Booranasubkajorn, S, Lumlerdkij, N, Chaisri, SMATM, Akarasereenont, P and Prieto Garcia, JM

Effects of the Thai Herbal Wattana Formula and Its Ingredients in the Human Hepatocarcinoma HepG2 Cells: Safety and Efficacy Considerations

Natural Product Communications
Volume 20(10): 1-16

DOI: 10.1177/1934578X251385040
journals.sagepub.com/home/npx

Effects of the Thai Herbal Wattana Formula and Its Ingredients in the Human Hepatocarcinoma HepG2 Cells: Safety and Efficacy Considerations

Suksalin Booranasubkajorn[1], Natchagorn Lumlerdkij[1,2], Sirinapa M.ATM. Chaisri[1], Pravit Akarasereenont[1,3] and Jose M. Prieto Garcia[2,4]

Abstract

Introduction: The Thai Herbal Wattana formula (WNF) is a multi-ingredient remedy used to promote overall health and mitigate age-related physiological degeneration, suggesting a potential adjuvant use in oncological treatments.

Aims: This ethnopharmacological study aimed to evaluate cytotoxic and antimigratory effects of the Ayurveda Siriraj WNF AVS073 variation (ASW) and its active/s botanical constituents in human liver cancer (HepG2) cells.

Methods: ASW and its ingredients were evaluated for cytotoxicity (Alamar blue), anti-migratory activity (2D gap closure). Mechanistic studies included cell death, apoptosis and cell cycle arrest (flow cytometry), and intracellular glutathione levels. Bioguided isolation was used to identify the active compound/s.

Results: ASW did not inhibit HepG2 cell proliferation at 200 μg/mL although it halved intracellular glutathione levels and reduced cell migration similarly to paclitaxel 0.01 nM. In contrast, the water extract from *Biancaea sappan* (syn. *Caesalpinia sappan L.*) (CSL) was the only ingredient showing cytotoxicity (IC50 = 44 μg/ml). It induces apoptosis and G2/M phase cell cycle arrest and significantly reduced 2D cell migration without modifying glutathione levels. Brazilein was dereplicated as the active cytotoxic component in CSL but it did not show any effect on glutathione levels or 2D cell migration.

Conclusion: This preclinical study demonstrates that the ASW formula lacks direct in vitro cytotoxicity towards HepG2 cells, but effectively reduced cell mobility and intracellular glutathione although at non-physiological concentrations. *B. sappan* L., exhibits potent cytotoxic and anti-migratory activities. Brazilein is its primary cytotoxic compound although is not endowed with the glutathione-depleting or anti-migratory effects observed in the ASW formula. These findings suggest that ASW's benefits in oncology may primarily link up with its established immunological and anti-inflammatory effects, while its lack of toxicity to HepG2 cells, a proxy for hepatocytes, plus its clinically proven lack of major adverse effects might indicate a positive safety profile.

Keywords

Cytotoxicity, cell migration, hepatocellular carcinoma, glutathione, herb-drug interaction, flavonoids

Received: June 18, 2025; accepted: September 19, 2025

Introduction

The Thai Herbal Wattana formula (WNF) is a multi-ingredient remedy traditionally employed for its purported benefits in enhancing appetite, promoting overall health, and mitigating age-related physiological degeneration. Clinical evidence supports several potential medicinal applications for WNF. A Phase II clinical trial demonstrated that WNF possessed comparable efficacy to diclofenac tablets in alleviating pain and improving knee function in patients with knee osteoarthritis.[1] Furthermore, WNF has been shown to exert protective effects

[1]Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[2]School of Pharmacy, University College London, London, UK
[3]Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[4]School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, UK

Corresponding Author:
Jose M. Prieto Garcia, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, UK.
Email: J.M.PrietoGarcia@ljmu.ac.uk

against UVA-induced melanogenesis,[2] as well as exhibit immunomodulatory,[3] anti-inflammatory,[3] and anti-amyloidogenic properties.[4]

The precise composition of WNF exhibits variability, with formulations reportedly containing up to 18 distinct ingredients, often with qualitative differences documented over time.[2–5] A set of core herbal ingredients are present in all the variations of this formula namely *Aegle marmelos*, *Boesenbergia rotunda*, *Brachypterum scandens*, *Cinnamomun* sp., *Cladogynos orientalis, Criptolepsis dubia, Cyperus rotundus*, *Ferula assa-foetida*, *Piper nigrum*, *Putranjiva roxburghii*, *Saussurea lappa, Terminalia chebula* and *Tinospora crispa*. Common additions to these are *Caesalpinia sappan*, *Carthamus tinctorius*, *Citrus* × *aurantius, Ligusticum sinense* and *Mallotus repandus*. The complete botanical details of the ingredients used in the recipe used for this work (hereby termed Ayurveda Siriraj WNF AVS073 variation or ASW) are presented in the materials and methods section (Table 1).

While traditional indications suggest that WNF's potential lies in its use as an adjuvant in oncological treatments, its direct anticancer activity remains uninvestigated. Advanced cancer patients frequently experience a range of physical and psychological symptoms, including significant anorexia, which aligns with WNF's traditional use for appetite stimulation. A recent work demonstrated that the in vitro exposure of cytokine-induced killer cells and dendritic cells concluded that WNF upregulated Th1 and Th17, but downregulated Th2 and Treg phenotypes within $CD3^+CD56^+$ cells.[3] This specific immune profile, particularly the balance shift away from Th2 and Tregs towards Th1, is generally associated with a more favorable immune response against liver cancer,[6] the third leading cause of cancer death worldwide.[7]

A survey of the literature unveiled that several of the ingredients such as *Boesenbergia rotunda,*[8] *Biancaea sappan* (syn. *Caesalpinia sappan*),[9,10] *Ligusticum sinense,*[11] and *Aucklacia lappa* (syn. *Saussurea lappa*)[12] are reported as exerting direct cytotoxicity upon HepG2 cells, the most researched liver cancer cell line.[13] Despite its lack of consistent expression of phase 1 enzymes,[14] the HepG2 cell line has been also a popular model for hepatocytes metabolism[15] and toxicology.[16] Consequently, this study primarily aimed to evaluate cytotoxicity and antimigratory effects of botanical constituents of the Ayurveda Siriraj WNF AVS073 variation (ASW) and its active/s botanical constituents in human liver cancer (HepG2) cells.

Materials and Methods

Plant Extract Preparation

All plant materials (Table 1) were authenticated according to the Thai Herbal Pharmacopoeia,[17] cleansed by rinsing under purified water, dried in air-circulating ovens at 40 °C, grounded and sifted into fine powders with various pharmaceutical-grade milling equipment all following the in-house PIC/S GMP-certified Standard Operating Procedures of the Ayurved Siriraj Manufacturing Unit of Herbal Medicine and Products (Centre of Applied Thai Traditional Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand)[18] and phytochemical analyses (HPTLC CAMAG system, Switzerland; LC-MS-MS system Xevo TQ-XS, Waters, UK) and characterization of powders for particle size distribution, flowability, and tapped density using a powder flow analyser (TA.HD plus, Stable Micro Systems, Surrey, UK) were performed at the Siriraj Herbal—Drug Examination and Analysis Central Laboratory (SiHAC).[19] Batch certificates may be supplied upon reasonable request to the Office of Applied Thai Traditional Medicine, Piyamaharajkarun Building, seventh Floor (SiPH), Tel. 02-419-1705.

Water extracts were prepared according to Thai traditional methods for decoctions.[17] Briefly, each herbal powder was boiled with bi-distilled water (1:10) until the volume reached about 1/3 of the initial volume. The decoction was then filtered and freeze-dried. The dry extracts were stored in a cool, dry place and protected from light until use. Before cell-based assays, the extracts were prepared into stock solutions in water, filtered through a Millipore© 0.22 µm syringe filter (Merck, UK), and kept at −20 °C until use.

Cell Lines

HepG2 cells (ACC No 85011430, Lot 11C013) were cultured in Minimal Essential Medium (MEM) Alpha supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin in a NuAire© DH Autoflow CO_2 Air-Jacketed incubator (Avidity Science Ltd., UK) at 37 °C/5% CO_2. All cell culture reagents were obtained from Gibco® (Thermo Fisher Scientific Inc., UK).

Cytotoxicity Assay

HepG2 cells (5000 cells/well) were incubated with the extracts, paclitaxel (≥95%, HPLC) from *Taxus brevifolia* (Sigma-Aldrich, UK), or medium as control for 48 h. The cell viability was measured using the AlamarBlue assay as recommended by Miret and co-workers[21] following the instructions of the manufacturer (Bio-Rad Abd Serotec Ltd., UK). The fluorescence intensity was measured at 560/590 nm using a Tecan Infinite© M200 microtiter plate reader (Tecan Trading AG, Switzerland). According to the American National Cancer Institute (NCI) criteria, crude plant extracts are considered cytotoxic if they exhibit an IC50 value less than 100 µg/mL. Extracts demonstrating an of 30 µg/mL or less are deemed particularly promising and warrant further pharmacological and phytochemical investigation The maximum non-toxic concentration (MNTC) was defined as the highest concentration of the compound that resulted in cell viability of >80% (IC20).[22–24] As for pure compounds, the maximum concentration for being considered within pharmacological reasonable limits is 25 µM.[25] In our experiments Paclitaxel was used as a positive reference drug and exhibited an IC50 = 60 nM and an IC20 (MNTC) = 0.01 nM.

Table 1. Summary of the Botanical Identity of the Ingredients of the ASW Formula.

Code	Most used scientific name in literature	Current accepted scientific name[20]	Family	Part
AM	*Aegle marmelos* (L.) Corrêa	=	Rutaceae	Fruit
BR	*Boesenbergia rotunda* (L.) Mansf.	=	Zingiberaceae	Rhizome
CT	*Carthamus tinctorius* L.	=	Asteraceae	Flower
CSL	Caesalpinia sappan L.	*Biancaea sappan* (L.) Tod.	Caesalpinioideae	Heartwood
CSC	*Cinnamomum siamense* Craib.	=	Lauraceae	Wood
CA	*Citrus* × *aurantium* L.	=	Rutaceae	Fruit peels
CO	*Cladogynos orientalis* Zipp.	=	Euphorbiaceae	Root
CD	*Cryptolepis dubia* (Burm.f.) M.R.Almeida	=	Asclepiadaceae	Stem
CR	*Cyperus rotundus* L.	=	Cyperaceae	Root
DS	*Derris scandens* (Roxb.) Benth	*Brachypterum scandens* (Roxb.) Wight & Arn. ex Miq.	Fabaceae	Stem
DR	*Drypetes roxburghii* (Wall.) Hurus. (Euphorbiaceae)	*Putranjiva roxburghii* Wall.	Putranjivaceae	Root
FA	*Ferula assa-foetida* L.	=	Apiaceae	Gum resin
LS	*Ligusticum sinense* Oliv. cv. Chuanxiong	*Conioselinum anthriscoides* (H.Boissieu) Pimenov & Kljuykov	Apiaceae	Root
MR	*Mallotus repandus* (Rottler) Müll.Arg.	=	Euphorbiaceae	Stem
PN	*Piper nigrum* L.	=	Piperaceae	Fruit
SL	*Saussurea lappa* Clark.	*Aucklandia lappa* DC.	Asteraceae	Root
TCH	*Terminalia chebula* Retz. var. *chebula*	=	Combretaceae	Fruit
TCR	*Tinospora crispa* (L.) Hook.f. & Thomson	=	Menispermaceae	Stem

The extracts with IC_{50} below 50 μg/ml were further investigated for apoptosis induction and cell cycle arrest. Maximum non-toxic concentrations (MNTC) were determined from the maximum concentrations with <20% cytotoxic effect on the cells.

