Integrative Cancer Therapies
Volume 23: 1–12

DOI: 10.1177/15347354241237519
journals.sagepub.com/home/ict
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Anti-proliferative Effects of Pinocembrin Isolated From *Anomianthus dulcis* on Hepatocellular Carcinoma Cells

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Abstract

Background: Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer. *Anomianthus dulcis* (Dunal) J.Sinclair (syn. *Uvaria dulcis*) has been used in Thai traditional medicine in various therapeutic indications. Phytochemical constituents of *A. dulcis* have been isolated and identified. However, their effects on liver cancer and the associated mechanisms have not been elucidated. **Methods:** Dry flowers of *A. dulcis* were extracted using organic solvents, and chromatographic methods were used to purify the secondary metabolites. The chemical structures of the pure compounds were elucidated by analysis of spectroscopic data. Cytotoxicity against HCC cells was examined using SRB assay, and the effects on cell proliferation were determined using flow cytometry. The mechanisms underlying HCC inhibition were examined by molecular docking and verified by Western blot analysis. **Results:** Among 3 purified flavonoids, pinocembrin, pinostrobin, and chrysin, and 1 indole alkaloid (3-farnesylindole), only pinocembrin showed inhibitory effects on the proliferation of 2 HCC cell lines, HepG2 and Li-7, whereas chrysin showed specific toxicity to HepG2. Pinocembrin was then selected for further study. Flow cytometric analyses revealed that pinocembrin arrested the HCC cell cycle at the G1 phase with a minimal effect on cell death induction. Pinocembrin exerted the suppression of STAT3, as shown by the molecular docking on STAT3 with a better binding affinity than stattic, a known STAT3 inhibitor. Pinocembrin also suppressed STAT3 phosphorylation at both Tyr705 and Ser727. Cell cycle regulatory proteins under the modulation of STAT3, namely cyclin D1, cyclin E, CDK4, and CDK6, are substantially suppressed in their expression levels. **Conclusion:** Pinocembrin extracted from *A. dulcis* exerted a significant growth inhibition on HCC cells *via* suppressing STAT3 signaling pathways and its downstream-regulated genes.

Keywords

cytotoxicity, flavonoid, pinocembrin, hepatocellular carcinoma, liver cancer

Submitted September 21, 2023; revised January 20, 2024; accepted February 20, 2024

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and was the third cause of death by cancer in 2020.[1] Standard treatments of HCC include surgical resection, targeted therapy, and chemotherapy, which are fairly effective at the early stage, where tumors are localized within the liver. However, HCC is naturally aggressive, while the gradual progression is usually silent.[2] Most patients present at the advanced stages with multiple tumors within the livers and have a local invasion or distant metastasis. Most therapeutic modalities have influential roles only for palliative purposes at this stage.[3] Local chemotherapy by transarterial chemoembolization and systemic chemotherapy may also help improve survival; however, undesired adverse effects are unavoidable. Therefore, alternative therapeutic options are always needed to improve the treatment outcomes for patients who were unsuccessful with standard treatments.

Anomianthus dulcis (Dunal) J.Sinclair (synonym *Uvaria dulcis* or "Nom Maew Son" in Thai) is a large climbing shrub belonging to the Annonaceae family. It is indigenous to Southeast Asia and can be found in woods or grasslands of the Northeast of Thailand.[4,5] The flowers are salmon-pink, with a strongly sweet smell, and the fruit is edible

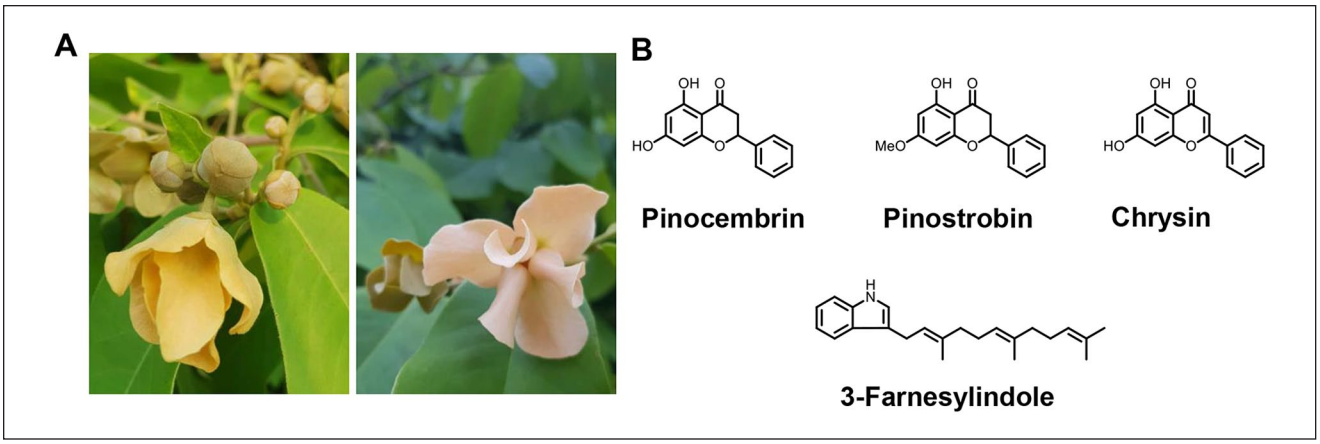

Figure 1. Anomianthus dulcis and the isolated secondary metabolites. The flowers of *A. dulcis* (A) were extracted using organic solvents. The most abundant secondary metabolites, which were used in the downstream experiments, consist of 3 flavonoids and 1 indole alkaloid (B).

