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Research Article

METABOLITE PROFILING, CYTOTOXICITY, AND PRO-APOPTOTIC EVALUATION OF *CINNAMOMUM MYRIANTHUM* MERR. IN HT-29 COLORECTAL CANCER CELLS

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ABSTRACT

Objective: To evaluate the metabolite profile as well as the cytotoxic and pro-apoptotic activities of *Cinnamomum myrianthum* on HT-29 colorectal cancer (CRC) cells *in vitro*.

Methods: The cytotoxic effects of *C. myrianthum* crude extract (CmCE) and its fractions were assessed against HT-29 CRC cells using the WST-8 assay. The half-maximal inhibitory concentration (IC_{50}) and selectivity index (SI) were determined. The most bioactive fraction was further subjected to untargeted metabolite profiling using ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry. Apoptosis induction was analyzed through Annexin V-FITC/PI staining through flow cytometry, while caspase-3/7 activation was measured using the Caspase-Glo® 3/7 luminescence assay.

Results: CmCE exhibited moderate cytotoxic activity against HT29 CRC cells with an IC_{50} value of 32.7 µg/mL ($p<0.001$). Among the tested fractions (Cm1A-Cm1E), Cm1C demonstrated the most potent cytotoxic effect, with an IC_{50} of 25.7 µg/mL ($p< 0.001$) and a SI of 3.63. Flow cytometry analysis revealed that Cm1C significantly induced late-stage apoptosis in HT29 cells. In addition, Cm1C markedly increased caspase-3/7 activity with 2.7-fold ($p<0.001$) at higher concentration, indicating activation of the apoptotic pathway. Metabolite profiling of Cm1C identified several polyphenolic compounds, including cinnamtannin B1, rutin, hyperin, and kaempferol glycosides.

Conclusion: These findings indicated that Cm1C, a polyphenol-rich fraction of *C. myrianthum*, exhibited selective cytotoxicity against HT29 CRC cells and induced apoptosis through caspase-3/7 activation. This fraction shows promise as a potential natural candidate for CRC therapy.

Keywords: Apoptosis, *Cinnamomum myrianthum*, Colorectal cancer, Cytotoxicity, Metabolomics.

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INTRODUCTION

Colorectal cancer (CRC) has emerged as a significant global health concern because of its high incidence and mortality rates [1]. The number of cases and deaths will increase substantially by 2040 [2,3]. According to a 2022 report, CRC is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide [1]. In that year alone, more than 1.93 million new CRC cases were reported, representing 9.6% of all cancer diagnoses, along with nearly 900,000 deaths, or 9.3% of all cancer-related fatalities [1]. In this context, the National Cancer Institute has called on the scientific community to rediscover the exploration of natural products as sources of novel anticancer agents from the rich biodiversity found worldwide [4]. This perspective underscores the untapped potential of bioresources for developing safer and more effective cancer treatments.

The Philippines is one of the 18 mega-biodiverse countries in the world, ranking fifth in plant species. It contains about 5% of the world's flora, including over 6,000 endemic species [5,6]. As the demand for natural product-based drug discovery and development increases, the need to conserve biodiversity becomes critical. The Convention on Biological Diversity supports the sustainable use of biological resources, particularly through bioprospecting [6]. Historically, humans have relied on natural resources, such as plants, for medicinal purposes due to their rich natural products, particularly secondary metabolites, such as flavonoids, tannins, and terpenoids [7,8]. Ancient civilizations, including the Native Americans, Chinese, and Indians, utilized plant-based remedies to address various health issues [9]. From 2900 BC, the Ebers Papyrus documented over 700 medicinal substances from nature, showcasing the early exploration of natural products for health benefits [10]. Many indigenous and endemic plants in the Philippines remain unexplored for their medicinal value, particularly in cancer research. The Philippine endemic *Cinnamomum* species are a promising source for medical applications.

The genus *Cinnamomum* is a fragrant medicinal herb known for its therapeutic benefits due to its vitamins, micronutrients, and antioxidants [11]. Twenty-five indigenous species exist in the Philippines, and *Cinnamomum myrianthum* is found only in Northwest Luzon [12]. Traditional uses include treating skin conditions, diabetes, pain, and gastrointestinal issues [13,14]. Recent studies show that cinnamon extracts can inhibit cancer cell growth, with cinnamaldehyde promoting apoptosis and reducing inflammation [15,16].

Given the limited access to effective, affordable, and targeted therapies for CRC with present treatments often being costly, non-specific, and associated with adverse effects, natural products have gained renewed interest for their therapeutic potential and lower toxicity [17]. Despite the Philippines being a recognized biodiversity hotspot, many of its endemic plant species, including *C. myrianthum*, remain underutilized and unexplored. To date, there are no published studies reporting the phytochemical composition or pharmacological properties of *C. myrianthum* based on available literature. This gap is especially critical, as habitat loss and human activities threaten the extinction

of potentially valuable medicinal plants. Hence, this study aimed to investigate the metabolite profile and *in vitro* cytotoxic and pro-apoptotic potential of *C. myrianthum* against CRC cells.

METHODS

Collection and preparation of the plant material

Two kilograms of mature leaves of the endemic *C. myrianthum* were collected from a forest terrain in Pasuquin, Ilocos Norte. A herbarium specimen (Herbarium of Northwest Luzon [HNUL] 0020807) was prepared and deposited at the HNUL, Ilocos Norte, Philippines. The plant was taxonomically identified, and a certificate of identification was issued.

The collected *C. myrianthum* leaves were rinsed thoroughly with running tap water to remove surface debris and contaminants, then air-dried under shade in a well-ventilated area for 24 h. Afterward, the leaves were oven-dried using a hot air oven (Memmert, Germany) at a temperature not exceeding 40°C to preserve thermolabile compounds. The dried leaves were then ground into a fine powder using a mechanical herbal grinder and stored in an airtight container until extraction.

