
Tamarind paste	17.05	16.12
Fish sauce	17.05	16.12
Sugar	21.24	20.15
Coconut sugar	21.24	20.15
Chili Sauce	8.48	8.05
Vinegar	8.48	8.05
Chili powder	1.70	1.61
Vegetable oil	4.76	-
Tea oil seed	-	9.67
Gac fruit aril powder	-	0.08

Source: *Onnompun (2012)

3.2 Ka Phrao sauce

Pad Ka Phrao is one of the most famous dishes in Thailand. It is a single-dish meal that can be stir-fried with multiple choices of meat. Table 2 presents a modified formula for Ka Phrao sauce that substitutes tea seed oil for vegetable oil and the prototype formula from Onnompun (2012).

Table 2 The ingredients of the prototype Ka Phrao sauce and the tea seed oil replacement formulation

Ingredients (%)	Prototype*	Tea seed oil replacement formulation
Chopped garlic	11.1	11.1
Chopped bird-chili	11.1	11.1
Holy basil	11.1	11.1
Fish sauce	11.1	11.1
Oyster sauce	5.6	5.6
Sugar	5.6	5.6
Water	22.2	22.2
Vegetable oil	22.2	-
Tea seed oil	-	22.2

Source: *Onnompun (2012)

3.3 Production process of ready-to-cook sauce

Each sauce was put into 250 ml glass bottles, which was pasteurized in boiling water at 90°C. They were then kept at room temperature until cooled, as indicated in Fig. 1.

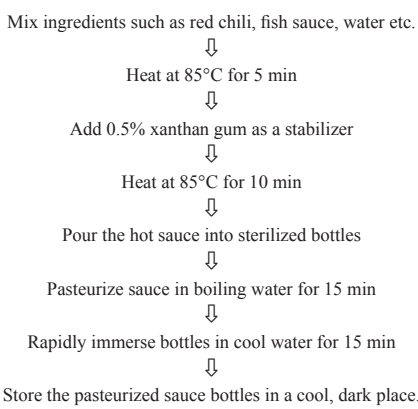

Fig. 1 Production process of ready-to-cook sauce (250 mL)

4. Sensory evaluation of ready-to-cook sauce

4.1 Preparation of ready-to-cook sauce

Pad Thai sauce

Noodles should be stir-fried with 60 g of Pad Thai sauce for every 100 g of noodles.

Ka Phrao sauce

Stir-fry chicken diced (1.0x1.0 cm) in Ka Phrao sauce with 50 g of sauce for every 100 g of chicken were stir-fried and served with rice.

4.2 Sensory evaluation

Sensory evaluation was conducted with 50 panelists who lived in Bangkok, Thailand. Target

panelists were chosen based on how frequently they used ready-to-cook sauce, at least once per month. They were asked to rate ready-to-cook sauce samples for a degree of liking regarding color, aroma, saltiness, sweetness, sourness, spiciness and overall liking using a 9-point hedonic scale (1: "Dislike extremely", 9: "Like extremely") (Lawless & Heyman, 1998).

5. Physical and chemical properties of ready-to-cook sauce

5.1 Color (L*, a*, b*) of the ready-to-cook sauce, measured using a colorimeter (MiniScan EZ, model MSEZ-4500L, USA).

5.2 Viscosity was performed with Brookfield Viscometer (Brookfield LVDV-E, US).

5.3 pH value was determined using the digital pH meter (Mettler Toledo, Switzerland).

6. Microbiological qualities of ready-to-cook sauce

Microbiological qualities of ready-to-cook sauce was determined by total plate count and yeast and mold according to AOAC. (2000).

7. Acceptability test

A survey was conducted to determine the acceptance of target consumers who consume ready-to-cook sauce at least once a month. The survey used questionnaires that included demographic information, data on consumer liking scores for the product using a 9-point hedonic scale, and data on the acceptance of 100 panelists using a binary (yes/no) scale. The sample preparation process for the acceptability test followed the same methods as the sensory evaluation section.

8. Statistical analysis

Formulation data of ready-to-cook sauce compared the means with t-test. The results of acceptance test comprised of the frequency (percentage) and the average "like" score of consumers toward the developed ready-to-cook sauce. Statistically difference was established at $p<0.05$.

Results and discussion

1. Formulation of healthy ready-to-cook sauce

1.1 Pad Thai sauce

Using the prototype Pad Thai sauce and the Pad Thai sauce with tea seed oil and gac fruit aril powder to prepare Pad Thai, the results from testing sensory characteristics using the 9 point hedonic scale (1: "dislike extremely", 9: "like extremely") (Lawless & Heyman, 1988) with 50 participants are shown in Table 3.

Table 3 Mean liking scores of the prototype ready-to-cook Pad Thai sauce and the developed ready-to-cook Pad Thai sauce with tea seed oil and Gac fruit aril powder

n=50

Sensory attributes	Mean liking score	
	Prototype formula	Developed formula
Color	7.0±1.1[b]	7.6±1.3[a]
Aroma [ns]	7.1±1.3	7.2±1.1
Saltiness [ns]	7.1±1.0	7.2±1.6
Sweetness [ns]	7.2±1.2	7.4±1.4
Sourness [ns]	7.1±1.1	7.2±1.5
Spiciness [ns]	7.2±1.3	7.2±1.4
Overall liking	7.1±1.3[b]	7.5±1.2[a]

Remark: Means in rows followed by different letters represent significant differences ($p\leq0.05$)

[ns] = Means in row do not have significant differences ($p>0.05$)

The results were statistically analyzed by comparing the means of the 2 Pad Thai sauce groups using T-test. The liking scores for the aroma, saltiness, sourness and spiciness have no significant difference between the 2 groups ($p>0.05$). Meanwhile, the liking scores for the color, sweetness and overall liking of the Pad Thai sauce with tea seed oil and gac fruit aril powder were higher than the prototype formula with statistical significance ($p\leq0.05$) since the added gac fruit aril powder provided a more appealing red-orange color to the Pad Thai which positively affects the overall liking of the participants (Do et al., 2019).

1.2 Ka Phrao sauce

Using the prototype Ka Phrao sauce and the tea seed oil-substituted Ka Phrao sauce to prepare Chicken Ka Phrao, the results from testing sensory characteristics using the 9 Point hedonic scale (1: "dislike extremely", 9: "like extremely") (Lawless & Heyman, 1988) with 50 participants are shown in Table 4.

Table 4 Mean liking scores of the prototype ready-to-cook Ka Phrao sauce and the developed ready-to-cook Ka Phrao sauce with tea seed oil

n=50

Sensory Attributes	Mean liking score	
	Prototype formula	Developed formula
Appearance	7.0±1.0[b]	7.4±1.2[a]
Aroma	7.0±1.4[b]	7.4±1.2[a]
Saltiness [ns]	7.1±1.1	7.1±1.3
Sweetness [ns]	7.2±1.2	7.4±1.2
Spiciness [ns]	7.1±1.3	7.2±1.2
Overall liking	7.2±1.3[b]	7.5±1.2[a]

Remark: Means in rows followed by different letters represent significant differences ($p\leq0.05$)

[ns] = Means in row do not have significant differences ($p>0.05$)

The results were statistically analyzed by comparing the means of the 2 Ka Phrao sauce groups using T-test. The liking scores of the saltiness, sweetness and spiciness had no significant differences ($p>0.05$). However, the liking scores of the appearance, aroma and overall liking for the tea seed oil-substituted Ka Phrao sauce were significantly higher than the prototype formula ($p\leq0.05$) due to the tea seed oil's sensory characteristics being accepted by the participants-colorless and has no undesirable flavors (Chen, 2007), which positively influenced the overall liking score for the tea seed oil-substituted Ka Phrao sauce.

2. Physical and chemical properties of ready-to-cook sauce

2.1 Pad Thai sauce

The Pad Thai sauce with tea seed oil and gac fruit aril powder underwent chemical and physical characteristics testing, including the pH value, color value and viscosity. As shown in Table 5, the pH value of the sauce was 3.2-3.4, which is due to acidic ingredients, i.e., tamarind paste, vinegar and chili sauce. The color value of the Pad Thai sauce is brown-orange; when measured with MiniScan EZ MSEZ-4500L colorimeter, the L* (perceptual lightness) value was 10.4-10.6 showing that the sauce was slightly dark in color since many ingredients were dark-colored; the a* value was marginally positive meaning the sauce had a slight red color, due to the addition of gac fruit aril powder which gave a red-orange color; the b* value was positive, signifying the yellowness of the sauce. The prototype formula sauce had a higher viscosity than the developed formula sauce, as indicated by the viscosity measurement with the Brookfield LVDV-E, since the formula had fewer liquid components than the other sauce.

