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Original Article

Syzygium aromaticum extract inhibits cell proliferation through targeting apoptosis, cell cycle, and cilia signal transduction pathway in HT-29 human colorectal cell line

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Abstract

Syzygium aromaticum exhibits diverse pharmacological activities due to its antioxidant potential. Therefore, this study addressed the mechanisms of *S. aromaticum* extract (SAE) treatment on HT-29 cells proliferation. SAE has an adequate content of phytochemicals. The IC_{50} of SAE for HT-29 cells was calculated to be 137.81 ± 1.25 µg/ml after 48 hours. Treatment with SAE showed significant increase in the percentage of apoptotic HT-29 cells, with significant increase in their count in the G0/1 and S-phases, along with significant decrease in the G2/M phase. Significant downregulations of the *Hh, Wnt-4,* and *PDGFR-β* genes was represented in the colorectal cell lines (HT-29) after treatment with SAE. Collectively, these results demonstrate that SAE inhibits HT-29 cell proliferation by activating apoptosis and interfering with cilia signal transduction, highlighting its potential as a natural therapeutic candidate against colorectal cancer.

Keywords: *Syzygium aromaticum* extract, Colorectal cancer, Cell proliferation, Apoptosis, Cell cycle, Cilia signal transduction

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Introduction

Rapid technological advances have resulted in a rise in genetic diseases including cancer, which can be life-threatening. One of the most devastating illnesses that can greatly reduce a person's quality of life is cancer, specifically, colon cancer. This cancer is caused by a combination of environmental, genetic, and nutritional factors. Environmental contributors include physical inactivity, smoking, alcohol consumption, and chronic inflammations. Genetic predispositions involve mutations and hereditary syndromes. Dietary influences include a high consumption of processed meats, which can change gut microbiota and promote carcinogenesis (Bray et al., 2024). According to reported estimates, colon cancer is the third most diagnosed cancer and the second leading cause of cancer-related deaths, with approximately 1.9 million new cases and 935,000 deaths reported in 2020. The burden of colorectal cancer (CRC) shows striking geographic variability: incidence rates are highest in developed regions, whereas substantially lower rates are observed in parts of Africa and South Asia (Morgan et al., 2023). The invasion of neoplastic epithelial cells beneath the intestinal wall's muscularis mucosae is a hallmark of colorectal cancer. Proliferative lesions emerge because of a malfunction in cell replication brought on by progressive alterations in the quantity or activity of proteins that regulate cell proliferation, differentiation, and survival (Nascimento-Gonçalves et al., 2021). Chromosome instability and microsatellite instability are two of the main processes that contribute to the development of CRC. Environmental factors that increase the risk of colon cancer include dietary factors that lead to obesity and high energy intake (Malki et al., 2020; Esmeeta et al., 2022). Chemotherapy is commonly used to treat colon cancer; however, resistance, high recurrence rates, and side effects restrict its therapeutic effectiveness. Therefore, the demand for safe and efficient treatments for CRC is urgent (Vodenkova et al., 2020; Wei et al., 2023).

Because of its antioxidant qualities, herbal medicines have been utilized to treat cancer all over the world (Khan et al., 2019). According to prior research, phytocompounds produced from plants are well known for their ability to regress colon cancer in various ways. These include slowing tumor development, controlling the negative effects of chemotherapy, and acting at the molecular level because of their pro-apoptotic and anti-angiogenic qualities (Talib et al., 2020; Mazumder et al., 2022). These plants increase superoxide dismutase levels, reduce oxidative stress-induced DNA damage, promote apoptosis, and slow the growth of colon cancer in a variety of ways (Greenwell and Rahman, 2015; Esmeeta et al., 2022).

Among the members of the Myrtaceae family, *Syzygium aromaticum* (Cloves) contain high concentration of bioactive components, including flavonoids and eugenol, which give it a variety of biological, biomedical, gastronomic, and traditional medical purposes (Kaur and Kaushal, 2019; Pandey et al., 2024). Clove leaves provide flavonoids and phenolic acids that suppress tumor progression by modulating oxidative stress and signaling pathways. The stem and bark are sources of tannins and triterpenoids, which demonstrate apoptosis-inducing, and anti-angiogenic effects. Additionally, its flower buds and essential oil are rich in eugenol, a phenolic compound that induces apoptosis and inhibits angiogenesis in multiple cancer cell types (Rudrapal et al., 2022; Abdulrahman and Hama, 2023). Sesquiterpenes, monoterpenes, hydrocarbons, and vital phytochemicals present in cloves. The two most crucial phytochemicals are caryophyllene and eugenol. Caryophyllene exhibits anticancer effects on pancreatic, cutaneous, lymphatic, and cervical cancers, while eugenol has demonstrated anticancer activities against various types of cancers (Mostafa et al., 2020; Aksono et al., 2022; Mergulhão et al., 2024). Ibrahim et al. (2023) reported the immunomodulatory and anti-cancer potentials of *S. aromaticum* suggesting that *S. aromaticum* extract (SAE) can be used as an immune-stimulatory agent to counteract tumor development. Therefore, this study evaluated the biochemical and molecular mechanisms of the SAE antitumor efficacy on the proliferation of human colorectal adenocarcinoma cell lines.

