Anti-angiogenic and anti-proliferative effects of Benja-ummarit extract in rats with hepatocellular carcinoma

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Abstract. The herbal extract Benja-ummarit (BU) is a traditional Thai medicine with a putative cancer-suppressing effect. However, this effect has only been tested *in vitro* in human hepatocarcinoma cell lines. The present study determined the efficacy of a BU extract to treat hepatocellular carcinoma (HCC) in rats *in vivo* and established its anti-angiogenic and anti-proliferative properties. The BU extract was prepared in 95% ethanol and its composition determined using liquid chromatography-mass spectrometry. HCC was induced in Wistar rats by an injection of diethylnitrosamine (DEN), followed 2 weeks later by injections of thioacetamide (TAA) thrice weekly for 4 weeks. Following 2 months, the DEN-TAA-treated rats were divided into 6 groups that were treated orally for another 2 months with: i) No treatment; ii) vehicle; iii) 30 mg/kg sorafenib (SF); iv) 1 mg/kg BU; v) 10 mg/kg BU; or vi) 50 mg/kg BU. Liver samples were collected for gross morphological, histological, reverse transcription-quantitative PCR and western blot analyses, and serum samples were collected for liver function tests. The size and number of the cancer nodules were reduced ~10-fold in BU-treated HCC groups and ~14-fold in the SF-treated group compared with the HCC group. Furthermore, the serum parameters of liver damage were lower in BU-compared with SF-treated rats. These results indicate that while each of these formulations strongly reduce HCC expansion, BU extract results in less liver damage. Vascular endothelial growth factor expression was reduced significantly in the BU-and SF-treated HCC groups compared with the HCC group ($P<0.05$). BU extract antagonizes HCC growth *in vivo* potently through inhibiting tumor angiogenesis. BU, therefore, qualifies as a promising medical herb requiring further evaluation as a treatment of HCC.

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Abbreviations: ALT, alanine aminotransferase; BU, Benja-ummarit; DEN, diethylnitrosamine; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PBS, phosphate buffered saline; PBS-T, phosphate buffered saline with Tween-20; PCR, polymerase chain reaction; TAA, thioacetamide; VEGF, vascular endothelial growth factor

Key words: hepatocellular carcinoma, Benja-ummarit, Thai traditional medicine, Sorafenib, vascular endothelial growth factor

Introduction

Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, is the third leading cause of cancer-associated mortality globally (1). It is the fifth most common cancer type in men and the seventh most common cancer type in women. The incidence of HCC varies topographically, with the majority of cases occurring in developing countries. Southeast Asia and Sub-Saharan Africa host >75% of HCC cases, with incidence rates exceeding 20 per 100,00 individuals (2). Southern European countries have intermediate incidence rates, whereas North America, South America and Northern Europe have the lowest incidence rates (<5 per 100,000 individuals) (2).

A previous report suggests that the incidence of HCC in areas with high and intermediate incidence may stabilize or even decrease due to vaccination programs for hepatitis B virus (HBV) and higher HBV treatment rates in China and Taiwan (3). The decrease in HCC incidence in Japan and Southern Europe may be associated with an aging cohort of patients with hepatitis C virus (HCV) infection (4). In contrast, there is a rapid increase in the incidence of HCC in low-incidence areas, including the United States (5). The two most important causes of the rise in incidence in the USA are growing populations of patients with an advanced HCV infection and non-alcoholic steatohepatitis (2). HCC also has one of the fastest growing mortality rates of all solid tumor types. While the prognosis for most solid cancer types improved between 1994 and 2003, the mortality rate for HCC almost doubled (6). These trends warrant a further search for an effective treatment. Numerous signaling pathways serve a function

in the development of HCC, but those involving vascular endothelial growth factor (VEGF) angiogenesis stand out (7,8).

Benja-ummarit (BU) ('Phaetsat Songkhro') is a Thai traditional medicine from the Thai scripture 'Tadbunjob' (9). BU extract is used to treat patients with asthma, cough, short breath, liver abscess and anorexia (9). In Thai traditional medicine, all of those symptoms are interpreted as signs of a malignant tumor (10). In addition, BU is also used as a laxative in Thai traditional medicine (10). It is composed of eight herbs [aloe vera (*Aloe barbadensis*), red physic nut (*Baliospermum montanum*), kaffir lime (*Citrus hystrix*), asafoetida (*Ferula assafoetida*), gamboge resin (*Garcinia hanburyi*), Javanese long pepper (*Piper Chaba*), black pepper (*Piper nigrum*), ginger (*Zingiber officinale*)] and epsom salt (magnesium sulfate) (9). A chemical component of gamboge resin, gambogic acid, inhibits the proliferation of numerous types of cancer cells *in vitro*, including HepG2 (11) and SMMC-7221 (12). In addition, evidence suggests that when gambogic acid is co-administered with docetaxel, a chemotherapy drug, an increased inhibitory effect is observed against the proliferation of gastric and colorectal cancer cell lines (13). It has also been revealed that gambogic derivatives inhibit the proliferation of the HepG2 and A549 cancer cell lines (14). Furthermore, gambogic acid has an anti-angiogenic effect in numerous cancer types (15-19), exhibits anti-inflammatory activity (20-22) and anti-invasion activity against A549 human lung cancer cells (23) and osteosarcoma cell lines (24). Furthermore, when used against human breast carcinoma MCF-7 cells or human chronic myelogenous leukemia K562 cells, the cells are arrested at the G2/M (25) and G0/G1 transitions of the cell cycle (26), respectively. It has also been revealed that the decreased adhesion of human cancer cells is an effect of gambogic acid (27). Previously, a crude BU extract has been claimed to exert an anti-proliferative effect on human lung cancer (A549) and liver cancer (HepG2) cells by inducing apoptosis via reactive oxygen species (ROS) generation (10).

To the best of our knowledge, only a single clinical report has been published about the beneficial effects of BU in patients with HCC (28). This prospective descriptive study was performed in patients with certified HCC. A total of 96 patients were treated with 300-1,200 mg BU twice daily in addition to standard drug treatment. Once BU had been administered continuously for 2 months, the quality of life of patients taking BU was significantly better compared with that of similar patients not receiving BU. The study was undertaken in 5 public hospitals in Thailand and used the Thai Modified Function Living Index Cancer Questionnaire Version 2 score. Subsequent to a follow-up of 1 year, the survival rate of this patient group was higher compared with the control group. No serious adverse effects were reported (28).