(Figure 1A). The decoction of its stems and roots is used as an antipyretic and galactagogues in traditional Thai medicine.[5] The different parts of *A. dulcis* are rich sources of various phytochemicals with a wide range of bioactivities, for example, alkaloids,[4-6] flavonoids,[4,7] phenolic compounds,[4,7,8] and terpenoids.[4,7] Examples of the bioactivities of the phytochemicals obtained from *A. dulcis* are antiplasmodial,[5] antispasmodic,[7] and cytotoxic effects against oral squamous cell carcinoma,[5] lung adenocarcinoma,[4] colon adenocarcinoma,[4] and cervical cancer cell lines.[4]

In our previous study, we reported 11 pure compounds isolated from the extracts of dried flowers of *A. dulcis*.[4] Among those purified compounds, 3 flavonoids, that is, pinocembrin, pinostrobin, and chrysin, together with 1 indole alkaloid, namely 3-farnesylindole, were the most abundant (Figure 1B). Their bioactivities are, however, less known, especially for the cytotoxicity against liver cancer cells. In the present study, the anti-proliferative effects against HCC cell lines were then screened for all candidates. The most effective compound without cell line-specific effects was then selected for a clarification of the underlying molecular mechanisms, which could be useful for further pharmacological developments for anticancer medications.

Materials and Methods

Plant Material, Extraction, and Structure Elucidation of the Isolated Compounds

Plant material, extraction, and structure elucidation were previously reported.[4] Briefly, 1.4 kg of air-dried flowers of *A. Dulcis* were extracted in *n*-hexane, ethyl acetate, and methanol (3 times × 3 L for each) at room temperature, successively. The crude extracts of each organic solvent were then purified using chromatographic methods. The structures of the isolated compounds were elucidated by analysis of spectroscopic data, that is, Fourier-transformed infrared spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. The NMR spectra of the known compounds were compared with previously reported values in the literature.

Species identification of the plant specimen was done by Professor Pranom Chantaranothai, Department of Biology, Faculty of Science, Khon Kaen University. The voucher

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specimen (#RL-041) was deposited in the KKU Herbarium at Khon Kaen University, Thailand.

Cell Lines and Cell Culture

Two HCC cell lines, HepG2 and Li-7, were purchased from the American Type Culture Collection (ATCC) Cell Bank (Manassas, VA) and the RIKEN BioResource Research Center Cell Bank (Tsukuba, Japan), respectively. HepG2 was cultured in Dulbecco Modified Eagle's Medium (Gibco, Carlsbad, CA), while Li-7 was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco). The culture media were supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic (Gibco). Cells were incubated at 37 °C with 5% CO2 in a humidified incubator and subcultured every 3 days or when the confluence reached 80% to maintain logarithmic growth. The protocols for using human-derived cell lines have been reviewed and approved by the Khon Kaen University Ethics Committee for Human Research (HE661358) based on the Declaration of Helsinki and the ICH-Good Clinical Practice Guideline.

Cytotoxic Assay

Cytotoxicity of the compounds was examined with sulforhodamine B (SRB) assay following standard protocol with some modifications as following details.[9] Cells (1×10^3 cells/well)[10,11] were seeded into a 96-well plate and incubated overnight. Then, the media were replaced with media containing various concentrations of the specified compounds and incubated further for 72 hours while 0.3% dimethyl sulfoxide (DMSO) was used as a vehicle control. Once incubation was completed, the media were removed. Then, cells were fixed with 10% cold trichloroacetic acid at 4°C and stained with 0.4% SRB (Gibco). Excessive staining was washed with 1% aqueous acetic acid, solubilized by 1% Tris-base pH 10.5, and measured OD540 using a microplate reader (Tecan, Männedorf, Switzerland). The effects of different concentrations of each tested compound were compared using the one-way ANOVA test.

Cell Cycle Analyses

Cell cycle analyses were performed using the flow cytometric method. Cells (1.5×10^5 cells/well) were seeded in a 6-well plate overnight, treated with the indicated concentrations of pinocembrin, and incubated for 72 hours. At the completed incubation time, cells were trypsinized and fixed with 70% ethanol in phosphate-buffered saline. Cells were stained with propidium iodide (Gibco) and then analyzed using a flow cytometer (BD Biosciences, Franklin Lake, NJ) and FlowJo software (BD Biosciences, Ashland, OR).

Sodium Dodecyl Sulfate Gel Electrophoresis and Western Blots

Western blot analyses were performed using specific antibodies against retinoblastoma protein (RB) (Santa Cruz Biotechnology, Santa Cruz, CA), p-RB (Santa Cruz), cyclin D1 (Cell Signaling, Cambridge, MA), cyclin E (Santa Cruz Biotechnology), cyclin-dependent kinase (CDK) 4 (Biorbyte, Cambridge, UK), CDK6 (Biorbyte), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling), signal transducer and transcription activator 3 (STAT3) (Cell Signaling), p-STAT3 (Tyr705) (Cell Signaling), p-STAT3 (Ser272) (Cell Signaling).

Cells (1.5×10^5 cells/well) were seeded in a 6-well plate overnight, treated with the indicated concentrations of pinocembrin, and incubated for 72 hours. Cell lysates were then collected using a radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Nacalai Tesque, Tokyo, Japan) and a phosphatase inhibitor (Roche, Mannheim, Germany). Total proteins (20-30 μg/well) were resolved in 10% sodium dodecyl sulfate gel electrophoresis and then electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK). The membranes were blocked using 5% skim milk in 1% Tween-20 (TCI, Tokyo, Japan) in Tris-buffered saline (TBST) for 1 hour at room temperature and incubated with primary antibody at 4°C overnight. After washing with TBST, a horseradish peroxidase-conjugated secondary antibody (Cell Signaling) was applied, and the membranes were further incubated for 1 hour at room temperature. The chemiluminescent signals were detected using the enhanced chemiluminescence (ECL) kit (GE Healthcare) and visualized using Image Quant Las 4000 (GE Healthcare, Uppsala, Sweden). Band intensities were quantified by Image J Software (National Institute of Health, Bethesda, MD) for further statistical analysis.

Molecular Docking

The crystal structure of human STAT3 (PDB ID: 1BG1)[12] with some modifications was obtained from a previous study.[13] The 3D structure of pinocembrin and stattic was downloaded from the PubChem database. The residues Lys591, Arg595, Arg609, Glu612, Trp623, and Gln635 of STAT3 were defined as binding sites with a docking sphere radius of 9.5 Å. The protein-ligand complexes were generated using the CDOCKER module implemented in Accelrys Discovery Studio 2.5 program with 100 docking runs. The docked complex with the lowest CDOCKER interaction energy was selected as a representative model.

