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Anti-Aging Activities and LC-MS Analysis of *Chrysophyllum cainito* Leaves Extract

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Abstract

Star apple (*Chrysophyllum cainito* L.), or Apple-nam in Thai, is a medium-sized tree in the Sapotaceae family. It is native to Middle America and distributed in Thailand and Southeast Asia. It has antioxidation, anti-inflammatory and anti-diabetic properties. The active compounds in star apples are flavonoids. This research aimed to study the biological activities and chemical constituents of crude extract and active fractions from the leaves of star apples. The leaf powder was defatted with dichloromethane and further extracted with ethanol using ultrasound-assisted extraction. The ethanol extract was separated by quick column chromatography given 7 fractions (CCE1-CCE7). The total phenolic content and antioxidant activity of the extract and chromatographic fractions were determined. The active fractions were further studied for tyrosinase inhibition, collagenase inhibition and cell proliferation properties. The result showed that the highest total phenolic content was observed in ethanol extract. The high polar fraction, CCE6, displayed the highest antioxidation activity with radical scavenging of 0.34 ± 0.01 mg AAE/mL in the DPPH assay and a FRAP value of 1.36 ± 0.01 mg AAE/mL in the FRAP assay. The CCE6 also exhibited the highest anti-tyrosinase and anti-collagenase activities with a percentage of 67.14 ± 1.34 and 70.32 ± 2.88, respectively and showed the best activity in cell proliferation with a percentage of 24.68 ± 2.94. The analysis of the components using the negative ionization LC-MS technique revealed that gallic acid and quercetin-3-galactoside are the major constituents in the ethanol extract. While the fraction CCE6 showed glucosyl (2E,6E,10x)-10,11-dihydroxy-2,6-farnesadienoate and *p*-mentha-1,3,5,8-tetraene as the predominant compounds.

Keywords: *Chrysophyllum cainito*, Leaves, Antioxidant, Anti-tyrosinase, Anti-collagenase, Cell proliferation, LC-MS

Introduction

Currently, the majority of cosmetics consumers seek formulations containing ingredients known for promoting skin brightening, preventing wrinkle formation, minimizing fine lines and delaying the aging process. The key factors contributing to undesired skin conditions in humans are primarily the excessive presence of free radicals. Free radicals are molecules with unpaired electrons, highly reactive in chemical

reactions. It develops as a result of cellular metabolic factors and chronic exposure to agents with damaging effects on the skin, such as pollutants, chemicals, nicotine and sunlight radiation, especially UV-B, which causes skin cancer and photoaging [1]. These factors may contribute to increased expression of matrix metalloproteinases (MMPs) in human skin. MMPs are responsible for the degradation of connective tissue and elastic fibers [2]. When free radicals bind to proteins or collagen in skin cells, they cause deterioration, leading to the formation of wrinkles and premature aging. This process stimulates the enzyme collagenase, ultimately increasing the destruction of collagen fibers [3]. Sunlight also stimulates the activity of the tyrosinase enzyme, leading to increased production of melanin, resulting in the development of dark spots and pigmentation on the skin. Therefore, introducing substances capable of inhibiting tyrosinase activity can reduce melanin production, resulting in a lighter skin tone.

Aging is a biological process characterized by a slow and continual deterioration in cellular and organismal function during a lifetime, culminating in senescence. Skin aging is caused by intrinsic aging, which is a natural process influenced by genetics, and extrinsic aging, which is the skin's reaction to environmental harm. Skin aging signs encompass hyperpigmentation, decreased elasticity and laxity, fine lines and wrinkles, telangiectasia, uneven texture, increased pores, puffy eyes and keratosis [1].

In aging prevention, antioxidants emerge as crucial players by mitigating the surplus of free radicals. It is worth noting that synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have raised concerns about their adverse effects on human health [4], including allergies. Consequently, there has been a growing quest for natural non-toxic antioxidants in recent years. Numerous natural products, including extracts, fractions and pure compounds, have demonstrated promising effects as antioxidants, anti-inflammatories [5] and agents with antiaging and anti-hyperpigmentation properties. Natural antioxidants, such as vitamin C, vitamin E, carotenoids, phenolics and flavonoids, have demonstrated their ability to scavenge free radicals efficiently [6,7].

Moreover, some of these natural antioxidants have been applied to preserve the quality and extend the shelf life of cosmetic products. The antioxidant or anti-free radical impact is attributed to phenolic constituents like flavonoids, phenolic acids and phenolic diterpenes. The effectiveness of these phenolic compounds primarily arises from their ability to undergo redox reactions, thereby absorbing and counteracting free radicals [8].

Chrysophyllum cainito, commonly known as star apple, is a tropical tree from the Sapotaceae family. It was indigenous to Central America and the West Indies. It has been used as a traditional remedy for laryngitis with inflammation, pneumonia and diabetes mellitus [9]. The biological activity of *C. cainito* has been extensively investigated. It exhibits a wide range of associated health benefits and properties, including antioxidants, antidiabetic, anti-inflammatory, anticancer and antihypertensive properties [9]. The fruit closely resembles apples, complete with a tender endocarp and exhibits a distinctive star-shaped pattern within its pulp. It is important to note that while the skin is latex-rich, the rind is inedible. The leaves of *C. cainito* are characterized by their dark green coloration and shiny surface, alternate, the underside shines with a golden color when seen from a distance. They are typically elliptical or ovate and exhibit a leathery texture.