The successful *in vitro* studies and the promising clinical study prompt interest in BU as an (adjuvant) treatment for HCC. Furthermore, the aforementioned anti-angiogenesis and anti-cancer effects of gambogic acid, which is a chemical component of gamboge resin which is present in BU, suggest that BU may inhibit the proliferation of cancer cells. However, since the majority of the cited mechanistic studies were performed *in vitro*, it is not easy to differentiate general from specific cytotoxic effects. For all these reasons, a relevant and reliable animal model is necessary to elucidate the putative functions of BU in a solid biochemical base. For this reason, the present study assessed the effects of BU extract in an established rat liver cancer *in vivo*. In this model, the putative anti-angiogenic effect of BU extract was also assessed.

Materials and methods

Preparation of a 95% ethanol extract of BU. The 95% ethanol extract of BU was provided by Dr. Arunporn Itharat from the Faculty of Medicine of Thammasat University (9). The BU formula consisted of aloe vera (2.48%), red physic nut (9.92%), kaffir lime (33.06%), asafoetida (2.48%), gamboge resin (4.96%), Javanese long pepper (2.48%), black pepper (2.48%), ginger (2.48%) and epsom salt (39.66%). Dried plant material (300 g) in a hot air oven at 50˚C was macerated in 95% ethanol for 3 days, filtered through a Whatman No. 1 filter paper and concentrated using an evaporator.

Composition of the ethanolic extract of BU. Liquid chromatography-mass spectrometry (LC-MS) was used to determine the compounds in the BU extract. The analysis was performed on an Agilent HPLC 1260 series consisting of a vacuum degasser, a binary pump, an autosampler and a column thermostat equipped with QTOF 6540 UHD accurate mass (Agilent Technologies GmbH). MassHunter Software B06.0 (Agilent Technologies GmbH) was used to control the LC-MS.

The separation of the sample solution was performed on a Luna C18, 150x4.6 mm, 5 μm column (Phenomenex, Torrance). A 10 μl sample of each filtrated extract at a concentration of 20 mg/ml was injected into the LC system with a solvent flow rate of 500 μl/min. The mobile phase consisted of a gradient elution between water (solvent A) and acetonitrile (solvent B), each containing 0.1% v/v formic acid. The linear gradient elution was 5-95% for solvent B starting at 0-35 min with holding for 5 min and post-run for 5 min. The column temperature was controlled at 35˚C. The mass analysis was performed using a QTOF 6540 UHD accurate mass spectrometer. The conditions for the negative electrospray ionization source were drying gas (N_2) at a flow rate of 10 l/min, a drying gas temperature of 350˚C, nebulizer 30 psi, fragmentor 100 V, capillary voltage 3,500 V and scan spectra from m/z 100-1,000 amu. The auto MS/MS for the fragmentation was set with collision energies of 10, 20 and 40 V. The positive mode was also set up with the same MS conditions as the negative mode.

Animals. All animal experiments were performed according to the Thai guidelines for the care and use of experimental animals, subsequent to being approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (Bangkok, Thailand; approval no. 3/2558). A total of 42 male Wistar rats (6-7 weeks old) weighing 200-250 g were obtained from the National Laboratory Animal Center of Mahidol University (Bangkok, Thailand). The animals were acclimatized for one week. All animals were maintained under standardized hygienic conditions throughout the experimental period, including a temperature of 21-22˚C, humidity at 55±5%, a standard 12 h light-dark regime and *ad libitum* access to standard diet and tap water.

Experimental design. The experimental protocol for HCC induction was based on El-Ashmawy *et al* (29). For the induction of HCC, 200 mg/kg diethylnitrosamine (DEN; Sigma-Aldrich; Merck KGaA) was injected intraperitoneally (i.p.) in a single dose. Following 14 days, the rats were subjected to i.p. injections of 300 mg/kg thioacetamide (TAA) (Sigma-Aldrich; Merck KGaA) 3 times weekly for 4 weeks. Then the rats were left for 2 further weeks without any treatment. At the end of the induction period (8 weeks), HCC rats were weighed and randomly divided into 6 groups: i) No treatment; ii) treatment with propylene glycol: Tween 80: deionized water (4:1:4), a solvent of BU; iii) treatment with 30 mg/kg Sorafenib (30-34); or treatment with iv) 1 mg/kg, v) 10 mg/kg or vi) 50 mg/kg BU. Doses of BU used in the present study were based on those previously used *in vitro* (9) and demonstrated to be safe in a toxicity test in rats (Intharit *et al*, preliminary study). During the time course of the experimental tumor study (16 weeks), a set of criteria was developed to follow the rats' condition. These included their external physical appearance, appearance of any visible lesions, changes in body weight and behavioral responses to external stimuli (including light or noise and so on), which reflect pain and distress in the animals. The humane endpoint in the present study was based on a weight loss exceeding 20% of the body weight of the rats in the control group. Subsequent to 16 weeks, experimental rats (n=7 per group) were anesthetized with an i.p. injection of 45 mg/kg pentobarbital sodium prior to sacrifice by decapitation with a rodent guillotine. Liver tissues and blood samples were collected for histological and immunohistochemical analyses, liver function tests, and reverse transcription-quantitative PCR (RT-qPCR) and western blot analyses.

Measurement of liver/body weight ratio. At the end of the treatment period, the body weight of all animals along with their respective livers were measured in order to determine the liver-to-body-weight ratio in each group.

Assay of serum alanine aminotransferase (ALT) and albumin. Blood samples, collected by cardiac puncture, were assayed in a standard clinical lab for serum ALT and albumin. The reagent kits for ALT (cat. no. 7D56-21) and albumin (cat. no. 7D53-23) were used (Abbott Pharmaceutical Co. Ltd.). The signals were detected by ARCHITEC model Ci16200 (Abbott Pharmaceutical Co. Ltd.).

Histopathological study. Liver tissues were fixed overnight at 4˚C in 4% (v/v) formaldehyde solution, dehydrated in an ascending series of ethanol (50, 70, 80, 90, 95 and 100%), cleared in xylene and embedded in paraffin. Specimens were sliced into sections that were 5 μm thick. The slides were stained with hematoxylin for 6 min and eosin for 1 min at room temperature and scanned with a panoramic digital slide scanner (3DHISTECH Ltd.). For each image, an area of 4,000x2,500 μm (10 mm^2) was randomly selected to locate and calculate the cancer area characterized histopathologically by the presence of thick-cell cords (35) using the CaseViewer software (v1.3.0.41885; https://www.3dhistech.com/caseviewer). A total of 21 images of each animal group were sampled, in which three specialists in liver histopathology identified the thick-cell cords and located the cancer areas. The mean cancer area in each group was then calculated.

