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Anti-cancer effects of the extracts of broad and spirale cultivars of *Codiaeum variegatum* (L.) Blume on MCF-7, HepG2, and HeLa cell lines

Phanida Suphiratwanich[1], Benjaporn Buranrat[2,3], Supavadee Boontha[3,4*]

[1]Faculty of Pharmacy, Payap University, Chiang Mai, 51000, Thailand
[2]Faculty of Medicine, Mahasarakham University, Maha Sarakham, 44000, Thailand
[3]Research Group in Herbal and Development of Formulation and Delivery Systems for Elderly Adults and Cancer Treatment, School of Pharmaceutical Sciences, University of Phayao, Phayao 56000, Thailand
[4]School of Pharmaceutical Sciences, University of Phayao, Phayao, 56000, Thailand

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ABSTRACT

Introduction: *Codiaeum variegatum* (L.) Blume is a well-known ornamental foliage plant used as a vegetable in northern Thailand, and it is the source of numerous bioactive substances. This work explored the effects of leaf extracts of broad (BCE) and spirale (SCE) cultivars of *C. variegatum* on three cancer cells, including human breast, human liver, and human cervical cancer cells.

Methods: Ethanolic plant extracts were prepared, and then, 2,2-diphenyl-1-picryhydrazyl (DPPH), ferrous iron chelating, and lipid peroxidation assays were used to examine the flavonoid and phenolic compounds. The proliferative inhibition, growth, and migration of MCF-7, HepG2, and HeLa cancer cells, as a result of exposure to the extracts, were investigated. The extracts were investigated for their anti-cancer activities using sulforhodamine B (SRB), clonogenic, and wound-healing methods.

Results: The data demonstrated that BCE and SCE contained high phenolic compounds. However, both extracts showed inactive anti-oxidant activities. Both extracts had high cytotoxicity on three types of cancer cells in a dose- and time-dependent manner after 24-72 hours of incubation with IC_{50} values in a range of 208-830 µg/mL. Moreover, the prepared extracts of *C. variegatum* significantly inhibited colony-forming ability and cell migration on all types of cancer cells. Compared with BCE, SCE showed more potent anti-cancer activities.

Conclusion: These findings revealed that SCE had higher anti-cancer activities on MCF-7, HepG2, and HeLa cancer cells than BCE. Consequently, the SCE might be used as an effective chemotherapeutic compound for the prevention and treatment of cancer.

Implication for health policy/practice/research/medical education:
This study provides novel insights into the anti-cancer effects of extracts prepared from the leaves of broad and spirale cultivars of *C. variegatu*m on breast (MCF-7), hepatoma (HepG2), and cervical (HeLa) cancer cell lines and their effective uses in treating hepatoma, breast, and cervical cancers.

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Introduction

In 2020, cancer caused 10 million deaths making it the second leading global cause of mortality (1,2). In Thailand, men are more likely to develop lung (16.5% of new cases) and prostate (9.2% of new cases) cancers, while women are more likely to develop breast (22.8% of new cases) and cervical (9.4% of new cases) cancers (2,3). The development of medication resistance by cancer cells and chemotherapy side effects are the primary causes of failure in the treatment of cancer (4,5). Herbal medicine provides a promising alternative approach to cancer treatment due to its less toxic and preferable treatments. Some phenolic and flavonoid compounds found in herbal plants exhibit notable anti-oxidant, anti-amoebic, anti-Alzheimer, and

***Corresponding author**: Supavadee Boontha,
Email: supavadee.bo@up.ac.th

anti-cancer properties (6-8).

