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Effects of different plant parts and solvents on bioactive compounds and antioxidation in large fruit Bird's eye chili (*Capsicum annuum* L. cv. Superhot)

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Abstract

The production chain is impacted by an overabundance of large fruit Bird's eye chili (*Capsicum annuum* L. cv. Superhot) on the market. Another way to make the most of the potential of raw materials for product development is to supplement the data on phytochemicals and antioxidative activity by segmentation (fruit, placenta, seed, and pericarp). In an experiment, distilled water, 60% ethanol and 90% ethanol were used as the primary solvents. The outcomes demonstrated that placenta and seeds using 90% ethanol as a solvent (PS-Et90) contained capsaicin, total phenolics, and total flavonoids at their most outstanding levels (16.30 µg/mg, 44.65 µg GAE/mg, and 21.77 µg CE/mg, respectively). At the same time, the Scoville Heat Unit was high at 244,550 SHU (very high pungency). Additionally, it demonstrated the highest antioxidative activity (129.57 µg TE/mg) when tested with the ABTS method, and total flavonoids were discovered to enhance the antioxidation effect when measured by ABTS and FRAP methods. PS-Et90 had a significantly different potency than the other groups when the extracts were grouped using Agglomerative Hierarchical Cluster Analysis based on phytochemical data and antioxidant activity. Therefore, the placenta and seeds appear to be preferable over others when utilizing large fruit Bird's eye chili for pharmacological purposes. Using 90% ethanol as a solvent will provide the best bioavailability.

1. Introduction

Large fruit Bird's eye chili (*Capsicum annuum* L. cv. Superhot) is a commercial species of pepper found throughout Thailand. The primary cultivation sources are in the Northeast, though they are partially distributed in the northern, eastern, central, and southern regions as well with a plantation area of 11,908.96 hectares (accounting for 55.61% of the chili planting area nationwide) and an annual yield of 138,652 tons (accounting for 55.09% of the total output) (Kraikruan, 2015; Sukhawatthanakun, 2022). However, the selling price of large fruit Bird's eye chili has dropped considerably in the past three years (2019 - 2021) due to oversupply in the market (Barean of Agricultural Commodities Promotion and Management, Department of Agriculture, 2022). Large fruit Bird's eye chili is mostly used fresh or dried and processed into chili sauce or other flavoring products. However, its higher spiciness compared to commercial chili, either raw, ripe, or dried (Thanawiroon and Homhual, 2011) makes it an interesting ingredient for use in pharmacological products. The bioavailability of large fruit Bird's eye chili, including the fruit, placenta and seed, and pericarp, is expected to help provide information on its potential use as a raw material.

Capsaicinoids are compounds in the phenolic family that give a pungency taste; they are distributed in the fruits, seeds, placenta, and pericarp, but are most common in the placenta. The most common type of capsaicinoid is capsaicin, which contains approximately 70% of the whole substances, followed by dihydrocapsaicin, nordihydrocapsaicin and homodihydrocapsaicin, and homocapsaicin (Castro-Munoz *et al.*, 2022). Capsaicin is used directly to determine the spice level, interpreted via the Scoville Heat Unit (SHU) (Hoffman *et al.*, 1983). Capsaicin has outstanding pharmaceutical properties, especially anti-

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inflammation, anti-cancer cell proliferation, antimicrobials, and antioxidation. Therefore, chili peppers are now used as an ingredient in gels, lotions, and plasters to relieve muscle pain and arthritis because they can reduce the amount of substance P, a neurotransmitter. As a result, capsaicin decreases the sensation of nerves, thereby reducing pain (Bureau of Drug and Narcotics, Department of Medical Sciences, 2020). Capsaicin also reduces the risk of cardiovascular and heart disease, such as by lowering insulin synthesis for diabetics (Sharma *et al.*, 2013; Vera-Guzmán *et al.*, 2017; Valim *et al.*, 2019; Wang *et al.*, 2022).

Although hexane is the best solvent for capsaicin, the commonly used solvents for extracting capsaicin from chili peppers are methanol, ethanol, acetonitrile, and distilled water because they are safer than hexane. Moreover, hexane is a dangerous chemical that often forms other product substances during extraction. However, capsaicinoid recovery by the replacement solvents was suitable at about 70 to 92% (Castro-Munoz *et al.*, 2022). Extraction techniques are also involved. It was reported that the capsaicin extraction with ethanol using the Soxhlet technique for 5 hrs had the highest recovery rate compared to Ultrasound-assisted extraction for 3 hrs and maceration for 15 hrs, based on percentages of recovery at 92, 87, and 79%, respectively (Boonkird *et al.*, 2008).