The leaves of *C. cainito* contain natural antioxidants, specifically flavonoids and tannins. In the literature review conducted by Luo *et al.* [10], seven polyphenolic antioxidants were identified, namely gallic acid, galloyl myricitrin, rutin, quercetin, myricitrin, myricetin and quercetin. Two triterpenoids namely β-amyrin and lupeol [10] together with ursolic acid, β-sitosterol, lupeol and gallic acid [11] were also reported in leaves. Additionally, *C. cainito* leaves contain triterpene antioxidants, including β-amyrin acetate and gentistic acid [12].

Many studies reported the antioxidation capacity of *C. cainito* leaf extract. Nevertheless, the information regarding their potential in tyrosinase and collagenase activities, as well as promoting cell proliferation, remains largely unexplored. Hence, these studies have focused on the total phenolic content and antioxidant capabilities of extracts and chromatographic fractions derived from star apples cultivated in Thailand. Furthermore, the investigation explores the inhibitory effects on tyrosinase, collagenase enzymes and cell proliferation, aiming to assess the potential applicability of the isolated substances in cosmetic formulations. The research extends to the analysis of components in both crude extract and selected fraction, employing liquid chromatography-mass spectrometry (LC-MS) techniques. The integration of these potential extracts with locally sourced herbs within the community not only enhances product quality and diversity but also contributes to the economic upliftment of the community through improved income.

Materials and methods

Chemical

The chemicals and reagents were analytical grade. Dimethyl sulfoxide (DMSO), absolute ethanol, Folin-Ciocalteu reagent and ascorbic acid were purchased from Fisher Scientific. Ferric chloride and trichloroacetic acid were acquired from Merck. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, L-tyrosine, mushroom tyrosinase, kojic acid, potassium ferricyanide, Matrix metalloproteinase-1 (MMP-1) and *N*-Isobutyl-*N*-(4-methoxyphenyl-sulfonyl)glycyl hydroxamic acid (NNGH) were supplied by Sigma-Aldrich. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), fibroblast and penicillin-streptomycin antibiotic solution were obtained from Gibco. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Bio Basic Inc. Quick column chromatography (QCC) was performed on silica gel 60H (230-400 Mesh ASTM, Merck). Solvents for extraction and chromatography were distilled at their boiling ranges before use.

Plant material

Leaves of *C. cainito* (**Figure 1**) were collected from the Dorninthanin temple in Nakhon Sri Thammarat province, Thailand in June 2022. The plant samples were accurately identified by a botanist at the Department of Biology, Faculty of Science and Digital Innovation, Thaksin University, Thailand. The dark green leaves were cleaned with tap water, cut into small pieces, air-dried and powdered. The resulting powder was then kept in a zip lock bag and stored at 4 °C until further use.

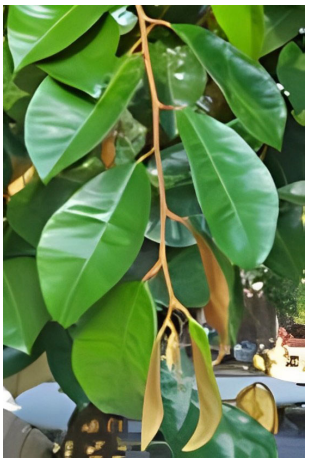

Figure 1 The leaves of *C. cainito*.

Extraction and separation procedures

The dried leaves powder (1 kg) of *C. cainito* was defatted with dichloromethane in a sonicator bath for 15 min at 40 °C and then filtered. The residue of the leaves powder was further extracted with 95 % ethanol for 5 days and then sonicated at 40 °C (600 W) for 30 min. After filtration and solvent removal using a vacuum rotary evaporator, a viscous ethanol extract (46.69 g) was obtained. Twenty grams of ethanol extract was subsequently loaded onto a silica gel quick column chromatography using silica gel 60H as a stationary phase and eluted with a stepwise gradient from 5 - 40 % ethanol in dichloromethane. The column was initially eluted with 200 mL of 5 % ethanol in dichloromethane (twice), increasing the ethanol concentration by 5 % each time. The total eluent was about 5 L. Polar compounds adsorb to silica more strongly than nonpolar compounds. Consequently, they will elute from the column more slowly. Fractions with the same major components were combined based on TLC analysis to yield 7 fractions (CCE1-CCE7). The extract and chromatographic fractions were dried using a freeze-drying process and then stored under refrigerated conditions until further use.

Sample preparation for biological testing

The extracts and chromatographic fractions of ethanol extract (50 mg) were dissolved in 10 mL of dimethyl sulfoxide (DMSO). The solubility of the sample solution was enhanced by subjecting it to a vortex mixer and then a sonicator bath for 5 min. Before analysis in all assays, the absorbance spectra of all samples were recorded using a microplate spectrophotometer at the lambda max to check for interference or impact on the experiment.