Immunohistochemistry. The deparaffinized tissue sections were treated with xylene and rehydrated in a descending series of ethanol (100, 95, 90, 80, 70 and 50%), followed by antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 10 min at 120˚C using an autoclave. The blocking solution TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin and 0.05% Tween 20; pH 8.0) containing 10% goat serum was applied for 30 min at room temperature to the slides to block any non-specific binding, and the slides were incubated with mouse anti-VEGF immunoglobulin G (IgG; cat. no. sc-53462; Santa Cruz Biotechnology, Inc., Dallas; 1:50) at 4˚C overnight. The slides were then incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG (cat. no. 11569520, Sigma-Aldrich; Merck KGaA; 1:100). Subsequent to washing in phosphate buffered saline (PBS), the sections were incubated in substrate containing nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (toluidine salt; Dako; Agilent Technologies GmbH) diluted in 100 mM Tris (pH 9.5), 100 mM NaCl and 50 mM $MgCl_2$ at room temperature for 30-120 min to visualize the immunopositive areas in the tissues. The staining reaction was stopped by washing with distilled water. The sections were dehydrated with ethanol, cleared in xylene and covered with Permount® prior to being examined and photographed under a light microscope (Olympus Corporation).

RNA isolation and RT-qPCR assays. The livers were homogenized with a sonicator (Sonics & Materials, Inc.) followed by total RNA isolation using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA with 2 μg of each RNA sample was reverse transcribed with the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol (25˚C for 10 min, 37˚C for 120 min, 85˚C for 5 min and hold at 4˚C). RT-qPCR was set up using the SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.), along with cDNA and commercial PrimePCR™ primers (Bio-Rad Laboratories, Inc.). The primer used was rat *Vegfa* (unique assay ID: qRnoCED0002159). The differences in sample RNA content were normalized to rat β-actin (*Actb*) expression (unique assay ID: qRnoCID0056984). The conditions of the reactions were as follows: 95˚C for 2 min of polymerase activation, 40 cycles of 95˚C for 5 sec for denaturation and 60˚C for 30 sec for primer annealing and extension. Each sample's mRNA expression was measured in triplicate to ensure the fidelity and accuracy of the results using a CFX96 Real-Time PCR Detection System and Bio-Rad manager™ software version 1.3.1 (Bio-Rad Laboratories, Inc.). The quantification of relative mRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method (36).

Protein extraction and western blot analysis. A total of 50 mg frozen liver was homogenized in 500 μl radioimmunoprecipitation lysis buffer (Santa Cruz Biotechnology, Inc.,). The lysate was centrifuged at 4˚C and 12,000 x g for 15 min to collect the supernatant fraction. Protein concentration was determined

Table I. Results of liquid chromatography-mass spectrometry and the identification of putative active components in the ethanolic Benja-ummarit extract monitored in the electrospray ionization negative mode.

Peak	Retention time, min	m/z [M-H]-	MS/MS fragmentation	Tentative identification	Formula	Error (ppm)
1	10.091	393.1197	273.0850, 203.0769, 59.0376	Aloesin or aloeresin B derivative	$C_{19}H_{22}O_9$	-1.51
2	11.67	393.1173		Aloesin or aloeresin B derivative	$C_{19}H_{22}O_9$	4.59
3	12.356	393.1171	273.0820, 203.0753, 125.0274, 59.0161	Aloesin or aloeresin B derivative	$C_{19}H_{22}O_9$	5.1
4	12.711	393.117	273.0829, 203.0757, 59.0175	Aloesin or aloeresin B derivative	$C_{19}H_{21}O_9$	5.36
5	13.699	479.1164[a]	433.1248, 270.0606, 187.9765	5-Hydroxyaloin A	$C_{21}H_{22}O_{10}$	6.47
6	14.108	539.1537	375.1186, 273.0837, 163.0445, 119.0541	2'-o-p-Coumaroylaloesin	$C_{28}H_{28}O_{11}$	4.05
7	14.731	417.1172	297.0841	Aloin A	$C_{21}H_{22}O_9$	4.57
8	15.165	417.1169	297.0854, 205.0195	Aloin B	$C_{21}H_{22}O_9$	5.29
9	16.859	553.1659	443.1397, 279.0694	2'-p-Methoxycoumaroylaloeresin	$C_{29}H_{30}O_{11}$	10.19
10	33.791	629.3115	461.1940, 392.1234, 337.0694	Dihydrogambogic acid derivative	$C_{38}H_{46}O_8$	0.78
11	34.499	629.3106	541.3295, 461.1939, 392.1246	Dihydrogambogic acid derivative	$C_{38}H_{46}O_8$	2.21
12	38.64	627.2951		Gambogic acid	$C_{38}H_{44}O_8$	1.27

[a][M+HCOO]-; MS, mass spectrometry.

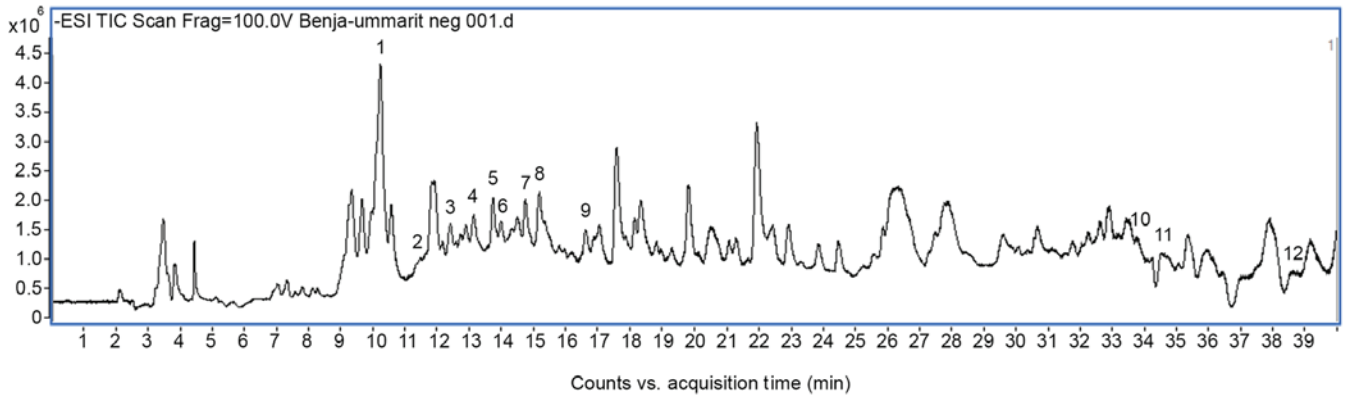

Figure 1. TIC chromatograms of 20 mg/ml Benja-ummarit extract monitored in the ESI negative mode. The peak number and compound identifications are summarized in Table I. TIC, total ion current; ESI, electrospray ionization.

