Inhibitory Effects of Green Tea Leaf Stalk Extracts on Colibactin-producing *Escherichia coli* and Colorectal Cancer Cells

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Abstract. Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide. Colibactin-producing *Escherichia coli* strains, particularly those carrying the *pks* gene cluster, have been increasingly detected in CRC patients, complicating treatment and highlighting the need for alternative therapeutic strategies. Tea (*Camellia sinensis* var. *assamica*) leaf stalks, a byproduct of tea processing, are often discarded during production, even though they are rich in bioactive compounds and have been less studied for their biological activities. This study evaluated young and mature green tea leaf stalk extracts for their chemical compositions, antioxidant activity, antibacterial activity, and cytotoxicity against HT-29 and Caco-2 colorectal cancer cells. The result revealed that tea leaf stalk extracts contained the phenolics and flavonoids compounds that related to antioxidant activity. In addition, young and mature green tea leaf stalks contained the bioactive compounds including epigallocatechin gallate (EGCG) and catechin by HPLC detection. For antibacterial activity, the young and mature green tea leaf stalk extracts exhibited the lowest MIC and MBC values at 62.5-250 mg/ml against all isolates of colibactin-producing *E. coli* (BA1, BA2, CA5, HA2, and VA2). Furthermore, cytotoxicity assay was showed that mature tea leaf stalk extract effectively inhibited HT-29 and Caco-2 cells, with lower IC_{50} values of 0.402 ± 0.029 mg/ml and 0.195 ± 0.028 mg/ml, respectively comparing to young tea leaf stalks (0.627 ± 0.044 mg/ml and 0.218 ± 0.035 mg/ml). These new findings suggested that young and mature green tea leaf stalks are a promising natural source of bioactive compounds with potential antioxidant, antibacterial and anticancer effects, providing a safer alternative or complementary approach to conventional CRC therapy while mitigating associated side effects.

1 Introduction

According to the World Health Organization, colorectal cancer (CRC) represents nearly 10% of all cancer diagnoses globally and is a major contributor to cancer-related deaths, ranking second worldwide [1,2]. A large amount of *Escherichia coli* has been detected in tissue samples from CRC patients, with most isolates belonging to phylogroup B2 and

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carrying the *pks* gene cluster, which encodes the genotoxin colibactin [3-5]. The *pks* genomic island spans approximately 54 kilobases and consists of 19 genes (*clb*A to *clb*S) responsible for colibactin biosynthesis. Previous studies have indicated that *E. coli* strains carrying the pks gene cluster are closely linked to colorectal carcinogenesis in humans [6]. In addition, an increasing number of studies have reported the presence of drug-resistant *E. coli* isolates from CRC patients worldwide, which further complicates current treatment approaches [7,8]. Derived from *Camellia sinensis*, tea is a globally consumed beverage with a long history that traces back to China [9]. From tea products processing, tea leaf stalks are the parts cut off from the tea leaves after processing, accounting for nearly 35% of the total dry biomass of the leaves [10]. A diverse array of compounds has been identified in these materials, such as polyphenols, saponins, theanine, caffeine, cellulose, hemicellulose, lignin, polysaccharides, proteins, pigments and vitamins [11]. Tea leaf stalks have a unique aroma, containing compounds such as pyrazine, pyrrole, and furan. Studies have shown that the presence of tea stems significantly increases the nerolidol content in oolong tea's aroma and enhances the umami flavor of oolong tea [12,13]. When brewing Lu'an Guapian tea, the taste differs depending on whether the stem or the leaf is used. Brewing from the leaves results in a more bitter and astringent taste, while brewing from the stems results in a sweeter and more umami-rich flavor [13-15]. Compared with tea leaves, the stems exhibit significantly greater concentrations of theanine, quinic acid, and sucrose, while also serving as a rich source of phenolics and caffeine [16,17]. These biologically active compounds found in tea are beneficial for health, helping to prevent and treat cardiovascular diseases, tumors, digestive system disorders, obesity, and diabetes [18,19]. Comparative studies on the antibacterial activity and underlying mechanisms of green, oolong, black, and Fuzhuan tea leaf extracts have demonstrated their inhibitory effects against Gram-positive bacteria, including *Staphylococcus aureus* and *Enterococcus faecalis*. These extracts also exhibit activity against Gram-negative bacteria, such as *E. coli* and *Salmonella* Typhimurium, although Gram-positive bacteria are generally more susceptible to tea-derived compounds [20]. Despite these findings, research focusing on tea leaf stalks remains limited, particularly in terms of their bioactive constituents and biological activities. This study investigated the phytochemical composition and antioxidant activity of extracts from young and mature green tea leaf stalks and further assessed their inhibitory effects against colibactin-producing E. coli and colorectal cancer cells.