Table 5 Chemical and physical characteristics of Pad Thai sauce with tea seed oil and Gac fruit aril powder

Chemical and physical characteristics	Prototype formula	Developed formula
pH^{ns}	3.2 ± 0.0	3.4 ± 0.0
Color values		
L* ^{ns}	10.6 ± 0.3	10.4 ± 0.5
a* ^{ns}	0.8 ± 0.3^{b}	1.0 ± 0.4^{a}
b* ^{ns}	8.0 ± 0.7^{a}	7.7 ± 0.9^{b}
Viscosity (centipoise; cP)	23212.25 ± 598.12^{a}	22758.55 ± 634.24^{b}

Remark: L* is the perceptual lightness, value in the range 0 – 100 where
0 signifies black-colored subject, 100 signifies white-colored subject
a* (+) signifies subject with red color, (–) signifies subject with green color
b* (+) signifies subject with yellow color, (–) signifies subject with blue color
Means in rows followed by different letters represent significant differences ($p\leq0.05$)
^{ns} = Means in row do not have significant differences ($p>0.05$)

2.2 Ka Phrao sauce

The tea seed oil-substituted Ka Phrao sauce was tested for its chemical and physical characteristics, which includes the pH value, color value and viscosity. As listed in Table 6, the pH value of the Ka Phrao sauce is 4.4-4.5 and the color of the sauce is brown. When measured with MiniScan EZ MSEZ-4500L colorimeter, the L* value is 17.0-17.3 meaning the sauce has a dark color; the a* is marginally positive meaning the sauce is slightly red; the b* value is positive signifying the yellowness of the sauce. The developed Ka Phrao sauce has a lighter color and a higher viscosity than the developed Pad Thai sauce, as the Ka Phrao sauce contains holy basil leaves, minced chili and garlic, which adds to the viscosity of the sauce.

Table 6 Chemical and physical characteristics of tea seed oil-substituted Ka Phrao sauce

Chemical and physical characteristics	Prototype formula	Developed formula
pH^{ns}	4.5 ± 0.0	4.4 ± 0.0
Color values		
L* ^{ns}	17.3 ± 1.4	17.0 ± 1.6
a* ^{ns}	2.1 ± 0.8	2.3 ± 0.9
b* ^{ns}	16.9 ± 1.0	16.7 ± 0.9
Viscosity (centipoise; cP)^{ns}	41178.23 ± 398.56	41229.33 ± 421.58

Remark: L* is the perceptual lightness, value in the range 0 – 100 where
0 signifies black-colored subject, 100 signifies white-colored subject
a* (+) signifies subject with red color, (–) signifies subject with green color
b* (+) signifies subject with yellow color, (–) signifies subject with blue color
^{ns} = Means in row do not have significant differences ($p>0.05$)

3. Microbiological qualities of ready-to-cook sauce

Microbiological qualities of the Pad Thai sauce with tea seed oil and gac fruit aril powder and tea seed oil-substituted Kra Phrao sauce were determined as shown in Table 7, which indicates the microorganisms growing and yeast and mold meets the standard of Thailand Ministry of Public Health Notification No 355 B.E. 2556 (2013) Food in a Hermetically Sealed Container.

Table 7 Microbiological qualities of Pad Thai sauce with tea seed oil and Gac fruit aril powder and tea seed oil-substituted Kra Phrao sauce

Microorganism type	Pad Thai sauce with tea seed oil and Gac fruit aril powder	Tea seed oil-substituted Kra Phrao sauce	Ministry of Public Health Notification No 355 B.E. 2556 (2013) Food in a Hermetically Sealed Container
Total Plate Count (CFU/g)	< 10	< 10	< $1x10^3$
Yeast and mold (CFU/g)	< 10	< 10	< $1x10^2$

4. Acceptability test

Table 8 presents the results of the study on consumer acceptance of the developed ready-to-cook Pad Thai sauce. The study included 100 panelists from the Bangkok Metropolitan Area and covered demographic information as well as consumer liking and acceptability tests.

Table 8 Demographic data

Demographic attributes	Frequency (percentage)
1. Gender	
- Male	50
- Female	50
2. Age	
- 20 – 31 years old	30
- 31 – 40 years old	25
- 41 – 50 years old	20
- More than 50 years old	25
3. Education level	
- High school diploma	7
- Bachelor's degree	48
- Higher than bachelor's degree	45
4. Occupation	
- Student	32
- Government official	34
- Housewives	5
- Private company employees	25
- Personal business	4
5. Average monthly income	
- Less than 10,000 THB	18
- 10,001 – 20,000 THB	41
- 20,001 – 30,000 THB	27
- More than 30,000 THB	14

4.1 Pad Thai sauce

The results of investigating consumers' liking on the 9-point hedonic scale of the developed ready-to-cook Pad Thai sauce in its color, aroma, saltiness, sweetness, sourness, spiciness and overall liking revealed that consumers' likings scored in every characteristic at the "like very much" level (7.7-8.2), as shown in Table 9.

Table 10 presents the results of customers' acceptability and purchase decisions about the created ready-to-cook Pad Thai sauce.

Table 9 Mean liking scores of the developed ready-to-cook Pad Thai sauce

n=100

Sensory attributes	Mean liking scores
Color	8.2 ± 1.4
Aroma	7.8 ± 1.6
Saltiness	7.7 ± 1.3
Sweetness	8.0 ± 1.1
Sourness	7.7 ± 1.5
Spiciness	7.8 ± 1.3
Overall liking	8.2 ± 1.3

According to the findings of the survey of customers' acceptance and decision to purchase the developed ready-to-cook Pad Thai sauce, 98% accepted the product and 92% chose to purchase the developed ready-to-cook Pad Thai sauce.

Table 10 Consumers' acceptance on the developed ready-to-cook Pad Thai sauce

n=100

Data	Frequency (percentage)
Consumers' acceptance on the developed ready-to-cook Pad Thai sauce	
- Accept	98
- Not accept	2
Decision on purchasing the developed ready-to-cook Pad Thai sauce	
- Purchase	92
- Not purchase	8

4.2 Ka Phrao sauce

Results of the investigation into consumers' preferences for the ready-to-cook Ka Phrao sauce in terms of color, aroma, saltiness, sweetness, spiciness and overall preference on 9-point hedonic scale revealed that consumers' preferences scored in each category at the "like moderately" to "like very much" level (7.5-7.9), as shown in Table 11.

Table 12 illustrates the findings of the survey of customers' acceptance and purchase decision regarding the developed ready-to-cook Ka Phrao sauce.

Table 11 Mean liking scores of the developed ready-to-cook Ka Phrao sauce

n=100

Sensory attributes	Mean liking scores
Appearance	7.8 ± 1.1
Aroma	7.9 ± 1.5
Saltiness	7.5 ± 1.3
Sweetness	7.6 ± 1.3
Spiciness	7.5 ± 1.0
Overall liking	7.8 ± 1.2

Table 12 Consumers' acceptance on the developed ready-to-cook Ka Phrao sauce

n=100

Data	Frequency (percentage)
Consumers' acceptance on the developed ready-to-cook Ka Phrao sauce	
- Accept	94
- Not accept	6
Decision on purchasing the developed ready-to-cook Ka Phrao sauce	
- Purchase	90
- Not purchase	10

A survey of customers' acceptance and decision to purchase the developed ready-to-cook Ka Phrao sauce found that 94% accepted the product and 90% decided to purchase the developed ready-to-cook Ka Phrao sauce.

Conclusion

This study incorporates the use of tea seed oil as a replacement of vegetable oil in various types of ready-to-cook sauce, to utilize the benefits of tea seed oil as it is trans-fat free and has high levels of unsaturated fatty acids, which reduces LDL cholesterol and increases HDL cholesterol. Moreover, Gac fruit aril powder was used to enhance the color consistency of Pad Thai sauce, while reducing rancidity and benefiting from antioxidants. Overall, the study has been able to develop a guideline in developing healthy ready-to-cook sauces. The acceptability test for the ready-to-cook sauces also showed consumer acceptability in the levels of "Moderately Like" to "Like Very Much", which confirms the feasibility of commercializing the production of these ready-to-cook sauces for Thai food, to satisfy the consumers' demand for convenience in food preparations and health benefits. Furthermore, the knowledge of ready-to-cook sauces for Thai food can be applied to cooking in both domestic and international Thai restaurants. The developed ready-to-cook sauces may also be utilized as alternatives for people who are concerned about their health.

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The Physicochemical Properties and Consumer Acceptance of Ricegrass Beverage Ready for Consumption

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Abstract

Juice extracted from riceberry ricegrass has exhibited higher phenol and antioxidant activity. The objectives of this research was to enhance the value of ricegrass and to develop a product that small community enterprises can follow through the entire production process. This study was designed to examine the physicochemical, microbiological properties and sensory evaluation of ricegrass juice and ricegrass juice mixed with soybeans and navy beans (SN) milk. Rice leaves were extracted with room temperature water at ratios of 10:1, 2:1, 1:1, 1:5 and 1:10 (w/v) and with hot water at ratios of 2:1 and 1:1 (w/v). The results revealed that ricegrass leaves extracted with room temperature water at a ratio of 1:5 (w/v) had a higher yield, total phenol and antioxidant activity. The sensory evaluation found that 30% of ricegrass juice that was mixed with 10% SN milk had the highest acceptance score on all attributes. Pasteurized products stored at 4°C for 8 days were closed near to the Thai community product standard (TCPS 529/2547), but total phenol and antioxidant activity decreased during storage ($p \leq 0.05$). Furthermore, ricegrass juice mixed with SN milk product is a functional drink which has plenty of phenol, antioxidant and nutrition.