Determination of total phenolic content

Total phenolic content (TPC) was analyzed using the Folin-Ciocalteu assay as reported by Vichit and Saewan, 2015 [13], with slight modifications. Briefly, 12.5 µL of the sample was mixed with 50 µL of deionized water and 25 µL of Folin-Ciocalteu reagent. The mixture was then allowed to stand for 6 min before adding 125 µL of a 7 % sodium carbonate solution. Subsequently, the mixture was incubated for 60 min at room temperature. The absorbance was measured at 750 nm using a microplate reader (UVM 340, Biochrom, USA). The result was determined using the gallic acid standard solution and expressed as gallic acid equivalents (mg GAE/mL).

DPPH radical scavenging activity assay

DPPH radical scavenging activity was assessed following the method outlined by Vichit and Saewan, 2015 [13], with some modifications. The reaction mixture, containing 5 µL of sample and 195 µL of 0.1 mM DPPH solution, was incubated for 30 min in the dark at room temperature. Absorbance was measured at 515 nm using a microplate reader. The scavenging activity of all extracts was determined using the calibration curve of ascorbic acid and expressed as ascorbic acid equivalents per mL of sample (mg AAE/mL).

Ferric reducing antioxidant power assay

Ferric-reducing antioxidant power (FRAP) activity was determined according to Kuda and Yano [14], with slight modifications. The sample (25 µL) was mixed with 25 µL of 0.1 M phosphate buffer (pH 7.2) and 50 µL of 1 % potassium ferricyanide and then stood for 60 min at room temperature. Then, 25 µL of 10 % trichloroacetic acid and 100 µL of deionized water were added. Absorbance was measured at 700 nm using a microplate reader and designated as absorbance A1. Then, 25 µL of 0.1 % ferric chloride was

introduced and the absorbance was recorded at 700 nm, denoted as absorbance A2. The optical density of each sample was calculated using the following equation:

$$\text{Optical density} = (A2 - A1)_{sample} - (A2 - A1)_{control}$$

The reducing power activity was determined using an ascorbic acid calibration curve and expressed as mg ascorbic acid equivalents per mL of sample (mg AAE/mL).

The extract and chromatographic fractions with high total phenolic content that displayed good antioxidant activity (CCE, CCE4, CCE5 and CCE6) were selected for further investigation.

Tyrosinase inhibition

Tyrosinase inhibitory activity of the highly potent antioxidant extract or fraction was determined according to the methods described by Rangkadilok *et al.* [15] and Saewan *et al.* [16], with some modifications, employing mushroom tyrosinase as the enzyme and L-tyrosine as the substrate. Briefly, 40 µL of 1.7 mM L-tyrosine solution was dissolved in 40 µL of 0.1 M phosphate buffer (pH 6.8) and then added to 40 µL of samples or DMSO (as control). After incubation for 15 min at room temperature, 40 µL of mushroom tyrosinase (245 U/mL in phosphate buffer at pH 6.8) was added and the mixture was then incubated for 25 min at room temperature. The amount of dopachrome was measured at 490 nm using a microplate reader. Kojic acid served as a standard tyrosinase inhibitor. The data were expressed as the percentage of tyrosinase activity inhibition, calculated using the following equation.

$$\text{Tyrosinase inhibition (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

where $A_{control}$ is the absorbance of dopachrome without extract and A_{sample} is the absorbance of dopachrome in the presence of the tested sample.

Fibroblast cell culture

Human fibroblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % Penicillin/Streptomycin. The cells were then seeded into a 96-well plate at a density of 2,000 cells/well and incubated at 37 °C in a 5 % CO_2 humidified incubator for 24 h before the test.

The sample solutions of the selected extract and fractions were diluted in DMEM without FBS to a concentration of 2 mg/mL, filtered and sterilized through a 0.22 µm filter (Sartorius, Germany) for experimental use.

Collagenase inhibition

The matrix metalloproteinase-1 (MMP-1) colorimetric drug discovery kit, designed to screen MMP-1 inhibitors using a thiopeptide as a chromogenic substrate, was used to determine collagenase inhibition activity as described by Saewan *et al.* [17]. The supernatant was mixed with 20 µL of 153 mU/µL MMP-1 and 20 µL of a 1.3 µM prototypic control inhibitor (NNGH). The mixture was incubated for 60 min at 37 °C to allow interaction between the inhibitor and the enzyme. Then, 10 µL of 100 µM thiopeptide was added to start a reaction, the absorbance was measured at 412 nm. The percentage of collagenase inhibition was calculated as:

Collagenase inhibition (%) = $[(A_{control} - A_{sample})/ A_{control}]\times100$

where $A_{control}$ is the absorbance of the control (without extract) and A_{sample} is the absorbance in the presence of the tested sample.