Cell Migration Assay

Bidimensional cell migration was measured using Oris™ Universal Cell Migration Assembly Kit (Platypus Technologies LLC, USA).[26] The assay utilizes proprietary stoppers made from a medical-grade silicone to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2-mm diameter unseeded region in the center of each well (the detection zone) into which the cells migrate offering more reproducibility than the classic "scratch assay".[27] For the migration assay, the sterile stoppers were introduced in a flat-bottom 96-well Nunclon™ plate (Thermo Fisher Scientific Inc., UK) before seeding HepG2 cells at a density of 10^5 cells per well. After 24 h of incubation the stoppers were removed, and the cells incubated with the maximum non-toxic concentrations (MNTC) of each treatment to avoid interference with any antiproliferative effect. We used complete cell media as control, and paclitaxel 0.1-0.01 nM (≥95%, HPLC) from *Taxus brevifolia* (Sigma-Aldrich, UK) as positive reference. At the end of the incubation period, the cells were fixed with 100 µl of cold 40% TCA. The plates were kept at 4 °C for 1 h and rinsed with water. The cells were stained by the addition of 50 µl of 0.4% trypan blue and incubated at room temperature for 1 h. Another washing step was performed, and the plate was then left to air-dry completely overnight. Measurement of zone closure was performed by digital images obtained by optical scanning (transmittance mode) by a Snapscan e50 (Agfa-Gevaert, Germany). Image J (National Health Institute, USA) was used to measure an average gap distance value and compare to the vehicle control to monitor the migratory capacity of the treated cells.[28] The migration rate was calculated as follows:

% Migration rate = [Cell-free area at 0 h before treatment − Cell − free area after 24 h treatment]/ [Cell−free area at 0 h before treatment − Cell−free area after 24 h treatment with control (solvent)]

Determination of Glutathione Cellular Levels

HepG2 cells (50,000 cells/ well) were incubated for 48 h with camptothecin 5 µM, WNF 200 µg/ml, or CSL 50 µg/ml. The assay was performed with minor modifications from Allen et al (2000), as well as the calculation.[29] Briefly, HepG2 cells (4×10^4 cells/well) were incubated for 24 h with buthionine sulfoximine (10 µM) or plant extracts (100 µg/mL), then washed with PBS after which 60 µL of 0.1% Triton-X was added to each well of the plates to lyse the cells. Twenty-five microliters of 5% sulfosalicylic acid was added to the cell lysates and plates were shaken for 2 min. Twenty-five microliters of glutathione reaction buffer containing NADPH 2.39 mM, 5-5′-dithiobis(2-nitrobenzoic acid) 0.01 M and 500UI of glutathione reductase in sodium phosphate buffer 143 mM containing EDTA 6.3 mM was added to the cell lysates. Absorbance was read in a kinetic cycle every 30 s for

5 min at 405 nm (11 readings) using a Tecan Infinite© M200 microtiter plate reader (Tecan Trading AG, Switzerland). Absorbances were converted into absolute amounts by means of the i-slopes method using known concentrations of reduced L-glutathione. All chemicals were from Sigma-Aldrich (Merck Ltd, UK).

Cell Death Detection Experiments via Annexin V—Propidium Iodide Staining in HepG2 Cells

Detection of early apoptosis by quantitation of apoptosis and necrosis by annexin V binding, propidium iodide uptake, and flow cytometry was performed according the principles described in literature[30] with the eBioscience™ Annexin V Apoptosis Detection Kit BMS500FI Kit according to the manufacturer's instructions (Thermo Fisher, UK) using HepG2 cells (100,000 cells/ well) incubated with the extracts, camptothecin 5 μM (Sigma-Aldrich, UK), or control for 48 h after which a flow cytometric analysis was performed using a MACSQuant© Analyzer (Miltenyi Biotec Ltd., UK), and the data were processed using MACSQuantifyTM© Software (Miltenyi Biotec Ltd., UK).

Cell Cycle Analysis

HepG2 cells (100,000 cells/well) were incubated with the extracts, paclitaxel 1 μM or control for 48 h. PI staining was performed according to Leonce et al[31] The flow cytometric analysis was performed using MACSQuant Analyzers (Miltenyi Biotec Ltd., UK) and the data were processed using MACSQuantifyTM Software (Miltenyi Biotec Ltd., UK). using a MACSQuant© Analyzer (Miltenyi Biotec Ltd., UK), and the data were processed using MACSQuantifyTM© Software (Miltenyi Biotec Ltd., UK).

Fractionation of B. sappan L. Water Extract

Bioguided isolation pf the cytotoxic principles from *B. sappan* was conducted according to authoritative guidelines of the field.[32,33] We fractionated 100 mg of CSL water extract using a glass column packed with Silica Gel 60 (Merck, UK). extract was eluted with 100 ml of Chloroform, Chloroform:Methanol (75:25; 50:50; 25:75), Methanol:Ethylacetate (75:25; 50:50; 25:75), and Ethylacetate, respectively. The fractions were analysed with a Waters™ HPLC consisting of a 2695-separation module and a 996-photodiode array detector (Waters Corporation, USA) using a Zorbax© RX-C18 column (5 μm; 4.6 × 250 mm, Agilent). The mobile phase consisted of 0.2% acetic acid in water (A) and methanol (B) were used in gradient mode from 0%(B) to 80%(B) in 60 min. Data were analysed using Empower© software (Waters Corporation, USA).

Statistical Analysis

The means ± standard deviation (SD) was calculated using Microsoft Excel 2010. Calculations of IC_{50}, MNTC, and one-way Analysis of Variance (ANOVA) were performed using GraphPad Prism 8 software (GraphPad Software, Inc.) followed by Bonferroni post-ANOVA multiple t-test. The level of significance was set at $p < 0.05$. Experiments were run in technical duplicates in three or more independent biochemical or biological replicates ($N \geq 3$). In all experiments a control (solvent as treatment) and positive drug treatment were added.

Results

In Vitro Cytotoxicity of ASW and its Ingredients to Human Hepatocellular Carcinoma HepG2 Cells

The screening of all individual ingredients (Figure 1) yielded three different types of effect: those with both IC50 and MNTC above 200 μg/mL (ASW, AM, BR, CT, CSC, CO, LS, PN and SL), extracts with IC50 ≈ 200 μg/mL and MNTC ≤ 50 μg/mL (CA, CR, DS, DR, FA, MR, TR, and TM), and finally the CSL extract was the only one exhibiting cytotoxicity in a linear dose-dependent manner with IC50 at 44 μg/ml and MNTC at 25 μg/ml. Therefore, the crude CSL extract was further assessed for its GSH depletion ability, anti-migratory activity, apoptotic effect, and cell cycle distribution as well as subjected to bioguided isolation.

In Vitro Effect of ASW and its Cytotoxic Ingredient to Glutathione Levels in HepG2 Cells

The treatment of HepG2 cells with WNF (200 μg/ml) significantly halved GSH control levels (21.17 + 1.0 down to 11.47 + 2.3 μM/mg cell protein; $p < 0.05$) similarly to camptothecin 5 μM. In turn, *B. sappan* water extract (50 μg/ml) showed no effect on total glutathione levels.

Effects of ASW and B. sappan Water Extract to Bidimensional Migration of Human Hepatocellular Carcinoma HepG2 Cells

We measured the effect of unrestrained proliferation and migration of liver cells in an in vitro bidimensional, membrane free model. Our findings are that treatments with WNF (at 200 μg/ml) or CSL (at 25 μg/ml) were as effective as the positive anticancer drug paclitaxel 0.01 nM in inhibiting this hallmark of cancer (Figure 2).

Apoptotic Effects of B. sappan Water Extract in Human Hepatocellular Carcinoma HepG2 Cells

Cancer cells frequently develop resistance to apoptosis, making the early circumvention of this barrier a potential strategy to

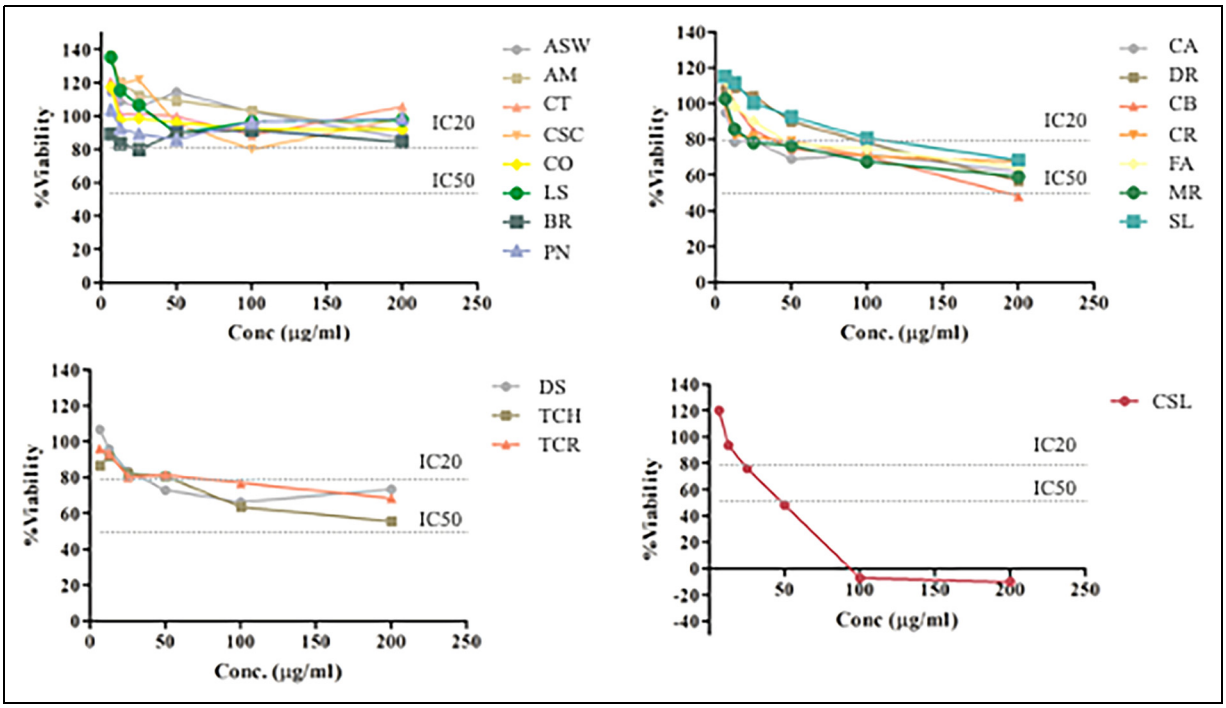

Figure 1. Dose-Dependent Inhibitory Effects of the Ayurveda Siriraj Wattana Formula (ASW; AVS073 Variation) and its Constituent Ingredients (see Table 1 for Abbreviations) on Mitochondrial Activity in HepG2 Cells, Assessed after 48 h Using the Alamar Blue Assay.

halt cancer progression. A hallmark event in early apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, exposing it to the extracellular environment. In our study, dual Annexin V/Propidium Iodide (PI) staining of HepG2 cells after 48-h incubations with varying concentrations of *B. sappan* water extract revealed that this cytotoxic ingredient primarily induced cell death via necrosis, whereas the reference anticancer drug camptothecin significantly induced apoptosis (Figure 3).

To understand how the cytotoxic ingredient affects cell cycle distribution, we synchronized HepG2 cells and analysed their DNA content using flow cytometry after 48 h of treatment (Figure 4). Both the positive drug of reference (paclitaxel) and the water extract of *B. sappan* significantly increased the fraction of cells in the G2/M phase, which was accompanied by a notable decrease in the S phase population. This data suggests that the *B. sappan* extract induces a cell cycle arrest or delay, primarily at the G2/M checkpoint.

