



Exploring traditional uses and biological activities related to liver cancer of Saban remedy extracts for potential liver cancer treatment

[Exploración de los usos tradicionales y las actividades biológicas relacionadas con el cáncer de hígado de los extractos del remedio Saban para su posible tratamiento]

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Abstract

Context: Saban remedy is used at Khampramong Temple to relieve adverse effects in liver cancer patients.

Aims: To analyze the taste and biological activities of Saban remedy and *Cassia garrettiana* as a principal component species in liver cancer treatment supported *in vitro* and *in vivo*.

Methods: Saban remedy and its ingredient extracts were tested for cytotoxic activity against hepatocellular carcinoma (HepG2, KKU-M156), cholangiocarcinoma cells, and normal human lung fibroblast cells (MRC-5) using the sulforhodamine B assay. Anti-inflammatory effects were evaluated through inhibition of inflammatory cytokine production (RAW 264.7 cell line), and mutagenicity and anti-mutagenicity were assessed using the *Salmonella typhimurium* strain TA98 and TA100 mutation assay. Both Saban remedy extracts and ethanolic *C. garrettiana* extract were tested *in vivo* for anti-hepatocarcinogenesis during the initiation stage in male Wistar rats.

Results: Saban remedy, traditionally used as a purgative, exhibited bitter and bitter-nauseating flavors. The remedy demonstrated *in vitro* cytotoxic activity and *in vivo* reduction of GST-P foci in liver tissue, indicating protective and therapeutic potential against liver cancer. Saban remedy ethanolic extracts showed significant anti-inflammatory effects through nitric oxide inhibition. Notably, the aqueous extract of Saban remedy effectively reduced GST-P foci, a preneoplastic liver lesion, though it exhibited fewer biological activities compared to the ethanolic extract. This aligns with Khampramong Temple's clinical use of the aqueous solution in cancer patients.

Conclusions: The taste and biological properties of Saban remedy are correlated, supporting its use in treating liver cancer through various bioactivities.

Keywords: cytotoxicity; inflammation; liver cancer; nitric oxide; Thai traditional medicine.

Resumen

Contexto: El remedio Saban se utiliza en el Templo Khampramong para aliviar los efectos adversos en pacientes con cáncer de hígado.

Objetivos: Analizar el sabor y las actividades biológicas de Saban y *Cassia garrettiana* como especies componentes principales en el tratamiento del cáncer de hígado soportado *in vitro* e *in vivo*.

Métodos: Se analizó la actividad citotóxica del remedio Saban y sus extractos de ingredientes contra células de carcinoma hepatocelular (HepG2, KKU-M156), de colangiocarcinoma y de fibroblastos de pulmón humano normal (MRC-5) mediante el ensayo de sulforodamina B. Los efectos antiinflamatorios se evaluaron mediante la inhibición de la producción de citocinas inflamatorias (línea celular RAW 264.7), y la mutagenicidad y antimutagenicidad se evaluaron utilizando el ensayo de mutación de la cepa TA98 y TA100 de *Salmonella typhimurium*. Tanto los extractos del remedio de Sabán como el extracto etanólico de *C. garrettiana* se sometieron a pruebas *in vivo* de antihepatocarcinogénesis durante la fase de iniciación en ratas Wistar macho.

Resultados: El remedio Saban, utilizado tradicionalmente como purgante, exhibió sabores amargo y amargo-nauseoso. El remedio demostró actividad citotóxica *in vitro* y reducción *in vivo* de los focos de GST-P en el tejido hepático, lo que indica un potencial protector y terapéutico contra el cáncer de hígado. Los extractos etanólicos del remedio Saban mostraron efectos antiinflamatorios significativos a través de la inhibición del óxido nítrico. En particular, el extracto acuoso del remedio de Sabán redujo eficazmente los focos de GST-P, una lesión hepática preneoplásica, aunque mostró menos actividades biológicas en comparación con el extracto etanólico. Esto concuerda con el uso clínico de la solución acuosa en pacientes con cáncer en el templo de Khampramong.

Conclusiones: El sabor y las propiedades biológicas del remedio Saban están correlacionados, apoyando su uso en el tratamiento del cáncer de hígado a través de diversas bioactividades.

Palabras Clave: cáncer de hígado; citotoxicidad; inflamación; medicina tradicional tailandesa; óxido nítrico.

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Abbreviations: DMEM: Dulbecco's modified eagle medium; DMSO: dimethylsulfoxide; HepG2: hepatocellular carcinoma cells; KKU-M156: cholangiocarcinoma (CCA); LPS: lipopolysaccharide; MRC-5: human lung fibroblast cells; MTT: thiazolyl blue tetrazolium bromide; NO: nitric oxide; TTM: Thai traditional medicine.

INTRODUCTION

Liver cancer ranks as the primary and fourth most prevalent form of cancer among males and females, respectively, in terms of global incidence. Liver cancer can be defined into two main types, which are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). According to the Liver Cancer Statistics (2025), HCC is a prominent contributor to global cancer mortality. The primary etiological factors contributing to hepatocellular carcinoma (HCC) are viral hepatitis B and C infections, alcohol consumption, and exposure to carcinogenic substances such as aflatoxin B1 and nitrosamines (El-Serag and Rudolph, 2007; Starley et al., 2010). Furthermore, hepatitis is known to induce an upregulation in the synthesis of inflammatory cytokines and an elevation in oxidative stress levels. These factors contribute to the progression of hepatitis, leading to the development of fibrosis, cirrhosis, and HCC.

Traditional medicine has been used for a long time to treat chronic diseases such as cardiovascular disease and cancer (Damery et al., 2011; Harikrishnan et al., 2018; Mwaka et al., 2020). Saban remedy (SB) is an herbal remedy mixture prescribed at Arokhayasala Khampramong Temple for cancer patients (Poonthananiwatkul et al., 2015). The remedy was first used to relieve adverse effects and symptoms in cancer patients by Phrapaponpatchara Pibanpaknatee. The remedy is composed of ten herbs, including *Baliospermum montanum* (Willd.) Muell. Arg. (BM), *Carissa carandas* L. (CC), *Cassia garrettiana* Craib (CG), *Croton oblongifolius* Roxb. (CO), *Harrisonia perforate* Merr. (HP), *Phyllanthus amarus* Schum. & Thonn. (PA), *Phyllanthus reticulatus* (PR), *Tinospora baenzigeri* L. (TB), *Tinospora crispa* Forman. (TC), and *Tiliacora triandra* (Colebr.) Diels. (TT).

Phrapaponpatchara Pibanpaknatee, Benjawan Poonthananiwatkul and Traditional Thai Herbal Medicine practitioners have revealed that patients responded with positive clinical signs and symptoms when they used herbal regimens. Previous studies have demonstrated that some of its ingredients have been studied for anti-cancer activity. For instance, *C. garrettiana* is a plant used as herbal medicine in folk doctor treatment. The stem of the plant is used to treat liver disease and provides protective hepatotoxicity. Cassigarol A and piceatannol isolated from *C. garrettiana* heartwood inhibit tumor growth and lung metastasis in LLC-bearing mice (Jeong et al., 2011; Kimura, 2003). The cassialoin from *C. garrettiana* admin-

istered orally can reduce the number of mice with an abdominal invasion of tumors (Kimura et al., 2008). *P. amarus*, an ingredient in Saban remedy, exhibits anti-tumour activity by increasing the life span of ascites tumor-harboring mice (Rajeshkumar and Kuttan, 2000).

Although Thai folk doctors are utilizing the Saban remedy to treat liver disease, there is little scientific evidence for its biological activity related to liver cancer treatment. Thus, our study focused on ethanolic and aqueous Saban remedy extracts. These extracts were studied using Thai traditional medicine wisdom and the biological activities related to liver cancer. The experiments included extractions of the remedy, cytotoxic activity, anti-inflammatory assay through inhibition of inflammatory cytokine production, anti-mutagenicity activity *in vitro*, and the effects of Saban extracts on the initiation stage of anti-hepatocarcinogenesis *in vivo*. We further investigated both extracts of the Saban remedy for their chemical constituent fingerprint by HPLC.

MATERIAL AND METHODS

Materials, chemicals, and reagents

Commercial-grade 95% ethanol was purchased from C.M.J. Anchor Co., Thailand. Analytical grade reagents. Ethanol, isopropanol, and dimethyl sulfoxide were purchased from Labscan® Ltd. Bangkok, Thailand. Fetal bovine serum (FBS), penicillin-streptomycin (P/S), trypan blue, trypsin-EDTA, Minimum Essential Medium (MEM), Nutrient Mixture F-12 Ham, trypsin-EDTA, and Dulbecco's modified eagle medium (DMEM) were purchased from Gibco® BRL Life Technologies (NY, USA). Phosphate buffer saline (PBS) was purchased from Amreso® (OH, USA). Lipopolysaccharide (LPS, Serotype: *Escherichia coli* O55:B5), sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, phosphoric acid, thiazolyl blue tetrazolium bromide (MTT), sulforhodamine B (SRB), Trisma base, trichloroacetic acid (TCA), sodium azide (NaN₃), histidine, biotin, and aflatoxin B1 (AFB1) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). A CO₂-humidified incubator was purchased from Shellab (OR, USA). A laminar airflow cabinet was purchased from Bosstech (Bangkok, Thailand). A microplate reader was purchased from Bio-Tek Instrument, Inc. (VT, USA). 2-Aminoanthracene (2-AA), 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2), and 2-amino-3,4 dimethylimidazo(4,5-f) quin-

olone (MeIQ) were purchased from Wako (Japan). Nutrient broth No. 2 was purchased from Oxoid (England). Bacto agar was purchased from Difco (USA).