For future pharmaceutical utilization, we extracted large fruit Bird's eye chili under different solvent conditions through plant part separation. Subsequently, we evaluated the level of capsaicin, pungency, phenolics, flavonoids, and antioxidation in each extract.

2. Materials and methods

2.1 Chemicals

Folin-ciocalteu's phenol, gallic acid, catechin, Trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma-Aldrich, St. Louis, USA. Purified capsaicin used Tokyo Chemical Industry Co., LTD, Tokyo, Japan. The chemicals used in the study were quality grades for analysis.

2.2 Materials preparation and extraction

Large fruit Bird's eye chili was grown from November 2021 to January 2022 under an organic cultivation system at Oumrak Supplier Social Enterprise Co., Ltd, Nakhon Ratchasima province, Thailand. Only mature fruits without insect or microbe disruption were sampled, cleaned, got rid of peduncles, and separated into three group samples, whole chili fruits without peduncles (WF), placenta and seeds (PS), and chili pericarp (CP) (Figure 1). After that, samples were dried at 65°C, ground to fine powder, and kept under -20°C in tight containers for further extraction. As with the extraction solvents, distilled water (Dw), 60% ethanol (Et60), and 90% ethanol (Et90) were used for continuous Soxhlet extraction for 5 hrs. After extraction, the extracts were centrifuged at 4,000 rpm, and then the supernatants were filtrated and evaporated solvents by using a rotating vacuum evaporator. Crude extracts were converted to powders by freeze-dry method and kept at a cool temperature until the study.

Figure 1. Large fruit Bird's eye chili: (a) fresh fruit, (b) whole chili fruits without peduncles, (c) placenta and seeds, and (d) chili pericarp.

2.3 Capsaicin analysis

Capsaicin measurement was carried out on High-performance liquid chromatography (HPLC) at conditions of column VertisepTM GES C18 (300 × 4.6 mm × 5 µm), equilibrated column temperature at 40°C, mobile phase: acetonitrile/1% acetic acid, 1:1 v/v, flow rate: 1 mL/min, and UV detection at 280 nm. The standard solutions were generated to chromatograph, and the standard curve was plotted between the peak areas against capsaicin concentrations. The initial sample concentrations started at 100 mg/L for HPLC analysis, and the obtained peak areas were compared with the standard curve. The results of capsaicin contents were re-calculated to Scoville Heat Units (SHU) according to Hoffman *et al.* (1983), SHU = capsaicin (%Dry weight) × 150,000. The identification of pungency levels followed by Scoville (1912), 0 - 700 SHU is non-pungent, 700 - 3,000 SHU is mildly pungent, 3,000 - 25,000 SHU is moderately pungent, 25,000 - 70,000 SHU is highly pungent, and > 80,000 SHU is very highly pungent.

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2.4 Total phenolic analysis

Total phenolic measurement followed Folin-ciocalteu's phenol test (Singleton *et al.*, 1999). The test started with pipetting 150 µL of extract sample solution, then adding 700 µL of distilled water and 150 µL of Folin (Folin-ciocalteu's phenol reagent: ethanol, ratio 1:1 v/v). The solution was mixed and left for 6 min. After that, 2 mL of 2% sodium carbonate solution was added into the solution, mixed well, and left for another 45 min. Absorbance was measured at a wavelength of 760 nm. The contents of total phenolics were calculated by comparing with the standard curve of gallic acid and interpreted as µg gallic acid equivalent/mg sample (µg GAE/mg).

2.5 Total flavonoid analysis

Total flavonoid measurement followed the Colorimetric test (Jia *et al.,* 1999). The test began with pipetting 250 µL of extract sample, then adding 1.25 mL of distilled water and 75 µL of 5% sodium nitrite solution. Then, the solution was mixed properly and left for 10 mins. After that, 150 µL of 10% aluminum chloride solution was added, mixed well, and left for 5 mins. Five hundred µL of 1M sodium hydroxide was added to the solution, and then the volume was adjusted to 3 mL. Absorbance was measured at a wavelength of 510 nm. The contents of total flavonoids were calculated by comparing with the standard curve of catechin and interpreted as µg catechin equivalent/mg sample (µg CE/ mg).