Bioguided Isolation of the Cytotoxic Component of B. sappan Water Extract

The extract was subjected to fractionation by vacuum liquid chromatography (VLC) prior to characterization/purification by high performance liquid chromatography (HPLC). Only two fractions, CSL1 (Chloroform/Ethylacetate 75:25; IC50 = 13 µg/ml; MNTC = 7 µg/ml) and CSL2 (Chloroform/Ethylacetate 50:50; IC50 = 41 µg/ml; MNTC = 24 µg/ml), retained the cytotoxic activity (Figure 5A). However, they did not significantly affect cell migration and GSH levels assays at MNTCs (Data not shown). Further HPLC analysis revealed that CSL1 (Figure 5B) consisted of a major peak with a characteristic UV (λ_{max} = 445 nm). After prep-HPLC enrichment and purification, a compound matching the ^{1}H NMR spectrum of brazilein (Figure 5C) was obtained.

Discussion

Cytotoxic Effects of the ASW Ingredients on HEPG2 Cells and Their Molecular Mechanisms

The ASW formula extract did not show any cytotoxicity towards HepG2 cells (IC50 > 200 µg/mL). The only cytotoxic herbal ingredient in the ASW formula was the water extract of *Biancaea sappan*. The double-digit IC50 of its water extract compares well with those of organic extracts reported in the literature: the methanolic extract is cytotoxic to HepG2 cells, with an IC50 of 20 µg/mL.[34] Meanwhile, petroleum ether root, leaf, and stem extracts were antiproliferative against HH-7 hepatoma cells (IC50 of 56 and 77 µg/mL, respectively) via intraperitoneal injections of petroleum ether extracts from the leaves and stems (20-65 mg/Kg) and oral administration (100-325 mg/kg, 12 days) in a H22 hepatoma-bearing mouse model,[35] thus confirming their antitumor activity.

Previous reports did not investigate the mechanisms behind these actions. Our study is therefore the first to provide data

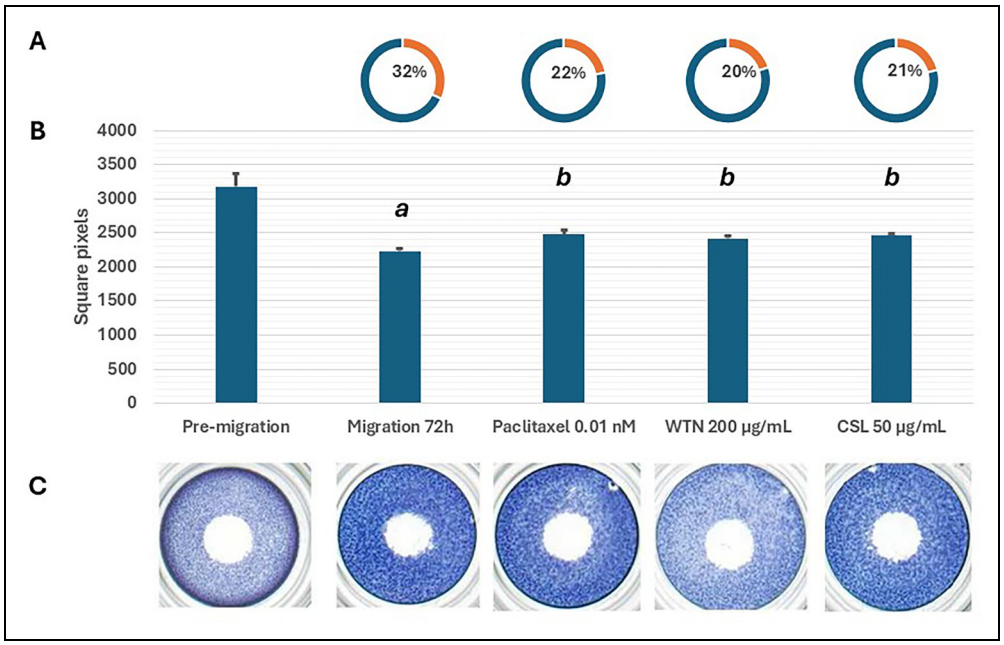

Figure 2. In Vitro Effect of the Water Extracts of the Ayurveda Siriraj Wattana Formula (ASW; AVS073 Variation) and its Cytotoxic Ingredient *B. sappan* (CSL) Compared with the Reference Drug Paclitaxel on the 2D Migration/Motility of HepG2 Cells. (A) Pie Chart Representing the Cell Migration Rate (%) for Each Treatment. (B) Bar Chart Showing Absolute Area of the Free Cell Gap before and after Treatments in Square Pixels (Average of Five Random Measurements Per Replicate, N = 3). (C) Representative Images of the Scratch/Wound before and after Treatment. (*a*) Difference is Significant ($p < 0.05$) Compared with Pre-migration Values; (*b*) Difference is Significant ($p < 0.05$) Compared with Control Migration Values (72 h).

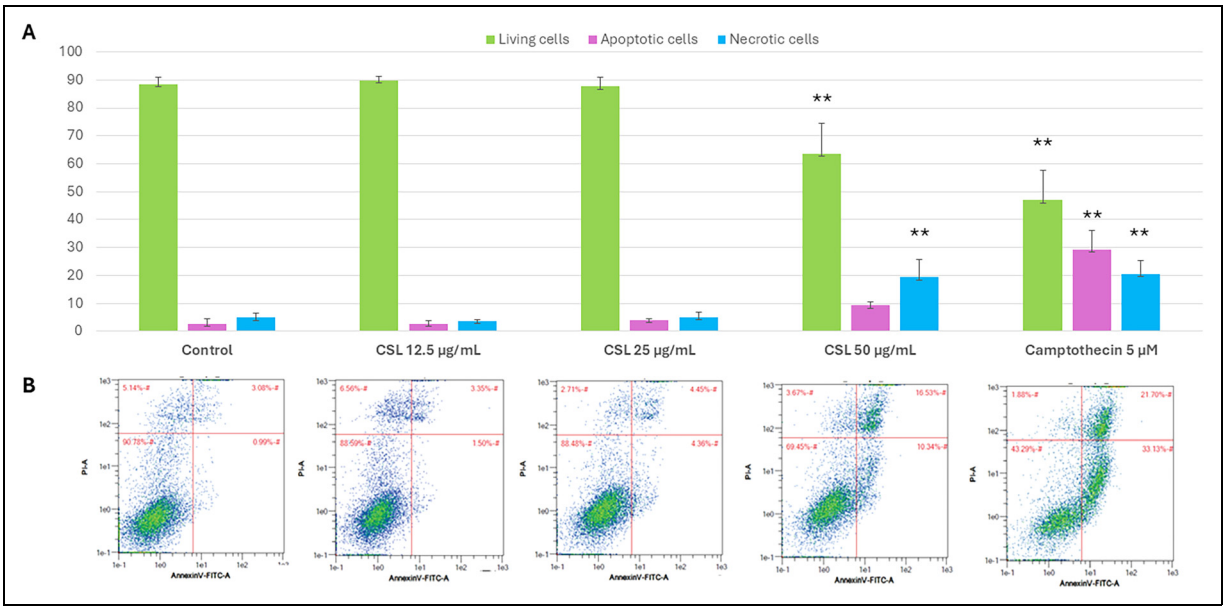

Figure 3. Apoptotic Effects of *B. sappan* (CSL) and the Reference Drug Paclitaxel on HepG2 Cells Assessed by Annexin V-FITC/PI Staining. (A) Quantitative Analysis of Data Presented as Mean + SD. (**) $p < 0.01$ Compared to Control ($N > 3$). (B) Representative Dot Plots of Cells for Annexin V-PI Counterstain Showing Annexin V-FITC and PI Channels (X and Y Axes, Respectively) Gated According to Untreated Cells for Analyses (Control, DMSO 0.08%). Each Plot Shows Cells Positive for Annexin V only (Lower-Right Quadrant, Early Apoptotic Cells), Positive for PI Only (Upper-Left Quadrant, Necrotic Cells), Positive for Both (Upper-Right Quadrant, Late Apoptotic Cells), and Negative for Both (Lower-Left Quadrant, Live Cells).

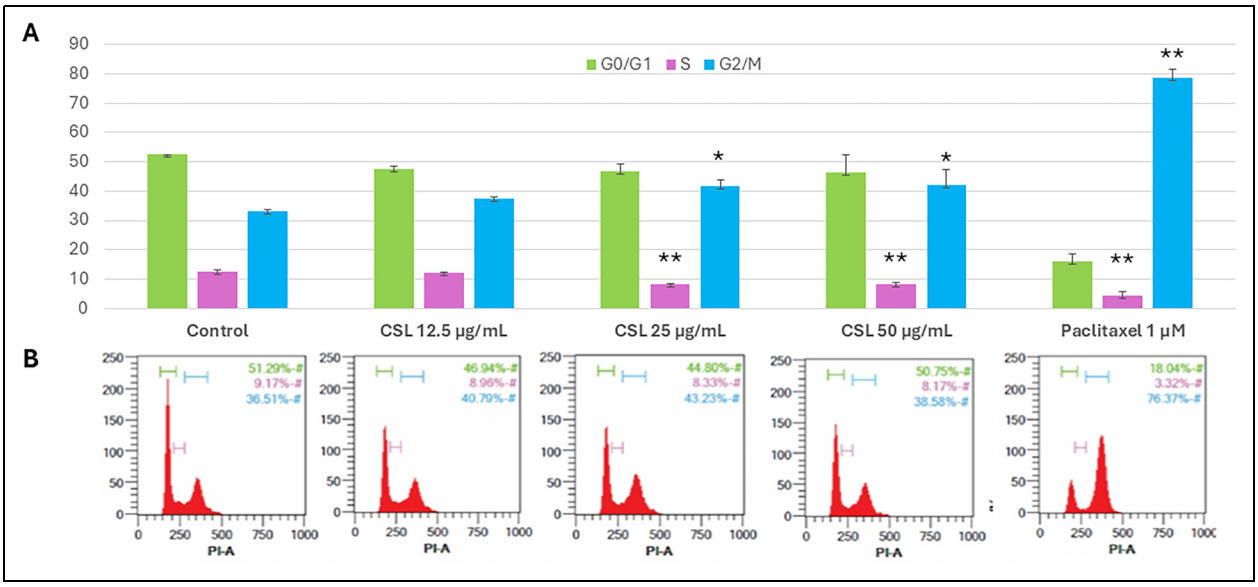

Figure 4. Representative Flow Cytometric Analysis of Cell Cycle Distribution in HepG2 Cells Treated with *B. sappan* (CSL) and the Reference Drug Paclitaxel. (A) Quantification of Cells in G0/G1, S, and G2/M Phases Using the PI Channel. Data are Presented as Mean ± SD (N > 3). (*) $p < 0.05$; (**) $p < 0.01$ Compared with Control (DMSO 0.08%). (B) Representative PI Signal Plots (20,000 Single Events) Showing the Area Under the Fluorescence Signal Versus the Height of the PI Signal.

