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# *Cratoxylum formosum* ssp. *pruniflorum* activates the TRAIL death receptor complex and inhibits topoisomerase I

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ABSTRACT

Six species of *Cratoxylum* are found in Thailand. Whether the *Cratoxylum formosum* ssp. *pruniflorum* (CFP) has anticancer properties requires investigation. CFP exhibited cytotoxicity against hepatocellular carcinoma HepG2 cells. Based on FTIR microspectroscopy, CFP raised the respective lipid and nucleic acid content and β-pleated sheet in HepG2 cells, suggesting a change in the secondary protein structure. CFP induced apoptosis by increasing the activities of various caspases. Overexpression of TRAILR2 indicated the extrinsic pathway while expression of Bax/Bcl-2 ratio indicated the intrinsic pathway and decreased expression of Bid indicated cross-talk between the two. CFP alkylated the DNA and caused DNA damage, which may be the initial step in the intrinsic pathway. CFP indirectly and directly inhibited Top IB enzyme activity and decreased PARP—the protein-related DNA repair process. CFP could, thus, protect the resistance mechanism of cancer by reversing the effect of Top as confirmed by apoptosis induction in the resistant HepG2 cells. The phytochemical analysis suggested that the compounds playing an important role in the anticancer activity of CFP are a group of xanthones.



## 1. Introduction

*Cratoxylum formosum* ssp. *pruniflorum* belongs to the family of Guttiferae, which is found throughout Southeast Asia (Boonnak et al., 2006). Six species are found in Thailand (Boonnak et al., 2006). Two subspecies (ssp.) of *C. formosum*—ssp. *formosum* and ssp. *pruniflorum*—are mistakenly mismatched due to their similarity and close Thai names. Ssp. *formosum* (CFF) is called Tiew khao in Thai, while spp. *pruniflorum* (CFP) is called Tiew kon. The more edible, *Cratoxylum* is ssp. *formosum* (CFF), is eaten as a side dish due to its sour taste. The edible part of CFF comprises fresh young leaves and these are commonly available in local markets in northeastern Thailand. By contrast, CFP is not so palatable because it has tiny hairy leaves and is astringent.

*Cratoxylum* genus has long been used in traditional medicine for relieving several diseases (Boonnak et al., 2006). Traditional use of bark or leaves of CFF is as a tonic, stomachic, diuretic (Grosvenor et al., 1995a, 1995b; Waiyaput et al., 2012), diarrhea, flatulence (Anderson, 1986), food poisoning, internal bleeding (Grosvenor et al., 1995a, 1995b) while the roots and leaves are used for relieving liver cirrhosis (Waiyaput et al., 2012; Issara-Amphorn and T-Thienprasert, 2014). The leaves of CFP are used for heat and thirst quenching, detoxifying, preventing obesity, reducing blood fat, and lowering blood pressure (Xiong et al., 2014). In addition, CFP has long been used to prepare infusions (viz., "Ku Ding tea") by Southern and Southwestern Chinese despite its bitter taste (Xiong et al., 2014).

Several biological activities of both subspecies have been reported but taxonomic authentication was not always provided. CFF was reported to possess anti-oxidant activity (Maisuthisakul et al., 2006; Kukongviriyapan et al., 2007; Waiyaput et al., 2012), anti-bacterial (Boonsri et al., 2006), and cytotoxic effect on MCF-7 (breast adenocarcinoma), HeLa and Caski (human cervical cancer) (Kuete et al., 2011), KB (human oral cancer), and HT-29 (colon cancer) (Boonsri et al., 2006). Ethanolic extract of CFF leaves showed cytotoxicity against HepG2 hepatocellular carcinoma cells (Prayong et al., 2008), a gastroprotective effect (Sripanidkulchai et al., 2010), and an inhibitory effect on HBV

*Abbreviations:* Bax, Bcl-2 associated X protein; Bcl-2, B cells lymphoma-2 protein; Bid, Bcl-2 homology interacting domain death against; CFP, *Cratoxylum formosum* (Jack.) Dyer ssp. *pruniflorum* (Kurz) Gogel; DISC, Death inducing signaling complex; FADD, Fas associated death domain; FTIR, Fourier transform infrared; GC–MS, Gas chromatography–mass spectrometry; HCC, Human hepatocellular carcinoma; HepG2, Hepatocellular carcinoma cell lines; PARP, Poly-ADP ribose polymerase; PVDF, Polyvinylidene difluoride; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; tBid, Truncated Bid; Top IB, Topoisomerase IB enzyme; TRAIL-R2, Tumor necrosis factor related apoptosis inducing ligand -receptor 2; Vero, African green monkey kidney cells.

