

## Research Article

# Growth inhibition of HCT116 induced by Thai herbal recipe is mediated via cyclin D1 and CDK4 suppression

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Three-root herbal recipe; Colon cancer; HCT116; *Cyclin D1*; *CDK4*

## ABSTRACT

Thai herbal recipe named “Sam Rak” is commonly used in the Northeastern part of Thailand for treating various malignancies, such as breast, liver, and colon cancers. Previously, we investigated the ED<sub>50</sub> of the herbal ethanolic extract on colon cancer cell line, HCT116, and found the significant growth inhibition. Thus, this herbal recipe was further investigated in this study to clarify the underlying mechanisms in HCT116. The clonogenic assay was performed to assure the antiproliferative activity of the herbal recipe at the concentrations of 35 and 70 µg/mL. In addition, the expression of three genes, i.e., *CCND1*, *CDK2*, and *CDK4*, which regulate the cell cycle progression, were also done using a real-time PCR (qPCR). The results showed that the herbal recipe could kill HCT116 cells, as shown in clonogenic assay, especially at day 7. Moreover, with qPCR techniques, the significant downregulation of both *CCND1* and *CDK4* were demonstrated. *CDK2* also showed downregulation, but not significant. We summarized that this three-root herbal recipe could inhibit growth and kill colon cancer cell line, HCT116, *in vitro*. The mechanisms of action involved in significant suppression of the expression of two genes, *CCND1* and *CDK4*. This study might be useful for the application of this herbal recipe in treating colon cancer in the future.

## 1. INTRODUCTION

A famous Thai herbal recipe in the Northeastern part of Thailand named “Sam rak” has long been used for treating liver cancer. Though the most common usage of “Sam Rak” in traditional medicine is for treating liver cancer, there are still some breast and colon cancer patients use this herbal recipe for treating themselves. This herbal recipe comprises three roots from three herbal plants, namely, *Eurycoma longifolia*, *Dipterocarpus obtusifolius*, and *Tamilnadia uliginosa*. *Eurycoma longifolia*, in Thai named “Pla-Lai-Puank”, is in the family of Simaroubaceae, whereas *Dipterocarpus obtusifolius*, named in Thai “Kung”, is in Dipterocarpaceae family. The third root is derived from *Tamilnadia uliginosa*, called “Ta-Lum-Puk”, which is in the family of Rubiaceae. Two of the three plants, *Eurycoma longifolia* and *Dipterocarpus obtusifolius* have higher potent growth inhibition on several cancer cell lines, than *Tamilnadia*

*uliginosa* has, according to the previous studies<sup>1-3</sup>. Interestingly, *Tamilnadia uliginosa* is prescribed as one of the components in this herbal recipe to treat patients for the purpose of adjusting the abnormal four body elements, i.e., earth, water, fire, and wind elements, to normal condition. Additionally, in traditional medicine, the root of *Tamilnadia uliginosa* could also be prescribed for curing some specific gastrointestinal symptoms, such as mucous bloody stool, including acute and chronic diarrhea<sup>4</sup>. For Thai traditional medical treatment, these three roots will be ground into powder and dissolved in drinking water. Cancer patients in this specific region drink this herbal mixture every day for treating themselves. Although most cancer patients claim that they have disease regression, the scientific clinical data on the efficacy and side effect of this herbal recipe have not yet been reported so far.

Based on our previous results, the ethanolic extract of this herbal recipe was investigated for growth inhibition and significant detection was demonstrated on five cancer cell lines, including colon cancer cell line (HCT116). The ED<sub>50</sub> value of HCT116 was found at 70 µg/mL<sup>5</sup>. Cell cycle determination of HCT116 treated cells showed significant G1/S phase arrest strikingly increasing from 8.05 to 52.81% when compared with the control cells<sup>5</sup>. Therefore, in this study, HCT116 treated cells was further investigated for the specific genes expression involving in cell cycle regulation for G1/S phase, i.e., cyclin D1 (CCND1), cyclin-dependent kinase 2 (CDK2), and cyclin-dependent kinase 4 (CDK4), using a real time PCR (qPCR). Moreover, clonogenic assay of HCT116 was also performed and evaluated on day 7 and day 10 to ensure the growth inhibition, including cytotoxic property, induced by this herbal recipe.