2.6 Antioxidative activity analysis

2.6.1 DPPH method

DPPH radical scavenging assay was determined following Sanchez-Moreno *et al.* (2003). Initially, 50 µL of sample solution was mixed with 1.95 mL of DPPH solution and left for 45 mins at a dark condition. After that, the mixture was measured absorbance at a wavelength of 515 nm. The median effective concentration (EC_{50}) was calculated from a linear graph equation between the sample concentrations against DPPH inhibitory percentages. Various concentrations of Trolox solutions were practiced similarly to the sample solution for creating a standard curve. Antioxidative potentials of the samples were compared with the Trolox potential and interpreted as µg Trolox equivalent/mg sample (µg TE/mg).

2.6.2 ABTS method

ABTS radical scavenging assay was determined following Re *et al.* (1999). ABTS solution was prepared by mixing 7 mM of ABTS solution with 2.45 mM of potassium sulfate solution at a ratio of 2:1 and then incubated at a dark condition for 14 hrs. The complete ABTS solution was ready to use when the absorbance was read at 1.500±0.05 at a wavelength of 734 nm. The sample was prepared in various concentrations for evaluating EC_{50}. The test began by mixing 150 µL of sample solution with 2.85 mL of ABTS solution and left for another 30 min at the dark condition. After that, the solution was measured absorbance at a wavelength of 734 nm. EC_{50} calculation was done via a linear graph equation between the sample concentrations against ABTS inhibitory percentages. Trolox was used as a standard solution, which was practiced similarly to the samples. The antioxidative activities of samples were compared with the Trolox potential and interpreted as µg Trolox equivalent/mg sample (µg TE/mg).

2.6.3 FRAP method

Ferric reducing antioxidant power was determined following Benzie and Strain (1996). FRAP solution was prepared by mixing 10 mM of TPTZ with 20 mM of ferric chloride solution and acetic acid buffer solution at a ratio of 1:1:10. The sample was prepared in various concentrations for evaluating EC_{50}. Approximately 100 µL of sample solution was pipetted and mixed with 2.9 mL of FRAP solution and left for 30 mins at room temperature. Then, the mixture was measured absorbance at a wavelength of 593 nm. EC_{50} calculation was performed through a linear graph equation between the sample concentrations against ferric reduction percentages. Various concentrations of Trolox solutions were done as same as a sample to obtain a standard curve. The antioxidative activity of samples was compared with the Trolox potential and interpreted as µg Trolox equivalent/mg sample (µg TE/mg).

2.7 Statistical analysis

Results were presented in the form of mean ± standard deviation (SD) for triplicate replication. Two-way analysis of variance (ANOVA), followed by Duncan's test, was used to determine significant differences (p-value < 0.05) in phytochemicals and antioxidative activities. The relationship between the two parameters was obtained from Correlation coefficient analysis (p-value < 0.01). Agglomerative Hierarchical Cluster Analysis was used to categorize extract samples.

3. Results

3.1 Capsaicin

From the results of capsaicin content analysis by the HPLC technique, it was found that the chromatogram of standard capsaicin showed a clear peak during the retention time of 12.193 mins, including chromatography of all extract samples (Figure 2). After comparison with

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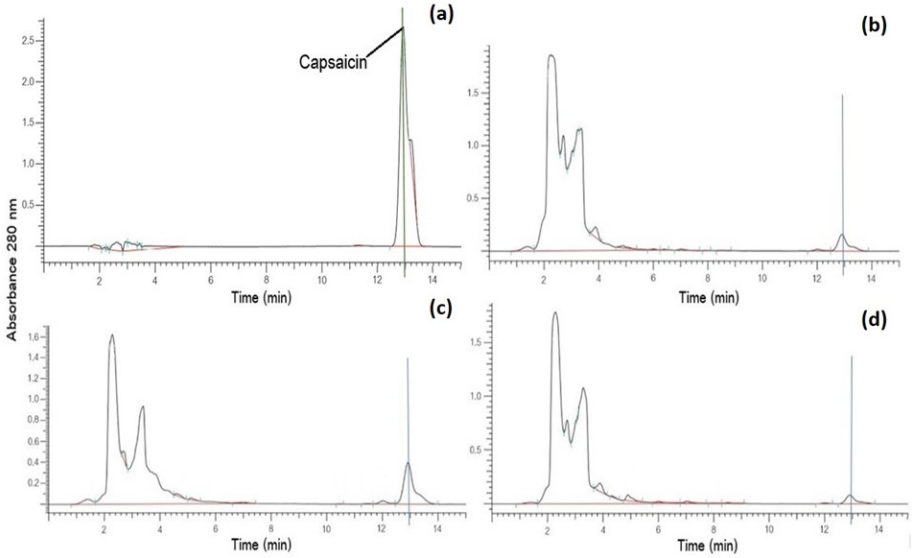

