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Centella asiatica Ethanolic Extract Induces Apoptosis in Colorectal Cancer Cells via Modulation of the Akt/p53/Bcl-2 Axis: An *In Vitro* and Network Pharmacology Study

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Abstract

This study aims to investigate the antiproliferative effects of *Centella asiatica* ethanolic extract (CAE) in colorectal cancer (CRC) cells and to explore its potential apoptosis-related molecular associations using an integrative network pharmacology and experimental approach. Five major reported compounds of *C. asiatica* were analyzed for CRC-related targets using network pharmacology and STRING analysis. The plant material was authenticated and extracted with ethanol to obtain CAE. Asiaticoside was identified as a marker compound by HPLC analysis. HT-29 (p53 mutant) and HCT-116 (p53 wild type) CRC cell lines were treated with CAE. Cell viability and clonogenic survival were evaluated by MTT and colony formation assays. Apoptosis and cell-cycle distribution were analyzed by flow cytometry. Protein expression levels of p53 and total Akt were assessed by immunofluorescence staining, while Bcl-2 expression was determined by western blotting. HPLC chromatographic analysis confirmed the presence of asiaticoside in CAE. Network pharmacology identified 538 CRC-related targets associated with the 5 dominant compounds, with STRING analysis indicating p53, Bcl-2, and Akt as central nodes. CAE significantly inhibited cell proliferation and colony formation, induced apoptosis, and altered cell-cycle distribution in a dose-dependent manner. These effects were accompanied by increased p53 expression and decreased total Akt fluorescence intensity and Bcl-2 expression. HCT-116 cells exhibited greater sensitivity to CAE compared with HT-29 cells. Therefore, CAE exerts antiproliferative and pro-apoptotic effects in CRC cells, which are associated with modulation of the p53/Akt/Bcl-2 signaling axis, with a more pronounced response observed in HCT-116 cells.

Keywords: *Centella asiatica*, Colorectal cancer, Proliferation, Apoptosis, p53, Akt, Bcl-2

Introduction

Colorectal cancer (CRC) arises from the uncontrolled proliferation of epithelial cells in the colon or rectum, most commonly through the adenoma-carcinoma sequence, in which benign polyps progressively transform into invasive adenocarcinomas over several years [1]. CRC is a multifactorial and heterogeneous disease influenced by genetic, environmental, and lifestyle factors [2-4]. It remains a major global health burden, ranking among the most commonly diagnosed cancers and a leading cause of cancer-related mortality worldwide [2,5,6].

Key molecular regulators of CRC progression include the serine/threonine kinase Akt, the tumor suppressor protein p53, and the anti-apoptotic protein Bcl-2 [7]. Akt is frequently hyperactivated in CRC, promoting cell survival, proliferation, and resistance to chemotherapy through downstream effectors such as mTOR and NF-κB. Its activation is associated with poor prognosis and increased metastatic potential. Conversely, p53 plays a pivotal role in maintaining genomic stability and inducing apoptosis in response to cellular stress. However, its function is often compromised in CRC due to mutations or regulatory suppression. Mutant p53 loses its tumor-suppressive function and may acquire oncogenic properties that further drive CRC progression. Bcl-2, a member of the Bcl-2 family of proteins, inhibits mitochondrial-mediated apoptosis and is commonly overexpressed in CRC, contributing to tumor cell survival and chemoresistance [7,8]. The balance between molecular regulators critically determines the fate of colorectal cancer cells and their response to therapeutic interventions [9,10].

Conventional treatment for CRC typically involves a combination of surgery, chemotherapy, and radiotherapy, with 5-fluorouracil (5-FU) remaining a fundamental chemotherapeutic agent. 5-FU functions by inhibiting thymidylate synthase, thereby disrupting DNA synthesis and inducing apoptosis in rapidly dividing cells [11]. However, despite its widespread use, 5-FU is associated with significant adverse effects, including gastrointestinal toxicity, myelosuppression, mucositis, and cardiotoxicity. Moreover, a substantial proportion of patients exhibit either intrinsic or acquired resistance to 5-FU, which compromises therapeutic efficacy and contributes to disease progression [12]. Non-responsiveness to treatment can lead to recurrence and metastasis, while severe toxicity may result in treatment discontinuation or even mortality in vulnerable populations. These limitations underscore the urgent need for alternative or adjunctive therapies that are both effective and better tolerated.

Centella asiatica (L.) Urban (APIACEAE), a medicinal plant widely used in traditional Asian medicine, has attracted considerable scientific interest due to its diverse pharmacological properties [13]. Major bioactive constituents of *C. asiatica*, including asiaticoside, asiaticoside B, asiatic acid, madecassoside, and madecassic acid, belong primarily to the triterpenoid saponin class and have been reported to exhibit antioxidant, anti-inflammatory, and anticancer activities [14]. Previous investigations have demonstrated cytotoxic and anti-proliferative effects of *C. asiatica* extracts or isolated constituents in colorectal cancer models, as well as modulation of cell cycle progression and apoptosis [15-17]. However, the molecular mechanisms underlying these effects remain incompletely characterized. In particular, the integration of multi-compound network pharmacology with experimental validation in colorectal cancer cells of distinct genetic backgrounds has not been sufficiently explored. Moreover, the potential association of key survival and apoptosis-related regulators, such as Akt, p53, and Bcl-2, in mediating the anticancer effects of *C. asiatica* extract warrants further investigation.

In this study, the antiproliferative and pro-apoptotic effects of an ethanolic extract of *C. asiatica* (CAE) were evaluated in HCT-116 and HT-29 colorectal cancer cell lines. An integrative approach combining network pharmacology analysis with *in vitro* experimental validation was employed to explore the potential involvement of the Akt/p53/Bcl-2 signaling axis associated with CAE treatment. The findings aim to provide mechanistic insights into the biological effects of *C. asiatica* in colorectal cancer models and to support its further investigation as a candidate complementary therapeutic strategy.