using a Bradford protein assay (Bio-Rad Laboratories, Inc.). Total protein samples (40 μg) were separated on 12% SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc.) and transferred onto a 0.2 μm polyvinylidene difluoride membrane. The membrane was blocked at room temperature for 1 h with 5% non-fat milk in 1X PBS-0.1%Tween 20 (PBS-T), then washed with 1X PBS-T, and incubated overnight at 4˚C with a primary mouse anti-VEGF antibody (cat. no. sc-53462; Santa Cruz Biotechnology; diluted 1:500). Subsequent to washing thrice in 1X PBS-T for 5 min, the membrane was incubated at room temperature for 90 min with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (cat. no. 7076S lot 32; Santa Cruz Biotechnology; diluted 1:10,000) followed by 3x10 min washes with 1X PBS-T. Protein bands were developed using enhanced chemiluminescence (Bio-Rad Laboratories, Inc.) and quantified using densitometry with Scion Image software version Beta 4.0.3 (Meyer Instruments, Inc.). β-actin was used as a loading control.

Statistical analysis. Data were analyzed using a one-way analysis of variance with a Tukey's post-hoc test (PSPP 0.10.4; http://gnu.org). All results were represented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Table II. Results of liquid chromatography-mass spectrometry and the identification of putative active components in the ethanolic Benja-ummarit extract monitored in the electrospray ionization positive mode.

Peak	Retention time, min	m/z [M+H]+	MS/MS fragmentation	Tentative identification	Formula	Error (ppm)
1	19.8	336.3314	290.2888, 81.0712	Pipericine	$C_{22}H_{41}NO$	-15.78
2	24.4	286.149	201.0579, 135.0458	Piperine	$C_{17}H_{19}NO_3$	-18.28
3	26.5	312.1637	227.0735, 169.0669, 112.0772	Piperettine	$C_{19}H_{21}NO_3$	-13.71
4	28.5	340.1959	179.1329, 112.0771, 103.0556	Dehydropipernoline	$C_{21}H_{25}NO_3$	-15.23
5	29.7	356.2274	255.1415, 135.0457	Pipercide	$C_{22}H_{29}NO_3$	-15.10

MS, mass spectrometry.

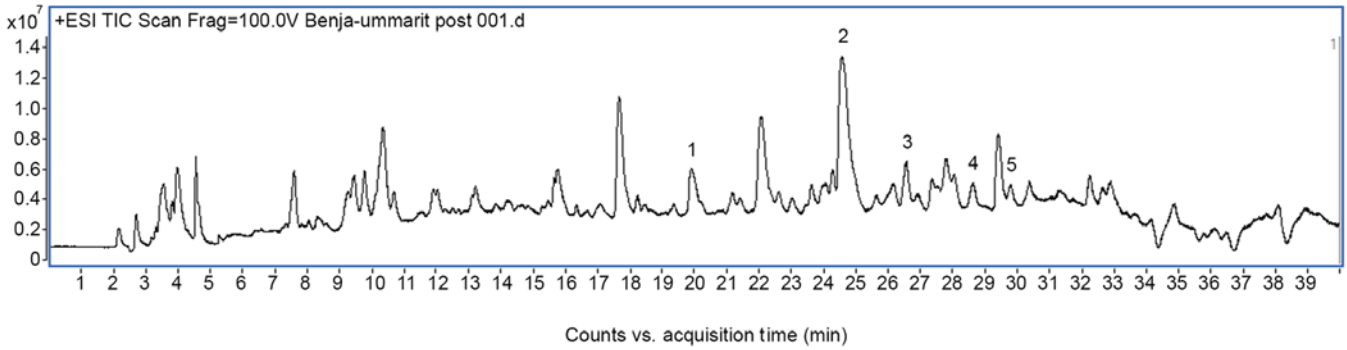

Figure 2. TIC chromatograms of 20 mg/ml Benja-ummarit extract monitored in the ESI positive mode. The peak number and compound identifications are summarized in Table II. TIC, total ion current; ESI, electrospray ionization.

Results

Composition of the ethanolic extract of BU. In the negative mode, 12 compounds were tentatively identified based on their molecular mass and fragmentation pattern (Fig. 1 and Table I). Aloin, aloesin and their derivatives together with dihydrogambogic acid and gambogic acid were indicated to be present in the extract. These compounds were identified in aloe vera and gamboge resin. In addition, five alkaloids from black pepper were identified in the positive mode (Fig. 2 and Table II). The chromatograms, mass spectra data and compounds proposal were presented in Figs. 1 and 2 and Tables I and II. This analysis confirms the previously published results for the original extract (9).

Changes in body weight and liver/body weight ratio. Fig. 3A presents the changes in the body weight in the respective groups of rats. Apart from the anticipated adverse effects of DEN administration at the beginning of the experiment and of thioacetamide during weeks 2-6, the experimental animals gained weight at the same rate as the control animals. To identify the potentially harmful general effect of the treatments on the liver, the liver/body weight ratio was determined (Fig. 3B). Rats treated with Sorafenib had a significantly reduced liver/body weight ratio compared with all other groups ($P<0.05$). There were no significant effects in the BU-treated groups when compared with the non-treated and vehicle-treated groups (Fig. 3).

Biochemical markers. Figs. 4 and 5 revealed the effects of BU on liver function tests (serum ALT activity and albumin content) in the experimental animals. The serum ALT level was significantly increased in the Sorafenib-treated group compared with all other groups ($P<0.05$; Fig. 4). On the other hand, the serum albumin concentration was significantly lower in the Sorafenib-treated group compared with all other groups ($P<0.05$; Fig. 5). The results demonstrate that Sorafenib causes liver injury.

Gross anatomy and histopathology. Fig. 6 revealed that Sorafenib (Fig. 6C) and BU (Fig. 6D-F) suppressed nodule growth compared with untreated and vehicle-treated rats with HCC (Fig. 6A and B). Sections revealed the characteristic histopathological thick-cell cord changes in the HCC nodules of non-treated (Figs. 7A and 8, 22±9%) and vehicle-treated rats (Figs. 7B and 8, 18±4%). The percentage of the cancer area was lowest in the Sorafenib-treated group compared with all other groups (Figs. 7C and 8, 1.5±0.7%). The percentage of the cancer area in the BU-treated group decreased dose-dependently from 2.7±1.6% (Figs. 7D and 8; 1 mg), to 2.6±1.4% (Figs. 7E and 8; 10 mg) to 2.1±0.8% (Figs. 7F and 8; 50 mg), and was significantly reduced when compared with the vehicle-treated group ($P<0.05$). These data reveal that BU reduces cancer growth *in vivo*.