2. Method

2.1 Green tea leaf stalks extraction

Dried young and mature green tea leaf stalks (*Camellia sinensis* var. *assamica*) were obtained from Tea Gallery Group (Thailand) Co., Ltd., Chiang Mai, Thailand. The samples were milled into a fine powder and subjected to ethanol extraction as previously described [21]. The resulting extracts were subsequently filtered, concentrated by solvent evaporation, and freeze-dried. All extracts were stored at −20 °C in the dark until further analysis.

2.2 Quantification of total phenolic compounds in green tea leaf stalk extracts

Total phenolic content in the tea leaf stalk extracts was measured by the Folin–Ciocalteu method [22]. The concentration was calculated using gallic acid as a standard and reported as mg GAE per gram of extract.

2.3 Quantification of total flavonoids in green tea leaf stalk extracts

Total flavonoid content in the tea leaf stalk extracts was measured by the aluminum chloride colorimetric assay [22], using quercetin as the reference standard and expressed as mg QE per gram of extract.

2.4 Evaluation of antioxidant activity of green tea leaf stalk extracts

The antioxidant potential of the extracts was determined by the DPPH assay [23]. Absorbance values of the control (A_1) and sample (A_2) were measured, and the free radical scavenging activity was calculated as percentage inhibition: percentage inhibition = $[(A_1 - A_2) / A_1] \times 100$. Results were quantified against a gallic acid calibration curve and expressed as mg GAE per gram of extract.

2.5 Detection of phytochemical compounds in green tea leaf stalk extracts by high performance liquids chromatography (HPLC)

The phytochemical compounds in green tea leaf stalk extracts such as catechin, epigallocatechin gallate (EGCG), caffeine, and theaflavin were detected by high performance liquids chromatography (HPLC). The HPLC conditions were performed according to Teppabut et al., (2025) [21].

2.6 Antibacterial activity of green tea leaf stalk extracts on colibactin-producing *E. coli*

The inhibitory activity of tea leaf stalk extracts against colibactin-producing *E. coli* was assessed by the broth dilution method, and minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were subsequently determined [24].

2.7 Cytotoxicity assessment of green tea leaf stalk extracts on colorectal cancer cells

Cytotoxicity of tea leaf stalk extracts against HT-29 and Caco-2 cells was determined by the MTT assay [25], with NCM460 cells serving as a normal control. After 48 hours of treatment, MTT reagent was added and incubated for an additional 4 hours. Formazan crystals were solubilized in DMSO, and absorbance was recorded at 540 and 630 nm using a microplate reader. Cell viability was expressed as a percentage relative to control cells.

2.8 Statistical Analysis

Experiments were conducted independently in triplicate, and the results are expressed as mean ± SD. Statistical analysis between control and treatment groups was performed using Student's t-test and one-way ANOVA implemented in IBM SPSS Statistics 20.