Molecular Dynamics (MD) Simulation

Partial atomic charges of pinocembrin were calculated with the HF/6-31G(d) level using the Gaussian 09 program.

Missing hydrogen atoms were introduced using the LEaP module in AMBER20. Subsequently, each system (apo form and bound form of STAT3) was immersed in a TIP3P water box, ensuring a distance of 12 Å between the solvation box edge and the protein surface. Hydrogen atoms and water molecules were subjected to minimization using 500 steps of the steepest descent algorithm followed by 1500 steps of the conjugated gradient method. The entire system was then minimized using the same procedures. The AMBER ff14SB force field and the generalized AMBER force field version 2 (GAFF2) were applied for protein and ligand, respectively. A 12 Å short-range cutoff was applied to nonbonded interactions, while long-range electrostatic interactions were handled using the particle mesh Ewald summation method. Subsequently, the systems were gradually heated to 310 K for 200 ps. After that, MD simulations with a time step of 2 fs were performed under the NPT ensemble (310 K and 1 atm) until reaching 50 ns. Principal component analysis (PCA) was performed using the CPPTRAJ module using 1000 snapshots extracted from the last 10 ns of the MD simulation.

Statistical Analysis

All quantitative data were shown as mean ± SD and compared by one-way analysis of variance (ANOVA) test using IBM SPSS ver 26.0. (IBM, Chicago, IL). Statistical significance was considered when $P < .05$.

Results

Pinocembrin Exerted Anti-Proliferative Effects on HCC Cell Lines

Two HCC cell lines were treated with various concentrations of pinocembrin, pinostrobin, chrysin, and 3-farnesylindole to screen for their cytotoxic effects. Among the tested compounds, only pinocembrin exerted anti-proliferative effects on both cell lines (HepG2 and Li-7) in a dose-dependent manner. Chrysin also significantly inhibited the growth of HepG2 ($P < .05$) with a higher dose compared with pinocembrin, while no effect was observed for Li-7 (Figure 2). The IC_{50} of each compound on HCC cell lines is shown in Table 1. As pinocembrin was the only compound that exerted undiscriminating anti-proliferative effects on different types of HCC cell lines, it was selected for further investigation for the underlying mechanisms.

Pinocembrin Arrested Growth and Induced HCC Cell Death

To clarify the anti-proliferative effect of pinocembrin on HCC cells, flow cytometric analyses were carried out using propidium iodide (PI) staining. HCC cells were significantly arrested at the G1 phase ($P < .05$) of the cell cycle after being treated with pinocembrin (Figure 3A and B). In addition to the effects of cell cycle arrest, pinocembrin also showed the induction of cell death in Li-7, as shown in the significant accumulation of cell population in a sub-G1 phase ($P < .05$).

Pinocembrin Suppressed the Expression of G1 Cell Cycle Machinery

Demonstrated by cell cycle analysis, HCC cells were arrested at the G1 phase of the cell cycle, suggesting manipulation of pinocembrin on cell cycle regulatory proteins at this first stage of cell division. Pinocembrin significantly suppressed the expression of cyclin D1, cyclin E, CDK4, and CDK6, the crucial proteins involved in the progression of the cell cycle from the G1 to the S phase, in accordance with the accumulation of total RB protein in HepG2 cells ($P < .05$) (Figure 4). The ratios of phosphorylated RB to the total RB (pRB/RB) appeared to decrease significantly in both cell lines ($P < .05$), suggesting the suppression of CDK4 and CDK6 activities resulting from their down-regulated levels (Figure 4A and B).

Pinocembrin Suppressed STAT3 Phosphorylation In Vitro

As the G1 regulatory proteins were regulated by various signaling pathways in which STAT3 has been shown for its roles in hepatocarcinogenesis and progression,[14,15] whether pinocembrin affects the STAT3 signaling pathway was then investigated. The phosphorylation of STAT3 at both Tyr705 and Ser727 residues was significantly reduced after the treatment by pinocembrin in both HCC cell lines ($P < .05$), as demonstrated in Figure 5A and B.

Prediction of the Binding Mode and Susceptibility of Pinocembrin Toward STAT3 SH2 Domain

To study the mechanisms of how pinocembrin interacts and inhibits the phosphorylation of STAT3, as shown by Western blots, molecular docking simulation and dynamics simulation were performed. As illustrated in Figure 6A, pinocembrin had a more energetically favorable interaction with the SH2 domain of STAT3 (CDOCKER interaction energy = −31.87 kcal/mol) than did the interaction of stattic, a known STAT3 inhibitor[16] (−17.81 kcal/mol). Based on the 2D interaction profile, pinocembrin formed more hydrogen bonds with STAT3 (4 bonds with Glu594, Leu607, Thr622, and Ser636) compared to stattic (2 bonds with Gln635 and Ser636). In addition, van der Waals and alkyl interactions found in the pinocembrin/STAT3 complex (10 interactions