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replication (Waiyaput et al., 2012). Xanthone V1 from CFF showed cytotoxicity against the Hela, Caski, MCF-7, PF-382 cell lines, were anti-angiogenesis, and arrested the cell cycle in the S phase (Kuete et al., 2011). Meanwhile, CFP was reported to have an anti-microbial effect (Boonnak et al., 2006; Kuvatanasuchati et al., 2011). The combination of two pure compounds isolated from the roots of CFP—macluraxanthone and garcinone B—showed a synergistic effect against both gram positive and gram negative bacteria (Boonnak et al., 2010). Compounds separated from CFP stem—i.e., pruniflorone Q, pruniflorone R, 1,7-dihydroxy-8-methoxyxanthone, 1,4,7-trihydroxy-8-methoxyxanthone, 1,7-dihydroxy-4-methoxyxanthone, 1,3,6-trihydroxy-7-methoxyxanthone, dulcisxanthone B, cudratricusxanthone E, cochinchinone B—were reported to inhibit the retinoid receptor RXR, which is related to the transcription factor associated with gene expression (Duan et al., 2010, 2011). In addition, xanthones from CFP leaves (i.e., toxyloxanthone B and vismione D) showed an anti-inflammatory effect through inhibition of NO production (Boonnak et al., 2014; Xiong et al., 2014) while toxyloxanthone B showed a significant neuroprotective effect against β-amyloid in neuroblastoma cells (Xiong et al., 2014). Hydroethanolic (50%) extract of CFP twig delayed amyloid-β induced paralysis in *C. elegans*, providing useful information for developing an anti-Alzheimer candidate (Keowkase and Weerapreeyakul, 2016).

According to the literature review, anticancer activity of both CFF and CFP has been reported. Our group previously reported the anticancer potential of 50% hydroethanolic extract from both CFF leaves (Prayong et al., 2008) and CFP twigs (Nonpunya et al., 2014). A polar solvent was selected for the extraction to emulate a decoction. CFP extract induced apoptosis in U937 leukemia cells as determined by FTIR microspectroscopy (Machana et al., 2012) and induced caspases-mediated apoptosis in HepG2 cells (Nonpunya et al., 2014). CFP extract has been shown to be a good candidate for anticancer activity in HepG2 cells; however, the detailed cellular effect of CFP on these cancer cells is lacking. Hepatocellular carcinoma (HCC) was the focus of the current study due to its high mortality rate among people in the Asia-Pacific region (Zhu et al., 2016). HCC patients were reported to have a poor prognosis due to a resistance mechanism by HCC against anticancer drugs and the limited choice of treatments because of the often advanced stage of the disease at diagnosis (Issara-Amphorn and T-Thienprasert, 2014). An alternative treatment (or the development of a new source of anticancer agents) was therefore needed. In the current study, we aimed to investigate the mechanisms of anticancer action against HCC. One useful technique for monitoring biological alterations and predicting the structure of molecules (such as proteins) is Fourier transformation infrared (FTIR) microspectroscopy (Srisayam et al., 2014; Junhom et al., 2016; Siriwarin and Weerapreeyakul, 2016). FTIR microspectroscopy was used to evaluate the biomolecular changes related to the IR spectra absorption in CFP extract-treated HepG2 cells.

