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Effect of Citrus Hystrix DC. Peel Extracts on The Cytotoxicity of Human Colorectal Cancer SW480 Cells in Vitro

Bowornluk Anutarapong
E-mail: 6752003266@lamduan.mfu.ac.th
Master of Science in Anti-Aging and Regenerative Science
School of Anti-Aging and Regenerative Medicine, Mae Fah Luang University
Vitoon Julratanaporn, Ph. D.
E-mail: vitoon.jul@mfu.ac.th
School of Anti-Aging and Regenerative Medicine, Mae Fah Luang University

Abstract

Background: Colorectal cancer (CRC) is a leading cause of cancer-related mortality globally. Conventional chemotherapies often present narrow therapeutic windows and severe side effects, prompting the search for novel, safe, and effective natural agents. *Citrus hystrix* DC. (Kaffir lime) is known for its pharmacological properties, yet the selective cytotoxicity of its peel extract against CRC remains to be fully elucidated. Objective: To evaluate the chemical profile and selective cytotoxic effects of *Citrus hystrix* DC. peel ethyl acetate extract against human colorectal cancer cells (SW-480) and normal colon epithelial cells (CCD-841 CoN) *in vitro*. Methods: The phytochemical composition of the extract was analyzed using Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS). The cytotoxicity and anti-proliferative activities were assessed using the MTT assay after 24 hours of treatment. The Selectivity Index (SI) was calculated to evaluate the safety profile. Results: LC-QTOF-MS profiling tentatively identified a complex matrix of 89 secondary metabolites (45 in positive and 44 in negative ionization modes). The prominent phytochemical groups included flavonoids (e.g., Apigenin, Hesperidin, Limocitrol), coumarins (e.g., Citropten, Umbelliferone, Dentatin), terpenoids (e.g., Limonin, β-Pinene), and phenolic compounds. The extract significantly inhibited SW-480 cell viability in a dose-dependent manner ($p < 0.0001$), exhibiting an IC_{50} value of 64.63 ± 15.15 µg/mL. In contrast, it demonstrated comparatively lower toxicity against normal CCD-841 CoN cells (CC_{50} = 181.60 ± 37.28 µg/mL). This resulted in a

Selectivity Index (SI) of 2.81, indicating a favorable therapeutic window in vitro. Conclusion: The ethyl acetate extract of *C. hystrix* peel demonstrates significant and selective cytotoxicity against SW-480 colorectal cancer cells while maintaining a favorable safety profile in normal cells. The observed anti-proliferative activity is likely associated with the diverse phytochemical matrix, particularly the presence of flavonoids and coumarins. By utilizing an agricultural by-product, this study highlights *C. hystrix* peel as a promising botanical source for natural chemopreventive agents. Further in vivo studies and mechanistic investigations are required to fully understand the apoptotic pathways and validate its potential for future functional health applications.

Keywords: Citrus hystrix DC., Colorectal cancer, Selective cytotoxicity, LC-QTOF-MS, Secondary metabolites, Agricultural by-product.

Introduction

Colorectal cancer (CRC) stands as a monumental global health challenge, ranking as the third most commonly diagnosed malignancy and the second leading cause of cancer-related mortality worldwide, responsible for over 1.9 million new cases and approximately 935,000 deaths annually (Sung et al., 2021; Bray et al., 2024). While incidence and mortality rates have begun to stabilize or decline in several high-income nations due to early screening interventions, the disease trajectory in transitioning economies depicts a starkly contrasting reality. In Thailand, the burden is particularly alarming; CRC currently stands as the third most common cancer in males and the fourth in females, accounting for approximately 11% of the national cancer incidence with over 17,000 new cases reported each year (Khuhaprema et al., 2013). More critically, while global mortality averages are heavily influenced by aging populations, CRC is the *only* malignancy in Thailand demonstrating a continuous, uninterrupted upward trend in incidence across both sexes (Chantarapanich et al., 2020). This escalating crisis mirroring the adverse impact of global lifestyle transitions highlights CRC as a severe health threat that demands immediate, scalable, and accessible therapeutic innovation.