Materials and methods

Target gene prediction

Pharmacological targets of each phytochemical were retrieved from 3 online databases: SwissTargetPrediction (https://www.swisstargetprediction.ch/), Super-PRED (https://prediction.charite.de/), and SEA Search Server (https://sea.bkslab.org/). Colorectal cancer (CRC)-related target genes were obtained from GeneCards (https://www.genecards.org/), DisGeNET (https://www.disgenet.org/), and OMIM (https://www.omim.org/). Venn diagram analysis was then performed using InteractiVenn to identify the overlapping genes between the *C. asiatica* phytochemical targets and the CRC-related genes [18].

Pathway enrichment analysis

Functional enrichment analysis of the overlapping target genes was conducted using the STRING database (https://string-db.org/), with the organism set to Homo sapiens. Reactome pathway enrichment analysis was performed to elucidate the biological pathways associated with the identified targets.

Protein-Protein Interaction (PPI) network construction

The overlapping genes were imported into the STRING database (https://string-db.org/) to construct the protein-protein interaction (PPI) network, with the confidence score set to 0.9 (highest confidence) and the species restricted to Homo sapiens. The resulting network was visualized and analyzed using Cytoscape (version 3.10.1). Hub genes were identified using the cytoHubba plugin in Cytoscape, applying the Maximal Clique Centrality (MCC) algorithm to rank the top 10 genes by importance in the network.

Extraction of *Centella asiatica*

Fresh whole plant and aerial parts of *C. asiatica* were collect in Pathumthani province, Thailand. A complete whole plant specimen was separated and prepared for herbarium deposition. The specimen was submitted to the Herbarium of Mahidol University (PBM) and assigned a voucher number (PBM-006734). The remaining fresh plant material was air-dried and ground into powder. A portion of the powder (20 g) was weighed and extracted with 95% ethanol (200 mL) under gentle shaking for 3 days at room temperature. The resulting extract was filtered through No. 0 filter paper. The filtrate was concentrated using a rotary evaporator and further dried on a heating mantle at 40 °C until a constant weight of the crude extract was obtained, after which the per cent yield was calculated. The crude extract was stored at −20 °C until further use.

HPLC analysis of asiaticoside in *Centella asiatica* extract

Phytochemical analysis of *Centella asiatica* extract was performed to determine the presence and quantity of asiaticoside, a major bioactive compound commonly reported in *C. asiatica*. High-performance liquid chromatography (HPLC) analysis was conducted with reference and slight modification from the method described by Prateek *et al.* [19]. The crude extract of *C. asiatica* was weighed and dissolved in 100% dimethyl sulfoxide (DMSO) to obtain a stock solution with a concentration of 300 mg/mL. The stock solution was subsequently diluted with methanol to obtain a working concentration of 1,000 µg/mL and filtered through a 0.45 µm syringe filter prior to HPLC injection. The asiaticoside standard was purchased from a commercial supplier (MedChemExpress, USA) and used as a reference compound. The standard was prepared by dissolving the compound in 100% DMSO to obtain a maximum concentration of 1,000 µg/mL. Serial dilutions were then performed with methanol to obtain concentrations of 200, 100, 50, and 25 µg/mL for calibration. Chromatographic separation and detection were performed using an HPLC system equipped with an absorbance detector (Waters 2695 equipped with Waters 2487 Dual λ Absorbance Detector, Waters, USA). The mobile phase consisted of 0.3% orthophosphoric acid (solvent A) and acetonitrile (solvent B). Separation was carried out on a C18 reversed-phase column (250×4.60 mm) (ACE®, Scotland). The mobile phase gradient program is shown in **Table 1**. The detector wavelength was set at 210 nm. The flow rate was maintained at 1.8 mL/min and the injection volume was 20 µL for each sample [19].

Table 1 Gradient program of the mobile phase used for HPLC analysis.

Time (min)	Solvent A (%)	Solvent B (%)
0.01	95	5
5.0	80	20
15.0	50	50
20.0	20	80

Time (min)	Solvent A (%)	Solvent B (%)
23.0	20	80
30.0	5 0	50
35.0	80	20

Cell culture

Human colorectal cancer cells HT-29 (ATCC® HTB-38™, USA) and HCT-116 (ATCC® CCL-247™, USA) were cultured in McCoy's 5A culture medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Capricorn Scientific, Germany), 10% non-essential amino acids, 1% L-glutamine, and 1% penicillin-streptomycin (Capricorn Scientific, Germany). The cells were incubated at 37 °C in a 5% CO_2 incubator [20].

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

The anti-proliferative effect of the *C. asiatica* ethanolic extract (CAE) was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [19]. Human colorectal cancer cell lines (HT-29 and HCT-116) were seeded in 96-well plates at 1.5×10^5 cells/well and cultured in complete McCoy's 5A medium. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. Subsequently, the cells were treated with various concentrations of CAE, previously diluted in complete medium (concentrations ranged from 200 - 1,000 µg/mL), and incubated for 48 h. After treatment, the medium was discarded and MTT solution (100 µL) was added to each well. Plates were incubated for an additional 1 h at 37 °C in 5% CO_2. The MTT solution was then removed, and dimethyl sulfoxide (DMSO, 100 µL) (Sigma-Aldrich, USA) was added to dissolve the formazan crystals. The plates were gently shaken until the solution turned uniformly purple, and absorbance was measured at 595 nm using a microplate reader (EMax Plus, Molecular devices, USA) [20]. Five-fluorouracil (5-FU) (Sigma-Aldrich, USA) concentration at 100 µM was used as positive control.

Clonogenic assay

The anti-colony formation effect of the *C. asiatica* ethanolic extract (CAE) was conducted by performing a clonogenic assay, adapted from the method of Praphasawat *et al.* [21]. HT-29 or HCT-116 colorectal cancer cells (1×10^3 cells) were seeded into 6-well culture plates and incubated for 24 h. After incubation, the medium was replaced with fresh medium containing various sub-lethal concentrations of CAE (25, 50 and 100 µg/mL), followed by incubation at 37 °C for 48 h. The treated medium was discarded, replaced with fresh medium, and the cells were incubated for a further 72 h. After the incubation period, the medium was removed, and the cells were washed with PBS and fixed with ice-cold methanol for 15 min. The cells were washed again with PBS and stained with 0.5% crystal violet. Excess stain was gently washed off with PBS. Images of the colonies adhering to the bottom of the culture wells were recorded, and colonies containing more than 50 cells were counted. The percentage colony formation was calculated and compared to the untreated control group (UT) while 5-FU was used as positive control [21].