Extraction process

The *C. myrianthum* crude extract (CmCE) was obtained through cold maceration. Specifically, the resulting powder from *C. myrianthum* leaves was soaked in absolute ethanol, a solid-to-liquid ratio of 1:6 (w/v) for 72 h, with occasional shaking, in a low-light area at room temperature. After the initial extraction, the mixture was filtered through muslin cloth to obtain the first filtrate. The residual plant material (marc) in the first stage was then re-macerated with fresh solvent under the same conditions for 72 h and filtered to obtain the second filtrate. The process was repeated once again to produce the third filtrate. All three filtrates collected were further subjected to filtration using a Whatman filter paper (Grade no. 2). The resulting filtrates were concentrated under reduced pressure at a temperature not exceeding 40°C using a rotary evaporator (Heidolph, Germany) and further concentrated using a speed vacuum (Eppendorf, Germany) to afford a dried CmCE. The percentage yield of the crude extract was computed using the following formula:

$$\text{Yield}(\%) = \frac{\text{weight of the extract after evaporating the solvent}}{\text{dry weight of the sample}} \times 100$$

Solvent partitioning and fractionation

The CmCE was suspended in distilled water (H_20) and partitioned with diethyl ether (Et_2O) using a separatory funnel to separate non-polar components. The resulting aqueous fraction was subjected to adsorbent open-column chromatography. The CmCE passed through an open column packed with macroporous resin (Diaion™ HP20, Mitsubishi Chemical Corp., Tokyo, Japan), and then it was successively eluted with appropriate solvent systems (H_2O, 20% MeOH, 60% MeOH, 100% MeOH, acetone) to afford five fractions, Cm1A through Cm1E. All fractions except Cm1A were concentrated *in vacuo* at not exceeding 40°C using a rotary evaporator (Eyela, Tokyo, Japan), further concentrated on a water bath sample concentrator (Biolab Scientific, Canada), and dried under a vacuum desiccator. Cm1A was freeze-dried using a lyophilizer (Eyela, Tokyo, Japan). The resulting fractions were stored at 4°C in airtight containers until further analysis. The percentage yield of the subsequent fractions was computed using the following formula:

$$\text{Yield}(\%) = \frac{\text{weight of the fraction after evaporating the solvent or freeze drying}}{\text{dry weight of the sample}} \times 100$$

Cell culture and maintenance

HT29 human colorectal adenocarcinoma cells (ECACC; catalog no. 91072201, Salisbury, UK) were cultured in McCoy's 5A medium (Gibco™, Thermo Fisher Scientific K.K., Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco™, Thermo Fisher Scientific K.K., Tokyo, Japan) and 1% (v/v) Antibiotic-Antimycotic Mixed Stock Solution (10,000 U/mL penicillin and 10,000 µg/mL streptomycin, 25 µg/mL Amphotericin B) (NacalaiTesque, Inc., Kyoto, Japan). Mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's Modified Eagle Medium (FUJIFILM, Wako Pure Chemical Corporation, Osaka, Japan) supplemented with the same concentrations of FBS and antibiotics. Both cells were kept in a humidified incubator (PHCbi, PHC Corporation, Japan) at 37°C with 5% CO_2. When cultures reached 80–90% confluence, cells were passaged by first rinsing with sterile phosphate-buffered saline (PBS) (FUJIFILM, Wako Pure Chemical Corporation, Osaka, Japan), followed by incubation with Accutase® (NacalaiTesque, Inc., Kyoto, Japan) solution to detach the monolayer. After detachment, the enzyme activity was neutralized by adding an equal volume of complete medium. Detached cells were centrifuged to form a pellet, resuspended in fresh medium, and seeded into new culture flasks for continued maintenance.

Cytotoxicity assay using WST-8

The cytotoxicity of the CmCE and its fractions against HT-29 human colorectal adenocarcinoma cells was measured using the WST-8 Cell Count Reagent SF (NacalaiTesque, Inc., Kyoto, Japan), which contains a water-soluble tetrazolium (WST) salt, according to the manufacturer's instructions and a previous protocol [18].

In detail, HT-29 cells were seeded in 96-well flat-bottom microplates at a density of 1×10^4 cells per well in 100 µL of complete McCoy's 5A medium supplemented with 10% FBS and 1% Antibiotic-Antimycotic Mixed Stock Solution. After an overnight incubation at 37°C to allow cell attachment, 10 µL of the test compounds were added to each well. The test compound was prepared at a stock concentration of 10 mg/mL and serially diluted fivefold to obtain final concentrations of 500 µg/mL, 100 µg/mL, 20 µg/mL, and 4 µg/mL. Irinotecan (IRI) used as a positive control for cytotoxicity. It was prepared at a stock concentration of 2000 µM, followed by five-fold serial dilutions to yield final concentrations of 100 µM, 20 µM, 4 µM, and 0.8 µM. Ethanol at 10% (v/v) was used as a vehicle control. Blank wells, containing only culture medium, test compound, and Cell Count Reagent SF (without cells), were included to correct for background absorbance. Following treatment, plates were incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. After incubation, 10 µL of Cell Count Reagent SF was added directly to each well. The plate was again incubated for another 2 h to allow the formazan dye to develop. Absorbance was measured at 450 nm using a microplate spectrophotometer (Corona Absorption Grating Microplate Reader SH-1300Lab, Corona Electric Co., Ltd., Ibaraki, Japan). The absorbance values were used to calculate relative cell viability, expressed as a percentage of the untreated control group. Three replicate wells were used per concentration in three independent trials.

The half-maximal inhibitory concentration (IC_{50}) values for the CmCE and its fractions and positive control (IRI) were determined using GraphPad Prism 10 (version 10.4.2; GraphPad Software, San Diego, CA, USA) based on a three-parameter non-linear regression model. Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test for comparisons against the control group.