The clinical management of CRC is heavily hindered by late-stage diagnoses. Because early clinical manifestations, such as altered bowel habits, unexplained weight

loss, and iron-deficiency anemia, are frequently subtle and nonspecific, the majority of cases in Thailand are detected at advanced or metastatic stages. This delayed medical intervention severely limits treatment outcomes and patient survival. Consequently, there is an urgent need to explore novel, safe, and effective alternative therapeutic options.

In the search for such agents, indigenous flora like *Citrus hystrix* DC. (Kaffir lime) have emerged as promising sources of bioactive compounds. Traditionally utilized in Thai medicine, *C. hystrix* is rich in volatile compounds and phytoncides that possess antimicrobial, antioxidant, and anti-inflammatory properties. Recent evidence suggests that these inherent pharmacological traits could harbor potent natural anticancer capabilities (Li et al., 2006; Peterfalvi et al., 2019).

Over the past decade, several studies have substantiated the cytotoxic effects of *C. hystrix* extracts against CRC, though research has predominantly focused on its leaves. Studies demonstrate that leaf extracts (such as ethanol and ethyl acetate extracts) exert strong apoptotic and cytotoxic effects on CRC cell lines like HT-29 and HCT-116. Mechanistically, these extracts drive cell death by upregulating pro-apoptotic genes and increasing reactive oxygen species (ROS) generation (Abolmaesoomi et al., 2023).

While the anti-proliferative efficacy of the leaves is well-documented, the pharmacological potential of the *C. hystrix* peel remains insufficiently explored. Often discarded as agricultural waste, the peel contains a similar profile of essential oils and secondary metabolites. Investigating its anticancer efficacy would not only fill a critical gap in current phytomedical research but also add value to an underutilized botanical resource.

Addressing this gap, the present study aims to evaluate the anti-colorectal cancer and cytotoxic activities of the *Citrus hystrix* DC. peel ethyl acetate extract against the SW480 colorectal cancer cell line *in vitro*. By analyzing its potential to inhibit cancer cell proliferation, this study seeks to establish a clear pharmacological profile and contribute to the development of accessible, natural-origin therapeutic agents for CRC.

Research Methodology

1. Study Design and Plant Material

This laboratory-based research evaluated the anti-colorectal cancer and cytotoxic activities of *Citrus hystrix* DC. (Kaffir lime) peel extract. Fresh fruits of the native "sweet" cultivar (10.05 kg) were collected from Ratchaburi, Thailand. Taxonomic identification was performed according to the Thai Herbal Pharmacopoeia (THP) 2021, and voucher specimens were deposited at the Medicinal Plants Innovation Center, Mae Fah Luang University.

2. Extraction of C. hystrix Fruits

Fresh peels were manually isolated, dehydrated in a hot-air oven at 50°C for 48 h, and pulverized into a coarse powder. The powdered biomass underwent solid-liquid maceration using analytical-grade ethyl acetate on a rotary shaker (100 rpm, 16 h) for three cycles to maximize the extraction of semi-polar to non-polar bioactive metabolites. The filtrates were combined, and the solvent was evaporated under reduced pressure at 50°C. The resulting crude extract (yield: 7.46% of dry weight) was stored at -20°C for subsequent analysis.

3. Phytochemical Profiling via LC-QTOF-MS

The chemical composition of the extract was analyzed using an Agilent 1290 Infinity II UHPLC system coupled to a 6540 Series Accurate-Mass Q-TOF LC/MS equipped with an electrospray ionization (ESI) interface. The extract (1 mg/mL in methanol) was injected (5 µL) onto an Inertsil ODS-3 column at 40°C. A gradient elution was applied using 0.1% (v/v) formic acid in water (Solvent A) and 0.1% (v/v) formic acid in acetonitrile (Solvent B). High-resolution mass spectra were recorded in both ESI-positive and ESI-negative modes (m/z 100-1,000). Data processing, tentative compound annotation, and semi-quantification were performed using Agilent MassHunter Qualitative Analysis software (Version 10.0) with an exact mass error threshold of $< \pm 5$ ppm.

