RESEARCH ARTICLE

The Effects of Thai Traditional Herbal Extracts on the Death of Cancer Cells

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Received: 23 October 2019; **Revised:** 18 May 2020
Accepted: 12 June 2020

Abstract

Cancer is a major health problem in Thailand; thus, there is a need to find and develop new active ingredients from plants for the inhibition of cancer cell proliferation. This paper reports an investigation into the effects on cancer cell proliferation of leaf extracts of five Thai traditional herbal plants: *Cratoxylum formosum*, *Limnophila geoffrayi*, *Aegle marmelos*, *Syzygium gratum* and *Fagraea fragrans*. The cytotoxic effects of the extracts on colon adenocarcinoma cells (HT-29), hepatocellular carcinoma cells (HepG2) and normal colon cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the cell morphology was assessed by using phase-contrast microscopy. The signaling protein levels in apoptosis pathway were investigated by western blot analysis. The results showed that *L. geoffrayi* extract significantly induced the deaths of HT-29 cells ($p<0.001$) and HepG2 cells ($p<0.05$) with IC_{50} levels of 0.57±0.03 mg/mL and 0.57±0.01 mg/mL, respectively. *C. formosum* extract significantly induced HT-29 cell death ($p<0.01$), with an IC_{50} of 0.87±0.03 mg/mL. *A. marmelos* extract significantly induced HepG2 cell death ($p<0.05$) with an IC_{50} of 0.76±0.02 mg/mL. Moreover, these three extracts showed no significant cytotoxic effects towards the normal colon cells. In apoptosis pathway, *L. geoffrayi* and *C. formosum* extracts decreased the level of X-linked inhibitor of apoptosis protein (XIAP) as well as induced caspase-3 activation in HT-29 cells. In HepG2 cells, *L. geoffrayi* and *A. marmelos* extracts up-regulated death receptor 5 (DR5) level and inhibited the XIAP in correlation with caspase-3 activation. This study indicated that *L. geoffrayi*, *C. formosum* and *A. marmelos* leaf extracts at the concentrations up to 1 mg/mL induced cancer cell deaths without any cytotoxic effects on normal colon cells.

Keywords: *Cratoxylum formosum, Limnophila geoffrayi, Aegle marmelos, Syzygium gratum, Fagraea fragrans*

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ฤทธิ์ของสารสกัดจากพืชสมุนไพรพื้นบ้านต่อการตายของเซลล์มะเร็ง

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รับบทความ: 23 ตุลาคม 2562; **แก้ไข:** 18 พฤษภาคม 2563
ตอบรับ: 12 มิถุนายน 2563

บทคัดย่อ

มะเร็งเป็นปัญหาด้านสุขภาพที่สำคัญในประเทศไทย ดังนั้น การค้นคว้าและพัฒนาสารสำคัญใหม่จากพืชที่มีฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งจึงมีความจำเป็น บทความนี้รายงานการศึกษาผลของสารสกัดจากใบของพืชสมุนไพรไทย 5 ชนิดคือ ติ้ว (*Cratoxylum formosum*) ขแยง (*Limnophila geoffrayi*) มะตูม (*Aegle marmelos*) เม็ก (*Syzygium gratum*) และกันเกรา (*Fagraea fragrans*) ต่อการเพิ่มจำนวนของเซลล์มะเร็ง โดยการศึกษาความเป็นพิษของสารสกัดต่อเซลล์มะเร็งลำไส้ใหญ่ (HT-29) เซลล์มะเร็งตับ (HepG2) และเซลล์ลำไส้ใหญ่ปกติด้วยวิธี 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay และศึกษาสัณฐานวิทยาของเซลล์โดยใช้ phase contrast microscopy ส่วนการศึกษาระดับโปรตีนส่งสัญญาณในวิถีอะพอพโทซิสใช้วิธี western blot ผลการศึกษาพบว่า สารสกัดจากขแยงเหนี่ยวนำให้เกิดการตายของเซลล์ HT-29 และ HepG2 อย่างมีนัยสำคัญ (p<0.001 และ p<0.05) โดยมีค่า IC_{50} เท่ากับ 0.57±0.03 มก./มล. และ 0.57±0.01 มก./มล. ตามลำดับ ในขณะที่สารสกัดจากติ้วเหนี่ยวนำให้เกิดการตายของเซลล์ HT-29 อย่างมีนัยสำคัญ (p<0.01) โดยมีค่า IC_{50} เท่ากับ 0.87±0.03 มก./มล. สำหรับสารสกัดจากมะตูมพบว่าเหนี่ยวนำให้เกิดการตายของเซลล์ HepG2 อย่างมีนัยสำคัญ (p<0.05) โดยมีค่า IC_{50} เท่ากับ 0.76±0.02 มก./มล. นอกจากนี้ สารสกัดทั้ง 3 ชนิดดังกล่าวไม่มีความเป็นพิษต่อเซลล์ลำไส้ใหญ่ปกติ ในวิถีอะพอพโทซิสพบว่า สารสกัดจากขแยงและติ้วสามารถลดระดับโปรตีน X-linked inhibitor of apoptosis protein (XIAP) และกระตุ้นการทำงานของ caspase-3 ในเซลล์ HT-29 ขณะที่ในเซลล์ HepG2 สารสกัดจากขแยงและมะตูมกระตุ้นการแสดงออกของ death receptor 5 (DR5) และลดระดับโปรตีน XIAP โดยสัมพันธ์กับการกระตุ้นการทำงานของ caspase-3 การศึกษานี้ชี้ให้เห็นว่าสารสกัดจากใบของขแยง ติ้ว และมะตูมที่ความเข้มข้นสูงถึง 1 มก./มล. เหนี่ยวนำให้เกิดการตายของเซลล์มะเร็งโดยไม่มีความเป็นพิษต่อเซลล์ลำไส้ใหญ่ปกติ