BU inhibits VEGF expression. Next, the mechanisms by which BU exerts its antitumor effect *in vivo* were investigated.

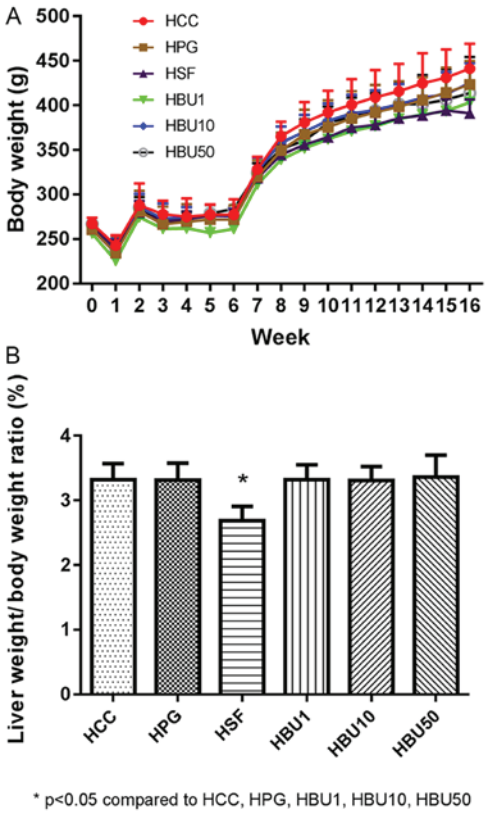

Figure 3. Effect of Sorafenib and BU on (A) the body weight and (B) the liver/body weight ratio of rats with HCC. Results were presented as the mean ± standard deviation. *P<0.05 vs. all other groups. HPG, rats with HCC only treated with BU solvent; BU, Benja-ummarit; HCC, hepatocellular carcinoma; HSF, rats treated with Sorafenib; HBU1, rats treated with 1 mg BU; HBU10, rats treated with 10 mg BU; HBU50, rats treated with 50 mg BU.

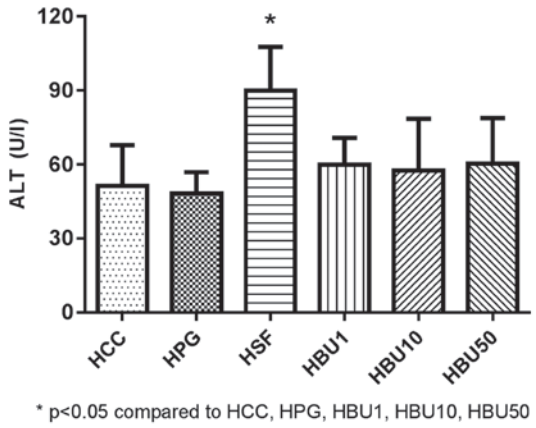

Figure 4. Effect of Sorafenib and BU on serum ALT activity in rats with HCC. Results were presented as the mean ± standard deviation. *P<0.05 vs. all other groups. ALT, alanine aminotransferase; HPG, rats with HCC only treated with BU solvent; BU, Benja-ummarit; HCC, hepatocellular carcinoma; HSF, rats treated with Sorafenib; HBU1, rats treated with 1 mg BU; HBU10, rats treated with 10 mg BU; HBU50, rats treated with 50 mg BU.

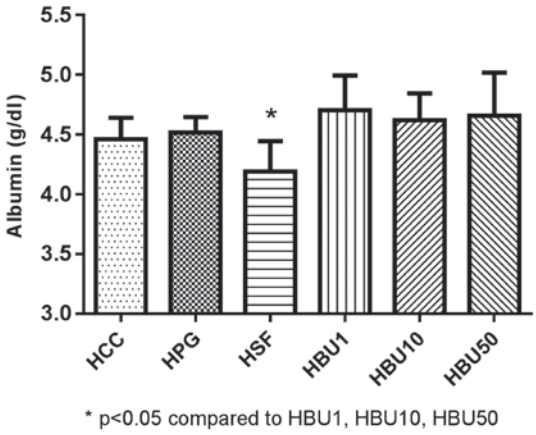

Figure 5. Effect of Sorafenib and BU on serum albumin activity in rats with HCC. Results were presented as the mean ± standard deviation. *P<0.05 vs. all other groups. HPG, rats with HCC only treated with BU solvent; BU, Benja-ummarit; HCC, hepatocellular carcinoma; HSF, rats treated with Sorafenib; HBU1, rats treated with 1 mg BU; HBU10, rats treated with 10 mg BU; HBU50, rats treated with 50 mg BU.

Immunohistochemical analysis revealed that the cytoplasmic VEGF concentration was markedly increased in cancerous areas (Fig. 9A; arrowheads) and that BU solvent alone did not change that result (Fig. 9B). In contrast, Sorafenib (Fig. 9C) and BU treatment prevented the formation of VEGF-positive cancer areas (Fig. 9D-F) in rats with HCC. In agreement with these results, VEGF mRNA expression was revealed to be significantly downregulated by Sorafenib compared with the control group (P<0.05) and an even stronger and dose-dependent downregulation by increasing doses of BU compared with the control groups (P<0.05; Fig. 10). Similarly, western blot analysis of the liver revealed that VEGF protein content was, compared with untreated and vehicle-treated rats with HCC, decreased significantly by Sorafenib treatment and treatment with the two highest doses (10 and 50 mg) of BU (P<0.05; Fig. 11). These results suggest that the anticancer activity of BU is mediated at least in part by the inhibition of VEGF expression in rats with HCC.

Discussion

BU is a traditional Thai herbal medicine containing a crude extract of eight plants and thus consists of numerous ingredients. The word 'Benja-ummarit' is a combination of the Thai words 'Benja' (five) and 'Ummarit' (holy compound or nectar). Although BU typically contains the extracts of eight plants and epsom salts ($MgSO_4$), its main active ingredients appear to be derived from only four plants (gamboge resin, aloe vera, asafoetida and red physic nut) and $MgSO_4$ (9). The other four plant extracts (kaffir lime, ginger, Javanese long pepper and black pepper) are added during the preparation of BU to reduce its toxicity, to eliminate the accumulation of gas in the alimentary canal and to increase appetite (9).