3. Result and Discussion

3.1 The physical appearance of green tea leaf stalk extracts

Extraction of young and mature green tea leaf stalks (100 g each, dry weight) with 95% ethanol for 3 days yielded dark green extracts. After solvent evaporation and lyophilization,

the mature leaf stalk extract exhibited a higher yield (4.63%) than the young leaf stalk (4.13%) (Table 1).

Table 1. Percentage yields of green tea leaf stalk extracts.

Extracts	Color of extract	Yield (%)
Young green tea leaf stalk	Dark green	4.13
Mature green tea leaf stalk	Dark green	4.63

3.2 Total phenolic compounds in green tea leaf stalk extracts

The total phenolic content of green tea leaf stalk extracts, measured by the Folin–Ciocalteu method, was significantly higher in the young extract (97.21 ± 0.017 mg GAE/g extract) than in the mature extract (71.92 ± 0.007 mg GAE/g extract) (Table 2). These findings are in agreement with Liu et al. (2020) [26], who demonstrated a decline in phenolic content with increasing leaf maturity. Comparable trends have also been reported for Assam tea (*C. sinensis* var. *assamica*), where young leaves contain approximately 1.2-fold higher phenolic levels than mature leaves [27].

3.3 Total flavonoids in tea leaf stalk extracts

The total flavonoid in green tea leaf stalks extract was analyzed using the aluminium chloride colorimetric method. The mature green tea leaf stalk extract exhibited a higher total flavonoids content (23.518 ± 0.024 mg quercetin/g extract) than the young green tea leaf stalk extract (19.162 ± 0.020 mg quercetin/g extract) (Table 2). Studies on flavonoids, especially flavanols, have demonstrated that mature tea leaves possess greater concentrations of cis-catechins (e.g., EGCG, ECG, and EGC) than young leaves, highlighting the impact of leaf maturity on catechin composition [26].

3.4 Antioxidant activity of tea leaf stalk extracts

Based on the IC_{50} values for DPPH radical scavenging activity, the young and mature green tea leaf stalk extracts showed values of 0.293 ± 0.009 mg/mL and 0.322 ± 0.012 mg/mL, respectively. The young leaf stalk extract exhibited stronger antioxidant activity, with a value of 37.599 ± 1.160 mg gallic acid equivalents (GAE)/g extract, followed by the mature leaf stalk extract (Table 2). These results indicate that the young green tea leaf stalk extract possesses stronger antioxidant activity than the mature extract, as evidenced by its lower IC_{50} value, reflecting a higher radical-scavenging capacity compared to the standard. This finding is consistent with Murdiono *et al*. (2025) [28] who reported that young tea leaves exhibit stronger antioxidant activity compared to mature leaves, which is reflected by their higher total phenolic content (TPC).

Table 2. Antioxidant activity, total phenolic content and flavonoid content of green tea leaf stalk extracts.

Antioxidant activity, total phenolic and flavonoid contents	Extracts	
	Young green tea leaf stalk	Mature green tea leaf stalk
Antioxidant activity (mg gallic acid/g extract)	37.599 ± 1.160*	34.179 ± 1.299
Total phenolic content (mg gallic acid/g extract)	97.21 ± 0.017*	71.92 ± 0.007
Total flavonoid content (mg quercetin /g extract)	19.162 ± 0.020	23.518 ± 0.024*

*The values are significantly different at $P < 0.05$. The results are presented as mean ± SD of triplicate independent experiments.