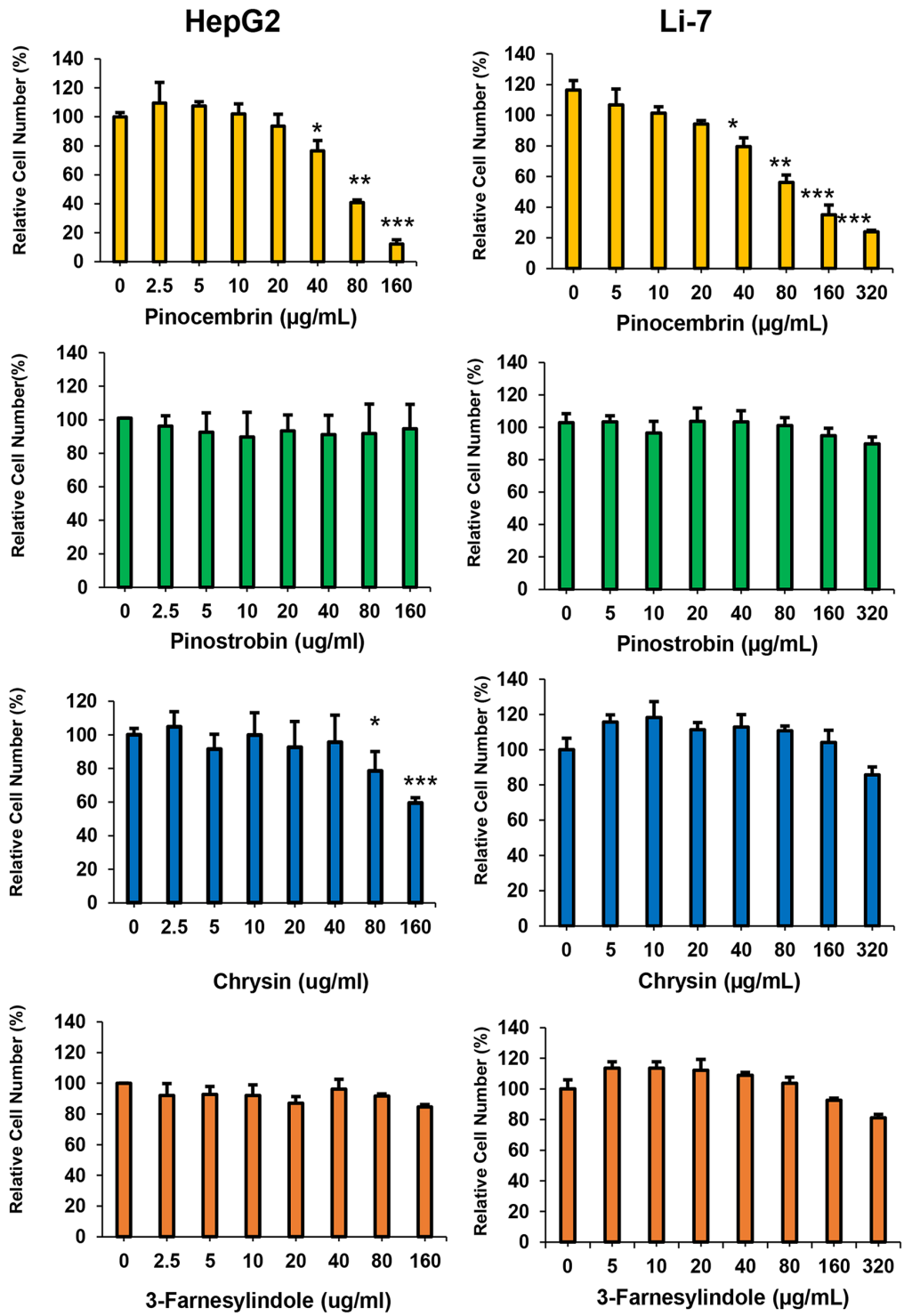

Figure 2. Effects of the isolated compounds from *Anomianthus dulcis* on the proliferation of hepatocellular carcinoma (HCC) cell lines. Among the tested compounds, only pinocembrin exerts significant anti-proliferative effects on both HepG2 and Li-7. A high dose of chrysin inhibits HepG2 cell growth but does not affect the growth of Li-7 cells. Data are shown as mean ± SD of a representative triplicated experiment from 3 biological replications. Statistical analyses were done using the One-way ANOVA test. *$P < .05$, **$P < .01$, ***$P < .001$.

Table 1. Half Maximal Inhibitory Concentration (IC_{50}) of the Isolated Compounds.

	IC_{50} (μg/mL)	
Compound	HepG2	Li-7
Pinocembrin	68.4 ± 2.5	156.7 ± 56.8
Pinostrobin	>160	>320
Chrysin	>160	>320
3-Farnesylindole	>160	>320

with Ile589, Ile597, Leu608, Arg609, Ser611, Thr620, Phe621, Ile634, Gln635, and Val637 residues) were higher than those found in the stattic/STAT3 system (3 interactions with Glu594, Gln633, Ile634). These findings suggested that pinocembrin has the potential to function as a STAT3 inhibitor.

Computational Simulations Suggest Conformational Changes of STAT3 After Binding With Pinocembrin

The structurally relevant motions of STAT3 induced by pinocembrin binding were investigated in comparison with the apoprotein using PCA. As shown in Figure 6B, the first principal component suggested that, as compared to the apo form, pinocembrin binding altered the direction of motions and increased the amplitude of motions in the STAT3 SH2 domain to move closer to the ligand. This effect was particularly notable in the residues located within the pY−X and pY+1 pockets of the SH2 domain. In addition, these conformational changes in STAT3 induced by pinocembrin also affected the movement of the Tyr705 residue, as evidenced by the superimposition of the docked structure with the MD final snapshot (Figure 6C), leading to the inhibition of STAT3 phosphorylation at Tyr705.

Discussion

HCC is an aggressive cancer with the highest incidence among all primary liver malignancies. Although many therapeutic modalities have been developed, a substantial group of patients still develop resistance to the treatment, resulting in a poor quality of life and short survival time. Effective alternative treatments are therefore needed to improve the therapeutic outcomes as well as the quality of living. Complementary and alternative medicines are an option to improve the treatment outcome in either curative or palliative aims of care. In the present study, we report the inhibitory effects of pinocembrin, a natural product isolated from *A. dulcis*—a plant growing in Asia, on the cell cycle of HCC cells. Pinocembrin shows an obvious suppressing effect on STAT3 phosphorylation, which leads to a down-regulation of cell cycle regulatory proteins playing roles in the G1 phase. A schematic summary of the findings in the current study is depicted in Figure 7.