Material and Methods

Preparation of plant extract

The flower buds of *S. aromaticum* were imported from Carrefour Market in Riyadh, Saudi Arabia. An expert confirmed that the plant complied with the institutional criteria. The *S. aromaticum* flower buds (SAFB) were washed by tap water and dried in the shade, then by using hydro-ethanol solution (70% ethanol), the exact amount was soaked for 3 days. After that, the *S. aromaticum* extract (SAE) solutions were filtered and left to dry, then the extract weight was determined. The extract was stored at 4 °C for further studies (El-Said et al., 2023). This procedure was repeated in triplicate with minimal batch-to-batch variation.

Phytochemicals and GC-MS profiling

The phytochemicals' ability to scavenge DPPH radicals was evaluated using the SAE (Aslam et al., 2023; Prieto et al., 1999). The phytochemicals in the SAE were identified using a Trace GC 1310-ISQ mass spectrometer. Following component identification, retention durations and mass spectra were compared to those in the NIST 11 and WILEY 09 mass spectra databases.

Cancer cell line and treatment protocols

In RPMI-1640 media (Cat. no. MT10040CM), HT-29 cells were cultivated with 10% FBS, amphotericin B (250µg/mL), HEPES buffer (238.3µg/mL), streptomycin (100µg/mL), and penicillin (100U/mL) as supplements. Each cell was cultivated in an environment with 5% CO_2 at 37 °C.

Cell viability

HT-29 cells were planted (1×10^4 cells/well) for a duration of 24 hours. After 48 hours of treatment with varying doses of SAE (0 to100µg/ml) or control, then absorbance was read at 570nm. The IC_{50} was evaluated and used for further studies (Urbinati et al., 2016). The percentage of cell viability and growth inhibition were calculated as follows:

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}}\right) \times 100$$

$$\text{Growth inhibition (\%)} = 100 - \text{Cell viability (\%)}$$

Clonogenic assay

Cells were exposed to a certain concentration of extract or vehicle (50, 100, 150, and 200µg/ml) for 24 hours to replating for colony formation., cells were trypsinized, plated at a density of approximately 1×10^4 cells per well in a 6-well plate, and allowed to develop for 14 days. Following a PBS wash, the cells were incubated for 30 minutes with a 0.5% crystal violet solution that included 3.7% formaldehyde. The plates were then left to dry after the crystal violet was removed with running tap water. To evaluate the death of the HT-29 cancer cells, cell density was assessed using Bio-Rad (Banerjee et al., 2016). For the clonogenic assay, biomass (colony formation efficiency) was calculated using:

$$\text{Plating efficiency (PE, \%)} = \left(\frac{\text{Number of colonies formed}}{\text{Number of cells seeded}}\right) \times 100$$

$$\text{Surviving fraction (SF)} = \frac{\text{Colonies counted after treatment}}{\text{Cells seeded} \times \text{PE (control)}}$$

Flow cytometry analysis

Apoptosis was assessed using apoptosis kit (catalog no. V13242), and a computer program and the flow cytometry histogram were generated. After being fixed and stained with Dye Cycle Violet stain (catalog no. V35003), the cells were examined using flow cytometry (Ali et al., 2014).

Biochemical analysis

The B-cell lymphoma 2 (Bcl-2) (cat. no. ab119506), Bcl-2 Associated X-protein (Bax) (cat. no. ab199080), and caspase-3 (cat. no. ab285337), were evaluated using their human ELISA kits from Abcam company (Cambridge, CB2 0AX, UK). PARP protein levels (cat. no. MBS455488) were measured using their human's ELISA kits from My-BioSource (Inc., San Diego, CA, USA).

Gene expression analysis

The mRNA expression levels of genes involved in the cilia signal transduction pathways, including Hh, Wnt, and PDGFR-β genes were evaluated in the HT-29 cells before and after the treatment with SAE using SYBR Green (Livak and Schmittgen, 2001). Normalization against the GAPDH reference gene was made, and the gene-specific primers were shown in table 1.

Table-1. Forward and reverse primer sequences for RT-PCR.

Gene	Accession number	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
Hh	NM_000193	CGGGAAGAGGAGGCACCCCA	GTACTTGCTGCGGTCGCGGT
Wnt-4	NM_030761.5	TGCCACTGAGGTGGAGCCAC	TCAGCCAGCTCCACCTGCGC
PDGFR-β	NM_001355016.2	CAACTTCGAGTGGACATACCC	AGCGGATGTGGTAAGGCATA
GAPDH	NM_001256799	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

Hh: Hedgehog; *Wnt-4*: Wingless-related integration site; *PDGFR-β*: platelet-derived growth factor beta; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

Statistical comparisons between groups were conducted using one-way ANOVA followed by Tukey's post hoc test. All analyses were performed using Graph Pad Prism software, version 10.5.0 (San Diego, CA). A p-value of < 0.05 was considered statistically significant.