Figure 2. Capsaicin chromatogram from high-performance chromatography: (a) standard capsaicin, (b) whole chili fruit extract, (c) placenta and seed extract, and (d) chili pericarp extract.

the peak area of the standard solution, it was found that PS extracts had the highest statistically significant capsaicin content, especially PS-Et90 (16.30 µg/mg). However, the capsaicin contents lowered when the solvents were changed to 60% ethanol and distilled water (Table 1). The direct extraction of capsaicin from the placenta and seeds was more efficient than the whole chili fruits by about 154.29%. Direct extraction of capsaicin from the chili pericarp was less efficient than extraction from the whole chili fruits (Figure 3). All WF and PS samples using ethanol as solvent had a Scoville Heat Unit > 80,000 SHU, which indicated a very high pungency level; particularly, PS-Et90 had the highest spiciness index (244,550 SHU). The distilled water extract, especially CP-Dw, had the lowest spiciness index (1,450 SHU) and was classified as mildly pungent (Table 1).

3.2 Total phenolics

The analysis of total phenolic compounds found that the solvent types were suitable for dissolving total phenolics from different plant parts. 90% ethanol was suitable for the placenta and seed extraction (44.65 µg GAE/mg). In contrast, 60% ethanol was suitable for whole chili fruit extraction (36.44 µg GAE/mg), and distilled water was suitable for chili pericarp extraction (31.54 µg GAE/mg) (Table 2). The isolated total phenolic extraction did not yield better results than the whole chili fruit extract, except PS-Et90 and CP-Dw (Figure 3).

3.3 Total flavonoids

Based on the total flavonoid content analysis, 60% and 90% ethanol were the best at dissolving total flavonoids out of the placenta and seeds (12.73 and 21.77 µg CE/mg, respectively). However, distilled water was most suitable for extracting flavonoids from chili pericarp (2.15 µg CE/mg) (Table 2). The fractionated extraction method for total flavonoids was appropriate rather than whole chili fruit extraction. Extraction efficiency was the highest when only the placenta and seeds were extracted, and a high ethanol concentration of 90% was used as the solvent (Figure 3).

Table 1. Capsaicin dosages, Scoville Heat Units, and spicy levels of various groups of chili extracts.

Sample	Solvent	Capsaicin (µg/mg)	Scoville Heat Unit (SHU)	Degree of Pungency
WF	Dw	0.40 ± 0.01^{Bc}	6,000	Moderately pungent
	Et-60	5.70 ± 0.11^{Bb}	85,500	Very highly pungent
	Et-90	6.41 ± 0.10^{Ba}	96,150	Very highly pungent
PS	Dw	0.80 ± 0.01^{Ac}	12,050	Moderately pungent
	Et-60	8.00 ± 0.22^{Ab}	120,000	Very highly pungent
	Et-90	16.30 ± 0.45^{Aa}	244,550	Very highly pungent
CP	Dw	0.09 ± 0.01^{Cc}	1,450	Mildly pungent
	Et-60	2.40 ± 0.09^{Cb}	36,000	Highly pungent
	Et-90	3.20 ± 0.05^{Ca}	47,950	Highly pungent

Values are presented as mean±SD. Values with different uppercase superscripts within the same column are statistically significantly different (p-value < 0.05) between groups and values with different lowercase superscripts within the same column are statistically significantly different (p-value < 0.05) within the groups.

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Table 2. Total phenolics and total flavonoids of chili extracts under different types of solvents.

Sample	Solvent	Total phenolics (µg GAE/mg)	Total flavonoids (µg CE/mg)
WF	Dw	24.32±0.94^{Bc}	1.38±0.50^{Bc}
	Et-60	36.44±0.42^{Ab}	6.19±0.54^{Bb}
	Et-90	41.11±0.67^{Ba}	8.50±1.37^{Ba}
PS	Dw	23.09±0.70^{Cc}	1.96±0.31^{Bc}
	Et-60	34.56±0.76^{Bb}	12.73±0.44^{Ab}
	Et-90	44.65±0.64^{Aa}	21.77±0.67^{Aa}
CP	Dw	31.54±0.09^{Ab}	2.15±0.25^{Ac}
	Et-60	35.50±1.66^{ABa}	4.37±0.49^{Cb}
	Et-90	34.37±2.29^{Ca}	5.90±0.49^{Ca}

Values are presented as mean±SD. Values with different uppercase superscripts within the same column are statistically significantly different (p-value < 0.05) between groups and values with different lowercase superscripts within the same column are statistically significantly different (p-value < 0.05) within the groups.