Introduction

Riceberry rice (*Oryza sativa* L.) is a native rice of Thailand with high nutrient values. In the same manner, rice is one of the cereal plants in the grass family (Poaceae) which is the same as wheat, rye and barley. Chomchan et al. (2016) found that plants at a younger stage produce higher levels of phytochemicals substances which can protect themselves away from risks, while these compounds exert various biological benefits for human health, especially 6 to 20 days young ricegrass has a higher level of antioxidant compounds (Akcan Kardas & Durucasu, 2014). Moreover, young leaves of monocotyledon plants are rich in vitamins, minerals, dietary fibre, antioxidants, superoxide dismutase (SOD) enzyme and other bioactive substances, such as tyrosinase and phosphodiesterase inhibitor in wheat, barley, oats, rye and rice (Hattori, 2002; Chen & Hsieh, 2008). Prior

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research reported on discovering in ricegrass juice phenolic compounds and several biological properties, which are antioxidant properties and DNA protective properties (Khanthapoka et al., 2015). Kapkum et al. (2011) claimed that the total phenolic content (TPC) of ricegrass juice from white rice cultivars varied in the range of 1.50 to 2.14 mg gallic acid equivalent (GAE)/g dry extract. Additionally, ricegrass juice from the Khao Dawk Mali 105 cultivar prevented lipid oxidation to a similar extent as wheatgrass juice. According to previous literature, ricegrass juice has been extracted from fresh leaves with room temperature water (Chomchan et al., 2016), dried rice leaves extracted in boiling water (80-98°C) (Phimphilai et al., 2021).

The introduction of young ricegrass juice as an innovative functional drink is fascinating. A previous study demonstrated that the extracts of Thai purple ricegrass at the jointing stage contained significantly higher levels of phytochemicals and antioxidant ability than those of white ricegrass (Khanthapok et al., 2015). Furthermore, black glutinous rice grain sprouts (cv. BGR) were more effective in suppressing the proliferation of human T-lymphocyte (Jurkat), human liver (HepG2) and human colon (HCT116) cancerous cells than white rice sprouts (cv. RD6), which is due to their antioxidant ability and greater polyphenol and anthocyanin contents (So et al., 2020).

It is well known that beans are an excellent source of phytochemicals, particularly polyphenols, as well as protein and dietary fibers (Chen et al., 2019). A number of health benefits have been reported from their consumption, including a reduction in obesity, diabetes and cardiovascular risks (Tharanathan & Mahadevamma, 2003). As a health beverage with potential nutraceutical properties, bean milk has gained popularity in recent years. In particular, it is suitable for children who are allergic to cow's milk and for adults who are not able to tolerate lactose in their systems (Meng et al., 2023). De et al. (2022) found that soymilk and soymilk products were not only effective nutraceutical adjuncts to treating hyperglycemia but also improved nutritional values. Ricegrass drinks have been produced to improve certain nutritional qualities and health benefits. The functional drink products have a global market value of US $ 125.36 billion in 2020 and are expected to raise to US $216.7 billion by 2028 (Department of International Trade Promotion, 2023). However, there are limited investigatory research studies that have been conducted (Phimphilai et al., 2021). In this study, total phenolic and antioxidant activities were determined by using Folin-Ciocalteu method and 2,2-diphenyl-1-picryl hydrazyl (DPPH), respectively. Additionally, microbiological properties, sensory evaluation and nutritional values of riceberry rice juice were evaluated.

Materials and methods

1. Plant materials

Riceberry rice seed (*Oryza Sativa* L.) was obtained from the community enterprise of organic agriculture for Life, Darn Chang, Suphun Buri Province, Thailand. The seeds were washed and soaked overnight in tap water. After washing with distilled water, seeds were planted in a soil bed under natural light for further growth. After 11 days of seed germination, fresh grasses were rapidly cut above ground, weighed and washed three times with tap water followed by juice extraction.

2. Extraction procedure

Rice leaves were extracted with room temperature water at ratios of 10:1, 2:1, 1:1, 1:5 and 1:10 (w/v) and hot water (80°C) at ratios of 2:1 and 1:1 (w/v). The samples were extracted by using a juice extracter (EM-11, Sharp, Japan) at level 2 speed for 3 min. Ricegrass extract were tested for % yield, color by using a colorimeter (Minolta chroma meter (CR-300), Konica Minolta, Inc., Tokyo, Japan). The pH value was measured using the digital pH meter (L855, Xylem, Germany). Total phenolic content was measured by using a Folin–Ciocalteu method from Waterman & Mole, 1994. Antioxidant activity was determined in using 2,2-diphenyl-1-picryl hydrazyl (DPPH). In the presence of an antioxidant, DPPH solution turns from purple to yellow, corresponding to the color of the hydrazine in the solution. The reducing ability of antioxidants toward DPPH can be determined by measuring the decrease of its absorbance at 517 nm. All results are expressed as % DPPH for a fixed antioxidant concentration (Brand-Williams et al., 1995). The % DPPH was calculated using the following equation:

$$\% \text{ DPPH} = \frac{(A\ control - A\ sample)}{A\ control}\ x\ 100$$

3. Development of ricegrass juice

3.1 Optimum concentration of ricegrass extract

The ricegrass juice (ricegrass extract mixed with sugar and water) was prepared by mixing 100 g of sugar with 1.5 L of water and the added 10, 20, 30 and 40% (v/v) ricegrass extract. All samples were assessed for

color, odor, taste and acceptance based on sensory attributes. The test panel consisted of 50 untrained panellists who participated in 9-Points Hedonic Scale as used in this experiment.

3.2 Optimum concentration of ricegrass juice mixed with SN milk

SN milk was prepared by mixing commercial soybeans with navy beans at ratio of 3:1 (w/w) and prepared by using soymilk production protocols (Yu et al., 2021). Ricegrass extract with the highest sensory score was selected and mixed with 0, 10, 20 and 30% (v/v) SN milk. The samples were evaluated by following section 3.1.

4. Pasteurization condition

Samples were boiled at 100°C for 2-3 min, followed by storage at 4±2°C for 8 days and sampling every 2 days (Day 0, 2, 4, 6 and 8). To assess the color of pasteurized products, a colorimeter was used (Minolta chroma meter (CR-300), Konica Minolta, Inc., Tokyo, Japan). The pH value of the samples was measured with a digital pH meter (L855, Xylem, Germany); the total phenolic content was determined using the Folin–Ciocalteu method (Waterman & Mole, 1994); and the antioxidant activity was determined using 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Brand-Williams et al., 1995). An analysis of the microbiological properties was conducted by using the total plate count to identify the presence of *Staphylococcus aureus* and *Escherichia coli* (AOAC, 2000). Sensory evaluations were estimated for color, odor, taste and acceptance based on sensory attributes. This experiment consisted of 50 untrained participants and 9-Points Hedonic Scale was used to analyse the results.

5. Physicochemical properties, antioxidant activity and nutritional value of ricegrass juice and ricegrass juice mixed with SN milk products

Pasteurized products were measured for nutritional value, total phenolic content by using a Folin–Ciocalteu method (Waterman & Mole, 1994) and antioxidant activity using 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Brand-Williams et al., 1995).

6. Statistical analysis

All experiments were conducted in triplicate and data were analyzed using ANOVA and significant differences were determined using Duncan's multiple range test ($p \leq 0.05$). Physicochemical and microbiological properties were assessed using a completely randomized design (CRD), while sensory evaluation was conducted using a randomized complete block design (RCBD). All analysis was performed using SPSS software, version 16 (SPSS Inc.).

Results and discussion

1. Effect of extraction process on qualities and biological properties of ricegrass extraction

Effects of extraction process on the yield and color of ricegrass extract are presented in Table 1. In accordance with the extraction process ratios were modified by Chomchan et al. 2016. The result of a study conducted on fresh leaves with room temperature water (10:1 w/v) showed the lowest yield, while greenness was the highest at 19.67 ($p \leq 0.05$). According to the results, there will be a higher cost for the final product when using fresh leaves as an ingredient compared to the other conditions since the fresh leaves have the lowest %yield. When comparing room temperature water and hot water (80°C) at the same ratio, it was found that using hot water significantly decreased the lightness and total phenol content in ricegrass juice. One of the factors that may affect the decomposition of bioactive substances is the higher water temperature. Tiwari et al. (2011) found that juices were extracted using water, which is a high-polarity solvent and that most compounds dissolved in the juice were hydrophilic. Besides, tannin, saponin and soluble phenolic compounds were presented in the solution using water as solvent in some of earlier reports. Wangcharoen & Phimphilai (2016) published that number and position of hydroxyl groups attached to ring structures of their molecules as the reason in affect to the antioxidant potential of phenolic compounds. It means that high antioxidant activities displayed in ricegrass can be detected by the high level of phenolic compounds. Khanthapoka et al. (2015) showed that riceberry, Kum Doisaket and Kum Noi pigmented rice juices displayed a higher percentage of DPPH radical scavenging activity and FRAP, which was compared with wheatgrass juice. The reason is because of the high anthocyanin content of riceberry. Moreover, compared with white-colored rice (RD6), colored rice had a higher TPC, DPPH scavenging activity and ferric reducing antioxidant power (So et al., 2020). As a result, the high productivity can be attributed to the juice obtained by squeezing fresh rice leaves with room temperature water at a ratio of 1:5. This variety had the highest total phenolic content as well as a high % yield. It had a good appearance as well as a high lightness and a bright green color. A ratio of 1:5 is significantly more potent than those in the other ratios, which will be explored in the next step.