Flow cytometric analysis

The apoptotic effect of the *C. asiatica* ethanolic extract (CAE) on colorectal cancer cells was evaluated through flow cytometric analysis of cells stained with Annexin V-fluorescein isothiocyanate (FITC) (Merck, Germany) and propidium iodide (PI) (Glentham, UK). This method was adapted from the protocol described by Tiptiri-Kourpeti *et al.* [22]. HT-29 or HCT-116 cells (1×10^6 cells) were seeded into 6-well culture plates and incubated for 24 h. After incubation, the medium was replaced with fresh medium containing 25, 50, and 100 µg/mL concentrations of CAE, followed by incubation for 24 h at 37 °C. Cells were then washed with PBS and detached using 0.25% trypsin (Capricorn Scientific, Germany). Trypsin activity was neutralized with medium containing 10% fetal bovine serum (FBS). The cells were centrifuged, washed with PBS, and

resuspended in binding buffer (100 µL) containing FITC (0.5 µL). The mixture was gently shaken and incubated in the dark at 4 °C for 15 min. After incubation, cells were re-centrifuged, the supernatant discarded and replaced with 1% paraformaldehyde (300 µL). Subsequently, PI (1 µL) was added before analysis by flow cytometry (FACSymphony A1, BD Biosciences, USA). Apoptotic cell populations were identified using quadrant-based gating parameters: lower left (viable cells), upper left (necrotic cells), lower right (early apoptotic cells), and upper right (late apoptotic cells). The percentage of apoptotic cells in the CAE-treated groups was compared to untreated cells (UT) and 5-FU was used as the positive control [22].

Cell cycle distribution analysis

Cell cycle distribution was determined by flow cytometry using propidium iodide (PI) staining. HT-29 and HCT-116 colorectal cancer cells were seeded in 6-well plates and allowed to attach for 24 h. Cells were subsequently treated with various concentrations of *C. asiatica* ethanolic extract (CAE) for 24 h. Untreated cells (UT) served as the negative control, while 5-FU was used as a positive control. After treatment, both adherent and floating cells were collected and washed twice with cold PBS. Cells were fixed in 70% ice-cold ethanol added dropwise with gentle vortexing to prevent cell clumping and stored at 4 °C overnight. Prior to analysis, fixed cells were centrifuged to remove ethanol and washed with PBS. The cell pellets were resuspended in PI/RNase staining solution containing propidium iodide and RNase A (MedChemExpress, USA) and incubated at room temperature in the dark for 30 min. Cell cycle distribution was analyzed using a flow cytometer (FACSymphony A1, BD Biosciences, USA), and at least 10,000 events per sample were acquired. Doublets were excluded by gating on forward scatter area versus forward scatter height. DNA content histograms were generated, and the percentages of cells in the G0/G1, S, and G2/M phases were quantified using cell cycle analysis software. Data were expressed as the percentage of cells in each phase relative to the total cell population and compared between CAE-treated and UT [23].

Western blot analysis

The effect of the *C. asiatica* ethanolic extract (CAE) on the expression of the anti-apoptotic protein Bcl-2 in colorectal cancer cells was assessed using western blot analysis, adapted from the method of Hassametto *et al.* [24]. HT-29 or HCT-116 cells (1×10^6 cells) were seeded into 6-well culture plates and incubated for 24 h. After incubation, the medium was replaced with fresh medium containing various concentrations of CAE (25, 50 and 100 µg/mL), followed by incubation for 24 h at 37 °C. Cells were then washed with PBS and detached using 0.25% trypsin. Trypsin activity was neutralized with medium containing 10% fetal bovine serum (FBS). Trypsinized and washed cells were lysed in RIPA buffer (Merck, Germany) supplemented with a protease inhibitor cocktail, followed by homogenization using a cell sonicator. The lysates were centrifuged at 14,000 rpm for 15 min at 4 °C to collect the supernatant. Total protein concentration was quantified using the Bradford assay. Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). Membranes were blocked with 5% skim milk at room temperature (RT) for 1 h and then incubated overnight at 4 °C with Bcl-2 primary antibodies (1:1,000) (Abcam, USA). After washing, the membranes were incubated with anti-rabbit (1:10,000) secondary antibodies (Abcam, USA) for 1 h at RT. Protein bands were visualized using Luminata Forte Western HRP substrate (Sigma-Aldrich, USA) for 5 min and quantified relative to GAPDH (Abcam, USA) using ImageJ software. Protein expression levels in CAE-treated cells were compared to those in untreated cells (UT) [24].

Immunofluorescent staining analysis

The expression of the total Akt and p53 proteins in colorectal cancer cells was assessed using immunofluorescent staining, adapted from the method of Rao *et al.* [25]. HT-29 or HCT-116 cells (2,000 cells) were cultured on sterile cover slips placed in 6-well plates and incubated for 24 h. After incubation, the medium was replaced with fresh medium containing *C. asiatica* ethanolic extract (CAE, concentrations at 25, 50 and 100 µg/mL), followed by incubation for 24 h at 37 °C. Cells were washed with PBS and fixed with cold methanol for 45 min. After fixation, the cells were re-

washed with PBS and permeabilized with 0.1% Triton X-100 for 15 min to enhance membrane permeability. Non-specific protein binding was blocked using 1% bovine serum albumin (BSA) (HiMedia, India) for 1 h, followed by PBS washing. Monoclonal primary antibodies against Akt (Abcam, USA) and p53 (Abcam, USA) were diluted in PBS at a ratio of 1:500 and added to the fixed and blocked cells. The cells were incubated at 37 °C for 2 h. Alexa Fluor® 488 and 594 (Abcam, USA)-conjugated secondary antibodies were then added to bind the primary antibodies and enable fluorescence detection under a fluorescence microscope. Nuclear staining was performed using Hoechst dye (Sigma-Aldrich, USA), followed by PBS washing. The stained cover slips were mounted onto glass slides and observed under a fluorescence microscope. Images were captured, and the fluorescence intensity of Alexa Fluor® 488 and 594 bounded to the primary antibodies targeting p53 and total Akt, respectively. Fluorescent emission of total Akt and p53 were analyzed using ImageJ software. Protein expression levels were quantified relative to Hoechst-stained nuclear intensity. Relative intensities of Akt or p53 were compared with the untreated cells (UT) [25].

