

## RESEARCH ARTICLE

# Anticancer Potential of *Cratoxylum formosum* Subsp. *Pruniflorum* (Kurz.) Gogel Extracts Against Cervical Cancer Cell Lines

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## Abstract

**Background:** Most northeast Thai vegetables may play roles in human health by acting as antioxidant and anticancer agents. Recent study showed that *Cratoxylum formosum* subsp. *pruniflorum* (Kurz.) Gogel. (Teawdang) could inhibit growth of liver cancer cell lines. Cervical cancer, which has human papillomavirus as its main cause, is found at high incidence in Thailand. Due to increasing drug resistance, searches for potential anticancer compounds from natural source are required. Therefore, our purpose was to evaluate the cytotoxicity of Teawdang extracts in cervical cancer cell lines. **Materials and Methods:** Teawdang edible parts, purchased from Khon Kaen market during July-October 2013 was extracted with organic solvent. Phenolic profiles of crude hexane (CHE), ethyl acetate (CEE), methanol (CME) and water (CWE) extracts were performed by high performance liquid chromatographic (HPLC) techniques. Their cytotoxic effects on cervical cancer cells were investigated with HPV-non infected (C-33A) and HPV-infected (HeLa and SiHa) cell lines. **Results:** HPLC profiles showed that all crude extracts contained caffeine, ferulic acid and resveratrol. CME and CEE had high contents of gallic acid and quercetin. Catechin was found only in CWE. Cytotoxicity test showed that CEE had the lowest IC<sub>50</sub> on HeLa (143.18±13.35 µg/mL) and SiHa cells (106.45±15.73 µg/mL). C-33A cells were inhibited by CWE (IC<sub>50</sub> = 130.95±3.83 µg/mL). **Conclusions:** There were several phenolic compounds in Teawdang extracts which may have cytotoxic effects on cervical cancer cell lines. Investigation of these bioactive compounds as new sources of anticancer agents is recommended.

**Keywords:** Cervical cancer - *Cratoxylum formosum* subsp. *pruniflorum* (Kurz.) Gogel - cytotoxic effect - phenolics

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## Introduction

Cervical cancer is common in the world with an estimated 527,624 new cases and 275,008 deaths of the total cancer among females in 2012 (Ferlay et al., 2013). In Thailand, it is the second diagnosed in patients compared with other cancers (Wilailak, 2009). Human papillomavirus (HPV), especially the high risk HPV type 16 and 18, is associated with cervical carcinogenesis (zur Hausen, 1999). Cisplatin and 5-fluorouracil (5-FU) are used to treat cervical cancer but there are reports about their drug resistance (Shen et al., 2012) and side effects throughout the treatment (Sun et al., 2014). Low expression of Raf kinase inhibitory protein (RKIP) and high expression of ERCC1 mRNA resistance to cisplatin treatment in cervical cells were related with tumor progression, metastasis and drug resistance (Britten et al., 2000; Martinho et al., 2013). Therefore, many studies are

carried out to search for new potential anticancer agents from natural sources (Sak, 2012). It was reported that phytochemicals in vegetables and fruits such as flavonoids can reduce risk of cancer (Romagnolo and Selmin, 2012). Quercetin can induce cell-cycle arrest in HeLa cell line (Vidya et al., 2010). Gallic acid have potential to inhibit cancer cell progression via PI3K/Akt pathway (Zhao and Hu, 2013) and to induce apoptosis (Palasap et al, 2014).

Northeast Thai vegetables are commonly used in traditional medicine because their phytochemical contents play role as antioxidants (Stewart et al., 2013), anti-inflammatory (Siriwatanametanon et al., 2010), antimicrobial and anticancer agents (Daduang et al., 2011, Stewart et al., 2013). *Cratoxylum* (Teaw), which belongs to Guttiferae family, contains several bioactive constituents especially chlorogenic acid which has radical scavenging activity (Maisuthisakul et al., 2006). Formosumone A, toxyloxanthone B and vismione D

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isolated from 95% Teaw leaves ethanolic extract play role as anti-neuroinflammatory agents (Xiong et al., 2014). The anticancer potential of Teaw extracts was reported on HepG2 cell line by increasing caspase 3/7, 8, and 9 related apoptosis pathway (Nonpunya et al., 2014) and decreased the level of circular DNA of hepatitis B virus (HBV). *Cratoxylum formosum* subsp. *pruniflorum* (Kurz.) Gogel., Thai name “Teawdang” (Figure 1) is a subspecies of *Cratoxylum*. As there is a limited information about Teawdang and its effect on cervical cancer, therefore, this study aims to investigate the cytotoxic effect of Teawdang extracts on cervical cancer cell lines. Phenolic contents of Teawdang were also determined.

