Chrysin: A Histone Deacetylase 8 Inhibitor with Anticancer Activity and a Suitable Candidate for the Standardization of Chinese Propolis

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ABSTRACT: Chinese propolis (CP) is a natural product collected by honeybees and a health food raw material. Previous studies have shown that CP exhibits a broad spectrum of biological activities including anticancer, antioxidant, antibacterial, anti-inflammatory, and antiviral activities. The focuses of the present study were the standardization of CP and the possible mechanisms of its active anticancer ingredients. Nine samples of CP were collected from different locations in China. Analyses of the CP samples revealed that all 9 had similar chemical compositions. Parameters analyzed included the CP extract dry weight, total phenolic content, and DPPH free radical scavenging activities. The active anticancer ingredient was isolated, characterized against human MDA-MB-231 breast cancer cells, and identified as chrysin, a known potent anticancer compound. Chrysin is present at high levels in all 9 of the CP samples, constituting approximately 2.52% to 6.38% of the CP extracts. However, caffeic acid phenethyl ester (CAPE), another potent active ingredient is present in low levels in 9 samples of CP, constituting approximately 0.08% to 1.71% of the CP extracts. Results from analyses of enzymatic activity indicated that chrysin is a histone deacetylase inhibitor (HDACi) and that it markedly inhibited HDAC8 enzymatic activity (EC50 = 40.2 μM). In vitro analyses demonstrated that chrysin significantly suppressed cell growth and induced differentiation in MDA-MB-231 cells. In a xenograft animal model (MDA-MB-231 cells), orally administered chrysin (90 mg/kg/day) significantly inhibited tumor growth. Despite the geographical diversity of the 9 samples’ botanical origins, their chemical compositions and several analyzed parameters were similar, suggesting that CP is standardized, with chrysin being the major active ingredient. Overall, in vitro and in vivo data indicated that chrysin is an HDAC8 inhibitor, which can significantly inhibit tumor growth. Data also suggested that chrysin might represent a suitable candidate for standardization of CP.

KEYWORDS: Chinese propolis, standardization, chrysin, histone deacetylase inhibitor, HDAC8 inhibitor

INTRODUCTION

Propolis is a natural product and resinous mixture collected by honeybees (Apis mellifera) from tree buds, fruits, sap flows, or other botanical sources. Bees use propolis as a general sealer, draft excluder, antibiotic, and as an emulsifying substance for the carcasses of hive invaders. They also use propolis as a sealant for unwanted open spaces in their hives. Currently, propolis is one of the most popular health foods in the world. The colors of propolis vary greatly due to its source and the season of collection. Numerous regions and countries of the world also use propolis as a traditional folk remedy. Propolis has a range of chemical compositions, and it exhibits a broad spectrum of biological activities, including antitumor, antioxidant, antibacterial, antiviral, antifungal, and anti-inflammatory activities.

Propolis contains phenolic and volatile compounds. Previous studies have demonstrated that the main active ingredients of propolis are flavonoids (such as flavanols, flavones, flavanones, and flavanones), phenolic compounds (such as cinnamic acids and their derivatives), and various aldehydes and ketones. Volatile compounds such as mono- or sesquiterpenoids, prenylated acetophenones, and essential oils are also major chemical components. The chemical composition of propolis can vary according to the location of its botanical origin and season of collection. Bankova conducted pioneering research in the field of propolis standardization, classifying the six main types of propolis: (a) poplar propolis (locations, Europe, North America, and the temperate zone of Asia; botanical origin, Populus nigra); (b) birch propolis (location, Russia; botanical origin, Betula verrucosa); (c) Brazilian green propolis (location, Brazil; botanical origins, Baccharis spp., predominantly Baccharis dracunculifolia DC); (d) red propolis (locations, Cuba and Venezuela; botanical origin, Clusia spp.); (e) pacific propolis (locations, Taiwan, Okinawa, and Solomon Islands; botanical origin, Macaranga tanarius); and (f) Canarian propolis (location, Canary Islands; botanical origin, unknown). Chinese propolis (CP) is mainly classified as poplar-type and is predominantly distributed in Europe, China, and North America (Canada and USA). Previous studies have isolated and identified several active ingredients from CP, classifying them into 3 structural types: (a) caffeic acid, cinnamic acid, p-coumaric acid, ferulic acid, and derivatives.
(3, 4-dimethoxy cinnamic acid, cinnamyl caffeate, caffeic acid phenethyl ester (CAPE), and cinnamylidene acetic acid); (b) pinobanksin, chrysin, pinocembrin, and derivatives (5-pinobanksin-5-methyl ether, pinobanksin-3-acetate, galangin, and techoctyrin); (c) racemate flavanol, [8(\text{E})-4-phenylprop-2-en-1-one]-[(2R, 3S)-2-(3-methoxy-4-hydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-5-methoxy-3,7-diol and [8(\text{E})-4-phenylprop-2-en-1-one]-[(2S,3R)-2-(3-methoxy-4-hydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-5-methoxy-3,7-diol]. To our knowledge, this is the first study aiming to classify and/or standardize CP. The standardization of CP is an important issue because of its popular use as a health food material; approximately 350 tons per year are collected in China alone. The biological activities of CP are reported to include anticancer, anti-inflammatory, antioxidant, and neuroprotective activities.