Statistical analysis

All experimental data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by an appropriate post hoc multiple comparison test. Untreated cells (UT) were used as the control group for comparison. All experiments were performed in 3 independent biological replicates. A *p*-value < 0.05 was considered statistically significant. Statistical analyses were conducted using SPSS software (version 18.0).

Results and discussion

Prediction of molecular targets and pathways of *Centella asiatica* metabolites in colorectal cancer

To explore the potential molecular targets of *Centella asiatica* in colorectal cancer (CRC), target genes associated with 5 previously reported major phytochemicals - asiaticoside, asiaticoside B, asiatic acid, madecassoside, and madecassic acid—were collected and compared with CRC-related genes. The chemical structures of these compounds are presented in **Figure 1(A)**. Venn diagram analysis (**Figure 1(B)**) identified 538 overlapping genes between CRC-associated targets and the predicted targets of the 5 phytochemicals, indicating potential shared molecular interactions involved in CRC progression. Reactome pathway enrichment analysis revealed that these overlapping targets were mainly associated with the PI3K/AKT signaling pathway, receptor tyrosine kinase signaling, and p53-related regulatory pathways (**Figure 1(C)**). A protein–protein interaction (PPI) network further identified top hub genes including TP53, BCL2, and AKT1 (**Figure 1(D)** and **Table 2**). These findings suggest that *C. asiatica* phytochemicals may exert anticancer effects through modulation of critical signaling pathways regulating cell proliferation and apoptosis. Previous studies have reported that p53 and Bcl-2 are central regulators of colorectal cancer cell survival and apoptosis, whereas Akt signaling is also implicated in promoting tumor cell survival [8,26], supporting the relevance of these predicted targets. Integrating network pharmacology with experimental validation offers a broader perspective on the multi-target potential of the extract, highlighting p53, Akt, and Bcl-2 as critical molecular nodes for further investigation.

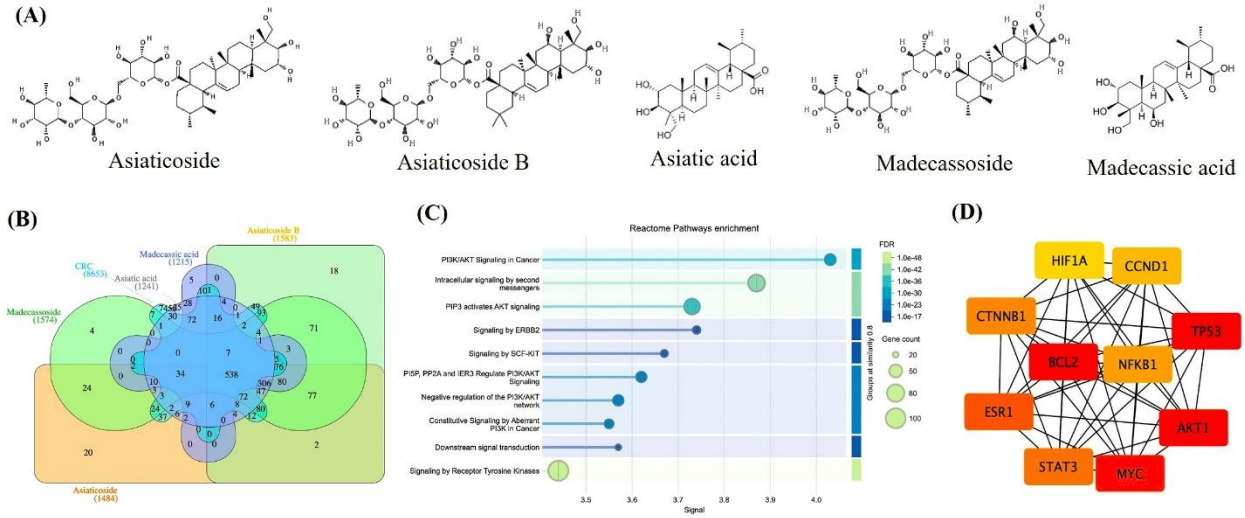


Figure 1 Network pharmacology analysis of major phytochemicals from *Centella asiatica* in colorectal cancer (CRC). (A) Chemical structures of the 5 reported major phytochemicals from *C. asiatica*: asiaticoside, asiaticoside B, asiatic acid, madecassoside, and madecassic acid. The structures were retrieved from the PubChem database. (B) Venn diagram showing the overlapping target genes between CRC-related genes and the predicted targets of the 5 phytochemicals. A total of 538 shared genes were identified. (C) Reactome pathway enrichment analysis of the overlapping genes. The bubble plot illustrates the significantly enriched signaling pathways. Bubble size represents the number of genes involved in each pathway, and color indicates the false discovery rate (FDR). (D) Protein–protein interaction (PPI) network identifies top hub genes (red) including TP53, BCL2, and AKT1.

Table 2 The top 10 hub genes in the protein–protein interaction (PPI) network identified by the MCC method using the STRING database.

Rank	Name	Score
1	TP53	1.3298228E+30
2	BCL2	1.3298227E+30
3	AKT1	1.3298226E+30
4	MYC	1.3298225E+30
5	ESR1	1.3298225E+30
6	STST3	1.3298221E+30
7	CTNNB1	1.3298211E+30
8	NFKB1	1.3297990E+30
9	CCND	1.3297403E+30
10	HIF1A	1.3296842E+30

***Centella asiatica* ethanolic extract inhibits the proliferation of colorectal cancer cells**

The whole plant of *C. asiatica* was authenticated (PBM-006734) (**Figure 2(A)**) and extracted with ethanol, yielding 13.575% crude extract. HPLC analysis confirmed the presence of asiaticoside at 6.231 µg per 1,000 µg of extract (**Figure 2(B)**). The anti-proliferative effect of the extract (CAE) was assessed using MTT assay in HT-29 and HCT-116 cells. CAE significantly reduced cell viability in a dose-dependent manner, with HCT-116 cells showing higher sensitivity (IC_{50} = 265.13 µg/mL) compared to HT-29 (IC_{50} = 800.43

μg/mL) (**Figure 2(C)**). IC_{50} values were calculated using nonlinear regression analysis of dose–response curves generated with GraphPad Prism software. Colony formation assays corroborated these results, demonstrating dose-dependent suppression of clonogenic survival, again with stronger effects in HCT-116 cells (**Figures 2(D)** and **2(E)**). These results indicate that CAE effectively inhibits proliferation and clonogenic potential in colorectal cancer cells. The differential sensitivity between HT-29 (mutant p53) and HCT-116 (wild-type p53) suggests a possible role of p53 status in mediating responsiveness. Consistent with previous reports, phytochemicals from *C. asiatica*, such as asiaticoside, have been shown to suppress CRC cell proliferation via inhibition of NF-κB and other pro-survival pathways [27], supporting the observed cytotoxic and anti-clonogenic effects.