Pinocembrin is a flavonoid that can be isolated from propolis (bee glue) and plants in several families, for example, Piperaceae, Lauraceae, and Asteraceae.[5] It can also be biosynthesized using microorganisms and chemical reactions.[17-19] Our previous study reported a successful isolation of several flavonoids, including pinocembrin, from *A. dulcis*, a species belonging to the Annonaceae family. Pinocembrin is known to display various bioactivities, namely antioxidant, anti-inflammatory, and cytotoxic activities.[20] For the latter, anti-proliferative effects against several cancer cell types were reported, for example, colon cancer,[21,22] non-small cell lung cancer,[23,24] retinoblastoma,[25] and gastric cancer.[26] Although pinocembrin showed non-mutagenicity and no effects on hepatocarcinogenesis in rats induced with a hepatoxic carcinogen,[27] the clarification of anti-proliferative effects on HCC remains to be understood. A computational study reported by Mo et al[28] predicted that pinocembrin might have anti-proliferative effects against HCC without being biologically validated in the same study. A report from Kumar et al[29] demonstrated that the fraction containing pinocembrin extracted from *Elytranthe parasitica* had anti-proliferative effects on HepG2, an HCC cell line, *via* the suppression of the MAPK pathway, resulting in the arrest of G2M and S phases of the cell cycle and the induction of cellular apoptosis. Our study is, therefore, the first report demonstrating the anti-proliferative effects of pinocembrin on 2 different HCC cell lines *via* the inhibition of a common STAT3 pathway. These effects result in suppressing the expression of G1 regulatory proteins, not only cyclin D1 and cyclin E but also their CDK4/6 partners.

In addition to cell cycle arrest, a higher dose of pinocembrin can induce both HCC cell lines to cell death, as shown by the increase in the sub-G1 population. Noteworthy is that Li-7 was more sensitive to the treatment of pinocembrin in the induction of cell death, and fewer cells were arrested at the G1 phase compared with HepG2. The discrepancy in response to pinocembrin might result from the genetic background of each cell line. Li-7 was established from HCC with *TP53* (encoding for p53) and *CDKN2A* (encoding for p16) mutations.[30] Since p53 and p16 play important roles in arresting the cell cycle by inhibiting the functions of the cyclin-CDK complex, the lack of competent functions in these proteins might thus lead to the defect of cell cycle inhibition. Moreover, Li-7 was reported with a lower basal level of phosphorylated pSTAT3 (Tyr705).[30] STAT3 is a known signaling pathway governing the expressions of anti-apoptotic proteins.[14,15] Inhibition of STAT3 phosphorylation in Li-7 could, thereby, lead to more cells undergoing apoptosis compared with HepG2. In line with this, Li-7 was also shown to be more sensitive to tyrosine kinase inhibitors.[30] These results suggested that the major inhibitory effects of pinocembrin on HCC cells in the presented models are likely

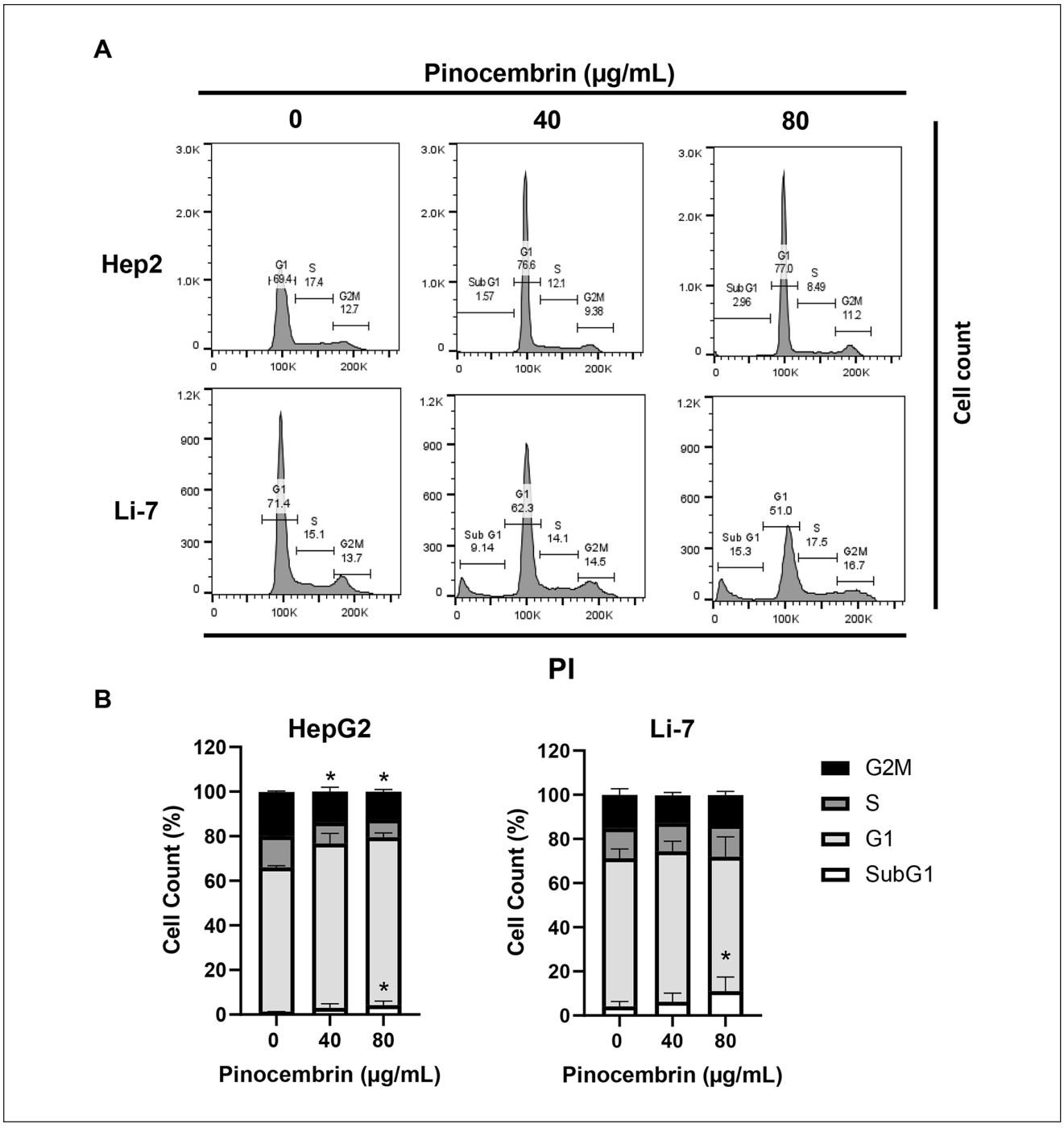

Figure 3. Pinocembrin arrests the growth of hepatocellular carcinoma cells at the G1 phase. (A)Flow cytometric analysis shows the cell cycle arrest of HepG2 and Li-7 at the G1 phase after treatment with pinocembrin. (B)A higher dose of pinocembrin also induces cell death, as indicated by the increase in cell population at the sub-G1 phase. Data are shown as mean ± SD of the average from 3 duplicated biological replications. *$P < .05$.

from the inhibition of STAT3. More investigations, nevertheless, remain needed to fully understand the mechanisms of pinocembrin's actions in HCC.