## Materials and Methods

### Chemicals and reagents

The organic solvents used for vegetable extraction, including hexane, ethyl acetate, and methanol were purchased from S.C. Science Co., Ltd. (Thailand). Methanol and phosphoric acid, HPLC grade, were purchased from RCI labscan (Thailand). Dimethyl sulfoxide (DMSO) was obtained from Amresco Inc. (USA). Catechin, quercetin, ferulic acid, rutin, caffeine, gallic acid and neutral red were obtained from Sigma-Aldrich Co. LLC (USA). Dulbecco’s Modified Eagle’s Medium (DMEM), bovine serum albumin (BSA), penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

### Preparation and extraction of vegetable materials

Teawdang was purchased from local markets in Khon Kaen province, Thailand during June to October 2013. The edible parts (stems and leaves) of Teawdang (2,870 g) were washed with distilled water and dried at 50°C in a hot air oven. Dried vegetable was grinded to fine powder (557 g) and extracted by soaking in one liter of hexane for 5 days at room temperature. Next it was filtered through Whatman® No. 4 paper and hexane was evaporated by using a rotary evaporator (Buchi, Switzerland). The residue obtained was “crude hexane extract (CHE)”. Then, the residue was further extracted with ethyl acetate followed by methanol respectively by the same procedure. “Crude ethyl acetate (CEE)” and “crude methanol extract (CME)” were obtained respectively. The residue left was macerated in deionized water for 5 days at room temperature. After that “crude water extract (CWE)” was obtained by removing water using a lyophilizer. All the extracts were stored at 4 °C for further analysis. The yield in percentage was calculated by using an equation below : % yield = (weight of extract after solvent evaporation/ weight of the vegetable powder) x 100

### High performance liquid chromatographic (HPLC) profile of crude extracts

The HPLC analysis of crude extracts was performed according to a method of Palasap et al (2014). Standard compounds including gallic acid, catechin, caffeine, caffeic acid, ferulic acid, resveratrol and quercetin and each crude extract were dissolved in methanol to make final concentration 1 mg/mL. Then 100 µL of each

sample was passed through a 0.22 µm pore size filter before injection to a column (C18 reversed phase, 5 µm particle size, 150x4.6 mm, Phenomenex). Mobile phase used was a step-gradient mixture of methanol and 0.5% phosphoric acid (methanol : 5% phosphoric acid ratio 5 : 95%, 70 : 30%, 90 : 10% and 5 : 95% at 0-17, 17-18, 18-20.5 and 20.5-25 min, respectively) with a flow rate of 1 mL/min. The compounds eluted were detected by using a spectrophotometer (Model 2489, Waters) at wavelength 270 nm. The retention time was recorded and peak areas of these compounds were calculated by using Clarity software.

### Cell culture

The cell lines used in this study were Vero (*Cercopithecus aethiops* kidney normal), HeLa (adenocarcinoma with HPV 18 positive), SiHa (squamous cell carcinoma grade II with HPV 16 positive) and C-33A (carcinoma with non HPV infection) cells. All cell lines were cultured in DMEM with 10% BSA and 1% penicillin-streptomycin.

### Cytotoxicity test

The cytotoxicity test of crude extracts on cervical cancer cell lines were performed in a 96-well plate. One hundred and sixty microliters of each suspension cell (8,000 cells/mL) was seeded into each well. Each crude extract (40 µL), diluted with 5%DMSO to make final concentration 50-400 µg/mL and filtered through a 0.22 µm pore size filter (Corning, USA), was added. After that, they were incubated at 37°C for 24 hrs under 5% carbon dioxide atmosphere. Negative control was 5%DMSO.

After incubation for 24 hrs, the existing media was removed and cell viability was detected by neutral red (NR) uptake assay. The pre-filtrate NR media (0.066 mg/mL) was passed through a 0.22 µm pore size filter and 200 µL of this solution was pipetted to each well in the plate. They were incubated for 3 hrs and then media was removed. Phosphate buffer saline (PBS) pH 7.4 was used for washing the cells for 2 times and 200 µL of lysis buffer (50% methanol and acetic acid at a ratio of 99:1) was added to break cell membrane. The absorbance at 540



Figure 1. *Cratoxylum formosum* Subsp. *Pruniflorum* (Kurz.) Gogel.

nm was recorded by using a plate reader (Rayto, China).