Determination of the selectivity index (SI) of the most bioactive fraction of *C. myrianthum*

The SI was calculated to evaluate the specificity of Cm1C toward cancer cells compared to normal cells. Cytotoxicity assays were performed on both HT-29 and MEF cells under identical conditions using the WST-8 assay, as previously described. IC_{50} values for the Cm1C were determined independently for each cell line using GraphPad Prism through a three-parameter non-linear regression model. The SI was calculated using the following formula:

$$\text{Selectivity Index}(\text{SI}) = \frac{\text{IC}_{50}\,(\text{MEF cells})}{\text{IC}_{50}\,(\text{HT29 cells})}$$

Untargeted ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UHPLC-QTOF-MS/MS)-based metabolite profiling of the most bioactive fraction of *C. myrianthum*

For the chromatographic analysis, 1 mg of Cm1C was dissolved in 1 mL of LC-MS grade methanol. The sonication process was used for 10 min, and the solution was filtered through a 0.2 µm polyvinylidene fluoride membrane syringe filter into a 1 mL LC autosampler vial.

The metabolite profiling of Cm1C was performed using a Shimadzu LCMS-9050 Q-TOF mass spectrometer equipped with a Nexera™ UHPLC system. Chromatographic separation was achieved using a Shim-pack Scepter Diol-HILIC-120, 1.9 µm, 2.1 × 100 mm, maintained at 40°C. The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), with a flow rate of 250 µL/min. The gradient elution was programmed as follows: 0.0–1.0 min (95% B), 1.0–8.0 min (50% B), 8.0–12.0 min (40% B), 12.0–14.0 min (30% B), 14.0–15.0 min (30% B), 15.0–16.0 min (95% B), and 16.0–22.0 min (95% B) for column re-equilibration. The injection volume was set at 1 µL. Mass spectrometric detection was conducted using a heated electrospray ionization interface in positive and negative ion modes. The interface, desolvation line, and heat block temperatures were set at 300°C, 250°C, and 400°C, respectively. The nebulizing, heating, and drying gas flows were maintained at 3 L/min, 10 L/min, and 10 L/min, respectively. Full-scan MS data were acquired over an m/z range of 100–1000, while data-dependent acquisition (DDA) was performed to acquire MS/MS data over m/z 100–1000 with a collision energy of 30 V (±25 V) and a loop time of 0.2 s.

The DDA data file was imported into the Analyze pane window of the software platform. Both MS and MS/MS options were selected to ensure that all spectra generated under the pre-defined triggering conditions were included. Upon analysis, the software generated a list of all pre-cursor ions, each associated with a corresponding DDA spectrum. A screening step was then applied using a pre-defined compound target list, which enables the identification of potential hits. These hits and all detected pre-cursors were displayed in the Pre-cursor Pane, where each pre-cursor's extracted ion chromatogram, retention time, and corresponding DDA spectrum can be reviewed. Individual DDA spectra of interest were then selected and subjected to spectral library matching using the National Institute of Standards and Technology (NIST) 2023 mass spectral database for compound identification.

Apoptosis assay using Annexin V-FITC/propidium iodide (PI) staining and flow cytometry

Apoptotic cell death was evaluated using the Annexin V-FITC Apoptosis Detection Kit (NacalaiTesque, Kyoto, Japan), following the manufacturer's instructions and previously described protocols [19-21]. In detail, HT-29 cells (1×10^5) were seeded in 6-well plates and exposed to the test compounds (IC_{50} values of Cm1C and IRI) for 24 h under standard culture conditions (37°C, 5% CO_2). After treatment, both adherent and non-adherent cells were collected using Accutase. Following detachment, cells were pooled, centrifuged at 200 × g for 5 min at 4°C, and washed twice with cold PBS. The resulting pellets were resuspended in 100 µL of Annexin V binding buffer. Each sample was stained by adding 5 µL of Annexin V-FITC and 5 µL of PI solution, followed by gentle vortexing and incubation in the dark at room temperature for 15 min. After staining, 400 µL of binding buffer was added to each tube before flow cytometric analysis. Test samples were analyzed using the Attune NxT Flow Cytometer (Invitrogen, USA) with a 488 nm blue laser. A minimum of 10,000 events was acquired per sample. The percentage of each cell population was calculated from the total gated events. An unstained control was included for gating accuracy. All assays were performed in triplicate, and two independent trials were conducted. Two-way ANOVA was performed with treatment and apoptosis category as factors, followed by Sidak's multiple comparisons test.

Caspase-3/7 activity assay using Caspase-Glo® 3/7 kit

Caspase-3/7 activity was assessed using the Caspase-Glo 3/7 Assay Kit (Promega, USA), a luminescence-based assay designed to quantify caspase activity related to apoptosis, following the manufacturer's instructions and previously described methods [22,23]. HT-29 cells were seeded in white, opaque 96-well plates at a density of 1×10^4 cells per well in 100 µL of McCoy's 5A medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were incubated overnight at 37°C with 5% CO_2 to allow proper adhesion. After 24 h of treatment with the test compounds, 100 µL of Caspase-Glo® 3/7 reagent was added to each well. After adding the reagent, the plate was gently mixed on an orbital shaker at 300 rpm for 30 s. It was then incubated in the dark at room temperature for 30 min to stabilize the luminescent signal. Luminescence was measured using a SpectraMax® iD5 Multi-Mode Microplate Reader (Molecular Devices, USA) in luminescence detection mode, without filters, using an integration time of 0.5–1.0 s per well. Wells containing only medium and reagent (no cells) served as blanks to account for background luminescence and were subtracted from sample readings. Caspase activity was reported as relative luminescence units, normalized to untreated control wells. Each experimental condition was assayed in triplicate and repeated across two independent biological experiments, with results expressed as mean ± standard error mean. Statistical significance was determined using one-way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 10.4.2 (GraphPad Software, San Diego, CA, USA). For cytotoxicity testing, IC_{50} values of the crude extract (CmCE), its fractions, and the positive control (IRI) were calculated using a three-parameter non-linear regression model, and statistical significance was evaluated using one-way ANOVA followed by Dunnett's *post hoc* test for comparisons with the untreated control. For the apoptosis assay, two-way ANOVA was conducted with treatment and apoptosis category as factors, followed by Sidak's multiple comparisons test. For caspase-3/7 activity analysis, statistical differences among groups were determined using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.