Codiaeum variegatum (L.) Blume (garden croton) is the second-largest genus in the Euphorbiaceae family. It is a well-known ornamental foliage plant with unusually wide variations in leaf size, shape, color, and pattern (9,10). The *C. variegatum* plant is grown throughout the tropical forest and is highly valued for its therapeutic qualities, including its anti-oxidant, anti-fungal, anti-viral, and cytotoxic effects (11,12). Two clone cultivars of *C. variegatum*, including broad leaf (Broad) and spiral leaf (Spirale), have been found in many regions of Thailand (8). *C. variegatum* cultivars are mainly used as vegetables in northern Thailand. Our previous study showed that the extract prepared from *C. variegatum* leaves was abundant in phenolics and flavonoids, and it displayed anti-oxidant and anti-acetylcholinesterase properties (8). However, there are little data about the anti-cancer effects of the extracts prepared from *C. variegatum*. Moreover, only their cytotoxicity on cancer cells have been reported in previous studies, and the results are described as follows. An ethanolic stem and leaf extract of *C. variegatum* was found to be cytotoxic to leukemia (Jurkat), prostate cancer, and breast cancer (MCF-7) cell lines (13). Furthermore, the methanolic leaf extract of *C. variegatum* cv. *petra* was found to be cytotoxic, with a range of 17.3% to 98%, to breast cancer, liver cancer, colon cancer, and lung carcinoma cells (14). Metastasis is the primary reason for cancer-related deaths. Metastasis allows cancer cells to spread to various locations throughout the body, typically via the lymphatic or circulatory systems (15). Therefore, a critical factor in anti-cancer action is the suppression of cell movement: the reduction in metastasis. The extracts' effects in relation to colony formation in/on cancer cells need to be evaluated because of the importance of preventing cell growth recurrence (16). Thus, colony formation and wound healing methods were investigated to explore the extract's anti-colony formation and anti-migratory activities on cancer cells.

This investigation aimed to study the anti-cancer activity (cytotoxic, anti-colony formation, and anti-migratory activities) of leaf extracts of broad and spirale cultivars of *C. variegatum* on breast cancer MCF-7, liver cancer HepG2, and cervical cancer HeLa cells.

Materials and Methods

Plant material

The broad and spirale cultivars of *C. variegatum* (L.) Blume leaves were collected from Lamphun province in Thailand. A specimen (voucher no. 00232621_1 (broad cultivar) and 00232621_2 (spirale cultivar)) was kept at the herbarium of the Department of pharmaceutical science, Faculty of Pharmacy, Chiang Mai University, Thailand.

Chemicals and cell culture reagents

Ammonium thiocyanate, dimethyl sulfoxide (DMSO), 2,2-diphenyl1-picryhydrazyl radical (DPPH), EDTA, ferrozine, and ferric chloride ($FeCl_2$), gallic acid, kojic acid, vitamin C (L-(+)-ascorbic acid), and vitamin E were provided by Sigma Chemical Co. (St. Louis, MO, USA). Cholesterol, linoleic acid, oleic acid, and 1,6 diphenyl 1,3,5-hexatriene were purchased from Wako Pure Chemical Industrial Ltd. (Osaka, Japan). Gamma linolenic acid was obtained from Tokyo Chemical Industrial Ltd. (Tokyo, Japan). DMEM medium, fetal bovine serum, and other cell culture supplies were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Plant extract preparation

Codiaeum variegatum (broad and spirale cultivars) leaves were dried at 45 °C in an oven for 48 hours. Then, the dried plants were ground with a Moulinex® grinder. The prepared dried powder (2 kg) was macerated with 50% ethanol for 72 hours, followed by filtering the extracts, and then evaporating the solvent from the extract under vacuum using a rotary evaporator and then a freeze dryer to obtain the dried extract. Thereafter, the extracts were stored at 4 °C for further use.

Total phenolic content evaluation

The total phenolic contents of the extracts were evaluated using the Folin–Ciocalteu assay with minor modifications from the prior study (17) as follows. The obtained extracts (20 µL) at a concentration of 100 mg/mL were mixed with Folin–Ciocalteu's solution (100 µL) and then left in the dark for 5 minutes. Then, 80 µL of 7.5% w/v sodium carbonate solution was added and mixed at ambient temperature. The resulting combination was then incubated for a further 30 minutes at room temperature. Next, the optical density was determined at 760 nm using a spectrophotometer (M200, Tecan, Switzerland). On the basis of a gallic acid standard curve, an equivalent of gallic acid per gram of the extract (mg GAE/g extract) was used to assess the total phenolic compound contents.

