

RESEARCH ARTICLE

Cytotoxicity of *Cratoxylum Formosum* Subsp. *Pruniflorum* Gogel Extracts in Oral Cancer Cell Lines

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Abstract

Background: Oral cancer is a health problem in Thailand. *Cratoxylum formosum* subsp. *pruniflorum* Gogel (Teawdang), normally consumed in northeast Thailand, has proven cytotoxic to cervical cancer cell lines including HeLa, SiHa and C-33A. Recently, Asian oral cancer cell lines, ORL-48 and ORL-136, were established. Therefore, we aimed to study cytotoxicity of Teawdang in these. Total phenolic (TPC) and flavonoid content (TFC), and antioxidant activity of Teawdang were also determined. **Materials and Methods:** Teawdang was purchased from Khon Kaen market during June-October 2013. Hexane (CHE), ethyl acetate (CEE) and methanol (CME) extracts of its edible part were analyzed for TPC by the folin-ciocalteau method and for TFC by an aluminium colorimetric method. Antioxidant activity and cytotoxicity in normal Vero cells and oral cancer cells were investigated. Cell viability was assessed using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assays. **Results:** CME and CEE had higher TPC and TFC and antioxidant activity than CHE. Both CME and CEE, at 200 µg dry wt/mL, were cytotoxic to the studied oral cancer cell lines. However, CME was cytotoxic to Vero cells whereas CEE was not. Compared to Vero cells, CEE significantly inhibited ORL-48 and ORL-136 growth (p=0.03 and p=0.02, respectively). **Conclusions:** CEE exhibited cytotoxic effects on the studied oral cancer cell lines but not normal Vero cells. The bioactive compounds in CEE should be further purified and elucidated for their mechanisms of action for development as anticancer agents.

Keywords: Antioxidant - cytotoxicity - *Cratoxylum formosum* subsp. *pruniflorum* Gogel - oral cancer cell line

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Introduction

Oral cancer becomes a health problem worldwide because of the increasing patient number each year. In 2012, there were about 67,000 new cases in Southeast Asian countries (Ferlay et al., 2013). In northeast Thailand, the incidence of oral cancer is the highest in country (Khuhaprema et al., 2010) and its incidence rates are increasing in female (Vatanasapt et al., 2011). The risk factors are tobacco smoking, alcohol drinking (Sturgis et al., 2004) and oral human papilloma virus (HPV) infection (Gillison et al., 2008). Oral sex behavior might cause HPV transmission (Heck et al., 2010). Oral carcinogenesis occurs from the imbalance between antioxidant and free radicals caused by oxidative stress (Patel et al., 2008). The molecular mechanisms of anticancer agents are induction of apoptosis and suppression of cancer cell cycle (Surh, 2003). A previous study reported side effects of several anticancer drugs, including neurotoxicity and electrolyte

disturbance (Dzagnidze et al., 2007). Moreover, multidrug resistance has been reported in oral cancer patients through the overexpression of drug efflux transporters induced from radiotherapy at plasma membrane (Ng et al., 1998; Perez-Sayans et al., 2010). At present, natural products have been focus as a potential source of anticancer agents.

Epidemiological studies reveal that daily consumption of vegetables can reduce cancer (Chen et al., 2014; Wang et al., 2014). The extracts of some Thai vegetables could effectively induce apoptosis in oral cancer (Manosroi et al., 2015) and cervical cancer cells (Palasap et al., 2014). Recently, *Cratoxylum formosum* subsp. *pruniflorum* Gogel., which its Thai name is Teawdang, has become an attractive functional food due to its phytochemical composition (Nonpunya et al., 2014). It is consumed in northeast Thailand as side dishes to relieve fever and stomach ache. In south and southwest China, it is used as ordinary tea (Xiong et al., 2014). The ethanolic extract of Teawdang leave contained toxyloxanthone B and

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vismione D which have anti-neuroinflammatory activity (Xiong et al., 2014). Teaw contained chlorogenic acid (Maisuthisakul et al., 2006) which had high antioxidant activity and high cytotoxic effect to oral squamous cell carcinoma (HSC-2 cell line) (Jiang et al., 2000). Teawdang also had *in vitro* cytotoxicity on liver cancer cell lines (HepG2) by effectively reducing hepatitis B virus (Waiyaput et al., 2012; Nonpunya et al., 2014). Recently, the Asian oral cancer cell lines, ORL-48 and ORL-136, were established as *in vitro* model for studying a disease prevalent in Asia (Hamid et al., 2007). We hypothesized that Teawdang may be cytotoxic to these oral cancer cell lines. Therefore, we aim to investigate cytotoxic effect of Teawdang extracts on these oral cancer cell lines. The phenolic content and antioxidant activity were also determined.