RESULTS AND DISCUSSION

Percentage yield obtained from CmCE and its subsequent fractions

Fig. 1 illustrates the percentage yield obtained at each stage of the extraction and fractionation process from powdered leaves of *C. myrianthum*. The schematic diagram highlights mass distribution across the crude ethanolic extract and subsequent solvent fractions.

The fractionation of *C. myrianthum* leaves was designed to obtain polar bioactive compounds through a polarity-based method. Although the diethyl ether phase had a higher yield in the initial biphasic separation, the aqueous phase was prioritized due to its potential to concentrate hydrophilic metabolites with known therapeutic importance. This approach aligns with previous studies on *Cinnamomum* species, where polar compounds, particularly flavonoids, polyphenols, tannins, and glycosides, are linked to significant biological activity, such as anticancer [8]. Five sub-fractions (Cm1A–Cm1E) were successfully obtained from the aqueous extract. Cm1C was the most abundant among these, accounting for over 40% of the aqueous fraction. The substantial yield of Cm1C suggests that it may contain a significant portion of the water-soluble secondary metabolites present in the original extract, positioning it as a promising candidate for further bioactivity evaluation.

Cytotoxic potential of the crude extract and fractions of *C. myrianthum* against HT-29 cancer cells

This study evaluated the cytotoxic effects of CmCE and the reference drug IRI *in vitro* against HT-29 CRC cells. As shown in Table 1, both treatments produced a concentration-dependent reduction in cell viability. Treatment with CmCE showed minimal cytotoxicity at

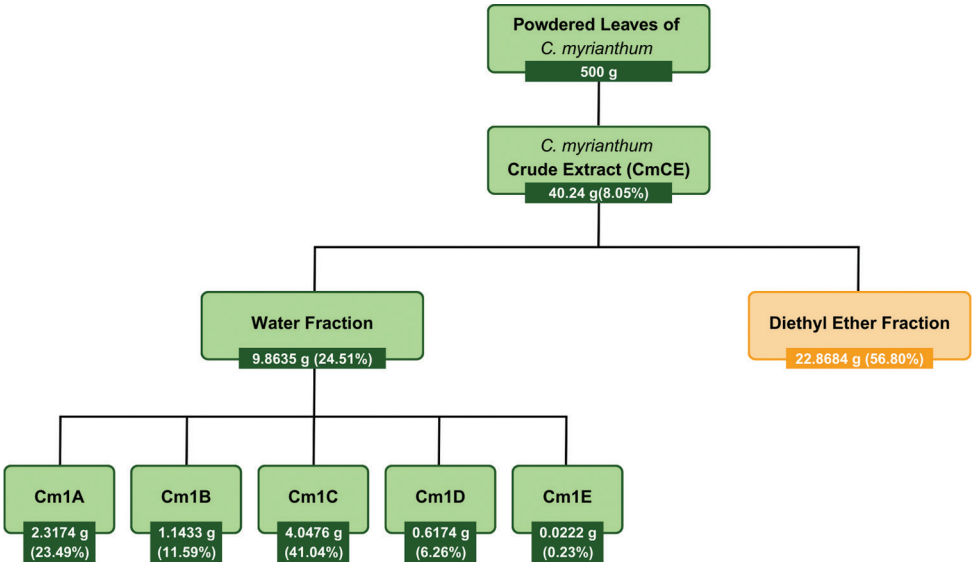

Fig. 1: Schematic representation of extraction and fractionation yields from powdered leaves of *Cinnamomum myrianthum*

Table 1: Concentration-dependent cytotoxic activity of CmCE (μg/mL) and irinotecan (μM) on HT-29 colorectal cancer cells expressed as mean percentage viability±standard error mean from n=3 independent experiments (each with 3 replicates)

CmCE Conc. (ug/mL)	% Viability	Irinotecan (μM)	% Viability
0 (control)	100	0 (control)	100
4	93.91±5.47	0.8	83.85±3.51*
20	54.28±5.06***	4	52.83±3.76***
100	32.97±3.13***	20	44.27±6.00***
500	2.95±0.83***	100	17.26±2.72***

Statistical significance from the one-way ANOVA, Dunnett's *post hoc* test: *p<0.05, ***p<0.001 versus control group, CmCE: *Cinnamomum myrianthum* crude extract

4 μg/mL (93.91±5.47%) but induced pronounced cell death at higher concentrations. Similarly, IRI reduced viability from 83.85±3.51% at 0.8 μM to 17.26±2.72% at 100 μM, demonstrating strong cytotoxic effects at elevated doses.

The IC_{50} values and corresponding cytotoxicity classifications for CmCE and IRI against HT-29 cells are summarized in Table 2. CmCE exhibited an IC_{50} of 32.70 μg/mL, which, based on the criteria established by the U.S. National Cancer Institute (NCI), falls within the moderate cytotoxicity range (IC_{50} 30–100 μg/mL) [24]. In comparison, IRI demonstrated a stronger cytotoxic response, with an IC_{50} of 8.23 μM.