Total flavonoid content evaluation

A colorimetric aluminum chloride assay was used to determine the extracts' flavonoid contents. An amount of 100 µL of 10% aluminum chloride was combined with the obtained extracts (100 µL). The resulting solution was then incubated at room temperature in the dark for 10 minutes. The optical density was determined at 415 nm. On the basis of a quercetin standard curve, an equivalent of quercetin per gram of extract (mg QE/g extract) was used to assess the total flavonoid contents.

Anti-oxidant activity evaluation

DPPH radical scavenging method

The DPPH method was used to assess the extracts' capacity to scavenge free radicals, which was adapted from a previous method (18) as follows. In brief, the extracts

were combined with DPPH solution (0.5 mM) at various concentrations. The resulting mixture was then left in the dark at room temperature for 15 min. The optical density was determined at 517 nm. Vitamin C was used as the positive control. The extract's capacity to scavenge the DPPH radical was calculated using the formula:

% DPPH radical scavenging capacity = [(AC - AT) / AC] × 100

where AC stands for the control sample's optical density (without the extract) and AT for the test sample's optical density. Calculating the sample concentrations using the graph plotting of the DPPH inhibition (%) and concentrations resulted in a 50% inhibition of DPPH radical scavenging (IC_{50}).

Ferrous iron chelating assay

The activity of metal chelation of the extracts was determined via the ferrous iron chelating method as already explained by Manosroi et al (19). In brief, the extracts were solubilized in DMSO to obtain the final concentrations of 0.001–10 mg/mL. Then, various concentrations of the extract (100 µL) were mixed with 2 µM of ferrous sulfate aqueous (50 µL), followed by adding 5 mM ferrozine (50 µL) and then adjusting the final volume to 300 µL with distilled water. After incubation for 15 minutes, the optical density was read at 570 nm. EDTA was used as a positive control, while the complex formation molecules of $FeCl_2$ and ferrozine were used as the negative control. The metal chelating activity (%) was determined via this equation:

% Metal chelating activity = [(AC – AT)/AC] × 100

where AC stands for the control sample's optical density (without the extract) and AT for the test sample's optical density. The graph plotting of the metal chelating activity (%) and the sample concentrations allowed for the calculation of the sample concentrations, which showed a result of 50% metal chelating activity (IC_{50}).

Lipid peroxidation inhibition activity

The activity of lipid peroxidation inhibition of the obtained extracts was determined using a ferric thiocyanate assay. In brief, the extract was solubilized in DMSO to obtain the extract with the final concentrations of 0.01–10 mg/mL. Then, 50 µL of the extract with various concentrations was mixed with 50 µL of linoleic acid in 50% DMSO, followed by the addition of 50 µL of NH_4SCN (5 mM) to 50 µL of $FeCl_2$ (2 mM), mixing them well together, and incubating for 60 minutes at 37 °C. The oxidation of linoleic acid was allowed to obtain peroxides, resulting in the oxidation of Fe^{2+} to Fe^{3+}. The complexation's absorbance was determined at 490 nm. Vitamin E was used as the positive control. The inhibition (%) of lipid peroxidation was determined via this equation:

(%) Inhibition of lipid peroxidation = [(AC – AT)/AC] × 100

where AC stands for the control sample's optical density (without the extract) and AT for the test sample's optical density. The graph plotting of the lipid peroxidation inhibition (%) and the sample concentrations allowed for the calculation of the sample concentrations, which showed a 50% inhibition of lipid peroxidation (IC_{50}).

Cytotoxicity test

Three types of cancer cells were used in this study composed of human breast cancer MCF-7, liver cancer HepG2, and cervical cancer HeLa cells. All cancer cells were purchased from the American Type Culture Collection (ATCC; USA). To determine the extracts on cancer cell viability, the sulforhodamine B (SRB) method was used. In 96-well plates, 1×10^4 cancer cell lines were cultivated overnight. The media were then removed and a new medium containing various extract concentrations (0-1000 µg/mL) was added for 24-72 hours. Following that, the cells were stained for 30 minutes with 0.4% SRB and then the cells were fixed with 10% trichloroacetic acid at 4 °C. The cells were lysed using 10 mM of Tris base buffer, and optical density was read at 540 nm. Comparing the treated and untreated control groups, the percentage of cell cytotoxicity (%) was determined.