Materials and Methods

Chemicals and reagents

The organic solvents including hexane, ethyl acetate, and methanol were purchased from S.C. Science (Thailand). Phosphoric acid and hydrochloric acid (HCl) were obtained from RCI labscan (Thailand). Dimethyl sulfoxide (DMSO) was supplied from Amresco Inc. (USA). 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrocortisone solution were obtained from Sigma-Aldrich (Missouri, USA). Iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) was purchased from AnalaR (England). Dulbecco's Modified Eagle Medium (DMEM), Ham F'12 and bovine serum albumin (BSA) were obtained from Gibco BRL (New York, USA).

Preparation of Teawdang

Teawdang was purchased from local markets in Khon Kaen province, Thailand during June to October 2013. Its edible part was cleaned with distilled water and dried in a hot air oven at 50°C till its dried. The dried Teawdang was ground to fine powder.

Extraction of Teawdang

Teawdang powder was macerated in 1 liter of hexane for 5 days. After filtration through Whatman No. 1 filter paper, the residue was re-extracted by the same procedure with ethyl acetate and methanol respectively. The organic solvents were removed by using a rotary evaporator (Buchi, Japan). Then, crude hexane, ethyl acetate, and methanol extracts (CHE, CEE and CME respectively) were kept at 4°C for further analysis.

Determination of total phenolic content (TPC)

Total phenolic content of crude extracts were determined according to a modified method of Daduang et al (2011). All crude extracts were dissolved in 5% DMSO and final concentration was adjusted to 1 mg/mL. One hundred microliters of the crude extract was mixed with 500 μL of 0.2 N Folin-Ciocalteu reagent and incubated in the dark for 30 min. Then 400 μL of 7% Na_2SO_4 was added. After standing in the dark for 5 min, the absorbance at 750 nm was recorded by using a spectrometer (Genesys 20, Thermo scientific, USA). Gallic acid, at a concentration range 10-100 $\mu\text{g}/\text{mL}$, was used for preparation of a

calibration curve. The concentration of total phenolic was expressed as milligram of gallic acid equivalent per gram dry weight (mg GAE/g dry wt.). The experiment was carried out in triplicate.

Determination of total flavonoid content (TFC)

The determination of total flavonoid content of crude extracts was performed by using a modified method of Patel et al (2010). Two hundred and fifty microliters of crude extracts were mixed with 75 μL of 5% NaNO_2 . After standing for 5 min, 150 μL of 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was added into the mixture and left for another 6 min. Five hundred μL of 1 M NaOH was added and total volume was adjusted to 2 mL with distilled water and incubated at room temperature for 30 min. The absorbance at 415 nm was read. A blank and standard compound were distilled water and quercetin (concentration range 50-800 $\mu\text{g}/\text{mL}$) respectively. Total flavonoid content was reported as milligram of quercetin equivalent per 1 g dry weight (mg QE/g dry wt.). All measurements were carried out in triplicate.

Ferric reducing antioxidant power (FRAP) assay

The determination of total antioxidant activity by FRAP method was performed by using a modified method of Benzie and Strain (1996). FRAP reagent was freshly prepared by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ at a ratio of 10:1:1. One milliliter of the crude extracts was dissolved in 5% DMSO and 1 mL of FRAP reagent was added and left at room temperature for 5 min. The absorbance at 593 nm was recorded. A calibration curve was prepared by using standard gallic acid at various concentrations (5-50 $\mu\text{g}/\text{mL}$). This assay was performed in triplicate.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

DPPH radical scavenging ability was analyzed by a modified method of Clarke et al (2013). In a 96-well plate, 120 μL of crude extract and 120 μL of 100 μM DPPH reagent were mixed in each well. The mixture was vigorously shaking for 2 min and left for 30 min at room temperature with light protection. After that the absorbance was measured at 540 nm by using a microplate reader (Tecan, France). All measurement was carried out in triplicate. The percentage inhibition activity was calculated as below:

$$\% \text{DPPH inhibition} = [(A^0 - A^1) / A^0] \times 100$$

A^0 = the absorbance of DPPH reagent with 120 μL of distilled water

A^1 = the absorbance of DPPH reagent with 120 μL of standard gallic acid or the extract