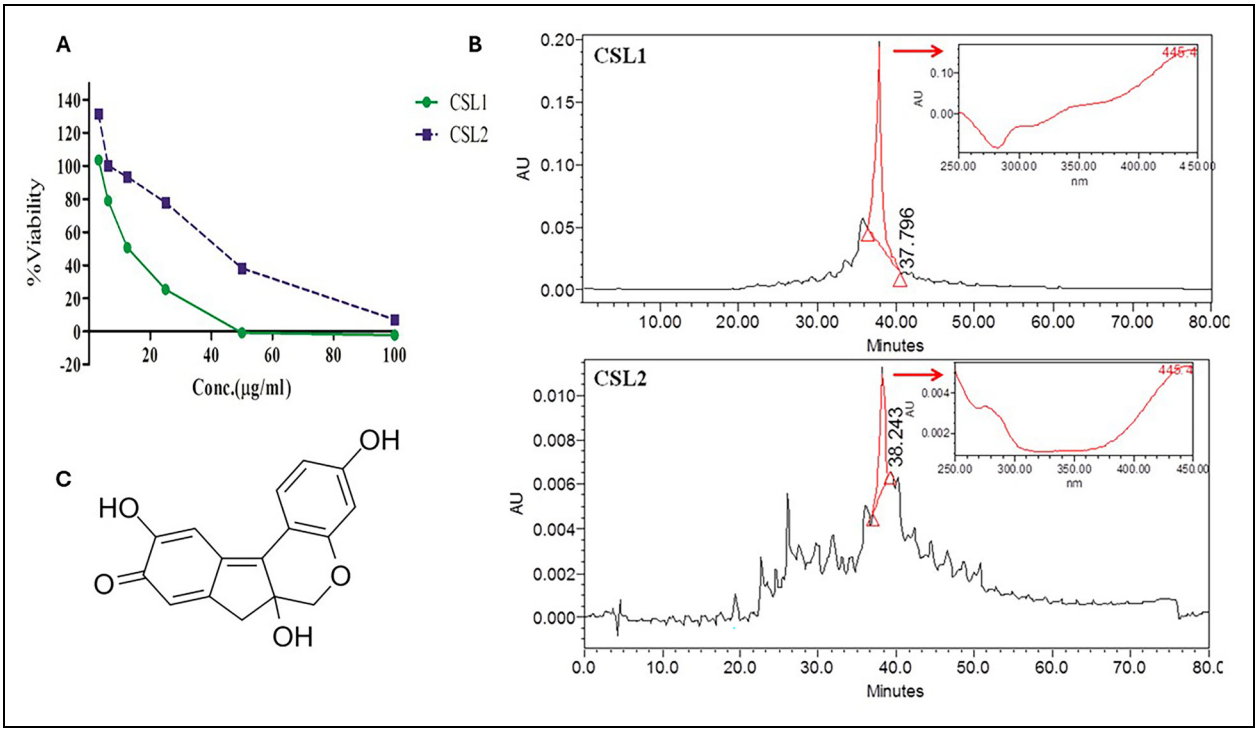

Figure 5. Bioguided Isolation of the *B. sappan* Water Extract (CSL). (A) Dose Dependent Cytotoxicity of CSL1 and CSL2 in the Alamar Blue Assay; (B) HPLC Chromatograms of CSL1 and CSL2 at 445 nm and UV spectra of the major Peaks; (C) 2D Chemical Structure of Brazilein.

on the effects of sappanwood water extracts on the cell cycle and to characterize their pro-apoptotic effects. Flow cytometry data suggests that the *B. sappan* extract induces a cell cycle arrest or delay, primarily at the G2/M checkpoint. This means that after DNA replication, cells are prevented from entering or progressing through mitosis. The corresponding reduction in the S

phase population is a direct consequence of this G2/M blockage, as fewer cells successfully complete mitosis to re-enter G1, thus decreasing the overall proportion of cells actively synthesizing DNA. This G2/M arrest could be triggered by the *B. sappan* water extract through mechanisms such as inducing DNA damage, interfering with DNA replication completion, or disrupting the machinery essential for proper mitotic entry. In this regard, the *B. sappan* water extract appears to mimic the action of the known cytotoxic natural drug paclitaxel. Unlike the apoptotic cell, which undergoes shrinkage and efferocytosis by neighboring living cells, necrotic cells swell and lyse, releasing their contents into the local tissue microenvironment.[36] This release subsequently recruits immune cells to survey damage and clear debris, triggering a pro-tumorigenic inflammatory process which may exacerbate pre-existing unresolved inflammation 39. However, some evidence suggests that the high inflammatory response characteristic of hepatocellular carcinomas might correlate with improved patient outcomes, highlighting the idiosyncratic nature of cancer biology.[37] Further studies are needed to fully characterize the pathways involved in these effects in HepG2. According to the literature, it may involve an increase in the expression of p53 and p21WAF1/CIP1 (as in head and neck cancer cells[38]) and seems to be selective as it induced apoptosis in oral cancer cells but not in normal epithelial cell lines.[39]

Our bioguided isolation efforts identified brazilein as the active principle of the *B. sappan* water extract, exhibiting an IC50 of 13 mg/mL. This finding aligns with a previous report on its cytotoxicity against human HepG2 cells and two additional hepatocellular carcinoma cell lines (SMMC7721 and SGC7901), with an IC50 of 10 μg/mL. Brazilein has also been shown to induce significant apoptotic cell death, as evidenced by DNA ladder assay, and growth inhibition in HepG2 cells, which the authors attributed to its effects via the inhibition of survivin protein and mRNA expression.[10] Other studies have reported cytotoxic activity on various cancer cells, including liver, MDA-MB-231, MCF-7, A549, and Ca9-22.[9] Brazilein is a natural, red-colored dye formed by the spontaneous oxidation of brazilin, a homoisoflavonoid found in the heartwood of trees such as brazilwood and sappanwood, which have been used for centuries as dyes for textiles, paints, and inks.[40] Our cell cycle analyses are consistent with the ability of the homoisoflavonoid brazilin to induce apoptosis and G2/M arrest through the inactivation of histone deacetylase in multiple myeloma U266 cells.[41] Furthermore, in other studies, the downregulation of survivin by brazilein was associated with strong and prominent activation of caspases-9 and −3, as well as PARP cleavage. Notably, similar homoisoflavonoid derivatives, such as sappanone A and protosappanin B, induce ferroptosis in hepatocellular carcinoma via the NRF2/xCT/GPX4 axis,[42] and pretreatment of H22 hepatoma cells (6.25 mg/mL) resulted in complete inhibition of tumor formation in KM mice,[43] respectively.

Other ingredients in our study's recipe, despite failing to show significant effects, have been reported to exhibit cytotoxic effects on HepG2 or other liver cells, typically as organic extracts or containing specific phytochemicals not readily soluble in water. For instance, *Aegle marmelos* fruit extracts inhibited chemically induced and promoted hepatocarcinogenesis in Wistar rats (25 mg/kg *bw*)[44] and Balb/c mice (100 mg/kg *bw*)[45] with positive effects on inflammatory (IL-1β, IL-6), anti-inflammatory (IL-4) cytokine expression, apoptosis (Bcl-2), and tumor-related (p53, c-jun) genes. The rhizomes of *B. rotunda* contain two chalcone derivatives with cytotoxic effects on HepG2 cells: Panduratin A induces G2/M phase arrest in cancer cells, significantly inhibits NF-κB[46] signaling pathways leading to decreased cancer cell viability, and shows anti-angiogenic effects,[47] while Boesenbergin A was cytotoxic to HepG2 (20 μg/mL) among other cell lines.[8] Hydroalcoholic extracts of *Citrus × aurantium* fruit peels are rich in bioflavonoids known to induce human HepG2 cell apoptosis via mitochondrial and death receptor pathways. Hesperidin activated and increased caspase-9, −8, and −3 activities in hesperidin-treated HepG2 cells, downregulated Bcl-xL protein, and upregulated Bax, Bak, and tBid protein levels in a dose-dependent manner,[48] and Wnt3a/β-catenin and Wnt5a pathways protected against chemically-induced early hepatocarcinoma models in rats.[49] Naringenin induced G0/G1 and G2/M phase arrests in HepG2 cells via the p53 pathway and induced apoptosis through the mitochondrial-mediated apoptosis pathway, shown by an increased Bax/Bcl-2 ratio, subsequent cytochrome C release, and sequential caspase-3 activation,[50] as well as via ROS-Mediated JAK-2/STAT-3 signaling pathways.[51] Nobiletin may reduce lipid accumulation by up-regulating the SIRT1-AMPK signaling pathway in HepG2 hepatocarcinoma cells, thereby preventing potential carcinogenic effects.[52] Naringin induces human hepatocellular carcinoma HepG2 cell apoptosis via mitochondria-mediated activation of caspase-9 and caspase-8-mediated proteolysis of Bid.[53] Sinensetin suppresses angiogenesis in liver cancer by targeting the VEGF/VEGFR2/AKT signaling pathway.[54]

The root ethanolic extracts of *Cladogynos orientalis* exhibited cytotoxicity, apoptosis induction, and moderate alkylating activity in HepG2 cells.[55] Farnesiferol C from *Ferula assa-foetida* induces ROS-dependent apoptotic pathway in HepG2 cells.[56] 58. The ethanol extracts of *Aucklandia lappa* (syn. *Saussurea lappa*) are rich in sesquiterpene lactones such as costunolide, dehydrocostus lactone, and lappadilactone, which inhibited HepG2 cell proliferation,[12] as well as showing antiangiogenic effects by inhibiting the VEGFR KDR/Flk-1 signaling pathway in HUVECs and reducing VEGF-induced neovascularization in vivo.[57] Mechanisms of action in other cancer cell lines have been described for cytotoxicity[58–61] and inhibition of cell migration.[62]

Hydroalcoholic extracts of the *Terminalia chebula* fruit are rich in chebulagic and ellagic acids, which possess cytotoxic activity against several cancer cell lines.[63,64] Chebulagic acid increased the cytotoxicity of doxorubicin in HepG2 cells by 20-fold via a COX-2-dependent mechanism, accompanied by the downregulation of MDR1 expression.[65] *Tinospora crispa* aqueous crude extract (IC50 = 165 μg/mL)[66] had no anti-angiogenic properties in the rat aortic ring assay, no effect in the tube formation assay using human umbilical vein endothelial cells (HUVEC) on Matrigel, and no acute cytotoxicity in selected human cancer

cell lines.[67] Non-mechanistic reports on HepG2 cytotoxicity were found for *Cyperus rotundus* (IC50 < 10 μg/mL)[68] and *Conioselinum anthriscoides* (*syn Ligusticum sinense*), whose ethanolic extracts reduced the viability of liver cancer cell lines CL-6, HepG2, and Hep-2 after treatment at 50 μg/ml by approximately 44%, 69%, and 27%, respectively.[11] Although no reports on direct cytotoxicity against HepG2 cells were found for *Cinnamomum siamense, Cryptolepis dubia, Brachypterum scandens* (syn. *Derris scandens*), *Putranjiva roxburghii* (syn. *Drypetes roxburghii*), or *Mallotus repandus*, it is plausible that some non-cytotoxic ingredients may act via other anticancer mechanisms such as immunological activities, increasing the bioavailability of other compounds in the recipe, or even inhibiting multidrug resistance. For example, *Carthamus tinctorius* dried flowers water extracts revert the avoidance of immune destruction of cancer cells by stimulating IFN-γ and IL-10 secretion of splenic T lymphocytes and enhancing the maturation of dendritic cells (DCs). Moreover, reduced tumor weight in tumor-bearing mice changing cytokine secretion toward the Th1 pathway and increasing the population of cytotoxic T lymphocytes ex vivo.[69] The seed oil extract also exhibited antitumor effects in a skin and breast cancer animal model and in a melanoma cell assay system.[69] Notably, *Piper nigrum* may help overcome low bioavailability of other plant metabolites and overcome P-glycoprotein-mediated multidrug resistance,[70,71] although piperidine and piperine have been reported to have potent anticancer in vitro and in vivo effects in other cancer cells,[72–76] as has β-caryophyllene oxide, an important component of black pepper's fragrance.[77]

All the above biomolecular and pharmacological effects of the components of the ASW formula in the most important mechanisms associated to the hallmarks of cancer[78] -as proposed by Li and Mansmann[79]—have been summarized in Figure 6.