Table 1 Effect of extraction process on the physicochemical properties of ricegrass extract

Methods (w/v)	%Yield (ml)	Color			Total phenol (mg/L)	DPPH (%)
		Lightness (L^*)	Redness-Greenness (a^*)	Yellowness-Blueness (b^*)		
Fresh leaves/room temperature water (10:1)	124.56^{c}±1.00	10.78^{c}±2.56	-19.67^{a}±2.05	15.61^{a}±3.56	261.85^{ab}±68.18	64.14^{b}±1.36
Fresh leaves /room temperature water (2:1)	378.23^{d}±6.04	10.67^{c}±0.61	-17.21^{ab}±1.10	13.42^{ab}±0.52	333.67^{a}±54.43	70.57^{a}±1.15
Fresh leaves /room temperature water (1:1)	598.34^{c}±15.21	7.17^{d}±2.11	-11.16^{c}±1.38	7.85^{c}±1.05	276.56^{ab}±25.46	65.18^{b}±1.21
Fresh leaves /hot water (2:1)	408.67^{d}±9.29	6.83^{dc}±0.85	-13.71^{bc}±1.97	9.95^{bc}±1.37	226.15^{b}±4.78	63.52^{b}±1.01
Fresh leaves /hot water (1:1)	589.56^{c}±32.05	5.41^{c}±1.39	-9.75^{c}±4.74	7.32^{c}±3.18	225.56^{b}±16.84	58.36^{c}±1.42
Fresh leaves /room temperature water (1:5)	2,671.17^{b}±46.17	20.67^{b}±2.32	-15.56^{b}±2.62	14.23^{ab}±4.67	311.84^{a}±10.67	54.21^{c}±1.93
Fresh leaves /room temperature water (1:10)	4,982.62^{a}±83.02	29.53^{a}±1.85	-13.43b^{c}±2.75	12.78^{ab}±4.56	149.26^{c}±7.51	45.79^{d}±2.17

Remark: Mean ± S.D. with different superscripts in the same column indicated significant different ($p \leq 0.05$)

2. Product development of drinking from ricegrass extract

2.1 Sensory evaluation of ricegrass juice

The ricegrass juice preparation was made from 10-40% of the ricegrass extract. Sensory attributes of ricegrass juice on color, odor, taste and overall acceptance are illustrated in Table 2. Ricegrass juice received the highest odor score (7.58). As observed from the sensory score, 40% of ricegrass extraction received the lowest score in terms of odor, taste and overall acceptance due to the unique smell of the product hence affecting the liking score. However, the 30 % of ricegrass juice reached the highest score for color, taste and overall acceptance. This concentration of ricegrass juice was used for further studies.

Table 2 Sensory evaluation scores of ricegrass juice derived from different extraction % of ricegrass mixed with sugar and water

Attributes	Ricegrass extraction (%)			
	10	20	30	40
Color	5.31^{d}±1.08	6.25^{c}±1.35	7.45^{a}±0.28	7.25^{b}±1.05
Odor	7.58^{a}±0.56	7.42^{ab}±1.26	7.21^{b}±0.31	5.76^{c}±1.26
Taste	7.21^{b}±0.47	7.57^{a}±1.09	7.32^{a}±0.48	5.96^{c}±1.21
Overall acceptance	7.19^{b}±0.65	7.25^{b}±1.05	7.35^{a}±0.53	6.95^{c}±1.16

Remark: Mean ± S.D. with different superscripts in the same row indicated significant different ($p \leq 0.05$)

2.2 Sensory evaluation of 30% ricegrass extract mixed with SN milk

The sensory evaluation of 30% ricegrass extract mixed with 10-30% SN milk and the effects on color, odor, taste and overall acceptance are presented in Table 3. The result showed that 30% ricegrass extraction mixed with 10% of SN milk achieved the highest score for all attributes. A prior report found that increased quantities of SN milk are a significant contributing factor to a decrease in overall acceptance of the product due to the characteristic smell of soybean milk, which is unappealing to consumers.

Table 3 Sensory evaluation scores of 30% ricegrass extract mixed with different % of SN milk

Attributes	30% ricegrass extract mixed with SN milk (%)			
	0	10	20	30
Color	7.45^{a}±0.32	7.73^{a}±0.73	6.78^{b}±1.32	6.11^{b}±1.12
Odor	7.03^{a}±0.15	7.43^{a}±0.67	6.58^{b}±1.16	5.78^{b}±1.24
Taste	7.23^{b}±0.58	7.67^{a}±0.82	6.32^{c}±1.05	6.28^{c}±1.07
Overall acceptance	7.21^{b}±0.16	7.69^{a}±0.54	6.25^{c}±1.01	6.21^{c}±1.03

Remark: Mean ± S.D. with different superscripts in the same row indicated significant different ($p \leq 0.05$)

3. Physicochemical, microbiological and sensory evaluation of pasteurized products

3.1 Physicochemical and microbiological properties of pasteurized products

The physicochemical properties of pasteurized products stored at 4±2°C for 8 days are exhibited in Table 4 and 5. It was found that color, total phenol and antioxidant activity were significantly reduced with storage time, similar to what had been observed in previous studies by Wojdyło et al., 2014; Mäkilä et al., 2017; Aaby & Amundsen, 2023. Whereas pH was not significant during the storage time. However, important changes in the color during the storage ($p<0.05$) and the values of L^* and b^* decreased indicating that the yellowness reduced and then the products became darker. Besides, the rise in a^* values signified an increase in a reddish tone which supports the color had

considerably changed. Koca et al. (2003) reported those trends and the results are consistent with their report. It was observed in this study that the color gradually turned to brown during prolonged storage. Wibowo et al. (2015) described the pasteurized orange juice color changes during storage. The value of *L**, *a**, pH and total phenol of ricegrass juice mixed with SN milk was higher than ricegrass juice due to be mixed with SN milk. However, the value of *b** and DPPH of ricegrass juice mixed with SN milk was higher.

Researchers have previously observed that the phytochemicals exhibited similar behavior in extracts of rice plants. A study by Kapkum et al., 2011, indicated that dried rice plants dried by vacuum-microwave at 2880 W and water extracted for 3 min had a total polyphenol content of 0.04 GAE/mL, antioxidant activity of 0.26 mg TEAC/mL and FRAP of 0.09 mg $FeSo_4$/ml, respectively. Khanthapok et al., 2015 also examined the antioxidant activity of juice squeezed from rice grasses prepared from grass juice and processed at a low temperature, demonstrating that the total phenolic content ranged from 1.9-4.3 mg GAE/g DE in colored rice cultivars, while the Kum Doisaket cultivar demonstrated the greatest radical scavenging activity with an EC_{50} of 0.11 mg DE/mL. Additionally, Wangcharoen & Phimphilai (2016) studied three rice varieties (Jusmine, Sukhothai 1 and Sukhothai 2) under sterilization conditions and stored ricegrass juices at room temperature for a period of one month. Researchers found that TPC varied between 10.50 and 23.04 mg gallic acid equivalent/200 mL, antioxidant activities such as ABTS varied between 25.44 and 35.18 mg vitamin C equivalent/200 mL, and FRAP varied between 16.00 and 23.70 mg $FeSo_4$ equivalent/200 mL. However, no studies have examined the physicochemical characteristics and consumer acceptance of ricegrass beverages at the ready-to-drink stage. The decrease of phenolic and antioxidant can be explained by the pasteurized process and storage time. The reason is that phenolics and antioxidants are sensitive to heating and the prolonged thermal treatment would lead to a loss of natural antioxidants due to most of these compounds are unstable (Kapkum et al., 2011; Aydar et al., 2023). Moreover, the total phenolic compound decreased, an effect that is inversely proportional to the storage time due to the fact that total phenolic compounds are highly sensitive to light, oxygen and temperature and as a result, the total phenolic content will decrease over time due to oxidative stress caused by pairing reactive oxygen free electrons with each other (Silva et al., 2023).

Table 6 demonstrates the microbiological analyses of pasteurized products, including total plate count, *Staphylococcus aureus* and *Escherichia coli.* The results show that in 8 days of storage, the total plate count was recorded at less than 3 CFU/mL. For the pathogenic bacteria, including *Staphylococcus aureus* and *Escherichia coli,* there was no detection in the pasteurized samples. The results were closed near to the Thai community product standard (TCPS 529/2547).

Table 4 Physicochemical properties of pasteurized ricegrass juice with different storage times

Day	color (CIE-Lab)			pH^{ns}	Total phenol (mg/L)	DPPH (%)
	*L**	*a**	*b**			
0	50.86^{a}±0.65	-24.31^{a}±0.62	34.87^{a}±1.02	6.45±0.35	86.09^{a}±13.01	61.05^{a}±2.21
2	50.41^{ab}±0.41	-24.08^{a}±0.46	33.67^{ab}±1.13	6.43±0.78	87.18^{a}±2.16	62.56^{a}±3.03
4	48.68^{bc}±1.32	-23.57^{ab}±0.21	32.53^{c}±1.23	6.51±0.11	84.59^{b}±6.03	52.35^{b}±1.21
6	47.62^{c}±1.27	-21.67^{c}±0.02	31.12^{c}±1.18	6.48±0.56	68.42^{c}±1.04	43.21^{c}±1.25
8	44.32^{d}±1.28	-20.89^{d}±0.05	29.28^{d}±0.67	6.44±0.47	65.71^{c}±3.19	31.67^{d}±1.19

Remark: Mean ± S.D. with different superscripts in the same column indicated significant different ($p \leq 0.05$)
Mean ± S.D. with ns in the same column indicated not significantly different ($p > 0.05$)

Table 5 Physicochemical properties of pasteurized ricegrass juice mixed with SN milk with different storage times

Day	Color (CIE-Lab)			pH^{ns}	Total phenol (mg/L)	DPPH (%)
	*L**	*a**	*b**			
0	92.05^{a}±0.11	-5.85^{a}±0.21	10.81^{a}±0.31	6.65±0.48	129.51^{a}±2.21	36.67^{a}±1.21
2	91.32^{b}±0.03	-5.69^{ab}±0.03	10.53^{ab}±0.59	6.68± 0.75	121.61^{b}±1.26	28.31^{b}±1.32
4	91.31^{b}±0.11	-5.33^{cd}±0.04	10.04^{c}±0.11	6.66± 0.51	93.94^{c}±1.25	21.04^{c}±1.05
6	91.25^{b}±0.14	-5.21^{d}±0.18	9.54^{d}±0.15	6.70± 0.84	81.21^{d}±1.13	11.05^{d}±1.01
8	90.58^{c}±0.02	-4.67^{c}±0.12	9.49^{d}±0.12	6.67± 0.65	80.42^{e}±1.17	12.71^{d}±1.05