คำสำคัญ: ติ้ว, ขแยง, มะตูม, เม็ก, กันเกรา

Introduction

Cancer has become one of the most crucial health problems in the world. In 2012 the World Health Organization (WHO) reported that cancer was a leading cause of death, responsible for around 8.2 million deaths with the number of new cases having risen to 14 million worldwide.[1] Moreover, these numbers are increasing annually. In Thailand, the information from Ministry of Public Health in 2013 showed that cancer and other tumors were the most common causes of death among Thai population. The top five types of cancer in Thai population include breast, lung, cervical, colorectal and liver cancers.[2] Colon carcinoma is a cancer that has its origin in the colonic or rectal areas. Some behaviors such as eating red meat, having low-fiber meals, or taking minimal exercise can increase individual risk of developing such cancers.[3] Liver cancer may start in the cells of the liver itself or spread from other parts of the body. Recently, it was found that viral infections such as hepatitis B or C may increase the risk of liver cancer. Patients with liver cancer may experience no symptoms until it develops to a later stage. These patients may feel pain around the abdominal area, especially the right-upper part, or they may notice a yellow coloring of the skin and eyes.[4]

Cancer is a disease that results from abnormal cell growth and/or a loss of apoptosis. Cancer cells have invasion potential, i.e., the ability to destroy nearby cells, angiogenesis and metastasis through the blood and lymphatic systems. Apoptosis or programmed cell death is an intracellular suicide program activated for eliminating the defective or malfunctioning cells from surrounding tissues. The loss of a functioning apoptotic program can lead to the formation of abnormal cells, which can develop to become malignant.[5] Chemotherapy commonly refers to a medication treatment procedure employed in cancer therapy. The medications involve not only targeting fast-growing cancer cells but also killing or inhibiting the proliferation of normal cells, especially those that divide rapidly, for example, hair root cells and mouth epithelial cells. As a result, such treatment produces several well-known side effects such as mouth ulcers, hair loss, vomiting and nausea as well as opportunistic infections.

At present, herbal plants represent one interesting cancer treatment alternative. Many studies have reported that certain plants have anticancer effects, for example, soybeans, garlic and onions.[6,7] The present study was designed to investigate the cytotoxic effects of crude leaf extracts of five Thai traditional herbal plants: *Cratoxylum formosum*, *Limnophila geoffrayi*, *Aegle marmelos*, *Syzygium gratum* and *Fagraea fragrans* on cancer cells as potential medicines for the treatment of cancers.

