PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol 11(1): 176–185
https://doi.org/10.14719/pst.2693

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RESEARCH ARTICLE

Antioxidant and lipid peroxidation inhibitory potentials of the unripe and ripe of *Citrus mitis* Blanco pulps and peels from south Thailand

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OPEN ACCESS

ARTICLE HISTORY
Received: 23 June 2023
Accepted: 04 September 2023
Available online
Version 1.0 : 15 October 2023
Version 2.0 : 01 January 2024

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Wanna C, Boonman N, Phakpaknam S. Antioxidant and lipid peroxidation inhibitory potentials of the unripe and ripe of *Citrus mitis* Blanco pulps and peels from south Thailand. Plant Science Today. 2024; 11(1): 176–185. https://doi.org/10.14719/pst.2693

Abstract

Citrus mitis fruit contains high levels of vitamin C, phenolics, and flavonoids, which exhibit a wide range of beneficial biological effects. The current research aimed to evaluate the levels of vitamin C, flavonoid, total phenolic contents, and antioxidant properties, as well as the inhibitory effects on lipid peroxidation, in both unripe and ripe fruit tissues (pulps and peels) of *C. mitis*. The fruit tissues were obtained from the Hat Som Paen subdistrict in Ranong province, located in the southern region of Thailand. Fruit tissues were extracted using maceration with 80% ethanol and water solvents. Vitamin C quantification was achieved using the 2,6-dichloro-indophenol titrimetric method. Total flavonoid content was determined using the aluminium chloride colorimetric method, while total phenolic content was assessed using the Folin-Ciocalteu procedure. Additionally, ABTS and DPPH assays were used to assess the antioxidant properties. The measurement of lipid peroxidation inhibitory activity was carried out using the thiobarbituric acid reactive substances (TBARS) assay. The findings indicated that the water extract exhibited higher levels of vitamin C compared to the 80% ethanolic extract. In contrast, the 80% ethanolic extract revealed greater amounts of total phenolic and flavonoid contents than the water extract. The water extract of unripe pulp exhibited the highest vitamin C content among all the extracts, reaching 36.74 mg/100 g fresh weight (FW). The 80% ethanolic extract of unripe peel demonstrated the highest level of total phenolic content (35.67 mg gallic acid equivalent (GAE)/g extract), while ripe peel exhibited the highest total flavonoid content (16.88 mg catechin equivalent (CE)/g extract). Both unripe and ripe fruit extracts exhibited antioxidant and lipid peroxidation inhibitory activities. The 80% ethanolic extract from unripe peel demonstrated the most significant antioxidant activities against DPPH (6.31 mg trolox equivalent (TE)/g extract) and ABTS (7.69 mg TE/g extract). Moreover, the 80% ethanolic extract of unripe peel showed the highest level of inhibitory activity against lipid peroxidation, with a value of 74.62%. These results underscore the potential bioactivities of unripe and ripe *C. mitis* fruit tissues, which can offer nutritional and health benefits. These properties make them particularly valuable in the food industry due to their excellent antioxidant and lipid peroxidation inhibitory characteristics.

Keywords

antioxidants; calamondin; *Citrus mitis* Blanco; lipid peroxidation; phenolics

Introduction

Vegetables and fruits play a crucial role in providing essential nutrients and phytochemicals that offer numerous health benefits to humans. These plant-based foods contain biologically active secondary metabolites, which have pharmacological effects, including the ability to neutralize reactive oxygen species (ROS) associated with the development of chronic diseases (1). The increased production of ROS can have damaging effects on biomolecules such as nucleic acids, proteins, and lipids, leading to oxidation damage. This oxidative damage is implicated in various pathophysiological conditions, including heart disease, neurological disorders, hypertension, diabetes, ageing, and even cancer (2). Previous research has demonstrated that long-term consumption of antioxidant-rich foods can reduce the risk of chronic degenerative diseases through various mechanisms, including ROS scavenging, quenching free radicals, and inhibiting key enzymes involved in disease pathogenesis (3). Synthetic antioxidants like BHT and BHA have faced significant restrictions due to concerns about their potential carcinogenic effects. Consequently, there is considerable interest in exploring alternative options to replace synthetic antioxidants. Vegetables and fruits have gained significant attention for being excellent sources of antioxidants and for the potential synergistic effects of their bioactive compounds (4).

Citrus fruits are extensively cultivated in tropical and subtropical regions, including Thailand, and belong to the Rutaceae family, which encompasses approximately forty citrus species (5). In addition to their delightful taste, these fruits offer significant nutritional value. Previous research has highlighted numerous bioactivities associated with citrus fruits, including antioxidant, anti-diabetic, anti-cancer, and anti-inflammatory properties (6-8). Many of these biological benefits can be attributed to specific bioactive compounds abundant in citrus fruits, such as phenolics, flavonoids, carotenoids, limonoids, glycosides, ascorbic acid, and fibree (8-9). Among these compounds, flavonoids, a subgroup of phenolic compounds, are particularly noteworthy as they are considered one of the primary sources of bioactive compounds in citrus fruits (10). Research has shown that a higher intake of phenolic compounds is associated with a reduced risk of developing heart disease or other degenerative conditions. Furthermore, citrus fruits have attracted the attention of researchers for their potential role in cancer prevention, as they inhibit lipid peroxidation (5).