## 2. MATERIALS AND METHODS

### 2.1. Plant collection and ethanolic extract

From our previous report, the roots of *Eurycoma longifolia*, *Dipterocarpus obtusifolius*, and *Tamilnadia uliginosa* were collected from Nong-bua-lum-pu province in the Northeastern part of Thailand<sup>5</sup>. All the plants were identified by comparing them with data and specimens at the Forest Herbarium (BKF nos. 141423, 143892 and 23913, respectively), Department of National

Parks, Wildlife and Plant Conservation, Bangkok, Thailand. The extraction process was based on our previous report<sup>5</sup>. In brief, ten grams of three roots were ground and macerated with 90% ethanol for one day before filtering. Dry herbal extract was obtained using evaporator. Finally, two hundred milligrams of the extract were sonicated and used for thin-layer chromatography. The ethanolic extract was diluted with 100% DMSO to make a 50 mg/mL and kept as a stock solution for further usage. For all the experiments, the final concentration was obtained by diluting the stock solution with complete medium DMEM before filtering through the syringe filtered membrane.

### 2.2. Cell culture condition of HCT116

HCT116, colon cancer cell line, was purchased from ATCC (American Type Culture Collection, USA). Cells were cultured in complete DMEM medium (Gibco, USA) in T75 flask. The complete medium was consisted of 4.5 g/L D-Glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Gibco, USA.), 100 U/mL penicillin (Gibco, USA.) and 100 µg/mL streptomycin (Gibco, USA.). Cells were incubated at 37°C, 5% CO<sub>2</sub>, with 90-98% humidity. After 3-4 days with 80% confluency, cells were harvested by washing twice with 1X PBS (Amresco®, USA), trypsinized with 0.5% trypsin in EDTA (Gibco, USA.) for 2 min and stopped the reaction with 0.2 ml FBS before transferring to the new culture medium in T75 flask.

### 2.3. Clonogenic assay

HCT116 cells were cultured in T75 flask in completed culture medium. The ethanolic extract of herbal recipe was added into the cells at concentrations of 35 and 70 µg/mL, which were equal to 0.5xED<sub>50</sub> and 1xED<sub>50</sub>, respectively. After 48-hour treatment, HCT116 cells were harvested and 5x10<sup>3</sup> cells were seeded in 60 mm dish in completed medium. The medium was changed every 3-4 days. After culturing until day 7 and day 10, the medium was removed. Cells were washed twice with PBS, fixed with absolute methanol for 10 min, and stained with 0.05% crystal violet (W/V) for 10 min. The colony was counted under light microscope<sup>6</sup>. Photographs of colonies in culture dishes were taken using Huawei G7 plus. The number of colonies was presented as mean ± SD.

#### 2.4. The expression of *CCND1*, *CDK2*, and *CDK4*, using real-time PCR

5.5x10<sup>6</sup> cells of HCT116 were cultured in T75 flask in complete DMEM medium. After 24 hours, the herbal extract was added to the cells at a final concentration of 70 µg/mL. The cells were incubated for further 48 hours in the same incubated condition. Untreated HCT116 cells were used as the control group. Then, HCT116 cells were harvested, collected and kept in Trizol solution (Invitrogen, USA) at -80 °C until the analysis. RNA was extracted from the cells following the manufacturer protocol Trizol® (Invitrogen, USA). Two micrograms of RNA were synthesized to cDNA using Transcriptor first strand cDNA synthesis kit® (Roche, Thailand). The expression of *CCND1*, *CDK2*, and *CDK4* were evaluated from cDNA of HCT116 using LightCycler®480 SYBR Green I master (Roche, Thailand). In brief, the 20 µL reaction consists of 10 µL of 2X LightCycler®480 SYBR Green I master, 5 µL cDNA, 0.5 µM of each forward and reverse primer. *GAPDH*

