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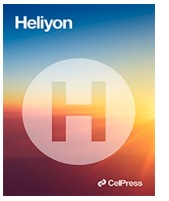

Research article

Effects of barakol from *Cassia siamea* on neuroblastoma SH-SY5Y cell line: A potential combined therapy with doxorubicin

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ABSTRACT

Management of neuroblastoma is challenging because of poor response to drugs, chemotherapy resistance, high relapse, and treatment failures. Doxorubicin is a potent anticancer drug commonly used for neuroblastoma treatment. However, doxorubicin induces considerable toxicities, particularly those caused by oxidative-related damage. To minimize drug-induced adverse effects, the combined use of anticancer drugs with natural-derived compounds possessing antioxidant properties has become an interesting treatment strategy. Barakol is a major compound found in *Cassia siamea,* an edible plant with antioxidant and anticancer properties. Therefore, barakol could potentially be used in combination with doxorubicin to synergize the anticancer effect, while minimizing the oxidative-related toxicities. Herein, the potential of barakol (0.0043–43.0 μM) to synergize the anticancer effect of low-dose doxorubicin (0.5 and 1.0 μM) was investigated. Results indicated that barakol could enhance the cytotoxic effect of low-dose doxorubicin by affecting the cell viability of the treated cells. Furthermore, the co-treatment with barakol and low-dose doxorubicin decreased the levels of intracellular ROS when compared with the control. Moreover, the antimetastatic effect of the barakol itself was studied through its ability to inhibit metalloproteinase-3 (MMP-3) activity and prevent cell migration. Results revealed that the barakol inhibited MMP-3 activity and prevented cell migration in time- and dose-dependent manners. Additionally, barakol was a non-cytotoxic agent against the normal tested cell line (MRC-5), which suggested its selectivity and safety. Taken together, barakol could

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be a promising compound to be further developed for combination treatment with low-dose doxorubicin to improve therapeutic effectiveness but decrease drug-induced toxicities. The inhibitory effects of barakol on MMP-3 activity and cancer cell migration also supported its potential to be developed as an antimetastatic agent.

1. Introduction

Neuroblastoma is one of the most common solid cancers found in early childhood [1]. The management of neuroblastoma is challenging for cancer specialists [2]. Conventional treatment of neuroblastoma includes several options (i.e., surgery, chemotherapy, radiotherapy, stem cell transplantation, and immunotherapy) [3–6]. Among all, chemotherapy, especially the combination drug therapy, was noted to be a treatment choice for patients whose extensive tumor resection is not recommended (i.e., immediate- and high-risk stages) [7,8]. Unfortunately, most of the cases are non-responsive to high-dosing chemotherapy and become prone to turning into the refractory stage [9]. Moreover, drug-induced severe toxicities, metastasis, and relapse are highly concerning factors causing treatment failure and mortality [10,11].

Doxorubicin, a member of the anthracyclines containing aglyconic and sugar moieties (Fig. 1), is an anticancer drug commonly used for several decades due to its high efficacy against a variety of solid and hematological cancers [12]. Doxorubicin is the most potent drug among the Food and Drug Administration (FDA)-approved chemotherapeutic drugs [13]. It is also well-known for its effectiveness in combating rapid-dividing cells [12]. However, the doxorubicin-induced toxicities (i.e., hepatotoxicity, cardiotoxicity, and neurotoxicity) [13,14], mainly caused by the oxidative damage to non-cancerous cells [15,16], are highly concerning and limit its clinical uses. Long-term cardiotoxicity (i.e., heart failure, cardiomyopathy, and ventricular dysfunction) can be observed in patients treated with doxorubicin, although the chemotherapy has been terminated for years [17]. Thus, the finding of treatment strategies to reduce doxorubicin-induced toxicities is considered one of the ongoing research issues. Of most, the combination use of natural-derived compounds and doxorubicin has gained considerable interest [12,18,19]. Natural-derived compounds are well-known for their antioxidant properties as well as their anticancer effects. Thus, the combination use of these natural compounds with conventional chemotherapeutic drugs would be beneficial for effective treatment, in which the dose of conventional drugs could be reduced to minimize drug-induced toxicities while the therapeutic effect could still be achieved by the synergistic effect of the combined natural compounds [20].