C. formosum (Tiew) is a middle-sized deciduous shrub or tree. According to a Thai folk medicine book, its root is used as a diuretic and its bark for the treatment of skin disease.[8] *L. geoffrayi* (Ka-Yang) is a round, hollow, long and slender plant. Its trunk is used for galactagogue, flatulence, tinea and fever as well as a laxative.[9] *A. marmelos* (Ma-Toom) is a small to medium-sized, aromatic tree. It possesses several pharmacological activities including anticancer effect.[10] Sain et al.[11] reported the induction of apoptosis by *A. marmelos* extracts in Jurkat cell line. *S. gratum* (Mek), its extract showed antihypertensive effect in N^{ω}-Nitro-l-arginine methyl ester (L-NAME) hypertensive rats.[12] Thummajitasakul et al.[13] found a very high anti-oxidant activity in the ethanolic extract of *S. gratum* using ABTS assay. *F. fragrans*

(Kan-Krao) is a native large evergreen tree. Its leaves are used for treating malarial fever, asthma and skin disease.[14] Apisantiyakom[15] reported that *naucledal*, a bioactive constituent of the root bark of *F. fragrans*, exhibited cytotoxicity towards lung cancer cells (NCI-H187) and a mild anti-tubercular effect.

Materials and methods

Preparation of crude extracts

The leaves of *C. formosum, L. geoffrayi*, *A. marmelos*, *S. gratum* and *F. fragrans* collected from Khon Kaen province were extracted twice by maceration with ethanol at a ratio of 1:5 (w/v) for 3 days. Concentrated crude extracts were obtained by filtration, followed by evaporation using a rotary evaporator. Then the concentrated extracts were lyophilized into powder and stored in airtight containers at 2-8°C until use. The stock solutions of each crude extract (10 mg/mL) were prepared in ethanol or DMSO prior to use.

Cell culture

Human colon adenocarcinoma (HT-29), human hepatocellular carcinoma (HepG2), and normal fetal human colon (FHC) cell lines were purchased from the American Type Culture Collection (ATCC®, Gaithersburg, Maryland, USA). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), and DMEM/F12 media (Invitrogen, Carlsbad, CA, USA), respectively, under a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Assessment of cytotoxicity of Thai herbal crude extracts using MTT assay

To investigate the cytotoxicity of the crude extracts, the cancer cell lines (1×10^4 cells/well) were incubated in 96-well plates for 24 hrs and then treated with each crude extract of *C. formosum, L. geoffrayi, A. marmelos, S. gratum* and *F. fragrans* at a concentration of 1 mg/mL for screening test. After incubation for 24 hrs, the cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Absorbance was measured at 570 nm using an M965+ microplate reader (Metertech, Taipei, Taiwan).[16] The cytotoxicity (expressed as percent cell viability) of the treatment groups were compared with the vehicle control and doxorubicin at the concentration of 20 µg/mL as a positive control. For concentration dependent test, the cancer cell lines and FHC cells were treated with the extracts that showed cytotoxic effect in previous screening tests at the concentrations of 0, 0.01, 0.1 and 1 mg/mL as well as with doxorubicin at the concentrations of 0, 5, 10 and 20 µg/mL. To confirm the results from MTT assay, the cell morphology was observed using a Nikon phase contrast microscope (Nikon Instruments, Tokyo, Japan).

Western blotting analysis of apoptotic proteins

In order to observe the mechanism of the herbal extracts induced apoptotic pathway in HepG2 and HT-29 cells, the cancer cells were grown in 6-well plates and treated with the 0.1 mg/mL extracts for 4 hrs. For cell lysate preparation, the cancer cells were washed with ice-cold PBS and added with lysis buffer (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10 µg/mL leupeptin,

10 µg/mL aprotinin and 1 mM dithiothreitol (DTT). After that, the cells were scraped and centrifuged at 14,000 rpm for 10 min. Then the pellets were discarded and the supernatants were collected. Total protein concentrations in lysates were determined with Bradford assay[17] using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Bangkok, Thailand). Western blot was employed to investigate the effect of the extracts on apoptotic proteins including death receptor 5 (DR5), X-linked inhibitor of apoptosis protein (XIAP) and caspase-3. In brief, the cell proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to membranes and then reacted with specific antibodies to DR5, XIAP, caspase 3, cleaved caspase-3 and actin (Cell Signaling Technology, Beverly, MA, USA). Finally, the membranes were treated with enhanced chemiluminescence (ECL) substrates (Life Science, CA, USA) for 1 min and then visualised by X-ray film exposure.

Statistical analysis

The data were analyzed using IBM SPSS statistics version 24 (IBM Corp, Armonk, NY, USA). The statistical techniques used for the analysis was one-way analysis of variance (ANOVA). All experiments performed in triplicate and the data were expressed as mean±SD. The *P*-values less than 0.05 were considered statistically significant.