was used as a house-keeping gene. The sequences of all primers are showed in Table 1<sup>7</sup>. The reaction was run in LightCycler®480. The PCR protocol as followed: Pre-incubation at 95 °C 10 min 1 cycle, 45 cycles of amplification consist of denaturation 95 °C 10 sec, annealing 60 °C 10 sec and extension 72 °C 10 sec, respectively. The duplicate reaction was performed and analyzed using LightCycler®480 Software version 1.5 (Roche, Thailand). The triplicated independent experiments were done. The calculation of the expression of gene was evaluated using the following formula<sup>8</sup>:

$$\begin{aligned} \text{Normalized target gene} &= 2^{-\Delta\Delta Ct} \\ &= 2^{-(TS-RS)-(TC-RC)} \end{aligned}$$

TS = Ct Target gene sample,

RS = Ct Reference gene sample,

TC = Ct Target gene Control,

RC = Ct Reference gene Control

The comparison of normalized target gene between sample and control groups were calculated by Student's paired *t*-test.

**Table 1.** The sequences of all primers were demonstrated

Gene	Nucleotide sequences
<i>GAPDH</i>	Forward : GTCAACGGATTTGGTCGTATTG
	Reverse : CATGGGTGGAATCATATTGGAA
<i>CCND1</i>	Forward : GGCGGAGGAGAACAAACAGA
	Reverse : GGGCGGATTGGAAATGAACT
<i>CDK2</i>	Forward : CCTGGACACTGAGACTGAG
	Reverse : AGGCATCCATGAATTTCTTGAG
<i>CDK4</i>	Forward : CTTCTGCAGTCCACATATGCAACA
	Reverse : CAACTGGTTCGGCTTCAGAGTTTC

### 3. RESULTS

#### 3.1. Thin-layer chromatography of three-root herbal recipe

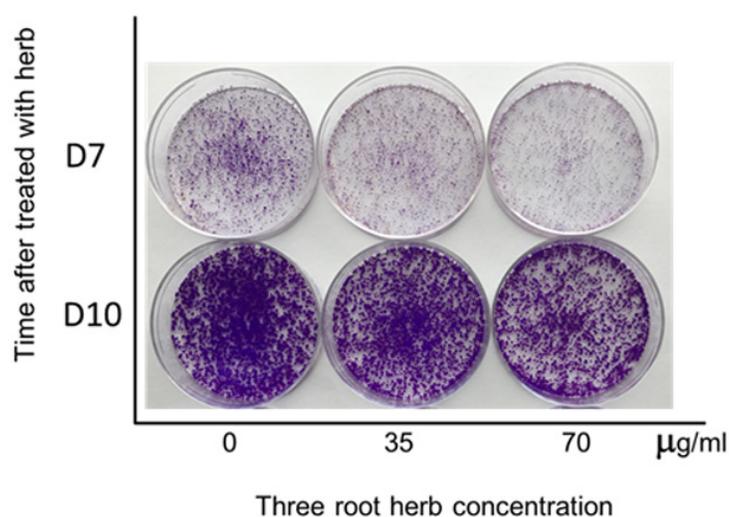
In this study, all the experiments were done using the same ethanolic extract as previously reported<sup>5</sup>. Thus, the thin-layer chromatography of this herbal recipe was already mentioned and demonstrated.

#### 3.2. The effect of ethanolic herbal extract on clonogenic assay of HCT116 cells

After incubated the cells with the herbal extract, the number of colonies of HCT116 was significantly decreased as showed in Figure 1. At day 7 of incubation, the colony counts of HCT116 treated with 35 µg/mL of the extract were 1366.67±33.31 colonies, which were significantly decreased when compared to the control (2017.33±125.26),

with  $P$  value  $< 0.05$ . After increasing the herbal extract concentration up to  $70 \mu\text{g/mL}$ , the number of colonies was markedly decreased to  $910.67 \pm 72.23$  colonies. This result showed the significant decrease with  $P$  value  $< 0.05$  when compared

to both 0 and  $35 \mu\text{g/mL}$  of the herbal extract, at day 7. However, the number of colonies at day 10 could not be counted because of too high number with over 500 colonies. All the counted number of HCT116 colonies are shown in Table 2.