Cell proliferation

The effect of the selected extracts on fibroblast proliferation was evaluated by MTT assay. The supernatant was added to cells and incubated for 72 h at 37 °C in a 5 % CO_2 humidified incubator. Then, the culture medium was removed and 50 µL of a filtered and sterilized MTT solution (0.1 mg/mL) was added and followed by incubation for 4 h. Subsequently, 100 µL of DMSO was introduced and incubated for an additional 30 min. The absorbance was measured at 570 nm by using the microplate reader. The keratinocyte proliferation was calculated according to the following equation:

Cell proliferation (%) = $[(A_{sample} - A_{control})/ A_{control})]\times100$

where $A_{control}$ is the absorbance of the control (without extract) and A_{sample} is the absorbance in the presence of the tested sample.

Equipment and procedure of Liquid chromatography-mass spectrometry (LC-MS) analysis

The chemical constituents of ethanol extract and the highest potential fraction of *C. cainito* were analyzed using a liquid chromatograph-Quadrupole Time-of-Flight Mass spectrometer (LC-QTOF MS), 1290 Infinity II LC-6545 Quadrupole-TOF, Agilent Technologies, USA. The analysis method was described by Ma *et al.* [18] and López-Fernández *et al.* [19], with slight modifications.

Separations were performed with a Zorbax Eclipse Plus C18 (1.8 µm, 150×2.1 mm i.d.) column. Mobile phases consisted of 0.1 % acetic acid in water (A) and 0.1 % acetic acid in methanol (B) with the following conditions: 1 - 25 min from 0 to 56 % B; 2 min with 56 % B; 27 - 30 min from 56 to 0 % B; 4 min with 0 % B. The column was equilibrated for 1 min before each analysis. The flow rate was 0.2 mL/min and the volume of the sample solution injected was 5 µL. Analysis was performed at 25 °C.

The conditions used for mass spectrometry for the Dual AJS ESI source were: Nebulizing gas (N_2) 35 psig, gas flow rate of 13 L/min, gas temperature, 325 °C; Nozzle voltage, 2.0 kV, capillary voltage, 4.0 kV. For full scan MS analysis, the spectra were recorded in the negative mode in the 50 - 1,200 m/z range. The constituents were identified through the Mass Hunter software analysis. The compound name probability was determined using the METLIN database (Personal Compound Database) and PCDL (Personal Compound Database and Library) version 8.

Statistical analysis

All measurements were conducted in triplicate. Results were presented as means ± SD. Data comparison between each sample was analyzed using one-way ANOVA (Duncan's new multiple range test), with significance determined at a *p*-value less than 0.05 using the SPSS program.

Results and discussion

One kilogram of dried star apple leaves, from Nakhon Si Thammarat province, Southern Thailand, was defatted with dichloromethane. The residue was further extracted with ethanol by maceration method for 5 days, followed by ultrasound-assisted extraction for 30 min. After filtration and evaporation using a

rotary evaporator, 15.90 g of dichloromethane extract (CCD) and 46.69 g of ethanol extract (CCE) were obtained. Twenty grams of ethanol extract (CCE) was subjected to Quick Column Chromatography (QCC) gave 7 chromatographic fractions (CCE1-CCE7).

Total phenolic content

The total phenolic content of the crude extracts and chromatographic fraction of star apple leaves were conducted by mixing the sample with Folin-Ciocalteu reagent. The total phenolic content was calculated from the standard gallic acid curve, $y = 2.4113x + 0.0214$ ($R^2 = 0.9993$). The results are expressed as mg GAE/mL (**Table 1**).

The dichloromethane extract showed a total phenolic content of 0.21 ± 0.01 mg GAE/mL, while the ethanol extract exhibited a higher phenolic content of 0.99 ± 0.01 mg GAE/mL (equivalent to 198.01 ± 2.0 mg GAE/g extract). The phenolic content in this study is more than the value reported by Ningsih *et al.* [12] (190.13 ± 0.457 mg GAE/g extract), possibly due to differences in the extraction methods. However, the age of plants and cultivation areas varies, leading to different quantities of phenolic compounds, it can still be concluded that star apple leaves grown in Thailand have a relatively high amount of phenolic compounds. Among the chromatographic fractions, CCE6 displayed the highest total phenolic content (0.69 ± 0.02 mg GAE/mL). The total phenolic content of CCE6 was lower than that of the crude extract. Additionally, CCE5 and CCE4 exhibited even lower phenolic content compared to CCE6.

Table 1 Total phenolic content and antioxidant activity of crude extract and fractions of star apple leaves.