These findings are consistent with studies of other *Cinnamomum* species, which also show marked antiproliferative effects on colon cancer cells in crude extracts and preparations rich in essential oil. Notably, a study reported that a cinnamaldehyde-rich cinnamon extract reduced cell viability in HT-29 and HCT116 cell lines, with IC_{50} values ranging from 9.3 to 18.2 μg/mL, indicating potent cytotoxic effects in a time- and concentration-dependent manner [25]. Similarly, Park *et al.* [26] found that hot water extracts from *Cinnamomum cassia* twigs significantly decreased the viability of HCT116 and SW480 CRC cells through apoptosis induction, with concentration-dependent reductions in metabolic activity confirmed by MTT assays.

The most cytotoxic fraction of *C. myrianthum* against HT-29 cancer cells

The cytotoxic effects of five fractions of *C.myrianthum* (Cm1A to Cm1E) on HT-29 CRC cells were examined using a dose-response viability

Table 2: Comparative IC_{50} values and cytotoxicity profiles of CmCE and IRI against HT-29 colorectal cancer cells after 24-h exposure

Sample	IC_{50} (μg/mL or μM)	95% CI	Interpretation
CmCE	32.70	22.65-47.36	Moderate Cytotoxicity
IRI	8.23***	4.19-16.28	Strong Cytotoxicity

***Statistically significant difference in logIC_{50} compared to CmCE (nonlinear regression analysis, p<0.001), CmCE: *Cinnamomum myrianthum* crude extract, IRI: Irinotecan

assay. The bar graph in Fig. 2 displays the percentage of cell viability after 24 h of treatment at various concentrations.

All tested fractions demonstrated a dose-dependent inhibition of cell viability, indicating that higher concentrations lead to increased toxicity. Cm1C showed the most potent effect, reducing cell viability to below 20% at the highest concentration. Cm1D and Cm1E followed a similar trend, though to a slightly lesser extent. In contrast, Cm1A showed the weakest effect, suggesting a lower potential for cytotoxic activity than the other fractions. The cytotoxic activity of five polar fractions (Cm1A to Cm1E) from CmCE was evaluated against HT-29 CRC cells. Dose-response data were analyzed using non-linear regression in GraphPad Prism 10, which generated individual IC_{50} values for each fraction.

The IC_{50} values derived from non-linear regression analysis are presented in Fig. 3. Among the different fractions, Cm1C showed the lowest IC_{50} value of 25.7 μg/mL, indicating strong cytotoxic activity against HT-29 CRC cells based on the NCI threshold (IC_{50} < 30 μg/mL) [20], which is lower than the IC_{50} value of CmCE.

These findings suggest that Cm1C is a potential lead fraction for further anticancer studies. A similar study was observed with polyphenolic fractions from *C. cassia* and *Cinnamomum zeylanicum*, where enriched fractions demonstrated stronger cytotoxic activity than the unfractionated extract [27]. Palmioli *et al.* [27] showed that polyphenol-enriched fractions had enhanced apoptotic and antiproliferative effects on CRC cells compared to crude hydroalcoholic extracts. They suggested using polyphenolic fractions from cinnamon extracts as adjuvants in CRC therapy.

On the other hand, our study focused on the aqueous fraction of *C. myrianthum* to highlight the potential bioactivity of polar compounds. Previous reports have shown that cinnamaldehyde-, polyphenol-, and flavonoid-rich fractions from *Cinnamomum* species

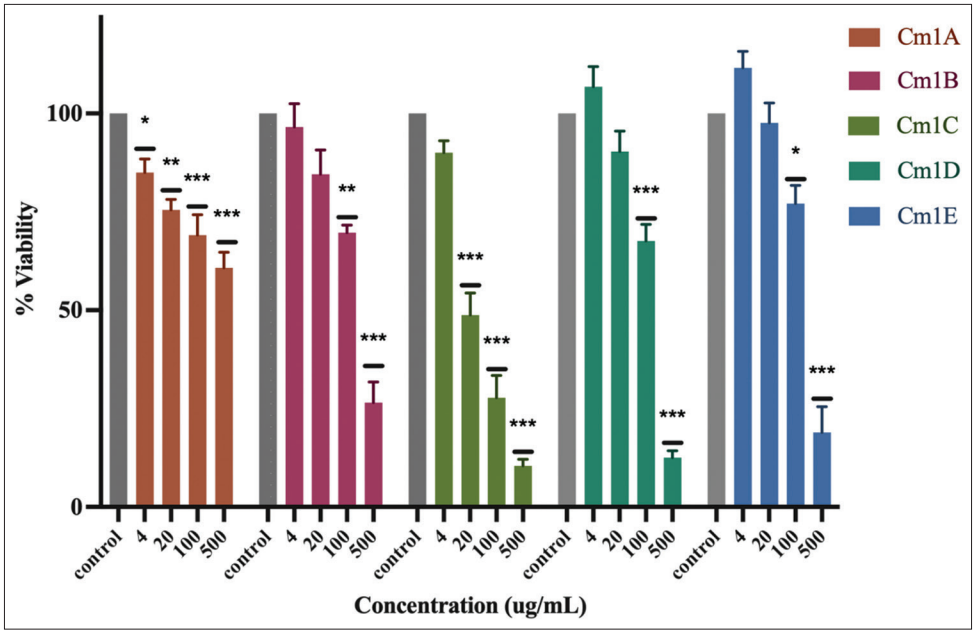

Fig. 2: Percent viability of HT-29 cells treated with varying concentrations (4–500 µg/mL) of Cm1A–Cm1E fractions by WST-8 Assay. Data are presented as mean±standard error mean from 3 independent experiments (each with technical triplicates). Statistical significance from the one-way analysis of variance, Dunnett's *post hoc* test: *p<0.05, **p>0.01, ***p<0.001 versus control group