3.5 Phytochemical compounds in green tea leaf stalk by high performance liquids chromatography (HPLC)

HPLC analysis was conducted to quantify major compounds in green tea leaf stalk extracts. Four compounds including catechin, EGCG, caffeine, and theaflavin, the mature green tea leaf stalks, EGCG was the predominant compound (490.27 μg/ml), followed by catechin (20.74 μg/ml), while caffeine and theaflavin were below the quantification limit. Similarly, in young green tea leaf stalks, EGCG exhibited the highest concentration (220.68 μg/ml), followed by catechin at 12.44 μg/ml, whereas caffeine and theaflavin remained undetectable (Table 3). The present findings are in agreement with previous work by Lee et al. (2014) [29], which demonstrated that the timing of tea leaf harvest significantly influences the composition of bioactive compounds. Specifically, earlier harvests were associated with higher levels of catechin, gallocatechin gallate (GCG), caffeine, theobromine and theanine, whereas later harvests resulted in increased concentrations of epicatechin (EC), EGCG, and epigallocatechin (EGC). In addition, non-gallated catechins and total catechin content were reported to increase with delayed harvesting, while gallated catechins exhibited no clear or consistent trend.

3.6 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of tea leaf stalk extracts against colibactin-producing *E. coli*

The antibacterial activity of young and mature green tea leaf stalk extracts against colibactin-producing *E. coli* isolates was evaluated using the broth dilution method to determine MIC and MBC values. The tested isolates included BA1, BA2, CA5, HA2, and VA2, with *E. coli* ATCC 25922 used as a positive control strain. The young leaf stalk extract exhibited MIC and MBC values of 250 mg/mL against most isolates, except for BA1, which was inhibited at 125 mg/mL. In contrast, the mature leaf stalk extract showed greater antibacterial activity, with MIC and MBC values of 125 mg/mL for most isolates and 62.5 mg/mL for CA5 (Table 4). These findings are consistent with those reported by Ahmed et al. (2018) [30], who observed MIC and MBC values of approximately 200 mg/mL and 400 mg/mL, respectively,

for green tea leaf extracts against uropathogenic *E. coli*. Overall, the results suggest that mature tea leaf stalk extracts exhibit stronger antimicrobial activity, as indicated by their effectiveness at lower concentrations compared to conventional green tea leaf extracts.

Table 3. Quantification of catechin, EGCG, caffeine, and theaflavin in tea leaf-stalk extracts by HPLC assay.

Phytochemical compounds (µg/ml)	Young green tea leaf stalk extract	Mature green tea leaf stalk extract
Catechin	12.44 ± 0.13	20.74 ± 0.71*
Epigallocatechin gallate (EGCG)	220.68 ± 17.14	490.27 ± 14.13*
Caffeine	ND	ND
Theaflavin	ND	ND

*The values are significantly different at $P < 0.05$. The results are presented as mean ± SD of triplicate independent experiments. ND; not detected.

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) value of green tea leaf stalk extracts against colibactin-producing *E. coli*.

MIC/MBC (mg/ml) of *E. coli* isolates*		Young green tea leaf stalk extract	Mature green tea leaf stalk extract
E. coli ATCC 25922	MIC	250	125
	MBC	250	125
E. coli BA1	MIC	125	125
	MBC	125	125
E. coli BA2	MIC	250	125
	MBC	250	125
E. coli CA5	MIC	250	62.5
	MBC	250	62.5
E. coli HA2	MIC	250	125
	MBC	250	125
E. coli VA2	MIC	250	125
	MBC	250	125

*The results are presented as mean ± SD of triplicate independent experiments.

3.7 Cytotoxicity of tea leaf stalk extracts on colorectal cancer cells

Cytotoxicity of the extracts toward HT-29 and Caco-2 cells was assessed relative to NCM460 normal intestinal cells. After 48 hours of treatment, the mature green tea leaf stalk extract exhibited no cytotoxicity toward NCM460 cells at 0.156 mg/ml, showing 100.45 ± 10% cell viability, while the viabilities of HT-29 and Caco-2 cells were 68.49 ± 10.9% and 57.85 ± 7.1%, respectively. Comparison of NCM460 normal intestinal cell viability showed that mature green tea stems were more effective than young stems, as they maintained higher cell survival at lower extract concentrations. In addition, the young green tea leaf stalk extract at