Colony formation test

To determine the extracts on the colony-forming ability, the colony formation test was used. Briefly, viable cells (500 cells) from cancer cell lines were plated in 6-well plates, grown for an additional day, and then exposed to varying doses of the extract (0-250 µg/mL) overnight. Next, the medium was discarded, and the cancer cells were reconstituted in a brand new full DMEM medium and cultivated for another 10 days in the case of the Hela cells, 14 days in the case of the MCF-7 cells, and 21 days in the case of the HepG2 cells. The medium was replaced every two to three days in between tests. The cells were subsequently fixed with absolute methanol and stained for 60 minutes in the dark with 0.25% crystal violet in methanol (25% v/v). Direct counting was conducted after viewing the colonies. Comparing treated control groups with untreated groups, the percentage of cell regrowth (%) was calculated.

Wound healing test

The wound healing method was employed to assess the effects of the extract on cancer cell migratory ability. Briefly, 2.5×10^5 of each of the cancer cell lines were plated in 24-well plates and cultivated for 24 hours. Next, a

wound was made in the cancer cells using a 0.2 mL sterile pipette tip, and then washed with PBS buffer. Images were captured using inverted microscopy from 0 to 72 hours after fresh media containing varied concentrations of the extract (0-1000 µg/mL) were introduced (4X). The image capturing of the exposed area allowed for the examination of wound closure. The calculated wound distance (%) was compared with control groups that were not subjected to any treatment.

Statistical analysis

The data are shown as the mean and standard deviation (SD) of measurements made in three replicates. One-way analysis of variance (ANOVA) was used to gather all data, and it was followed by the LSD multiple range test.

Results

Plant extraction, phenolic compound, flavonoid content, and anti-oxidant activity evaluation

The percentage yields of the BCE (broad) and SCE (spirale) cultivars of *C. variegatum* were approximately 14% and 13 % of dry weight, respectively. Both extracts had high yields regardless of the extraction technique used for the leaves of *C. variegatum*. BCE and SCE contained total phenolic compounds of 25.73 ± 0.52 and 28.52 ± 0.19 mg GAE/g extract, respectively. The total flavonoid contents of BCE and SCE were 26.88 ± 0.38 and 34.96 ± 0.79 mg QE/g extract, respectively. These results revealed that SCE contained more total phenolic and total flavonoid contents than BCE. Since both BCE and SCE contained phenolic and flavonoid compounds, both extracts may contribute to providing medicinal properties to the plant. Therefore, the anti-oxidant activities of BCE and SCE were studied. BCE and SCE exhibited inactive anti-oxidant activity with IC_{50} >10 mg/mL when compared with that of vitamin C using DPPH radical scavenging assay (Table 1) (20-22). Using the ferrous iron chelating and lipid peroxidation tests, the anti-oxidant effects of both extracts were not identified due to the inhibition being below 50% at 10 mg/mL (Table 1).

Cytotoxicity effects

The cytotoxicities of BCE and SCE extracts on three cancer cell lines, including MCF-7, HepG2, and HeLa cells were determined. The results showed that BCE and SCE extracts had cytotoxicity on cancer cells in a concentration- and time-dependent way, as depicted in Figures 1A (BCE) and 1B (SCE). Based on the cytotoxicity results of linear regression, the IC_{50} values of BCE and SCE extracts were calculated and are shown in Table 2. The results revealed that SCE had more cytotoxicity on HepG2 and HeLa cells than BCE. This might be due to that SCE contained more phenolic and flavonoid contents than BCE, while BCE showed more cytotoxicity on MCF-7 cells than SCE. However, there was no significant difference between IC_{50} values of BCE and SCE in MCF-7 cells (Table 2).

Inhibition of cell growth effects

Following the determination of inhibition cell growth effects, BCE showed a capacity to inhibit cell growth at concentrations of 25 µg/mL (MCF-7), 50 µg/mL (HepG2), and 50 µg/mL (HeLa) (Figure 2A). SCE showed a capacity to inhibit cell growth at a concentration of 25 µg/mL (MCF-7), 25 µg/mL (HepG2), and 50 µg/mL (HeLa) (Figure 2B). SCE showed more potential to inhibit HepG2 cell growth than BCE.