Histone deacetylase inhibitors (HDACi’s) displayed pharmacological activity in several types of human cancers. The histone deacetylases (HDACs) catalyze deacetylation of the ε-amino group of lysines in the N-terminal tails of histones. Histone acetylation and deacetylation play very important roles in the expression of genes of eukaryotic cells, causing chromatin remodeling. The degree of acetylation of histones is regulated by 2 types of enzymes: the histone acetyltransferases (HATs) and the HDACs. Previous studies have identified 11 human HDACs. Imbalance in histone acetylation can trigger changes in chromatin conformation, leading to dysregulation of genes involved in cell-cycle progression, differentiation, and apoptosis. The anticancer mechanism of HDACi’s is HDAC-induced hyperacetylation of core histones to trigger chromatin remodeling, then reactivation of silent genes, such as tumor suppressor genes, to suppress tumor cell growth. Studies have observed HDAC overexpression in several human tumor types. Inhibition of the class I and II HDACs enzymes has a potential anticancer therapeutic strategy.

In the present study, we analyzed the active ingredients, chemical composition, and biological activities of CP collected from 9 locations. Chrysin is found to be the active ingredient in all samples. More importantly, we have shown that chrysin possesses anticancer properties due to its specificity in HDAC8 inhibition.

**MATERIALS AND METHODS**

**Origin of Propolis.** Chinese propolis (CP) was collected in late summer (August to October) from 2006 to 2010. Propolis samples were obtained from 9 different locations in China, including Beijing, Henan, Jiangsu, Anhui, Hunan, Guangxi, Gansu, and Ningxia (Figure 1). Samples were maintained at −20 °C before processing.

**Extraction of CP.** All 9 of the CP samples (200 g) were alone treated and homogenized by stirring at 4°C. The sample was then washed 3 times with 1.0 L deionized water and the residue was extracted 3 times with 95% ethanol. The filtered ethanol extract was evaporated to dryness under reduced pressure to obtain a brown powder, which was stored at −20 °C until further purification.

**Chrysin Purification.** The powder was dissolved in methanol and applied to an open silica column (Kiesel gel 60 A, E. Merck, Darmstadt, Germany) for the purification of chrysin. An n-hexane/ETOAc solvent system was used as the eluting solvent. All fractions, including those obtained from the chromatography procedures, were used to evaluate the effect on cell proliferation in human MDA-MB-231 breast cancer cells. The fraction possessing the highest antiproliferative effect, SF7 (n-hexane/ETOAc, 40:60), went through a second fractionation using the same system. Table 1 displays the purification of the most active fraction (SF7SF7, n-hexane/ETOAc, 30:70). Reversed phase preparative high performance liquid chromatography (HPLC) was further performed on the SF7SF7 fraction. Fractions with retention times of 16.5 min were collected as chrysin. The conditions were as follows: column, Luna Phenomenex (C18, 250 × 4.6 mm; USA); solvent system, methanol:water (65:35); flow rate, 1.0 mL/min; detection, UV 254 nm; injection volume, 10 μL; 1H NMR (400 MHz, CDCl3) δ 12.87 (1H, s), 10.93 (1H, s), 8.04 (1H, d, J = 7.1 Hz), 8.03 (1H, d, J = 7.1 Hz), 7.57 (1H, m), 7.56 (2H, m), 6.77 (1H, s), 6.56 (1H, d, J = 3.9 Hz), 6.26 (1H, d, J = 3.8 Hz); 13C NMR (400 MHz, CDCl3) δ 183.8 (s), 166.2 (s), 165.4 (s), 164.1 (s), 159.6 (s), 133.5

**Figure 1.** Map of China displaying the regions of Chinese propolis sample collection: 1, Beijing; 2, Henan; 3, Jiangsu; 4, Anhui; 5, Hubei; 6, Hunan; 7, Guangxi; 8, Gansu; and 9, Ningxia.