Sample (5 mg/mL)	Total phenolic content (mg GAE/mL)	DPPH radical scavenging (mg AAE/mL)	Ferric reducing power (mg AAE/mL)
CCD	0.21 ± 0.01^{c}	0.11 ± 0.01^{d}	0.20 ± 0.01^{e}
CCE	0.99 ± 0.01^{a}	0.28 ± 0.03^{b}	1.34 ± 0.03^{a}
CCE1	0.09 ± 0.01^{d}	0.04 ± 0.01^{e}	0.08 ± 0.01^{f}
CCE2	0.10 ± 0.00^{d}	0.09 ± 0.01^{d}	0.09 ± 0.01^{f}
CCE3	0.25 ± 0.01^{c}	0.15 ± 0.01^{c}	0.33 ± 0.01^{d}
CCE4	0.53 ± 0.01^{b}	0.28 ± 0.01^{b}	1.21 ± 0.03^{b}
CCE5	0.58 ± 0.00^{b}	0.30 ± 0.03^{b}	1.25 ± 0.01^{b}
CCE6	0.69 ± 0.02^{b}	0.34 ± 0.01^{a}	1.36 ± 0.01^{a}
CCE7	0.35 ± 0.01^{c}	0.18 ± 0.01^{c}	0.41 ± 0.03^{c}

The same letters (a-f) indicate statistically insignificant differences ($p > 0.05$) within the same column.

DPPH radical scavenging activity

The DPPH radical scavenging activity of the extract and fractions at the concentration of 5 mg/mL was determined by calculation from the standard ascorbic acid calibration curve, $y = 254.3x + 5.509$ ($R^2 = 0.9971$). The results were reported in mg of ascorbic acid equivalent per mL of sample, as shown in **Table 1**. The results indicated that the ethanol extract, with a concentration of 0.125 mg/mL in the well, exhibited strong DPPH free radical scavenging activity of 0.28 ± 0.03 mg AAE/mL (73.19 % inhibition), while the dichloromethane extract demonstrated a lower antioxidant activity of 0.11 ± 0.01 mg AAE/mL (34.79 % inhibition). The extraction process in this study produces ethanol extract (CCE) with a higher percentage of inhibition (73.19 %) compared to the extraction method reported by Ningsih *et al.* [20] (53.25 %). This

variance can be attributed to differences in the extraction technique and the concentration of the tested solution. The antioxidant activity of the extract, however, correlates with the phenolic content.

Fraction CCE6 exhibited lower phenolic content compared to the ethanol extract but demonstrated the highest DPPH free radical scavenging activity. At a 0.125 mg/mL concentration in the well, CCE6 demonstrated a DPPH free radical scavenging activity of 0.34 ± 0.01 mg AAE/mL (90.42 % inhibition) followed by CCE5 with 0.30 ± 0.03 mg AAE/mL (78.90 % inhibition) and CCE4 with 0.28 ± 0.01 mg AAE/mL (74.85 % inhibition). This corresponds to the order of phenolic content in the fractions, suggesting that CCE6 contains effective antioxidant compounds.

Ferric reducing antioxidant power activity

The ability of the extract and fractions at the concentration of 5 mg/mL to reduce ferric iron (Fe^{3+}) to ferrous ion (Fe^{2+}) was determined by calculation from the standard ascorbic acid calibration curve, generated from the reaction of ascorbic acid with Fe^{3+}, $y = 2.0970x - 0.0193$ ($R^2 = 0.9976$). The results were reported in mg of ascorbic acid equivalent per mL of extract. Higher FRAP values indicated a greater ability of the sample to reduce Fe^{3+} to Fe^{2+}.

The results revealed that the FRAP values of dichloromethane and ethanol extracts were found to be 0.20 ± 0.01 mg AAE/mL and 1.34 ± 0.03 mg AAE/mL, respectively. Additionally, the fractions CCE1-CCE7 derived from ethanol extract, demonstrated the ability to reduce ferric iron, as indicated by their FRAP values in the range of 0.08 - 1.36 mg AAE/mL. Notably, CCE6 exhibited the highest ferric-reducing capability, with a FRAP value of 1.36 ± 0.01 mg AAE/mL, which was close to that of the ethanol extract. Following in rank were CCE5 (1.25 ± 0.01 mg AAE/mL) and CCE4 (1.21 ± 0.03 mg AAE/mL), respectively, as depicted in **Table 1**. The ethanol extract and CCE6 displayed high FRAP values, suggesting the chemical constituents in the extract exhibit potent reducing capabilities, consistent with their total phenolic content and DPPH assay results.

The results from the total phenolic content, DPPH and FRAP assays indicated that ethanol extract and fractions CCE4-CCE6 exhibited significant antioxidant properties. Consequently, they were further analyzed for their inhibitory effects on the enzymes tyrosinase and collagenase along with the study for cell proliferation stimulation.

Anti-tyrosinase activity

The tyrosinase inhibitory activity of the ethanol extract and selected fractions was assessed using the dopachrome method with L-tyrosine as the substrate. An extract that inhibits the activity of the tyrosinase enzyme leads to a decrease in the synthesis of dopachrome. At the initial concentration of 5 mg/mL, the ethanol extract exhibited a low inhibitory effect of only 15.42 %. In contrast, the fractions CCE4-CCE6 demonstrated enzyme inhibitory percentages ranging from 41.12 - 67.14 %. Notably, CCE6 exhibited the highest inhibition of tyrosinase, as shown in **Table 2** and **Figure 2**(**a**).

Table 2 % Tyrosinase, collagenase inhibition and % cell proliferation of ethanol extract and selected fractions.