**Figure 1.** The colonies of treated HCT116 cells were showed. The cells were treated with various concentrations of herbal extract at concentrations of 35 and  $70 \mu\text{g/mL}$  for 48 hours. After day 7 and day 10, treated cells were stained with 0.05% crystal violet (W/V). The colonies of HCT116, at the dosage of  $70 \mu\text{g/mL}$ , was significantly decreased in number on day 7 and day 10. This could be observed from the stained colonies in the photo. However, at day 10, colonies could not be directly counted because of the numerous colonies, over 500 colonies

**Table 2.** The number of colonies of HCT116 was presented. The cells were treated with two concentrations of herbal extract for 48 hours. Then cells were stained with 0.05% crystal violet (W/V) after continuous culturing for 7 and 10 days

Cell line	Concentration of herbal extract	Colony count (colonies; mean $\pm$ SD)	
		D7	D10
HCT116	$0 \mu\text{g/mL}$	$2017.33 \pm 125.26$	$>500$
	$35 \mu\text{g/mL}$	$1366.67 \pm 33.31^*$	$>500$
	$70 \mu\text{g/mL}$	$910.67 \pm 72.23^{*,a}$	$>500$

\* Significantly decreased ( $P < 0.05$ ) of HCT116 colonies when compared to control (herbal extract =  $0 \mu\text{g/mL}$ ).

<sup>a</sup> Significantly decreased ( $P < 0.05$ ) of HCT116 colonies when compared to treated with  $35 \mu\text{g/mL}$  of herbal extract.

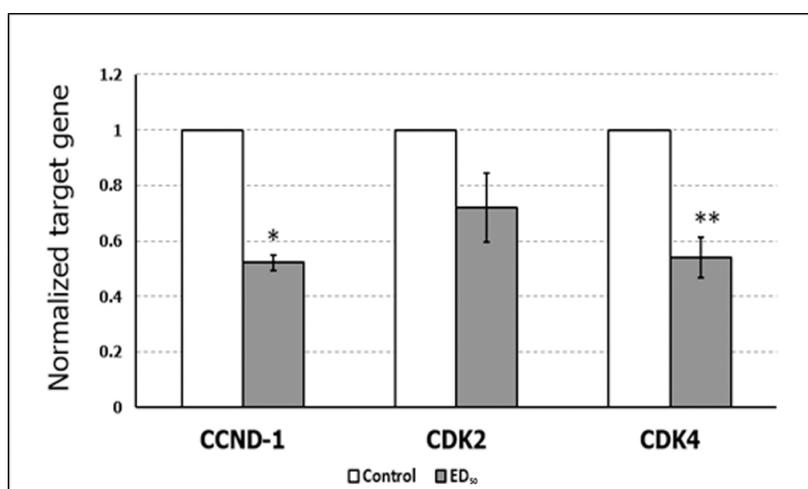
### 3.3. The expression of *CCND1*, *CDK2* and *CDK4* of HCT116 after treated with the extract $70 \mu\text{g/mL}$

The expression of *CCND1*, *CDK2* and *CDK4* of HCT116 after the herbal extract

treatment compared to the control was showed in Figure 2 (n=3 experiments). There was a decrease of these gene expression after treated with  $70 \mu\text{g/mL}$  of the extract when compared to the control. The mean  $\pm$  SD values of gene expression ratio

of *CCND1* and *CDK4* of treated HCT116 were  $0.52 \pm 0.02$  and  $0.54 \pm 0.07$ , respectively. These results were significantly lower than the control

( $P = 0.001$  and  $P = 0.015$ ). Slight decreasing in gene expression ratio of *CDK2* was found with  $0.72 \pm 0.12$ , but no significant detection.