Inhibition of cell migration effects

The wound healing test was used to determine the capability of cancer cells to migrate or metastasize.

Table 1. Evaluation of 2,2-diphenyl1-picryhydrazyl (DPPH), ferrous iron chelating, and lipid peroxidation assays in the broad (BCE) and the spirale (SCE) cultivars of *Codiaeum variegatum* extracts

Samples	IC_{50} (mg/mL)		
	DPPH radical	Ferrous iron chelating	Lipid peroxidation
BCE	>10	N/D	N/D
SCE	>10	N/D	N/D
Positive control	0.01 ± 0.00 (Vitamin C)	0.13 ± 0.01 (EDTA)	0.04 ± 0.01 (Vitamin E)

IC_{50} stands for the concentration of the test sample that provided 50% inhibition, N/D stands for not determined since the inhibition was less than 50% at the tested concentration (10 mg/mL).

Table 2. The cytotoxicity of the broad (BCE) and spirale (SCE) cultivars of *Codiaeum variegatum* extracts on three cancer cell viability at 72 hours

Samples	IC_{50} (mg/mL)		
	MCF-7	HepG2	HeLa
BCE	214.77 ± 4.63	829.27 ± 68.05	352.30 ± 33.82
SCE	228.67 ± 56.40	208.26 ± 120.24*	239.03 ± 20.99*

The cytotoxicity test of each cell was done in separated three experiments; *$P < 0.05$ vs BCE.

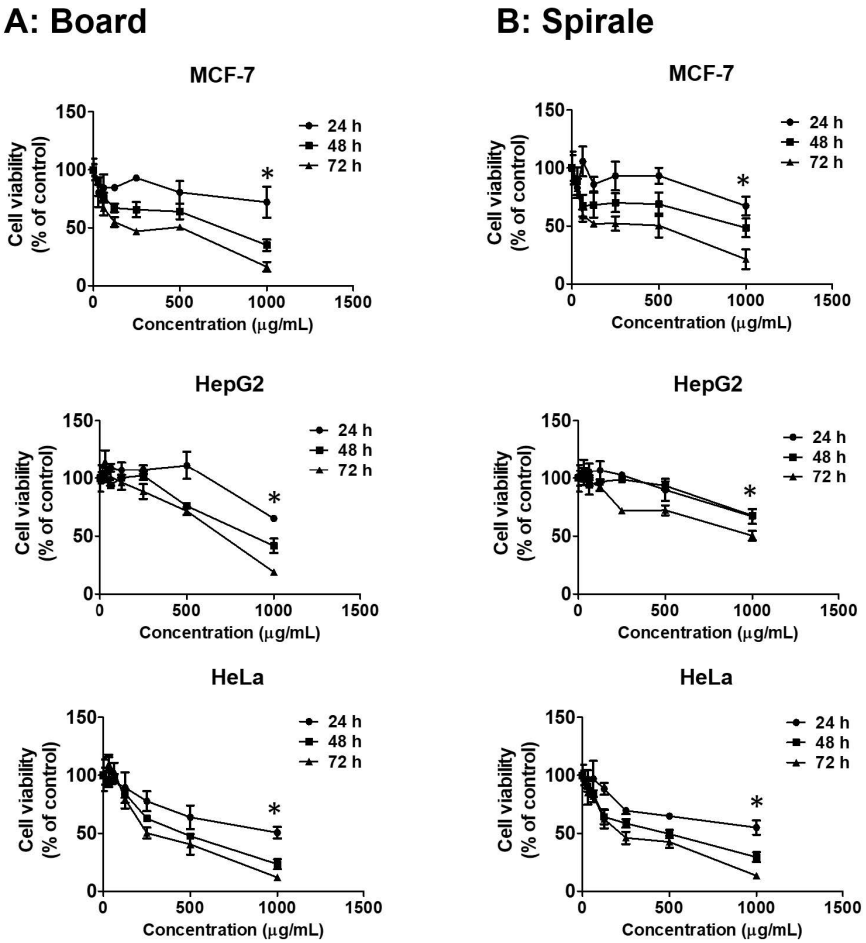

Figure 1. The effects of the extracts of (A) broad and (B) spirale cultivars of *Codiaeum variegatum* on cancer cell viability at 24-72 h. *$P < 0.05$ vs 24 h.