Inflammation plays crucial roles in the development and progression of cancers, as well as their responsiveness to treatment [21,22]. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that act as key regulators of the inflammatory process [23]. Extracellular matrix (ECM) degradation and remodeling are essential events in tumor metastasis. These events are facilitated by the enzymatic functions of the MMPs [24]. Among other MMP subtypes, matrix metalloproteinase-3 (MMP-3), also known as stromelysin-1, is noted to play essential roles in promoting cancer metastasis [25,26] and neuronal damage [27]. An increase in MMP-3 expression was reported in experimental models of Parkinson's disease, and neuronal death is alleviated in the presence of an MMP-3 inhibitor [28]. Accordingly, the development of MMP-3 inhibitors has gained recent interest in cancer management [26,29,30]. Previous studies indicated that natural products are good sources for the discovery of MMP-3 inhibitors [26,31,32]. However, studies regarding the MMP-3 inhibitory effect of natural-derived compounds are still scarce.

Cassia siamea, also known as *Senna siamea* (Lam.) Irwin & Barneby, is commonly used in Thai dishes (i.e., Khi Lek curry) and traditional Thai herbal medicine for treating insomnia. *C. siamea* is well-known for its various pharmacological effects [33], including anxiolytic [34,35], sedative [34], antidepressant [36], antidiabetic, antilipidemic [37], analgesic, anti-inflammatory, antipyretic [38], and vasorelaxation [35,39].

Currently, the discovery of chemotherapeutic agents has been directed toward drug repurposing, focusing on several classes of anti-inflammatory drugs [40], especially the non-steroidal anti-inflammatory drugs (NSAIDs) [41]. Crude extracts from various parts of *C. siamea* were reported to exhibit *in vivo* anti-inflammatory activity [38,42]. It was also suggested that the ethanolic aerial part extract of *C. siamea* could serve as a promising natural source for the discovery of NSAIDs [42]. The key bioactive compounds responsible for NSAID-like activity were noted to be flavonoids, triterpenes, anthraquinones, and phytosterols [38]. However, the previous studies mostly focused on the biological effects of the crude plant extracts rather than those of the pure isolated compounds. Particularly, the studies regarding the bioactivities of the barakol are still in their infancy.

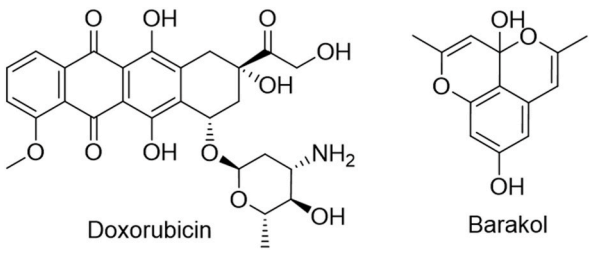

Fig. 1. Chemical structures of doxorubicin (DOX) and barakol.

Barakol, a derivative of dioxaphenalene (Fig. 1), is a key bioactive compound found in flowers and young leaves of the *siamea* plants [43,44]. Barakol possessed several bioactivities, such as laxative [45], CNS depressant [46], and antioxidant activities [47,48]. Additionally, the barakol exhibited the anticancer effect against embryonic carcinoma cell lines derived from an embryo-derived teratocarcinoma (P19 cells), in which possible mechanisms of anticancer action were noted to be via reactive oxygen species (ROS) generation, mitochondrial dysfunction, and caspase-9 activation [49]. Accordingly, barakol is a promising bioactive food ingredient that is noteworthy to be investigated for potential combined therapy with the conventional drug (i.e., doxorubicin) as well as for further development as a novel antimetastatic agent. Despite the medicinal values of this edible plant, scientific studies regarding the medicinal values of this plant are still scarce compared to others, particularly in the anticancer area. Most of the previous studies mainly focused on the biological effects of crude extracts and the characterization of chemical compositions rather than the effects of the isolated major compounds. This motivated the design of this work to reveal the potential of barakol as a lead compound for anticancer management.

In this study, a neuroblastoma SH-SY5Y cell line was used as a model for investigating the synergistic effect of barakol co-treated with low-dose doxorubicin on cell viability and intracellular reactive oxygen species generation. Furthermore, the barakol itself was studied for cytotoxicity, inhibitory effect on cancer cell migration, and MMP-3 inhibition. In summary, this study revealed the potential of barakol as a lead compound to be further developed for improving neuroblastoma treatment.