the concentration of 0.625 mg/ml showed a markedly higher toxicity on colorectal cancer cells of HT-29 and Caco-2 cells, while also toxic on NCM460 normal intestinal cells that exhibited the cell viability of 5.14 ± 1.35%, 9.07 ± 4.67%, and 28.08 ± 4.67% cells, respectively (Fig. 1 and 2). The enhanced activity observed in mature stems may be attributed to their higher content of flavonoids, catechins, and EGCG, as well as their greater antioxidant capacity. Notably, EGCG has been shown to exert anti-proliferative and anti-migratory effects on colorectal cancer cell lines, including SW480, SW620, and LS411N, through the downregulation of STAT3, highlighting its potential as a natural adjuvant for colorectal cancer therapy [31]. The anticancer effects of flavonoids are associated with their ability to regulate ROS-scavenging systems, induce cell cycle arrest, and promote apoptosis and autophagy, thereby suppressing cancer cell proliferation and invasion [32]. The enhanced cytoprotective and anticancer effects observed in mature green tea leaf stalks may be attributed to their higher levels of bioactive compounds compared to young leaf stalks.

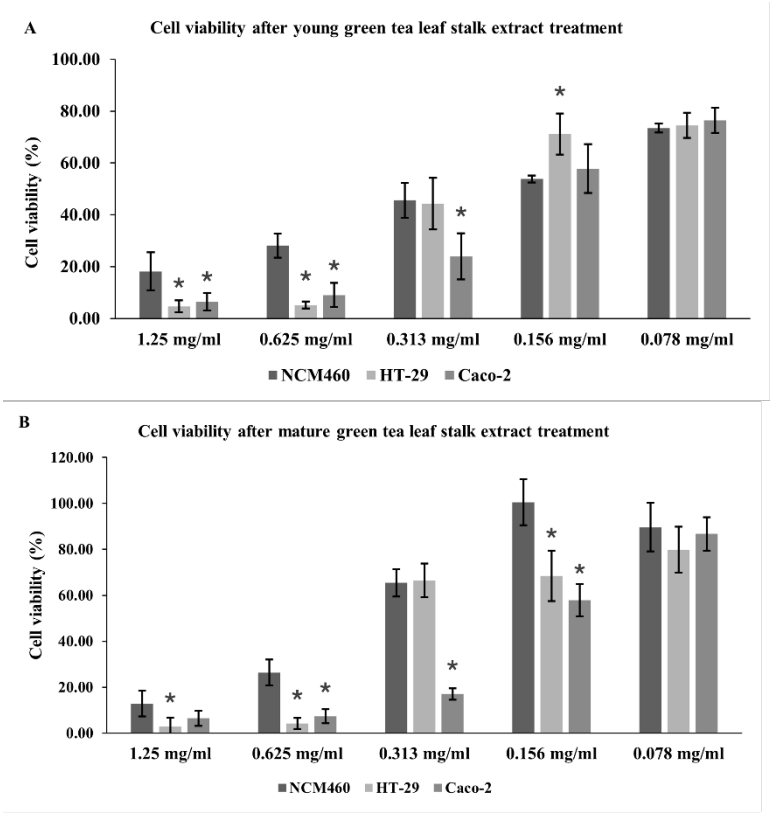

Fig. 1. (A) Comparison of cell viability between normal intestinal cells (NCM460) and colorectal cancer cells (HT-29 and Caco-2) after treatment with young green tea leaf stalk extracts. * Indicates significant difference at $P < 0.05$ compared with NCM460 cells. (B) Comparison of cell viability between normal intestinal cells (NCM460) and colorectal cancer cells (HT-29 and Caco-2) after treatment with mature green tea leaf stalk extracts. * Indicates significant difference at $P < 0.05$ compared with NCM460 cells.

