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Citrus sinensis Peel Extract Synergistically Enhances the Cytotoxic Effect of Chemotherapeutic Agents on HepG2 Cells

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Abstract

Doxorubicin (DOX) and cisplatin (Cis), non-specific chemotherapeutic agents used for hepatocellular carcinoma (HCC), are frequently combined with synthetic or natural agents to enhance their cytotoxic effects. *Citrus sinensis* peel extract (CPE) serves as a natural source of flavonoids, including sinensetin (SIN), which has the potential to increase the efficacy of DOX and Cis. This study aimed to observe the effect of CPE and SIN one of CPE compounds, in enhancing liver cancer cell susceptibility to doxorubicin and cisplatin. The assays conducted in this study included a phytochemical analysis of CPE using TLC, cell viability assays against HepG2 cells using MTT assay in both single and combination forms, and cell viability assays on Vero cells. The result confirmed the presence of SIN as one of the compounds in CPE. Both CPE and SIN, when used individually, exhibited moderate cytotoxic effects on HepG2 cells with IC_{50} of 101.09 µg/mL and 83.13 µM, respectively, while showing no cytotoxic effect on Vero cells. Cis demonstrated significant cytotoxicity against HepG2 cells with an IC_{50} of 7.86 µM. DOX exerted a strong cytotoxic effect on both HepG2 and Vero cells, with the IC_{50} of 2.52 µM and 13.98 µM. It was observed that CPE was able to synergistically enhance the cytotoxic effects of DOX, and SIN synergistically increased the cytotoxicity of Cis, particularly against HepG2 cells, with CI<1.0.

Keywords: *CPE, SIN, Cisplatin, Doxorubicin, HCC.*

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INTRODUCTION

Hepatocellular carcinoma is a type of liver cancer with leading mortality due to its aggressiveness and the less effective chemotherapy (Cox and Weinman, 2016). Doxorubicin (DOX) and Cisplatin (Cis) are standard chemotherapeutic agents for various types of cancer, including hepatocellular carcinoma (Li, *et al.*, 2023; Cox and Weinman, 2016) that can induce double-strand breaks (DSBs) in cancer cells, leading them to undergo apoptosis (Chen, *et al*., 2022; van der Zanden, *et al*., 2021; Mizutani, *et al*., 2005). However, the use of DOX and Cis as anticancer agents can generate free radicals that promote DNA damage in normal cells, resulting in some side effects such as nephrotoxicity and cardiotoxicity, while also reducing the specificity of the chemotherapeutic agents (Haryanti, *et al*., 2022; Salsabila, *et al*., 2021; Makovec, 2019). These issues highlighted the urge to utilize different agents in combination with DOX or Cis to enhance their efficacy and inhibit side effects.

Several natural agents and compounds are known for their cytotoxic activity against cancer cells, which can enhance the effects of standard chemotherapeutic drugs (Handayani, *et al*., 2022; Anwanwan, *et al*., 2020). They are also recognized for their antioxidant capacity, countering ROS, and attenuating the side effects on normal cells in various organs (Haryanti, *et al*., 2022). For instance, *Annona muricata* leaf extract exhibited potent cytotoxicity and synergism with DOX in 4T1, but the cytotoxic effect was not due to ROS induction and senescence (Salsabila, *et al*., 2021). Citronella and lemongrass oil were known to have a low cytotoxic effects on normal cells (NIH-3T3 and Vero) and inhibited DOX-induced senescence by reducing ROS levels in these cells (Salsabila, *et al*., 2023). Several citrus flavonoids have also been explored to increase the cytotoxic effects of chemotherapeutics agents against several types of cancers (Meiyanto, *et al*., 2012). Hesperidin, for example, enhanced DOX to synergistically kill the 4T1 cells in relation to cell cycle arrest and ROS generation (Amalina, *et al*., 2023), while hesperetin and naringenin show synergistic effects with DOX on T47D and MCF-7 cells (Sarmoko, *et al*., 2014; Fitriasari, *et al*., 2010; Junedi, *et al*., 2010). Interestingly, almost all the phyto-flavonoids exhibit an antioxidant capacity, reducing ROS production in normal cells (Meiyanto, *et al*., 2012). However, the use of pure compounds of natural origin was costly and less effective, prompting the search for more affordable materials with increased effectiveness as co-chemotherapeutic agents.