**Figure 2.** The expression of *CCND1*, *CDK2* and *CDK4* of HCT116 (n=3) after treated with the herbal extract compared to the control (herbal extract = 0 µg/mL) was showed. There was significant decreased of *CCND1* and *CDK4* expression ratio of HCT116 after treated with herbal extract at 70 µg/mL when compared to the control. The expression of *CDK2* was decreased, but not significant. The mean ± SD of gene expression ratio of *CCND1* was  $0.52 \pm 0.02$  ( $*P = 0.001$ ) and *CDK4* was  $0.54 \pm 0.07$  ( $**P = 0.015$ )

#### 4. DISCUSSION

From our previous investigation, we found the significant ED<sub>50</sub> value from the ethanolic herbal extract on HCT116 colon cancer cell line. Thus, we continued our experiments to search for the underlying mechanisms of this herbal recipe on HCT116 cells. The three-root herbal ethanolic extract could inhibit growth in HCT116 colon cancer cell line at ED<sub>50</sub> value of 70 µg/mL<sup>5</sup>. Previously, that study also showed that HCT116 was inhibited predominantly by cell cycle inhibition. Therefore, we conducted the experiments to find out specific gene expression that regulate and inhibit growth of HCT116 cells.

According to the ED<sub>50</sub> value previously shown, the clonogenic assay was performed. After incubation with the herbal extract, HCT116 colonies were decreased significantly, especially at day 7, as shown in Figure 1 and Table 2. Our results showed that the herbal ethanolic extract could, not only inhibit growth, but also definitely kill the cancer cells. Only cells that still retain capacity to grow can form colonies after continuous culturing,

thus, it can determine the potent cytotoxic property of this three-root herbal recipe *in vitro*<sup>9</sup>. However, in our study, it is difficult to count directly the colony number on day 10, since there were over 500 colonies in the cell culture dishes.

From this study, the three-root herbal ethanolic extract inhibit growth proliferation of HCT116 colon cancer cells by significantly downregulating *CCND1* and *CDK4*, as shown in Figure 2. *CCND1* is in the cyclin family which has the motif cyclin box for binding to the appropriate CDKs<sup>10</sup>. *CCND1* expression is responsive to a group of growth factors, including cytokines, such as Her2/Neu<sup>11</sup>. This gene is also a key target for β-catenin to drive colon cancer development<sup>12</sup>. After stimulating with mitogenic growth factors, the *CCND1* expression is upregulated through some upstream specific signaling pathways. The misregulation and accumulation of cyclin D1 can lead to uncontrolled cell cycle progression, abnormal cell division and cell proliferation, consequently, cancers pursue<sup>13</sup>. In addition, the activation of cyclin D1 needs specific group of

enzymes called cyclin-dependent kinases or CDKs, which are protein kinases. Binding of cyclins with CDKs will form dimers which activate the kinase function and trigger cell cycle progression<sup>14</sup>. The partner of cyclin D1 is commonly CDK4/6, which forms the dimer with cyclin D1 and regulates cell cycle transition through Rb phosphorylation<sup>15</sup>. The activity of cyclin D1-CDK4/6 complex is usually overexpressed in cells, which can be found in many kinds of cancers, including colorectal carcinoma. CDK2 is also the choice of cyclin D1 partner<sup>16,17</sup>. Downregulating of *CDK2* was demonstrated in our study, as shown in Figure 2, but not significant.

## 5. CONCLUSIONS

In conclusion, this study demonstrates that three-root herbal recipe has cytotoxic property and can inhibit growth of HCT116, colon cancer cell line, by significantly downregulating *CCND1* and *CDK4*. This support in ongoing usage of three-root herbal recipe in the Northeastern part of Thailand. Moreover, this study might be useful for the application of this herbal recipe in treating colon cancer in the future.

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### Conflict of interest

Authors declare no conflict of interest.

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