3.8 Half Maximal Inhibitory Concentration (IC_{50}) of tea leaf stalk extracts against colorectal cancer cells

The cytotoxic effects of green tea leaf stalk extracts on HT-29 and Caco-2 colorectal cancer cells were assessed using the MTT assay. The mature leaf stalk extract exhibited significant inhibitory activity, with IC_{50} values of 0.402 ± 0.029 mg/mL for HT-29 cells and 0.195 ± 0.028 mg/mL for Caco-2 cells. Similarly, the young leaf stalk extract also demonstrated cytotoxic effects, with IC_{50} values of 0.627 ± 0.044 mg/mL and 0.218 ± 0.035 mg/mL for HT-29 and Caco-2 cells, respectively (Table 5). Overall, both extracts showed greater inhibitory effects against Caco-2 cells compared to HT-29 cells, indicating higher sensitivity of Caco-2 cells. This observation is consistent with previous reports demonstrating differential sensitivity of these cell lines to 5-fluorouracil (5-FU), where lower IC_{50} values were observed in Caco-2 cells (353.4 ng/mL) than in HT-29 cells (543.3 ng/mL) [33]. Furthermore, a comparison between extracts revealed that the mature leaf stalk extract exhibited stronger cytotoxic activity against HT-29 cells than the young extract, as indicated by its lower IC_{50} value (0.402 ± 0.029 mg/mL vs. 0.627 ± 0.044 mg/mL). Similarly, for Caco-2 cells, the IC_{50} values were 0.218 ± 0.035 mg/ml for young green tea leaf stalks and 0.195 ± 0.028 mg/ml for mature green tea leaf stalks, also showing a significant difference. These results indicated that mature green tea leaf stalks exhibited greater cytotoxicity toward HT-29 and Caco-2 cells than young green tea leaf stalks, as they achieve 50% cell growth inhibition at lower concentrations. This enhanced activity is likely due to the higher contents of flavonoids, antioxidant activity, catechins, and EGCG in mature stalks. Previous *in vitro* and *in vivo* studies have demonstrated that green tea catechins (GTCs) exert anticancer effects on gastric and colorectal cancers by inhibiting tumor angiogenesis, DNA methylation, cancer cell proliferation, and by promoting programmed cell death. Specifically, EGCG plays a crucial role in suppressing cancer cell formation and invasion through modulation of cellular signaling pathways. Additionally, previous studies have been reported that EGCG has been shown to induce G1 cell cycle arrest, regulate the phosphorylation of GSK-3 and PP2A, and suppress the activation of EGFR, HER2, and the Wnt/β-catenin signaling pathway [34,35]. However, these mechanisms were not directly investigated in our study and should be confirmed in future work.

Table 5. IC_{50} values of green tea leaf stalk extracts on HT-29 and Caco-2 cells

Cancer cells	IC_{50} of the extracts (mg/ml)	
	Young green tea leaf stalk	Mature green tea leaf stalk
HT-29 cells	$0.627 \pm 0.044^{a,A}$	$0.402 \pm 0.029^{a,B}$
Caco-2 cells	$0.218 \pm 0.035^{b,A}$	$0.195 \pm 0.028^{b,B}$

[a,b] The values were significantly different between the IC_{50} of HT-29 and Caco-2 cells ($P < 0.05$).
[A,B] The values were significantly different between the extracts ($P < 0.05$).

4 Conclusions

Both young and mature green tea leaf stalk extracts demonstrated notable antioxidant, antibacterial, and anticancer activities. The mature green tea leaf stalk extract contained higher levels of flavonoids, catechins, and EGCG, leading to greater antioxidant and cytotoxic effects. Both extracts exhibited significant inhibitory effects against colibactin-producing *E. coli* and reduced the viability of HT-29 and Caco-2 colorectal cancer cells, with the mature green tea leaf stalk extract demonstrating greater potency. Overall, green tea leaf stalks represent a promising natural candidate for colorectal cancer prevention and adjunct

therapy. Nonetheless, further studies involving molecular mechanisms and in vivo models are essential to substantiate their therapeutic potential and safety.

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