4. Cell Culture and Treatment Protocols

Human colorectal adenocarcinoma cells (SW-480) and normal colon epithelial cells (CCD-841 CoN) were maintained in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. For treatments, a 50 mg/mL stock solution of the extract in DMSO

was prepared. Cells were seeded and incubated overnight before being exposed to varying concentrations of the extract (15.625 to 1,000 µg/mL) diluted in complete medium for 24 h. The vehicle control consisted of medium with 1% DMSO.

5. Cytotoxicity Assay

Cell viability was assessed using the colorimetric MTT assay. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h of treatment, the medium was replaced with fresh medium containing 0.5 mg/mL MTT reagent, followed by a 2-h incubation. The formed formazan crystals were dissolved in DMSO (100 µL/well), and absorbance was measured at 570 nm using a Spark multimode microplate reader.

6. Statistical Analysis

Data are presented as the mean ± standard deviation (SD) of three independent biological replicates (n = 3). Non-linear regression using a four-parameter logistic model in GraphPad Prism (version 8.0.1) was employed to calculate the half-maximal inhibitory concentration (IC_{50}) for SW-480 cells and the half-maximal cytotoxic concentration (CC_{50}) for CCD-841 CoN cells. The Selectivity Index (SI) was calculated as the ratio of CC_{50} to IC_{50}. Statistical differences were evaluated using one-way ANOVA followed by Tukey's or Dunnett's post hoc tests, or the Kruskal-Wallis test with Dunn's post hoc test, as appropriate. A p-value < 0.05 was considered statistically significant.

Results

This section reports the findings regarding the chemical composition and cytotoxic activity of the ethyl acetate extract from *Citrus hystrix* DC. peel, detailed as follows:

The evaluation of the chemical composition using Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS) in both positive and negative ionization modes tentatively identified a total of 89 secondary metabolites (45 compounds in the positive mode and 44 compounds in the negative mode). The major phytochemical groups identified included flavonoids (e.g., Apigenin, Hesperidin, Kaempferol, Isorhamnetin, Limocitrol, and Clitoriacetal), coumarins (e.g., Citropten,

Umbelliferone, and Dentatin), terpenoids (e.g., Limonin and β-Pinene), and phenolic compounds (e.g., Caffeic acid).

Table 1 Tentative identification of the top three major phytochemical compounds per ionization mode in *Citrus hystrix* peel extract using LC-QTOF-MS.

Compound Name	RT[1] (min)	m/z[2]	Abund[3]	Formula	Error[4] (ppm)	Ref.[5]
Negative Ionization Mode						
(+)-Syringaresinol	19.207	417.1538	59,937,315	$C_{22}H_{26}O_8$	-3.94	Zhao et al., 2023
Arachidoside	17.856	349.0918	23,839,287	$C_{16}H_{16}O_6$	-3.58	Purda et al., 2024
(-)-Epicatechin	17.856	349.0918	23,816,058	$C_{15}H_{14}O_6$	-3.76	Zhao et al., 2023
Positive Ionization Mode						
(+)-Oxypeucedanin	19.726	287.0923	204,574,753	$C_{16}H_{14}O_5$	3.22	Zhao et al., 2023
Caffeic acid	22.693	203.0309	128,797,214	$C_9H_8O_4$	-3.06	Zhao et al., 2023
Arachidoside	17.846	305.1016	113,053,315	$C_{16}H_{16}O_6$	-1.38	Purda et al., 2024

Note [1]RT (Retention time) indicates the elution time of the substance (minutes), [2]*m/z* represents the mass-to-charge ratio of the charged particles, [3]Abund indicates the abundance of the detected substance, [4]Mass error (ppm) indicates that the chemical formula was determined using a mass difference tolerance of ±5 ppm, [5]Ref indicates references.