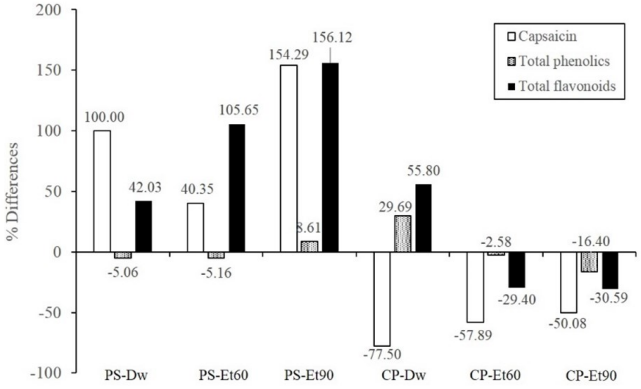

Figure 3. Phytochemicals from placenta and seed (PS) and chili pericarp (CP) compared with the whole chili fruits (WF).

3.4 Antioxidative activity

Antioxidant activities by DPPH, ABTS, and FRAP analyses showed that PS-Et90 demonstrated the highest antioxidant activity at 31.44, 129.57, and 102.81 µg TE/mg (EC_{50} 4.74, 1.14, and 1.28 mg/mL, respectively). Furthermore, these antioxidative activities decreased when the solvent was converted to 60% ethanol and distilled water, except for the chili pericarp extract (CP). Although the ethanol concentration of CP extracts was reduced (from 90% to 60%), the antioxidative activity was not significantly different (p > 0.05). The ABTS method was the most suitable for testing the antioxidant activity in large fruit Bird's eye chili because the antioxidant activity was the highest among all test samples (Table 3). The expression of antioxidant activity in the placenta and seed was particularly prominent when using the fractional approach and the solvent extraction was ethanol. Most of the chili pericarp were unsuitable for fractionation methods because they showed lower

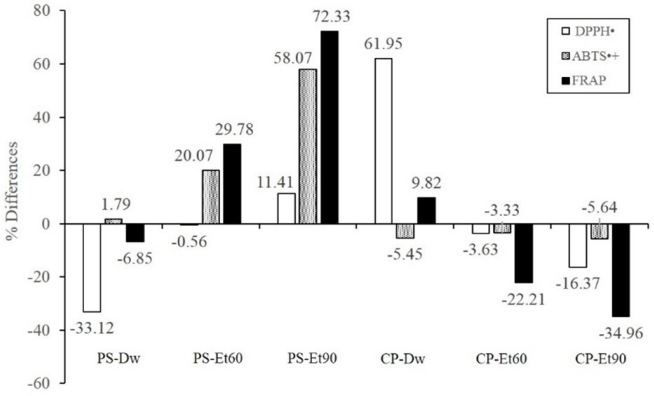

Figure 4. Antioxidative activities from placenta and seed (PS) and chili pericarp (CP) compared with the whole chili fruits (WF).

Table 3. Antioxidative activities found in different groups of chili extracts.

Sample	Solvent	Antioxidant activity (µg TE/mg)		
		DPPH•	ABTS•+	FRAP
WF	Dw	12.59±1.39^{Bc}	75.99±0.77^{Bc}	29.33±0.38^{Bc}
	Et-60	23.14±1.63^{Ab}	79.69±1.64^{Bb}	51.01±1.33^{Bb}
	Et-90	28.22±1.48^{Ba}	81.97±1.21^{Ba}	59.66±1.71^{Ba}
PS	Dw	8.42±2.20^{Cc}	77.35±0.60^{Ac}	27.32±0.77^{Cc}
	Et-60	23.01±0.42^{Ab}	95.68±2.70^{Ab}	66.20±2.67^{Ab}
	Et-90	31.44±0.28^{Aa}	129.57±2.49^{Aa}	102.81±3.23^{Aa}
CP	Dw	20.39±1.11^{Ab}	71.85±1.05^{Cb}	32.21±1.69^{Ab}
	Et-60	23.21±2.13^{Aab}	77.04±0.76^{Ba}	39.68±1.98^{Ca}
	Et-90	23.60±0.97^{Ca}	77.35±1.79^{Ca}	38.80±1.42^{Ca}

Values are presented as mean±SD. Values with different uppercase superscripts within the same column are statistically significantly different (p-value < 0.05) between groups and values with different lowercase superscripts within the same column are statistically significantly different (p-value < 0.05) within the groups.