During the processing of citrus fruits for juice production, the primary byproduct is the fruit peel, which accounts for approximately 50-65% of the fruit and is often discarded, resulting in a significant environmental burden. Traditionally, these byproducts have been utilized for animal feed, fibre extraction (pectin), and fuel production (11). However, citrus peels contain some of the highest levels of natural antioxidants, including flavonoids, phenolics, ascorbic acid, carotenoids, and reducing sugars. Literature has shown that citrus peel extracts exhibit stronger antioxidant effects compared to seeds or fruit pulp (8-12). Consequently, the phenolic compounds found in citrus peels and fruit byproducts can be effectively employed as active ingredients or substitutes for synthetic preservatives in food products. Furthermore, these compounds have been associated with health benefits.

In the southern region of Thailand, specifically in Ranong province, a common citrus variety cultivated is known as Calamondin or Musk lime (*Citrus mitis* Blanco). It is a hybrid cultivar of *Citrus reticulata* Blanco and *Fortunella* spp., known for its small fruit size. This variety has a long history of traditional use in Taiwan, where it is often used to make warm beverages using immature fruits with green peel, primarily due to its potential health benefits. These benefits can be attributed, at least in part, to bioactive substances, including flavonoids, found in the citrus peel (13). Calamondin's peel, leaves, and juice sac contain significant amounts of DGPP (3',5'-di-C-β-glucopyranosylphloretin) (14). DGPP, along with compounds such as naringin and hesperidin, has been confirmed as an important flavonoid found in the water extract obtained from immature calamondin peel (15). Moreover, polymethoxyflavones like nobiletin and tangeretin have been isolated and identified in the calamondin peel (16). Fruit ripening leads to various changes in biochemical, physiological, and structural properties, all of which can impact fruit quality (17). In the case of calamondin, as it ripens, its color transitions from green to orange, corresponding to changes in chlorophyll concentration as well as carotenoid content levels. During ripening, carotenoid levels tend to increase, influenced by alterations occurring in other bioactive substances (18).

To the best of our knowledge, comprehensive studies thoroughly investigating the antioxidant and lipid peroxidation inhibitory properties of unripe and ripe fruit tissues, including pulps and peels, of *C. mitis* are limited in the literature. With this in mind, the current research sought to conduct a comparative assessment of various biologically active compounds as well as the antioxidant and lipid peroxidation inhibitory potentials of the pulps and peels from unripe and ripe *C. mitis* fruits. The goal is to promote the global *C. mitis* industry, provide valuable information about the health-enhancing compounds present in Thai citrus fruits, and highlight the significant contribution of citrus as a major dietary source of antioxidants.

Materials and Methods

Chemicals

Sigma Chemical Co. (St. Louis, Missouri, United States) supplied the Folin-Ciocalteu reagent, catechin, gallic acid, thiobarbituric acid, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The reagents and solvents employed in this research were all of analytical grade.

Collection and preparation of fruit samples

In March 2022, unripe and ripe fruits of *C. mitis* (Figure 1) were collected from the Hat Som Paen subdistrict, Mueang District, Ranong province, in the southern region of Thailand. The collection site co-ordinates were 9°57'8.446"N, 98°41'31.547"E (Figure 2). To ensure their botanical authenticity, the plant materials were carefully identified and verified with the assistance of experts at the Plant Varieties Protection Department of the Department of Agriculture in Bangkok, Thailand. One plant sample underwent preservation at the Bangkok Herbarium and was assigned the classification code BK No. 085008. Separation of the peels from the pulp was done manually, and the peels were dried at 50 °C using a hot-air oven for 48 hours. After drying, the fruit samples were finely powdered with a blender and stored in vacuum-sealed packaging at 4 °C for future analysis.

Fig. 1. *Citrus mitis* Blanco (Photographed by Narumon Boonman)

Preparation of ethanolic and aqueous extracts

The extraction process was carried out following previously published methods with minor modifications (4). In brief, ten grams of powdered peels and pulps were subjected to maceration with 200 ml of 80% ethanol and water solvents at approximately 25 °C for 48 hours. The resulting mixture underwent filtration using Whatman No.1 filter paper, and the residue was subjected to two additional extraction cycles using the same procedure. The filtrates from each extraction were concentrated under reduced pressure using a rotary evaporator, yielding crude extracts. For analysis, the crude extracts were re-dissolved in dimethyl sulfoxide (5% DMSO) to a concentration of 100 mg/mL. The solution was then transferred to glass bottles and stored at 4 °C until use. The percentage yield of crude extracts from *C. mitis* was determined and expressed as % w/w of the dry sample.