Results

The effects of Thai traditional extracts on cancer cell viability

The cytotoxic effects on HT-29 and HepG2 cell lines of the crude leaf extracts of *C. formosum*, *L. geoffreyi*, *A. marmelos*, *S. gratum* and *F. fragrans* at 1 mg/mL were performed with MTT assay. The results showed that *C. formosum* and *L. geoffrayi* significantly decreased HT-29 cell viability (Figure 1A), whereas *L. geoffrayi* and *A. marmelos* significantly reduced HepG2 cell viability (Figure 1B). Doxorubicin (positive control) revealed toxicity on the survival of both cell lines.

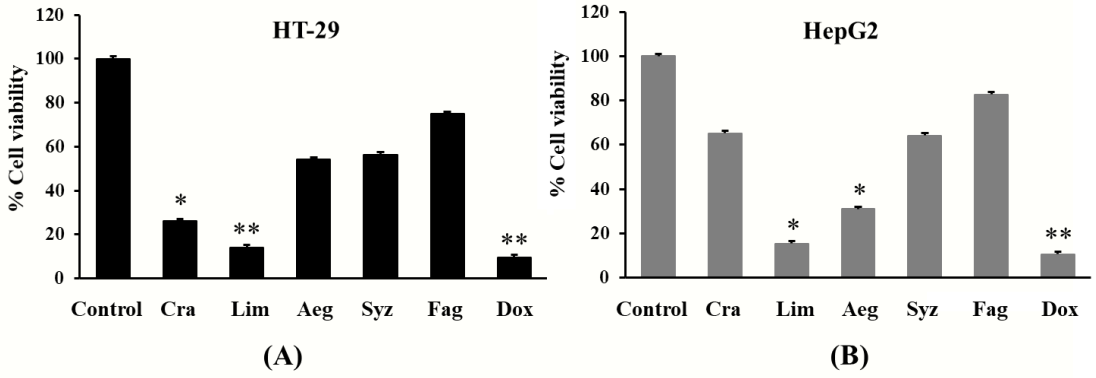

Figure 1. The cell viability of (A) HT-29 and (B) HepG2 cell lines treated with the crude leaf extracts (1 mg/mL) of *C. formosum* (Cra), *L. geoffrayi* (Lim), *A. marmelos* (Aeg), *S. gratum* (Syz) and *F. fragrans* (Fag) and 20 µg/mL doxorubicin (Dox). Data are expressed as mean±SD. **p*<0.01 and ***p*<0.001 compared to untreated control cells.

Further study revealed that the three potential extracts of *C. formosum*, L. *geoffreyi* and *A. marmelos* possessed cytotoxic effects against HT-29 (Figure 2) and HepG2 (Figure 3) cells in a concentration dependent manner with the IC_{50} values as shown in Table 1.

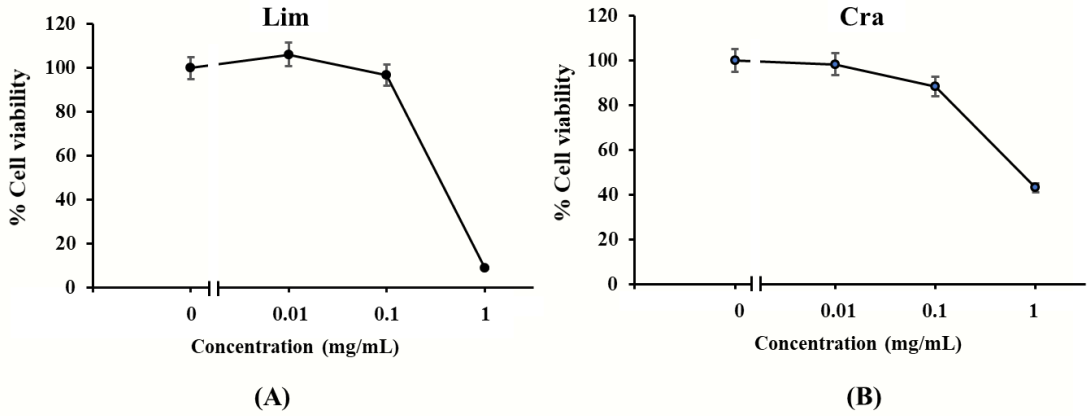

Figure 2. The viability of the HT-29 cell line treated with the crude leaf extracts of (A) *L. geoffreyi* (Lim) and (B) *C. formosum* (Cra). Data are expressed as mean±SD.