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Table 4. Correlation matrix between phytochemicals and antioxidative activities of chili extracts.

	Capsaicin	Total phenolics	Total flavonoids	DPPH•	ABTS•+	FRAP
Capsaicin	1					
Total phenolics	0.80*	1				
Total flavonoids	0.99*	0.77*	1			
DPPH•	0.76*	0.96*	0.74*	1		
ABTS•+	0.95*	0.62*	0.95*	0.58*	1	
FRAP	0.99*	0.82*	0.98*	0.77*	0.94*	1

*means the correlation of two variables in the matrix is statistically significantly correlated ($p < 0.01$).

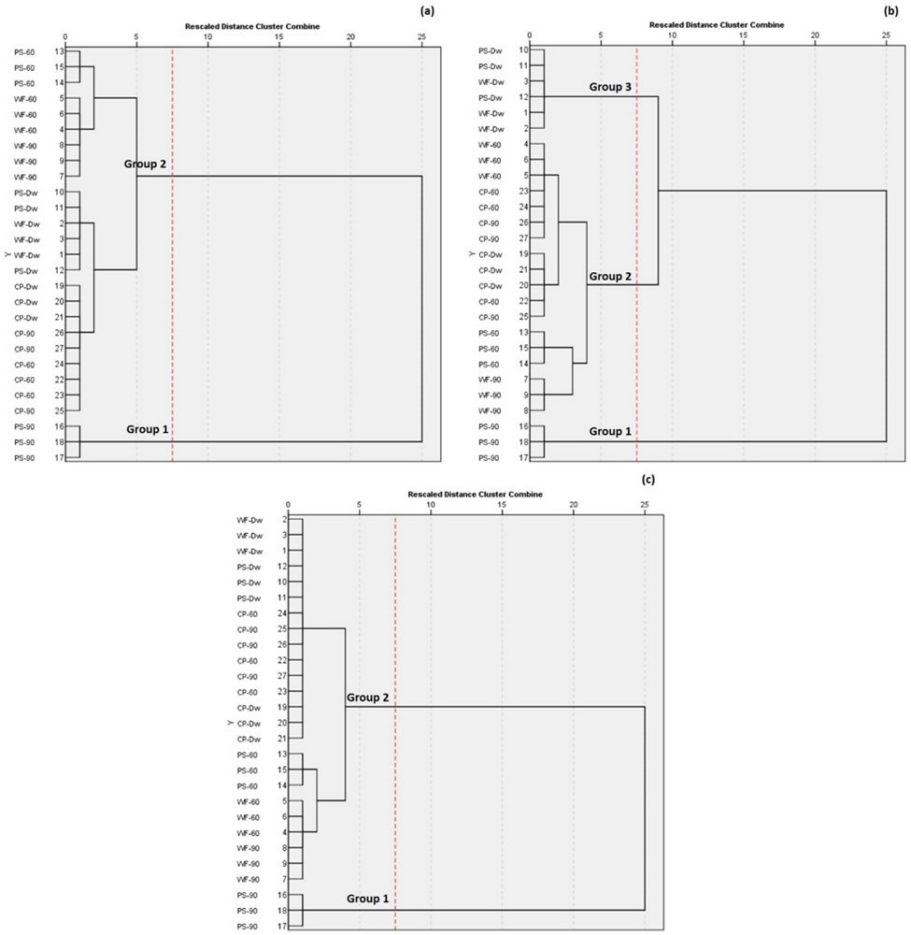

Figure 5. Dendrogram from Agglomerative Hierarchical Cluster analysis based on (a) all parameters, (b) capsaicin, total phenolics, and total flavonoids, and (c) antioxidative activities.

antioxidant activity than whole chili fruit extraction (Figure 4).

3.5 Correlation analysis

The correlation analysis revealed that phytochemicals and antioxidative activities were significantly and positively correlated ($p < 0.01$). In particular, capsaicin, total flavonoids, and antioxidative activities from ABTS and FRAP analyses showed correlation coefficients close to 1 ($R \geq 0.94$). The presence of total phenolics and antioxidative activities tested by the DPPH method also showed a similar result ($R = 0.96$) (Table 4).