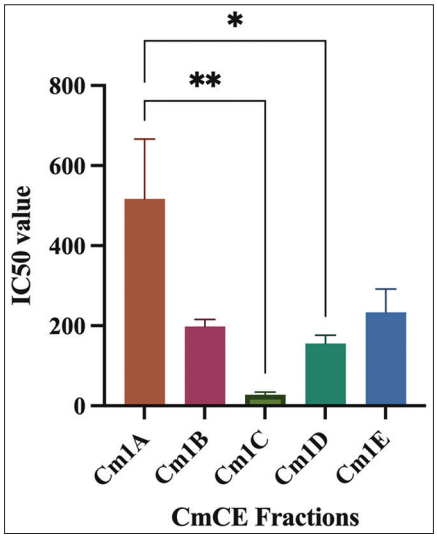

Fig. 3: Half-maximal inhibitory concentration values of *Cinnamomum myrianthum* fractions (Cm1A–Cm1E) against HT-29 colorectal cancer cells. Data are presented as mean±standard error mean from n=3 independent experiments (each with technical triplicates). Statistical significance from the one-way analysis of variance, Tukey's *post hoc* test: *p<0.05, **p<0.01 between groups

exhibit selective cytotoxicity, induce apoptosis, and inhibit metastasis in CRC models [25,27-29]. These studies reinforce the potential of polar phytochemicals as promising candidates in anticancer research.

SI of the most bioactive fraction, Cm1C (HT-29 cancer cells and MEF normal fibroblast cells)

The SI of Cm1C was obtained by comparing its IC_{50} values in HT-29 cancer cells and MEF normal fibroblast cells. Table 3 shows that Cm1C was more effective at killing cancer cells than normal cells, with an IC_{50} of 25.7 µg/mL for HT-29 cells and 93.32 µg/mL for MEF cells. This gave a SI of 3.63, which is above the threshold of 2.0 used to indicate selective toxicity [30,31].

Table 3: Selectivity index of Cm1C against HT-29 colorectal cancer cells and MEF normal cells

Cell line	IC_{50} (µg/mL)	Selectivity index
HT-29	25.70	3.63
MEF	93.32	

These results suggest its selective cytotoxicity and potential for therapeutic use. This aligns with earlier findings on cinnamon extracts, which have demonstrated preferential toxicity toward cancer cells. Wondrak *et al*. [32] found that cinnamic aldehyde and standardized cinnamon extracts helped protect normal colon cells while killing cancerous ones. Similarly, polyphenol-rich fractions from *C. cassia* selectively reduced the viability of CRC cells without significantly affecting normal colon epithelial cells, yielding favorable SIs of more than 2.0 [27,33].

UHPLC-QTOF-MS/MS-based metabolite profiling of Cm1C, the most bioactive fraction

Untargeted UHPLC-QTOF-MS/MS profiling of the Cm1C fraction yielded 137 hits in positive and 19 in negative ion modes. However, metabolites that matched the NIST database with an isotope score (ISO) of ≥85%, an accurate mass of ≤3 ppm, and an MS^2 spectrum of ≥70% similarity were assigned a level 2 confidence annotation and reported in this study [34-36]. In addition, compounds not biologically relevant or likely introduced as contaminants (e.g., polyethylene glycols or instrument-derived artifacts) were excluded.

Confidently annotated metabolites of the Cm1C fraction revealed a wide range of polar metabolites, such as flavonoid glycosides, anthocyanins, condensed tannins, and simple phenolics, as shown in Table 4. Notable compounds included cinnamtannin B-1 (precursor ion *m/z* 865.1974; RT 3.992 min) with characteristic fragments (*m/z* 301.0708, 533.1078, 575.1184), along with rutin, hyperin, narcissin, and 7-O-methylrutin (*m/z* 593–625; RT 2.7–6.9 min), all showing aglycone-based fragmentation patterns (quercetin, kaempferol). Two juncein isomers (*m/z* 449.1078) at RT 5.983 and 3.341 min suggested stereochemical or glycosidic differences. Cyanidin-3-β-glucoside (*m/z* 449.1078; RT 5.780 min) was confirmed by its diagnostic cyanidin fragment (*m/z* 287.0558). In addition, syringaldehyde (*m/z* 183.0652)

Table 4: Confidently annotated metabolites of the Cm1C fraction using UHPLC-QTOF-MS/MS

S. No.	Compound	Formula	Retention time (min)	Precursor m/z	Fragments m/z	Mass error (ppm)
		Positive Ion Mode				
1	Syringaldehyde	$C_9H_{10}O_4$	1.721	183.0652	95.0499 123.0452 155.0716	0.119
2	4H-1-Benzopyran-4-one, 3-[[6-O-(6-deoxy-L-mannopyranosyl) hexopyranosyl] oxy]-5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl-	$C_{28}H_{32}O_{16}$	2.704	625.1763	317.0654	0.272
3	7-O-Methylrutin	$C_{28}H_{32}O_{16}$	2.869	625.1763	317.0658 479.1192	0.720
4	Juncein	$C_{21}H_{20}O_{11}$	3.341	449.1078	85.0282 287.0546	0.000
5	2,8-Bis (3,4-dihydroxyphenyl)-4-(2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-8-yl)-3,4-dihydro-2H,14H-8,14-(Cinnamtannin-B1)	$C_{45}H_{36}O_{18}$	3.992	865.1974	301.0708 453.1181 533.1078 575.1184	0.069
6	Cyanidin-3-β-glucoside (cation)	$C_{21}H_{21}O_{11}$	5.780	449.1078	287.0558	0.089
7	Juncein	$C_{21}H_{20}O_{11}$	5.983	449.1078	85.0282 287.0546	0.000
8	Rutin	$C_{27}H_{30}O_{16}$	6.906	611.1607	303.0502 465.1039 611.1612	0.491
9	Hyperin	$C_{21}H_{20}O_{12}$	6.906	465.1027	91.0400 303.0500	0.839
		Negative Ion Mode				
10	Afzelin	$C_{21}H_{20}O_{10}$ (-)	1.529	431.0982	151.0038 284.0324 285.0401 327.0507	0.232
11	Narcissin	$C_{28}H_{32}O_{16}$(-)	2.917	623.1621	314.0414 315.0490	0.642
12	Kaempferol 3-rungioside	$C_{27}H_{30}O_{15}$ (-)	3.098	593.1515	285.0406	0.331
13	5,7-Dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-3-yl 6-O-(6-deoxyhexopyranosyl) hexopyranoside	$C_{27}H_{30}O_{15}$ (-)	3.360	593.1512	285.0403	0.068

All metabolites were annotated at confidence Level 2 (putative identification based on spectral similarity, without authentic standards or orthogonal validation), UHPLC-QTOF-MS/MS: Ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry

and afzelin (*m/z* 431.0982) were identified among the early-eluting polar phenolics.