2. Materials and methods

2.1. Preparation of barakol

Barakol was extracted from the young leaves of *Cassia siamea*, and purified as described previously [39]. Fresh leaves of *C. siamea* were purchased from the local market in Bangkok, with reliable source of cultivation. The plant materials were identified for confirmation by a botany specialist at Srinakharinwirot University. The leaves were chopped and boiled in 0.5 % sulfuric acid at 60 °C for 2 h. The boiled mixture was filtered and alkalinized using sodium hydrogen carbonate before extraction using dichloromethane as an extraction solvent. The mixture was concentrated by evaporation under reduced pressure, followed by shaking with 5 % acetic acid, and neutralizing with 25 % ammonium hydroxide. The greenish-yellow crude extract was obtained, then re-crystallized from aqueous methanol, and further purified by chromatographic method on a silica gel to obtain the isolated barakol. Chemical structure of the barakol was confirmed by nuclear magnetic resonance [50]. The compound was dissolved in vehicle, 0.1 % dimethyl sulfoxide (DMSO), and further diluted with the DMSO to obtain various concentrations used for the assays.

2.2. SH-SY5Y human neuroblastoma cells

The SH-SY5Y neuroblastoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was cultured as monolayers in a 1:1 ratio of ATCC-formulated Ham's F-12 Nutrient Mixture (F12) and Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) containing 10 % heat-inactivated fetal bovine serum (FBS, GIBCO, Gaithersburg, MD, U.S.A.), essential amino acid, sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5 % CO_2 in an incubator along the experiments. Cells were seeded at an initial density of 10^4 cells/cm^2 in culture dishes (Corning, NY, U.S.A.). The medium was changed every 48 h. The cells were used at a low passage number (<35).

2.3. Cell viability assay

The cytotoxic activity of the barakol was assessed by a rapid 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability screening assay as previously described [51]. The cytotoxic effect of barakol against neuroblastoma SH-SY5Y cells was investigated at varying doses using a colorimetric MTT assay. SH-SY5Y cells were harvested by 0.25 % trypsin containing 1 mM EDTA, then plated on a 96-well plate at a density of 3×10^4 cells/well and allowed overnight to attach. The cells were pretreated with various concentrations of barakol: 0.0043, 0.043, 0.43, 4.3 and 43.0 μM in 0.1 % DMSO for 8 h, then treated with or without doxorubicin (0.5 and 1 μM) and further incubated for 24 h at 37 °C. Then, the MTT dye (0.25 mg/mL) was added to each well. After the cells were incubated for 4 h, the supernatant was carefully discarded. The formazan crystals in each well were dissolved in DMSO 100 μL/well. The amount of purple formazan was determined by measuring the optical density using an ELISA microplate reader at 550 nm. All measurements were carried out in triplicate.

2.4. Intracellular reactive oxygen species (ROS) assay

The determination of intracellular ROS was performed by monitoring the fluorescent intensity of 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF). Cells were harvested with 0.25 % trypsin containing 1 mM EDTA and plated on a 96-well plate at a density of 3×10^4 cells/well. After the incubation at 37 °C overnight, cells were pretreated for 8 h with varying concentrations of barakol: 0.0043, 0.043, 0.43, 4.3, and 43.0 μM in 0.1 % DMSO, followed by treatment with or without doxorubicin (0.5 and 1 μM), and then incubated for 24 h at 37 °C. The medium was aspirated and washed twice with PBS. Then, 0.05 mM DCDHF was added to a 96-well plate (200 μL/well) and incubated at 37 °C for 30 min. Then, cells were washed twice with PBS, and 100 μL of PBS was added to each well. Fluorescent intensity was measured at excitation/emission 485/528 nm using a fluorescence plate reader (BioTek® Instruments, Inc.).

2.5. MMP-3 activity and inhibition assay

The cells were grown in F12: MEM containing 10 % FBS in a 6-well culture plate at a density of 1×10^6 cells/well. After the incubation at 37 °C overnight, cells were treated with varying concentrations of barakol: 0.043, 0.43, 4.3, and 43.0 μM and incubated for 24 h.

MMP-3 activity was performed using the MMP-3 activity fluorometric assay kit (Biovision), which followed the manufacturer's instructions. Briefly, cells were homogenized in the ice-cold MMP-3 assay buffer and then centrifuged to remove insoluble materials at 13,000 *g* for 10 min. The 50 μL samples and 50 μL of reaction mixture were added to each well of the 96-well plate. Fluorescent intensity was measured at excitation/emission 325/393 nm using a fluorescence plate reader (BioTek® Instruments, Inc.).