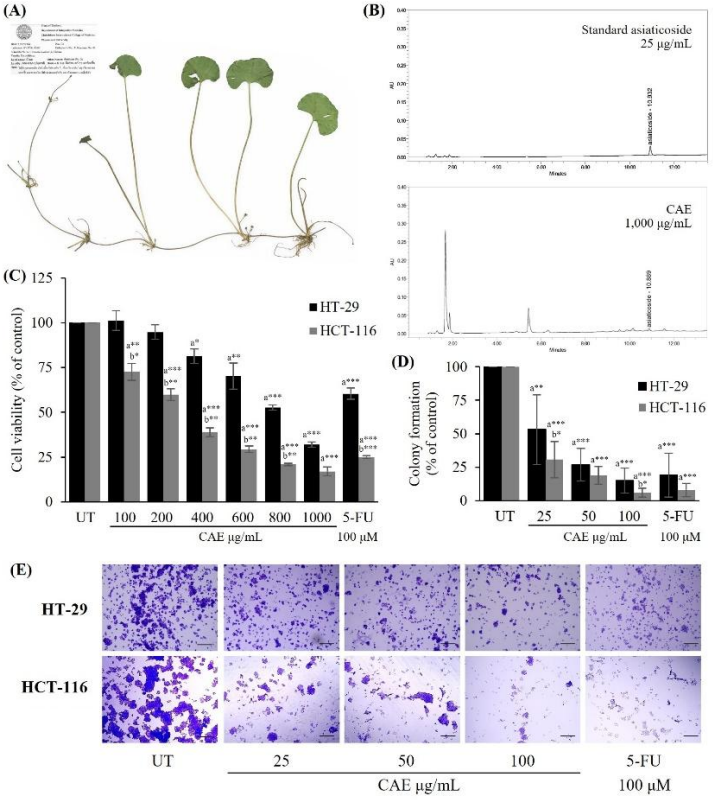


Figure 2 Authentication of *Centella asiatica* and the anti-proliferative effects of its ethanolic extract (CAE) on colorectal cancer cells. (A) Herbarium specimen of *C. asiatica* deposited at the Herbarium of Mahidol University (PBM-006734). (B) HPLC chromatograms showing the presence of asiaticoside in CAE compared with the asiaticoside standard. (C) Cell viability of HT-29 and HCT-116 cells following 48-h treatment with CAE at concentrations of 100 - 1,000 μg/mL, determined by MTT assay. (D) Quantitative analysis of colony formation after treatment with CAE (25 - 100 μg/mL). (E) Representative images of colony formation in HT-29 and HCT-116 cells after treatment. Five-Fluorouracil (5-FU, 100 μM) was used as a positive control. Data are presented as mean ± SD (n = 3). *, **, ** indicates $p < 0.05$, 0.01, 0.001, respectively versus untreated (UT) control. [a]: compared with UT; [b]: compared with HT-29 cells at the same concentration.

***Centella asiatica* ethanolic extract induces apoptosis with minimal effects on cell-cycle distribution**

Flow cytometry using Annexin V-FITC/PI staining revealed that CAE significantly induced apoptosis in both HT-29 and HCT-116 cells in a dose-dependent manner, with the highest apoptotic response observed at 100 μg/mL (**Figure 3(A)**). The apoptotic response was more pronounced in HCT-116 cells than in HT-29 cells, indicating greater sensitivity of HCT-116 cells to CAE treatment. In contrast, 5-fluorouracil (5-FU) induced substantially higher apoptotic cell populations in both cell lines. Notably, the concentration of CAE used in the apoptosis experiments (100 μg/mL)

was selected to investigate apoptosis-related alterations while minimizing excessive cytotoxicity. Consequently, the apoptotic response induced by CAE appeared less pronounced than that observed with 5-FU treatment. Cell-cycle analysis demonstrated only modest alterations in phase distribution following CAE treatment (**Figure 3(B)**). In HT-29 cells, CAE treatment slightly increased the S-phase population with minor reductions in G1 and G2/M populations, without marked cell-cycle arrest. Similarly, HCT-116 cells exhibited moderate increases in S-phase and G2/M populations following CAE treatment, whereas 5-FU induced marked G1-phase accumulation in both cell lines. These findings suggest that CAE primarily triggers apoptotic cell death rather than suppressing proliferation through classical cell-cycle checkpoint arrest. The observed increase in S-phase cells may reflect early disturbances in DNA replication; however, the relatively subtle changes in cell-cycle distribution may be attributed to the shorter treatment duration (24 h), as previous studies have reported more pronounced S-phase accumulation following longer exposure periods (48 - 96 h) to *C. asiatica* extracts [16]. This suggests that cell-cycle arrest may require prolonged exposure to become evident, whereas apoptotic responses can occur at earlier time points. Notably, HCT-116 cells were more sensitive to CAE-induced apoptosis, consistent with their wild-type p53 status and suggesting a possible p53-associated mechanism; however, this requires further investigation. Moreover, high concentrations of CAE exhibited antiproliferative effects comparable to those of 5-FU, particularly in the cell viability and colony formation assays, supporting the inhibitory effects of CAE on CRC cell growth.