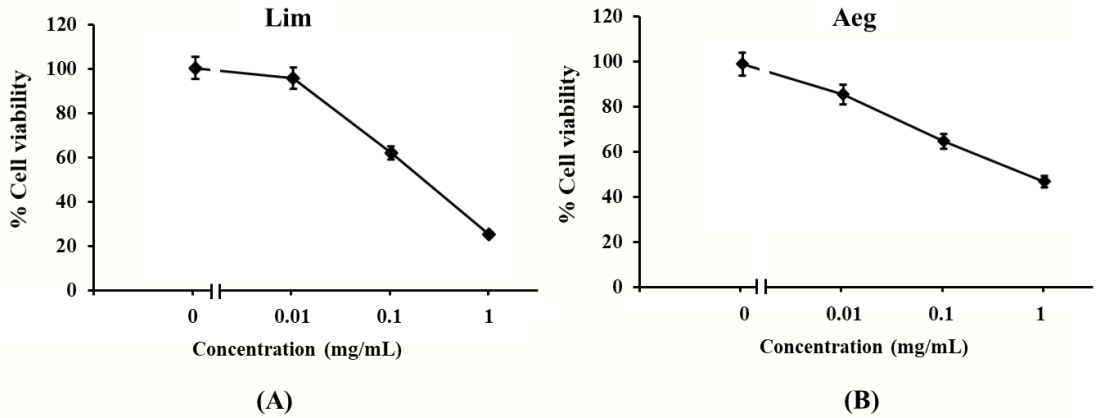

Figure 3. The viability of the HepG2 cell line treated with the crude leaf extracts of (A) *L. geoffreyi* (Lim) and (B) *A. marmelos* (Aeg). Data are expressed as mean±SD.

Table 1. IC_{50} values for potential herbal extracts and doxorubicin on HT-29 and HepG2 cell lines.

Extracts	**IC_{50} (mean±SD; mg/mL)**	
	HT-29	**HepG2**
L. geoffreyi	0.57±0.03	0.57±0.01
C. formosum	0.87±0.03	-
A. marmelos	-	0.76±0.02
Doxorubicin (positive control, µg/mL)	4.39±0.12	3.26±0.15

The cytotoxicity study in FHC cells showed that these three extracts had no significant effects on their viability. In contrast, doxorubicin significantly reduced FHC normal cell viability (p<0.05) (Figure 4).