MMP-3 inhibitory effect was investigated using the MMP-3 inhibitor screening kit (Fluorometric, Biovision), which followed the manufacturer's instructions. Briefly, various concentrations of barakol (0.043, 0.43, 4.3, and 43.0 μM) were added 50 μL/well into a 96-well plate. Inhibition control (2 μL) was used and diluted to 50 μL with an assay buffer. The assay buffer was used as a blank control. Subsequently, the reaction was added with 50 μL of MMP-3 enzyme solution to each well and incubated at 37 °C for the indicated time (10, 20, 30, 40, 50, and 60 min). Then, the substrate (10 μL) was added to each well and measured at excitation/emission 325/393 nm using a fluorescence plate reader (Synergy; BioTek Instruments, Inc.).

2.6. Cell migration assay

The effect of barakol on SH-SY5Y cell migration was determined by the monolayer wound healing assay. The cells were grown in F12: MEM containing 10 % FBS in a 6-well culture plate at a density of 1×10^6 cells/well. After the cells formed a confluent monolayer, the cells were scratched with a 10 μL sterile pipette tip from side to side of a 6-well culture plate. Subsequently, the cells were washed three times with F12: MEM media to remove cell debris. The medium was immediately replaced with varying concentrations of barakol (0.043, 0.43, 4.3, and 43.0 μM in 0.05 % DMSO) in each well. Cell migration was monitored and imaged under an inverted microscope with the ocular grid for 0, 12, 24, and 36 h. The distance of the wound (compared with the control at 0 h) was measured at two independent wound sites per group. Relative cell motility was calculated as the wound width at 12, 24, and 36 h.

2.7. Cytotoxicity assay

The cytotoxicity of barakol was examined on normal embryonic lung cells (MRC-5) using the MTT assay [52–54]. MRC-5 cells were cultured in DMEM medium supplemented with 100 U/mL penicillin–streptomycin and 10 % fetal bovine serum. In brief, the suspended cell lines were seeded in a 96-well microtiter plate (3599; Corning Incorporated, Corning, NY, USA) at a density of 5×10^3 to 2×10^4

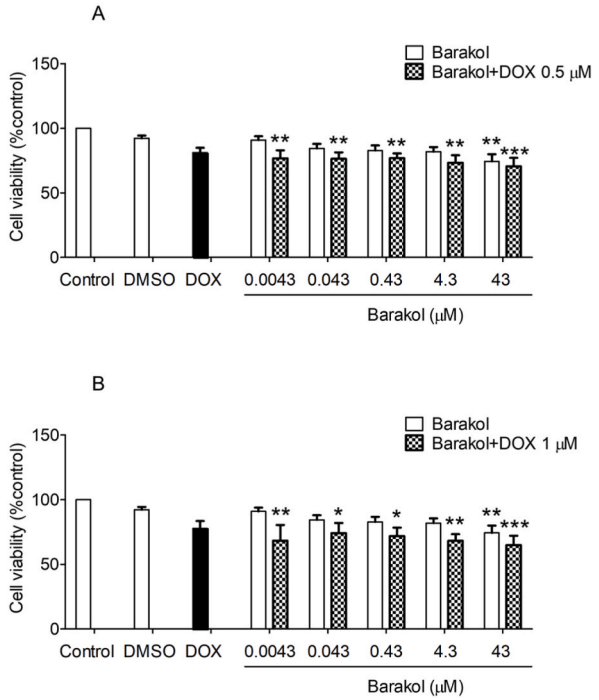

Fig. 2. Effects of barakol (0.0043, 0.043, 0.43, 4.3, and 43.0 μM) on SH-SY5Y neuroblastoma cell death were determined by MTT assay after the incubation with or without barakol following with or without doxorubicin (0.5 μM, A; 1 μM, B) and 0.05 % DMSO. Mean ± S.E.M. values of triplicate tests are shown, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the control.

cells/well, and incubated at 37 °C under a humidified atmosphere (95 % air, 5 % CO_2) for 24 h. Medium containing the serial dilutions of barakol, positive control (doxorubicin), or negative control (DMSO) was added in an equivalent volume to obtain the final concentrations, and the plates were incubated for 48 h. Then, MTT staining solution (10 μL/100 μL medium) was added, and the plates were further incubated (with the conditions mentioned above) for 2–4 h. Subsequently, DMSO was added to dissolve the produced formazan using sonication, and the plates were read by a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) using a test wavelength of 550 nm and a reference wavelength of 650 nm. Finally, IC_{50} values were determined as a concentration of a compound or drug that inhibits 50 % of cell growth in comparison with the negative control.