Citrus sinensis peel is acknowledged for its significant cytotoxic and antioxidant effects (Tajaldini, *et al*., 2020; Ikawati, *et al*., 2019; Meiyanto, *et al*., 2011). The peel harbors various compounds, such as sinensetin (SIN), a polymethoxyflavonoid, known for its anticancer and cytoprotective properties (Li, *et al.*, 2023; Hermawan and Putri, 2021; Kim, *et al.*, 2020; Samidurai, *et al*., 2020). In this study, *Citrus sinensis* peel extract (CPE) or SIN was combined with DOX or Cis to examine the potential of CPE in enhancing the therapeutic efficacy of DOX or Cis on HepG2 cells, representing hepatocellular carcinoma (HCC).

MATERIALS AND METHODS

Ethical Concern

All of the *in vitro* assay in this study was approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing of Universitas Gadjah Mada under the reference number KE/FK/1421/EC/2023.

Materials and Cell Lines

Citrus sinensis peel obtained from the local market in Yogyakarta-Indonesia underwent determination at the Pharmacognosy Laboratory, Pharmaceutical Biology Department, Faculty of Pharmacy, Universitas Gadjah Mada,

under the entry number 51.14.8/UN1/FFA.2/BF/PT/2023. Sinensetin was procured from Sigma, St. Louis, Missouri, USA, while Doxorubicin and Cipslatin were purchased from Wako, Japan. HepG2 and Vero cells were sourced from the Cancer Chemopreventive Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia.

Preparation of Citrus Peel Extract

Citrus peel was cleaned, dried in the oven at 60°C for 6 h, ground, and then passed through a 60-mesh sieve to produce citrus peel powder. The CPE was generated by macerating the citrus peel powder with 70% ethanol at a 1:10 ratio, stirring for 24 h, followed by centrifugation, and evaporation until a brown viscous extract was obtained.

Phytochemical Analysis

The phytochemical analysis was performed using the thin-layer chromatography (TLC) method as previously described with slight modification (Kartini, *et al*., 2023). CPE was applied at a concentration of 50,000 ppm, while sinensetin, serving as the standard compound, was applied at a concentration of 20,000 ppm. These samples were spotted 1 cm above the longitudinal side of 12x3 cm silica gel plate F254 (Merck, Darmstadt, Germany). Elution was carried out using a mixture of toluene and ethyl acetate in 5:7 ratio, and the results were observed under visible light, UV light at 254 nm, and 366 nm.

Cell Line Culture

The HepG2 and Vero cells were obtained from the CCRC, Faculty of Pharmacy, Universitas Gadjah Mada. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). The cells were then incubated under standard conditions at 37°C and 5% CO_2 until they reached confluence.

Cytotoxicity Assay

The cytotoxic assay was conducted using MTT, as in previous studies (Ikawati, *et al*., 2019). HepG2 cells ($5x10^3$ cells/well) and Vero cells (10^4 cells/well) were cultured a 96-well plate with a complete medium for 48 h. The medium was replaced with various concentrations of CPE, SIN (Sigma), Cis (Wako), and DOX (Wako) either alone or in combination, followed by a 24 h incubation period, with each concentration tested in triplicate. 0.5 mg/mL of 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, Eschenstraße, Taufkirchen, Germany) in phosphate buffer saline (PBS) was added and incubated for 4 h. Control wells contained untreated cells, whereas blank wells had no cells. A stopper comprising 10% SDS (Sigma Aldrich) in 0,01 N HCl (Sigma Aldrich) was added to dilute the formed formazan, allowing measurement using a microplate reader at a wavelength of 595 nm. The absorbance data were then utilized to determine the IC_{50} values for CPE, SIN, Cis, and DOX in a single form, while the combination index (CI) for combined CPE and DOX, as well as SIN and Cis, was calculated based on Blumenthal, *et al*., (2005).