Murine macrophage cell line (RAW 264.7: ATCC® TIB-71™), hepatocellular carcinoma cell line (HepG2: ATCC® HB-8065™), and the normal lung fibroblast cell line (MRC5: ATCC® CCL-171™) were purchased from the American Type Culture Collection (VA, USA). Cholangiocarcinoma cell line (KKU-M156) was obtained from Dr. Piti Aungareewithaya and Prof. Dr. Veeraphol Kukongviriyapan, Faculty of Medicine, Khon-Kaen University. The *S. typhimurium* tester strains were kindly provided by Dr. Kei-ichi Sugiyama, National Institute of Health Science, Tokyo, Japan.

Plant materials

The plants were collected from Sakon Nakhon Province, Thailand, in 2016 (GPS coordinates: 17.1595° N, 104.1252° E) and identified by Prof. Dr. Arunporn Itharat. The specimen voucher was deposited at the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand (Table 1). The Biosafety Committee of the University of Thammasat, Thailand, approved this experiment under Biosafety Level 2 (BSL II, Number 161/2561).

The preparation of extracts

Each plant material was cleaned, sliced, dried at 50°C, and ground to powder. The Saban remedy and its ingredients were extracted using maceration and decoction methods. For maceration, the ingredients were mixed as described in Table 2, extracted with 95% ethanol for three days, filtered, and dried using an evaporator. This process was repeated, and all extracts were combined and their yield percentages calculated. Similarly, each individual plant ingredient was macerated and extracted. For decoction, the remedy and ingredients were boiled in distilled water for 15 minutes, repeated three times, and filtered using a No. 1 Whatman® membrane filter. The filtrates were combined and concentrated using a lyophilizer. The crude extracts were stored at -20°C until use. Yield percentages are provided in Table 1.

Determination of chemical compounds in both extracts of Saban remedy using HPLC

The study utilized a high-performance liquid chromatography (HPLC) system with an ultraviolet-

visible (UV-Vis) detector and automatic injector. The stationary phase was a C18 column (Phenomenex analytical column, 4.6 × 150 mm, 5 µm) with a guard column. A gradient mobile phase elution was composed of water (A) and methanol (B) using a three-stage linear gradient: 0–5 min, 95% A, 5–55 min, 30% A–70% A, and 55–60 min, 95%. Firstly, the extracts of Saban remedy (10 mg/mL) were dissolved in methanol and were filtered through a 0.45 µm filter before injection. The 10 µL of solution was injected into the HPLC column. The gradient elution was performed at a 1 mL/min flow rate and monitored at 320 nm. All experiments were repeated in triplicate and presented as the mean ± standard deviation of mg/g of crude extract.

Biological activities of the Saban remedy extract and its ingredients related to liver cancer

Determination of cytotoxic activity assay using the sulforhodamine B (SRB) assay

The Saban remedy extracts, their ingredients, and 5-FU as a reference drug were determined for cytotoxic activity using the SRB assay against liver cancer cells, HepG2, KKU-M156, and a normal fibroblast cell line, MRC-5. The SRB protocol is described using the method of Thongdeeying et al. (2016). The selective index (SI) compared the cytotoxicity of the samples against normal cells and cancerous cells using the following equation [1].

$$SI = \frac{IC_{50} \text{ of normal cells}}{IC_{50} \text{ of cancer cells}} \quad [1]$$

Where: IC₅₀ was the half maximal inhibitory concentration of the drug that inhibited 50% of cell growth, IC₅₀ of normal cells was the IC₅₀ value for the reference drug or sample when tested against normal, non-cancerous cells, and IC₅₀ of cancer cells was the IC₅₀ value for the reference drug or sample when tested against cancerous or tumor cells.

A higher selectivity index indicates that the drug or sample is more selective in killing cancer cells (lower IC₅₀ for cancer cells) while sparing normal cells (higher IC₅₀ for normal cells).

Determination of inhibition of nitric oxide production, TNF-alpha production, and PGE2 production from RAW 264.7 cell line

The Saban remedy extracts and their ingredients, along with the reference drug prednisolone, were investigated for their effects on LPS-induced NO production, TNF-alpha production, and PGE2 production using the RAW 264.7 cell line, following the method of Makchuchit et al. (2017).

Table 1. Yields (%w/w) and voucher specimen number of Saban extracts and their ingredients.

Plant materials	Plant family	Thai name	Voucher specimen number	Place of collection	Part of used	Proportion (%w/w)	Code*	Extraction yield (%)
Saban remedy	-	Saban remedy	None	-	Mixed herbs	-	SBE	3.24
							SBW	7.74
<i>Baliospermum</i> Müll.Arg. (Synonym of <i>Baliospermum solanifolium</i> (Burm.) Suresh)	<i>Euphorbiaceae</i>	Tong-Tak	SKP 071 02 13 01	Kanchanaburi	Root	11.11	BME	2.62
							BMW	7.83
<i>Carissa carandas</i> L.	<i>Apocynaceae</i>	Nam-Dang	SKP 072 03 03 01	Kanchanaburi	Root	11.11	CCE	1.48
							CCW	7.78
<i>Cassia garrettiana</i> Craib (Synonym of <i>Senna garrettiana</i> (Craib) H.S.Irwin & Barneby)	<i>Caesalpiniaceae</i>	Sa-Mae-Sarn	SKP 034 03 07 01	Kanchanaburi	wood	11.11	CGE	3.24
							CGW	4.78
<i>Croton oblongifolius</i> Sieber ex Spreng. (Synonym of <i>Chrozophora tinctoria</i> (L.) A.Juss.)	<i>Euphorbiaceae</i>	Plao-Yai	SKP 071 03 15 01	Kanchanaburi	Stem	11.11	COE	3.34
							COW	3.84
<i>Harrisonia perforate</i> (Blanco) Merr.	<i>Rutaceae</i>	Khon-Tha	SKP 178 08 16 01	Kanchanaburi	Root	11.11	HPE	2.87
							HPW	4.97
<i>Phyllanthus amarus</i> Schum. & Thonn.	<i>Phyllanthaceae</i>	Luk-Tai-Bai	SKP 071 16 01 01	Kanchanaburi	Whole plant	11.11	PAE	3.57
							PAW	9.94
<i>Phyllanthus reticulatus</i> Poir	<i>Phyllanthaceae</i>	Kang-Pla-Dang	SKP 071 16 18 01	Kanchanaburi	Root	11.11	PRE	2.02
							PRW	3.76
<i>Tinospora crispa</i> (L.) Hook.f. & Thomson	<i>Menispermaceae</i>	Bo-Ra-Pet	SKP 114 20 03 01	Kanchanaburi	Stem	5.56	TCE	6.03
							TCW	14.95
<i>Tinospora baenzigeri</i> Forman	<i>Menispermaceae</i>	Ching-Cha-Cha-Lee	SKP 114 20 02 01	Kanchanaburi	Stem	5.56	TBE	3.29
							TBW	16.40
<i>Tiliacora triandra</i> (Colebr.) Diels	<i>Menispermaceae</i>	Ya-Nang	SKP 114 20 20 01	Kanchanaburi	Root	11.11	TTE	5.53
							TTW	8.09

*E: Maceration, W: Decoction.

Table 2. The relationship between taste and biological activities of Saban remedy and their ingredients.

Thai remedy group drugs	Plant material	Flavor	Folk doctor used for	Scientific evidence
1. Main drug (Bitter and bitter-nauseating flavors) The function of the main drugs is to decrease fire <i>dhatu</i>, which causes cancer cell proliferation in cancer patients. Fire <i>dhatu</i> can be decreased by using a purgative drug to reduce the temperature in the body's cancer patients	<i>Cassia garrettiana</i> Craib (Synonym of <i>Senna garrettiana</i> (Craib) H.S.Irwin & Barneby) <i>Baliospermum montanum</i> Müell.Arg. (Synonym of <i>Baliospermum solanifolium</i> (Burm.) Suresh)	Bitter Bitter nauseating	Wood is used as a purgative drug for relieving fever symptoms Roots have bitter-nauseating flavor, which cures jaundice symptoms and anemia and is anti-inflammatory	• Antitumor and antimetastatic activities (Kimura, 2003; Kimura et al., 2008) • Cytotoxic activity (Cherian et al., 2015; Ogura et al., 1978) • Anti-inflammatory activity (Kakatum et al., 2012; Pipatrattanaseree et al., 2019) • Hepatoprotective activity (Wadekar et al., 2008; Kumar and Mishra, 2009; Kumar and Mishra, 2012; Suresh Kumar and Mishra, 2014)
2. Secondary drug (Bitter-cold flavor) Function of secondary drugs reduced temperature and pain-relieving symptoms. Reducing fever, enhancing appetite, and anti-inflammatory were properties of secondary drugs for cancer patients.	<i>Tiliacora triandra</i> Colebr. Diels. <i>Tinospora crispa</i> (L.) Hook.f. & Thomson <i>Tinospora baenzigeri</i> Forman <i>Phyllanthus amarus</i> Schumach. & Thonn.	Bitter Bitter-cold Bitter-cold Bitter	Roots are used to treat fever, maintain elements, and anti-hangover symptom Root reduced fever, maintained fire element, cured stomach disease, reduced pain from toothache, and enhanced appetite Roots are used to cure cancer, reduce pain, antidote, maintain health, cure fever, treat fatigue, and anti-inflammatory The whole plant treated toxic fever symptoms that present high temperature, jaundice, hemorrhoid, diuretic, and ascites	• Anti-inflammatory activity (Dechayont et al., 2021) • Cytotoxicity (Ibrahim et al., 2011; Zulkhairi et al., 2008) • Anti-inflammatory activity (Adnan et al., 2019; Kamarazaman et al., 2012) • Hepatoprotective activity (Rakib et al., 2020; Sutrisna et al., 2017) • Cytotoxic activity (Hanthanong et al., 2021) • Cytotoxic activity (Abhyankar et al., 2010; Lawson-Evi et al., 2008; Lee et al., 2011; Nguyen et al., 2017) • Antitumor (Rajeshkumar and Kuttan, 2000) • Anti-inflammatory activity (Alagan et al., 2019; Harikrishnan et al., 2018; Yuandani et al., 2016) • Hepatoprotective activity (Naaz et al., 2007; Ogunmoyole et al., 2020; Pramyothin et al., 2007)

Table 2. The relationship between taste and biological activities of Saban remedy and their ingredients (continued...)