Determination of total vitamin C content

Assessment of total vitamin C content in the sample extracts was conducted using the 2,6-dichloroindophenol titrimetric approach (19). Initially, plant matter (100 g) was broken into tiny fragments before being mixed with 150 mL of a solution containing 3% (w/v) metaphosphoric acid and 8% (v/v) acetic acid using a Waring blender. The resulting mixture underwent filtration, and suitable dilutions were performed with the metaphosphoric acid–acetic acid solution based on the color intensity of the extract. Subsequently, a titration of the solution (7 mL) was performed against a standard solution of indophenol. The findings were quantified and reported as mg/100 g FW (milligrams of ascorbic acid per 100 g of fresh weight).

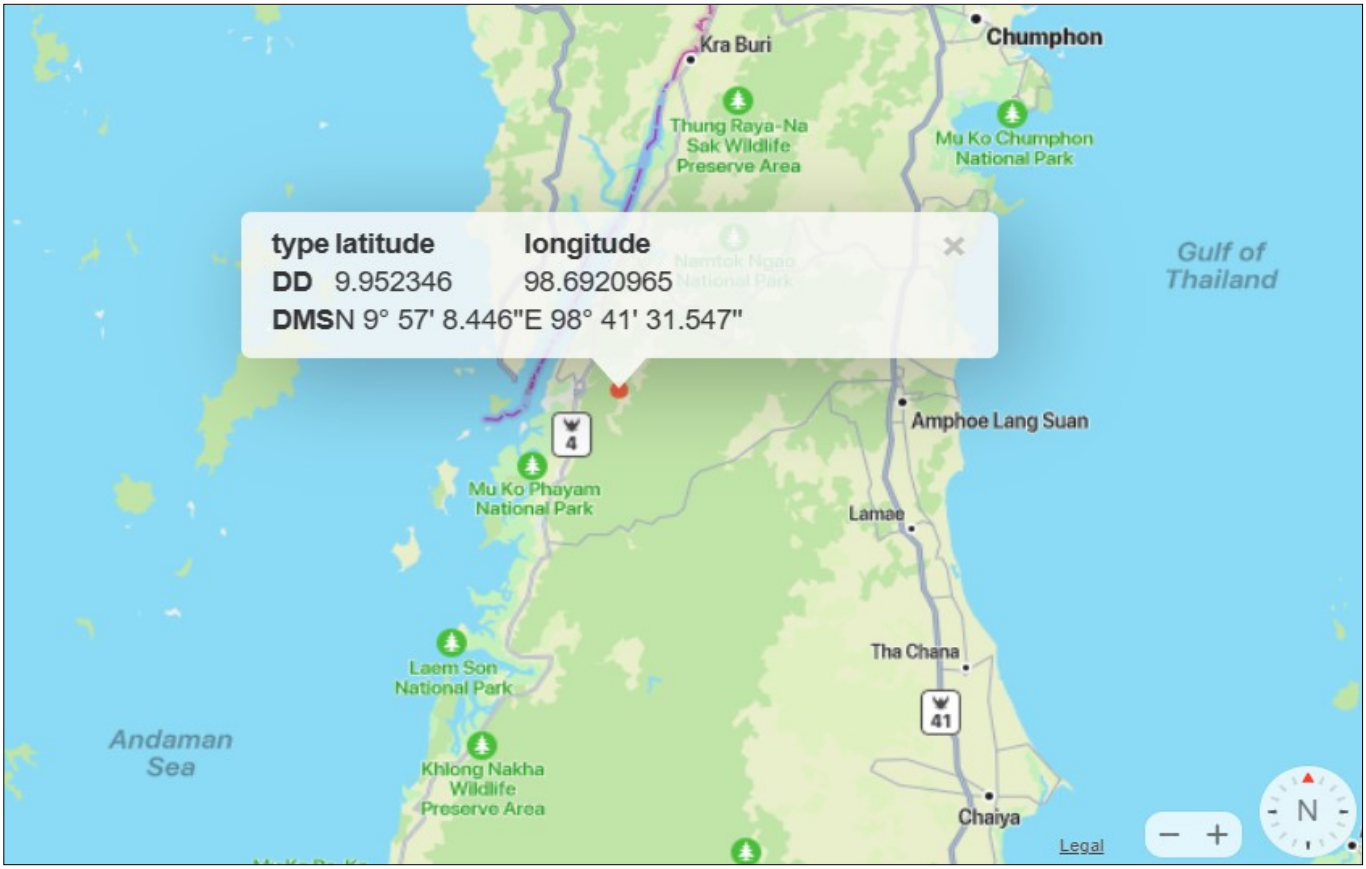

Fig. 2. The collection site of *Citrus mitis* Blanco is situated in the Hat Som Paen subdistrict, Mueang District, Ranong province.

Determination of total phenolic content

The TPC (total phenolic content) of the sample extracts could be evaluated via the modified Folin-Ciocalteu procedure (20). In summary, 100 µL of fresh 10% Folin-Ciocalteu reagent was combined with 20 µL of 30 mg/mL extracts and placed in 96-well microplates, where the reaction was permitted to take place for a period of 5 minutes at a temperature of around 25 °C. Subsequently, 80 µL of 7.5% (w/v) Na_2CO_3 (sodium carbonate) solution was introduced to the original mixture and incubated at 25 °C for half an hour. Measurements of the absorbance were taken for the incubated solution at 765 nm with a microplate reader (SpectraMax M2). Trials were performed three times, whereupon the total phenolic content could be quantified and reported as mg GAE/g extract (milligrams of gallic acid equivalent per gram of extract). This quantification was achieved by employing a calibration curve constructed on the basis of gallic acid dilution series in the range of 5-100 mg/L.