The evaluation of cytotoxic effects revealed that the *C. hystrix* peel ethyl acetate extract significantly inhibited the viability of human colorectal cancer cells (SW-480) in a dose-dependent manner ($p < 0.0001$). A marked reduction in cell viability was clearly observed starting from a concentration of 31.25 µg/mL.

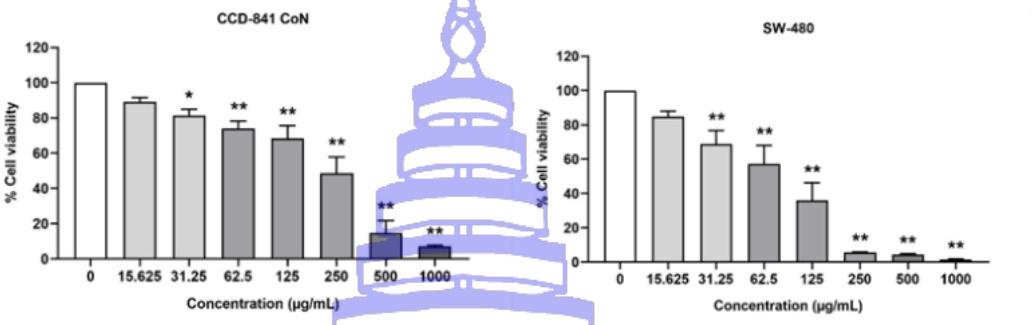

Figure 1 Percentage of cell viability of CCD-841 CoN and SW-480 cells following 24 h treatment with the ethyl acetate extract of *Citrus hystrix* DC. peel. Bar graphs represent the mean ± SD (n = 3). $p < 0.01$ compared with the vehicle control (0 µg/mL). (* = $p < 0.05$, ** = $p < 0.01$)

Table 2 The half-maximal cytotoxic concentration (CC_{50}), half-maximal inhibitory concentration (IC_{50}), and selectivity index (SI) of the *Citrus Hystrix* extract

Sample	CCD-841 CoN (CC_{50}; µg/mL) *	SW-480 (IC_{50}; µg/mL) *	Selectivity index
The ethyl acetate extract of *Citrus hystrix* DC. peel	181.60 ± 37.28	64.63 ± 15.15	2.81

Note * Data are presented as mean ± SD from three replicates.

As shown in Table 2, IC_{50} required to inhibit the growth of SW-480 colorectal cancer cells was 64.63 ± 15.15 µg/mL. Meanwhile, CC_{50} against normal human colon epithelial cells (CCD-841 CoN) was 181.60 ± 37.28 µg/mL.

When comparing the cytotoxic effects between the normal cells and colorectal cancer cells, SI was found to be 2.81. This finding demonstrates that the survival rate of the SW-480 was significantly lower than that of the normal cells CCD-841 CoN when treated at the same extract concentrations, indicating the extract's selective action against the cancer cells.

Discussion

This study provides profound insights into the potential of the ethyl acetate extract from *Citrus hystrix DC.* peel as an effective inhibitory agent against the SW-480 human colorectal cancer cell line, while concurrently demonstrating a highly satisfactory safety profile in normal cells. These findings not only present novel knowledge that contributes to the valorization of *C. hystrix* peel an agricultural by-product frequently discarded as waste but also underscore the significance of indigenous flora in the development of alternative, natural chemo preventive agents.

Compared to previous studies that have predominantly focused on *C. hystrix* leaves, the current findings reveal that the peel extract exhibits comparably potent biological activities. Prior research has indicated that leaf extracts (e.g., ethyl acetate and ethanol fractions) can effectively inhibit HCT-116 and HT-29 colorectal cancer cells, with IC_{50} values generally ranging from 60 to over 100 ug/mL (Abolmaesoomi et al., 2023). Concurrently, the present study provides clear evidence that the peel extract significantly suppresses the proliferation of SW-480 cancer cells (IC_{50} = 64.63 ± 15.15 µg/mL) when compared to the untreated control group, thereby directly substantiating our primary hypothesis.