| Table 1. Antiproliferative Activity of CP Extracts and Fractions against Human MDA-MB-231 Breast Cancer Cells |
|---|---|
| Extracts | Antiproliferative Potency |
| SF1 (100% Hex; 0% EtoAc) | − |
| SF2 (90% Hex; 10% EtoAc) | − |
| SF3 (80% Hex; 20% EtoAc) | − |
| SF4 (70% Hex; 30% EtoAc) | + |
| SF5 (60% Hex; 40% EtoAc) | ++ |
| SF6 (50% Hex; 50% EtoAc) | ++ |
| SF7 (40% Hex; 60% EtoAc) | +++ |
| SF8 (30% Hex; 70% EtoAc) | ++ |
| SF9 (20% Hex; 80% EtoAc) | ++ |
| SF10 (10% Hex; 90% EtoAc) | + |
| SF11 (0% Hex; 100% EtoAc) | − |
| SF7SF1 (100% Hex; 0% EtoAc) | − |
| SF7SF2 (80% Hex; 20% EtoAc) | − |
| SF7SF3 (60% Hex; 40% EtoAc) | − |
| SF7SF4 (55% Hex; 45% EtoAc) | ++ |
| SF7SF5 (50% Hex; 50% EtoAc) | ++ |
| SF7SF6 (45% Hex; 55% EtoAc) | +++ |
| SF7SF7 (40% Hex; 60% EtoAc) | +++ |
| SF7SF8 (35% Hex; 65% EtoAc) | +++ |
| SF7SF9 (30% Hex; 70% EtoAc) | +++ |
| SF7SF10 (0% Hex; 100% EtoAc) | − |

*A portion of the CP extract was fractionated two times by gel chromatographic analysis using silica gel. The elution solvents that were used were n-hexane (Hex) and ethyl acetate (EtoAc). All fractions were analyzed by MDA-MB-231 cells as described in detail in the Materials and Methods section. Cells were treated with a fixed concentration (20 μg/mL) of fraction for 72 h. The antiproliferative potency was evaluated: −, non-antiproliferation activity; +, 10–20% antiproliferation activity; ++, 30–40% antiproliferation activity; ++++, 50–70% antiproliferation activity; ++++, 80–100% antiproliferation activity.
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(d), 133.2 (s), 131.0 (d), 131.0 (d), 128.1 (d), 128.1 (d), 107.0 (d), 106.3 (s), 101.1 (d), 95.7 (d) data from chrysin.

**Analytical Conditions for High-Performance Liquid Chromatography.** The chemical compositions of chrysin and CAPE in the nine CP sample extracts were analyzed using reversed phase preparative HPLC. The separation conditions were as follows: column, Luna Phenomenex C18 (250 mm x 4.6 mm; USA); mobile phase, methanol:water (55:45); flow rate, 1.0 mL/min; detection, UV 280 nm; injection volume, 20 µL.

**Cell Culture and Determination of Cell Number.** MDA-MB-231 human breast cancer cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in L-15 medium (10% FBS, 1% penicillin-streptomycin, and 2 mM l-glutamine) in a humidified 37 °C incubator with no CO₂. The generic substrate was a fluorogenic HDAC9 substrate (1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol. The free radical scavenging capability can be detected spectrophotometrically by a decrease in absorbance at 517 nm, which transforms the deep violet DPPH radical into its pale yellow nonradical form. The mixtures of chrysin/CAPE and DPPH were shaken vigorously and left undisturbed at room temperature for 30 min in the dark. Absorbance at 517 nm was measured using methanol as a blank. The free radical scavenging activity was indicated by EC₅₀ (the concentration required to scavenge 50% of free radicals). Results are expressed as the mean ± standard deviation (SD). The free radical scavenging activity of DPPH was calculated using the following equation:

\[
\text{scavenging effect} = \left[1 - \frac{A_{517 \text{ of sample}} - A_{517 \text{ of control}}} {A_{517 \text{ of control}}} \right] \times 100%
\]

**Analysis of the Cell Cycle.** MDA-MB-231 cells (1.0 x 10⁶) in a 100-mm dish were treated with various concentrations of chrysin (10, 20, and 40 µM), CAPE (5, 10, and 20 µM), and suberoylanilide hydroxamic acid (SAHA) at concentrations of 1.25, 2.5, and 5.0 µM for 48 h. Cells were trypsinated and collected in ice cold PBS. The cells were resuspended in 200 µL of PBS and fixed by adding 800 µL of iced 100% ethanol then incubating overnight at -20 °C. The cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 1 µg/mL RNase A), and incubated at 37 °C for 30 min. One milliliter of propidium iodide (PI) solution (50.0 µg/mL) was added, and the mixture was allowed to stand at 4 °C for 30 min. Cellular DNA content was then analyzed using FACScan cytometry (Becton Dickinson).