Effects of the ASW Ingredients on HEPG2 Cells Migration and Their Molecular Mechanisms

This is the first study to report on *B. sappan* water extract's ability to reduce 2D cell migration in HepG2 cells. Its active compound, brazilin, may be directly responsible for this effect, as it has previously been reported to suppress the migration and invasion of MDA-MB-231 breast cancer cells.[9] The progression of hepatocellular carcinoma is driven by the unrestrained proliferation and migration of liver cells, a complex process in which multiple signal transduction pathways are critically involved.[78,79] A significant portion of existing therapeutic approaches are designed to inhibit these pathways. Specifically, pathways such as Wnt/β-catenin, MAPK, PI3K/Akt/mTOR, PKB/Akt, HGF/c-MET, JAK, and VEGF contribute to hepatocellular carcinoma development to varying extents, often in concert with gaseous signaling molecules like nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S).[80] Based on the work of Hsieh et al, brazilin inhibits cancer cell metastasis primarily by disrupting some of these specific signaling pathways, namely by suppressing the phosphorylation of p38 MAPK, PI3K, and Akt, which are crucial for cell survival and proliferation. This inactivation of the p38 MAPK and PI3K/Akt pathways, in turn, inhibits the activation of NF-κB. The result of this cascade is the suppression of MMP-2 expression, a key protein involved in breaking down the cellular matrix and allowing cancer cells to spread. The authors also explicitly state that brazilin does not affect the phosphorylation of ERK1/2 and JNK.[9] While a comprehensive investigation, like that conducted by Hsieh et al, would be needed to formally establish whether these mechanisms apply to the effects of *B. sappan* and brazilin on the HepG2 cell line, the conservation of these signaling pathways across different cancer types suggests that a similar outcome is highly probable. A dedicated study would therefore likely serve to confirm, rather than discover, the underlying mechanisms as illustrated in Figure 7.

Modulatory Effects of Glutathione Levels of the ASW Ingredients on HEPG2 Cells

Glutathione (GSH), the most abundant tripeptide antioxidant, is crucial for maintaining cellular redox homeostasis and plays diverse roles in detoxification, proliferation, differentiation, and programmed cell death. In malignant neoplasms, disruptions in GSH synthesis and the GSH/GSSG ratio are common, significantly impacting tumor cell viability, initiation, progression, and drug resistance.[81] Elevated GSH levels in various cancer types result from increased expression of key γ-glutamyl cycle enzymes and altered precursor amino acid transport.[82] Beyond its direct antioxidant function, GSH-related enzymes like glutathione peroxidases (GPxs) reduce hydroperoxides crucial for cell viability, while glutathione S-transferases (GSTs) catalyze GSH conjugation to electrophiles and regulate vital cellular signaling pathways, including metabolism and anti-cancer drug resistance via S-glutathionylation.[82,83] Given that high GSH levels in cancer cells confer protection against oxidative stress and xenobiotics, GSH depletion strategies are emerging as promising therapeutic targets to enhance the efficacy of various cancer treatments, including chemotherapy, radiotherapy, and ROS-based therapies like ferroptosis.[83–86]

Although the treatment of HepG2 cells with ASW water extract (200 μg/ml) significantly halved GSH control levels similarly to camptothecin 5 μM this was considered as a modest result precluding further screening of the ingredients. It came as a surpise given the inhibition of UVA-induced glutathione depletion observed in G361 skin cells incubated with WTN.[2] In these skin cells, WTN appears to safeguard GST activity and the expression of γ-GCLC, γ-GCLM, and GST mRNA under radiation stress. Further research is warranted to determine if this differential bioactivity can be harnessed for future therapeutic applications.

On the other hand, *B. sappan* water extract (50 μg/ml) showed no effect on total glutathione levels. Other ingredients of the formula, such as *A. marmelos* hydro-alcoholic extract are

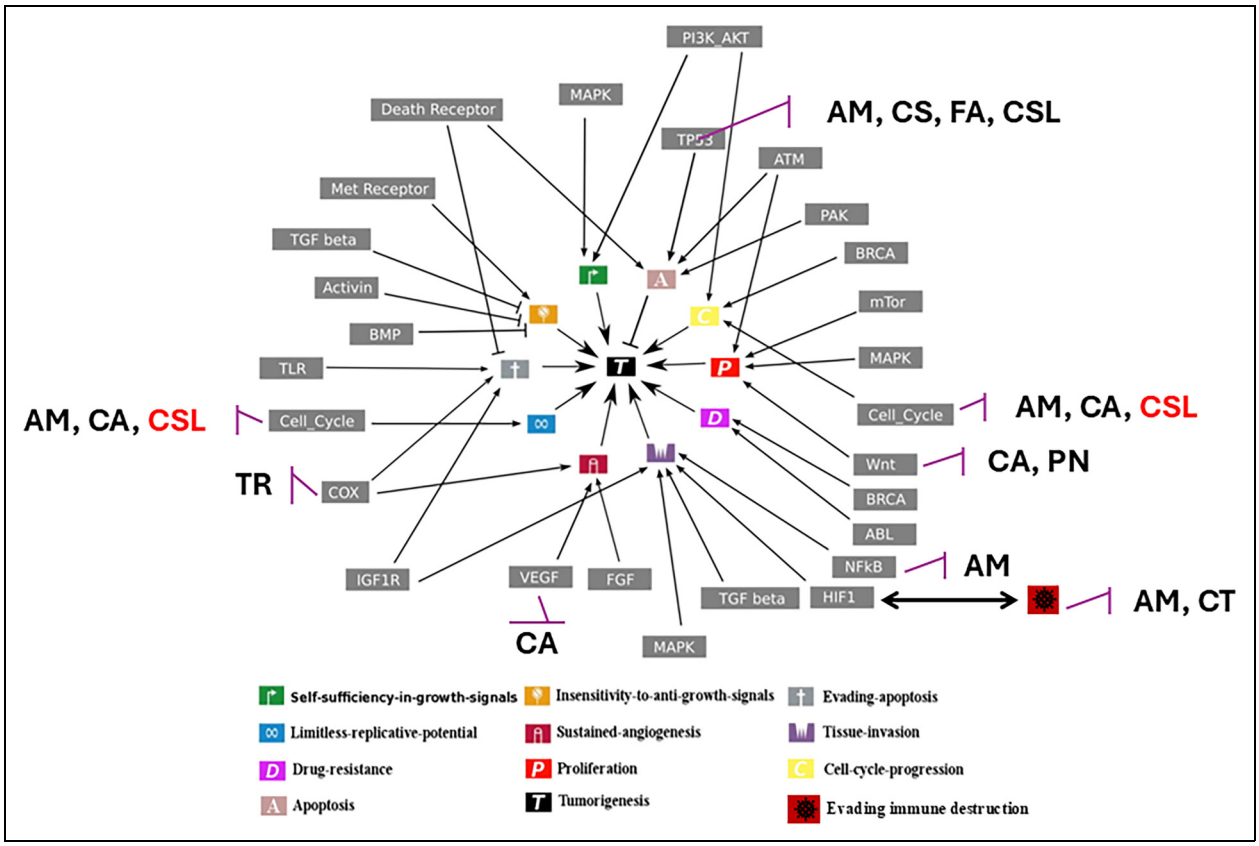

Figure 6. Summary of the Molecular Mechanisms of the Ayurveda Siriraj Wattana Formula (ASW; AVS073 Variation) and its Constituent Ingredients on a Map Linking Hallmarks of Cancer with Cell Signalling Pathways. The Abbreviation CSL in red Denotes new Information Provided by the Present Work on the Effects of *B. sappan* Water Extract. See Table 1 for plant species abbreviations.

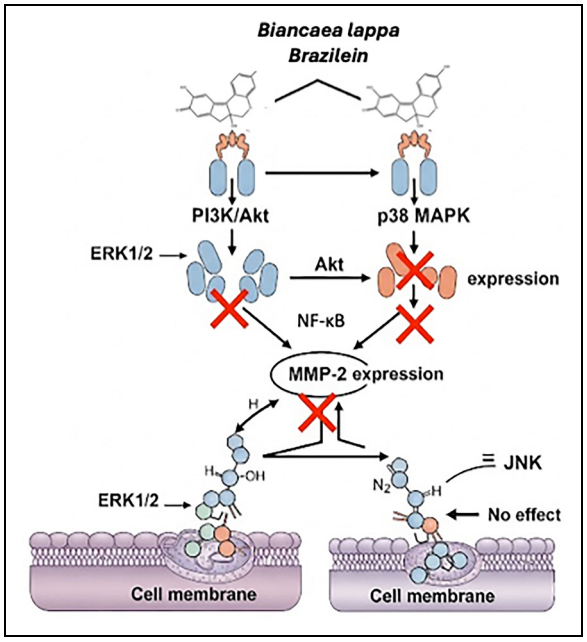

Figure 7. Summary of the Molecular Mechanisms of B. *sappan* Extract and its Active Principle Brazilein on Cell Pathways Related to Cell Migration.

reported to significantly reduced glutathione[87] and *B. rotunda* is known to contain metabolites that modulate glutathione levels, such as Panduratin A which reduced the (GSH) depletion caused by t-BHP in HepG2 cells, thus providing cell protection to oxidative stress in a similar dose-dependent (10-15 μM) effect than silybin[88] but their contribution to the effect of ASW seems to be dimisnish eiter by a dilution effect or by changes in the solubility of their bioactive metabolites.

Implications for the use of Herbal Formulae in Thai Traditional Medicine and Other Traditional Medicines in Clinical Setups

The Thai Herbal Wattana formula has been traditionally used for promoting overall health in Thailand, but clinical evidence supports potential medicinal applications and Phase II clinical trials demonstrated that WNF possessed a good safety profile in patients with knee osteoarthritis.[1] The precise composition of traditional recipes exhibits both complexity and variability, in this case containing up to 18 distinct ingredients, often with qualitative differences documented over time.[2–5] Our study documents the in vitro effects of ASW in HepG2 cells in a similar way to the study carried out by Mahavorasirikul et al with 28

ingredients and 5 Thai traditional recipes who reported on the missing activity against human cholangiocarcinoma (CL-6) of the Pra-Sa-Prao-Yhai recipe. Moreover, these authors discuss how the human hepatocarcinoma HepG2 cell line proved to be the most resistant to most of the tested extracts.[89] This finding underscores the difficulty in unravelling the effects of complex traditional recipes, suggesting that the synergistic or combinatorial effects of multiple ingredients—a key principle in traditional Thai medicine—may be a crucial area for future investigation.

Traditional Chinese medicine also appears to utilize formulae to "soothe the liver" as an adjuvant therapy for liver cancer patients. An example is the Da-Chai-Hu-Tang formula, which dates back almost two millennia. The formula contains eight herbals: *Bupleurum chinense*, *Scutellaria baicalensis*, *Paeonia lactiflora*, *Pinellia ternata*, *Rheum officinale*, *Citrus* × *aurantium*, *Zingiber officinale*, and *Ziziphus jujuba*. Clinical studies have demonstrated the effectiveness of this formula in hepatocellular carcinoma patients both directly and boosting their immunity. In vitro studies corroborated that the formula could induce cell cycle arrest and apoptosis in HepG2 cells by regulating the expression of E-cadherin, N-cadherin, p53, Bax, Bcl-2, PI3K, p-AKT, AKT, and STAT3 through the PI3K/AKT/STAT3 signaling pathway.[90] Apparently, in China the concept of "soothing the liver" may transcend liver cancer and be used as a therapeutic approach for breast cancer. In a study to investigate this concept, Zhao et al developed a herbal formula using three liver-soothing herbs: *Cyperus rotundus* L., *Citrus medica* L. var. *sarcodactylis* Swingle, and *Rosa rugosa* Thunb, the first ingredients two being identically or similarly present in ASW. The results from both in vivo and in vitro experiments demonstrated significant anti-cancer effects. In vitro studies on MCF-7 and T47D breast cancer cells confirmed these findings. The formula exhibited a dose-dependent inhibitory effect on cell growth by downregulating serum estradiol, upregulated apoptosis rates, and significantly decreased the protein expression of SNCG, ER-α, p-AKT, and p-ERK.[91]

In India and Persia, Triphala, a fundamental traditional Ayurveda formulation consisting of *Terminalia chebula*, *Terminalia bellirica*, and *Phyllanthus emblica* combined with honey, is also a remedy for malignant and chronic diseases. The cytotoxic activity of its hydroalcoholic extract significantly reduces HepG2 cell survival in a concentration-dependent manner, with the extract exhibiting a mild IC50 of 78 μg/mL.[92] This has inspired a recent network pharmacology approach unveiled the potential in silico mechanisms of *Terminalia chebula* fruit extracts in the treatment of hepatocellular carcinoma[93] illustrating the importance of traditional use to inform cutting-edge drug-discovery computational approaches.