The half maximal inhibitory concentration of crude extract (IC<sub>50</sub>) against cervical cancer cell lines were calculated by plotting the percent of cell inhibition (as in an equation below) versus the crude extract concentrations (Machana et al., 2011).

$$\%inhibition = [(A_{control} - A_{test}) / A_{control}] \times 100$$

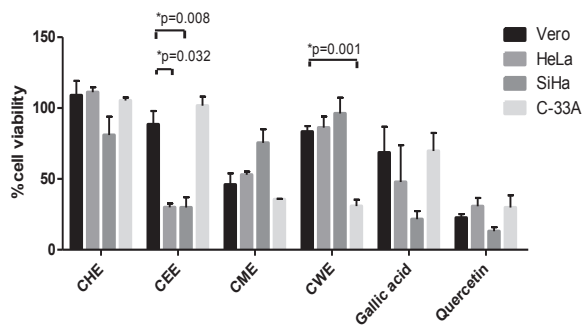
$A_{control}$  = Absorbance at 540 nm of well with negative control,  
 $A_{test}$  = Absorbance at 540 nm of well with crude extract

#### Statistic analysis

The results were expressed in mean±SD. Statistical significance was analyzed by one-way ANOVA using the SPSS software (version 17). P-values less than 0.05 (p<0.05) was considered statistically significant.

## Results

### High performance liquid chromatographic profile of crude extracts



**Figure 2. Cell Viability of Various Cervical Cancer Cell Lines, After Treated with Crude Teawdang Extracts (200 µg/mL), Compared to Vero Cell Line.** Data are expressed as mean±SD; \* = statistically significant (p<0.05)

**Table 1. Retention Time of Standard Phenolic Compounds and Percentage of Peak area of Teawdang Crude Extracts that Matched with Retention Time of Phenolic Compounds**

Standard compounds	RT (min)	CHE		CEE		CME		CWE	
		%PA	Conc.	%PA	Conc.	%PA	Conc.	%PA	Conc.
Gallic acid	7.45	ND	-	1.45	14.48	1.00	10.02	0.88	8.78
Catechin	11.06	ND	-	ND	-	ND	-	5.01	50.07
Caffeine	12.35	1.15	11.47	13.23	132.26	12.57	125.7	19.75	197.55
Caffeic acid	13.38	2.45	24.49	ND	-	ND	-	18.45	184.46
Ferulic acid	15.57	3.01	30.08	5.28	52.81	5.38	53.81	10.43	104.32
Resveratrol	17.02	2.86	28.63	7.29	72.97	7.61	76.08	4.08	40.82
Quercetin	19.59	ND	-	2.46	24.64	2.26	22.55	0.96	9.61

Conc. = concentration of phytochemicals (µg/mL); ND = no peak was detected; %PA = percent peak area; RT = retention time

**Table 2. IC<sub>50</sub> of Teawdang Crude Extracts and Standard Compounds on Cervical Cancer Cell Lines**

Crude extracts	IC <sub>50</sub> (µg/mL)			
	Vero	HeLa (HPV type 18 positive)	SiHa (HPV type 16 positive)	C-33A (non-HPV infection)
CHE	>400	>400	>400	>400
CEE	>400	143.18±13.35	106.45±15.73	>400
CME	151.67±42.33	208.32±5.21	338.06±25.28	107.74±3.45
CWE	>400	>400	>400	130.95±3.83
Gallic acid	270.05±77.69	131.57±32.62	42.69±5.02	250.27±53.30
Quercetin	143.23±6.87	49.25±20.16	87.62±15.70	46.25±12.95

IC<sub>50</sub> = the half maximal inhibitory concentration

The yield of CHE, CEE, CME and CWE were 5.49, 2.95, 5.33 and 3.37% respectively. Their HPLC retention time and percentage of peak area of standard phenolic compounds and crude extracts were shown in Table 1. The total peak area of CHE, CEE, CME and CWE were 14675.76, 36396.23, 39307.13 and 59008.64 mV·s respectively. Area under curve (AUC) was analyzed for phenolic concentration. The results demonstrate that all crude extracts contain caffeine, ferulic acid and resveratrol. Gallic acid was found in CEE, CME and CWE (14.48, 10.02, 8.78 µg/mL respectively) whereas catechin was found only in CWE (50.07 µg/mL). CEE and CME showed peaks that matched with the retention time of gallic acid, caffeine, ferulic acid, resveratrol and quercetin.