Reductive wound closure represented the ability to suppress cell migration of the extract. Following the determination of the inhibition of cell migration effects, BCE showed significant inhibition of cell migration at the concentrations of 1000, 250, and 250 µg/mL on MCF-7, HepG2, and HeLa cancer cells (Figure 3A). SCE showed a significant ability to inhibit cell growth at the doses of 500, 250, and 250 µg/mL on MCF-7, HepG2, and HeLa cancer cells (Figure 3B). Compared with BCE, SCE showed a more potent suppression of MCF-7 cell migration than BCE. For the determination of the ability to inhibit cell migration of the extracts on HepG2, HepG2 cell death was observed; the cells could not migrate after 72 hours of incubation as shown in Figures 3A and 3B. The results showed that the relative closure of the wounds treated with 250-1000 µg/mL of the BCE and SCE was more than 100%.

Discussion

The use of medicinal plants as a strategy in cancer prevention and cancer treatment has been practiced for a long time and has shown progress; numerous therapeutic plants with anti-cancer effects have been documented in the literature (23,24). Plant-based medicine is becoming more popular as interest in organic and minimalist living increases (25). Additionally, side effects and drug resistance are significant barriers to the development of synthetic anti-cancer medications; as a result, plants have been researched globally as unique and potential sources of anti-cancer medicines. Previous research on *C. variegatum* revealed therapeutic qualities such as anti-inflammatory and anti-cancer effects (13,14,26). It has been noted that variations in the fatty acid, amino acid, phenolic, and flavonoid makeup of the extracts could account for the variable cytotoxic actions of herbal extracts on a range of cancer cell lines (27,28). In this study, the total phenolic and flavonoid contents of two clone cultivars of *C. variegatum* (broad and spirale) in 50% ethanol extracts were determined. Thereafter, the cytotoxic potential of the two clone cultivar extracts on cancer cells was assessed. The extracts prepared from *C. variegatum* leaves of BCE and SCE were shown to display phenolic and flavonoid compounds similar to previous studies.

Codiaeum variegatum leaves extracts have shown phenolic contents with approximately 40 mg GAE/g extract (13) and 50 mg GAE/g extract (26). The present