Thai remedy group drugs	Plant material	Flavor	Folk doctor used for	Scientific evidence
	<i>Harrisonia perforata</i> (Blanco) Merr.	Bitter	Roots cured toxic fever .	• Cytotoxic activity (Itharat et al., 2010) • Anti-inflammatory activity (Choodej et al., 2013) • Mutagenic and anti-mutagenic activity (Singharachai et al., 2011)
3. Component drug (astringent, hot, and flavorless flavors)	<i>Phyllanthus reticulatus</i> Poir.	Astringent	Roots treated toxic fever, common cold, and herpes zoster.	• Cytotoxic activity (Aarthi and Babu, 2017; Deivayanai et al., 2019; Pojchaijongdee et al., 2010) • Hepatoprotective activity (Das et al., 2008)
The function of component drugs is to cure other cancer patients' symptoms , such as anti-mutagenicity, hepatoprotective activity, and anti-oxidation.	<i>Croton oblongifolius</i> Sieber ex Spreng. (Synonym of <i>Chrozophora tinctoria</i> (L.) A.Juss.)	Hot	Roots cure skin diseases and cancer.	• Cytotoxic activity (Adam et al., 2018; Aye et al., 2019; Poofery et al., 2020; Pudhom et al., 2007; Roengsumran et al., 2001; Weerapreeyakul et al., 2012) • Hepatoprotective activity (Ahmad et al., 2016)
	<i>Carissa carandas</i> L.	Flavorless	Roots have a flavorless taste, which treats skin diseases and reduces toxic fever.	• Anti-inflammatory activity (Galipalli et al., 2015)

Determination of mutagenicity and anti-mutagenicity using the Salmonella mutation assay

The extracts were investigated for mutagenicity and anti-mutagenicity using *Salmonella* mutation assay (AMES test) in *Salmonella typhimurium* strain TA98 and TA100 with the metabolic activation required by additional rat enzyme (S9 fraction) from rat liver. The *Salmonella* mutation assay used is as described by Tejs (2008).

Experimental animals

Male Wistar rats (4 weeks old, 80-120 g) were purchased from Nomura Siam International Co, Ltd., Bangkok, Thailand, to evaluate the potential protective effect on the initiation stage of hepatocarcinogenesis. Animals remained under standard environmental conditions with a temperature of 25°C under a 12 h dark-light cycle and free excess for drinking water and pelleted diet at the laboratory animal facility of the Faculty of Medicine, Chiangmai University.

The Animal Ethics Committee approved the experiment protocol of the Faculty of Medicine, Chiang Mai University (Protocol No. 05/2563), and the Animal Care and Use Committee of Thammasat University (Protocol No. 018/2562).

The doses of drugs

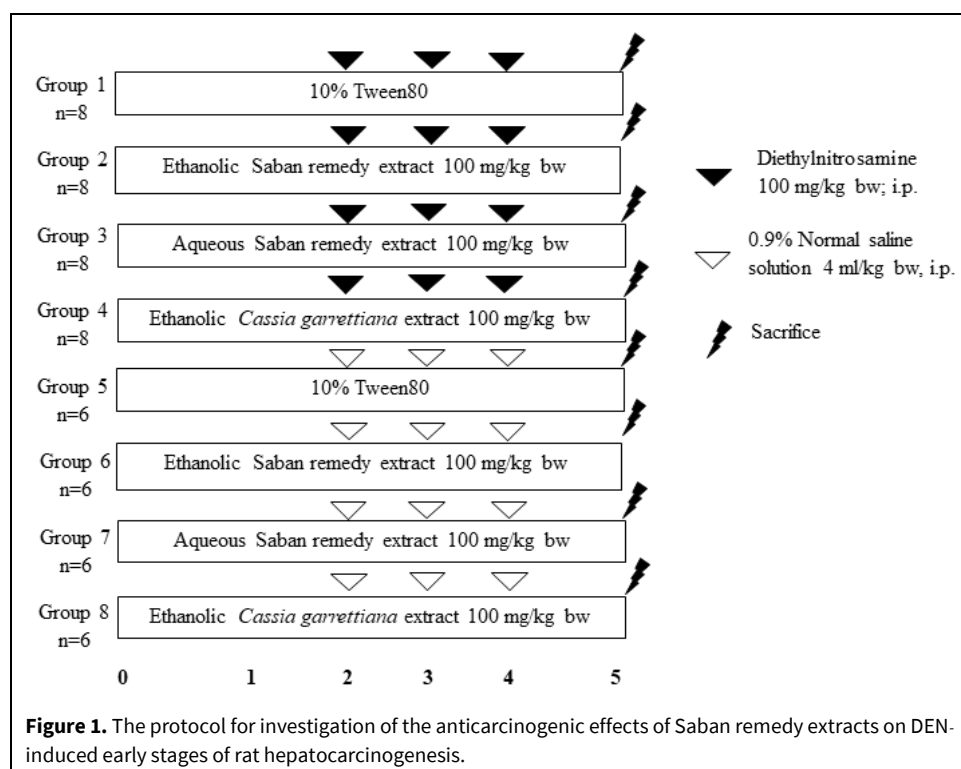
This Thai-patented No. 14572 (Itharat et al., 2018) remedy has been used for decades in patients at Khampramong Temple. The traditional use of 1000 mg/day divided into three doses is a standard clinical human dose corresponding to a dose in rats of 100 mg/kg. The 100 mg/kg body weight (bw) dosage was used for preliminary experiments and additional scientific data to support remedy use. This dosage subsequently serves as a suitable no-observable toxic effect level similar to other herbal substances for further investigation (Accord Healthcare, 2021; Dokkaew et al., 2019; Inprasit et al., 2024; Pibonpaknatee, 2014; Poonthananiwatkul et al., 2015; Prajuabjinda et al., 2012).

The anti-hepatocarcinogenesis on initiation stage testing *in vivo*

Preliminary experimental protocol of the Saban remedy extracts

The preliminary experimental protocol was used to study the effect of the Saban remedy and ethanolic *C. garrettiana* extract on the early stages of diethyl nitrosamine (DEN) -induced hepatocarcinogenesis in rats. In this work, we focused on the chemopreventive effects of Saban remedy extracts rather than the therapeutic effect of Saban remedy extracts at the early stage of carcinogenesis, when there is no tumor mass of liver cancer in an animal model. At this point, our major goal was to determine the impact of Saban remedy extracts on preneoplastic lesion development rather than established tumors. Thus, the therapeutic drug for liver cancer was not used as a reference drug in this model (Dokkaew et al., 2019; Hagiwara et al., 2011; Lto et al., 2003).

Male Wistar rats (4 weeks old, 80-120 g) were randomly separated into six groups. Eight rats per group for groups 1 to 4 and six rats per group for groups 5 to 8, as shown in Fig. 1. Groups 1 to 4 were intraperitoneally injected with 100 mg/kg BW of DEN at weeks 2, 3, and 4, whereas groups 5 to 8 were intraperitoneally injected with normal saline. Groups 1 and 5 were positive and negative control groups, respectively. Two weeks before DEN injection, Groups 1 and 5 were treated with 10% Tween 80 as vehicle control groups, respectively. The 100 mg/kg BW of ethanolic Saban remedy extract was orally administered to groups 2 and 6. Groups 3 and 7 were orally given aqueous Saban remedy extract (100 mg/kg BW), and groups 4 and 8 were also administered orally with the ethanolic *Cassia garrettiana* extract (100 mg/kg BW). Food and water consumption and body weight were measured twice a week throughout the study. At the end of the fifth week of the experiment, all rats were sacrificed to study the effect of Saban remedy extracts on the initiation stage of hepatocarcinogenesis. Blood was collected to measure serum alanine transaminase activity. All rats were anesthetized with isoflurane, and blood was collected to determine liver enzyme activity and internal organs (i.e., liver, spleen, kidney). The internal organs were removed and weighed. The livers were cut and fixed in 10% formalin for glutathione-S-transferase placental positive foci examination (Fig. 1).



Determination of glutathione-S-transferase placental positive foci

Immunohistochemically staining for glutathione-S-transferase placental (GST-P) positive foci was undertaken to determine pre-neoplastic lesions in the rat liver tissue. GST-P foci evaluation was conducted using the avidin-biotin complex method reported by Puatanachokchai et al. (2006). Liver sections were soaked in 3% H₂O₂ and skim milk to block endogenous peroxidase activity and non-specific protein binding, respectively, after deparaffinization. Then, the slides were incubated with polyclonal anti-rabbit GST-P antibody (1:1000) for 2 hours at room temperature. After that, the slides were loaded with goat anti-rabbit IgG biotinylated antibody conjugated with the avidin-biotin-peroxidase complex for 30 min. Finally, the peroxidase binding sites were envisioned with 3,3-diaminobenzidine hydrochloride. The tissue sections were counterstained with hematoxylin for microscopic examination. The total area of liver sections and numbers of GST-P positive foci, some of which contain more than 12,000 μm^2 per foci, were counted under a light microscope.