STAT3 is an emerging pathway responsible for HCC carcinogenesis and progression. It is also a potential and promising protein for HCC-targeted therapy.[14,15] Previous studies showed that pinocembrin can inhibit the phosphorylation of STAT3 at Ser727 in keratinocytes[31] and at Tyr705 in microglial[32] and non-small cell lung cancer cells.[23] In the present study, we showed that pinocembrin

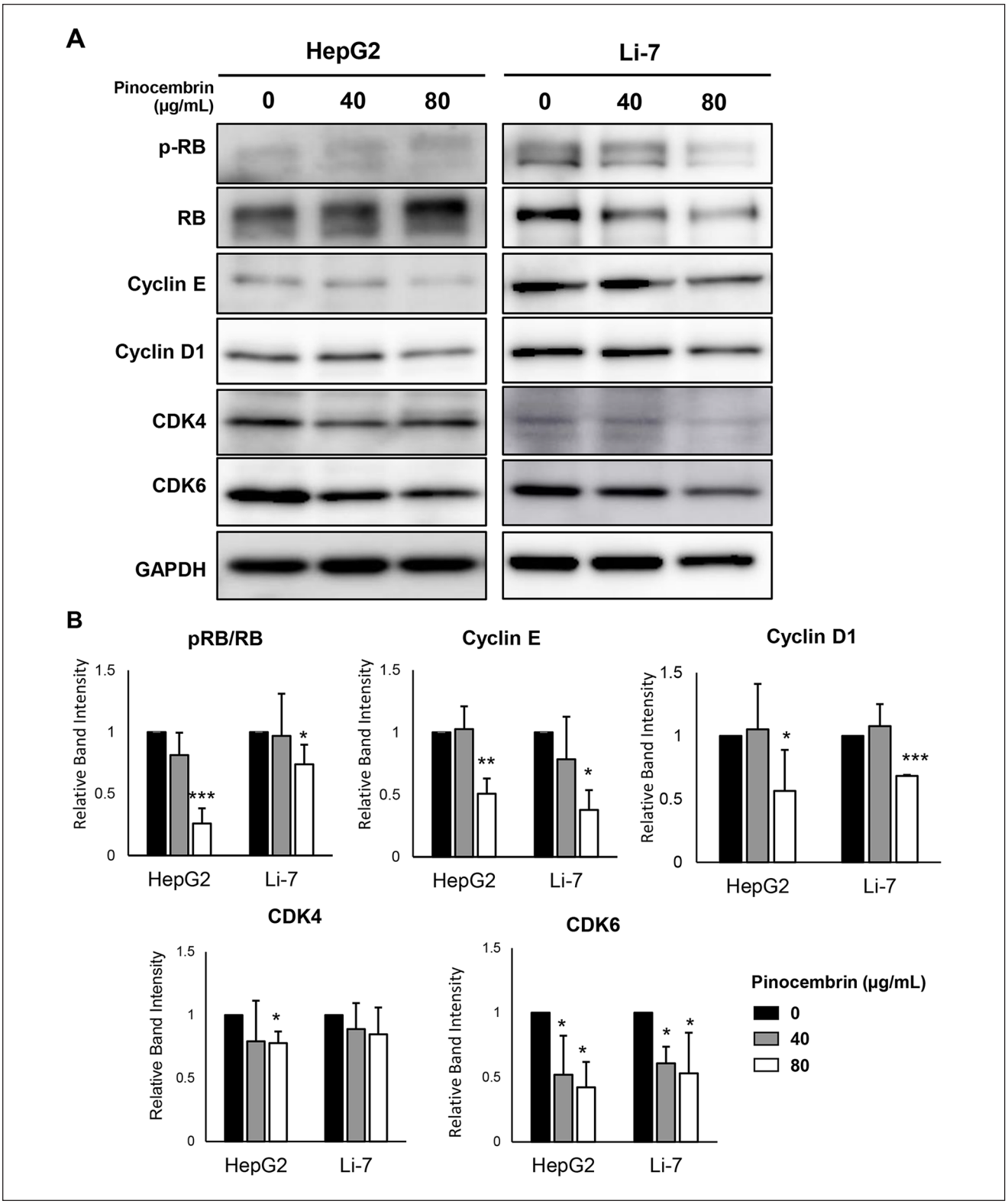

Figure 4. Pinocembrin suppresses the expression of G1 cell cycle machinery. (A)The expression of cyclin D1 and cyclin E, and their cyclin-dependent kinase partners in the G1 phase, CDK4 and CDK6, are significantly suppressed. The down-regulation of G1 cell cycle machinery corresponds to the G1 arrest, as shown by flow cytometry. The Western blot is representative of 3 biological replications with the same trend of results. (B)Graphs show the average band intensities of 3 biological replications, given the control as a factor of 1. Statistical analyses were done using the One-way ANOVA test. *$P < .05$, **$P < .01$, ***$P < .001$.