## 2. Materials and methods

### 2.1. Materials

Human hepatocellular carcinoma cells (HepG2) (ATCC#8065), and African green monkey kidney (Vero) (ATCC#CCL-81) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The reagents used in the cell culture were biological grade and obtained from GIBCO, Invitrogen Corporation (Grand Island, NY, USA). Neutral red, melphalan, and 5-FU were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The BCA assay kit was obtained from Pierce Biotechnology (Rockford, IL, USA). The primary antibodies—Bax, Bcl-2, TRAILR, PARP, Bid and β-actin—were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The goat anti-mouse IgG1 heavy chain (horseradish peroxidase) was purchased from Abcam (Cambridge, UK). The protease inhibitor cocktail was purchased from Amresco® (solon, OH, USA). The Apoalert™ caspase fluorescent assay kit was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). The enhanced chemiluminescence (ECL) reagent was bought from GE Healthcare UK Limited (Buckinghamshire, UK). FITC-conjugated annexin V and Propidium iodide (PI) were purchased from eBioscience (San Diego, CA, USA). The purified topoisomerase IB (Top IB) enzyme was provided by Professor Alessandro Desideri, Department of Molecular Biology, Faculty of Science, University of Rome Tor Vergata, Italy. The reagents for the NP-40 buffer (NaCl, TritonX, Tris and EDTA) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany).

### 2.2. Plant extraction

Twigs of CFP were collected in northeastern Thailand in 2013. The herbarium collection (voucher specimen TTOC-SK-862) was kept at the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. A 50% hydro-ethanolic crude extract was prepared as per Nonpunya et al. (2014).

### 2.3. Phytochemical analysis

The major phytoconstituents—tannins, xanthones, terpenoids, saponins, steroids, alkaloids, and glycosides—in 0.05 g of CFP extract were identified as per previous reports with some modification (Ukoha et al., 2011; Manosroi et al., 2012; Gangwar et al., 2014). Briefly, the CFP extract (0.05 g) was dissolved in 2 mL of ethanol, incubated in a water bath (40 °C) for 10 min, centrifuged at 268.3 × *g* for 10 min, and the supernatant collected. For tannin, 2 mL of 15% $FeCl_3$ was added and a dark green or blue-black precipitate indicated the presence of tannin. Xanthones were indicated by a yellow precipitate, after adding 100 μL of 5% KOH to the supernatant (Manosroi et al., 2012). For terpenoids, CFP extract was dissolved in 2 mL of chloroform and dried in a water bath. After that, 2 mL of concentrated sulfuric acid was added and heated for another 2 min. A dark-gray color indicated the presence of terpenoids (Ukoha et al., 2011). While steroids were indicated after dropping concentrated sulfuric acid sidewise into CFP dissolved in chloroform. The red formation in the chloroform layer indicated the presence of steroids (Gangwar et al., 2014). Saponins were indicated by permanent foam at RT after CFP was dissolved in deionized water for 1 h and shaken vigorously for 30 min (Ukoha et al., 2011). CFP extract was dissolved in 1 mL of methanol and filtered. The supernatant was kept and 2 mL of 1% HCl added, followed by 1 drop of Dragendorff's reagent (potassium bismuth iodide). A reddish-brown precipitate with turbidity indicated the presence of alkaloids (Ukoha et al., 2011). Glycosides were indicated by Fehling reagent mixture (Fehling A: Fehling B = 1:1), as per Manosroi et al. (2012); and Ukoha et al. (2011). Fehling's solution A was a mixture of 3.5 g of copper II sulfate in 50 mL deionized water; while Fehling's solution B comprised 5 g sodium hydroxide, 17.5 g potassium sodium tartrate and 50 mL of deionized water. The CFP extract was dissolved in 1 mL of deionized water then heated with 1 mL of Fehling reagent mixture for 10 min. A brick-red precipitate indicated the presence of reducing sugar in the glycosides.

### 2.4. Standardization and phytochemicals analysis of 50% hydroethanolic extract of CFP extract

GC–MS analysis was performed as previously described (Weerapreeyakul et al., 2016) on an Agilent 6890 N gas chromatograph (Agilent Technologies, Shanghai, China) coupled to an Agilent 5973 N mass selective detector (Agilent Technologies, Shanghai, USA) to determine the extract composition for further standardization. Capillary GC analysis was performed using a DB-5 ms (3 m × 0.25 mm id, 0.25 μm) capillary column from Agilent Technologies (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas. The column initially flowed at 80 °C for 6 min at a rate of 2 mL/min and an average velocity of 52 cm/s. The temperature was increased to 280 °C (at a rate of 5 °C/min) for 24 min. The total run-time was 70 min. The injector temperature was maintained at 250 °C and the injection volume was 2.0 μL in the splitless