**HDAC Activity.** Determination of the enzymatic activity of the HDACs 1–11 was performed by Reaction Biology Corp. (Malvern, PA, USA). Chrysin and trichostatin A (TSA, a pan-HDACi, used as the positive control) were tested in the platform. Both compounds were assayed in serial dilution from 100 µM to 0.0005 µM (TSA). The generic substrate was a fluorogenic peptide from p53 residues 379 to 382 RHKK(Ac), and the HDAC8 substrate was a fluorogenic peptide from p53 residues 379 to 382 RHK(Ac)K(Ac). The class IIA substrate was a fluorogenic HDAC9 class 2A substrate Boc-Lys (trifluoroacetyl)-AMC. The fluorescence intensity was measured using a fluorometric reader with excitation at 360 nm and emission at 460 nm.

**Western Blotting.** MCF-7 cells (1.5 x 10⁶) on 100-mm dishes were treated with chrysin and SAHA at a fixed concentration for 1, 2, and 4 h. After treatment, cells were collected and resuspended in 100 µL of gold lysis buffer. Equal amounts of protein (30.0 µg) were mixed with 2x sample buffer and resolved by 12.5% SDS–PAGE for the detection of β-actin, p21, and Ac-histone 3. Proteins were electrotransferred to an immobilon membrane (PVDF; Millipore Corp.). This was followed by blocking with a solution composed of 20 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, and 3% BSA overnight. Specific antibodies were used anti-Ac-histone 3 (1:1000, rabbit polyclonal; Cell Signaling Technology, Inc.), anti-p21 (1:1000, mouse monoclonal; BD Pharmingen Technology, Inc.), and anti-β-actin (1:5000, mouse monoclonal; Cell Signaling Technology, Inc.). These proteins were detected using chemiluminescence (ECL, Amersham).

**Real Time Quantitative PCR Analysis.** MDA-MB-231 cells were treated with chrysin (40 µM to 60 µM) or SAHA (5.0 µM) for 24 h. Cells were lysed, and tRNA was extracted using a Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, and USA). The real time quantitative PCR (Q-PCR) primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA) based on GenBank sequence data. Each real time quantitative PCR reaction contained 30 ng of cDNA, each primer at 100 nM, and 7.5 µL of SYBR Green PCR Master Mix (1-step kit, Applied Biosystems) in a total volume of 15 µL. Each sample was tested 3 times on an Applied Biosystems Prism 7500 Fast Sequence Detection System, and the relative mRNA levels were calculated using the comparative threshold cycle method with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal control. Multiple PCR was performed using the following primers for humans: p21 forward, 5′-CAGACCAAGCAGACGAATTC-3′, and reverse, 5′-TTAAGGCTTCTCCTTGAGA-3′; CTP synthase (CTPS) forward, 5′-TGCAATTGGCAGTGTGA-3′, and reverse, 5′-TGTCAGCCGACACCGAGTA-3′; and GAPDH forward, 5′-ATGCTGGCCTGTAACGT-3′, and reverse, 5′-AGGCC-CAGCTTCTCCAT-3′. After the reverse transcription step at 48 °C for 30 min, the enzyme activation reaction was performed at 95 °C for 10 min. Initial denaturation was performed at 95 °C for 15 s; annealing/extension steps were performed at 60 °C for 1 min. Forty cycles were performed.

**Xenograft Model.** Twenty one female BALB/c nude mice (5 weeks old) were subcutaneously injected with MDA-MB-231 cells (5 x 10⁶ cells/mouse) suspended in 2.40 mL L15/10% FBS and mixed with 0.60 mL Matrigel (BD Bioscience, Bedford, MA, USA) in a 15 mL tube. After 2 week to 3 week of treatment, tumors (approximately 100 mm³ in size) were apparent in all mice. Animals were then allocated at random to one of 3 groups (n = 7). Two groups of nude mice were orally administered chrysin (45 mg/kg/day and 90 mg/kg/day). The other group of nude mice was treated with vehicle as the control group. Mice were treated every day for 42 days with chrysin or the vehicle. The tumor size was measured on a weekly basis and tumor weight was calculated after the mice had been sacrificed.