Phytochemically, the *C. hystrix* peel is enriched with flavonoids, coumarins, terpenoids, and phenolic compounds, which act synergistically to confer robust antioxidant and anti-cancer properties, with mechanistic insights from external literature substantiating that each of these compound groups plays a systematic role in cancer suppression. Prominent flavonoids directly modulate crucial cell survival pathways; for instance, apigenin impedes the Wnt/β-catenin signaling pathway and generates reactive oxygen species (ROS) through interference with the mitochondrial electron transport chain (Banerjee et al., 2015; Xu et al., 2016), while hesperidin and isorhamnetin upregulate the Nrf2/HO-1 pathway and suppress the *PI3K*/*AKT*/*mTOR* signaling cascades to induce apoptosis (Xia et al., 2018; Wang et al., 2018). Kaempferol actively downregulates *survivin* and upregulates pro-apoptotic *Bax* (Imran et al., 2019), and furthermore, citrusosides, limocitrol, durlettone, clitoriacetal, and buddleoflavonoloside act collectively as robust cellular antioxidants and suppress tumor cell viability by modulating intracellular survival signals, inducing metabolic stress, and activating caspase-mediated apoptosis in malignant cells (Benavente-García

et al., 2008; Ito et al., 2004; Tewtrakul et al., 2007; Liao et al., 1999). Coumarins are also highly effective in inducing DNA damage and cell cycle arrest; umbelliferone and (+)-oxypeucedanin cause DNA strand breaks, induce G0/G1 and G2/M phase cell cycle arrest, and regulate *Bax* and *Bcl-2* proteins (Muthu et al., 2016; Kang et al., 2013). Dentatin markedly downregulates the anti-apoptotic NF-κB signaling pathway and induces DNA fragmentation (Arbab et al., 2013), and additionally, citropten, isobergaptol, and paradisin C display targeted cytotoxicity by neutralizing ROS, inhibiting specific cytochrome P450 enzymes associated with tumor promotion, and triggering early-stage apoptosis (Conforti et al., 2009; Paya et al., 1992; Girennavar et al., 2007). Regarding terpenoids and phenolic compounds, terpenoids such as limonin and elemol trigger mitochondrial-mediated apoptosis by activating Caspase-3 and Caspase-9 cascades while downregulating Bcl-2 (Han et al., 2021; Chen et al., 2014). β-Caryophyllene acts as a selective CB2 receptor agonist that suppresses the STAT3 activation pathway (Fidyt et al., 2016), while β-pinene disrupts the mitochondrial membrane potential (Salehi et al., 2019). These effects are complemented by phenolic compounds like caffeic acid, which exerts strong ROS-scavenging capabilities and induces G1/S phase cell cycle arrest (Espíndola et al., 2019).

Furthermore, the high Selectivity Index (SI = 2.81) observed in this study suggests that the extract not only exerts cytotoxicity against malignant cells but may also confer protective effects on normal colon epithelial cells (CCD-841 CoN). Based on the recognized mechanisms of the identified phytochemicals, it is hypothesized that this selective protection could be mediated through the inhibition of cellular senescence pathways, such as the *mTOR, p38, and AKT* signaling cascades. By attenuating these senescence-inducing signals, the bioactive compounds within the *C. hystrix* peel may protect normal colon cells from oxidative damage and stress, thereby preserving their natural renewal capacity and preventing severe tissue inflammation.