Determination of total flavonoid content

TFC (total flavonoid content) for the sample extract could be assessed via the adjusted aluminum-chloride colorimetric approach (21). A total volume comprising 0.5 mL of 30 mg/mL extracts was added to 0.15 mL of 5% $NaNO_2$ (sodium nitrite) and 2 mL of distilled water. The mixture underwent incubation for 6 minutes, followed by adding 0.15 mL of 10% $AlCl_3$ (aluminum chloride). After allowing it to stand for a further 6 minutes, 1 mL of 1 M NaOH (sodium hydroxide) was introduced before adjusting the volume using distilled water to reach 5 mL. The mixing of the obtained solution was then carried out using a vortex, whereupon 200 µL of the resulting mixture was moved to a 96-well plate. The absorbance was then evaluated at 510 nm with a microplate reader (SpectraMax M2). Each sample was assessed in triplicate, with findings presented as mg CE/g extract (milligrams of catechin equivalent per gram of extract). This quantification was determined via a calibration curve produced from different catechin concentrations (in the range of 30-300 mg/L).

DPPH radical scavenging activity

Evaluation of the sample extract DPPH radical scavenging activity could be conducted through the application of an adjusted traditional technique (22). Initially, the preparation of the DPPH stock solution (0.1 mM) utilized 0.4 mg of DPPH powder, which was dissolved in 9.6 mL of 80% ethanol. Precisely, 20 µL drawn from the 30 mg/mL sample extracts could be mixed in the 96-well microplates with 180 µL of 0.1 mM DPPH solution. This resulting mixture underwent vigorous shaking before being left in darkness at a temperature of around 25 °C for half an hour. Subsequently, reaction mixture measurements were taken for the wavelength of 515 nm against a blank (ethanol) using a microplate reader (SpectraMax M2). DPPH radical scavenging inhibition percentages were duly determined for the samples investigated using the formula given below:

$$\%\ \text{DPPH radical scavenging inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} X100$$

Where, $A_{control}$ = DPPH absorbance without samples

A_{sample} = DPPH absorbance with samples

Relevant values were determined based on the trolox standard curve, constructed using concentrations ranging from 1 to 10 mg/mL and presented in the format of mg TE/g extract (milligrams of trolox equivalent per gram of extract). Data shown represent the average of triplicate tests.

ABTS radical cation scavenging activity

Scavenging of the ABTS radical cations followed a methodology outlined in the work of González-Palma *et al*. (2016) (22), albeit slightly modified. To prepare the necessary stable stock solution of ABTS radical cation, an aqueous ABTS solution (4 mM) was used for the reaction involving 2.45 mM $K_2S_2O_8$ (potassium persulfate) at a ratio of 1:1 (v/v) at room temperature in darkness for a period of 12 to 16 hours prior to its use in further investigation. This ABTS solution then underwent dilution using distilled water to achieve an absorbance level of 1.0 ± 0.02 at 734 nm. Subsequently, 20 µL of sample extracts (30 mg/mL) were added to 150 µL of working solution in a 96-well microplate. Following incubation for 15 minutes in the dark at around 25 °C, measurements of the absorbance were immediately taken for the wavelength of 734 nm using a microplate reader (SpectraMax M2). The ABTS radical cation scavenging inhibition percentages could be determined via the formula given below:

$$\%\ \text{ABTS radical cation scavenging inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} X100$$

Where, $A_{control}$ = ABTS absorbance without samples

A_{sample} = ABTS absorbance with samples

All outcomes could be determined via the use of a calibration curve of Trolox within a concentration range of 5-100 mg/mL. Reported findings took the form of mg TE/g extract. The experimental procedures were conducted in triplicate.

Lipid peroxidation inhibition assay

The inhibition of lipid peroxidation due to the actions of the sample extracts and ascorbic acid underwent assessment via the use of the thiobarbituric acid reactive substances (TBARS) assay, following the protocol outlined by Badmus *et al*. (2011) (23). Egg yolk homogenate, a lipid-rich medium, was combined with the extract (30 mg/mL) in a 0.1 mL to 0.5 mL ratio, respectively. The volume was adjusted to a value of 1.0 mL using distilled water. The addition of 0.05 mL of $FeSO_4$ (iron (II) sulfate) followed, with the subsequent incubation of the resulting mixture for a period of half an hour at a temperature of 37 °C. Acetic acid (1.5 mL) and TBA in SDS (1.5 mL) were then sequentially added to the mixture. Following vortex mixing, the solution was heated at 95 °C for 60 minutes. Once cooled, butanol (5 mL) was introduced, whereupon the resulting mix

was placed in a centrifuge for 10 minutes at 3,000 rpm. Measurements of the organic upper layer absorbance were taken at 532 nm, whereupon the lipid peroxidation inhibition percentage could be determined using the equation given below:

$$\% \text{ lipid peroxidation inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} X100$$

Where, $A_{control}$ = the control absorbance without samples

A_{sample} = the test absorbance with samples

Analysis of the data

Each analysis was conducted in triplicate (n = 3). Data were presented in the form of mean ± standard deviation and subjected to further analysis via the use of the Statistical Package for Social Sciences (SPSS version 21). One-way ANOVA allowed the determination of significant differences between means, while Duncan's new multiple tests allowed the identification of the least significant differences with 95% confidence ($p < 0.05$).