3.6 Agglomerative hierarchical cluster analysis

The analysis of the extract grouping with Agglomerative Hierarchical Cluster Analysis found that using all parameters or specific data on the antioxidative properties gave the same clustering results. As a result, the classification could categorize the extracts into two groups: group 1 consisted of PS-Et90, and group 2 consisted of all remaining extract samples (Figures 5A and 5C). When using the specific properties of capsaicin, total phenolic, and flavonoids, it was found that the extracts could be sorted into three groups: group 1 consisted of PS-Et90, group 2 consisted of WF-Et60, WF -Et90, PS-Et60, CP-Dw, CP-Et60, and CP-Et90, and group 3 contained WF-Dw and PS-Dw (Figure 5B).

4. Discussion

Capsaicin was the primary compound in capsaicinoids that were synthesized approximately 20 days post-anthesis by the regulation of the Pun1 gene and EST or AT3-type cofactors (Usman *et al.*, 2014) and

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deposited in the placental epidermis (Stewart Jr. *et al.*, 2005). Therefore, the placenta and surrounding regions are often found to have a higher amount of capsaicin than other structures, such as those found in this study, where capsaicin contents in PS extracts were the greatest. Furthermore, cultivars and physiological metabolisms (the timing of fruit ripening) also affect capsaicin contents. It was found that the amount of capsaicin in the large fruit Bird's eye chili increased when the fruit was ripe, and decreased when the fruit was dry (3.88 > 3.72 > 3.24 mg/g dry weight, respectively). Meanwhile, Thongdam peppers, which are also commercialized in Thailand, were found to have higher capsaicin content in raw fruit than dried and ripe (2.79 > 2.77> 2.35 mg/g dry weight, respectively) (Thanawiroon and Homhual, 2011). At the genotype level, it was also found to influence capsaicin content. The genotypes of Mexican chili peppers AVPP9703, AVPP0512, AVPP0307, AVPP0803, and AVPP0102 were at the non-pungent level (0 SHU) because capsaicin contents were not found, while the genotypes AVPP0705, AVPP0506, AVPP0104, AVPP0002, C05573, and AVPP0805 were very highly pungent (> 80,000 SHU), especially the AVPP0705 genotype with a very high capsaicin content of 1.49% (237,245 SHU). Therefore, AVPP0705 is not recommended for use in the pharmaceutical industry because the capsaicin content exceeds the British Pharmaceutical Codex standard of 0.5 – 0.9%, which could harm consumers (Usman *et al.*, 2014). For pain relief products sold in Thailand, capsaicin doses are usually listed in three levels according to drug potency: 0.0125, 0.025, and 0.075%, of which 0.025% is the preferred concentration used in products (Bureau of Drug and Narcotic, Department of Medical Sciences, 2020). In this study, the amount of capsaicin was in the range of 0.04 – 1.63%, which was enough concentration for an ingredient in pharmaceutic products (except CP-Dw extracts). However, PS-Et90 could offer concentrated capsaicin for cost-effective production, while others such as whole chili fruits and pericarp could not because of including no capsaicin reservoirs. Nevertheless, chili pericarp was a reservoir of other vital substances, such as glycosylated compounds and terpenoids (Cervantes-Hernández *et al.*, 2019).