Cell lines and culture condition

The cell lines used in this study were Vero cells (*Cercopithecus aethiops* normal kidney) which obtained from Faculty of Associated Medical Sciences, Khon Kaen University and two oral cancer cell lines including ORL-48 and ORL-136 which obtained from the Cancer Research Initiatives Foundation (CARIF, Malaysia). All cell lines were cultured in the mixture of DMEM and

Table 1. Total Phenolic Content, Total Flavonoid Content and Antioxidant Activity of Teawdang Extracts

Extracts	TPC (mg GAE/g dry wt)	TFC (mg QE/g dry wt)	FRAP (mg GAE/g dry wt)	%DPPH inhibition*
CHE	26.16±1.55	53.67±17.79	8.30±1.66	32.63
CEE	83.93±6.06	238.67±55.64	241.59±10.15	50.79
CME	161.63±12.85	495.00±55.49	435.25±15.87	60.23

TPC, TFC and FRAP values are expressed as mean ± SD, (n = 3); * = tested concentration at 1 mg dry wt /mL

Ham F¹² at the ratio 1:1 with 10% BSA, 1% penicillin-streptomycin and hydrocortisone solution (1.16 mL/40 mL complete medium).

Cytotoxicity test

The cytotoxicity of Teawdang extracts on Vero and oral cancer cell lines were performed in a 96 well plate. One hundred microliter of resuspend cells (2×10^5 cells/mL) were seeded into each well and allowed to attach the plate at 37°C in 5% carbondioxide atmosphere for 24 hrs. Then 100 µL of each crude extract (final concentration 50-400 µg dry wt/mL), which were diluted with 1%DMSO and filtrated with 0.22 µm pore size filter (Agela Technologies, USA), was added to the cells. After that, they were incubated for 24 hrs. Cell treated with 1%DMSO was negative control.

Cell viability assay

After incubation for 24 hrs, cell viability was detected by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mahavorasirikul et al., 2010). Cells were treated with 5 mg/mL MTT reagent (50 µL/well) for 4 hrs at 37°C and then 200 µL of 1x phosphate buffer saline (PBS) pH 7.4 was added to each well to dissolve the formazan crystals. The absorbance was recorded at 492 nm by using a microplate reader (Tecan, France). Percentage of residual cell inhibition was calculated by a formula below. Cell viability >50% at 400 µg dry wt/mL was considered as non-cytotoxic.

$$\% \text{Cell viability} = [(A^{\text{sample}} - A^{\text{control}}) / A^{\text{of cell without treatment}}] \times 100$$

(A^{control} = absorbance at 492 nm of well with 100 µL 1% DMSO,

A^{sample} = absorbance at 492 nm of well with crude extract)

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Cell viability was analyzed (one way ANOVA) by using SPSS windows version 17. Values of $p < 0.05$ was considered as statistically significant.

Results

Total phenolic and total flavonoid content

Total phenolic and total flavonoid content of CHE, CEE and CME are shown in Table 1. The highest content of TPC and TFC was found in CME. Among all extracts, TPC were well correlated with TFC of the extracts ($r=0.99$).

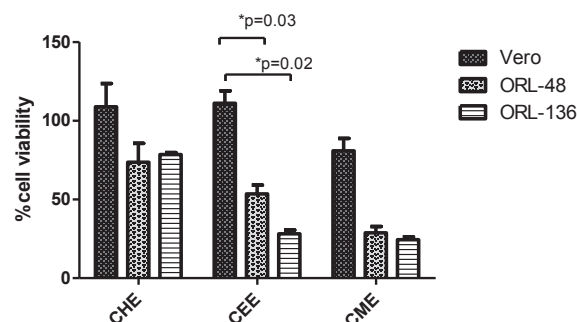
Antioxidant activity of Teawdang extracts

CME had the highest reducing power (435.25±15.87 mg GAE/g dry wt) follow by CEE and CHE respectively

Table 2. Cytotoxicity of Teawdang Extracts on Oral Cancer Cell Lines

Extracts	IC50 value (µg dry wt/mL)		
	Vero	ORL-48	ORL-136
CHE	>400	>400	>400
CEE	>400	290.27±13.85	167.43±11.67
CME	192.62±41.67	209.20±8.14	44.82±27.95

The IC50 values are shown as mean ± SD (n=3), IC50 > 400 µg dry wt/mL was non cytotoxic

**Figure 1. Cell Viability of Vero, ORL-48 and ORL-136 after Treated with Teawdang Extracts (200 µg dry wt/mL)**

(Table 1). At the same concentration (1 mg dry wt/mL), CME showed higher %DPPH inhibition than CEE and CHE. In addition, DPPH radical scavenging activity of CME was higher than 1 mg/mL gallic acid (60.23 vs 57.49 %). There was a relationship between DPPH radical scavenging activity and ferric reducing power ($r=0.98$).