While the crude ethyl acetate extract demonstrated significant anti-proliferative activity, the exact nature of the interactions among its constituents remains to be fully elucidated. The current findings strongly imply a combination effect, where multiple secondary metabolites work in concert to achieve the observed cytotoxicity. Future investigations should focus on isolating the most highly abundant bioactive compounds identified in the LC-MS profiling (e.g., (-)-Epicatechin, Hesperidin, and

(+)-Oxypeucedanin). Evaluating their single-agent cytotoxic efficacies alongside combination assays (synergism testing) will be crucial to identifying optimal synergistic combinations that could yield a significantly higher SI and improved targeted therapeutic outcomes compared to single-agent treatments.

Applications to Anti-Aging and Regenerative Science

From the perspective of anti-aging and regenerative medicine, these findings present highly actionable applications. Aging of the gastrointestinal tract is heavily characterized by "inflammaging" a chronic, low-grade systemic inflammation that drives cellular senescence and significantly increases the risk of age-related pathologies, including colorectal dysplasia and cancer. The bioactive matrix found in *C. hystrix* peel acts at the intersection of oncology and longevity in two primary ways:

First, the selective cytotoxicity observed suggests potential *senolytic* or *senomorphic* capabilities. By effectively triggering apoptosis in mutated, rapidly dividing cells (SW-480) while sparing normal epithelial cells, the extract mirrors the goals of *senolytic* therapies: clearing damaged or dysfunctional cells from the tissue microenvironment before they can secrete harmful pro-inflammatory cytokines (the Senescence-Associated Secretory Phenotype, or SASP).

Second, the high tolerance of the normal colon epithelial cells (CC_{50} = 181.60 ± 37.28 µg/mL) indicates that the extract may possess cytoprotective properties for healthy tissues. Preserving the integrity and renewal capacity of healthy mucosal stem and progenitor cells by mitigating intracellular ROS is a cornerstone of regenerative gastrointestinal health. Therefore, *C. hystrix* peel extract holds significant promise for translation into anti-aging functional foods, targeted nutraceuticals, or longevity supplements designed to maintain gut homeostasis, prevent inflammaging-induced mutations, and extend the healthspan of the digestive system.

Despite the promising findings, this study has several limitations that should be acknowledged. First, the research was conducted exclusively in vitro using 2D cell culture models, which cannot fully replicate the complex tumor microenvironment, pharmacokinetics, and bioavailability found in vivo or in human subjects. Second, due to time constraints, specific intracellular mechanisms such as exact caspase activation levels and precise cellular senescence mechanisms were not empirically validated

within the current scope of this study. Finally, since colorectal cancer progression is closely linked to chronic inflammation, the extract's potential anti-inflammatory efficacy, a crucial factor in preventing advanced disease stages, was not directly evaluated.

Conclusion and Future Directions

In conclusion, this study demonstrates that the ethyl acetate extract of *Citrus hystrix* DC. peel is a potent and selective botanical candidate for colorectal cancer research. The extract effectively inhibited the viability of SW-480 human colorectal cancer cells in a dose-dependent manner (IC_{50} = 64.63 ± 15.15 µg/mL), while demonstrating an excellent safety profile against normal human colon epithelial cells (SI = 2.81). This efficacy is driven by a complex matrix of 89 bioactive secondary metabolites identified via LC-QTOF-MS, which act synergistically to confer substantial antioxidant and anti-proliferative properties.

By expanding the research focus from the extensively studied leaves to the peel, this study highlights the valorization of a common agricultural by-product. To build upon these foundational findings, it is suggested that future studies prioritize in-depth mechanistic in vitro investigations to validate the specific apoptotic and anti-senescence pathways (e.g., evaluating *Bax/Bcl-2* ratios, *NF-kappaB, mTOR, AKT* expression, and SASP suppression). Subsequent in vivo studies using animal models should be pursued to evaluate systemic safety, pharmacokinetics, and gut microbiome interactions. Ultimately, *C. hystrix* peel establishes itself as a highly promising botanical candidate for the development of natural chemopreventive agents, geroprotectors, and functional anti-aging nutraceuticals aimed at preserving gastrointestinal longevity.

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