2.8. Statistical analysis

Data were analyzed by GraphPad Prism version 5.01 software and shown as means ± S.E.M. Statistical significance was calculated using one-way ANOVA followed by Dunnett's multiple comparison Post Hoc test, except for data obtained from the cell migration study, which was evaluated by two-way ANOVA. A p value of 0.05 was considered statistically significant.

3. Results

3.1. Effects on cell viability of neuroblastoma and normal cell lines

Effects of barakol alone (0.0043–43.0 μM) and varying dose combinations of barakol-doxorubicin (barakol 0.0043–43.0 μM and doxorubicin 0.5 or 1.0 μM) were determined against the cell viability of the SH-SY5Y cell lines (Fig. 2). Results indicated that treatment with low-dose doxorubicin alone (0.5 or 1.0 μM) did not significantly affect the cell viability of the tested cell line when compared with the control. However, the cytotoxic effects of low-dose doxorubicin (0.5 and 1.0 μM) were enhanced when the cells were co-treated with varying concentrations of barakol (0.0043–43.0 μM, Fig. 2A-B). Treatment with barakol alone showed no significant effects on cell viability at all tested doses when compared with the control, except for the treatment with the highest dose at 43.0 μM (Fig. 2A). Additionally, the cytotoxicity assay towards the normal cell line (MRC-5) showed that barakol was a relatively non-cytotoxic agent (less than 10 % cytotoxicity at 215.29 μM) when compared with doxorubicin (IC_{50} = 2.3449 ± 0.37 μM).

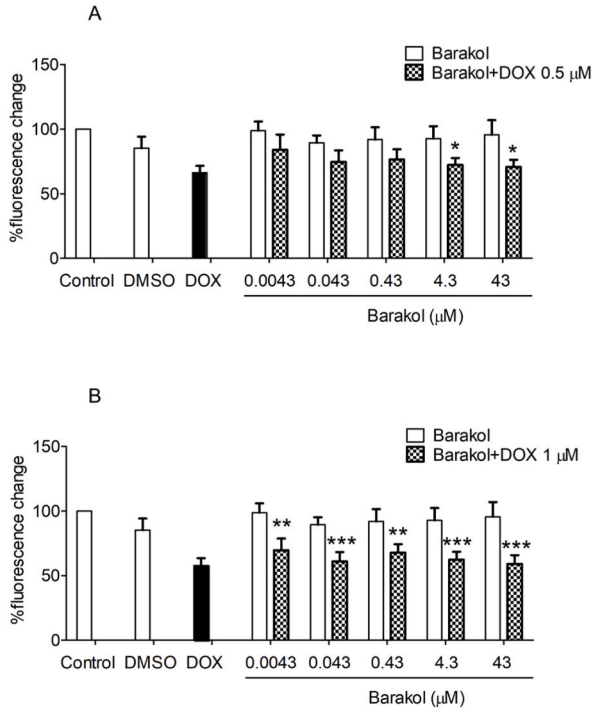

Fig. 3. Effects of barakol (0.0043, 0.043, 0.43, 4.3, and 43.0 μM) on ROS production in SH-SY5Y cells were determined by 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF) after the incubation with or without barakol, subsequent with or without doxorubicin (0.5 μM, A; 1 μM, B) and 0.05 % DMSO. The fluorescence intensity ($DCFH_2$-DA) was measured at excitation/emission 485/528 nm using fluorescence plate reader. Mean ± S.E.M. values of triplicate tests are shown, *p < 0.05, **p < 0.01, and ***p < 0.001 versus the control.

3.2. Effects on intracellular reactive oxygen species (ROS)

Effects of barakol (0.0043–43.0 μM) alone and varying dose combinations of barakol-doxorubicin (barakol 0.0043–43.0 μM and doxorubicin 0.5 or 1.0 μM) on intracellular ROS generation of the SH-SY5Y cell line were investigated. The cells treated with barakol (0.0043–43.0 μM) alone or doxorubicin alone (0.5 or 1.0 μM) showed no significant changes in intracellular ROS levels after 24 h of exposure when compared with the control (Fig. 3). However, significant reductions in intracellular ROS levels were observed for the cells co-treated with 0.5 μM doxorubicin combined with 4.3 or 43.0 μM barakol (Fig. 3A). Notably, the decreased intracellular ROS were more noticeable when the dose of doxorubicin was increased to 1.0 μM, as observed for the cells co-treated with 1.0 μM doxorubicin and any doses of barakol (0.0043–43.0 μM) when compared with the control (Fig. 3B).