Sample	% Tyrosinase inhibition	% Collagenase inhibition	% Cell proliferation
CCE	15.42 ± 2.04^{c}	57.60 ± 3.42^{b}	17.42 ± 3.11^{b}
CCE4	41.12 ± 1.64^{b}	45.28 ± 2.14^{c}	10.76 ± 2.66^{c}
CCE5	46.86 ± 2.76^{b}	63.54 ± 3.06^{b}	11.68 ± 3.16^{c}
CCE6	67.14 ± 1.34^{a}	70.32 ± 2.88^{a}	25.68 ± 2.94^{a}

Different superscript letters (a-c) denote significant differences within the same column.

The highest percentage of tyrosinase inhibition by the fraction CCE6 indicates that CCE6 possesses active compounds with significant potential in inhibiting the tyrosinase enzyme. This aligns with their antioxidant properties demonstrated in both the DPPH and FRAP assays. Despite not having the highest phenolic content, CCE6 exhibited the highest inhibitory effects on tyrosinase, consistent with its excellent performance in both antioxidant assays.

The result indicated that CCE6 probably contains highly polar substances, particularly phenolic compounds or alcohols with hydroxyl groups in their structure. The inhibition of tyrosinase enzyme activity by CCE6 is likely attributed to the hydroxyl groups (-OH) in the phenolic compounds or alcohols, forming hydrogen bonds with the active site of the enzyme. This interaction results in reduced enzyme activity. Alternatively, the hydroxyl groups may form hydrogen bonds with regions near the active site of the enzyme, inducing conformational changes and consequently reducing enzyme efficiency. This is the first report on the inhibitory properties against the activity of tyrosinase enzymes by star apple leaf extract.

Anti-collagenase activity

The inhibitory effect of ethanol extract and selected fractions (CCE4-CCE6) from star apple leaves on matrix metalloproteinase-1 (MMP-1) was studied. MMP-1 is a key enzyme in responding to the degradation of collagen caused by ultraviolet radiation on the skin [22]. Thiopeptide was used as an indicator of color changes resulting from enzyme reactions. A decrease in absorption values at 412 nm indicates the ability of the extract to inhibit the activity of collagenase enzymes. The percentage of collagenase inhibition at an initial concentration of 50 µg/mL is presented in **Table 2**.

The ethanol extract was able to inhibit *in vitro* collagenase activity, with an inhibition of 57.60 %. Fractions CCE6 and CCE5, which contain a higher amount of phenolic compounds, also demonstrated a greater ability to inhibit collagenase enzymes than the extract, with inhibitions of 70.32 and 64.54 %, respectively. This is the first report on the inhibitory property against the activity of collagenase enzyme by star apple leaf extract. The chemical constituents in the extract consist of flavonoids such as quercetin and quercetin-3-galactoside together with gallic acid. The effect of phenolics as collagenase inhibitors has been reported by Sin and Kim [21]. By comparing different structures, they concluded that the hydroxylation pattern in the B-ring of the flavonoid structure might be an important factor for the inhibitory action of the enzyme. Additionally, Sim *et al.* [22] investigated the structure-activity relationship of various flavonoids on MMP-1 gene expression in UV-A irradiated human dermal fibroblasts and demonstrated that the inhibitory effect became stronger as the number of OH groups on the B-ring increased.

Cell proliferation

The analysis of cell proliferation was performed using the MTT colorimetric assay. The MTT assay measures cellular metabolic activity, acting as an indicator of cell proliferation. This colorimetric assay is based on the reduction of a yellow tetrazolium salt or MTT to form purple crystals of formazan by metabolically active cells. The darker purple color of the solution at 570 nm indicates a greater number of viable, metabolically active cells.

The preliminary screening test showed that cell division decreases when the concentration is either lower or higher than 50 µg/mL (**Figure 2**). At a concentration of 50 µg/mL, the evaluation of the potential of ethanol extract and selected fractions CCE4-CCE6 on the proliferation and growth processes of keratinocyte cells revealed that the samples exhibited the ability to stimulate both cell division and growth by 10.76 - 25.68 %, as shown in **Table 2** and **Figure 2(a)**.

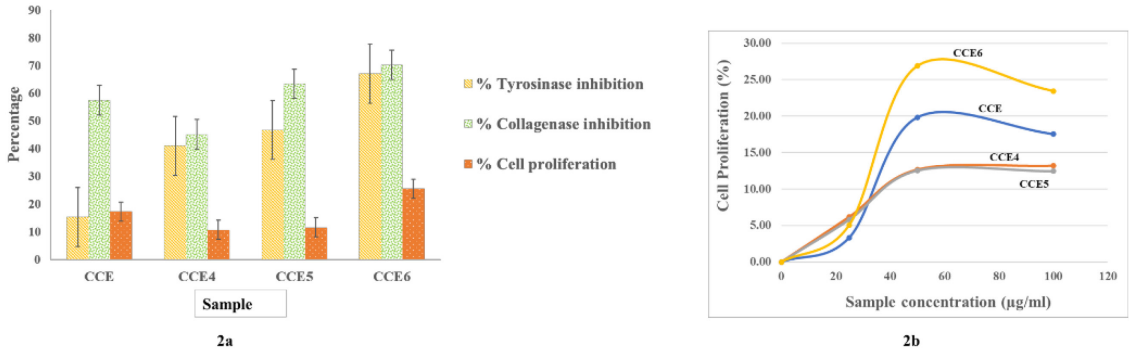

Figure 2 (a) The percentage of tyrosinase inhibition, collagenase inhibition and cell proliferation of extract and selected fractions. (b) The percentage of cell proliferation at different concentrations.