Remark: Mean ± S.D. with different superscripts in the same column indicated significant different ($p \leq 0.05$)
Mean ± S.D. with ns in the same column indicated not significantly different ($p > 0.05$)

Table 6 Microbiological properties of pasteurized ricegrass juice and ricegrass juice mixed with SN milk with different storage times

Day	Ricegrass juice			Ricegrass juice mixed with SN milk		
	Total plate count (CFU/mL)	*S. aureus* (CFU/mL)	*E.coli* (Per 100 mL)	Total plate count (CFU/mL)	*S. aureus* (CFU/mL)	*E.coli* (Per 100 mL)
0	< 3	Not detected	Not detected	< 3	Not detected	Not detected
2	< 3	Not detected	Not detected	< 3	Not detected	Not detected
4	< 3	Not detected	Not detected	< 3	Not detected	Not detected
6	< 3	Not detected	Not detected	< 3	Not detected	Not detected
8	< 3	Not detected	Not detected	< 3	Not detected	Not detected

3.2 Sensory evaluation of ricegrass juice and ricegrass juice mixed with SN milk after pasteurization

Ricegrass juice and ricegrass juice mixed with SN milk were sensory evaluated by 50 untrained panellists illustrated in Table 7. The ricegrass juice and ricegrass juice mixed with SN milk had no major differences among all attributes. Observing from the sensory score, the overall acceptance score from ricegrass juice mixed with SN milk received a higher score than ricegrass juice due to the unique smell of the product. Kongjaroon (2019) claimed that the aroma of ricegrass powder can be turned into a unique fragrance for green tea. In the most recent studies that compared different types of grass juices, blends of wheatgrass juice with fruit juices, or different treatments, which proved that better acceptability occurred when formulated with other juices (Rexhepi & Renata, 2015; Rodríguez et al., 2022).

Table 7 Sensory evaluation scores of ricegrass juice and ricegrass milk mixed with SN milk

Attributes	ricegrass juice	ricegrass milk mixed with soy and navy milk
Color^{ns}	7.21±0.73	7.73±0.82
Odor^{ns}	7.23±1.02	7.46±0.95
Taste^{ns}	7.11±0.93	7.71±0.91
Overall acceptance^{ns}	7.07±1.75	7.73±0.95

Remark: Mean ± S.D. with ns in the same column indicated not significantly different ($p > 0.05$)

4. Physicochemical properties and Nutritional value of ricegrass juice and ricegrass juice mixed with SN milk

The nutritional values of ricegrass juice and ricegrass juice mixed with SN milk were calculated from 100 mL of consumption and results are displayed in Table 8. The nutritional value of ricegrass juice mixed with SN milk was analysed as follows: Calories 54.5 kilocalories, Calories from fat 12.2 kilocalories, Total fat 1.36 g, Saturated fat 0.25 g, No Cholesterol, Protein 2.91 g, Carbohydrate 7.65 g, Dietary Fiber 0.16 g, Sugar 6.64 g, Sodium 0.91 g, Vitamin B1, 2 Calcium and Iron. The results revealed that the nutritional value of ricegrass juice mixed with SN milk was better than other mixtures of ricegrass juice because SN milk had sources of nutrition, such as protein and vitamins.

Table 7 Nutritional content of ricegrass juice and ricegrass juice mixed with SN milk

Nutrient	Products	
	Ricegrass juice (per 100 ml)	Ricegrass juice mixed with SN milk (per 100 ml)
Calories (kilocalories)	23.3	54.5
Calories from fat (kilocalories)	0.00	12.2
Total fat (g)	0.00	1.36
Saturated fat (g)	Not Detected	0.25
Cholesterol (mg)	Not Detected	Not Detected
Protein (Nx6.25) (g)	<0.20	2.91
Total carbohydrate (g)	5.82	7.65
Dietary Fiber (g)	0.01	0.16
Sugars (g)	5.69	6.64
Sodium (mg)	2.99	0.91
Vitamin A (ug RE)	12.5	Not Detected
Vitamin B1 (mg)	Not Detected	0.05
Vitamin B2 (mg)	Not Detected	0.1
Calcium (mg)	4.12	15.8
Iron (mg)	0.12	0.44
Ash (g)	0.08	0.38
Moisture (g)	94.1	87.7
Total phenol (mg/L)	85.33±13.61	129.95±2.93
DPPH (%)	61.00±3.61	37.33±2.52

Conclusion

This study demonstrated that 30% ricegrass juice and ricegrass juice mixed with 10% of SN milk could be considered as the best functional drinks with antioxidant potential, due to their total phenol, antioxidant activity and other nutrients. All the pasteurized products had a low number of microbial contents. A high preference of the sensory panellists towards the ricegrass juice mixed with 10% of SN milk created a unique flavor. However, the flavors from the product will be investigated in further studies.

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Health Benefit Potentials Offered by Soy Isoflavones as a Consequence of the Various Phytochemical Properties

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Abstract

Bioactive soy isoflavones found in soybeans and other soy products offer antioxidant and anti-inflammatory properties and have been shown to present similar structural characteristics to 17-*β*-estradiol. Moreover, they have the ability to form bonds with estrogen receptors known as phytoestrogen. They are able to serve as the estrogen agonists of the relevant antagonists, depending on the estrogenic level of endocrine, although it is understood that isoflavones are involved in many complex processes based on their activity modes and compound structures. Isoflavones are now a matter of considerable interest to researchers due to their potential health benefits, especially in addressing the onset of cardiovascular disease, hormone-dependent cancers, type 2 diabetes, problems related to menopause, osteoporosis and cognitive decline due to the aging process. This study provides an overview based on prior research of the various benefits that might be offered by soy isoflavones.

Introduction

Asia has consumed soybeans (*Glycine max* L.) and other soy products in large quantities for hundreds of years in the form of soy milk, tofu, natto, miso, tempeh and many other traditional dishes. In contrast, the West has typically consumed soy extract, soy flour and other soy proteins as ingredients in a wide range of food products (O'Keefe et al., 2015; Kim et al., 2005; Messina, et al., 2006; Riswanto et al., 2021; Qu et al., 2021). Soybeans serve as an excellent source of fatty acids, carbohydrates, proteins, oil and amino acids. Furthermore, they also provide isoflavones which are phytochemical components that play key roles in the biological defense mechanism (Messina, 1999; Chen & Chen, 2021). There are 12 different kinds of isoflavones found in soybeans, which can be categorized into the following four types: i) *β*-glucosides (glycitin, daidzin

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and genistin); ii) 6″-*O*-malonyl-*β*-glucosides (6″-*O*-malonylgenistin, 6″-*O*-malonyldaidzi and 6″-*O*-malonylglycitin); iii) 6″-*O*-acetyl-*β*-glucosides (6″-*O*-acetylglycitin, 6″-*O*-acetyldaidzin and 6″-*O*-acetylgenistin) and iv) aglycone (genistein, glycitein and daidzein) (Jung et al., 2020; Lim et al., 2021; Qu et al., 2021). The main soybean component among these various isoflavones are the 6″-O-malonyl-*β*-glucosides, while *β*-glucosides and 6″-O-acetyl-*β*-glucosides play a lesser role and aglycone is not often seen (Hoeck et al., 2000, Qu et al., 2021). Genistein, daidzein and glycitein along with their associated glucosides are the most important isoflavone aglycones, accounting for respectively 50%, 40% and 10% of the overall isoflavone concentration found within soybeans (Hassan, 2013; Riswanto et al., 2021). The capacity of the human body to absorb these isoflavones is dependent on their chemical structure (Chang & Choue, 2013; Kuligowski et al., 2022). Isoflavones exhibit similarities in their structure to that of 17-*β*-estradiol, which is a form of estrogen and accordingly, this leads to mild estrogenic activity in addition to other similar biological properties and for this reason, they are known as phytoestrogen (Chen et al., 2019). They can also be considered as a flavonoid sub-class found in plants, and offering strong antioxidant qualities (Ramachandran 2020). In recent years, the food sector has found a use for soybeans and other soy products as functional foods while numerous studies have sought to apply soybeans in the treatment of various conditions related to cognition, menopause, obesity, diabetes, cancer, osteoporosis and cardiovascular disease (Messina, 2016; Riswanto et al., 2021; Ramachandran et al., 2020). Fig. 1 shows some of the health benefits associated with isoflavones from soybeans. This current study places emphasis on soy isoflavones and offers a summary of their biological potential along with their nutritional significance and specific properties pertaining to their antioxidant qualities, anti-cancer properties and effectiveness against type 2 diabetes mellitus, osteoporosis, heart diseases, cognitive abnormalities and symptoms of menopause which have previously been detailed. It is anticipated that this work will be of interest to an audience whose interests lie within the fields of agricultural or food science and the applications of nutritional food products.

Antioxidant Properties of Soy Isoflavones

The soybean plant is a source of soy isoflavones which offer notable antioxidant properties and can deliver important health benefits. Isoflavones are compounds whose basic structure comprises a pair of benzyl rings connected by a three-carbon bridge, which is sometimes but not always closed to form a pyran ring. Compounds of this type are collectively known as flavonoids which provide a large and diverse set of plant phenolics (Liu, 1997; Erickson, 1995).