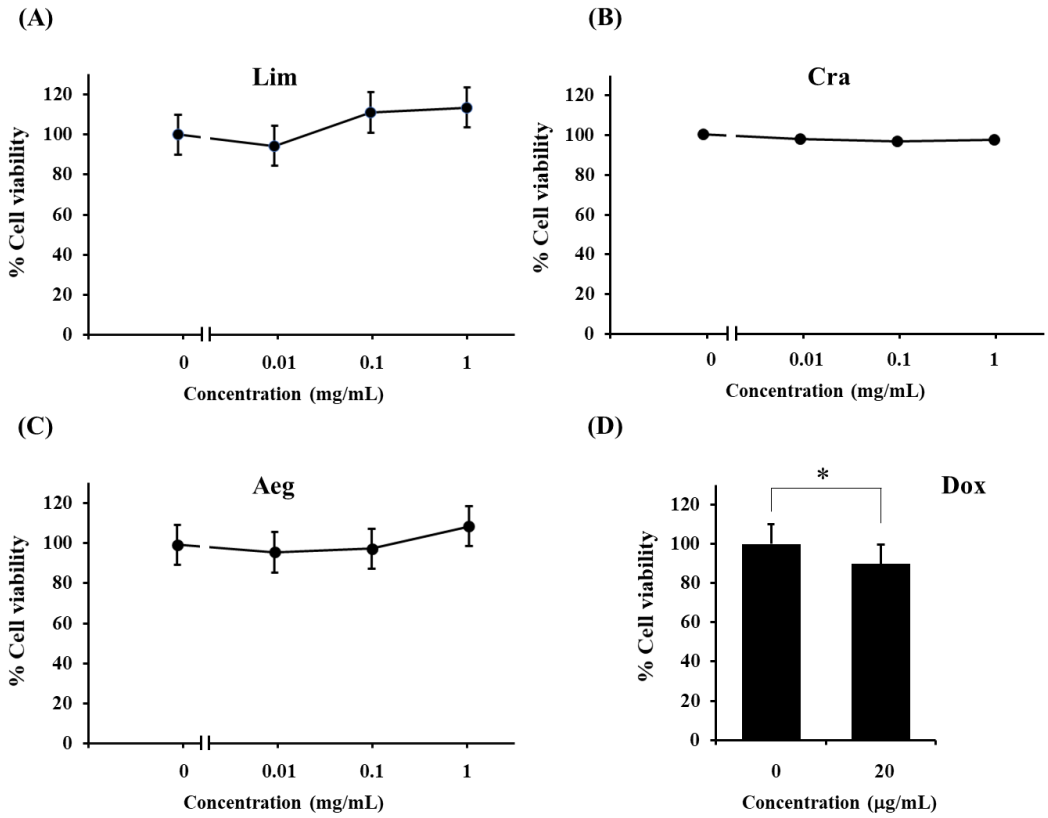

Figure 4. The cytotoxic effect of the crude leaf extracts of (A) *L. geoffreyi* (Lim), (B) *C. formosum* (Cra) and (C) *A. marmelos* (Aeg), and (D) doxorubicin on FHC normal cells. Data are expressed as mean±SD. *p<0.05 compared to untreated control cells.

The effect of Thai traditional extracts on cancer cell morphology

To examine whether the reduced cell viability was caused by apoptosis, phase contrast microscopy was employed to observed cell morphology. Figures 5 and 6 showed the morphologies of HT-29 and HepG2 cells after treating with 1 mg/mL of the crude leaf extracts of *L. geoffreyi*, *C. formosum* and *A. marmelos* compared with those treated with DMSO (control) and 20 µg/mL doxorubicin (positive control). The morphological changes included cell rounding and shrinkage. Specifically, the extracts with the highest cytotoxicity against HT-29 were *L. geoffreyi* and *C. formosum* extracts. However, *L. geoffrey*i extract and *A. marmelos* extracts had the highest cytotoxic effect on the HepG2 cell line.

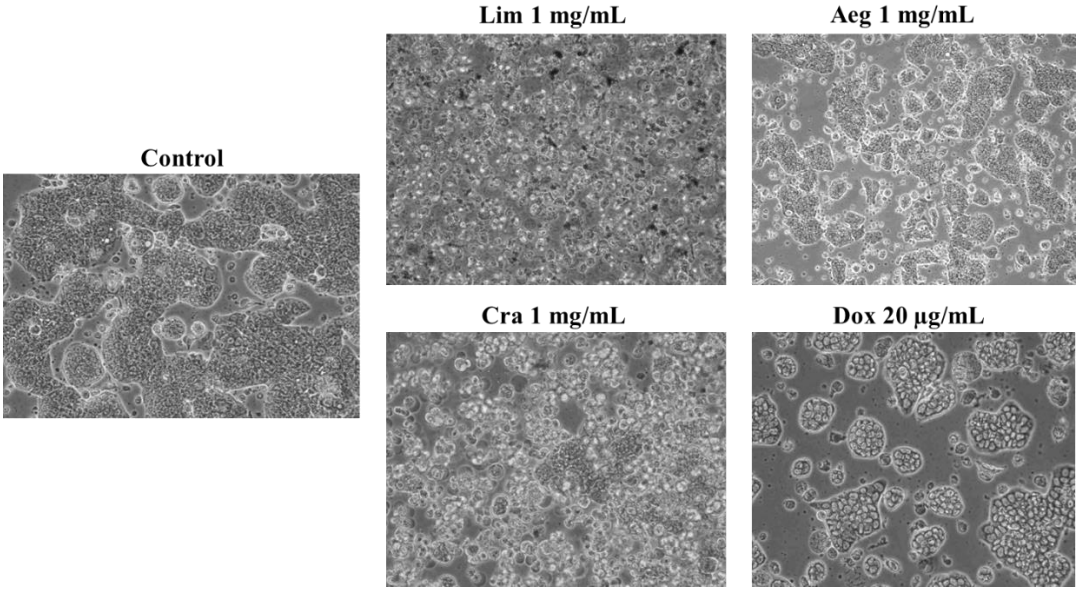

Figure 5. The morphologies of the HT-29 cell lines (magnification, ×20) treated with DMSO (negative control), the crude leaf extracts of *L. geoffreyi* (Lim), *C. formosum* (Cra) and *A. marmelos* (Aeg), and doxorubicin (Dox, positive control).