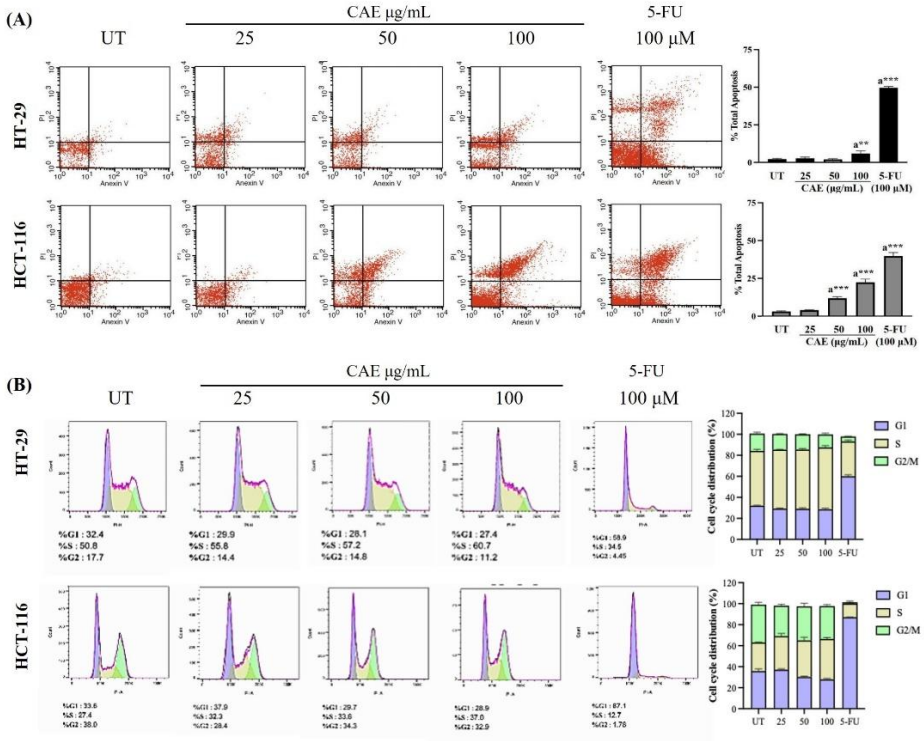


Figure 3 Apoptosis-induction and cell cycle alteration effects of *Centella asiatica* Ethanolic Extract (CAE). (A) Representative dot plots from flow cytometric analysis using Annexin V-FITC/PI staining illustrate apoptotic cell populations in HT-29 and HCT-116 colorectal cancer cells following treatment with CAE at concentrations of 25, 50, and 100 µg/mL, as well as 5-FU (100 µM, positive control). Early apoptotic cells (Annexin V^+/PI^-) and late apoptotic cells (Annexin V^+/PI^+) are located in the lower right and upper right quadrants, respectively. Quantification of total apoptosis (sum of early and late apoptotic cells) is presented in the accompanying bar graphs. (B) Alterations of cell-cycle distribution in both HT-29 and HCT-116 cells after treatment with CAE for 24 h, indicating slight reductions in G1 and G2/M populations with a modest increase in the S-phase fraction compared with untreated cells (UT). Data are expressed as mean ± SD (n = 3). **, *** Statistical significance was at $p < 0.01$, and $p < 0.001$, respectively. [a]: Compared to untreated cells (UT).

***Centella asiatica* ethanolic extract modulates Akt, p53, and Bcl-2 expression**

Immunofluorescence analyses showed that CAE treatment altered Akt and p53 fluorescence intensities in both CRC cell lines (**Figure 4(A)**). In HT-29 cells, p53 intensity significantly increased following CAE treatment, particularly at 25 - 100 μg/mL, whereas the reduction in Akt intensity was less pronounced. In HCT-116 cells, CAE treatment reduced Akt fluorescence intensity in a dose-dependent manner, while p53 intensity was markedly increased, especially at 100 μg/mL. Western blot analysis demonstrated that Bcl-2 expression was decreased in a dose-dependent manner in HCT-116 cells, whereas only slight changes were observed in HT-29 cells (**Figure 4(B)**). Akt is a known regulator of cell survival through interactions with Bcl-2 family proteins [8,28], while p53 activation promotes mitochondrial apoptosis [29]. Consistent with the network pharmacology analysis identifying AKT1, TP53, and BCL2 as major hub targets, CAE treatment resulted in decreased Akt and Bcl-2 expression together with increased p53 levels compared with untreated (UT) cells. These findings suggest the possible involvement of apoptosis-related signaling pathways in the antiproliferative and pro-apoptotic effects of CAE, particularly in p53 wild-type HCT-116 cells. This aligns with prior studies showing that *C. asiatica* phytochemicals (ECa233) can influence multiple apoptosis-related pathways, underscoring the potential of crude extracts to exert multi-target anticancer effects [30-31]. Overall, CAE inhibited CRC cell growth and induced apoptosis, with stronger antiproliferative and pro-apoptotic effects observed in HCT-116 cells than in HT-29 cells. This differential response may be associated with p53 status, as HCT-116 cells harbor wild-type p53 whereas HT-29 cells contain mutant p53. Notably, TP53 was identified as one of the top-ranked hub genes in the network pharmacology analysis, further supporting the possible involvement of p53-related signaling in the response to CAE treatment. The integration of network pharmacology prediction with experimental validation provides supportive evidence for the potential of CAE as a source of bioactive compounds for anticancer therapy. However, the present findings are primarily associative and do not establish direct causal regulation of the Akt/p53/Bcl-2 signaling pathway. Further mechanistic studies are warranted to validate the underlying molecular mechanisms.