Results and discussion

Extraction yields

The extraction efficiencies of 80% ethanol and water for obtaining unripe and ripe *C. mitis* pulps and peels are presented in Table 1. The extract yields, expressed as percentages, ranged from 6.54% to 11.57% for 80% ethanol and from 5.49% to 9.58% for water. Notably, the highest crude extract was obtained from the unripe peel using 80% ethanol, yielding 11.57%. Following closely was the ripe peel extracted in 80% ethanol, yielding 10.56%. Conversely, the lowest yield was observed in the ripe peel extracted using water, with a yield of 5.49%. Considering its favorable properties in extracting polyphenolic compounds and its non-toxic nature for the environment and human consumption, a solvent consisting of aqueous ethanol (80% v/v) was chosen for the extraction process (24). Consequently, the extraction process successfully obtained water-soluble and ethanol-soluble compounds.

Table 1. The extraction yields of unripe and ripe of *C. mitis* pulps and peels with different solvents based on weight

Samples	Yields (% w/w)	
	80% Ethanol	Water
Unripe pulp	6.54	8.32
Ripe pulp	7.23	9.58
Unripe peel	11.57	6.27
Ripe peel	10.36	5.49

Vitamin C content of C. mitis Blanco fruit tissues

Citrus fruits are known for their high ascorbic acid content, which can be understood to serve as a crucial indicator of their efficacy in preventing scurvy, cancer, cardiovascular diseases, and chronic nervous system diseases (25). The study on *C. mitis* revealed significant variations in the ascorbic acid content based on fruit ripening stages, fruit parts, and extraction solvents, as presented in Table 2. Comparing the fruit parts and ripening stages, it was found that the pulp contained higher levels of ascorbic acid than the peel, and this concentration decreased gradually as the fruit ripened. Among the solvents used for extraction, water extracts exhibited higher levels of ascorbic acid than 80% ethanolic extracts. Notably, the unripe pulp extracted with water displayed the highest ascorbic acid content, with a value of 36.74 mg/100 g FW, whereas the ripe peel extracted with 80% ethanol had a value of 7.39 mg/100 g FW. These findings concur with earlier research examining alternative cultivars involving citrus fruits, in which the pulp of various commercial citrus fruits in Brazil contained more ascorbic acid than peel (26). In contrast to these results, the peel of fruits, including lemon, grapefruit, or orange, contained higher levels of vitamin C than their inner parts, including the pulp and seeds (27). This variation could be attributed to different cultivars, maturity stages, extraction solvents, or other environmental factors.

Phenolic compositions of C. mitis Blanco fruit tissues

Polyphenolic compounds, encompassing phenolics and flavonoids, are secondary metabolites present in plants that exhibit notable antioxidant activity and are widely distributed in different parts of fruits. The current research sought to establish the TPC (total phenolic content) and TFC (total flavonoid content) of unripe and ripe *C. mitis* pulps and peels via the respective use of the Folin-Ciocalteu and aluminum chloride colorimetric methods. Results of TPC and TFC in 80% ethanol and water extracts for unripe and ripe *C. mitis* pulps and peels are provided in Table 2. The TPC ranged from 3.58 to 35.67 mg GAE/g extract in 80% ethanol and 2.32 to 29.86 mg GAE/g extract in water. Similarly, the TFC varied from 0.86 to 14.07 mg CE/g extract in 80% ethanol and 1.75 to 16.88 mg CE/g extract in water. The findings demonstrated significant variations in TPC and TFC based on fruit ripening periods, fruit tissues, and extraction solvents.

Interestingly, the TPC decreased as the fruit rip-

Table 2. Vitamin C, total phenolic and total flavonoid contents was detected in unripe and ripe of *C. mitis* pulp and peel extracts in different solvents

Treatments	Vitamin C content (mg/100 g FW)		Total phenolic content (mg GAE/g extract)		Total flavonoid content (mg CE/g extract)	
	80% Ethanol	Water	80% Ethanol	Water	80% Ethanol	Water
Unripe pulp	29.64 ± 0.10 ^{f}	36.74 ± 0.07 ^{h}	6.41 ± 0.06 ^{d}	5.32 ± 0.07 ^{c}	1.75 ± 0.04 ^{b}	0.86 ± 0.03 ^{a}
Ripe pulp	24.25 ± 0.08 ^{e}	31.36 ± 0.09 ^{g}	3.58 ± 0.07 ^{b}	2.32 ± 0.04 ^{a}	3.44 ± 0.05 ^{d}	1.96 ± 0.03 ^{c}
Unripe peel	11.25 ± 0.07 ^{c}	15.49 ± 0.08 ^{d}	35.67 ± 0.09 ^{h}	29.86 ± 0.05 ^{g}	11.07 ± 0.06 ^{f}	9.77 ± 0.06 ^{e}
Ripe peel	7.39 ± 0.08 ^{a}	10.20 ± 0.05 ^{b}	24.49 ± 0.08 ^{f}	21.24 ± 0.05 ^{e}	16.88 ± 0.06 ^{h}	14.07 ± 0.05 ^{g}