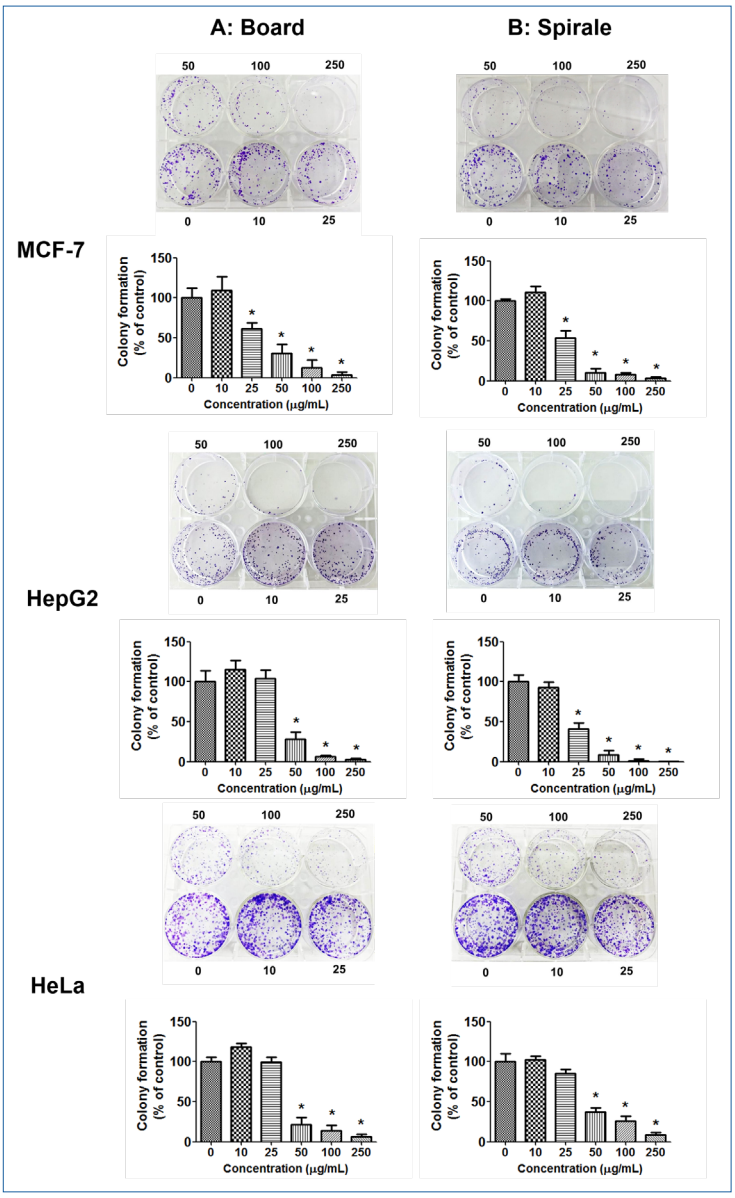

Figure 2. The effects of the extracts of (A) broad and (B) spirale cultivars of *Codiaeum variegatum* on the growth of three types of cancer cells. *$P < 0.05$ vs the untreated group.

study revealed lower phenolic contents (≅ 26 mg GAE/g extract for BCE and ≅ 28 mg GAE/g extract for the SCE) when compared with previous studies (13,26). This could be due to 50% ethanol being used as the extraction solvent in this study, whereas 95% ethanol was used in previous studies. The data of this study are in agreement with another report, as follows. Pechangou et al (26) reported that more phenolic contents were found in the extraction of *C. variegatum* leaves with ethanolic solvent (95% ethanol) than in the extraction of *C. variegatum* leaves with hydroethanolic solvent (70% ethanol). Moreover, the extracts prepared from *C. variegatum* leaves exhibited lower IC_{50} values of radical scavenging activity, metal chelating activity, and lipid peroxidation inhibition than those of the previous studies. Although the flavonoid contents in BCE showed a similar pattern, a dissimilar trend was observed for the flavonoid contents in SCE. The BCE and SCE extracts showed cytotoxicities on MCF-7 cells with IC_{50} of 214.77 µg/mL and 228.67 µg/mL, respectively (Table 2). A previous study showed the extract prepared from *C. variegatum* leaves had cytotoxicity on MCF-7 with an IC_{50} value of 84.44 µg/mL (13). This difference could be due to more phenolic content and anti-oxidant activity, resulting in more cytotoxic effects. Our results confirmed the cytotoxic effect of *C. variegatum* leaf extract on MCF-7 and HepG2 cells (14). Moreover, our study showed that *C. variegatum* leaf extracts had cytotoxicity on HeLa cells with an IC_{50} of 352 µg/mL for BCE and IC_{50} of 239 µg/mL for the SCE. A critical factor in anti-cancer action is the suppression of cell movement or