The present study performed experiments with the crude extract of BU as opposed to admixing highly purified components of BU, as the aim was to initially demonstrate that a crude BU extract exerts a similar effect *in vivo* as was demonstrated *in vitro* previously in a HepG2 HCC cell line (9,10). Furthermore, the ingredients of BU may have additive or even synergistic effects, as the cytotoxicity of crude extracts of BU was higher compared with that of each of its ingredients separately in the HepG2 cell line (9). In this respect, it is encouraging that the *in vitro* may be extrapolated *in vivo*. Following the same experimental protocol as used in the previous study and comparing treatment with 1 mg/kg

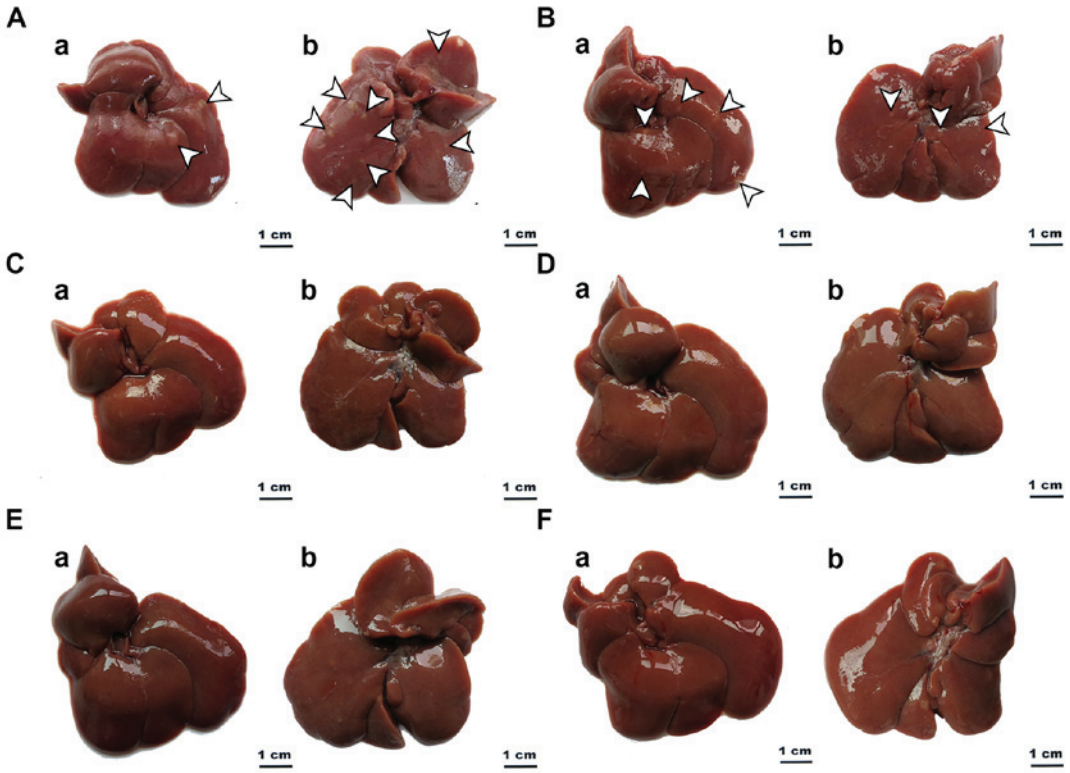

Figure 6. Gross appearance of the (a) diaphragmatic and (b) visceral surfaces of the livers of rats with hepatocellular carcinoma. Nodules were more prominent (white arrowheads) in the (A) untreated and (B) vehicle-treated groups compared with the (C) Sorafenib- or (D) 1 mg, (E) 10 mg or (F) 50 mg Benja-ummarit-treated groups.

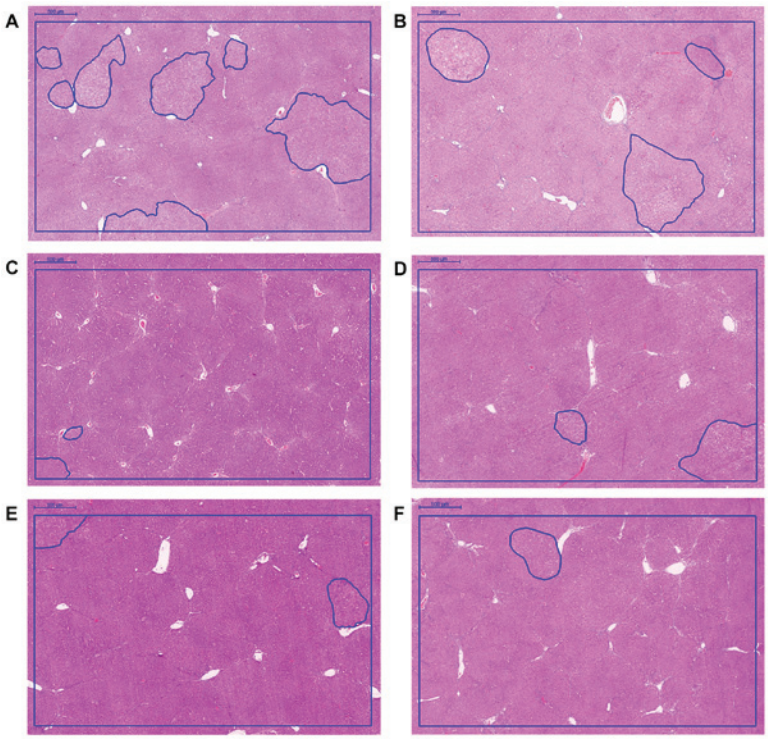

Figure 7. Histopathologically identified cancer areas in the liver of rats with HCC subsequent to 8 weeks of treatment with Sorafenib or BU. Compared with the (A) untreated and (B) vehicle-treated groups, the percentage of the cancer area in rats with HCC treated with (C) Sorafenib or (D) 1 mg, (E) 10 mg or (F) 50 mg BU was decreased. HCC, hepatocellular carcinoma; BU, Benja-ummarit.

crude BU extract with 1 mg/kg gamboge resin revealed that the cancer areas in the liver of rats with HCC were ~1.5-fold larger in the gamboge resin-treated group compared with the BU-treated animals (preliminary data not shown).