Flavonoids are classified as nutraceuticals. The most common types of chili peppers are quercetin, luteolin, kaempferol, catechin, epicatechin, rutin, myricetin, and apigenin, though quercetin and luteolin are the major flavonoids in chili peppers (Lee *et al.*, 2006; Vera-Guzmán *et al.*, 2017). Most plant flavonoids combine with sugars in the form of glycosides, but their antioxidant potential by a hydroxyl group (OH^-) is reduced (Rodriguez De Luna *et al.*, 2020). Conversely, these glycosides are more stable and resistant to enzymatic degradation in the digestive system (Xie *et al.*, 2022). Furthermore, the C-glycosides form showed more excellent antioxidant activity than the O-glycosides and aglycones (Xiao *et al.*, 2016). For example, luteolin 6-C-glucoside, which was the main flavonoid isolated from *C. annuum* L. var. Capel Hot, showed potent antioxidant activity against superoxide anions (O_2•-). It also inhibited xanthine oxidase activity, thereby reducing the risk of gout from degrading purin bases into uric acid by xanthine oxidase (Materska, 2015). The chemical structures of flavonoid types also affected solubility. For example, quercetin, isoquercetin, and rutin presented double bonds at the C2-C3 position in the C ring and a torsion angle *q* of -25° that caused insolubility in a low polar solvent such as acetone or acetonitrile (Rodriguez De Luna *et al.*, 2020). Nevertheless, they could become soluble in a highly polar solvent such as ethanol or ethanol and distilled water. In this experiment, distilled water and ethanol were selected as the primary solvents for large fruit Bird's eye chili extraction, which suggested being able to dissolve such vital substances in the flavonoid group. As a result, total flavonoid contents in the ethanol extracts (WF-Et, PS-Et, and CP-Et) were several times higher (4.37 – 21.77 > 1.38 – 2.15 µg CE/ mg) than the aqueous extract (WF-Dw, PS-Dw, and CP-Dw). Moreover, it was found that the flavonoids in large fruit Bird's eye chili were highly and positively correlated with the antioxidative activity as measured by the ABTS and FRAP methods (Table 4). The ABTS method is used to investigate the ability of both polar and non-polar compounds in reducing $ABTS^{•+}$ radicals (Cano *et al.*, 2000), while the FRAP method is used to determine the reducing ability of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) (Antolovich *et al.*, 2002; Shah and Modi, 2015). In the human body, iron is found mostly in the heme of red blood cells in the form of divalent Ferro-iron of hemoglobin (Hb), an oxygen-carrying form. Nevertheless, this image is easily oxidized to trivalent Ferri-iron of methemoglobin (MetHb) under stress conditions or the consumption of high-nitrogen foods. Even though scarce research links *In Vitro* FRAP results and *In Vivo* MetHb prevention because of the complexes of organism metabolisms, the antioxidation analysis in large fruit Bird's eye chili showed a good direction.

Other total phenolics correlated significantly and positively with the DPPH antioxidant results. The highest phenolic compounds were presented in PS-Et90 (44.65 µg GAE/mg), and it illustrated the most excellent DPPH antioxidant at the same location (31.44 µg TE/ mg). Total phenolics from this extract were expected to be water-soluble or lipid-soluble electron donors to DPPH• radicals due to the highlight of the DPPH method (Prior *et al.* 2005). Although this study did not

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differentiate between the placenta and the seed, it was estimated that the total phenolic content in the placenta was higher than in the seeds. The placenta was the source of capsaicinoid generation, which was one of the phenolic compounds. Consistent with Zamljen *et al.* (2021), a separate analysis of seed, placenta, and fruit pericarp in *C. baccatum*, *C. chinense*, and *C. chinense* × *C. frutescens*, found high total phenolics in the placenta of all three pepper varieties. Total phenolics of the three chili species were the highest in the placenta (15.83, 30.04, and 36.07 µg GAE/mg, respectively), with 6.28 – 7.71 times more in seeds and 1.38 – 1.70 times more in fruit pericarp. In addition, the placenta of these three chili peppers also contained many organic acids such as citric acid, ascorbic acid, malic acid, quinic acid, succinic acid, fumaric acid, and oxalic acid, which have a role in antioxidant promotion. Moreover, citric acid was the most common organic acid in the placenta of the three peppers (58.67, 43.01, and 51.06 µg/mg, respectively), followed by malic acid (10.88, 35.09, and 35.16 µg/mg, respectively) and quinic acid (2.49, 11.52 and 14.36 µg/mg, respectively). When comparing the color of chili peppers, it was found that green *C. annuum* contained more total phenolic compounds than red and yellow varieties (30.15, 28.73, and 27.68 mg GAE/g, respectively) and included more incredible organic acids. As a result, the best organic acid found among the three colors was tannic acid (Salamatullah *et al.*, 2022).

The grouping of chili extracts based on phytochemical and antioxidative activity data (Figure 5) confirmed that the extraction location of the plant and the appropriate solvent type strongly influenced the bioactivity of chili extracts. Even when using all parameter data or only antioxidant data or antioxidative activity data in the grouping, it was impossible to combine PS-Et90 with other extract groups because PS-Et90 was outstanding in terms of both phytochemicals and antioxidative activity.

5. Conclusion

The placenta and seeds of large fruit Bird's eye chili were sources of capsaicin, total phenolics, and total flavonoids. They had the highest antioxidative activity compared to whole chili fruits and fruit pericarp. Furthermore, 90% ethanol was the most suitable solvent to extract such active substances compared to distilled water and 60% ethanol. This antioxidative activity in large fruit Bird's eye chili was partly due to the action of capsaicin, total phenolics, and total flavonoids found in chili peppers.

Conflict of interest

The authors declare no conflict of interest.

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