The values are presented as mean ± standard deviation (n = 3). Different superscript lowercase letters in column denote significant differences at $p < 0.05$ using one-way ANOVA followed by Duncan's new multiple range test (DMRT). **FW** = fresh weight; **GAE** = gallic acid equivalent; **CE** = catechin equivalent

ened, whereas a rising trend could be seen in the case of flavonoids, with increases taking place as the fruit matured in all fruit tissues. Regardless of the solvent, unripe extracts consistently exhibited higher TPC than ripe extracts. Notably, the unripe peel extracted with 80% ethanol and water displayed the highest TPC, with values of 35.67 and 29.86 mg GAE/g extract, respectively. Conversely, the ripe extracts contained higher TFC than the unripe extracts, with the ripe peel in 80% ethanol and water exhibiting the highest TFC, with values of 16.88 and 14.07 mg CE/g extract, respectively. Furthermore, the ripe pulp showed the lowest TPC value, whereas the unripe pulp displayed the lowest TFC value across all solvents. Among the solvents used, 80% of ethanolic extracts consistently demonstrated significantly higher TPC and TFC than water extracts.

Our research has confirmed that the ripening process impacts phenolic compounds in different fruit tissues, and these compounds exhibit variations in antioxidant content depending on various factors, including components, cultivars, species, maturation stages, harvest times, climatic conditions, and storage durations (28). Wang *et al*. (2022) (29) reported that phenolic compounds such as catechin, ferulic acid, chlorogenic acid, and coumaric acid can be found in significant quantities in immature fruit, although the amounts decrease as the fruits mature. Generally, the total phenolic content (TPC) was higher in unripe fruits compared to ripe fruits, which can be a consequence of the lower levels of tannins and various other phenolic compounds as the fruits ripen to enhance their palatability (30). These findings align with previous studies that observed a high concentration of TPC in non-edible parts of orange plants, such as the leaves, seeds, or peel, in contrast to the parts which can be eaten, such as the juice or pulp (31-33). Similarly, it has been reported that phenolic compounds have an uneven distribution throughout the flesh of the fruit and are primarily found within the epidermis and sub-epidermis (34). However, this finding contradicts a previous study by Huda-Faujan *et al*. (2015) (35), which suggested that the water extracts derived from certain vegetables from Malaysia offered greater phenolic content than was the case for the ethanolic extracts. It can be explained that the discrepancy might possibly be due to the inability of pure ethanol to extract polar phenolic compounds. It could be dependent on the extract concentration along with the sample-to-solvent ratio employed. Overall, it has been shown that aqueous solvents offer greater phenolic compound yields than is the case for alternative solvents (36).

Flavonoids, the primary phenolic compounds in oranges, play a crucial role as antioxidants through free radical scavenging, lipid peroxidation inhibition, and the chelation of metal ions through those phenolic hydroxyl groups that are attached to their ring structures (31,33,37). Additionally, flavonoids exhibit effective regulation of various human diseases through their antioxidant, anti-inflammatory, and immunomodulatory effects (38). The findings of this study align with previous research studies indicating that orange peel contains flavonoids at high concentrations in contrast to the rest of the fruit (33,39). Moreover, when extracted using aqueous ethanol, TFC (total flavonoid content) for calamondin peels exceeds that of the pulp (40). The main flavonoids, such as naringin and hesperidin, have a higher solubility in aqueous ethanol, resulting in lower TFC values in water extracts compared to extracts with 80% ethanol (40). Similarly, in line with the present study, extracting TFC from *C. grandis* using aqueous ethanol was more favorable and efficient (41). Furthermore, *C. reticulata* peel exhibited a high content of flavonoids, which strongly influenced the fruit's color (42). It is worth noting that flavonoid synthesis requires light, and the full sunlight exposure may have induced their accumulation in the peel of this particular cultivar (43).

DPPH and ABTS radicals scavenging activities of C. mitis Blanco fruit extracts

The DPPH assay is widely used for assessing antioxidant activity *in vitro* since it is simple, cheap, fast, and the findings are easily reproduced. DPPH radicals are organic free radicals with a nitrogen center, exhibiting purple in ethanolic solutions. When they interact with materials capable of donating protons, or antioxidants, this changes the radical to form DPPH, resulting in alterations in the color to appear yellow rather than the original purple (diphenylpicrylhydrazine). During the course of this study, antioxidant activity against DPPH was compared between unripe and ripe *C. mitis* pulps and peels, and it was also compared to the well-known antioxidant, ascorbic acid, as presented in Table 3. The extracts from the analyzed fruit tissues demonstrated significant antioxidant properties, ranging from 2.63 to 6.31 mg TE/g extract. Consistent with the total phenolic content, the unripe peel exhibited the highest DPPH radical scavenging activity, with 6.31 mg TE/g extract in 80% ethanol and 5.49 mg TE/g extract in water. In