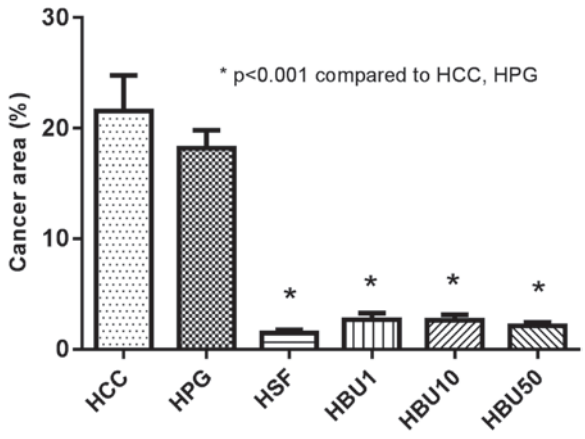

Figure 8. Quantified percentage cancer area in the livers of rats with HCC following 8 weeks of treatment with Sorafenib or different doses of BU. Results were presented as the mean ± standard deviation. *P<0.05 vs. HCC and HPG groups. HPG, rats with HCC only treated with BU solvent; BU, Benja-ummarit; HCC, hepatocellular carcinoma; HSF, rats treated with Sorafenib; HBU1, rats treated with 1 mg BU; HBU10, rats treated with 10 mg BU; HBU50, rats treated with 50 mg BU.

The anti-angiogenic effect of BU has been ascribed to numerous components of the BU extract, including gambogic acid in the gamboge resin (17,19,22), aloe-emodin and aloin in aloe vera (37,38), galbanic acid in asafetida (39), 6-gingerol in ginger (40-42), piplartine in Javanese long pepper (43) and piperine in black pepper (44). Among these substances, gambogic acid appears to have the strongest anti-angiogenic activity (16,17,19,22) and was present in easily detectable amounts in the extract used in the present study. Gambogic acid is claimed to function via the inhibition of the VEGF receptor 2 (22) and its downstream protein kinases SRC proto-oncogene, non-receptor tyrosine kinase, protein tyrosine kinase 2, extracellular signal-regulated kinase (ERK), p38 and protein kinase B (AKT) (16,19) or by inhibiting the egl-9 family hypoxia inducible factor 1-von Hippel-Lindau tumor suppressor-hypoxia inducible factor-1α pathway (17).

The anti-proliferative property of a crude extract of BU has been ascribed to much the same components as its anti-angiogenic effects, including gambogic acid in gamboge resin (13,16,45), aloe-emodin in aloe vera (46,47), galbanic acid in asafetida (39), 6-gingerol or zingerone in ginger (40,41,48,49), piplartine in Javanese long pepper (50) and piperine in black pepper (51-55). 6-Gingerol or zingerone in ginger reportedly inhibits cell proliferation via cell cycle arrest at the G1 phase (40) via the downregulation of cyclin D1 expression (48,49) or inhibition of nuclear factor-κβ activation (41). Similarly, piperine in black pepper may induce cell cycle arrest via the downregulation of cyclin D1 (51,52,54). Piperine from black pepper stops cell proliferation in breast cancer stem cells by inhibiting Wnt/β-catenin signaling (51). In contrast, aloe-emodin in aloe vera appears to inhibit cell proliferation via the phosphorylation of AKT and ERK (46). Piplartine in Javanese long pepper inhibits cell-cycle progression in various tumor cells by inactivating cyclin-dependent kinase 2 and destabilizing cyclin D1 (50).

In addition to the anti-angiogenic and anti-proliferative properties of BU, each ingredient may have further effects. Aloe-emodin in aloe vera, 6-gingerol in ginger and piperine in black pepper are all reported to inhibit tumor invasion and metastasis through the suppression of expression of matrix metalloproteinase-2/9 (38,42). All ingredients of BU, except epsom salts, have been revealed to exert apoptotic activity in various cancer cell lines, including SMMC-7721 (56), a human glioblastoma cell line (U87MG) (57), prostate cancer (58), H460 non-small cell lung carcinoma (59), adriamycin-resistant human leukemia (K562/ADR) (60), MCF-7 (61), IOMM-Lee and CH157MN (62) via the overexpression of BCL2 associated X, apoptosis regulator (60-62), caspase 3 (57,62), caspase 8 (57), caspase 9 (59), induced ROS accumulation (56,61) or via the AKT/mitogen-activated protein kinase pathway (60).

A note of caution with respect to the validity of all these reported effects of the components of BU is required, as nearly all these experiments have been performed *in vitro*. It is well known that general and specific effects may be difficult to separate *in vitro* unless careful dose-response associations have been established. Dose-response association of mixtures are, however, problematic, as non-effective compounds may have a low median lethal dose. The alternative approach is to perform experiments *in vivo*, as in the present study (63,64). In the present study, it was revealed that the effects of BU extract are selective (only liver cancer cells are inhibited in their growth, as demonstrated by the normal liver-body weight ratio, while normal liver cells are not affected as revealed by the unaltered ALT and albumin concentrations in serum). Furthermore, the present study attributed a selective mechanism of action to the BU extract, as the beneficial effect of BU extract corresponded with a decreased VEGF expression in the cancerous areas. This indicates that the respective components of BU must, therefore, contain active ingredients and should allow their isolation and characterization by established reductionist schemes, including testing the effectivity of mixtures which have one component removed.

Sorafenib is an oral multikinase inhibitor that is used globally for the treatment of advanced or metastatic HCC (65). The dose of Sorafenib used in the present study (30 mg/kg/day) was based on previous studies (30-34). This dose of Sorafenib produces complete tumor growth inhibition in mice (30) and reduced tumor angiogenesis in a mouse HCC xenograft model (34), with a skin rash at the beginning of the treatment as a minor side-effect in mice (31). The present study revealed that Sorafenib at the dose administered decreased the liver-to-body weight ratio and serum albumin concentration, and increased serum ALT activity more than the BU extract at any of the three concentrations used. These results indicate that Sorafenib caused hepatocyte injury in rats. Accordingly, Kuroda *et al* (66) reported that patients with HCC who were treated with 400 mg Sorafenib twice daily for 2 months had increased serum concentrations of transaminases and bilirubin. Histologically, these livers exhibited hepatocyte degeneration, necrosis, lymphocyte infiltration and cholestasis (66). The comparable effectivity of reducing tumor growth and the lesser degree of cytotoxicity, even at the highest concentration used, make BU extract or its active principle an attractive candidate to support or even replace Sorafenib in the treatment of HCC.