3.3. Effects on MMP-3 activity

Effects of barakol on MMP-3 activity of the treated SH-SY5Y cell line were investigated by dose-varying (0.043–43.0 μM) and time-varying exposures (10, 20, 30, 40, 50, and 60 min). Results indicated that the barakol significantly inhibited MMP-3 activity only at the highest dose (43.0 μM), whereas the compound was an inactive inhibitor at the lower concentrations (0.043, 0.43, 4.3 μM) when compared with the control, Table 1. The inhibitory effect of 43.0 μM barakol was observed after 30 min of compound exposure, and the effect was increased when the exposure time was prolonged. These results suggested that the barakol exhibited an MMP-3 inhibitory effect both in time-dependent and dose-dependent manners. However, the barakol showed weaker activity when compared with the inhibitor control.

3.4. Effects on cell migration

Effects of barakol (0.043–43.0 μM) on SH-SY5Y cell migration were studied after exposure times of 24 and 36 h (Fig. 4). Results revealed that significant inhibition of cell migration was observed for the cells exposed with the highest concentration of barakol (43.0 μM) for 24 h, whereas the longer exposure time (36 h) was required for inhibiting cell migration of the cells treated with the lower concentrations (0.43 and 4.3 μM), Fig. 4. From the scratch wound healing assay, it was suggested that the barakol could inhibit cell migration in dose- and time-dependent manners (Fig. 5).

4. Discussion

Results demonstrated that a single treatment with low-dose doxorubicin (0.5 or 1 μM) alone or barakol at low concentrations (0.0043–4.3 μM) alone showed no significant effects on cell viability of the treated SH-SY5Y cells, but the cytotoxic effect was enhanced when the cells were exposed with combined barakol (0.043–43.0 μM) and doxorubicin (0.5 or 1 μM), Fig. 2. This suggested that barakol could synergize the effect of low-dose doxorubicin to provide better cytotoxic potency when compared to the group treated with either drug or barakol alone. From the results, it was noted that an effective cytotoxic dose (IC_{50} value of more than 431 μM, % cell survival 80.91 ± 1.27, n = 4) of the barakol against SH-SY5Y cell survival is much lower than those previously reported for the HepG2 cell line (IC_{50} values = 5.70, 0.96, and 0.77 mM at 24, 48, and 72 h of exposure, respectively) [55]. Therefore, it may imply that barakol was a relatively non-hepatotoxic agent when administered at an effective cytotoxic dose to neuroblastoma cells. Additionally, the barakol itself showed no cytotoxicity against the tested normal MRC-5 cell line, which suggested that the compound is relatively safe and possibly less harmful to non-cancerous cells.

Results from the intracellular ROS investigation indicated that a single treatment with low-dose doxorubicin (0.5 or 1 μM) alone or barakol (0.0043–43.0 μM) alone showed no significant effects on the intracellular ROS levels. However, significant reductions were observed under the following combination treatments: 0.5 μM doxorubicin + high dose barakol (4.3 or 43.0 μM)/or 1.0 μM doxorubicin + barakol (0.0043–43.0 μM), Fig. 3. Accordingly, it is suggested that the production of intracellular ROS might not be the main cytotoxic mechanism of low-dose doxorubicin and barakol. This result was contradictory to the previous literature, which suggested that the production of intracellular ROS could be a mechanism driving the cytotoxic effect of doxorubicin (4.0 μM) against the SH-SY5Y cell line [56]. This might be due to the lower doses of doxorubicin (0.5 and 1 μM) used in this study. Moreover, barakol is an

Table 1
Inhibitory effect of barakol (%) on MMP-3 activity.

	Measuring at time (min)					
	10	20	30	40	50	60
Blank control	0.00	0.00	0.00	0.00	0.00	0.00
Inhibitor control	75.18 ± 33.62	91.19 ± 6.55$^{\#}$	101.77 + 3.52$^{\#}$	105.06 ± 2.93$^{\#}$	109.66 ± 3.44$^{\#}$	113.08 ± 3.74$^{\#}$
Barakol 0.043 μM	8.64 ± 29.88	18.46 ± 16.29	14.30 ± 10.93	5.02 ± 11.50	6.02 ± 10.16	5.55 ± 5.84
Barakol 0.43 μM	−4.78 ± 30.11	7.00 ± 17.52	7.08 ± 8.38	−1.98 ± 8.85	3.93 ± 9.14	2.14 ± 3.71
Barakol 4.3 μM	−40.81 ± 38.74	−1.35 ± 13.70	1.45 ± 5.52	4.81 ± 6.30	9.48 ± 5.69	9.11 ± 4.10
Barakol 43.0 μM	−19.70 ± 31.41	24.06 ± 10.46	34.03 ± 3.99*	38.59 ± 3.92*	44.94 ± 6.76$^{\#}$	47.45 ± 4.69$^{\#}$

Effects of barakol (0.043, 0.43, 4.3, and 43.0 μM) and MMP-3 inhibitor (positive control) on MMP-3 activity were determined using *in vitro* assays at 10, 20, 30, 40, 50, and 60 min after incubation. Mean ± S.E.M values of triplicate tests are shown, $^{*}p < 0.01$ and $^{\#}p < 0.001$ versus the control.