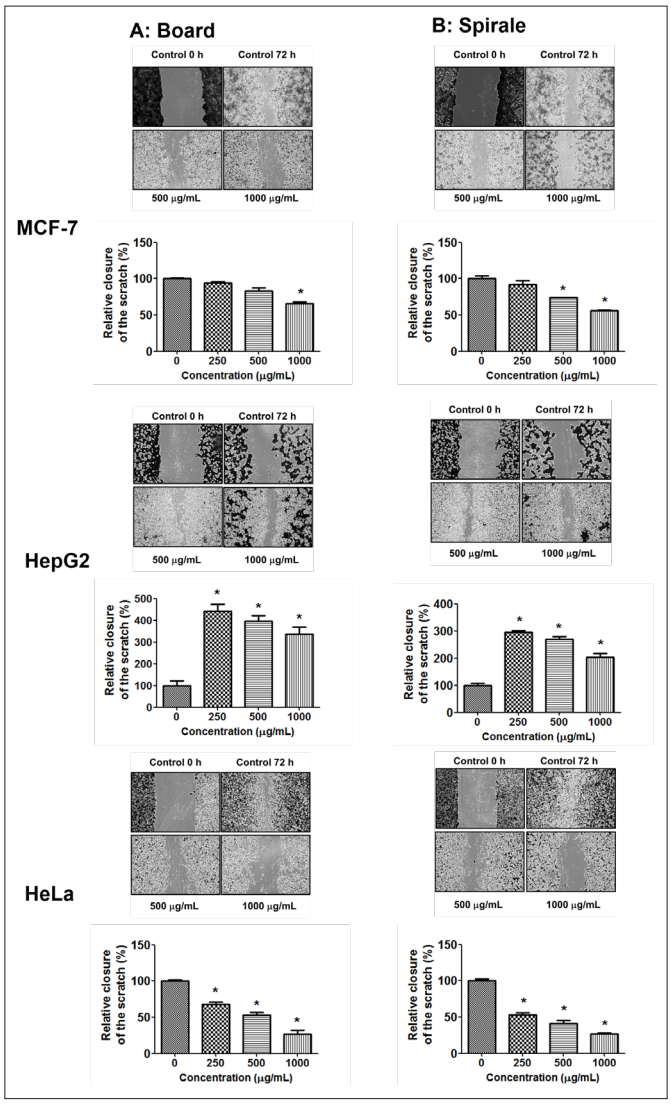

Figure 3. The effects of the extracts of (A) broad and (B) spirale cultivars of *Codiaeum variegatum* on the migration of three types of cancer cells. **P* < 0.05 vs the untreated group.

the reduction in metastasis. Therefore, the anti-migratory effects of the extracts, due to the prevention of cancer cell metastasis, need to be evaluated and the extracts' anti-colony formation effects, due to the prevention of cancer cell growth recurrence, need to be evaluated.

In addition to the cytotoxic effect, our study reported the anti-colony formation and anti-migratory effects of the extracts prepared from *C. variegatum* leaves on three types of cancer cells. The results are in agreement with the results of earlier research on the effects of *C. variegatum* extract on cancer cell lines in terms of its ability to inhibit colony formation and migration (12,13). The results showed the extract prepared from *C. variegatum* leaves had more cytotoxicity on MCF-7 cells ($IC_{50} \cong 215$ µg/mL for BCE and $IC_{50} \cong 230$ µg/mL for the SCE), followed by HeLa cells ($IC_{50} \cong 350$ µg/mL for BCE and $IC_{50} \cong 240$ µg/mL for the SCE) and HepG2 cells ($IC_{50} \cong 830$ µg/mL for BCE and $IC_{50} \cong 210$ µg/mL for the SCE), respectively. Whereas the extract prepared from *C. variegatum* leaves had more anti-colony formation and anti-migratory effects on HepG2 cells and HeLa cells, followed by MCF-7 cells. This could be due to the specificity of the anti-cancer activities (anti-colony formation and anti-migratory effects) of the extracts on different cancer cells. Theoretically, these data support the potential use of BCE and SCE as a target for chemotherapeutic drugs and chemo-preventive agents in the treatment of breast, liver, and cervical cancers. However, further investigation is necessary in order to completely understand the underlying mechanisms of actions.

Conclusion

It can be concluded from this study that *C. variegatum* leaves contain phenolic and flavonoid compounds as well as anti-oxidant and anti-cancer activities on breast cancer, liver cancer, and cervical cancer cells. Further studies of

the plant's biological properties, active compounds, and toxicity would be valuable to support the use of the broad and spirale cultivars of *C. variegatum* as useful agents for the prevention of cancer.

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Authors' contributions

PS designed the experiments and prepared and characterized the extract. SB conducted the project and prepared the manuscript. BB was responsible for the cancer cell study. All authors read the final version of the manuscript and confirmed its publication.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

Ethical issues related to plagiarism have been carefully observed by the authors. All the experiments were performed in accordance with relevant guidelines and regulations. The protocol was approved by the Research Committee of the Faculty of Medicine, Mahasarakham University, Thailand (MED_BBL_2566).

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