Statistical analysis

The biological activity results were reported as mean $\pm$ standard error of the mean (SEM) from three replicated experiments. IC₅₀ values were calculated using GraphPad Prism software (CA, USA). The animal test results were expressed as mean $\pm$ standard

deviation (SD). Statistical comparisons between groups were calculated via independent sample t-test and one-way analysis of variance (ANOVA) followed by an LSD post-hoc test. Differences were measured as significant for $p < 0.05$.

RESULTS

The association between tastes and biological activity of Saban remedy in liver cancer disease

In Thai Traditional Medicine (TTM), a mixture of herbs, known as an herbal remedy, is commonly used by folk doctors to treat various diseases, including cancer. TTM practitioners view cancer as a challenging, often uncontrolled, chronic ulcer disease. Many cancer patients also suffer from chronic ulcers, tumors, and rashes and frequently experience fever as a symptom. In TTM, cancer can manifest either as a chronic ulcer disease, which is considered a benign tumor, or as a malignant tumor (Olaku and White, 2011). In the context of TTM, cancer is referred to as "Kasai" in Thai, meaning a degenerative disease (Zhou et al., 2016). Hepatocellular carcinoma (HCC) is one of the most common types of liver cancer and has a variety of known etiologies, including viral hepatitis B and C infections, alcohol consumption, and toxin exposure (Liver Cancer Treatment - NCI). Furthermore, chronic hepatitis can exacerbate liver disease, progressing to conditions like fibrosis, cirrhosis, and, ultimately, the development of HCC (Dhiman and Chawla, 2005). From a TTM perspective, when the

body is frequently exposed to toxic dietary substances such as alcohol, aflatoxin, and some xenobiotics, these toxins stimulate the fire *dhatu* (*Pit-ta*) and wind *dhatu* (*Va-ta*), leading to their heightened activity. In contrast, the water *dhatu* (*Sem-ha*) is reduced, which is believed to contribute to liver disease (Xiang et al., 2019). TTM posits that prolonged exposure to these toxic substances ultimately leads to a reduction in *Pit-ta* function, disrupting the balance of the *Tri-Dhatu* system (earth, water, fire, and wind). Consequently, the water *dhatu* increases, leading to the manifestation of symptoms associated with liver disease (Tang et al., 2020). According to TTM theory, with aging, the body’s health maintenance diminishes, and the earth *dhatu*, which plays a key role in balancing the *Tri-Dhatu* system, becomes less effective (Nootim et al., 2020). As the earth *dhatu* loses its ability to maintain balance, it is believed to give rise to masses or tumors (Fig. 2).

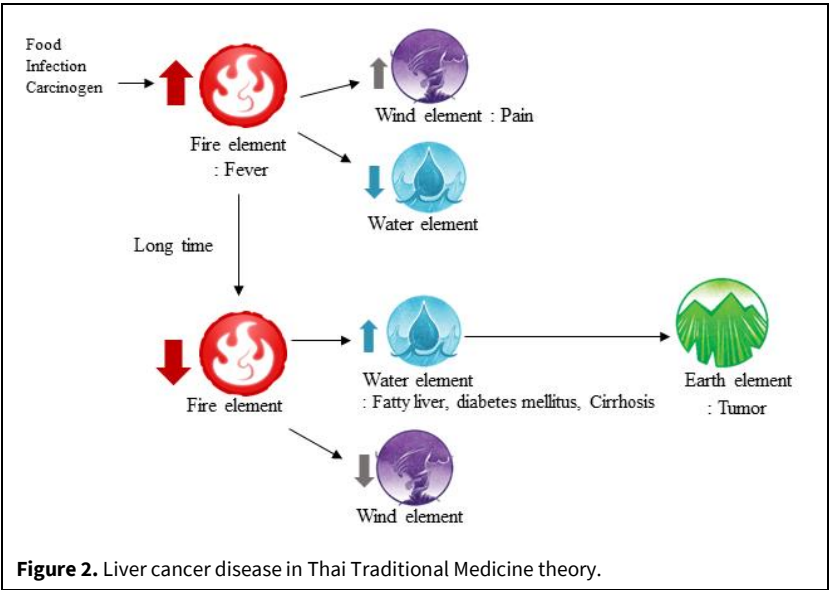
TTM theory for treating cancer disease is described via reducing high body temperature or fire *dhatu* with a purgative Thai herbal remedy. In its wisdom, bitter, bitter-nauseating, and bitter-cold taste is still considered an important flavor for reducing temperature by catharsis (Poopong et al., 2022). Astringent and spicy tastes are used as a medicine for maintaining wind *dhatu* and to treat other symptoms of cancer patients (Poopong et al., 2022). Flavor tastes are used to maintain *Tri-dhatu* through antioxidant activity to support other patient symptoms (Poopong et al., 2022). The TTM wisdom describes the Thai remedy pharmacotherapy structure as consisting of three groups: the

main therapeutic, the secondary therapeutic, and the component therapeutic. 1. The main treatment uses herbs, which have properties for directly treating cancer disease. 2. The secondary treatment uses herbs, which have the benefit of curing other symptoms of the disease. 3. The component therapy is used for protective purposes, allowing adjustment of the remedy to be easily consumable for the cancer patient (Bhaccha, 2005).

Saban remedy is an herbal remedy mixture for liver cancer patients in Arokhayasala, Khampramong Temple, which is being used to relieve adverse symptoms for cancer patients (Poonthananiwatkul et al., 2015). The remedy has a bitter flavor and is composed of ten herbs (Itharat, 2018; The Aphinyana Arokhayasala Foundation, 2017). TTM wisdom uses the properties of Saban remedy, including bitter, astringent, spicy, and flavorless taste, which has a liver tonic effect and enhanced appetite and reduces pain in cancer patients (Itharat, 2018; The Aphinyana Arokhayasala Foundation, 2017). The ethnopharmacological analysis of the association between taste and biological activity is described in TTM wisdom (Table 2).

Extract yields of plant extracts

The yields of 95% EtOH and aqueous extracts of SB were 3.24 and 7.74, respectively. The percentage of yield of ethanolic extracts was in the range of 1.48-6.03, while the percentage of yield of aqueous extracts was in the range of 3.76-14.95 (Table 1).



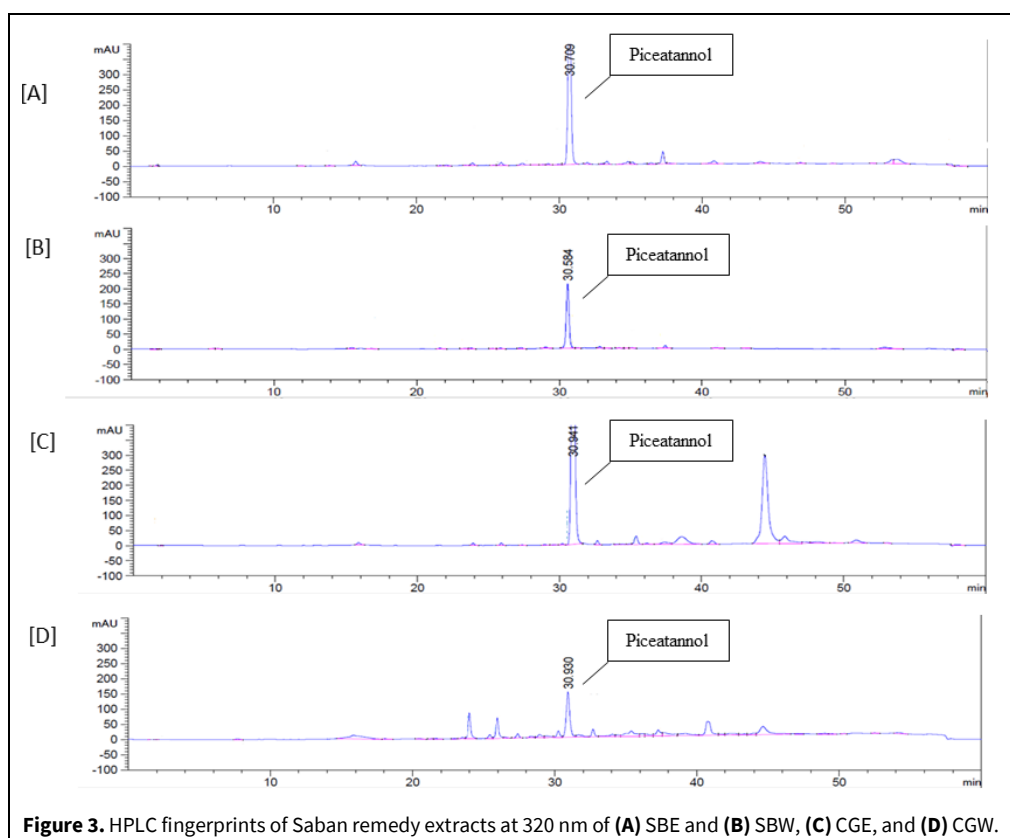


Figure 3. HPLC fingerprints of Saban remedy extracts at 320 nm of (A) SBE and (B) SBW, (C) CGE, and (D) CGW.