It is known that progression from chronic liver disease to hepatocellular carcinoma involves a complex interplay of molecular mechanisms, often driven by the dysregulation of key signaling pathways. In this context, the inhibitory effects of brazilein[9] on the MAPK and PI3K/AKT/mTOR pathways, which are highly relevant to hepatocellular carcinoma pathogenesis.[94] The PI3K-AKT signaling pathway has been identified as a significant target for other compounds, such as aloe-emodin, in the treatment of HCC,[95] providing a strong molecular rationale for the historical inclusion of *B. sappan* in ASW, an important formula among the above mentioned traditional Asian "liver formulas". For instance, research could focus on whether the recipe enhances the efficacy of existing treatments, such as gelation embolism agents used in TACE to combat pro-metastatic microenvironments.[96] Furthermore, its interaction with cellular pathways could be explored in conjunction with compounds like talazoparib, particularly in the context of specific genetic vulnerabilities like the loss of heterozygosity of CYP2D6.[97] Research could also explore whether the recipe's effects are mediated through a gut-liver axis, similar to how *Lactobacillus acidophilus* produces valeric acid to suppress HCC.[98]

This paper, like other ethnopharmacological studies, seeks to elucidate the mechanisms of traditional remedies. The goal is to facilitate and guide future pre-clinical and clinical strategies that integrate natural compounds with established efficacy and long-standing safety with contemporary chemotherapy. An example of this approach is the synergistic cytotoxic and migration-inhibitory effect observed when brazilein is combined with cisplatin in 4T1 breast cancer cells[99] or how chebulagic acid is combined with doxorubicin in HepG2 cells.[65] However, systematic combination studies following strict mathematical protocols such as the one proposed by Chou-Talalay[100] are hampered by the number of ingredients of a typical herbal formula which offers hundreds of thousands of potential combinations.

Impact and Limitations of This Study

The present study advances the preclinical understanding of the Thai Herbal Wattana formula (ASW variation) providing a scientific basis for its traditional use in the context of hepatocarcinoma. Its uniqueness stems from its focus on ASW as a specific formulation with traditional applications, rather than its value in the field of drug discovery. A key impact is the identification and dereplication of brazilein as the primary cytotoxic principle within *B. sappan*, thereby linking traditional efficacy to specific secondary metabolites. The observed glutathione modulation occurs at concentrations considered non-physiological, therefore suggesting the safety of the formula in patients with compromised liver function and informing practical implications for integrative oncology. These findings suggest that the positive effects of ASW in treating cancer may be primarily linked to its established ability to modulate the immune system and reduce inflammation, an important facet in many tumor patients when the cancer-immunity cycles fail to run optimally, leading to tumor development and even endangering the host's life.[101]

Despite these contributions, this study is subject to several limitations. Foremost, its exclusive reliance on an in vitro HepG2 cell model restricts the direct generalizability of findings to complex in vivo physiological systems, thereby emphasizing the critical need for subsequent animal and clinical studies. However, the genetic and phenotypic diversity of hepatocellular carcinoma (HCC) is here acknowledged. Different HCC cell

lines, such as Huh-7 and Hep3B, carry distinct genetic mutations (eg, in *TP53* and viral integrations) and display varying growth rates and signaling pathway activities, which can lead to differential responses to therapeutic agents. As comprehensively surveyed by Nwosu and co-workers, poorly differentiated HCC cells, which exhibit an aggressive phenotype, show altered metabolic pathways, including a higher reliance on glutamine for energy, making them uniquely susceptible to treatments that target the glutamine pathway.[102] Future work would certainly benefit from expanding the cell panel to include more HCC lines to validate and extend our observations. Such an approach would provide greater translational relevance by offering insight into how our findings might apply to different HCC subtypes. This is a critical next step, as the varied genetic backgrounds of cell lines can influence everything from chemosensitivity to the activation of key signaling cascades like the PI3K/Akt/mTOR pathway.[102] Ultimately, incorporating a diverse panel of cell lines will not only confirm our initial results but also allow us to identify specific genetic or phenotypic markers that predict a positive response to our agent, moving us closer to a personalized medicine approach for liver cancer treatment.

The limited direct cytotoxicity observed for the overall ASW formula, coupled with a modest reduction in glutathione levels that precluded broader screening, suggests a need for further optimization or exploration of its synergistic potential. Furthermore, the exclusive use of water extracts may have overlooked the bioactivity of lipophilic compounds present in the original herbs, but at the same time reduces the toxicity of the formula.

Future work should confirm the apoptotic pathway through molecular assays by investigating the effects of WNF and its constituents on an extended panel of liver cancer cells as well as normal hepatocytes or liver slices,[103] but we may avoid certain experimental work with in silico approaches such as exploring the anti-liver cancer mechanisms of natural entities using network pharmacology.[95]

Finally, to advance preclinical testing, the Wattana recipe could be evaluated using sophisticated models like patient-derived precision-cut tissue slices,[104] which more accurately replicate the tumor microenvironment. If the recipe's effects are specific to certain tumor subtypes, a DNA methylation-based classifier could be used to differentiate tumor types and select patients who may benefit most.[105] In a clinical setting, NIR-II fluorescence-guided surgery could be used to track the recipe's effects or enhance tumor removal.[106]

Conclusion

This preclinical study demonstrates that while the ASW formula lacks direct in vitro cytotoxicity towards HepG2 cells, it effectively inhibits their mobility and depletes intracellular glutathione although at non-physiological concentrations. *Biancaea sappan* (syn. *Caesalpinia sappan*), a core ingredient of ASW, exhibits potent cytotoxic and anti-migratory activities, with brazilein being its primary cytotoxic compound. However, brazilein is not endowed with the glutathione-depleting or anti-migratory effects observed in the full ASW formula. Further in vivo studies should be carried out to ascertain if long-term treatments with ASW may prevent invasion and metastasis of HepG2 cells without causing any significant cytotoxic effect. These findings suggest that ASW's benefits in oncology may primarily link up with its established clinical immunological and anti-inflammatory effects, while its lack of toxicity to HepG2 cells, a proxy for hepatocytes, plus its clinically proven lack of major adverse effects might indicate a positive safety profile.

Acknowledgements

We are grateful to Prof. Simon Gibbons (Liverpool John Moores University, UK) for kindly reading this paper and providing with invaluable feedback.

ORCID iDs

Suksalin Booranasubkajorn https://orcid.org/0000-0002-3192-878X
Natchagorn Lumlerdkij https://orcid.org/0000-0001-5142-8507
Sirinapa M.ATM. Chaisri https://orcid.org/0000-0001-7433-2110
Jose M. Prieto Garcia https://orcid.org/0000-0002-2649-1691

Ethical Considerations

Ethical Approval is not applicable for this article.

Author Contributions

Suksalin Booranasubkajorn: Supervision, Writing- Original draft preparation, Methodology. **Natchagorn Lumlerdkij**: Methodology, Visualization, Writing- Original draft preparation. **Sirinapa Chaisri**: Resources. **Pravit Akarasereenont**: Funding acquisition, Supervision. **Jose M. Prieto-Garcia**: Conceptualization, Supervision, Visualization, Methodology, Writing- Reviewing and Editing.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Declaration of Conflicting Interests

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

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

References

1. Pengkhum T, Chatsiricharoenkul S, Akarasereenont P, Charoencholvanich K. Phase II clinical trial of Ayurved Siriraj Wattana Recipe for symptomatic relief in patients with osteoarthritis of the knee. *J Med Assoc Thai*. 2012;95(3):452-460.
2. Panich U, Pluemsamran T, Tangsupa-a-nan V, et al. Protective effect of AVS073, a polyherbal formula, against UVA-induced melanogenesis through a redox mechanism involving glutathione-related antioxidant defense. *BMC Complement Altern Med*. 2013;13(1):159. doi:10.1186/1472-6882-13-159
3. Wongkajornsilp A, Numchaisermsuk N, Sa-Ngiamsuntorn K, et al. Effects of the Ayurved Siriraj Wattana recipe on functional and phenotypic characterization of cytokine-induced killer cells and dendritic cells in vitro. *BMC Complement Altern Med*. 2016;16(1):489. doi:10.1186/s12906-016-1480-7
4. Htoo HH, Limsuvan S, Thamsermsang O, et al. The polyherbal wattana formula displays anti-amyloidogenic properties by increasing α-secretase activities. *PLoS One*. 2017;12(1): e0170360. doi:10.1371/journal.pone.0170360
5. Tripatara P, Chotitham P, Paraput T, Akarasereenont P. Effects of the polyherbal wattana formula on food intake, intestinal transit, and ileum contraction in Wistar rats. *Tradit Integr Medi*. 2024;9(2):177-189. doi:10.18502/tim.v9i2.15871
6. Yu S, Wang Y, Hou J, et al. Tumor-infiltrating immune cells in hepatocellular carcinoma: tregs is correlated with poor overall survival. *PLoS One*. 2020;15(4):e0231003. doi:10.1371/journal.pone.0231003
7. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249. doi:10.3322/caac.21660
8. Isa NM, Abdelwahab SI, Mohan S, et al. In vitro anti-inflammatory, cytotoxic and antioxidant activities of boesenbergin A, a chalcone isolated from Boesenbergia rotunda (L.) (fingerroot). *Braz J Med Biol Res*. 2012;45(6):524-530.
9. Hsieh C-Y, Tsai P-C, Chu C-L, et al. Brazilein suppresses migration and invasion of MDA-MB-231 breast cancer cells. *Chem-Biol Interact*. 2013;204(2):105-115. doi:10.1016/j.cbi.2013.05.005
10. Zhong X, Wu B, Pan YJ, Zheng S. Brazilein inhibits survivin protein and mRNA expression and induces apoptosis in hepatocellular carcinoma HepG2 cells. *Neoplasma*. 2009;56(5):387-392. doi:10.4149/neo_2009_05_387
11. Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K. Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro. *BMC Complement Altern Med.*. 2010;10(1):55. doi:10.1186/1472-6882-10-55
12. Sun CM, Syu WJ, Don MJ, Lu JJ, Lee GH. Cytotoxic sesquiterpene lactones from the root of *Saussurea lappa*. *J Nat Prod*. 2003;66(9):1175-1180. doi:10.1021/np030147e
13. Arzumanian VA, Kiseleva OI, Poverennaya EV. The curious case of the HepG2 cell line: 40 years of expertise. *Int J Mol Sci*. 2021;22(23):13135. doi:10.3390/ijms222313135
14. Stanley LA, Wolf CR. Through a glass, darkly? HepaRG and HepG2 cells as models of human phase I drug metabolism. *Drug Metab Rev*. 2022;54(1):46-62. doi:10.1080/03602532.2022.2039688
15. Quintás G, Castell JV, Moreno-Torres M. The assessment of the potential hepatotoxicity of new drugs by in vitro metabolomics. *Front Pharmacol*. 2023;14(1):1155271. doi:10.3389/fphar.2023.1155271
16. Weaver RJ, Betts C, Blomme EAG, et al. Test systems in drug discovery for hazard identification and risk assessment of human drug-induced liver injury. *Expert Opin Drug Metab Toxicol*. 2017;13(7):767-782. doi:10.1080/17425255.2017.1341489
17. Kānphæt TKW, Pharmacopoeia S. *Thai Herbal Pharmacopoeia 2020*. Department of Medical Sciences, Ministry of Public Health; 2020.
18. University M. *Drug and Herbal Product Manufacturing Unit*. Mahidol University; 2025, https://www.si.mahidol.ac.th/th/department/thaimed/product.asp.
19. University M. Siriraj Herbal - Drug Examination and Analysis Central Laboratory (SiHAC). https://www.si.mahidol.ac.th/th/department/thaimed/analyze.asp.
20. WFO. Accessed Accessed on: 01 Jun 2025. http://www.worldfloraonline.org/taxon/wfo-0001228223.
21. Miret S, De Groene EM, Klaffke W. Comparison of in vitro assays of cellular toxicity in the human hepatic cell line HepG2. *J Biomol Screen*. 2006;11(2):184-193. doi:10.1177/1087057105283787
22. Ab Shukor N, Merrina A, Stanslas J, Sreeramanan S. Cytotoxic potential on breast cancer cells using selected forest Species found in Malaysia. *Int J Cancer Res.*. 2008;4(3):103-109. doi:10.3923/ijcr.2008.103.109
23. Suffness M, Pezzuto J. Assays related to cancer drug discovery. In: Hostettmann K, ed. *Methods in Plant Biochemistry: Assays for Bioactivity*. Academic Press; 1990:71-133.
24. Gruber S, Nickel A. Toxic or not toxic? The specifications of the standard ISO 10993-5 are not explicit enough to yield comparable results in the cytotoxicity assessment of an identical medical device. *Front Med Technol*. 2023;5(1):1195529. doi:10.3389/fmedt.2023.1195529
25. Cos P, Vlietinck Aj Fau - Berghe DV, Berghe Dv Fau - Maes L, Maes L. Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. (0378-8741 (Print)).
26. Brescia PJ, Banks P. *Investigation of Cell Migration using a High Density Cell Exclusion Assay and Automated Microplate Imager*. Platypus Technologies, LLC.; 2013, p. 1-8.
27. Walters A, Hulkower KI, Gehler S. *Comparison of the Oris™ Cell Migration Assay to the Scratch Assay*. Platypus Technologies, LLC; 2013, p. 1-2.
28. Gehler SR. *The use of ImageJ to Quantify Cell Number in the orisTM Cell Migration Assay*. Platypus Technologies, LLC; 2013, p. 1-2.
29. Allen S, Shea JM, Felmet T, Gadra J, Dehn PF. A kinetic microassay for glutathione in cells plated on 96-well microtiter plates. *Methods Cell Sci*. 2000;22(4):305-312. doi:10.1023/a:1017585308255
30. Crowley LC, Marfell BJ, Scott AP, Waterhouse NJ. Quantitation of apoptosis and necrosis by Annexin V binding, propidium iodide uptake, and flow cytometry. *Cold Spring Harb Protoc*. Nov 1 2016;2016(11):pdb.prot087288. doi:10.1101/pdb.prot087288