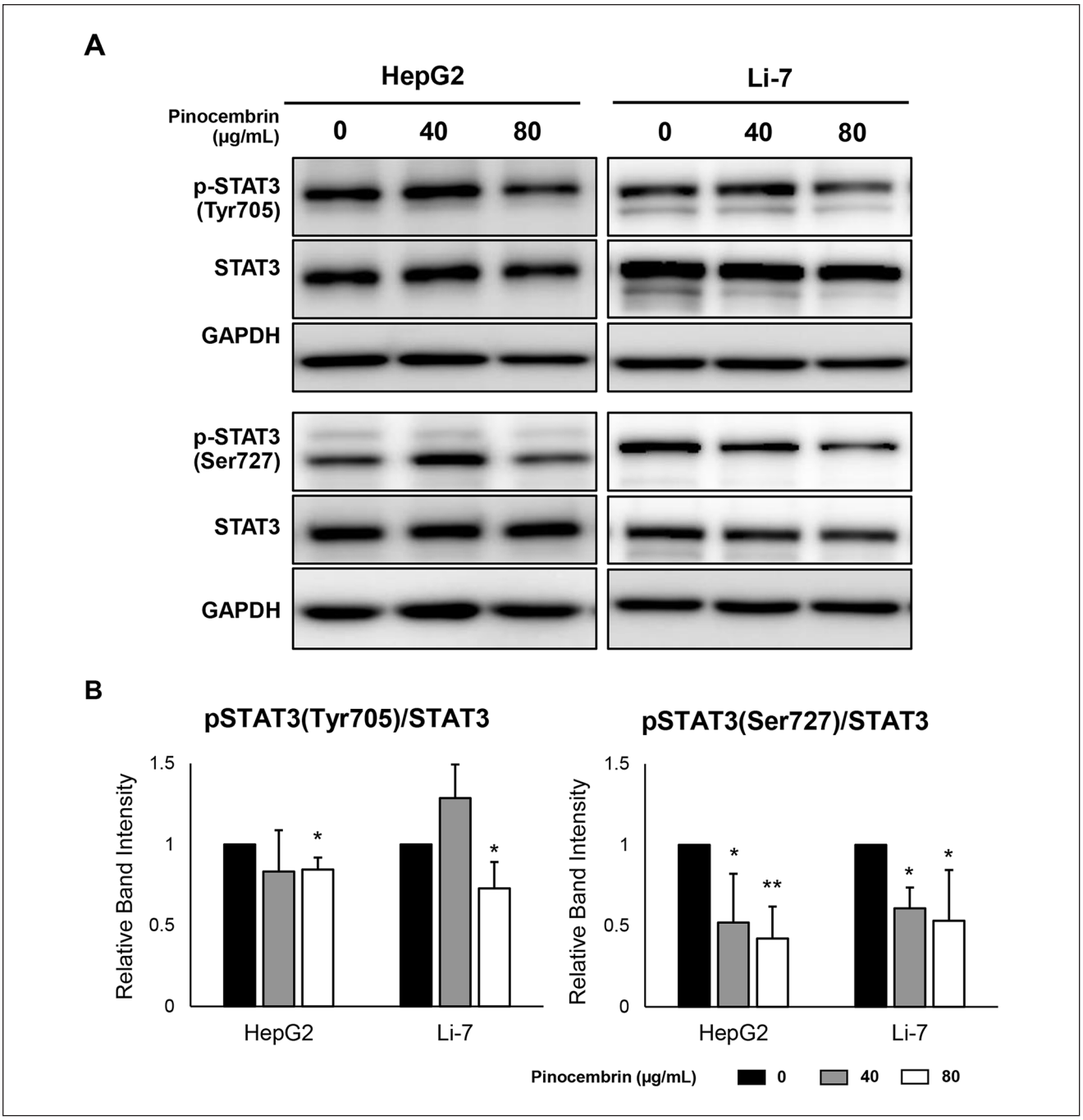

Figure 5. Pinocembrin suppresses the phosphorylation of STAT3 in hepatocellular carcinoma cells. (A)Pinocembrin suppresses the phosphorylation of STAT3 at both Tyr705 and Ser727 residues in HepG2 and Li-7. The Western blot is representative of 3 biological replications with the same trend of results. (B)Graphs show the average band intensities of 3 biological replications, given the control as a factor of 1. Statistical analyses were done using the One-way ANOVA test. *$P < .05$, **$P < .01$.

can inhibit the phosphorylation at both tyrosine and serine residues. Since phosphorylation at both amino acid residues is required for maximal activation and function of STAT3 in cell proliferation and survival,[33] our findings suggest that pinocembrin has the potential to be used as a STAT3 inhibitor as it forms hydrogen bonds, van der Waals, and alkyl interactions with the STAT3 SH2 domain, resulting in conformational change and prevention of phosphorylation at Tyr705. As pinocembrin displayed hepatoprotective effects on normal livers,[34] our study shows that it is a promising compound that should be studied as an alternative treatment for HCC.