RESULTS

Determination of Compound in the *Citrus sinensis* Peel Extract (CPE)

CPE contains various flavonoid compounds, such as hesperidin, hesperetin, tangeretin, nobiletin, and sinensetin, which possess the potential to augment the efficacy of chemotherapy agents (Hermawan and Putri, 2021; Wardani, *et al*., 2021). The existence of flavonoids in CPE was confirmed using TLC techniques, employing toluene: ethyl acetate (5:7) as the mobile phase and silica gel F254 as the stationary phase (Kartini, *et al*., 2023) (Figure 1A). The spots generated during the elution of the extract were compared with the sinensetin

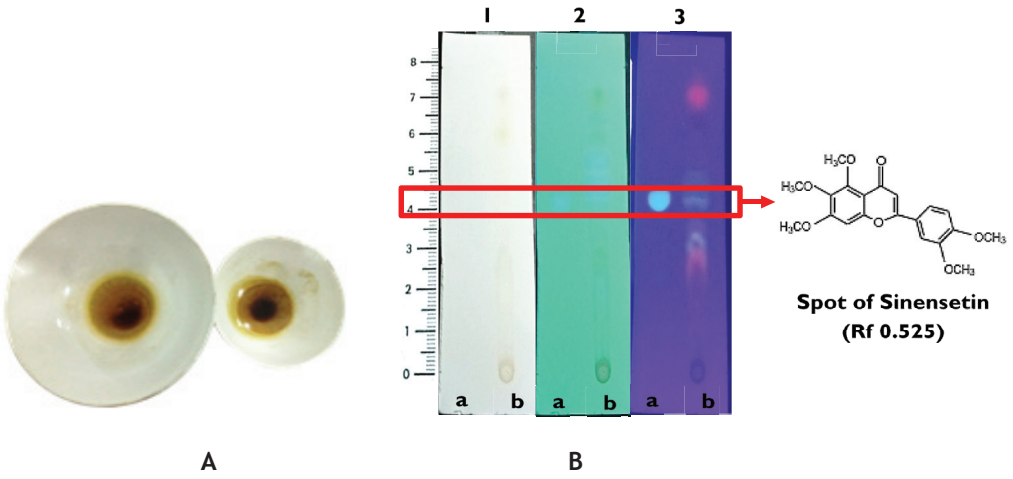

Figure 1. *Citrus sinensis* peel extract and TLC profile. *Citrus sinensis* peel extract (A) was prepared by macerating *Citrus sinensis* peel powder in a ratio of 1:10 with 70% ethanol, shaking for 24 h, and evaporating for 3 days, resulting in a high-viscosity extract yielding 16.74% (w/w). The TLC profile (B) was obtained by elution of sinensetin standard (a) and Citrus sinensis peel extract (b) using a combination of toluene and ethyl acetate (5:7) with silica gel F254 (Merck) as the stationary phase. The chromatograms were visualized under visible light (1), UV light at 254 nm (2), and 366 nm (3).

compound spot, serving as the standard. According to the TLC results (Figure 1B), there is a suspicion of the presence of sinensetin compounds in the CPE. This suspicion arises from observing a spot in the CPE column that aligns with sinensetin, both exhibiting the same retention factor (RF) of 0.525.

Cytotoxic Activity of *Citrus sinensis* Peel Extract (CPE), Sinensetin (SIN), Cisplatin (Cis), and Doxorubicin (DOX) on HepG2 and Vero cells

The cytotoxic activity of CPE, SIN, Cis, and DOX in their single form against HepG2 and Vero cells was assessed using the MTT method