**Densitometric Analyses of Protein Expression.** Western blot data were scanned using a BioImaging system (UVP, Inc., CA). In each case, bands were subjected to multiple exposures to ensure that the band density was in the linear range. The data are presented in terms of fold change relative to that of the internal control (β-actin) for each treatment. All presented data were derived from 2 or 3 independent experiments with similar results.

**Statistical Analysis.** Real time quantitative PCR analysis results were presented as the mean ± SD. Student’s t-test was used to calculate the differences between each group and the control group. Statistically significant was defined as p < 0.05.

**RESULTS**

**Purification and Identification of Chrysin from CP.** Previous studies on CP isolated and characterized several of its...
biologically active components. The present study aimed to isolate and identify the major anticancer ingredients of CP. Following chromatographic separation of the CP extracts, fractions were assayed for their effects on human MDA-MB-231 breast cancer cell proliferation. Table 1 presents the antiproliferative activities of the fractions. The SF7 fraction displayed higher antiproliferative activity than the other fractions. Separation of SF7 using a second silica column and analysis of the fractions obtained revealed SF7SF6, SF7SF7, SF7SF8, and SF7SF9 as potent cell growth inhibitors as shown in Figure 3.

Figure 3. Isolation of chrysin and analysis of CP extracts using HPLC. (a) Isolation and identification of chrysin from several fractions using HPLC. (b) The chemical compositions of chrysin and CAPE in 9 samples of CP extracts were analyzed using HPLC. The conditions were Luna Phenomenex column (C18, 250 × 4.6 mm); solvent system, methanol/water (55:45); flow rate, 1.0 mL/min; detection, UV 280 nm; and injection volume, 20 μL. The details are described in Materials and Methods.
in Table 1. HPLC was then applied to analyze their chemical compositions. As shown in Figure 3a, these fractions had a common major peak at 16.5 min. Further separation and purification of the SF7F7 fraction, using reversed phase preparative HPLC, collected fractions with retention times of 16.5 min as shown in Figure 3a. Following purification, the major peak, chrysin, could be isolated and identified as shown in Figure 2.

**Standardization of CP from Nine Samples Collected from Different Locations in China.** Standardization and characterization of propolis were two very important topics. Analysis of the nine CP extracts using HPLC revealed that their HPLC profiles were similar, as shown in Figure 3b. The nine CP samples, therefore, had similar chemical compositions. The common major peak (retention time of 44.0 min) was chrysin, the minor peak (retention time of 49.0 min) was CAPE as shown in Figure 3b. The CP samples collected from Hunan, Hubei, Anhui, Beijing, and Gansu contained high levels (>4.0%) of chrysin than the other CP samples, as shown in Table 2. The CP collected from Gansu contained higher levels of chrysin than CP collected from all other locations. The CP collected from Henan, however, contained lower levels of chrysin than CP collected from all other locations. The CP samples collected from Hubei, Anhui, Henan, Beijing, and Gansu contained high levels (>1.0%) of CAPE than the other CP samples as shown in Table 2. The CP collected from Hunan contained lower levels (0.08%) of CAPE than CP collected from all other locations. This result shows that the levels of chrysin are higher than those of CAPE in these 9 samples we collected. On the other hand, chrysin was present at low levels in Brazilian propolis. CAPE was not detectable in Brazilian propolis as shown in Table 2. Despite these minor variations, the results indicated that chrysin might be a suitable candidate for the standardization of CP; it is the active ingredient and also the major substance occurring in CP collected from all of the sources.

Table 3. Extraction of Percentage, Total Phenolic Content, and Radical Scavenging Activity of the Chinese Propolis Extracts

<table>
<thead>
<tr>
<th>locations</th>
<th>extract dry weight (%)</th>
<th>total phenolic content (mg)</th>
<th>total phenolic content (mg)</th>
<th>DPPH radical scavenged (EC50 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anhui (2006)</td>
<td>68.5</td>
<td>339</td>
<td>317</td>
<td>27.0 ± 3.0</td>
</tr>
<tr>
<td>Beijing (2006)</td>
<td>74.8</td>
<td>334</td>
<td>312</td>
<td>25.5 ± 2.2</td>
</tr>
<tr>
<td>Hunan (2009)</td>
<td>53.8</td>
<td>184</td>
<td>146</td>
<td>58.4 ± 5.0</td>
</tr>
<tr>
<td>Ningxia (2010)</td>
<td>53.7</td>
<td>265</td>
<td>235</td>
<td>26.4 ± 2.0</td>
</tr>
<tr>
<td>Gansu (2010)</td>
<td>71.0</td>
<td>323</td>
<td>300</td>
<td>29.4 ± 3.5</td>
</tr>
<tr>
<td>Jiangsu (2009)</td>
<td>60.9</td>
<td>194</td>
<td>194</td>
<td>70.0 ± 6.2</td>
</tr>
<tr>
<td>Hubei (2009)</td>
<td>79.6</td>
<td>249</td>
<td>218</td>
<td>47.5 ± 5.1</td>
</tr>
<tr>
<td>Henan (2010)</td>
<td>49.3</td>
<td>303</td>
<td>277</td>
<td>37.5 ± 2.6</td>
</tr>
<tr>
<td>Guangxi (2010)</td>
<td>65.5</td>
<td>260</td>
<td>233</td>
<td>49.1 ± 4.6</td>
</tr>
</tbody>
</table>