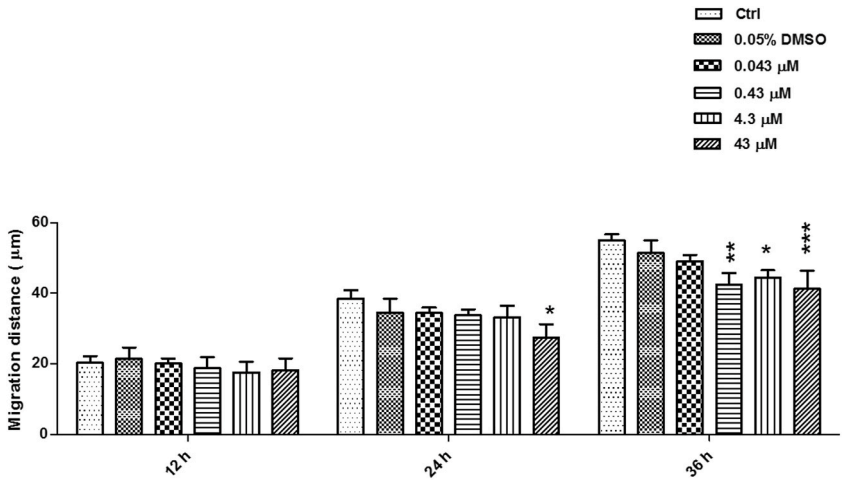

Fig. 4. Effects of barakol (0.043, 0.43, 4.3, and 43.0 μM) on SH-SY5Y cell migration were determined by scratch wound healing assay at observation time of 12, 24 and 36 h, as shown by migration distance. Mean ± S.E.M. values of triplicate tests are shown, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the control.

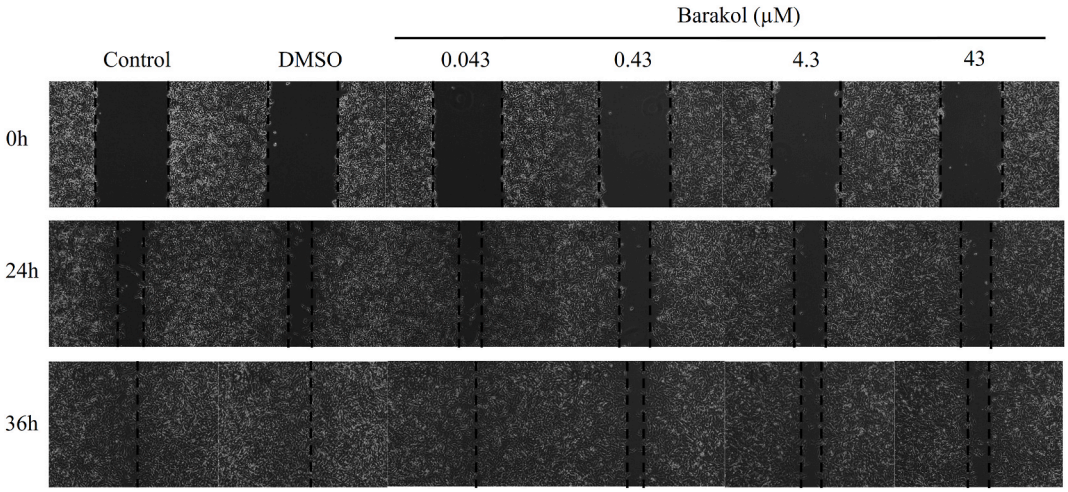

Fig. 5. Effects of barakol (0.043, 0.43, 4.3, and 43.0 μM) on SH-SY5Y cell migration were determined by scratch-wound-healing assay. SH-SY5Y cells were incubated with or without barakol at various doses. Migration was observed at 0, 12, 24, and 36 h after wounding under an inverted phase-contrast microscope and the morphology of SH-SY5Y cells are shown at 0 (A), 24 (B), and 36 (C) h after the incubation. Mean ± S.E.M. values of triplicate tests are shown, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the control.

antioxidant agent bearing a phenolic moiety (Fig. 1). It is expected that the barakol could scavenge the free radicals, leading to a decrease in the net intracellular ROS level. From our findings, the ability of barakol to reduce intracellular ROS production in the presence of doxorubicin indicated the protective potential of this compound against doxorubicin-induced oxidative toxicities.