An alcoholic extract of the traditional Thai remedy BU decreased HCC growth and cancerous VEGF expression *in vivo*, without exhibiting a measurable degree of

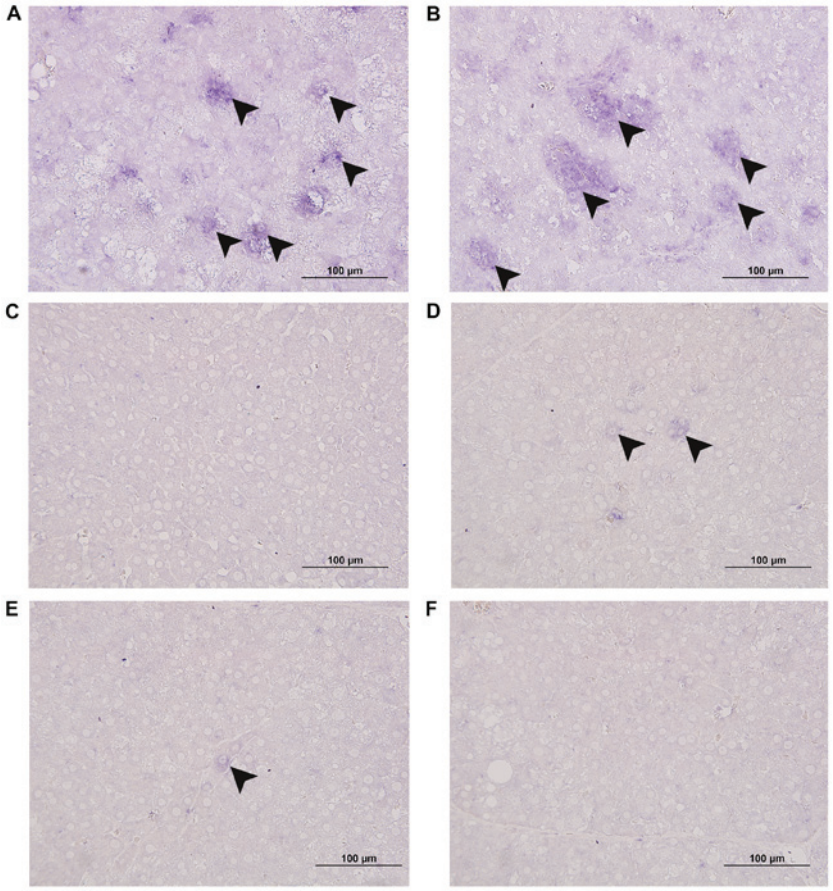

Figure 9. Immunohistochemical demonstration of cytoplasmic VEGF expression in the livers of rats with hepatocellular carcinoma. Compared with the (A) untreated and (B) vehicle-treated livers (arrowheads), the appearance of VEGF-positive cancer areas was suppressed in (C) Sorafenib- and (D) 1 mg, (E) 10 mg or (F) 50 mg Benja-ummarit-treated livers. VEGF, vascular endothelial growth factor.

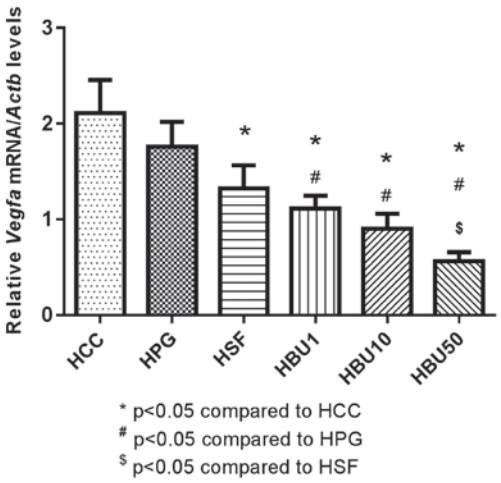

Figure 10. Effect of Sorafenib and BU on hepatic VEGF expression in rats with HCC. A significant downregulation of VEGF expression was caused by Sorafenib and a stronger, dose-dependent downregulation of VEGF was caused by increasing doses of BU. Results were presented as the mean ± standard deviation. *P<0.05 vs. HCC group; #P<0.05 vs. HPG group; $P<0.05 vs. HSF. VEGF, vascular endothelial growth factor; HPG, rats with HCC only treated with BU solvent; BU, Benja-ummarit; HCC, hepatocellular carcinoma; HSF, rats treated with Sorafenib; HBU1, rats treated with 1 mg BU; HBU10, rats treated with 10 mg BU; HBU50, rats treated with 50 mg BU.

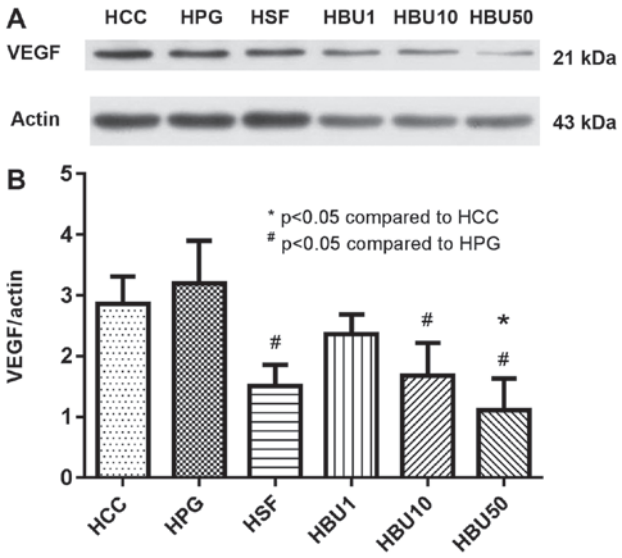

Figure 11. Effect of Sorafenib and BU on hepatic VEGF protein content in rats with HCC. (A) Western blots of VEGF protein expression in the liver of rats with HCC. (B) Bar graph demonstrating the densitometric quantification of the VEGF bands. BU extract at 10 and 50 mg/kg was revealed to be equally effective as Sorafenib in inhibiting VEGF protein expression levels. Results were presented as the mean ± standard deviation. *P<0.05 vs. HCC group; #P<0.05 vs. HPG group. VEGF, vascular endothelial growth factor; HPG, rats with HCC only treated with BU solvent; BU, Benja-ummarit; HCC, hepatocellular carcinoma; HSF, rats treated with Sorafenib; HBU1, rats treated with 1 mg BU; HBU10, rats treated with 10 mg BU; HBU50, rats treated with 50 mg BU.

hepatotoxicity. The present study, to the best of our knowledge, for the first time, reveals a selective anti-neoplastic effect and,

therefore, qualifies as a promising medical herb for further evaluation as a form of treatment of HCC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article excluding raw data, which are available from the corresponding author on reasonable request.

Authors' contributions

NK designed the experiments, treated the rats with drugs, conducted reverse transcription-quantitative PCR and western blot analyses, and analyzed and interpreted the data. AI performed the Benja-ummarit extraction. SP identified the tumor portions and performed the histopathological examination. CN collected, processed and stained the liver biopsies. VK collected specimens, processed and immunostained the liver samples. WP designed the experiments, identified the tumor portions, supervised the research project, interpreted the data and edited the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of the Faculty of Medicine of Srinakharinwirot University (Bangkok, Thailand; approval no. 3/2558).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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