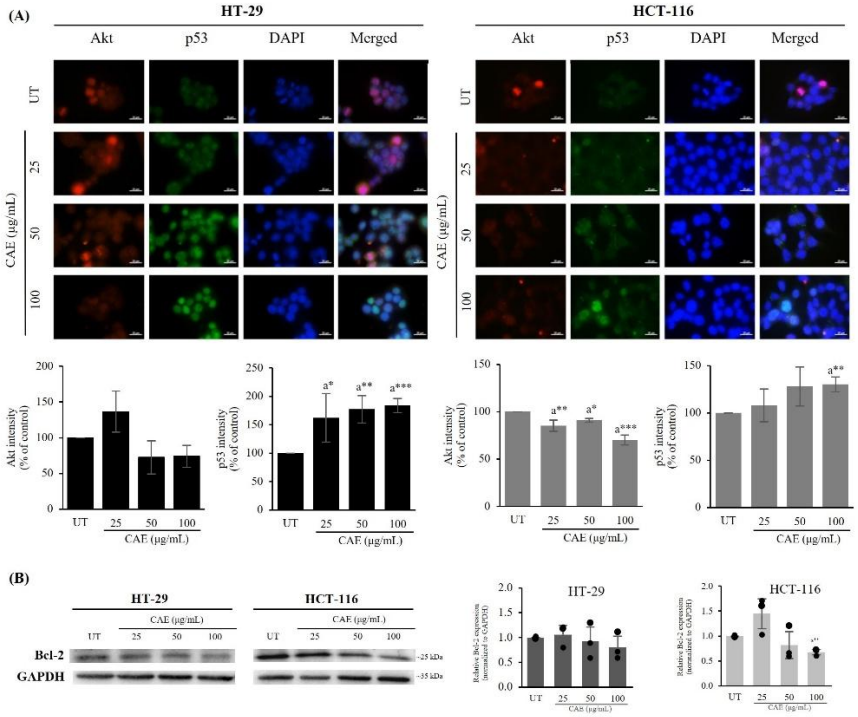


Figure 4 Effects of *C. asiatica* ethanolic extract (CAE) on Akt, p53, and Bcl-2 expression in colorectal cancer cells. (A) Representative immunofluorescence images of HT-29 and HCT-116 cells treated with CAE (25 - 100 μg/mL). Akt (red), p53 (green), and nuclei stained with DAPI (blue). Quantification of fluorescence intensity normalized to DAPI is shown in the lower panels. (B) Western blot analysis of Bcl-2 protein expression in HT-29 and HCT-116 cells following CAE treatment. Protein levels were normalized to GAPDH and quantified by densitometric analysis. Data are expressed as mean ± SD (n = 3). *, **, *** indicate statistical significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared with untreated cells (UT). [a]: Compared to untreated cells (UT).

Conclusions

The present study demonstrates that *Centella asiatica* ethanolic extract (CAE) exhibits significant antiproliferative and pro-apoptotic effects in colorectal cancer cells. Treatment with CAE markedly reduced cell viability and colony-forming ability while increasing apoptotic cell populations in both HT-29 and HCT-116 cells. Notably, high concentrations of CAE demonstrated antiproliferative effects comparable to those of 5-FU in cell viability and colony formation assays, supporting the inhibitory effects of CAE on CRC cell growth. Cell-cycle analysis revealed only modest alterations in phase distribution, including slight increases in the S-phase and G2/M populations following CAE treatment, which may reflect early cellular stress associated with apoptosis induction. Immunofluorescence and western blot analyses demonstrated that CAE treatment altered Akt, p53, and Bcl-2 expression patterns in colorectal cancer cells. In HCT-116 cells, CAE reduced Akt fluorescence intensity and decreased Bcl-2 expression while markedly increasing p53 intensity, whereas HT-29 cells exhibited less pronounced changes in Akt and Bcl-2 expression. Consistent with these findings, HCT-116 cells harboring wild-type p53 exhibited greater sensitivity to CAE compared with HT-29 cells, indicating that functional p53 may contribute to the observed effects. Notably, these experimental findings were aligned with network pharmacology predictions identifying AKT1, TP53, and BCL2 as major hub targets potentially associated with the anticancer effects of CAE. These findings provide preliminary evidence supporting the possible involvement of apoptosis-related signaling pathways and the multi-target nature of CAE. Together, these results suggest that *C. asiatica* extract represents a promising source of bioactive compounds with potential therapeutic relevance for colorectal cancer. The crude ethanolic extract, containing multiple bioactive constituents, may contribute to the observed multi-target antiproliferative and pro-apoptotic effects. Furthermore, the differential responses observed between HT-29 and HCT-116 cells suggest that p53 status may influence cellular responsiveness to CAE treatment. Nevertheless, the present mechanistic findings should be interpreted as preliminary and associative observations regarding the possible mechanisms underlying the anticancer effects of CAE. Additional investigations, such as gene expression analyses, upstream/downstream signaling mediators, and pathway-specific inhibition studies, are still required to further clarify and validate the molecular mechanisms involved.

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Declaration of generative AI in scientific writing

Generative AI tool (Microsoft Copilot) was used to improve the readability and language of a manuscript with the authors' oversight and control. There are no content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

CRediT author statement

Chonnapat Naktubtim: Writing - Original Draft; Methodology; Software; Investigation; Data Curation; Funding acquisition **Waristha Sriniang**: Investigation. **Thanaphat Worawichitchaikun**: Investigation. **Kitthisak Khlaeo Chansukh**: Investigation. **Witchuda Payuhakrit**: Resources; Validation. **Paween Kunsorn**: Conceptualization; Methodology; Investigation; Data Curation; Visualization; Writing - Reviewing and Editing; Supervision; Project administration; Funding acquisition.

References

[1] American Cancer Society. *Colorectal cancer facts & figures 2023 - 2025.* American Cancer Society, Maryland, United States, 2023.

[2] EP Weledji. The etiology and pathogenesis of colorectal cancer. *Clinical Oncology* 2024; **9**, 2046.

[3] FDE De Palma, V D'Argenio, J Pol, G Kroemer, MC Maiuri and F Salvatore. The molecular hallmarks of the serrated pathway in colorectal cancer. *Cancers* 2019; **11(7)**, 1017.

[4] N Murphy, V Moreno, DJ Hughes, L Vodicka, P Vodicka, EK Aglago, MJ Gunter and M Jenab. Lifestyle and dietary environmental factors in colorectal cancer susceptibility. *Molecular Aspects of Medicine* 2019; **69**, 2-9.

[5] W Chen, R Zheng, P D Baade, S Zhang, H Zeng, F Bray and others. Cancer statistics in China, 2015. *CA: A Cancer Journal for Clinicians* 2016; **66(2)**, 115-132.

[6] International Agency for Research on Cancer. *Global cancer observatory: Thailand 2025*. International Agency for Research on Cancer, Lyon, France, 2025.

[7] R Ahmad, J K Singh, A Wunnava, O Al-Obeed, M Abdulla and S K Srivastava. Emerging trends in colorectal cancer: Dysregulated signaling pathways. *International Journal of Molecular Medicine* 2021; **47(3)**, 14.