Notably, CCE6 exhibited the highest potential at 25.68 %. The results indicated that fractions with higher polarity (CCE6) had a superior capacity to stimulate the division and growth processes of human keratinocyte cells. This is the first report on the cell proliferation activity of star apple leaf extract.

LC-MS profiles of ethanol extract and fraction CCE6

The chromatograms of the ethanol extract and fraction CCE6 were examined using LC-MS techniques in both negative and positive modes. The negative mode chromatogram is depicted in **Figure 3**. The LC-MS chromatogram of the ethanol extract in the negative mode showed that the 2 major components in the extract are gallic acid and quercetin-3-galactoside, which appear at retention times (RT) of 7.88 and 23.27, respectively. The compounds at RT 7.88 and 23.27 produced $(M-H)^-$ ion at m/z 169.0146 and 463.0897, identified as gallic acid and quercetin-3-galactoside (hyperoside), respectively. These identifications were confirmed by Singh *et al.* [23] (m/z 169.0142) and by Zhang *et al.* [24] (m/z 463.0886), respectively. The LC-MS data of the ethanol extract is consistent with comparatively high phenolic content and favorable antioxidation and anti-collagenase properties.

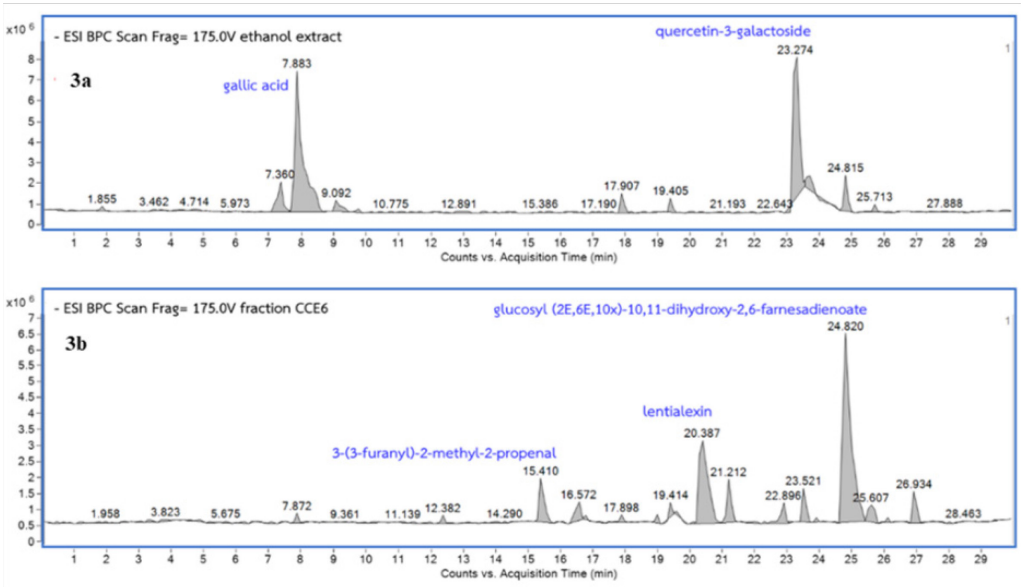

Figure 3 Negative mode LC-MS chromatogram of ethanol extract (a) and fraction CCE6 (b) from *C. cainito* leaves.

The CCE6, eluted with a highly polar solvent and exhibiting superior antioxidative properties, includes a major component identified as glucosyl (2E,6E,10x)-10,11-dihydroxy-2,6-farnesadienoate. This particular constituent is not classified as a phenolic compound. The result implied that the unidentified minor components may belong to the phenolic compound category. The positive mode chromatogram of the ethanol extract and fraction CCE6 is depicted in **Figure 4**.