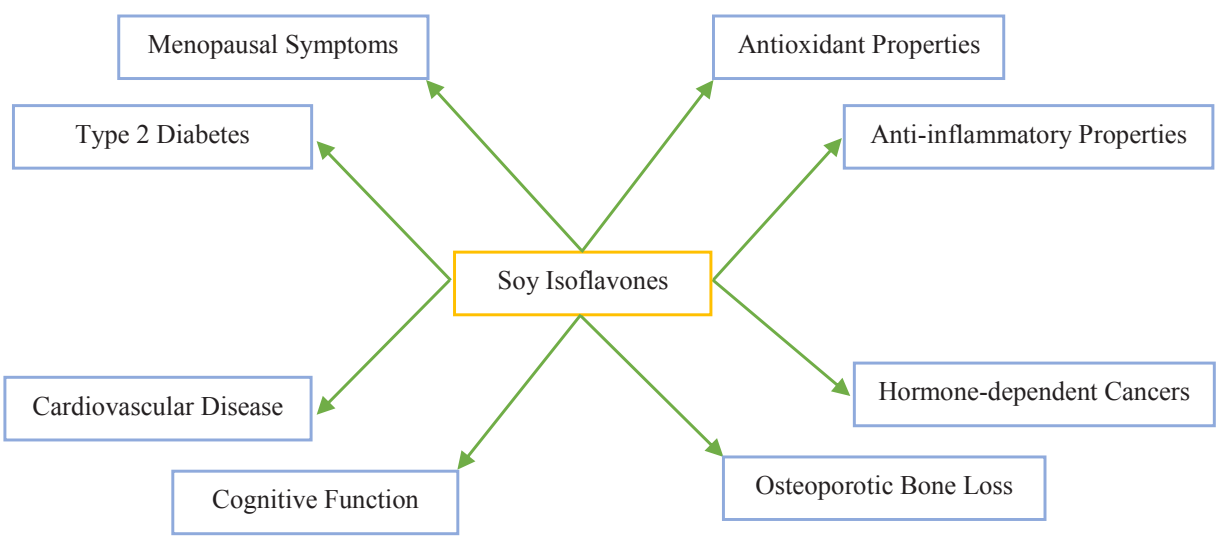

Fig. 1 The health benefits potentially offered by soy isoflavones.

The antioxidant activities of isoflavones include the scavenging of free radicals along with the capacity to reduce low-density lipoprotein (LDL). DNA is susceptible to oxidative stress and can support and enhance antioxidant enzyme expression and activity (Erba et al., 2012). Phenolic compounds can act directly as antioxidants through the scavenging of free radicals as well as act indirectly through the modulation of intracellular pro-oxidant or anti-oxidant enzymes (Schewe et al., 2008). For example, genistein inhibits radicals, thus acting as an antioxidant and can also act against cancer via the chelation of metals and the foraging of radicals. By donating hydrogen atoms from the hydroxyl group connected to the benzene ring, direct antioxidant activity takes place, thus ensuring the oxidative damage is reduced (Sawa et al., 1999; Sierens et al., 2002).

Rats that underwent treatment with daidzein exhibited greater catalase and superoxide dismutase (SOD) activity, whereas if they were treated with genistein, the SOD activity would also rise but not to the same extent (Banz et al., 2004). When mice were given soy isolate supplements that contained 400 mg/g isoflavone aglycones (226 mg/g genistein and 174 mg/g daidzein), their hepatic malondialdehyde (MDA) and conjugated dienes were found to be significantly reduced. The soy isolate served to increase catalase and SOD activity in the liver, but this was not the case for the activity of glutathione peroxidase (GSH-px) (Ibrahim et al., 2008).

The introduction of isoflavone supplements can increase antioxidant activity, which is evident through the decline in TBARs, the rise in plasma triglyceride and the reduced accumulation of body fat. However, the results of antioxidant activity which can be attributed to isoflavones could actually be in part a consequence of enhanced antioxidant enzyme activity (Yoon & Park, 2014).

Anti-inflammatory Properties of Soy Isoflavones

The inflammatory response is typically triggered by irritants, pathogens, or cell damage and serves the purpose of halting infection, repairing tissue, or removing necrotic cells (Barton, 2008). It is essential that the process is fast, specific to the target and controlled effectively to ensure that the innate immune response is not over-activated, since this could lead to severe tissue damage or the potential for chronic infections or inflammatory disorders (Garcia-Lafuente et al., 2009). The field of immunology has seen many recent advances and there has been considerable interest in the relationship between innate immunity and various diseases. It is understood that an immune response can result due to different triggering factors, but the response may occur at different levels of severity (Bernatoniene et al., 2021; Basson et al., 2021; Hariri et al., 2021). In a healthy individual or when facing a weak infection, the inflammatory response can be readily controlled and a return to normal health can be expected. However, when the response is not controlled, inflammation can be damaging and the outcome can be problematic (Hariri et al., 2021). To control inflammation, diet is a vital factor. For example, the consumption of acidic foods such as meats, which contain cholesterol and saturated fatty acids, leads to the generation of ROS (reactive oxygen species) as a part of the digestion process and as a consequence, inflammatory reactions can occur in different parts of the body (Jubaidi et al., 2021). In contrast, alkaline foods such as fruits, vegetables and legumes contain antioxidants and phytonutrients capable of eliminating ROS, thus contributing to weaker inflammatory responses (Tan et al., 2018; Xu et al., 2017).

It has been demonstrated that isoflavones can lower nitric oxide (NO) synthase 2 expression through the inhibition of nuclear factor kappa B (NF-κB) production which would normally be increased as a consequence of radiation and proinflammatory cytokines (interleukin-6, IL-6; interleukin-1 beta, IL-1β; tumor necrosis factor-alpha, TNF-α; and interferon gamma, IFN-γ) (Yu et al., 2016). Simultaneously, the activity of the anti-inflammatory enzyme arginase-1 is promoted, while radiation-induced neutrophils are prevented from gaining access to the lungs (Abernathy et al., 2015; Abernathy et al., 2017). Studies in human subjects have shown that when a soy nut diet (340 mg isoflavones/ 100 g soy nut) is followed for a period of two months, inflammation markers show signs of decline, in the case of interleukin-18 (IL-18) and C-reactive protein, while plasma nitric oxide levels are increased in post-menopausal females who have metabolic syndrome (Azadbakht et al., 2007). For post-menopausal females suffering hypertension, there is an improvement in endothelial function as well as the underlying inflammatory process in response to dietary soy nuts (25 g soy protein and 101 mg aglycone isoflavones) (Nasca et al., 2008). Furthermore, soy foods rich in isoflavones

cause a decline in the C-reactive protein serum levels for patients with end-stage renal failure, while interferon γ (IFN-γ) concentrations are decreased in healthy individuals (Fanti et al., 2006; Ferguson et al, 2014).

Effects of Soy Isoflavone on Hormone-dependent Cancers

It is understood that stronger endogenous estrogens have adverse consequences in terms of breast and endometrial cancer, but it may be the case that soy isoflavones and similar phytoestrogens may mitigate this through weak estrogenic activity. In Asian nations where soy products are a major dietary component, fewer cases of breast and endometrial cancers are observed, while animal models have also provided evidence of soy isoflavones acting against cancer (Peeters et al., 2003; Sarkar & Li, 2003; Magee & Rowland, 2004).

The incidence of breast and mammary gland cancer has shown reduced levels when animals receive a diet rich in soybeans (Barnes et al., 1990). Similarly, reduced rates were seen in postmenopausal women who consumed isoflavones (Ingram et al., 1997), while there were also observable declines in the density of the mammary glands (Atkinson et al., 2004) and the proliferation of mammary gland cells (Palomares et al., 2004). This may be due to the effects upon the sex hormone binding globulin concentration exerted by the isoflavones, leading to lowered sex hormone bioavailability in tissues that depend upon those hormones (Kurzer, 2002). Furthermore, in the tissues around the periphery enzymes which promote cell proliferation, such as tyrosine kinase, can be inhibited by isoflavones (Gerosa et al., 1993; Blair et al., 1996), while estradiol availability is also reduced as a consequence of the inhibition of aromatase P450 (Kao et al., 1998).

In healthy females, studies have revealed that women using isoflavones exhibit lower rates of cancer associated with estrogen. One Japanese study showed that a diet containing soybeans would lead to a reduced incidence of breast cancer in younger women, but after menopause, there was no benefit recorded (Hirose et al., 2005). Research conducted over a three-month period showed that while minor ultrasonographic breast alterations could be observed following the consumption of soy isoflavones, the changes were shown to be sub-clinical and required no further attention upon completion of the study (Alipour et al., 2012).

Chen et al. (2014) conducted a meta-analysis examining the findings of 35 previous investigations concerning the correlations between the intake of soy isoflavone and the risk of developing breast cancer. The analysis showed that soy isoflavone reduced that risk for Asian women both pre- and post-menopause. In Western countries, however, no evidence of such a link could be found to support a similar conclusion in either pre- or post-menopausal females. Published data were sought from PubMed, Embase and the Cochrane Library with the focus on prospective cohort studies assessing the influence of isoflavone in the diet upon subsequent breast cancer onset. The data analysis included 16 research studies, comprising 648,913 sample participants and 11,169 cases of breast cancer. It was demonstrated by Zhao et al. (2019) that there was no change in breast cancer risk when women consumed soy-based foods in moderation, while a higher intake was linked to a lower level of breast cancer risk. The meta-analysis indicated that females whose dietary consumption of soy-based foods was high could enjoy a statistically significantly lower risk of developing breast cancer.