[8] S Qian, Z Wei, W Yang, J Huang, Y Yang and J Wang. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. *Frontiers in Oncology* 2022; **12**, 985363.

[9] MT Hemann and SW Lowe. The p53-Bcl-2 connection. *Cell Death & Differentiation* 2006; **13(8)**, 1256-1259.

[10] AJ Watson. Apoptosis and colorectal cancer. *Gut* 2004; **53(11)**, 1701-1709.

[11] M Barathan, AK Zulpa, SL Ng, Y Lokanathan, MH Ng and JX Law. Innovative strategies to combat 5-fluorouracil resistance in colorectal cancer: The role of phytochemicals and extracellular vesicles. *International Journal of Molecular Sciences* 2024; **25(13)**, 7470.

[12] J Casale and P Patel. *Fluorouracil*. StatPearls Publishing, Florida, United States, 2024.

[13] MS Bandara, EL Lee and JE Thomas. Gotu kola (*Centella asiatica* L.): An under-utilized herb. *American Journal of Plant Science and Biotechnology* 2011; **5(2)**, 20-31.

[14] R Sabaragamuwa, CO Perera and B Fedrizzi. *Centella asiatica* (Gotu kola) as a neuroprotectant and its potential role in healthy ageing. *Trends in Food Science & Technology* 2018; **79**, 88-97.

[15] P Bunpo, K Kataoka, H Arimochi, H Nakayama, T Kuwahara, Y Bando, K Izumi, U Vinitketkumnuen and Y Ohnishi. Inhibitory effects of Centella asiatica on azoxymethane-induced aberrant crypt focus formation and carcinogenesis in the intestines of F344 rats. *Food and Chemical Toxicology* 2004; **42(12)**, 1987-1997.

[16] P Bunpo, K Kataoka, H Arimochi, H Nakayama, T Kuwahara, Y Ohnishi and U Vinitketkumnuen. Centella asiatica extract induces cell cycle arrest in Caco-2 human colon cancer cells. *Chiang Mai Medical Journal* 2005; **44(1)**, 21-28.

[17] PD Tanvi, NG Nilanjan, VM Chandrashekar and SV Rajendra. Aqueous extract of aerial parts of Centella asiatica arrests cell cycle at G0/G1 on colorectal cancer cell lines. *Journal of Pharmaceutical Research* 2016; **15(2)**, 45-50.

[18] H Heberle, GV Meirelles, FR da Silva, GP Telles and R Minghim. InteractiVenn: A web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics* 2015; **16(1)**, 169.

[19] KJ Prateek and KA Ram. High performance liquid chromatographic analysis of asiaticoside in *Centella asiatica* (L.) Urban. *Chiang Mai Journal of Science* 2008; **35(3)**, 521-525.

[20] R Tanomrat, C Naktubtim, P Aimvijarn and P Suwannalert. N-acetylcysteine improves the inhibitory effect of quercetin-rich onion extract on HT-29 and HCT-116 colorectal cancer migration and invasion through iNOS suppression. *International Journal of Medical Sciences* 2023; **20(9)**, 1123-1134.

[21] R Praphasawat, S Palipoch, P Suwannalert, W Payuhakrit, P Kunsorn, S Laovitthayanggoon, S Thakaew, N Munkong and W Klajing. Red rice bran extract suppresses colon cancer cells via apoptosis induction/cell cycle arrest and exerts

antimutagenic activity. *Experimental Oncology* 2023; **45(2)**, 220-230.

[22] A Tiptiri-Kourpeti, K Spyridopoulou, V Santarmaki, G Aindelis, E Tompoulidou, EE Lamprianidou and others. Lactobacillus casei exerts anti-proliferative effects accompanied by apoptotic cell death and up-regulation of TRAIL in colon carcinoma cells. *PLoS One* 2016; **11(2)**, e0147960.

[23] CY Huang, JH Chien, KF Chang, CY Hsiao, YC Huang, YT Chen, MY Hsu, MC Hsieh and NM Tsai. Cedrus atlantica extract exerts antiproliferative effect on colorectal cancer through the induction of cell cycle arrest and apoptosis. *Food Science & Nutrition* 2022; **10(5)**, 1638-1648.

[24] A Hassametto, R Tanomrat, T Muangthong, S Worawichawong and P Suwannalert. Role of oxidative stress-dependent C/EBPβ expression on CAF transformation inducing HCT116 colorectal cancer cell progression, migration and invasion. *Asian Pacific Journal of Cancer Prevention* 2023; **24(11)**, 3825-3835.

[25] V Rao, V Kansal, S Stoupa and D Agrawal. MMP-1 and MMP-9 regulate epidermal growth factor-dependent collagen loss in human carotid plaque smooth muscle cells. *Physiological Reports* 2014; **2(2)**, e00224.

[26] DY Lim and JHY Park. Induction of p53 contributes to apoptosis of HCT-116 human colon cancer cells induced by the dietary compound fisetin. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2009; **296(5)**, G1060-G1068.

[27] X Zhou, C Ke, Y Lv, C Ren, T Lin, F Dong and Y Mi. Asiaticoside suppresses cell proliferation by inhibiting the NF-κB signaling pathway in colorectal cancer. *International Journal of Molecular Medicine* 2020; **46(4)**, 1525-1537.

[28] JL Coloff. 2010, Akt, glucose metabolism, and the Bcl-2 family. Ph. D. Dissertation. Duke University, North Carolina, United States.

[29] FJ Reyes-Zurita, G Pachón-Peña, D Lizárraga, EE Rufino-Palomares, M Cascante and JA Lupiáñez. The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism. *BMC Cancer* 2011; **11(1)**, 154.

[30] S Manmuan, P Manmuan, P Yoykaew, P Thuetong, P Asipong, N Riantong and MH Tantisira. Evaluation of standardized extract of *Centella asiatica* on cell viability and repressive cancer migration in metastatic colorectal cancer cells in vitro. *Walailak Journal of Science and Technology* 2021; **18(5)**, 9016.

[31] MH Tantisira. Research and development of standardized extract of *Centella asiatica* ECa 233. *In*: Proceedings of the International Conference on Herbal and Traditional Medicine, Khon Kaen, Thailand. 2015.