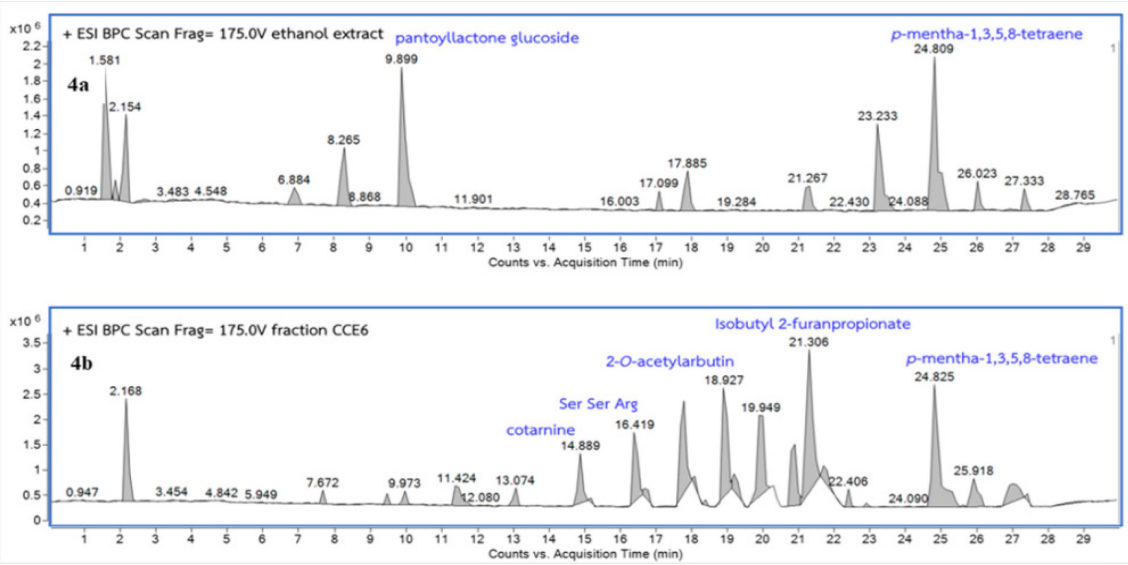

Figure 4 Positive mode LC-MS chromatogram of ethanol extract (a) and fraction CCE6 (b) from *C. cainito* leaves.

The LC-MS chromatogram of the ethanol extract in the positive mode showed the different compounds in the negative mode. The major components in the ethanol extract are pantoyllactone glucoside and *p*-mentha-1,3,5,8-tetraene, which appear at retention times of 9.89 and 24.81, respectively. However, the compound eluting at a retention time of 1.58 remains unidentified by this analytical technique. For fraction CCE6, the major components, namely isobutyl-2-furan propionate, *p*-mentha-1,3,5,8-tetraene, 2-*O*-acetyl arbutin, ser-ser-arg and cotarnine, are not classified as phenolic compounds. The results are consistent with those previously reported by Doan and Le [9]. The structure of gallic acid, quercetin-3-galactoside, pantoyllactone glucoside and *p*-mentha-1,3,5,8-tetraene, as illustrated in **Figure 5**.

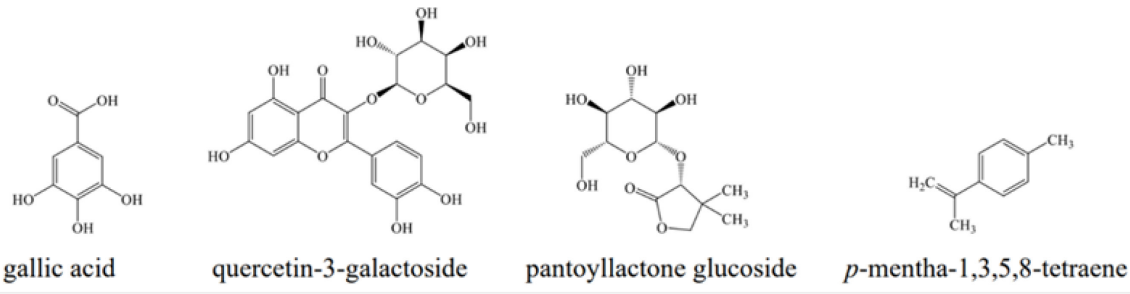

Figure 5 Chemical structure of major compounds from ethanol extract of *C. cainito* leaves.

The major compound observed in the ethanol extract in this study differed from that found in the ethanol extract in methanol solvent, as reported by Ma'arif *et al.* [25]. The metabolite profiling analysis of the ethanol extract in methanol solvent of *C. caimito* leaves using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry/mass spectrometry (UPLC-QToF-MS/MS) revealed compound 4-[2-(isopropylamino)-2-oxoethoxy]-3-methoxy-*N*-[2-(1-piperazinyl)ethyl]benzamide hydrochloride as the major component. While cetylamine was identified as the major compound in ethanol extract in dichloromethane solvent. The different major compounds in these studies could arise from variations in cultural regions, plant age and conditions during analysis. Based on the biological activity of the leaf extract and its most active fraction, further studies will focus on the biological activities of isolated compounds.

Conclusions

The dried leaves of the star apple were defatted with dichloromethane, followed by subsequent extraction with ethanol assisted by ultrasonication. The ethanol extract was separated into seven fractions (CCE1-CCE7) using quick column chromatography. The total phenolic content and biological activities of the extract and chromatographic fractions were investigated. Fraction CCE6 demonstrated superior antioxidant, anti-collagenase and cell proliferation properties despite containing less total phenolic content than ethanol extract. LC/MS analysis identified gallic acid and quercetin-3-galactoside as the major compounds in the ethanol extract. Notably, the major components within the CCE6 were determined not to be phenolic compounds.

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