Prostate cancer cell growth can be inhibited by genistein and biochanin A at a high dosage (Peterson & Barnes, 1993), while isoflavones brought about a reduction in the prostate weight and volume in rats (Lund et al., 2001; Fritz et al., 2002). Meanwhile, men have been shown to exhibit reduced rates of prostate cancer when greater plasma concentrations of genistein are present (Travis et al., 2009).

Effects of Soy Isoflavone on Osteoporotic Bone Loss

Osteoporosis is a condition that results in bone loss. Its effects are amplified with age and it is found in both males and females, although older women are more significantly affected due to the decline in estrogen levels after the menopause. In around 30% of elderly females, bone loss causes severe orthopedic difficulties (Zheng et al., 2016; Bone et al., 2000). In 2001, the first reports were published concerning the potential benefits of isoflavones in this context (File et al., 2001). Whether menopause is surgical or natural, it is followed by a rapid bone loss in the early stages, whereupon further skeletal decline tends to subsequently occur more gradually (Gallagher, 1990). Several studies have shown that soy isoflavones have a direct influence on certain conditions affecting the bones, with one metaanalysis finding a notable increase in bone mineral density of up

to 54% as a result of the influence of soy isoflavones, while urinary deoxypyridinoline, which is a bone resorption marker, declined by as much as 23% in women in comparison to the baseline. The influence of soy isoflavones on the density of bone minerals and deoxypyridinoline was demonstrated to be significant via sensitivity analysis (Wei et al., 2012; Nielsen et al., 2004; Seeman, 2004). When post-menopausal females received genistein over a period of six months, the outcome was a notable rise in bone density along with the appropriate decline in the concentration of biochemical bone resorption markers (Turhan et al., 2008). At twelve months of treatment, the bone density reached levels similar to those expected following estrogen hormonal replacement therapy (Potter et al., 1998; Morabito et al., 2002). Furthermore, the adverse consequences of estrogen therapy were not observed with treatment using isoflavones (Cornwell et al. 2004). It was noted by Polkowski & Mazurek (2000) that there may be two mechanisms that mediate the influence of isoflavones on bone metabolism. These are the effects on osteoclasts when apoptosis occurs and tyrosine-kinase activity inhibition which takes place via the modulation of membrane estrogen receptors (ERs) as consecutive changes arise in alkaline phosphatase activity. Based on this idea, it was argued by Blair et al. (1996) that when a genistein concentrate was used to wash cell osteoclast cultures, the tyrosine-kinase activity declined, followed by lower levels of bone remodeling.

Effects of Soy Isoflavone on Cognitive Function

It is believed that soy isoflavones might have a positive effect on cognitive function since they are phytoestrogens, offering activity similar to that of estrogen (Cui et al., 2019). In preclinical research, soy isoflavones have proven valuable in the removal of amyloid beta and reduction of tau phosphorylation, thereby addressing a number of diseases with pathologies similar to that of Alzheimer's (Bonet-Costa et al., 2016). In addition, the mitochondrial apoptotic pathway can be inhibited due to the anti-inflammatory and antioxidative effects of soy isoflavones, thus preventing Alzheimer's disease (Ye et al., 2017; Wang et al., 2016). Studies in animals confirm the ability of isoflavones to enhance cognitive function, while research in humans has also reported cognitive benefits derived from the use of soy isoflavones (Ozawa et al., 2013; Okubo et al., 2017; Nakamoto et al., 2018). Over the past twenty years, randomized controlled trials have been used to examine cognition function in the context of soy isoflavones, although the findings have lacked consistency (Henderson et al., 2012; File et al., 2005; Duffy et al., 2003; Fournier et al., 2007). Systematic reviews have also failed to report consistent patterns in the effects on cognition (Thaung-Zaw et al., 2017; Sumien et al., 2013). A meta-analysis was carried out by Cheng et al. (2015) concerning the influence of soy isoflavones upon females after menopause, finding positive effects on cognition. However, when males or younger females were investigated, the results which were indicative of positive outcomes for cognition were omitted from the overall analysis (Gleason et al., 2009; Thorp et al., 2009; File et al., 2001). Not all trials which involved postmenopausal females were included and the analysis also included research involving red clover, which has soy isoflavone content of only around 2% (Woo et al., 2003; Maki et al., 2009).

Effects of Soy Isoflavones on Cardiovascular Disease

In Western nations, cardiovascular disease is a leading cause of death, whereas Asian countries have a far lower incidence of heart disease (Beaglehole, 1990). While hereditary factors play a role, it is believed that nutrition may explain the huge difference between the regions. The Asian diet makes significant use of soy products, ensuring high levels of soy isoflavone consumption in Asia. It may therefore be the case that isoflavones can protect the cardiovascular system through various mechanisms (Adlercreutz, 1990; Anderson et al., 1999; Yamori, 2006). These processes are not yet understood in detail, however. Earlier research has revealed that isoflavones are able to support the amelioration of systemic arterial compliance in females undergoing menopause and those in the postmenopausal phase, albeit without affecting plasma lipids (Nestel et al., 1997). Meanwhile, research carried out in 2007 involving the use of 60 mg isoflavone daily for three months showed no significant difference in lipoprotein levels (Cheng et al., 2007). It is therefore likely that the preventive influence of isoflavones on cardiovascular disease may arise through alternative mechanisms (Brzezinski & Danenberg, 2008). For instance, daidzein and genistein release nitric oxide which causes the arteries to relax (Brzezinski & Danenberg, 2008). Even so, Wong et al. (2012) revealed no significant difference when blood pressure measurements were taken following

6 weeks of daily soy isoflavone use (80 mg). Animal studies, however, showed that in vessels with a preceding change of atherosclerosis, both soybeans and HRT bring about adverse consequences, with neither treatment capable of lowering the rates of myocardial ischemia or reperfusion injuries in either ovariectomized or diet-induced atherosclerotic monkeys. In the case of local ischemia, it has been shown that combining HRT with soybeans can increase myocardial changes (Suparto et al., 2008). It is important to note, however, that animal studies must be considered with care before concluding that human females should avoid soybean consumption following incidences of cardiovascular events. Further study would be necessary in order to make more reliable inferences (Brzezinski & Danenberg, 2008).

Effects of Soy Isoflavones on Type-2 Diabetes

T2D, or type-2 diabetes, poses a significant challenge for healthcare providers around the world. It leads to chronic insulin resistance along with the loss of functional pancreatic β-cell mass (Garg et al., 2016). A number of researchers have found that among the qualities of genistein is its antidiabetic activity, which arises from its interactions with β-cell proliferation, glucose-stimulated insulin secretion, apoptosis prevention and capacity for serving as an estrogen receptor (ER) agonist, antioxidant and inhibitor of tyrosine kinase (Gilbert & Liu, 2013). Although few studies have gathered data concerning the influence of genistein in human subjects with diabetes, animal studies and examinations of cell culture have shown that there is a direct effect upon β-cells exerted by genistein in concentrations that might arise in the body (<10 μM) (Gilbert & Liu, 2013; Wang et al., 2013). Furthermore, it has been observed that type-2 diabetes is less likely to develop in the presence of fermented soy products when compared to the effects of the unfermented version (Kwon et al., 2010). Meanwhile, Wagner et al. (2008) reported that the introduction of isoflavones can increase the insulin response to glucose challenge, as well as lower the expression of peroxisome proliferator-activated receptors (PPARs), which regulate the expression of genes. In addition, the plasma adiponectin concentration is also reduced in male monkeys, depending on the dosage. There was also an increase in adenosine monophosphate protein kinase (AMPK) activation due to soy isoflavones and this acts to regulate lipid and glucose metabolism, thus reducing the likelihood of type-2 diabetes (Stallings et al., 2014).

Effects of Soy Isoflavones on Symptoms of Menopause

For females undergoing the menopause, the symptoms which cause the greatest discomfort include sweating, hot flushes and palpitations, or vasomotor symptoms (VMS). Women suffering from such symptoms will often seek treatment (Chen & Chen, 2021). The work of St Germain et al. (2001) showed that females who consumed soybeans containing isoflavones for 24 weeks experienced decreased levels of hot flush, although this was also true of women who received soybeans without isoflavones and those who received whey proteins, so the placebo effect was apparent. The effect of the soy isoflavones is to enact the competitive inhibition of the enzyme 17-hydroxysteroid oxidoreductase (type 1), which plays the role of converting inactive estrone to significantly more active estradiol (Biniwale et al., 2022). Endothelial nitric oxide synthase (eNOS) transcription is activated when soy isoflavones bind to estrogen receptors, ultimately causing eNOS synthesis and the production of nitric oxide (NO). Heightened NO production permits the cutaneous dissipation of heat via vasodilatation. Accordingly, isoflavones are able to mitigate the problematic vasomotor symptoms (Hairi et al., 2019).

A systematic review was carried out by Jacobs et al. (2009) covering 17 randomized, placebo-controlled trials examining hot flushes and the influence of soy isoflavones. It was found that hot flushes could be reduced although consistency was not reported. There was no meta-analysis involved due to the similarity of the studies and their poor overall quality. Meanwhile, Bolanos et al. (2010) performed a meta-analysis making use of placebo-controlled randomized controlled trials which showed that vasomotor symptoms could be reduced using soy isoflavones when compared to the placebo. The systematic review by Taku et al. (2012) reached similar conclusions from the examination of 19 trials assessing the effects of synthesized soybean isoflavones as supplements that were able to reduce the incidence and strength of hot flushes. Finally, it was reported by Tranche et al. (2016) that 50 mg of soy milk isoflavones could bring about a significant decline in vasomotor symptoms for females undergoing menopause when receiving treatment for 12 weeks.