Free radicals are well-known for their roles in the activation of MMP-3 in microglia cells [57]. In recent years, MMP inhibitors have been developed based on the two main properties of compounds, including an ability to scavenge the ROS [58,59] and the ability to inhibit the zinc active site in the MMP molecule [60,61]. Our results demonstrated that barakol, at a high dose (43.0 μM), inhibited MMP-3 activity in dose- and time-dependent manners. Regarding the antioxidant property of the barakol [47,48], it is hypothesized that the barakol could neutralize the intracellularly produced free radicals, leading to an inhibition of MMP-3 activity. Moreover, previous studies indicated that many naturally derived compounds containing benzopyran structure (i.e., flavonoids) exhibit metal chelating properties [62–64]. Accordingly, barakol, as a related compound containing oxygen lone pair electrons in its structure (Fig. 1), could elicit the metal chelating effect and might be able to chelate zinc ions within the active site of the enzyme, leading to an inhibition of the MMP-3 activity. MMP-3 was reported for its roles in cancer cell migration and metastasis [65]. Our cell migration study indicated that barakol can suppress the migration of SH-SY5Y cells in dose- and time-dependent manners. This indicated that barakol could potentially prevent the metastasis of neuroblastoma. However, it should be noted that the inhibitory effects of the combined doxorubicin-barakol on MMP-3 activity and cell migration were not investigated in this work and should be further studied.

The previous study reported that doxorubicin inhibited hepatic cancer cell (HepG2) migration by suppressing MMP-3 expression, and the inhibition was synergized by co-treatment with a natural-derived compound, *trans*-ferulic acid [66]. From this evidence, it is hypothesized that barakol, as an MMP-3 inhibitor, may potentiate the anti-metastatic effect of doxorubicin similarly.

5. Conclusion

Most of the doxorubicin-induced drug toxicities are involved with the overproduction of free radicals leading to oxidative damage of non-cancerous cells. Thus, several strategies were noted to minimize toxicity, including optimizing the dosage (using lower doses) and finding potential compounds, such as naturally derived antioxidants, for co-administration with the drug to minimize the oxidative-related toxicities. *Cassia siamea* (or Khi Lek) is an edible plant used in daily Thai dishes, but the therapeutic effects of its major compound, barakol, are rarely studied. In the present study, bioactivities relating to the anticancer effect of barakol alone (cytotoxicity, MMP-3 inhibition, and cell migration inhibition) and as a synergistic agent with low-dosed doxorubicin (i.e., cytotoxic activity and intracellular ROS production) against the SH-SY5Y cell line were investigated. Results demonstrated that barakol, as a relatively non-cytotoxic compound to the normal cell line (MRC-5), could be a promising compound to be further developed for combination therapy with doxorubicin to allow the use of lower drug dosing while maintaining preferable anticancer effects as well as minimal drug-induced oxidative toxicities. Moreover, the barakol itself showed inhibitory effects on MMP-3 activity and cell migration, which suggested its potential for further development as an antimetastatic agent. However, it is recommended that the effects of combined doxorubicin-barakol on MMP-3 activity and cell migration inhibition be further explored. Additionally, further studies on the mechanisms of action, pharmacokinetics, toxicities, and appropriate combination dosing are recommended for future successful development.

Ethics declarations

Review and/or approval by an ethics committee was not needed for this study because none of the animal or human subjects were used in this work.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Orapin Wongsawatkul: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis, Conceptualization. **Paiwan Buachan:** Visualization, Validation, Investigation. **Yamaratee Jaisin:** Resources, Investigation. **Panaree Busarakumtragul:** Resources, Investigation. **Sunan Chainakul:** Resources, Investigation. **Ramida Watanapokasin:** Resources, Investigation. **Veda Prachayasittikul:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Funding acquisition, Formal analysis. **Supaluk Prachayasittikul:** Writing – review & editing, Supervision, Conceptualization. **Somsak Ruchirawat:** Supervision, Resources. **Virapong Prachayasittikul:** Supervision, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used QillBot AI website to screen and check minor grammatical errors. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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