Table 3. The antioxidant and lipid peroxidation inhibitory activities of unripe and ripe *C. mitis* pulp and peel extracts obtained using various solvents were compared to ascorbic acid with the concentration set to 0.5 mg/mL

Treatments	DPPH scavenging activity (mg TE/g extract)		ABTS scavenging activity (mg TE/g extract)		Lipid peroxidation inhibitory activity (%)	
	80% Ethanol	Water	80% Ethanol	Water	80% Ethanol	Water
Unripe pulp	4.13 ± 0.06 ^{d}	3.15 ± 0.03 ^{b}	6.50 ± 0.07 ^{e}	5.63 ± 0.05 ^{c}	53.56 ± 0.09 ^{e}	38.65 ± 0.13 ^{b}
Ripe pulp	3.66 ± 0.04 ^{c}	2.63 ± 0.05 ^{a}	5.44 ± 0.04 ^{b}	4.72 ± 0.05 ^{a}	47.87 ± 0.05 ^{c}	31.74 ± 0.06 ^{a}
Unripe peel	6.31 ± 0.03 ^{h}	5.49 ± 0.06 ^{g}	7.69 ± 0.04 ^{g}	7.04 ± 0.03 ^{f}	74.62 ± 0.05 ^{h}	59.67 ± 0.11 ^{f}
Ripe peel	5.33 ± 0.04 ^{f}	4.74 ± 0.02 ^{e}	7.06 ± 0.03 ^{f}	6.19 ± 0.02 ^{d}	68.15 ± 0.08 ^{g}	50.27 ± 0.09 ^{d}
Ascorbic acid	-	12.36 ± 0.04 ^{i}	-	14.52 ± 0.03 ^{h}	-	89.26 ± 0.05 ^{i}

The values are presented as mean ± standard deviation (n = 3). Different superscript lowercase letters in column denote significant differences at $p < 0.05$ using one-way ANOVA followed by Duncan's new multiple range test (DMRT). **TE** = trolox equivalent

contrast, the ripe pulp exhibited the lowest scavenging activity across all solvents.

Concerning the antioxidant potential of *C. mitis* extracts evaluated using the ABTS assay, the results were consistent with the DPPH radical scavenging activity. The data presented in Table 3 demonstrate notable variations in the ABTS cationic radical scavenging of *C. mitis* extracts acquired with a variety of solvents across various fruit tissues, ranging from 4.72 to 7.69 mg TE/g extract. The unripe peel, extracted with 80% ethanol, exhibited significantly higher ABTS scavenging ability than the other groups, with a 7.69 mg TE/g extract value. The ripe peel, unripe, and ripe pulp demonstrated progressively lower scavenging activities. When considering the different polar solvents, the water extracts displayed similar antioxidant activity to the 80% ethanolic extracts, albeit with lower values for each fruit tissue and ripening stage. Ethanol, an intermediate-polar solvent utilized in extraction, can effectively extract a wide range of compounds, from hydrophilic to hydrophobic, including phenolic compounds. Consequently, it provides higher antioxidant properties compared to water extraction (44).

However, the scavenging activities of the extracts using the DPPH and ABTS assays were lower than those of ascorbic acid, which served as the positive control, with values of 12.36 and 14.52 mg TE/g, respectively. Interestingly, a significant decrease in antioxidant capacity was observed in both the pulp and peel as they ripened. This decline in antioxidant activity during ripening could be attributed to a reduction in the total phenolic content or the activity of antioxidant enzymes like ascorbate and glutathione in the tissues (45). Furthermore, the high content of phenolics and flavonoids in *C. mitis* peel contributed to its greater antioxidant potential than the pulp. Our findings support previous studies indicating that citrus peel exhibits robust antioxidant activity due to its abundance of bioactive compounds such as phenolics, flavonoids (DGPP, naringin, and hesperidin), polymethoxyflavones (nobiletin and tangeretin), and limonoids (15,16). These compounds likely play a crucial role in scavenging free radicals in the extracts, particularly naringin and hesperidin, which possess 4'-OH and 3'-OH groups, respectively, known to enhance the antioxidant power of flavonoids. Immature calamondin peel extracts demonstrated good oxygen radical scavenging capacity (46). Most studies have indicated that phenolic compounds exert a greater influence on antioxidant activity compared to ascorbic acid (47,48).

Moreover, the total polyphenol content has been identified as the major contributor to the antioxidant capacity of citrus fruits (49). The variation in antioxidant capacity observed among different citrus fruit tissues may be attributed to differences in their phenolic composition (50). However, it should be noted that the extracts are complex mixtures containing numerous compounds with specific activities (51). Finally, the health benefits associated with citrus fruits are primarily attributed to their antioxidant activity.