HPLC fingerprints of Saban remedy extracts

The profiles of Saban remedy extracts are shown in **Fig. 3** at 320 nm. The HPLC profile of Saban remedy extracts showed a high peak corresponding to a major peak in the chromatogram of *C. garrettiana* extracts, piceatannol at 320 nm. The peak was piceatannol, which was used as a chemical marker in this study. The ethanolic extract of Saban remedy had 264.36 ± 91.65 μg piceatannol/g extract or 1.082 μM , while the aqueous extract had piceatannol content as valued at 46.34 ± 2.91 μg piceatannol/g extract or 0.188 μM . The ethanolic and aqueous extract of *C. garrettiana* showed the content of piceatannol valued as 3068.65 ± 683.85 μg piceatannol/g extract or 12.564 μM and 41.61 ± 1.67 μg piceatannol/g extract or 0.17 μM , respectively.

Cytotoxic activity against the HepG2 and MRC5 cell lines

The aqueous extracts showed less cytotoxic activity than the ethanolic extract on the HepG2 and KKKU-M156 cell lines. This study used the anti-cancer metabolite 5-Fluorouracil (5-FU) as the positive control. The results demonstrated that the aqueous extract of Saban remedy and its aqueous extract ingredients showed cytotoxic activity above 100 $\mu\text{g}/\text{mL}$ in the HepG2 cell line and 50 $\mu\text{g}/\text{mL}$ in KKKU-M156. On the other hand, the Saban remedy ethanolic extract had cytotoxic activity to HepG2 with IC_{50} of 26.85 ± 7.60

$\mu\text{g}/\text{mL}$ while its ethanolic extracts ingredients, *Cassia garrettiana*, *Croton oblongifolius*, *Baliospermum montanum*, *Phyllanthus amarus* and *Harrisonia perforate* showed potent cytotoxicity against HepG2 with IC_{50} values of 57.15 ± 19.07 , 9.39 ± 1.56 , 2.52 ± 1.28 , 37.16 ± 4.98 and 56.99 ± 9.63 $\mu\text{g}/\text{mL}$, respectively. The stilbene piceatannol was determined to have cytotoxic activity against the HepG2 cell line. Piceatannol had an IC_{50} value of 80.34 ± 2.91 $\mu\text{g}/\text{mL}$, and the positive control, 5-FU, had cytotoxic activity to HepG2 with IC_{50} of 0.39 ± 0.17 $\mu\text{g}/\text{mL}$. The result of cytotoxicity against KKKU-M156 of Saban remedy ethanolic extract exhibited an IC_{50} value of 0.26 ± 0.04 $\mu\text{g}/\text{mL}$. The fibroblast cell, MRC5, was selected to study cytotoxic activity to determine the toxicity of extracts to normal non-cancerous cells. The result of cytotoxicity activity against MRC5 is shown in **Table 3**. Most extracts exhibited moderate cytotoxic activity to normal cells.

Anti-inflammatory assays through inhibition of inflammatory cytokine production (nitric oxide, TNF-alpha, and PGE₂)

Saban remedy and its ingredients were evaluated for anti-inflammatory activity by inhibiting inflammatory cytokine production (nitric oxide, TNF-alpha, and PGE₂). Nitric oxide (NO), TNF-alpha, and PGE₂ are mediators released in inflammatory events (Son et al., 2020). The ethanolic and aqueous extract of Saban remedy exhibited moderate activity ($\text{IC}_{50} = 49.67 \pm$

9.14 $\mu\text{g/mL}$) and lack of appreciable anti-inflammatory activity through nitric oxide inhibition, respectively (Table 4). Ethanolic extracts of *Baliospermum montanum* and *Croton oblongifolius* possessed inhibitory activity on NO release with an IC_{50} of 10.40 ± 1.56 , 29.77 ± 15.05 $\mu\text{g/mL}$, while ethanolic extracts of *Cassia garrettiana* and *Phyllanthus amarus* displayed moderate activity with IC_{50} of 43.68 ± 9.89 and 50.20 ± 4.48 $\mu\text{g/mL}$. Piceatannol, a stilbene determined in the aqueous extract, had no detectable anti-inflammatory activity at 1 $\mu\text{g/mL}$. All extracts showed an IC_{50} greater than 100 $\mu\text{g/mL}$ on inhibition of TNF-alpha and PGE_2 production and less activity than the positive steroid control, prednisolone, which exhibited potent activity with an IC_{50} of 0.19 ± 0.04 $\mu\text{g/mL}$.

Mutagenic and anti-mutagenic activities using *Salmonella* mutation assay

The mutagenicity of Saban remedy extracts was determined using *S. typhimurium* strains TA98 and TA100 in the presence and absence of metabolic activation (+S9 and -S9). The concentration at 1 and 5 mg/plate of 95% ethanolic extract of Saban remedy (SBE) had a killing effect, but not the aqueous extract (SBW). On the other hand, the concentration at 0.2 mg/plate of SBE, and SBW did not increase the number of revertant colonies of *S. typhimurium* TA98 and TA100 in the presence and absence of S9 mix when compared to their negative control (Table 5).

Table 3. Cytotoxic activity and selective index (SI) of Saban remedy and its ingredients against HepG2 and MRC-5 cell lines.

Herbal plant/compound	Solvent	HepG2 (Cancer cells)		KKU-M156 (Cancer cells)		MRC-5 (Normal cells)
		IC_{50} ($\mu\text{g/mL}$)	SI	IC_{50} ($\mu\text{g/mL}$)	SI	
5-Fluorouracil (5-FU)		0.39 ± 0.17	NA	NA	NA	NA
Piceatannol		80.34 ± 2.91	0.61	NA	NA	48.90 ± 21.60
Saban remedy	95% Ethanol	26.85 ± 7.60	1.36	0.26 ± 0.04	140.11	36.43 ± 12.04
	Aqueous	>100	NA	>50	NA	NA
<i>Baliospermum montanum</i>	95% Ethanol	2.52 ± 1.28	39.73	0.16 ± 0.02	625.00	>100
	Aqueous	>100	NA	>50	NA	NA
<i>Cassia garrettiana</i>	95% Ethanol	57.15 ± 19.07	0.92	30.83 ± 0.60	1.70	52.32 ± 14.52
	Aqueous	>100	NA	>50	NA	NA
<i>Croton oblongifolius</i>	95% Ethanol	9.39 ± 1.56	5.12	1.31 ± 0.31	36.69	48.06 ± 18.78
	Aqueous	>100	NA	>50	NA	NA
<i>Harrisonia perforata</i>	95% Ethanol	57.00 ± 9.63	1.75	25.53 ± 2.96	3.92	>100
	Aqueous	>100	NA	>50	NA	NA
<i>Carissa carandas</i>	95% Ethanol	>100	NA	NA	NA	NA
	Aqueous	>100	NA	>50	NA	NA
<i>Phyllanthus amarus</i>	95% Ethanol	37.16 ± 4.98	2.69	39.25 ± 0.60	2.55	>100
	Aqueous	>100	NA	>50	NA	NA
<i>Phyllanthus reticulatus</i>	95% Ethanol	>100	NA	73.39 ± 4.32	NA	NA
	Aqueous	>100	NA	>50	NA	NA
<i>Tinospora crispa</i>	95% Ethanol	>100	NA	76.39 ± 4.32	NA	NA
	Aqueous	>100	NA	>50	NA	NA
<i>Tinospora baenzigeri</i>	95% Ethanol	>100	NA	47.80 ± 2.04	NA	NA
	Aqueous	>100	NA	>50	NA	NA
<i>Tiliacora triandra</i>	95% Ethanol	>100	NA	25.36 ± 0.94	NA	NA
	Aqueous	>100	NA	>50	NA	NA

Data are presented as mean $\pm$ SEM (n = 3). NA: Not analyzed.

Table 4. Inhibitory effects of Saban remedy and its ingredient on NO production from RAW 264.7 cell lines.