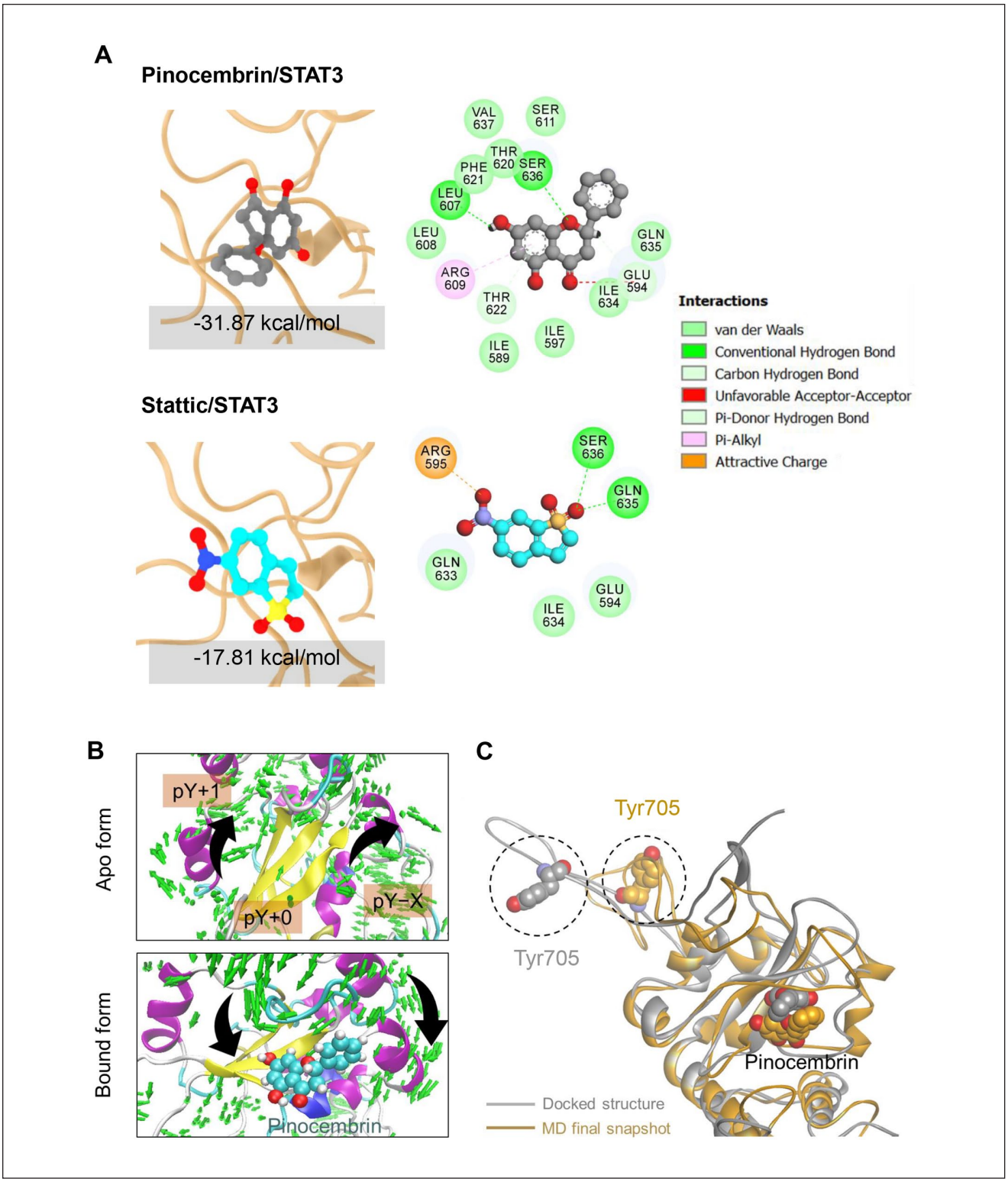

Figure 6. Molecular docking and molecular dynamic simulation of pinocembrin and stattic against STAT3 SH2 domain. (A) Pinocembrin binds to the SH2 domain of STAT3 with a higher binding affinity than stattic. The pinocembrin/STAT3 complex exhibited higher hydrogen bond and alkyl interactions than the stattic/STAT3 system. (B) Porcupine plot of apo and bound forms of STAT3 SH2 domain. The arrow and its length indicate the direction and amplitude of motions. (C) Superimposed structures between the docked structure and the MD final snapshot.

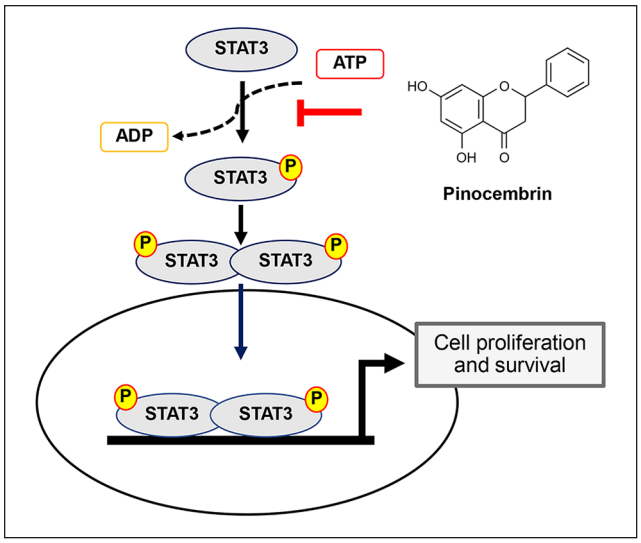

Figure 7. Schematic summary. Pinocembrin suppresses the phosphorylation of STAT3, resulting in the down-regulation of STAT3-downstream target genes, namely cyclin D1, which further inhibits the expression of cyclin E. The inhibition of STAT3 results in the suppression of cell proliferation and survival.

The present study, however, has some limitations. First, the effects of pinocembrin on the other signaling pathways underlying HCC progression have yet to be explored. Second, pinocembrin has some effects on the induction of cell death in Li-7, indicating that additional effects are present in a different cell line. Therefore, pinocembrin should be evaluated, and its mechanism of action should be studied in additional cell lines to clarify the mechanisms of cell death involved. Moreover, the inhibitory effects of pinocembrin on the other cancer hallmarks in HCC, as well as the toxicity toward non-malignant cells, are worth further study. Eventually, *in vivo* experiments should be undertaken to translate anti-proliferative activities into anti-tumor activities, a prerequisite to using pinocembrin in a clinical study.

Conclusions

Pinocembrin isolated from *A. dulcis* exerts anti-proliferative effects on HCC cells *via* inhibition of the STAT3 pathway, resulting in the suppression of the G1 cell cycle machinery and cell cycle arrest. Pinocembrin is, therefore, a promising compound that should be further studied to, perhaps, become an alternative treatment for HCC and improve the therapeutic outcomes.

Acknowledgments

We would like to acknowledge Professor Pranom Chantaranothai, Department of Biology, Faculty of Science, Khon Kaen University, for the taxonomic identification of the plant specimen.

Author Contributions

Conceptualization: CS, RL; Methodology: CS, PM, AK, SW, FTS; Investigation: CS, KT, JT, PM, RL, TC; Formal analysis: CS, PM, AA, SW, RL, TC; Visualization: CS, KT; Computing Resources: TR; Funding acquisition: CS, RL; Writing - Original draft: CS, PM, RL; Writing - Review & Editing: CS, FTS. All authors read and approved the final version of the manuscript.

Availability of Data and Material

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was financially supported by the Fundamental Fund of Khon Kaen University of the year 2565 BE (FF65) with funding support from The National Science, Research, and Innovation Fund (NSRF) of Thailand.

Ethics Approval

Approval from the Khon Kaen University Ethics Committee for Human Research (HE661358).

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

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