31. Leonce S, Perez V, Lambel S, et al. Induction of cyclin E and inhibition of DNA synthesis by the novel acronycine derivative S23906-1 precede the irreversible arrest of tumor cells in S phase leading to apoptosis. *Mol Pharmacol*. 2001;60(6):1383-1391. doi:10.1124/mol.60.6.1383
32. Sarker SD, Nahar L. *Natural Products Isolation*. 1. Springer; 2012.
33. Butterweck V, Nahrstedt A. What is the best strategy for preclinical testing of botanicals? A critical perspective. *Planta Med*. May 2012;78(8):747-754. doi:10.1055/s-0031-1298434
34. Manosroi A, Akazawa H, Kitdamrongtham W, Akihisa T, Manosroi W, Manosroi J. Potent antiproliferative effect on liver cancer of medicinal plants selected from the Thai/lanna medicinal plant recipe database "MANOSROI III". *Evid Based Complement Alternat Med*. 2015;2015(1):397181. doi:10.1155/2015/397181
35. Li Y, Dong M, Wu Z, Huang Y, Qian H, Huang C. Activity screening of the Herb Caesalpinia sappan and an analysis of its antitumor effects. *Evid Based Complement Alternat Med*. 2021;2021(1):9939345. doi:10.1155/2021/9939345
36. Yang YM, Kim SY, Seki E. Inflammation and liver cancer: molecular mechanisms and therapeutic targets. *Semin Liver Dis*. 2019;39(1):26-42. doi:10.1055/s-0038-1676806
37. Oshi M, Chida K, Roy AM, et al. Higher Inflammatory Response in Hepatocellular Carcinoma is Associated with Immune Cell Infiltration and a Better Outcome. *Hepatol Int*. 2024;18(4):1299-1309. doi:10.1007/s12072-024-10678-2
38. Kim EC, Hwang YS, Lee HJ, et al. *Caesalpinia sappan* induces cell death by increasing the expression of p53 and p21WAF1/CIP1 in head and neck cancer cells. *Am J Chin Med*. 2005;33(3):405-414. doi:10.1142/s0192415(05003016
39. Lee YM, Jeong GS, Lim HD, An RB, Kim YC, Kim EC. Isoliquiritigenin 2'-methyl ether induces growth inhibition and apoptosis in oral cancer cells via heme oxygenase-1. *Toxicol In Vitro : Int J Published Assoc BIBRA*. 2010;24(3):776-782. doi:10.1016/j.tiv.2009.12.024
40. Dapson RW, Bain CL. Brazilwood, sappanwood, brazilin and the red dye brazilein: from textile dyeing and folk medicine to biological staining and musical instruments. *Biotech Histochem*. 2015;90(6):401-423. doi:10.3109/10520295.2015.1021381
41. Kim B, Kim S-H, Jeong S-J, et al. Brazilin induces apoptosis and G2/M arrest via inactivation of histone deacetylase in multiple myeloma U266 cells. *J Agric Food Chem*. 2012;60(39):9882-9889. doi:10.1021/jf302527p
42. Xing Y, Yang H, Dai C, Qiu Z, Guan Y, Zhang L. Investigating the mechanism of ferroptosis induction by sappanone A in hepatocellular carcinoma: NRF2/xCT/GPX4 axis. *Eur J Pharmacol*. 2024;983(1):176965. doi:10.1016/j.ejphar.2024.176965
43. Yang X, Ren L, Zhang S, Zhao L, Wang J. Antitumor effects of purified protosappanin B extracted from Lignum Sappan. *Integr Cancer Ther*. Mar 2016;15(1):87-95. doi:10.1177/1534735415588929
44. Khan TH, Sultana S. Effect of Aegle marmelos on DEN initiated and 2-AAF promoted hepatocarcinogenesis: a chemopreventive study. *Toxicol Mech Methods*. Jul 2011;21(6):453-462. doi:10.3109/15376516.2011.564677
45. Verma S, Bahorun T, Singh RK, Aruoma OI, Kumar A. Effect of Aegle marmelos leaf extract on N-methyl N-nitrosourea-induced hepatocarcinogensis in Balb/c mice. *Pharm Biol*. Oct 2013;51(10):1272-1281. doi:10.3109/13880209.2013.786100
46. Wang Y, Wen J, Liu F, et al. Traditional usages, chemical metabolites, pharmacological activities, and pharmacokinetics of Boesenbergia rotunda (L.) Mansf.: a comprehensive review. *Front Pharmacol*. 2025;16(1):1527210. doi:10.3389/fphar.2025.1527210
47. Lai SL, Cheah SC, Wong PF, Noor SM, Mustafa MR. In vitro and in vivo anti-angiogenic activities of Panduratin A. *PLoS One*. 2012;7(5):e38103. doi:10.1371/journal.pone.0038103
48. Banjerdpongchai R, Wudtiwai B, Khaw-On P, Rachakhom W, Duangnil N, Kongtawelert P. Hesperidin from Citrus seed induces human hepatocellular carcinoma HepG2 cell apoptosis via both mitochondrial and death receptor pathways. *Tumour Biol*. Jan 2016;37(1):227-237. doi:10.1007/s13277-015-3774-7
49. Zaghloul RA, Elsherbiny NM, Kenawy HI, El-Karef A, Eissa LA, El-Shishtawy MM. Hepatoprotective effect of hesperidin in hepatocellular carcinoma: involvement of Wnt signaling pathways. *Life Sci*. Sep 15 2017;185:114-125. doi:10.1016/j.lfs.2017.07.026
50. Arul D, Subramanian P. Naringenin (citrus flavonone) induces growth inhibition, cell cycle arrest and apoptosis in human hepatocellular carcinoma cells. *Pathol Oncol Res*. Oct 2013;19(4):763-770. doi:10.1007/s12253-013-9641-1
51. Zhang M, Lai J, Wu Q, et al. Naringenin induces HepG2 cell apoptosis via ROS-mediated JAK-2/STAT-3 signaling pathways. *Molecules*. Jun 1 2023;28(11):4506. doi:10.3390/molecules28114506
52. Shokri-Afra H, Yousefi Abdolmaleki E, Mousavi Sadr Jadidi ES, et al. Nobiletin potentially reduce lipid accumulation by up-regulating the SIRT1-AMPK signaling pathway in HepG2 hepatocarcinoma cells. *Mol Biol Rep*. May 25 2025;52(1):503. doi:10.1007/s11033-025-10587-z
53. Banjerdpongchai R, Wudtiwai B, Khawon P. Induction of human hepatocellular carcinoma HepG2 cell apoptosis by Naringin. *Asian Pac J Cancer Prev*. 2016;17(7):3289-3294.
54. Li X, Li Y, Wang Y, et al. Sinensetin suppresses angiogenesis in liver cancer by targeting the VEGF/VEGFR2/AKT signaling pathway. *Exp Ther Med*. May 2022;23(5):360. doi:10.3892/etm.2022.11287
55. Machana S, Weerapreeyakul N, Barusrux S, Nonpunya A, Sripanidkulchai B, Thitimetharoch T. Cytotoxic and apoptotic effects of six herbal plants against the human hepatocarcinoma (HepG2) cell line. *Chin Med J (Engl)*. 2011;6(1):39. doi:10.1186/1749-8546-6-39
56. Alafnan A, Alamri A, Alanazi J, Hussain T. Farnesiferol C exerts antiproliferative effects on hepatocellular carcinoma HepG2 cells by instigating ROS-dependent apoptotic pathway. *Pharmaceuticals (Basel)*. Aug 28 2022;15(9):1070. doi:10.3390/ph15091070
57. Jeong SJ, Itokawa T, Shibuya M, et al. Costunolide, a sesquiterpene lactone from *Saussurea lappa*, inhibits the VEGFR KDR/Flk-1 signaling pathway. *Cancer Lett*. 2002;187(1-2):129-133.
58. Kretschmer N, Rinner B, Stuendl N, et al. Effect of costunolide and dehydrocostus lactone on cell cycle, apoptosis, and ABC transporter

expression in human soft tissue sarcoma cells. *Planta Med*. 2012;78(16):1749-1756. doi:10.1055/s-0032-1315385