Herbal plants/ compound	Extract	% Inhibition at various concentrations (µg/mL) (% Toxicity at various concentrations)							IC ₅₀ (µg/mL)	
		0.001	0.01	0.1	1	10	30	50	100	
Prednisolone		-	-0.26 ± 2.86 (-6.52 ± 2.36)	28.64 ± 4.79 (-7.86 ± 1.18)	54.68 ± 1.34 (10.62 ± 3.17)	59.45 ± 1.35 (16.17 ± 3.92)	-	72.75 ± 1.35 (21.63 ± 9.62)	-	0.19 ± 0.04
Piceatannol		-	0.19 ± 0.92 (7.00 ± 2.70)	3.67 ± 0.326 (7.602 ± 5.20)	12.84 ± 7.60 (8.99 ± 4.60)	97.99 ± 7.02* (33.79 ± 19.17*)	-	97.63 ± 0.00* (56.38 ± 0.00*)	95.09 ± 0.00* (70.68 ± 0.00*)	>1
Saban remedy	95% Ethanol	-	-	-5.96 ± 1.73 (-25.32 ± 17.91)	-6.33 ± 1.44 (-18.16 ± 12.65)	3.53 ± 3.29 (-1.25 ± 16.97)	-	53.01 ± 8.88 (11.60 ± 5.37)	79.63 ± 5.71 (21.64 ± 4.27)	49.67 ± 9.14
	Aqueous	1.62 ± 1.48 (3.43 ± 8.63)	0.65 ± 2.93 (3.21 ± 3.01)	-0.81 ± 3.11 (8.52 ± 8.75)	4.79 ± 4.69 (15.04 ± 6.32)	62.47 ± 0.00** (2.08 ± 0.00**)	-	88.29 ± 0.00** (1.44 ± 0.00**)	93.28 ± 0.00** (1.61 ± 0.00**)	>1
<i>Baliospermum montanum</i>	95% Ethanol	-	-	-	2.09 ± 1.57 (4.25 ± 4.17)	50.32 ± 5.22 (22.88 ± 2.35)	-	66.45 ± 8.34 (20.88 ± 3.18)	75.44 ± 4.62 (24.72 ± 4.93)	10.40 ± 1.56
	Aqueous	-	-	2.55 ± 5.213 (3.097 ± 0.717)	12.27 ± 3.91 (21.00 ± 3.09)	71.72 ± 0.00** (31.79 ± 0.00**)	92.96 ± 0.00** (40.209 ± 0.00**)	94.90 ± 0.00** (9.785 ± 0.00**)	97.74 ± 0.00** (4.099 ± 0.00**)	>1
<i>Carissa carandas</i>	95% Ethanol	-	-	-	-12.21 ± 1.29 (5.05 ± 5.20)	3.40 ± 3.59 (1.15 ± 7.26)	-	60.02 ± 10.03 (-5.20 ± 6.72)	81.69 ± 7.85 (4.70 ± 4.68)	>100
	Aqueous	-	-	-	-	-	-	1.11 ± 4.48 (-41.49 ± 1.75)	8.635 ± 5.26 (-43.71 ± 20.88)	>1
<i>Cassia garrettiana</i>	95% Ethanol	-	-	-	-12.21 ± 1.29 (5.047 ± 5.19)	3.40 ± 3.59 (1.145 ± 7.26)	-	60.02 ± 10.03 (-5.20 ± 6.72)	81.69 ± 7.85 (4.701 ± 4.68)	43.68 ± 9.89
	Aqueous	-	-	-	-	-	-	1.11 ± 4.48 (-41.49 ± 1.75)	8.64 ± 5.26 (-43.71 ± 20.88)	>100

Table 4. Inhibitory effects of Saban remedy and its ingredient on NO production from RAW 264.7 cell lines (continued...)

Herbal plants/ compound	Extract	% Inhibition at various concentrations (µg/mL) (% Toxicity at various concentrations)								IC ₅₀ (µg/mL)
		0.001	0.01	0.1	1	10	30	50	100	
<i>Croton oblongifolius</i>	95% Ethanol	-	-	-	-14.65 ± 4.82 (-2.39 ± 1.89)	24.21 ± 8.51 (15.20 ± 3.94)	-	68.99 ± 12.06 (22.13 ± 3.82)	85.85 ± 7.27 (20.69 ± 2.62)	29.77 ± 15.05
	Aqueous	-	-	-3.84 ± 6.41 (7.20 ± 11.09)	-3.68 ± 6.06 (9.73 ± 8.93)	-0.96 ± 0.00** (-19.51 ± 0.00**)	21.93 ± 0.00** (-2.00 ± 0.00**)	61.91 ± 0.00** (9.65 ± 0.00**)	83.00 ± 0.00** (9.06 ± 0.00**)	>1
<i>Harrisonia perforata</i>	95% Ethanol	-	-	-	-	-	-	12.05 ± 4.70 (24.094 ± 0.13)	26.46 ± 8.25 (24.291 ± 11.03)	>100
	Aqueous	-	-	-	-	0.97 ± 2.52 (14.06 ± 4.30)	2.74 ± 1.67 (22.15 ± 5.50)	18.46 ± 0.00** (-22.62 ± 0.00**)	33.75 ± 0.00** (-25.52 ± 0.00**)	>30
<i>Phyllanthus amarus</i>	95% Ethanol	-	-	-	-8.09 ± 1.11 (3.57 ± 1.53)	2.58 ± 3.76 (29.182 ± 2.70)	-	49.32 ± 4.06 (26.74 ± 7.63)	82.01 ± 1.59 (25.96 ± 4.18)	50.20 ± 4.48
	Aqueous	-8.00 ± 1.90 (7.34 ± 5.56)	-2.34 ± 9.95 (7.95 ± 5.85)	21.63 ± 15.94** (28.41 ± 4.57**)	75.02 ± 0.00** (46.81 ± 0.00**)	94.87 ± 0.00** (42.14 ± 0.00**)	94.03 ± 0.00** (45.32 ± 0.00**)	99.09 ± 0.00** (5.84 ± 0.00**)	93.09 ± 0.00** (15.17 ± 0.00**)	>0.01
<i>Phyllanthus reticulatus</i>	95% Ethanol	-	-	-	-	-	-	-4.97 ± 7.12 (11.81 ± 6.05)	4.18 ± 2.36 (14.84 ± 3.45)	>100
	Aqueous	-	-	-	-	-	-	15.44 ± 17.85 (-20.77 ± 3.14)	9.90 ± 4.60 (-19.93 ± 4.44)	>100
<i>Tinospora crispa</i>	95% Ethanol	-	-	-	-	-	-	13.33 ± 1.89 (15.75 ± 6.90)	41.13 ± 6.30 (18.57 ± 9.28)	>100
	Aqueous	-	-	-	-2.21 ± 5.35 (9.55 ± 14.70)	1.37 ± 4.15 (15.88 ± 4.83)	5.01 ± 0.00** (17.68 ± 0.00**)	24.57 ± 0.00** (0.54 ± 0.00**)	54.84 ± 0.00** (3.34 ± 0.00**)	>10

Table 4. Inhibitory effects of Saban remedy and its ingredient on NO production from RAW 264.7 cell lines (continued...)

Herbal plants/ compound	Extract	% Inhibition at various concentrations (µg/mL) (% Toxicity at various concentrations)								IC ₅₀ (µg/mL)
		0.001	0.01	0.1	1	10	30	50	100	
<i>Tinospora baenzigeri</i>	95% Ethanol	-	-	-	-	-	-	10.21 ± 2.22 (13.14 ± 8.78)	39.97 ± 2.79 (22.90 ± 4.04)	>100
	Aqueous	-	-	-	-	-	-	2.57 ± 0.16 (-14.23 ± 4.03)	-2.02 ± 4.55 (-26.87 ± 13.50)	>100
<i>Tiliacora triandra</i>	95% Ethanol	-	-	-	-	-	-	-1.38 ± 4.51 (-17.48 ± 14.33)	34.95 ± 9.20 (-22.62 ± 12.04)	>100
	Aqueous	-	-	-	-	-	-	-1.81 ± 1.69 (11.64 ± 15.36)	-3.52 ± 2.03 (-10.87 ± 5.82)	>100

*Toxicity, **Substance-induced cells to generate nitric oxide.

Table 5. Mutagenic activity of Saban extracts using *Salmonella* mutation assay.

Treatment	Concentration (mg/plate)	Average of His ⁺ revertant colonies per plate (Mutagenic Index; MI)			
		TA 98		TA 100	
		+S9	-S9	+S9	-S9
DMSO (50 µL/plate)		25.73 ± 2.94 (1.00)	18.30 ± 0.70 (1.00)	102.10 ± 1.60 (1.00)	106.23 ± 1.45 (1.00)
Distilled water (50 µL/plate)		26.00 ± 5.67 (1.00)	20.19 ± 0.03 (1.00)	119.01 ± 3.24 (1.00)	112.40 ± 6.51 (1.00)
2-AA (0.5 µg/plate)		487.59 ± 83.93 (18.82)	NA	779.94 ± 115.19 (7.72)	NA
AF-2 (0.1 µg/plate for TA98) AF-2 (0.01 µg/plate for TA100)		NA	365.72 ± 26.12 (20.04)	NA	473.29 ± 2.04 (4.54)
SBE	0.2	25.78 ± 7.64 (1.20)	16.73 ± 1.71 (1.15)	107.00 ± 12.25 (1.01)	81.22 ± 3.13 (0.84)
	1	22.83 ± 3 24 (1.06) (K)	18.05 ± 2.68 (0.95) (K)	101.22 ± 10.02 (0.92)	81.56 ± 8.98 (0.78) (K)
	5	19.67 ± 4.22 (1.50) (K)	17.47 ± 3.22 (1.22) (K)	84.89 ± 1.09 (0.78) (K)	52.89 ± 9.94 (0.50) (K)

Table 5. Mutagenic activity of Saban extracts using *Salmonella* mutation assay (continued...)