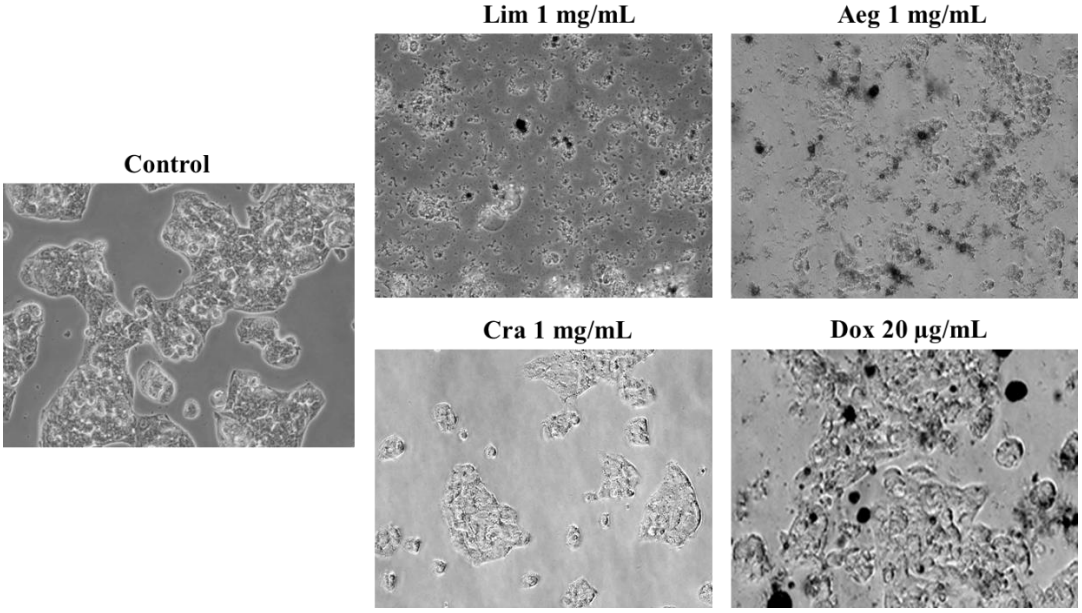

Figure 6. The morphologies of the Hep G2 cell lines (magnification, ×20) treated with DMSO (negative control), the crude leaf extracts of *L. geoffreyi* (Lim), *C. formosum* (Cra) and *A. marmelos* (Aeg), and doxorubicin (Dox, positive control).

Mechanisms of cancer cell apoptosis induced by Thai traditional herbal extracts

In order to study the molecular effects of the three extracts (*L. geoffrayi, C. formosum* and *A. marmelos*) on apoptosis in HT-29 and HepG2 cell lines, the levels of apoptotic proteins including DR5, XIAP, caspase-3, cleaved caspase-3 and actin were evaluated by western blot analysis. The results demonstrated that all three extracts induced DR5 upregulation in HepG2 comparing with the control. *L. geoffrayi* extract was the most effective one, followed by *C. formosum* and *A. marmelos* extracts (Figure 7). In contrast, they showed no effect on HT-29 cells. For XIAP, all three extracts could inhibit the expression of the proteins in HT-29 and HepG2 cells except for *C. formosum* extract, which had no inhibitory effect on HepG2 cells. *L. geoffrayi* extract showed the strongest inhibition of XIAP expression in both cancer cells. In HepG2 hepatocellular carcinoma cell line, *L. geoffrayi* and *A. marmelos* extracts both inhibited the expression of XIAP and induced DR5 upregulation at the same time.

Caspase-3 activation is a marker of apoptosis which can be investigated using cleaved caspase-3. In this study, treatment of *L. geoffrayi* extract caused the strongest activation of caspase-3 in both cell lines. *C. formosum* extract slightly induced the increase of cleaved caspase-3 in HT-29 cells but not in HepG2 cells. *A. marmelos* extract activated cleavage of caspase-3 in HepG2 cells but had no effect on HT-29 cells when compared with doxorubicin, which activated cleavage of caspase-3 in both cell lines. These results indicated that the extracts of *L. geoffrayi*, *C. formosum* and *A. marmelos* could induce cell death through interactions with the proteins in the apoptotic pathway.