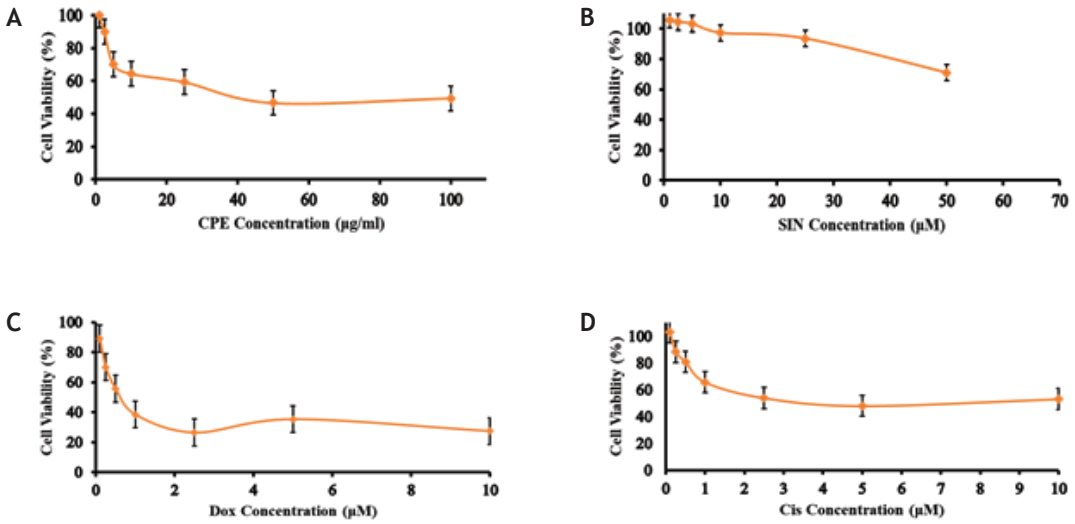

Figure 2. Cytotoxicity profile of single CPE (A), SIN (B), Cis (C), and DOX (D) on HepG2 cells. 5×10^3 HepG2 cells were grown in each well of a 96-well plate and treated with a serial concentration of CPE (1-100 µg/mL) (A), SIN (1-50 µM) (B), Cis (0.1-10 µM) (C), and DOX (0.1-10 µM) (D) for 24 h.

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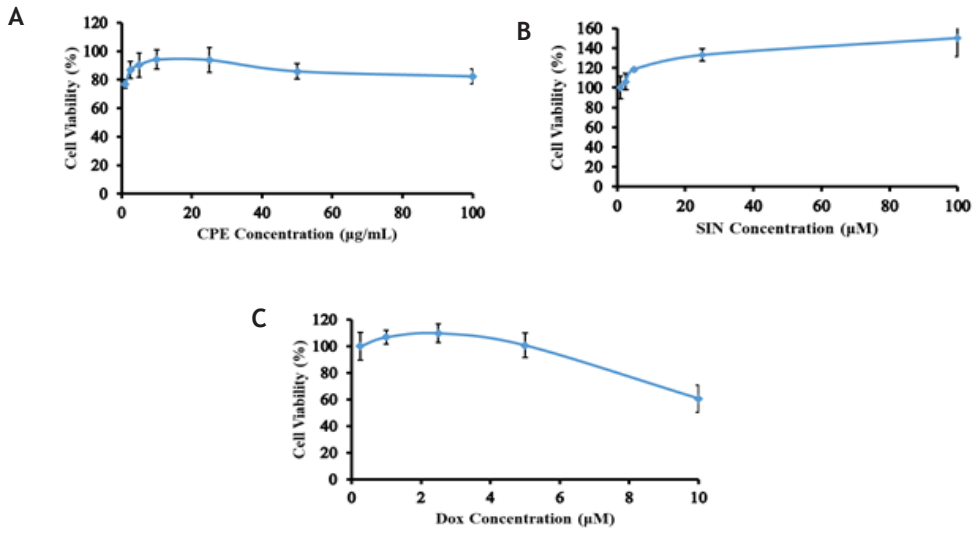

Figure 3. Cytotoxicity profile of single CPE (A), SIN (B), and DOX on Vero cells. Vero cells (104/well) were grown in a 96-well plate and treated with various concentrations of CPE (1-100 µg/mL) (A), SIN (1-100 µM) (B), and DOX (0.1-10 µM) (C) for 24 h.