Treatment	Concentration (mg/plate)	Average of His ^r revertant colonies per plate (Mutagenic Index; MI)			
		TA 98		TA 100	
		+S9	-S9	+S9	-S9
SBW	0.2	23.44 ± 6.25 (1.11)	15.78 ± 2.61 (0.78)	122.67 ± 5.42 (1.08)	107.22 ± 7.78 (1.03)
	1	33.67 ± 12.08 (1.56)	17.56 ± 1.31 (0.87)	103.33 ± 8.49 (0.91)	95.78 ± 12.28 (0.91)
	5	23.89 ± 5.06 (1.16)	17.44 ± 2.44 (0.86)	107.67 ± 10.39 (0.93)	117.56 ± 5.61 (1.12)
BME	0.2	27.50 ± 2.02 (1.13)	14.67 ± 1.59 (0.86)	110.56 ± 11.36 (1.02)	NA
CCE	0.2	25.00 ± 2.00 (1.05)	16.17 ± 2.85 (0.97)	105.56 ± 7.86 (0.97)	NA
CGE	0.2	20.33 ± 2.46 (0.82)	25.83 ± 1.01 (1.50)	115.11 ± 8.52 (1.06)	101.83 ± 4.44 (0.92)
COE	0.2	25.33 ± 0.88 (1.04)	19.33 ± 3.00 (1.15)	119.11 ± 8.40 (1.10)	NA
HPE	0.2	21.17 ± 3.00 (0.86)	15.50 ± 2.52 (0.88)	108.89 ± 11.95 (1.00)	95.17 ± 12.96 (0.88)
PAE	0.2	23.50 ± 5.48 (0.95)	18.67 ± 2.32 (0.86)	109.11 ± 12.75 (0.88)	NA
PRE	0.2	23.67 ± 0.82 (0.98)	14.67 ± 5.72 (0.92)	101.78 ± 6.80 (0.94)	NA
TCE	0.2	25.33 ± 2.74 (1.02)	16.33 ± 5.13 (0.94)	123.00 ± 10.49 (1.13)	NA
TBE	0.2	25.83 ± 5.09 (1.03)	18.67 ± 3.11 (1.04)	114.44 ± 14.18 (1.05)	NA
TTE	0.2	25.17 ± 0.73 (1.04)	13.50 ± 0.76 (0.80)	112.89 ± 12.53 (1.04)	NA
Piceatannol	0.0075	26.33 ± 1.59 (0.93)	NA	114.38 ± 9.44 (1.14)	NA
	0.03	26.50 ± 3.25 (0.94)	13.67 ± 1.69 (0.92)	112.63 ± 12.05 (1.13)	99.67 ± 6.65 (0.97)

Values are mean ± S.E.M. The data are the mean of three independent experiment. NA: Not analysed, K: Killing effect, 2-AA: 2-aminoanthracene, and AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide.

Furthermore, the extract's mutagenic index at 0.2 mg/plate was not greater than 2. The results indicated that the extracts at 0.2 mg/plate did not present mutagenicity in *S. typhimurium* TA98 and TA100 in the presence and absence of rat metabolic enzymes. According to mutagenicity data, the concentration of ethanolic of Saban remedy extract at 5 and 1 mg/plate displayed toxicity for bacteria, while the concentration of aqueous Saban remedy extract at 5 and 1 mg/plate was not toxic. Therefore, the maximum concentration for the anti-mutagenicity assay of the ethanolic extract was set at 0.2 mg/plate. The anti-mutagenicity of the extracts is summarized in Table 6. In the presence of metabolic activation, the extracts decreased the number of revertant colonies induced by aflatoxin B1 (AFB1) in TA98 and 2-amino-3,4 dimethylimidazo (4,5-f)quinolone (MeIQ) in TA100, whereas SBW at 0.2 mg/plate in TA100 was not decreased the number of revertant colonies. On the other hand, all the extracts in the absence of metabolic activation slightly decreased the number of revertant colonies induced by 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2) in TA98 and sodium azide (NaN₃) in TA100. The aqueous extract of the Saban remedy demonstrated antimutagenicity at high concentrations in TA98 (Table 6).

The anti-hepatocarcinogenesis on initiation stage testing of the Saban remedy extracts.

The findings showed that rats given an oral dose of 100 mg/kg of all extracts experienced no mortality, did not exhibit aberrant behavior, or experienced a change in their gross morphology. Body weight and food and water consumption were not significantly different in treatment groups compared to controls. The administration of 100 mg/kg of the Saban remedy ethanolic extract did not induce the number or size of hepatic GST-P positive foci in DEN-initiated rats. Remarkably, the aqueous extract of the Saban remedy decreased both the number and area of GST-P positive foci (Table 7).

DISCUSSION

Saban remedy contains ten herbs used for treating cancer patients' symptoms in TTM. The flavor of the Saban remedy is bitter. According to Joshi et al. (2007), the tradition of combining herbs depending on their flavor is an essential component of TTM, aimed at utilizing synergistic effects in the treatment of many diseases. The Saban remedy can be categorized into three treatment groups followed by Thai remedy

group drugs: main drug therapy, secondary drug therapy, and component drug therapy through their biological activity.

1. Main therapeutic drugs of Saban remedy are bitter and bitter-nauseating flavors, which decrease fire dhatu, which are purgative components to reduce the cancer patients' body temperature (Bhaccha, 2005). Our studies revealed that Saban remedy had potent the cytotoxic activity in vitro and GST-P foci in liver tissue *in vivo* was decreased. Saban remedy was utilized to protect and cure liver cancer in patients (Poonthananiwatkul et al., 2015). The biological activity of *B. montanum* demonstrated a strong cytotoxic effect against a liver cancer cell line at an IC₅₀ less than 10 µg/mL. In particular, *B. montanum* ethanolic extract displayed significant selective toxicity on HepG2 compared to MRC5. These studies further confirmed previous investigations that demonstrated the antitumor effects of constituents in *C. garrettiana*, showing activity against colon cancer, gastric carcinoma, human tongue cancer, human melanoma, and lung cancer cells. (Shang et al., 2022). Some chemical constituents in *C. garrettiana* have shown inhibition of hepatocellular carcinoma via anti-oxidation (Lu et al., 2017; Sáez et al., 2018; Seyed et al., 2016). The ethanolic extract of *C. garrettiana* at 100 mg/kg could reduce the number and size of GST-P positive foci. Alanine aminotransferase enzyme (ALT enzyme) was decreased by the ethanolic extracts of *C. garrettiana* when compared with the control group. Interestingly, Lebda et al. (2013) determined that serum levels of liver increased in acetaminophen-treated rats, which was attributed to the damaged structural integrity of the liver. Our results revealed that the ethanolic extract did not damage the structural integrity of the liver and are consistent with the use of *C. garrettiana* as traditional medicine for treating hepatocellular carcinoma which in part is modulated by the presence and activity of the stilbene piceatannol (Roupe et al., 2006).

Some additional reports of Saban remedy's ingredients having cytotoxic activity against various cancer cell lines such as liver cancer hepatocellular carcinoma (HepG2), cholangiocarcinoma cell line (KKU-M156), human gastric carcinoma (KATO-3), colon adenocarcinoma (SW480), breast ductal carcinoma (BT474), and undifferentiated lung carcinoma (CHAGO) have been reported (Aarthi and Babu, 2017; Ibahim et al., 2011; Itharat et al., 2010; Lawson-Evi et al., 2008; Nandagaon and Kulkarni, 2013; Weerapreeyakul et al., 2012; Zulkhairi et al., 2008).

Table 6. Anti-mutagenic activity of Saban extracts using Salmonella mutation assay in *S. typhimurium* strain TA98 and TA100 with the presence and absence of metabolic activation.

Extract code	Bacteria strains	TA98		TA100	
	Conditions	+S9	-S9	+S9	-S9
SBE at 0.2 mg/plate	Number of revertants	170.50 ± 33.79	363.17 ± 135.99	236.36 ± 17.86	787.11 ± 180.50
	% inhibition	89.04 ± 2.70	24.20 ± 17.73	88.17 ± 3.68	20.11 ± 23.89
SBW at 0.2 mg/plate	Number of revertants	1099.17 ± 375.85	329.83 ± 78.89	1301.33 ± 52.21	769.22 ± 64.12
	% inhibition	22.05 ± 21.84	30.01 ± 11.80	-30.29 ± 23.51	22.64 ± 7.84
SBW at 1 mg/plate	Number of revertants	956.00 ± 279.06	173.67 ± 14.15	875.67 ± 67.62	982.22 ± 222.71
	% inhibition	45.14 ± 8.01	53.41 ± 4.78	18.72 ± 17.92	17.45 ± 11.59
SBW at 5 mg/plate	Number of revertants	129.50 ± 21.72	99.67 ± 17.55	171.33 ± 29.13	1067.33 ± 178.39
	% inhibition	94.03 ± 1.77	72.28 ± 7.14	95.53 ± 1.52	7.69 ± 6.06
BME at 0.2 mg/plate	Number of revertants	1169.00 ± 261.86	341.17 ± 109.55	723.67 ± 159.25	NA
	% inhibition	13.05 ± 16.50	26.39 ± 14.45	54.64 ± 6.68	NA
CCE at 0.2 mg/plate	Number of revertants	876.00 ± 152.11	356.67 ± 72.57	867.78 ± 52.67	NA
	% inhibition	35.37 ± 2.93	20.85 ± 4.31	43.28 ± 1.84	NA
CGE at 0.2 mg/plate	Number of revertants	99.38 ± 12.38	370.67 ± 95.33	152.00 ± 7.00	1259.67 ± 149.66
	% inhibition	89.85 ± 5.60	33.38 ± 9.71	95.87 ± 2.47	-7.20 ± 18.14
COE at 0.2 mg/plate	Number of revertants	205.83 ± 28.42	380.00 ± 94.02	419.33 ± 98.89	NA
	% inhibition	86.39 ± 1.62	16.40 ± 8.44	78.36 ± 4.77	NA
HPE at 0.2 mg/plate	Number of revertants	486.50 ± 61.34	515.58 ± 103.08	173.56 ± 19.40	NA
	% inhibition	63.49 ± 6.16	3.89 ± 4.78	97.07 ± 0.77	NA
PAE at 0.2 mg/plate	Number of revertants	229.50 ± 67.66	377.33 ± 72.50	415.56 ± 142.72	NA
	% inhibition	84.92 ± 5.20	15.75 ± 1.35	79.54 ± 8.37	NA
PRE at 0.2 mg/plate	Number of revertants	1031.67 ± 182.04	383.17 ± 63.68	947.56 ± 199.63	NA
	% inhibition	23.39 ± 3.38	11.99 ± 24.40	37.97 ± 1187	NA
TCE at 0.2 mg/plate	Number of revertants	427.00 ± 191.65	381.00 ± 123.78	376.00 ± 68.71	NA
	% inhibition	72.16 ± 10.32	18.83 ± 13.65	80.85 ± 3.31	NA
TBE at 0.2 mg/plate	Number of revertants	398.50 ± 59.67	335.83 ± 79.71	444.22 ± 91.20	NA
	% inhibition	71.40 ± 3.68	26.49 ± 6.14	76.38 ± 4.18	NA
TTE at 0.2 mg/plate	Number of revertants	373.33 ± 53.80	289.83 ± 116.13	415.56 ± 35.61	NA
	% inhibition	73.58 ± 0.65	39.84 ± 15.91	77.46 ± 2.78	NA
Piceatannol at 0.03 mg/plate	Number of revertants	26.50 ± 3.25	13.67 ± 1.69	112.63 ± 12.05	99.67 ± 6.65
	% inhibition	-131.69 ± 58.84	8.14 ± 15.45	15.38 ± 14.19	13.12 ± 4.18

Values are mean ± S.E.M (n = 3). The data are the mean of three independent experiments. NA: Not analyzed.