59. Kim EJ, Lim SS, Park SY, Shin HK, Kim JS, Park JH. Apoptosis of DU145 human prostate cancer cells induced by dehydrocostus lactone isolated from the root of *Saussurea lappa*. *Food Chem Toxicol: Int J Published Br Ind Biol Res Assoc*. 2008;46(12):3651-3658. doi:10.1016/j.fct.2008.08.038
60. Kim EJ, Hong JE, Lim SS, et al. The hexane extract of *Saussurea lappa* and its active principle, dehydrocostus lactone, inhibit prostate cancer cell migration. *J Med Food*. 2012;15(1):24-32. doi:10.1089/jmf.2011.1735
61. Cho JY, Kim AR, Jung JH, Chun T, Rhee MH, Yoo ES. Cytotoxic and pro-apoptotic activities of cynaropicrin, a sesquiterpene lactone, on the viability of leukocyte cancer cell lines. *Eur J Pharmacol*. 2004;492(2-3):85-94. doi:10.1016/j.ejphar.2004.03.027
62. Kim EJ, Hong JE, Lim SS, et al. The hexane extract of *Saussurea lappa* and its active principle, dehydrocostus lactone, inhibit prostate cancer cell migration. *J Med Food*. 2012;15(1):24-32. doi:10.1089/jmf.2011.1735
63. Saleem A, Husheem M, Harkonen P, Pihlaja K. Inhibition of cancer cell growth by crude extract and the phenolics of *Terminalia chebula* retz. fruit. *J Ethnopharmacol*. 2002;81(3):327-336.
64. Reddy DB, Reddy TC, Jyotsna G, et al. Chebulagic acid, a COX-LOX dual inhibitor isolated from the fruits of *Terminalia chebula* Retz., induces apoptosis in COLO-205 cell line. *J Ethnopharmacol*. 2009;124(3):506-512. doi:10.1016/j.jep.2009.05.022
65. Achari C, Reddy GV, Reddy TC, Reddanna P. Chebulagic acid synergizes the cytotoxicity of doxorubicin in human hepatocellular carcinoma through COX-2 dependant modulation of MDR-1. *Med Chem (Shariqah, United Arab Emirates)*. 2011;7(5):432-442.
66. Zulkhairi Jr A, Abdah MA, Kamal NHM, et al. Biological properties of *Tinospora crispa* (Akar Patawali) and its antiproliferative activities on selected human cancer cell lines. *Malays J Nutr*. 2008;14(2):173-187.
67. Ng KW, Salhimi SM, Majid AM, Chan KL. Anti-angiogenic and cytotoxicity studies of some medicinal plants. *Planta Med*. 2010;76(9):935-940. doi:10.1055/s-0029-1240813
68. Mannarreddy P, Denis M, Munireddy D, Pandurangan R, Thangavelu KP, Venkatesan K. Cytotoxic effect of Cyperus rotundus rhizome extract on human cancer cell lines. *Biomed Pharmacother*. 2017;95(1):1375-1387. doi:10.1016/j.biopha.2017.09.051
69. Chang JM, Hung LM, Chyan YJ, Cheng CM, Wu RY. Carthamus tinctorius enhances the antitumor activity of dendritic cell vaccines via polarization toward Th1 cytokines and increase of cytotoxic T lymphocytes. *Evid Based Complement Alternat Med: ECAM*. 2011;2011(1):1-10. doi:10.1093/ecam/nen068
70. Lee G, Joung JY, Cho JH, Son CG, Lee N. Overcoming P-glycoprotein-mediated multidrug resistance in colorectal cancer: potential reversal agents among herbal medicines. *Evid Based Complement Alternat Med*. 2018;2018(1):3412074. doi:10.1155/2018/3412074
71. El-Shehawy AA, Elmetwalli A, El-Far AH, et al. Thymoquinone, piperine, and sorafenib combinations attenuate liver and breast cancers progression: epigenetic and molecular docking approaches. *BMC Complement Med Ther*. Mar 4 2023;23(1):69. doi:10.1186/s12906-023-03872-6
72. Hwang YP, Yun HJ, Kim HG, et al. Suppression of phorbol-12-myristate-13-acetate-induced tumor cell invasion by piperine via the inhibition of PKCalpha/ERK1/2-dependent matrix metalloproteinase-9 expression. *Toxicol Lett*. May 30 2011;203(1):9-19. doi:10.1016/j.toxlet.2011.02.013
73. Lai LH, Fu QH, Liu Y, et al. Piperine suppresses tumor growth and metastasis in vitro and in vivo in a 4T1 murine breast cancer model. *Acta Pharmacol Sin*. 2012;33(4):523-530. doi:10.1038/aps.2011.209
74. Li S, Lei Y, Jia Y, Li N, Wink M, Ma Y. Piperine, a piperidine alkaloid from *Piper nigrum* re-sensitizes P-gp, MRP1 and BCRP dependent multidrug resistant cancer cells. *Phytomed: Int J Phytother Phytopharmacology*. 2011;19(1):83-87. doi:10.1016/j.phymed.2011.06.031
75. Hwang YP, Yun HJ, Kim HG, et al. Suppression of phorbol-12-myristate-13-acetate-induced tumor cell invasion by piperine via the inhibition of PKCalpha/ERK1/2-dependent matrix metalloproteinase-9 expression. *Toxicol Lett*. May 30 2011;203(1):9-19. doi:10.1016/j.toxlet.2011.02.013
76. Selvendiran K, Thirunavukkarasu C, Singh JP, Padmavathi R, Sakthisekaran D. Chemopreventive effect of piperine on mitochondrial TCA cycle and phase-I and glutathione-metabolizing enzymes in benzo(a)pyrene induced lung carcinogenesis in Swiss albino mice. *Mol Cell Biochem*. 2005;271(1-2):101-106.
77. Park KR, Nam D, Yun HM, et al. beta-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. *Cancer Lett*. 2011;312(2):178-188. doi:10.1016/j.canlet.2011.08.001
78. Hanahan D, Weinberg Robert A. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674. https://doi.org/10.1016/j.cell.2011.02.013
79. Li J, Mansmann UR. A molecular signaling map and its application. *Cell Signal*. 2014/12/01/ 2014;26(12):2834-2842. https://doi.org/10.1016/j.cellsig.2014.08.022
80. Zhao H, Zhao L, Wu L, Hu S, Huang Y, Zhao W. Hydrogen sulfide suppresses H(2)O(2)-induced proliferation and migration of HepG2 cells through Wnt/β-catenin signaling pathway. *Med Oncol*. 2023;40(8):214. doi:10.1007/s12032-023-02091-w
81. Kalinina EV, Gavriliuk LA. Glutathione synthesis in cancer cells. *Biochemistry (Mosc)*. 2020;85(8):895-907. doi:10.1134/s0006297920080052
82. Kalinina E. Glutathione-dependent pathways in cancer cells. *Int J Mol Sci*. 2024;25(15):8423. doi:10.3390/ijms25158423
83. Bansal A, Simon MC. Glutathione metabolism in cancer progression and treatment resistance. *J Cell Biol*. 2018;217(7):2291-2298. doi:10.1083/jcb.201804161
84. Niu B, Liao K, Zhou Y, et al. Application of glutathione depletion in cancer therapy: enhanced ROS-based therapy, ferroptosis, and chemotherapy. *Biomaterials*. 2021;277(1):121110. doi:10.1016/j.biomaterials.2021.121110

85. Cheng X, Xu HD, Ran HH, Liang G, Wu FG. Glutathione-Depleting nanomedicines for synergistic cancer therapy. *ACS Nano*. 2021;15(5):8039-8068. doi:10.1021/acsnano.1c00498
86. Ozben T. Mechanisms and strategies to overcome multiple drug resistance in cancer. *FEBS Lett*. 2006;580(12):2903-2909. doi:10.1016/j.febslet.2006.02.020
87. Agrawal A, Jahan S, Goyal PK. Chemically induced skin carcinogenesis in mice and its prevention by *Aegle marmelos* (an Indian medicinal plant) fruit extract. *J Environ Pathol Toxicol Oncol*. 2011;30(3):251-259.
88. Sohn JH, Han KL, Lee SH, Hwang JK. Protective effects of panduratin A against oxidative damage of tert-butylhydroperoxide in human HepG2 cells. *Biol Pharm Bull*. Jun 2005;28(6):1083-1086. doi:10.1248/bpb.28.1083
89. Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K. Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro. *BMC Complement Altern Med*. 2010;10(1):55. doi:10.1186/1472-6882-10-55
90. Duan ZW, Liu Y, Zhang PP, et al. Da-Chai-Hu-Tang formula inhibits the progression and metastasis in HepG2 cells through modulation of the PI3K/AKT/STAT3-induced cell cycle arrest and apoptosis. *J Ethnopharmacol*. 2024;331(1):118293. doi:10.1016/j.jep.2024.118293
91. Zhao Y, Zhao L, Wang T, et al. The herbal Combination Shu Gan Jie Yu regulates the SNCG/ER-a/AKT-ERK pathway in DMBA-induced breast cancer and breast cancer cell lines based on RNA-Seq and IPA analysis. *Integr Cancer Ther*. 2024;23(1):15347354241233258. doi:10.1177/15347354241233258
92. Sahragard A, Alavi Z, Abolhassanzadeh Z, et al. Assessment of the cytotoxic activity of triphala: a semisolid traditional formulation on HepG(2) cancer cell line. *Biomed Res Int*. 2021;2021(1):6689568. doi:10.1155/2021/6689568
93. Jiang J, Yang Z, Hou G, Yao X, Jiang J. The potential mechanism of chebulae Fructus in the treatment of hepatocellular carcinoma on the basis of network pharmacology. *Ann Hepatol*. Jul–Aug 2022;27(4):100701. doi:10.1016/j.aohep.2022.100701
94. Diniz PHC, Silva SDC, Vidigal PVT, et al. Expression of MAPK and PI3K/AKT/mTOR proteins according to the chronic liver disease etiology in hepatocellular carcinoma. *J Oncol*. 2020;2020(1):4609360. doi:10.1155/2020/4609360
95. Zhu M, He Q, Wang Y, et al. Exploring the mechanism of aloe-emodin in the treatment of liver cancer through network pharmacology and cell experiments. *Front Pharmacol*. 2023;14(1):1-19. doi:10.3389/fphar.2023.1238841
96. Song L, Zhu C, Shi Q, et al. Gelation embolism agents suppress clinical TACE-incited pro-metastatic microenvironment against hepatocellular carcinoma progression. *EBioMedicine*. 2024;109(1):105436. doi:10.1016/j.ebiom.2024.105436
97. Zhang X, Rameika N, Zhong L, et al. Loss of heterozygosity of CYP2D6 enhances the sensitivity of hepatocellular carcinomas to talazoparib. *EBioMedicine*. 2024;109(1):105368. doi:10.1016/j.ebiom.2024.105368
98. Lau HC, Zhang X, Ji F, et al. Lactobacillus acidophilus suppresses non-alcoholic fatty liver disease-associated hepatocellular carcinoma through producing valeric acid. *EBioMedicine*. 2024;100(1):104952. doi:10.1016/j.ebiom.2023.104952
99. Handayani S, Susidarti RA, Udin Z, Meiyanto E, Jenie RI. Brazilein in combination with cisplatin inhibit proliferation and migration on highly metastatic cancer cells, 4T1. *Indones J Biotechnol*. 2016;21(1):38-47.
100. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*. 1984;22(1):27-55. doi:10.1016/0065-2571(84)90007-4
101. Sun LY, Zhang KJ, Xie YM, Liu JW, Xiao ZQ. Immunotherapies for advanced hepatocellular carcinoma. *Front Pharmacol*. 2023;14(1):1138493. doi:10.3389/fphar.2023.1138493
102. Nwosu ZC, Battello N, Rothley M, et al. Liver cancer cell lines distinctly mimic the metabolic gene expression pattern of the corresponding human tumours. *J Exp Clin Cancer Res*. Sep 3 2018;37(1):211. doi:10.1186/s13046-018-0872-6
103. Lerche-Langrand C, Toutain HJ. Precision-cut liver slices: characteristics and use for in vitro pharmaco-toxicology. *Toxicology*. Nov 16 2000;153(1-3):221-253. doi:10.1016/s0300-483x(00)00316-4
104. Jagatia R, Doornebal EJ, Rastovic U, et al. Patient-derived precision cut tissue slices from primary liver cancer as a potential platform for preclinical drug testing. *EBioMedicine*. 2023;97(1):104826. doi:10.1016/j.ebiom.2023.104826
105. Dragomir MP, Calina TG, Perez E, et al. DNA methylation-based classifier differentiates intrahepatic pancreato-biliary tumours. *EBioMedicine*. 2023;93(1):104657. doi:10.1016/j.ebiom.2023.104657
106. Wang B, Tang C, Lin E, et al. NIR-II fluorescence-guided liver cancer surgery by a small molecular HDAC6 targeting probe. *EBioMedicine*. 2023;98(1):104880. doi:10.1016/j.ebiom.2023.104880