(Figure 2 and 3). CPE in single form exhibited moderate cytotoxicity with an IC_{50} of 101.09 µg/mL (Figure 2A) against HepG2 cells but did not induce cytotoxic effects on Vero cells (Figure 3A). SIN also demonstrated a modest cytotoxic effect on HepG2 cells with an IC_{50} of 83.13 µM (Figure 2B) and displayed no effect on Vero cells (Figure 3B). Meanwhile, cisplatin generated a strong cytotoxic effect against HepG2 cells with an IC_{50} of 7.86 µM (Figure 2C), while DOX in its single form showed strong cytotoxicity on both HepG2 and Vero cells, with IC_{50} value of 2.52 µM and 13.98 µM, respectively (Figure 2D and Figure 3C).

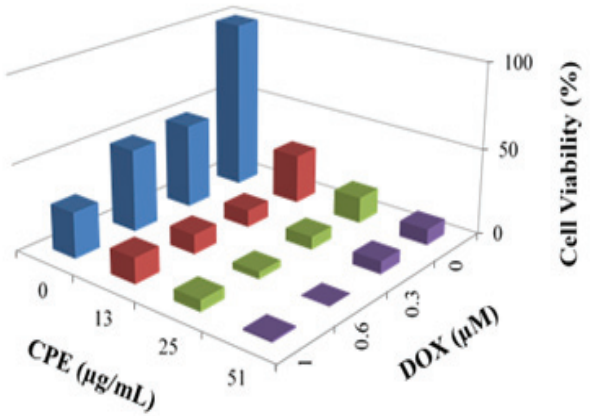

Figure 4. Cytotoxicity profile of CPE and doxorubicin (DOX) on HepG2. 5 x10^3 HepG2 cells were grown in each well of a 96-well plate and treated with the combination of CPE and DOX for 24 h.

Cytotoxic Effect of Combined *Citrus sinensis* Peel Extract (CPE) and Doxorubicin (DOX)

To evaluate the synergy between CPE and DOX, a combined cytotoxicity test was carried out using MTT (Figure 4 and Table 1). This test combined CPE and DOX extracts in proportions of ½ IC_{50}, ¼ IC_{50}, and ⅛ IC_{50}.

Table 1. Combination Index (CI) of CPE and Doxorubicin on HepG2. Two compounds can be categorized as synergic if their combination exerts CI<1.0.

CPE (µg/mL)	DOX (µM)		
	0.3	0.6	1
13	0.10	0.13	0.18
25	0.16	0.18	0.22
51	0.31	0.31	0.35

The test results indicated a reduction in the viability of HepG2 cells as the concentration of the CPE and DOX combination increased (Figure 4). Moreover, the calculation of the CI revealed that all combinations of CPE with DOX showed synergy (Table 1). This conclusion was drawn based on the CI value for each combination of CPE and DOX extracts, all of which were less than 1 (Blumenthal, *et al*., 2005; Ikawati, *et al*., 2023).

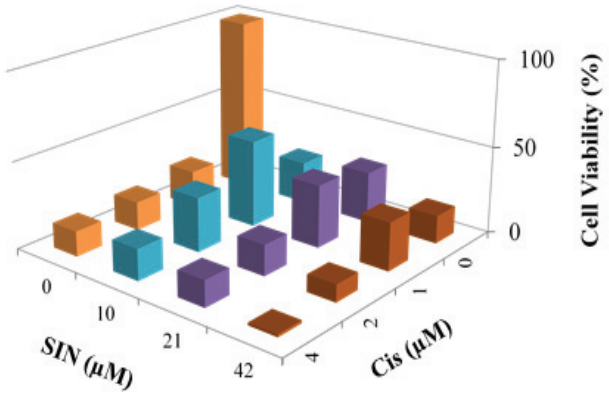

Figure 5. The influence of combined sinensetin (SIN) and cisplatin (Cis) on HepG2. HepG2 cells (5x10^3/well) were exposed to a mixture of CPE and Cis in a 96-well plate for 24 h.