Values are expressed as mg of dry extract/1000 mg x100% of raw propolis. 
Values are expressed as mg catechin equivalent per gram of propolis extract. 
Values are expressed as mg quercetin equivalent per gram of propolis extract. 
Values are expressed as the mean ± standard deviation. CAPE was used as a positive control with EC50 at a concentration of 8.0 μg/mL.

in Table 1. Article propolis. A gram of propolis extract. The concentration of 8.0 propolis extract. shown in Figure 3b. The CP samples collected from Hunan, the minor peak (retention time of 49.0 min) was CAPE as common major peak (retention time of 44.0 min) was chrysin, CP samples, therefore, had similar chemical compositions. The common major peak, chrysin, could be isolated and identified as shown in Figure 3a. Following purification, the major peak, chrysin, could be isolated and identified as shown in Figure 2. Chrysin, the major peak of propolis, is the active ingredient and also the major substance occurring in CP collected from all of the sources.

**Total Phenolic Content of CP Samples.** Table 3 shows the dry extract yields, total phenolic contents, and DPPH free radical scavenging activities of the nine different CP samples. The yield of dry extract ranged from 49.3% to 74.8%. CP collected from Beijing had a higher dry extract yield (74.8%) than the samples collected from other locations. The CP collected from Henan (49.3%), however, contained a lower yield than the samples collected from other locations. The average dry extract yield of the nine samples was 64.1%. As shown in Table 3, the two standards catechin and quercetin determined the total phenolic content of the CP extracts. Using catechin as a standard provided, we determined a total average phenolic content of the CP extracts of 276.1 mg (catechin equivalent per gram of propolis extract). Using quercetin as a standard provided a total average phenolic content of CP extracts of 248.0 mg (quercetin equivalent per gram of CP extract). The two different standards, therefore, provided similar results for total phenolic content. Several investigators have suggested that quercetin is an appropriate standard for the determination of total phenolic content of CP. The CP collected from Anhui, Beijing, and Gansu demonstrated the highest total phenolic content (>300 mg). The CP samples...
collected from Jiangsu and Hunan demonstrated the lowest total phenolic content. Evaluations of the DPPH free radical scavenging activities of the nine CP extracts identified the CP samples collected from Anhui, Beijing, Ningxia, and Gansu as displaying higher potency DPPH free radical scavenging activities ($EC_{50} < 30 \mu g/mL$) than those of the other CP samples. The CP collected from Hunan and Jiangsu displayed poor antioxidant activity. Among the CP extracts analyzed, those with higher total phenolic content also showed stronger free radical scavenging activity.

**Free Radical Scavenging Activity of Chrysin.** Figure 4 displays the free radical scavenging activities of various concentrations (1 $\mu g/mL$ to 32 $\mu g/mL$) of chrysin and CAPE, presenting the $EC_{50}$ values. CAPE extracts exhibited stronger free radical scavenging activity than chrysin. The $EC_{50}$ value of CAPE was 10 $\mu g/mL$ (34 $\mu M$); that of chrysin was 16 $\mu g/mL$ (64 $\mu M$). Although chrysin exerts antioxidant activity, its DPPH free radical scavenging activity was, therefore, lower than that of CAPE.

**Inhibition of Human MDA-MB-231 Breast Cancer Cell Growth by Chrysin.** Investigation of the antiproliferative effects of chrysin on human MDA-MB-231 breast cancer cells involved the treatment of cells with a fixed concentration (40.0 $\mu M$) of chrysin for 24 to 72 h. Chrysin markedly suppressed MDA-MB-231 cell growth in a dose-dependent manner (Figure 5b) and induced dendrite outgrowth in the MDA-MB-231 cells (Figure 5c). Morphological features of cell shrinkage and plasma membrane blebbing did not occur in chrysin-treated cells. This suggested that the cells might not have died and that cell growth

![Figure 5](image-url)
suppression might have occurred through the induction of differentiation.