### Cytotoxicity of crude extracts on cervical cancer cell lines

In Table 2, CHE was not toxic to all the studied cell lines whereas CEE had cytotoxic effect on HeLa and SiHa. CME was toxic to all tested cell lines and its IC<sub>50</sub> for C-33A was lower than the other extracts. CWE exhibited cytotoxic only for C-33A. All extracts, except CME, were not toxic to Vero. Phytochemical compound including gallic acid and quercetin were cytotoxic to all cell lines with lower IC<sub>50</sub> for SiHa and HeLa than CEE and CME. The inhibitory efficacy of each crude extract (200 µg/mL) on cancer cells is shown in Figure 2. Compared to Vero cell, CEE can inhibit HeLa and SiHa cell lines significantly (p=0.032 and 0.008 respectively). For C-33A cell line, CWE significantly decreased the cell viability with slightly effect on Vero cell (p=0.001).

## Discussion

In Thailand, the average incidence of cervical cancer was about 16.7 per 100,000. There were 21.7, 16.4, 14.9

and 14.4 per 100,000 in the north, central, northeast and south of Thailand respectively. In the northeast area, Ubon ratchathani, Nakon Phanom and Khon Kaen were only 13.4, 13.8 and 15.1 per 100,000 respectively (Khuhaprema et al., 2013). As there is lower incidence rate of cervical cancer in northeast Thailand where Teawdang is normally consumed as side dish, therefore, phytochemicals in Teawdang and the other northeast Thai vegetables may involve in preventing and/or cytotoxic to cervical cancer.

In the present study, Teawdang was extracted by increasing polarity of solvents, from nonpolar (hexane) to high polar solvent (water). According to a previous study, gallic acid was soluble in many organic solvents used including methanol, ethyl acetate and water (Daneshfar et al., 2008). Hence, gallic acid was detected in CEE, CME and CWE in HPLC profile analysis (Table 1). Only catechin was found in CWE. This was similar to a previous report that catechin was found in aqueous extracts of *Cladogynos orientalis* (Machana et al., 2011). The high concentration of caffeine and caffeic acid were found in CWE followed by CME and CEE because of increasing solvent polarity. Caffeic acid can form ester with quinic acid to give a compound called “chlorogenic acid”, which has antioxidant activity (Xu et al., 2012). This result agreed with a report that Teaw ethnolic extract contained chlorogenic acid, ferulic acid and dicaffeoylquinic acid (Maisuthisakul et al., 2007). The highest resveratrol found in CME was similar to the *Gouania leptostachya* methanolic extract (Dung et al., 2014). As the extracted phenolic compounds are related to polarity of the solvent used, this implies that the edible part of Teawdang contains more polar phenolic compounds than non-polar compounds. On the other hand, Teaw root extracted with hexane had anthraquinones such as formoxanthone, xanthone, gerontoxanthone I and madagascin which had antibacterial activity (Boonsri et al., 2006; Lee et al., 2014).

Different phenolic content in each crude extracts may cause different effects on cervical cancer cells (Di Domenico et al., 2012). Both CME and CEE contained gallic acid. Gallic acid was also found in *Caesalpinia mimosoides* Lamk (Kaya) extracts which had cytotoxic effect to cervical cancer cell lines (Palasap et al., 2014). In the present study, CEE was cytotoxic to SiHa and HeLa, which are HPV-infected cell lines, respectively. The study of Waiyaput et al. (2012) that Teaw can decrease hepatitis B virus and it was cytotoxic to liver cancer cell lines (HepG2 cell) supported our results that Teawdang extracts were cytotoxic to HPV infected cell lines.

Among Teawdang extracts, CME showed the lowest IC50 on C-33A cells, which is HPV- non infected cell lines, but not Vero cells. For standard treatment of cervical cancer, it was reported that C-33A cells were the most response to combination of chemotherapy and radiation (Saxena et al., 2005) which may cause side effects to the patients. In conclusion, Teawdang extracts had cytotoxic effect on both HPV-infected and HPV-non infected cervical cancer cell lines. Purification and identification of active compounds from CME and CEE should be further investigated.

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