The results demonstrate that the Cm1C fraction is enriched with polyphenolic compounds known for their promising anticancer properties. Cinnamtannin B-1 has been reported to induce apoptosis in colon cancer cells [37]. Rutin was shown to disrupt mitochondrial membrane potential, activate caspase pathways, and reduce the viability of CRC cells [38]. Hyperin (quercetin-3-O-galactoside) inhibited SW620 cell proliferation through activation of the p53 pathway [39]. Kaempferol derivatives, including afzelin, promote apoptosis, enhance antioxidant defenses, and suppress tumor growth [40]. Although direct evidence for narcissin and 7-O-methylrutin in CRC is limited, their structural similarity to active flavonoids suggests a supportive role in the bioactivity of the Cm1C fraction.

Although some polyphenolic compounds identified in this study have been previously reported for their anticancer properties, this is the first report of their presence and biological activity in *C. myrianthum*. As an underexplored local cinnamon species, *C. myrianthum* may exhibit a distinct phytochemical profile in which the collective effect of multiple polyphenolic constituents likely mediates the observed bioactivity. Future investigations should involve the isolation and structural elucidation of individual metabolites, followed by combinatorial assays to validate potential synergistic or additive effects. Such studies will not only substantiate the mechanistic basis of the bioactivity but also highlight the pharmacological relevance of underutilized endemic plant species as promising sources of therapeutic lead compounds.

Apoptosis detection of the polyphenolic fraction, Cm1C, via annexin V-FITC/PI dual staining assay

To investigate the pro-apoptotic potential of the polyphenolic fraction from *C. myrianthum*, an Annexin V-FITC/PI dual staining assay was used, based on its IC_{50} value. This assay is a widely validated method for characterizing cell death pathways by flow cytometry [28,29].

Based on Fig. 4, quantitative analysis of Annexin V-FITC/PI-stained HT-29 cells showed that treatment with Cm1C significantly increased the proportion of both early and late apoptotic cells compared to the control group ($p<0.05$). Cm1C induced a higher late apoptotic response than IRI, while both treatments significantly reduced the viable cell population. No significant differences were observed in necrotic populations across all treatments.

These results are consistent with previous studies. Nguyen and Kim [28] found that 2′-hydroxycinnamaldehyde increased apoptosis in SW480 and SW620 colon cancer cells in a dose-dependent manner, as shown by Annexin V-FITC flow cytometry. Similarly, Li *et al*. [29] observed that cinnamaldehyde significantly elevated apoptotic populations in SW480, HCT116, and LoVo cells after 24 h, confirming a consistent pro-apoptotic effect. Park *et al*. [26] further demonstrated increased apoptosis in HT-29 and other CRC cells following treatment with hot water extracts from *C. cassia* twigs. Kwon *et al*. [41] also reported similar findings in Caco2 cells, showing dose-dependent increases in early and late apoptosis following cinnamon extract treatment. More recently, Nile *et al*. [25] showed that a cinnamaldehyde-rich extract induced time- and dose-dependent apoptosis in HT-29 and