**Chrysin Is a HDAC8 Inhibitor.** The trypan blue exclusion assay identified that chrysin significantly inhibited cell growth but did not induce cytotoxic effects in MDA-MB-231 cells. The present study evaluated the HDAC inhibitory activity of chrysin by testing the enzymatic activities of HDACs following treatment with chrysin. As shown in Table 4, chrysin treatment inhibited only HDAC2 and HDAC8 enzymatic activities. The enzymatic activities of the other HDACs did not change significantly. Our data demonstrated that chrysin is a potent HDAC8 inhibitor with an EC$_{50}$ value of 40.2 μM. TSA

**Figure 6.** Effects of chrysin on cell cycle regulation. Flow cytometric analysis of chrysin-, CAPE-, and SAHA-treated MDA-MB-231 cells stained with propidium iodide (PI) as described in Materials and Methods. Following the flow cytometric analyses, the cellular DNA profiles were analyzed using Cell Quest software.

**Figure 7.** Western blot analysis showing that chrysin significantly upregulated p21 protein expression. The MDA-MB-231 cells were treated with chrysin (40.0 μM) and SAHA (5.0 μM) for 1, 2, and 4 h. Cell lysates were prepared and subjected to SDS–PAGE and immunoblotting using antibodies specific for p21, Ac-histone 3, and β-actin.
protein expression. Chrysin, however, did not significantly indicated that SAHA significantly upregulated Ac-Histone 3 protein expression. Previous research has demonstrated that HDAC8i had minimal effects on the upregulation of Ac-Histone 3 protein expression, while inhibition of HDAC1, -2, and -3 markedly upregulated Ac-Histone 3 or Ac-Histone 4 protein expression. Next, quantitative PCR evaluated p21 (waf1/cip1) and CTPS synthase 1 (CTPS) gene expression following treatment with chrysin (40 μM and 60 μM) and suberoylanilide hydroxamic acid (SAHA, ZOLINZA/Vorinostat [5.0 μM]) for 24 h. As shown in Figure 8, p21 (waf1/cip1) gene expression was significantly upregulated after treatment with chrysin at concentrations of 40 μM and 60 μM. However, only a subtle difference was observed when treating cells with SAHA under the same condition (Figure 8a). After cell treatment with chrysin and SAHA, CTPS gene expression markedly decreased as shown in Figure 8b. These data suggested that chrysin might function as an HDAC8 inhibitor.

**Chrysin Inhibits Tumor Growth in Mice.** Immunodeficient mice received a once daily oral dose of chrysin for 42 days. As shown in Figure 9a, their body weight did not significantly change following treatment with various doses of chrysin. Figure 9b shows that tumor sizes significantly decreased 2 weeks to 6 weeks after chrysin treatment (90 mg/kg/day). Tumor morphologic characteristics are shown in Figure 9c and Figure 9d displays the tumor weights, analyzed using Student’s t-test. The activity displayed by chrysin in this MDA-MB-231 xenograft model suggests that it might have potential use in the development of novel HDAC8i for targeted cancer therapies.

### DISCUSSION

Previous studies from our group isolated and characterized 10 prenylated flavonoids from Taiwanese green propolis (TGP, “Pacific propolis”). The present study is the first one to investigate CP, isolate and identify the compound chrysin in CP samples, and determined its chemical structure and biological activities. The chemical composition data from the previous studies showed that TGP contains high levels of propolins A to J; these have not been identified in CP previously. The botanical origins of TGP and CP differ; TGP is derived from Macaranga tanarius; and CP is from Populus nigra. The amount of TGP produced is greater than 2.0 tons per year, while the amount of CP produced is greater than 350 tons per year. CP is, therefore, produced in higher amount than TGP and plays an important role within the field of propolis research.