Additional investigations have been conducted on compounds in Saban remedy that also have cytotoxicity against hepatocellular carcinoma. For instance, the *C. oblongifolius* had labdanes, furanocembranoid, and croblongifolin (Adam et al., 2018; Aye et al., 2019; Roengsumran et al., 2001), while the *C. carandas* had steroids and coumarins (Galipalli et al., 2015). Saban remedy is known to have polyphenol constituents

that regulate liver metabolism and prevent liver cancer progression. Phenolic compounds can activate peroxisome proliferator-activated receptor alpha (PPARα) and sterol regulatory element binding protein-1 (SREBP-1) by up-regulating the expression levels of peroxisome proliferators. The genes involved in hepatic lipid metabolism may ameliorate non-alcoholic fatty liver disease and affect liver cancer.

Table 7. The results of the total area of liver sections and numbers of GST-P positive foci.

Group	Chemical	Treatment	GST-P Number/ Liver area (cm ²)	GST-P area (mm ²)/ Liver area (cm ²)
1	DEN	10% Tween 80 (control group) (n = 7)	4.48 ± 3.07	0.13 ± 0.09
2	DEN	SBE at 100 mg/kg bw (n = 4)	6.55 ± 1.71	0.17 ± 0.06
3	DEN	SBW at 100 mg/kg bw (n = 5)	2.99 ± 1.54	0.07 ± 0.04
4	DEN	CGE at 100 mg/kg bw (n = 4)	1.54 ± 1.0*	0.03 ± 0.02*

The results are shown as mean ± SD. *p<0.05 statistically significant difference from group 1. DEN: diethylnitrosamine; GST-P: glutathione-S-transferase placental; SBE: ethanolic Saban extract; SBW: aqueous Saban extract; CGE: ethanolic *Cassia garrettiana* extract.

Flavonoids improve non-alcoholic fatty liver disease (NAFLD)-related lipid buildup, inflammation, fibrosis, nitrosation/oxidative stress, and insulin resistance. The stilbene polyphenol piceatannol is known to enhance heme oxygenase-1 (HO-1) expression, an antioxidant and anti-inflammatory enzyme, to activate nuclear factor erythroid 2-related factor 2 (Nrf2) transcriptional activity. Piceatannol is a metabolite of resveratrol that can reduce alcohol and hepatotoxic drug-induced liver damage, improve lipid metabolism, debase lipid buildup, protect HepG2 cells, boost antioxidant capacity, and restore mitochondrial respiratory chain activity (Arora et al., 2019; Kershaw and Kim, 2017; Li et al., 2023). Therefore, Saban remedy ethanolic extract may potentially have cytotoxic activity against hepatocellular carcinoma (HepG2) through the polyphenol compounds found in Saban remedy ingredients.

2. Secondary drugs of Saban remedy are bitter in flavor which reduces temperature and pain-relieving symptoms through anti-inflammatory activity as measured by NO inhibition (Itharat, 2018). Some plant ingredients in Saban remedy are used as anti-inflammatory treatments in TTM (Itharat, 2018). The results revealed that the ethanolic extracts of Saban remedy and its ingredients had anti-inflammatory effects through nitric oxide inhibition, which is consistent with the reports of Kakatum et al. (2012) and Pipatrattanaseree et al. (2019). These investigations showed the ethanolic extracts of the Saban remedy *Baliospermum montanum* demonstrated high NO inhibition activity (Kakatum et al., 2012; Pipatrattanaseree et al., 2019). Our investigation agrees with that published by Panthong et al. (2020), who demonstrated that while *C. garrettiana* extract may decrease NO production, but it was unable to inhibit TNF-α production. The other reports showed a contrasting result in anti-inflammatory effects through the inhibition of TNF-alpha and PGE₂. Dechayont and Kamarazaman teams reported that the alcoholic extract of *T. triandra* showed inhibitory activity against TNF-alpha production (Dechayont et al., 2021; Kamarazaman et al.,

2012). *P. amarus* extract (80% ethanolic extract) significantly reduced the production of pro-inflammatory mediators (TNF-α, IL-1β, PGE₂) and COX-2 protein expression in LPS-induced U937 human macrophages (Alagan et al., 2019; Harikrishnan et al., 2018). This previous publication found that some of the compounds in Saban remedy inhibit NO production, such as propiophenones, ethyl 8-hydroxy-8-methyl-tridecanoate, 1,7,8-trihydroxy-2-naphtaldehyde, and harperfolide (Banik et al., 2020; Choodej et al., 2013; Jayasooriya et al., 2013; Pipatrattanaseree et al., 2019; Yuandani et al., 2016). The isolated propiophenones from a crude ethanolic extract of *B. montanum* root had anti-inflammatory activity by nitric oxide inhibition, while the ethyl 8-hydroxy-8-methyl-tridecanoate and 1,7,8-trihydroxy-2-naphtaldehyde compounds from *P. amarus* exhibited strong inhibition on nitric oxide production. In the same way, the harperfolide compound from *H. perforate* demonstrated inhibition against NO production in LPS-stimulated macrophages. However, the main active compound of the ethanolic and aqueous extracts of the Saban remedy was determined to be -piceatannol, which was found to be about 26.44% and 4.63% w/w of the dried extract, respectively. Other active compounds in Saban remedy’s ingredients, for example, propiophenones, ethyl 8-hydroxy-8-methyl-tridecanoate, 1,7,8-trihydroxy-2-naphtaldehyde, and harperfolide, may also affect the anti-inflammatory activity of this remedy.

3. Component drugs are various flavors that are easily edible for the patient by seasoning, function as a remedy and can alleviate other cancer patient symptoms (Bhaccha, 2005). The Saban remedy has three component drug flavors: astringent, hot, and flavorless (Itharat, 2018; The Aphinyana Arokhayasala Foundation, 2017). The various biological activities, such as anti-inflammatory and cytotoxic activity, are related to liver cancer treatment. According to our findings, most extracts at 0.2 mg/plate exhibited anti-mutagenicity against indirect mutagens in the *Salmonella* mutation experiment but presented co-

mutagenicity on SBW. The results also demonstrate that the anti-mutagenic property of the extracts was related to metabolic activation. The studies of Singharachai et al. (2011) demonstrated that the ethanolic and aqueous extracts of *T. triandra* and *H. perforate* at 5 mg/plate were non-mutagenic directly. Most of them were mutagenic indirectly under nitrosation (nitrite treated and acidic conditions), although they showed a high percentage of anti-mutagenic activity. However, this assay differed from the current study by not using metabolic activation and 1-aminopyrine (1-AP) as a positive mutagenic.

In summary, these current data and investigations indicate that the Saban remedy extracts may have the ability to decrease and relieve symptoms in liver cancer patients through its demonstrated *in vitro* and *in vivo* bioactivity. The aqueous and ethanolic extracts of Saban remedy have a potent ability to reduce GST-P foci, which is a pre-neoplastic lesion in liver tissue. These effects appear to correlate with our clinical experience at the Arokhayasala Khampramong temple using Saban remedy in cancer patients. The underlying scientific evidence and rationale found in this study are consistent with the TTM's claim that the Saban remedy has properties that can reduce the formation of pre-cancerous cells in liver cancer tissue. Further, a mechanistic understanding of the decreasing presence of GST-P foci and cytotoxic activity against the HepG2 and KKU-M156 in cell lines is undergoing. Based on the current findings, further understanding of the synergistic combination of medicinal herbs in Saban remedy for enhanced potency should be further investigated.

CONCLUSION

Saban remedy aqueous extract has been used to treat liver cancer patients in Arokhayasala, Khampramong Temple by TTM folk doctors. Saban remedy use is consistent with TTM wisdom in relieving symptoms in liver cancer patients with cytotoxicity, anti-inflammatory, and anti-mutagenic properties. Our results provide initial scientific evidence and analysis, underpinning and consistent with the use of Saban remedy in liver cancer in TTM.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Concepts or ideas	x	x	x	x	x	x	x		x	x	x			x	x	
Design	x	x	x	x	x		x	x	x	x	x	x	x			
Definition of intellectual content	x	x														
Literature search	x	x	x	x	x	x	x							x	x	
Experimental studies	x		x	x	x	x	x	x	x	x	x	x	x			
Data acquisition	x	x	x	x	x			x	x	x	x	x	x	x	x	
Data analysis	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Statistical analysis	x	x				x		x	x	x	x	x	x			
Manuscript preparation	x	x				x										x
Manuscript editing		x	x	x	x	x	x									x
Manuscript review	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

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