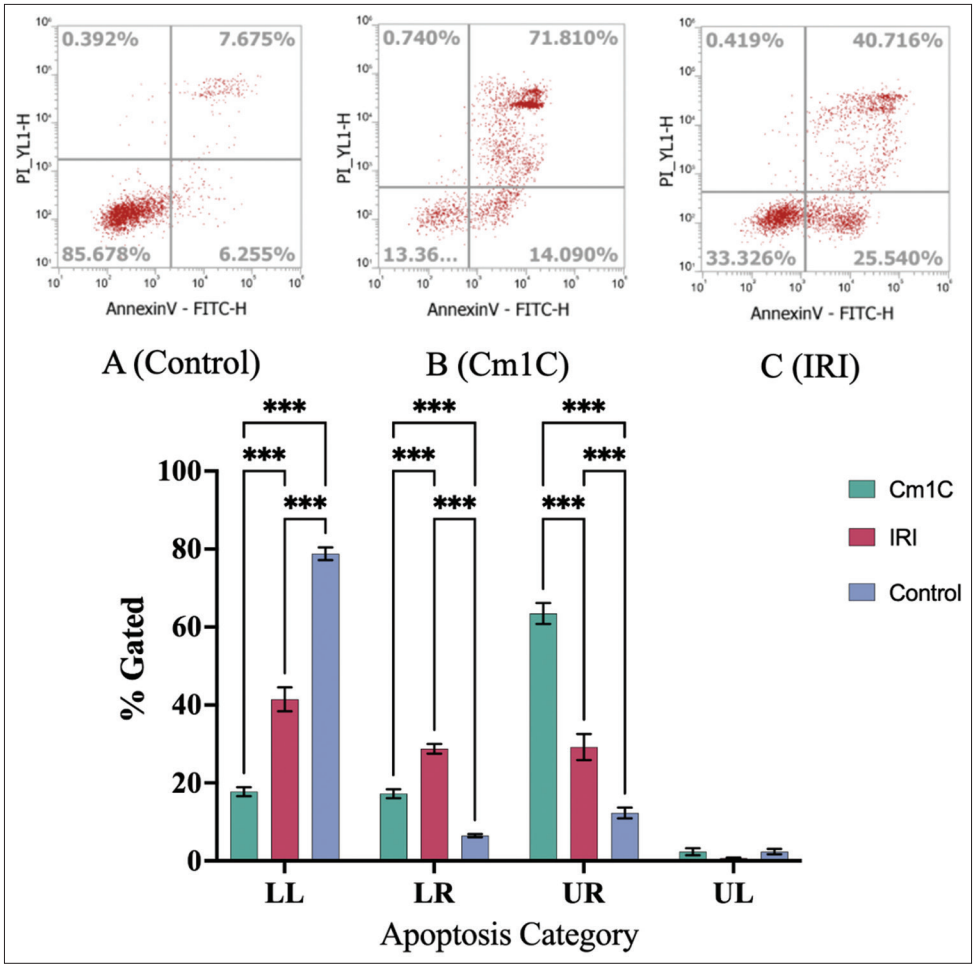

Fig. 4: Representative dot plots (a-c) and quantitative analysis (bar graph) of apoptosis in HT-29 cells treated with Cm1C, irinotecan (IRI), or control using Annexin V-FITC/PI staining. Cells were gated as live (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL). Statistical comparisons were performed with treatment (Cm1C, IRI, Control) and apoptosis category (LL, LR, UR, UL) as the two independent factors using two-way analysis of variance with Sidak's *post hoc* testing; ***p<0.001 (n=2 independent experiments with technical triplicates)

HCT116 cells, reinforcing the therapeutic relevance of *Cinnamomum* compounds in CRC therapy.

The alignment between these findings and the effects observed with the Cm1C fraction, which induced a controlled and selective apoptotic response in HT-29 cells with minimal necrosis, indicates that its cytotoxic activity is primarily mediated through programmed cell death, thereby supporting its potential as a therapeutic anticancer agent.

Assessment of apoptotic activity of Cm1C via caspase 3/7 activation

The Caspase 3/7 assay was used to investigate the pro-apoptotic effects of the experimental compound Cm1C compared to the standard chemotherapeutic agent IRI at various concentrations. As executioner caspases, caspase-3 and -7 serve as key indicators of apoptosis, with their enzymatic activity correlating with the extent of programmed cell death. In this context, the luminescence-based assay quantitatively measures caspase activation, normalized to untreated control cells.

The observed increase in caspase-3/7 activity following Cm1C treatment indicates activation of the execution phase of apoptosis in HT-29 CRC cells. Fig. 5 demonstrates that Cm1C treatment led to a 2.7-fold increase at 50 µg/mL, comparable to the 2.6-fold increase observed with IRI (16 uM). At lower concentrations, fold changes remained elevated for both treatments, ranging from 1.9 to 2.2. No significant differences were detected between Cm1C and IRI at any concentration. This dose-dependent response suggests that Cm1C facilitates programmed cell death through a caspase-dependent mechanism. The activation level

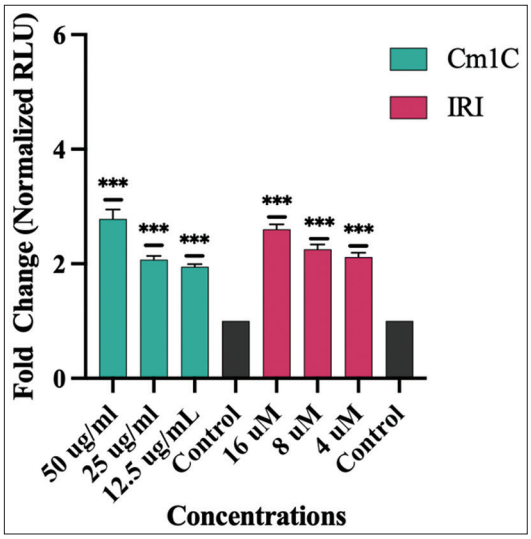

Fig. 5: Caspase 3/7 activity expressed as fold change in HT-29 cells treated with Cm1C and irinotecan across varying concentrations. Bars represent mean±standard error mean (n=2 independent experiments with technical triplicates). At each concentration, fold change was normalized to the corresponding untreated control. Statistical significance from the one-way analysis of variance, Dunnett's *post hoc* test: ***p<0.001 versus control group

was comparable to the standard chemotherapeutic agent IRI, which further supports the pro-apoptotic potential of Cm1C.

The induction of caspase 3/7 activity observed in response to Cm1C treatment in CRC cells is consistent with prior reports on *Cinnamomum* species and their phytoconstituents. Several bioactive components derived from *Cinnamomum verum* and *C. cassia* have been shown to activate intrinsic apoptosis by disrupting the mitochondria. Nile *et al*. [25] observed a 3.5-fold increase in caspase-3 activity in HCT-116 cells treated with a cinnamaldehyde-rich extract (40 µg/mL). At the same time, Banerjee and Banerjee [16] reported 3.2-fold caspase-3 and 2.9-fold caspase-7 activation with 50 µM cinnamaldehyde in HCT116 and SW480 cells. Similarly, Tsai *et al*. [42] demonstrated that cuminaldehyde, isolated from *C. verum*, induced a 2.8-fold increase in caspase-3/7 activity in COLO 205 cells, and Palmioli *et al*. [27] observed a 1.8-fold increase following treatment with polyphenolic fractions from cinnamon in SW480 cells.

CONCLUSION

The findings of this study demonstrate that Cm1C, a polyphenol-rich fraction from *C. myrianthum*, exhibits significant cytotoxic activity and induces apoptosis in HT-29 CRC cells through the activation of caspase-3/7. These results may suggest the potential of Cm1C as a natural anticancer agent and underscore the promise of *C. myrianthum*, an underexplored local cinnamon species, as a valuable source of bioactive compounds for the development of novel therapeutics against CRC.

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AUTHORS' CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the present journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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