Cytotoxic effect of Teawdang extracts on Vero and oral cancer cell lines

In Table 2, CME and CEE could inhibit ORL-48 and ORL-148 cells whereas CHE was non-cytotoxic to all tested cell lines. Although CEE had higher IC50 than CME for both oral cancer cell lines, it was not toxic to Vero cell lines. Cell viability of the studied cell lines treated with CME, CEE and CME was shown in Figure 1. When CEE was used to treat these cell lines, there were statistically significant of %cell viability between Vero cell and oral cancer cell lines ($p=0.03$ for ORL-48 and $p=0.02$ for ORL-136).

Discussion

Northeast Thai vegetables have potential in prevention of chronic diseases including cardiovascular diseases (Kukongviriyapan et al., 2007) and cancer (Nonpunya et al., 2014). Thai medicinal plants such as *Gloriosa superba*

L. (Colchicinaeae) and *Albizia chinensis* (Osbeck) Merr. were reported to have *in vitro* anti-oral cancer potential (Manosroi A et al., 2015). Antioxidant activity of the vegetables is one factor involving in prevention/treatment of these diseases. It was reported that the ethanolic extract of Teaw leave, which contained chlorogenic acid, had high antioxidant activity (Maisuthisakul et al., 2007). In the present study, CME had the highest antioxidant activity which related to their phenolic and flavonoid contents (Table 1). The correlation between TPC and FRAP ($r=0.98$), TPC and DPPH radical scavenging of the extracts ($r=0.92$) were agreed with previous studies (Ramkissoon et al., 2013; Sebai, 2013). TFC also had correlation with both FRAP and DPPH assays ($r=0.92$).

More than 90% of oral cancer is squamous cell carcinoma (Pathak et al., 2014). ORL-48 and ORL-136 cell lines were established from oral squamous cell carcinoma of Asian subjects (Hamid et al., 2007). Both of them were non HPV infection. Characterization of ORL-48 showed the overexpression of MDM2 which is a negative regulator of tumor suppressor gene (p53), whereas overexpressed EGFR, involve in cell proliferation and inhibit of apoptosis, were observed in ORL-136 cells (Hamid et al., 2007; Zanaruddin et al., 2013). A previous study reported that *Brucea* spp. had strong cytotoxic effect on ORL-48 cell line (Majid et al., 2014). In the present study, CME had IC50 value for ORL-48 ($209.20 \pm 8.14 \mu\text{g dry wt/mL}$) higher than ORL-136 ($44.82 \pm 27.95 \mu\text{g dry wt/mL}$) (Table 2). However, cytotoxicity of CME on Vero cell line is a limitation of using this extract as anticancer agent. Cytotoxicity of CEE on oral cancer cells, but not on normal Vero cells, indicated that CEE is a candidate source of anti oral cancer agents. Most phenolics play role as anticancer agents (Domenico et al., 2012). Potential anticancer activities of Teaw on MCF-7 (Woraratphoka et al, 2012) and HepG2 cells (Nonpunya et al., 2014) were reported. Teaw could inhibit HepG2 cell lines by activation of p53 protein, down-regulation of NF- κ B and cyclin D1 proteins and activation of caspase cascade pathway (Waiyaput et al., 2012; Issara-Amphorn and T-Thienprasert, 2014; Nonpunya et al., 2014). The phenolics compound in Teawdang extracts might induce apoptosis in ORL-136 through p53-dependent and p53 independent (Kang and Jang., 2012). However, the lower expression of p53 in ORL-48 might lead to activation of the other proteins at late stage of apoptosis. From our previous report, CEE and CME had cytotoxic effect on both HPV-infected (HeLa and SiHa) and HPV-non infected (C-33A) cervical cancer cell lines. CEE contained gallic acid, resveratrol and quercetin (Promraksa et al., 2015). Gallic acid and quercetin can inhibit migration and invasion of oral squamous cell carcinoma, SCC-4 and SAS, through inhibition of NF- κ B and matrix metalloproteinase-2 and -9 (Lai et al., 2013; Kuo et al., 2014). Resveratrol was reported to suppress cell growth and KB human oral cancer cells (Kim et al., 2011). Further purification and of active compounds from CEE and elucidating of their anticancer mechanism should be done to develop a novel anti oral cancer agents.

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