Ingredients from various propolis samples have displayed potential for use in the development of novel drugs. The color of CP is dark brown, and its main botanical origin is the black poplar (*Populus nigra*). CP contains typical poplar bud phenolic substances. Figure 1 shows the nine CP collection locations for the present study. These locations all lie within the temperate zone. Poplar trees are commonly distributed in the temperate regions and are rarely present in Taiwan (tropical zone). In general, the botanical origin of CP is similar to that of European propolis (EP), which is also derived from the black poplar tree (*Populus nigra*). CP and EP might, therefore, have similar chemical compositions. Standardization of CP is an important issue because of its popular use as a health food raw material. In this study, HPLC analysis of the nine different CP samples provided chemical composition profiles of the CP extracts. Figure 3b shows the nine different CP samples collected from different locations. All samples displayed the
same HPLC profile, and the major peak was identified as chrysin. Chrysin was present at low levels (0.09%) in Brazilian propolis (Table 2). This result suggests that chrysin may be a suitable candidate for the standardization of CP. Chrysin can also serve as an index for distinguishing propolis collected from China or Brazil. CAPE, another famous active ingredient was present at low levels in nine samples of CP. Reports have demonstrated that CAPE was better than chrysin in several biological activities, including antioxidant, anticancer, and anti-inflammatory activities. However, CAPE is not a good candidate for the standardization of CP because CAPE levels from nine CP samples varies over 21.4-fold (0.08% vs 1.71%), as shown in Table 2. However, CAPE was present at low levels in CP; it is not a major contributor to the biological activity of CP extract. Although previous research has described the occurrence of chrysin in CP, no study has identified chrysin as a suitable candidate for the standardization of CP. To standardize CP, a compound must possess three key properties: (a) it must be a potent active ingredient with a spectrum of biological activities; (b) it must occur at high levels in all CP samples collected from different sources in China; and (c) it must be chemically and physically stable. Chrysin fulfilled all of these conditions. Overall, results indicated that chrysin is a better candidate for the standardization of CP than CAPE.

On the determination of total phenolic content, we used two standards to evaluate the total phenolic content, as shown in Table 3. The data demonstrated that total average phenolic content (276.1 mg vs 248.0 mg) of the CP extract from nine samples did not significantly differ when using these two different standards. In China, quercetin is an appropriate standard used by the government to determine the total phenolic content of CP. Next, we evaluated the DPPH free radical scavenging activities of the nine CP extracts. Table 3 showed that CP samples collected from Anhui, Beijing, Ningxia, and Gansu display higher potency of DPPH free radical scavenging activities (EC<sub>50</sub> < 30 μg/mL) compared to those of others. However, the correlation was poor between DPPH free radical scavenging activity and the content of chrysin or CAPE of the CP extract because many of the active ingredients were present in the CP extract as shown in Table 2. Among the CP extracts analyzed, we can find a correlation between higher total phenolic content and stronger free radical scavenging activity. Chrysin is a potent antioxidant agent, anticancer agent, antiinflammatory agent, and aromatase inhibitor. This study is the first to identify a potential mechanism for its anticancer activity. HDAC8-specific inhibition is shown in Table 4. The EC<sub>50</sub> value of chrysin was 40.2 μM. As shown in Figures 5 and 6, treatment of MDA-MB-231 cells with 40.0 μM chrysin significantly suppressed cell growth and induced cell differentiation (dendrite outgrowth). Flow cytometric data also revealed noncytotoxic effects as shown in Figure 6. The US FDA approved HDACi (SAHA and FK-228) to treat cutaneous T cell lymphoma (CTCL). Both SAHA and FK-228 have been classified as pan-HDACi’s (nonselective HDACi’s). SAHA potently and nonselectively inhibited HDAC1, -2, -3, -8, -6, and -10 with EC<sub>50</sub> values in the nanomolecular range. Chrysin,
however, is a selective inhibitor of HDAC8. Inhibition of HDAC8 activity might, thus, represent the mechanism by which chrysin exerts its antiproliferative effects on cancer cells. The enzyme HDAC8 is a class I HDAC (HDAC1, -2, -3, and -8). Class I HDACs have ubiquitous distribution in organs or tissues, with the exception of HDAC8. Previous research has identified that HDAC8 is associated with smooth muscle differentiation and indicated that it could potentially provide a target for brain cancer therapy.

This study evaluated the antitumor activity of chrysin in a nude mice xenograft model implanted with MDA-MB-231 cells. As shown in Figure 9, in vivo data indicated that mice receiving an oral dose of chrysin (90 mg/kg/day) showed significantly suppressed tumor growth in comparison with that of untreated mice. A low dose of chrysin (45 mg/kg/day), however, did not significantly suppress tumor growth in comparison with that of the controls. These data suggest that orally administered chrysin might enter the blood circulation and then reach the tumor tissue, where it suppresses MDA-MB-231 cell proliferation. Orally administered chrysin potently inhibits tumor growth in mice. The mechanism by which chrysin exerts anticancer or antitumor activities might be the inhibition of HDAC8 enzymatic activity.

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


(10) Bankova, V. Chemical diversity of propolis and the problem of standardization. *J. Ethnopharmacol.* 2005, 100, 114–117.


(26) Chen, C. N.; Weng, M. S.; Wu, C. L.; Lin, J. K. Comparison of radical scavenging activity, cytotoxic effects and apoptosis induction in