Lipid peroxidation inhibitory activity of C. mitis Blanco fruit extracts

Lipid peroxidation typically involves a free radical chain reaction, causing damage to biological membranes and a loss of their functioning. Lipid radicals are formed when lipids react with free radicals, which combine with molecular oxygen to form lipid peroxyl radicals, which in turn will serve to extend the process of peroxidation through the extraction from the various lipid molecules of hydrogen atoms. This process of propagation can be disrupted by hydrogen-donating antioxidants, while lipid peroxidation is also inhibited (23). Our study assessed the potential of unripe and ripe *C. mitis* fruit extracts to inhibit lipid peroxidation, as shown in Table 3. The extracts from unripe fruit demonstrated a stronger inhibitory effect on lipid peroxidation than those from ripe fruit. Both unripe and ripe peels exhibited significantly higher inhibition of lipid peroxidation than the pulp extracts in egg yolk. However, the inhibitory effect of ascorbic acid was significantly greater than that of the extracts.

Interestingly, the unripe peel extracted with 80% ethanol displayed the highest inhibitory activity, with values of 74.62%, while the water extract of ripe pulp showed the lowest activity, with values of 31.74%. These findings align with a previous study that reported higher lipid peroxidation inhibition in the peel extract of *C. limonum* compared to the pulp extract (52). The high lipid peroxidation inhibition observed in the 80% ethanol extract of the peel may be attributed to the existence of the phenolic compounds known to show a good correlation with the antioxidant properties of citrus fruits. Additionally, the higher lipid content and lower levels of endogenous antioxidant components in the peel may contribute to the lower inhibition of lipid peroxidation (53).

Correlation between TPC and TFC with antioxidant and lipid peroxidation inhibitory activities

Pearson's correlation coefficients were considered significantly negative when they fell within the $-0.61 \leq r \leq -0.97$ range and significantly positive when they fell within the $0.61 \leq r \leq 0.97$ range (54). The results of this study, depicted in Table 4, examined the relationship between TPC and TFC with antioxidant and lipid peroxidation inhibitory activities in extracts from unripe and ripe *C. mitis* pulps and peels using different solvents. The findings of this study revealed a strong positive correlation, indicating that TPC and TFC in citrus samples contribute significantly to their

Table 4. The correlation coefficient was determined for the examination of the association between the inhibition of antioxidant properties and lipid peroxidation (LP) activities and the TPC (total phenolic content) and TFC (total flavonoid content) of unripe and ripe *C. mitis* pulp and peel extracts obtained using different solvents

Assays	DPPH	ABTS	LP	TPC	TFC
DPPH	1				
ABTS	0.960	1			
LP	0.957	0.957	1		
TPC	0.956	0.888	0.856	1	
TFC	0.866	0.784	0.704	0.959	1

antioxidant and lipid peroxidation inhibitory activities. Earlier research has also found that TPC shows a positive correlation with the antioxidant activity of citrus peel, although there have also been contrasting reports (40,55). For instance, de Moraes Barros *et al*. (2012) (8) found a lack of correlation linking TPC and antioxidant activity for extracts of citrus peel, while Rahman *et al*. (2018) (56) observed that DPPH values were negatively correlated with TPC. These findings might be attributed to the various different citrus species studied as well as the extraction procedures employed. The citrus peel contains various phenolic compounds that can be found in citrus peel, including flavonoids and phenolic acids, and different species may exhibit different compositions (9,40). While most phenolic compounds exhibit intense antioxidant activity, some may have weaker activity (57). Additionally, the antioxidant qualities of the extracts can also be affected by the presence of a number of different bioactive compounds, including mineral elements, vitamins, terpenoids, fatty acids, amino acids, or pectin (8,12).

Conclusion

In summary, the extracts obtained from *C. mitis* or calamondin fruit showed varying levels of antioxidant activity, including scavenging DPPH and ABTS radicals and lipid peroxidation inhibition. These activities were influenced by certain factors, such as the ripening stages of the fruit, the specific fruit tissues utilized, and the choice of solvent used for extraction. In comparison to the ripe fruit extract, the unripe fruit extract exhibited stronger antioxidant and lipid peroxidation inhibitory activities. Particularly, the fruit peel contained higher levels of bioactive compounds and exhibited greater antioxidant potential, making it a promising source of compounds for developing food products with health benefits. The observed bioactivities can be attributed to ascorbic acid, flavonoids, and phenolic compounds detected in the fruit extracts. These bioactive compounds contribute to free radicals' scavenging and lipid peroxidation inhibition. Therefore, the current study provides valuable insights into the potential of *C. mitis* fruit to counteract oxidative damage caused by free radicals. The findings have significant implications for agricultural practices and the food industry, offering important guidance for developing new products that prioritize safety and health promotion.

Acknowledgements

The authors gratefully acknowledge the Institute for Research and Development for the full funding of this research project and providing all the necessary support for this work through the Faculty of Science and Technology, Suan Sunandha Rajabhat University, Bangkok, Thailand.

Authors contributions

CW conducted the design of the study, carried out the experiments and drafted the manuscript. NB performed the statistical analysis and revised the manuscript. SP participated in the plant collection and revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors here with declare no conflict of interest.

Ethical issues: None.

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