Soy Isoflavone Dosage and Health Risks

Isoflavones have long been considered a safe dietary ingredient throughout Asia, with research indicating that three-quarters of all Asians have an average daily consumption level of 65 mg of isoflavones. It can be concluded from this evidence that there are no adverse consequences to isoflavone consumption in the short term and that isoflavones are safe for human consumption (Lee & Kim, 2007; Kim, 2021).

One important consideration, however, is the potential long-term influence of isoflavones, as this may be of concern for health reasons as the outcomes are not immediately apparent. The work of Chen & Rogan (2004) argued that there are adverse consequences associated with isoflavones, having shown that the use of soy-based infant formula can hinder the long-term development of children. Exposure to genistein over the longer term can facilitate the growth of cells causing breast cancer and tumors associated with soy protein isolate show a tendency to be non-regressive, instead of growing aggressively (Andrade et al., 2015).

The dosage is another factor that can affect the health risks associated with isoflavone consumption, as one recent meta-analysis revealed that for women in Korea, the risk of cancer recurrence in epidermal growth factor receptor-2 (HER-2)-positive breast cancer is heightened due to an elevated intake of soy isoflavones (Woo et al., 2012). Tests in mice showed that genistein supplements at a high dosage of 150 mg/BW throughout a gestation period of 21 days will result in damage to the reproductive organs of the immediate offspring from the weanling stage (Lofamia et al., 2014).

It can be argued that the quantities of isoflavones typically consumed in the Asian diet are appropriate, based on the US FDA daily recommended intake of 50 mg (Chen et al., 2019; Chen & Chen, 2021). In terms of health risks, the consequences of isoflavone consumption remain a matter for ongoing debate and research and it should be taken into consideration that this study has mentioned only positive outcomes. There have not, however, been any reports of side effects of a severe nature as a consequence of isoflavone consumption to date.

Conclusion

This research examined the role of soy isoflavones in addressing a number of health conditions and diseases. The isoflavones obtained from soybeans offer anti-tumor, antioxidant, anti-inflammatory and anti-menopausal properties and can delay the process of osteoporosis. Furthermore, they can improve the memory and learning abilities of females undergoing menopause and support the management of cancer, cardiovascular disease and diabetes. Having assessed these health conditions in the context of isoflavones, it can be concluded that the phytoestrogen and antioxidant properties are the principal factors driving the relevant mechanisms. However, while soy isoflavones are understood to deliver notable health benefits when used to prevent or treat certain diseases, concerns have been raised about the potential for adverse reactions. It will therefore be necessary to conduct further studies to assess in detail the role of isoflavones in disease management, whether used independently or as part of a regimen involving combination with other treatments.

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7. The revised version is granted the University's recognition of "Accepted" for publication status with the Journal of Food Health and Bioenvironmental Science Stamp on every page. Information regarding publication status (Accepted) is located on the journal's website (http/ research dusit ac.th/new/e-Journal)
8. The editorial tearm conducts an accuracy check for all articles before sending the manuscripts to the printer to create a draft journal issue.
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Publication Criteria

1. The original manuscript is concise and interesting to the academic community.
2. The content of the manuscript represents quality and theory of the discipline and also possesses knowledge with practical applications.
3. The manuscript's content is consistent with the aim and scope of the journal.

4. Manuscripts submitted to Journal of Food Health and Bioenvironmental Science must not have been published previously in or actively involved in the publication process of another journal.

5. All content within the manuscript must be the product of the author himself. Any use of intellectual property within must be appropriately credited to its original authors.

6. The author must comply with the writing style established by Journal of Food Health and Bioenvironmental Science.

7. There are four levels of assessments given to reviewed manuscripts:

7.1 Requires minor or no revisions prior to publication.

7.2 Requires moderate revisions prior to publication.

7.3 Requires intensive editing and revisions followed by a future evaluation. 7.4 Unsuitable for publication

In order to be assigned the "Accepted" status, an article must be assessed as "Requires minor or no modification prior to publication" by two of the three experts from the peer review process.

Formatting Guidelines

It is the author's responsibility to format manuscripts to the standards of Journal of Food Health and Bioenvironmental Science. The details of format style are contained herein,

1. Format

1.1 Single page printing on A4 paper with a width of 19 cm and height of 26.5 cm. The vertical and horizontal spacing from the margins must be 3.5 cm and 2.5 cm, respectively.

1.2 Typefaces and layout: English must be typed using Time New Roman using Microsoft word. Specific font format guidelines are as follows.

1.2.1 The header contains the page number, aligned on the right side, in 12 pt. font.

1.2.2 The title in English languages must be 12 pt. font, bolded, and center aligned. The title should not exceed two lines of text.

1.2.3 The author's name in English language must be typed 9.5 pt. font and centered below the title. Asterisks (*) should proceed the authors' names which is correspond to the appropriate author.

1.2.4 Affiliations should match each author with their appropriate affiliated institutions and organizations. In case of different affiliations, superscript numbers should follow the surname a and affiliation a.

1.2.5 A footnote must be placed on the first page of the article with the text "*Corresponding Author", and the next line of text should contain "e-mail".

1.2.6 "Abstract" in English must be 9.5 pt. font, bolded, left aligned, and placed below the Thai keywords section. Abstract text must be 9 pt. font, with 1 tab indentation from left and right margins.

1.2.7 "Keywords:" should appear in English language in 9.5 pt. font, placed beneath the English abstract text and be aligned with the left margin. English keywords must be 9 pt. font, and should not exceed four words. Each keyword should be separated by a comma (,) and space.

1.2.8 Regardless of language choice, the main text headings used throughout the paper must be 9.5 pt. font, bolded, and aligned with the left margin.

1.2.9 Bulleted items must appear as 9 pt. font, bolded, and be indented 1.5 tabs from the left margin.

1.2.10 Body text must appear as 9 pt. normal font, and be indented 1 tab from the left and right margins.

1.2.11 "References" must be 9.5 pt. font, bolded, and be aligned with the left margin. Individual entries must be 9 pt. font and should follow American Psychological Association (APA) formatting guidelines. Any lines of text for a single entry that exceed the first line should use a "hanging indent" of 1.5 tabs from the left margin.

1.3 An appropriate page length for publication in the Journal is approximately 15 pages.

2. Citing

Should follow American Psychological Association (APA) formatting guidelines. Click http://jfhb.dusit.ac.th/flie/Ref%20Guidelines. pdf to see the example.

3. Ordering of Titles in Journal of Food Health and Bioenvironmental Science

The written manuscript may contain only English. The content should be easy to understand and clear. If the author uses abbreviation, full word must appear before any abbreviation.

3.1 The title should be brief, the length should not exceed 100 characters.

3.2 The authors if there are more than six authors only the first author is listed, followed by "et al."

3.3 Affiliated entities associated with the author should appear in English languages.

3.4 The abstract must be written in English language. The abstract should briefly summarize the research and not exceed 250 words or 15 lines of text.

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3.6 The "Introduction" section should provide background information relevant to the research, provide information regarding the manuscript's content and state the objectives of the work.

3.7 The "Materials and methods" section delineates the procedures, how the research was conducted, sampling method (i.e. simple random samples) and population, and the creation and development of research tools used for data collection and analysis.

3.8 The "Results" section or "Results and Discussion" presents data obtained during the research and may be displayed as tables, graphs, illustrations, and accompanying explanations. Tables should be not have left and right borders and are normally black and white printed. No more than five tables should be present in the "Results" section. Pictures within the section should be clear and use simple black and white coloring with an accompanying caption, the author wishes to use colors for any item they may do so; however, the author will be responsible for the additional costs of color printing.

3.9 The "Discussion" section or "Result and Discussion" should explore the significance of the results of the work and address whether or not the data support the research hypothesis and compare research findings to other similar research works.

3.10 The "Conclusions" section should summary of the main topic covered or a re – statement of the research problem.

3.11 The "Acknowledgements" (if any) section should provide help during the research (e.g., providing materials, laboratory, equipment, etc.) and funding.

Sending Original manuscript

1. Compose the manuscript using the format of the Journal of Food Health and Bioenvironmental Science.

2. Send the manuscript via ScholarOne website https://mc03.manuscriptcentral.com/jfhb

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Author Regulations

- The authors should write the manuscript related to the theme of Food, Health, biological and environmental disciplines. The research manuscript should contained relevant background information, efficient methodology, APA style citation, accurate results, and reasonable discussion.
- The authors should follow the journal guidelines strictly.
- Any opinion or perspective made in the manuscript must be explicitly highlighted as "opinion" or "perspective"
- The authors must be careful and aware that fraudulent information and omission of important information are unethical author behaviors.
- The authors must be able to provide research data if the Editor see needed.
- Authors must reference other works properly. Any work involved in the manuscript also must be well credited.
- The authors must make sure that the manuscript has not been published elsewhere before and is not currently in the publication process in other journals.

- The person must have made significant contributions to the manuscript, participate and give important efficient content during revisions and provide approval for publication in order to be listed as an author. Researchers who do not meet the above criteria should be listed in the Acknowledgements section.
- Author should identify any conflicts of interest that might have influenced the data and/or interpretations of data.
- To make the efficient revision, the authors should respond to all the given critiques and suggestions during the revision.
- If the authors find errors in their works that need to be correct, the author should inform the editors immediately.

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