Cytotoxic Effect of Combined Sinensetin (SIN) and Cisplatin (Cis)

SIN has been identified as one of the compounds contained in CPE. This study aimed to assess the synergistic potential of SIN in enhancing the cytotoxic effect of Cis on HepG2 cells using MTT assay. The conducted assay revealed that SIN increased the sensitivity of HepG2 cells to Cis in a dose-dependent manner (Figure 5). Additionally, SIN exhibited synergistic behavior with Cis, as evidenced by the CI value being less than 1.0 as mentioned earlier (Table 2) (Blumenthal, *et al*., 2005; Ikawati, *et al*., 2023).

Table 2. The combination index (CI) of sinensetin (SIN) and cisplatin (Cis) on HepG2 cells. The combination is interpreted as synergist if the CI has a value <1.0.

SIN (μM)	Cis (μM)		
	1	2	4
10	0.32	0.29	0.37
21	0.34	0.32	0.44
42	0.49	0.44	0.51

DISCUSSION

SIN was suspected to be one compound present in CPE based on its retention factor (Rf). SIN is a pentapolymethoxyflavone with several methoxy groups located on 5, 6, 7, 3', and 4' of the flavone backbone (Han Jie, *et al*., 2021). It is known for its cytotoxic effect on several cancer types and its ability to alleviate pyroptosis caused by chemotherapeutic agents such as Cis, suggesting that SIN and all the SIN-containing natural sources could potentially serve as co-chemotherapeutic agents (Li, *et al*., 2023; Han Jie, *et al*., 2021). This study focuses on exploring the potential of CPE and SIN as co-chemotherapeutic agents with DOX or Cis.

Initially, both CPE and SIN exhibited moderate cytotoxicity against HepG2 cells, while showing no effect on Vero cells, indicating high selectivity and safety for normal cells. This outcome provides promising prospects as a source of chemopreventive agents, inviting further exploration for cytotoxic screening to indentify the most valuable active compounds. However, this endeavor will require substantial effort and resources, including budget and technical support. Alternatively, this potential herb could be employed in co-chemotherapeutic applications alongside chemotherapeutic agents. In this context, we employed DOX in conjunction with CPE and Cis with SIN. Notably, CPE synergistically enhanced the cytotoxic effect of DOX, while SIN exhibited a similar effect with Cis on HepG2 cells. Considering HepG2 as a representative of malignant HCC, these

findings raise hope that CPE might be developed as a co-chemotherapeutic agent for malignant liver cancer. This study revealed superior co-treatment activity compared to *Alpinia galangal* and *soursop* leaf extract, both combined with DOX. In this study, concentrations of 13-51 µg/mL were used, while *Alpinia galangal* and *soursop* leaf extract utilized concentrations of 50-100 µg/mL and 13-25 µg/mL respectively (Salsabila, *et al*., 2023; Ahlina, *et al*., 2019). However, it's crucial to ensure the safety of CPE as a therapeutic agent. Therefore, further exploration regarding its safety profile and more detailed efficacy, including its impact on cellular physiology and molecular changes, is needed.

CPE holds better promise than SIN as an anticancer agent or co-chemotherapy agent in HCC due to its composition containing multiple active compounds from both flavonoid and other compound groups. Future studies are recommended to delve deeper into the active compound in CPE and and explore its application in other types of cancer or with different chemotherapeutic agents for co-chemotherapeutics purpose.

CONCLUSION

The study suggest that CPE is likely to contain SIN among its constituents. Both CPE and SIN exhibit moderate cytotoxicity against HepG2 cells and show non-cytotoxicity towards Vero cells. Cis demonstrates potent cytotoxicity against HepG2 cells. Conversely, DOX, shows strong cytotoxic effect on both HepG2 and Vero cells in single treatment. CPE, comprising SIN as one of its compounds, appears to enhance the cytotoxic effect of DOX, while SIN itself augments the toxicity of Cis on HepG2 cells. Both in exhibit a synergistic effect on HepG2 cells.

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