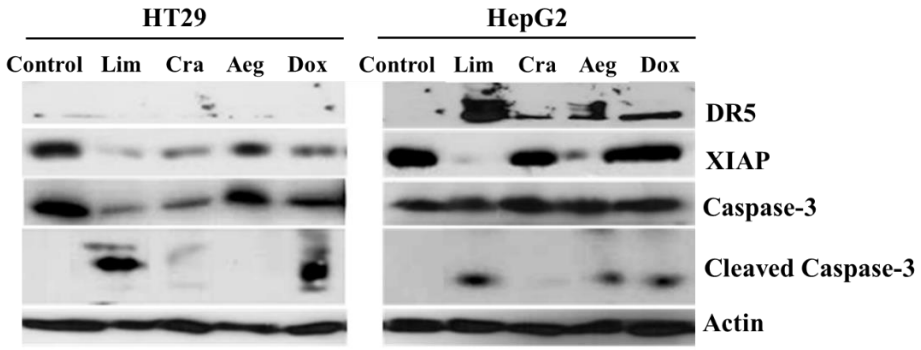

Figure 7. The effect of crude leaf extracts of *L. geoffreyi* (Lim), *C. formosum* (Cra) and *A. marmelos* (Aeg) (0.1 mg/mL) and doxorubicin (Dox, 20 µg/mL) on apoptosis signaling proteins in HT-29 and HepG2 cell lines.

Discussion

This study examined the cytotoxic effects of crude leaf extracts from *C. formosum, L. geoffrayi, A. marmelos, S. gratum* and *F. fragrans* on HT-29 and HepG2 cell lines. These herbal plants were selected because all of them are traditional plants found in the Northeast region of Thailand and previous studies showed their interesting properties such as antioxidant, cytotoxic and antiproliferative effects.

A previous study showed that *C. formosum* extract in DMSO exhibited a significant cytotoxic effect on HT-29 but had no effect on HepG2 cells.[18] Nonpunya

et al.[19] used an ethanol-water extract of twigs and Waiyaput et al.[20] used buffer and hydroalcoholic extracts, while our study used the ethanolic extract of *C. formosum* leaves. Hence, the different results are likely to be due to the differences in part of the plants used in each study as well as solvent for extraction.

This study revealed that *L. geoffrayi* extract possessed the strongest cytotoxic effect on both HT-29 and HepG2 cells, suggesting that the extract induced apoptosis through the receptor-mediated caspase-3 activation. The apoptosis process is controlled by many apoptotic proteins. *L. geoffrayi* extract (1 mg/mL) suppressed the expression of XIAP, resulting in activation of caspase-3 in both cells. However, the extract increased the level of DR5 only in HepG2 cells. The results indicated that *L. geoffrayi* extract has different cytotoxicity mechanisms in different cells. Further studies are required to confirm this hypothesis.

Bhatti et al.[21] demonstrated that the extract from *A. marmelos* leaves had an antiproliferative effect on colon adenocarcinoma CoLo-205 cells. However, in this study the extract provided no evidence of this effect on colon adenocarcinoma HT-29 cells. CoLo-205 is COX-2 negative with p53 wild-type colorectal cancer cells, while HT-29 is COX-2 positive with p53 mutant colorectal cancer cells. These differences in the cell type and cell characteristics may result in different responses to the treatment. Taken together, this study revealed that *L. geoffrayi, C. formosum* and *A. marmelos* crude leaf extracts could induce cancer cell death without any cytotoxic effects on FHC normal colon cells.

Conclusion

This study investigated the cytotoxic effect of the crude leaf extracts of Thai traditional herbal plants: *C. formosum, L. geoffrayi, A. marmelos, S. gratum* and *F. fragrans,* on HT-29 colon adenocarcinoma cells and HepG2 hepatocellular carcinoma cells. The results demonstrated the cytotoxicity of three extracts of *L. geoffrayi, C. formosum* and *A. marmelos* on cancer cells. Whereas *L. geoffrayi* extract showed the most cytotoxic effect on both HT-29 and HepG2, the extracts of *C. formosum* and *A. marmelos* only reduced the cell viability of HT-29 and HepG2 cells, respectively. However, these three extracts caused no harm to FHC normal cells.

Moreover, the results demonstrated that the *L. geoffrayi, C. formosum* and *A. marmelos* extracts were able to induce cell deaths through induction of apoptosis. We found that the extracts affected proteins in the pathways differently depending upon the type of the cancer cells.

Acknowledgement

This work was supported by Ubon Ratchathani University and Thailand Research Fund, Thailand (grant number BDG6080006).

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