Results

Phytochemical constitutes *S. aromaticum flower buds*

The findings demonstrated that the SAFB produces an extract (12%). They also revealed that SAE had total flavonoid and phenolic contents of 19.88 ± 1.85 mg QE/g DW and 34.69 ± 2.85 mg GAE/g DW, respectively. The extract's DPPH scavenging activity (%) was 81% ± 3.15 and its total antioxidant capacity (TAC) was 65.89 ± 3.24 mg AAE/g DW. A concentration of 5.67 ± 0.83mg/mL of extract was sufficient to suppress 50% of DPPH, and 375 ± 4.89mg/g DW of saponin was present (Table 2).

Table-2. Phytochemicals analysis of *S. aromaticum* flower buds (SAFB).

Phytochemicals	SAFB
Total phenolic content (mg GAE/g DW)	34.69 ± 2.85
Total flavonoids contents (mg QE/g DW)	19.88 ± 1.85
Total antioxidant capacity (TAC) (mg AAE/g DW)	65.89 ± 3.24
DPPH scavenging activity (%)	81 % ± 3.15
IC_{50} of DPPH (mg/ml)	5.67 ± 0.83
Saponin (mg/g DW)	375 ± 4.89

SAFB: *S. aromaticum* flower buds; GAE: Gallic acid equivalent; QE: Quercetin equivalents; TAC: Total antioxidant capacity; AAE: Ascorbic acid equivalent; DW: Dry weight; IC_{50}: Inhibitory concentration of 50%; DPPH: Diphenyl-1-picrylhydrazyl.

GC-MS analysis of SAE

Ethyl-α-D-glucopyranoside, eugenol, caryophyllene, and humulene were the most common phytochemicals in SAE. Their retention times (RT) were 7.64, 12.25, 15.82, and 16.54 minutes, respectively. The peak area percentages (PA%) were 2.27%, 64.17%, 18.07%, and 3.83% as shown in Figure 1 and Table 3.

Table-3. GC-MS analysis for phytochemicals composition in SAE.

No.	RT (min.)	Name	MF.	P.A (%)
1	7.64	Ethyl-alpha-D-glucopyranoside	$C_8H_{16}O_6$	2.27
2	12.25	Eugenol	$C_{10}H_{12}O_2$	64.17
3	15.82	Caryophyllene	$C_{15}H_{24}$	18.07
4	16.54	Humulene	$C_{15}H_{24}$	3.83
5	19.50	Caryophyllene oxide	$C_{15}H_{24}O$	0.86
6	21.55	10-Heptadecen-8-ynoic acid, methyl ester	$C_{15}H_{24}O$	0.67

SAE: *Syzygium aromaticum* extract; RT: Retention time; MF: Molecular formula; P.A%: Peak area percentage.

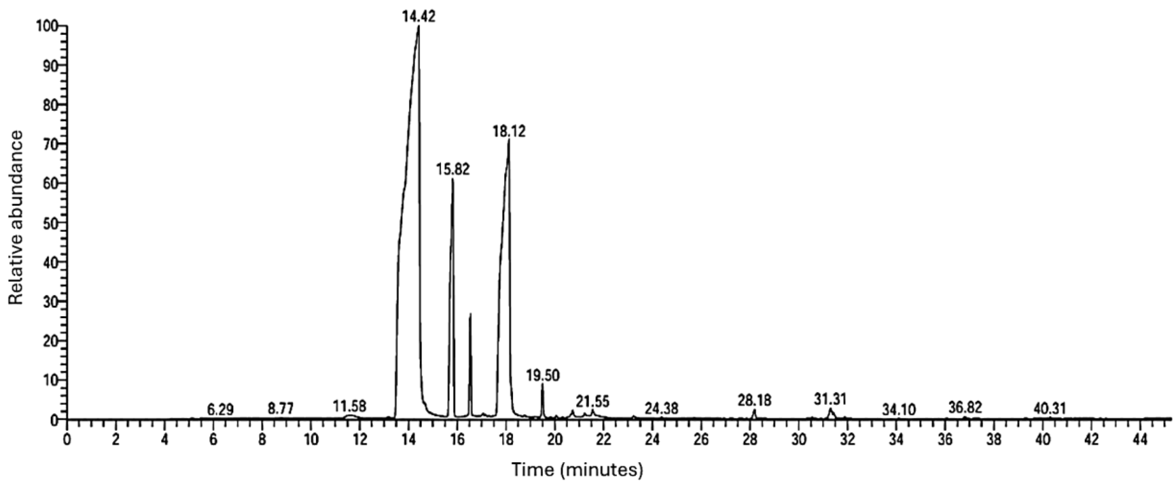

Figure-1. Chromatogram shows the relative abundance of phytochemicals detected in SAE. Several peaks are labeled with their retention times (RT): 6.29, 8.77, 11.58, 14.42, 15.82, 18.12, 19.50, 21.55, 24.38, 28.18, 31.31, 34.10, 36.82, and 40.31 minutes.

Cytotoxic effect of SAE treatment on HT-29 colorectal cell line

The SAE showed potential anticancer action in a dose-dependent manner towards the colon cancer cell line (HT-29). After 48 hours, the SAE IC_{50} for HT-29 cells was determined to be 137.81 ± 1.25µg/ml (Figure 2).

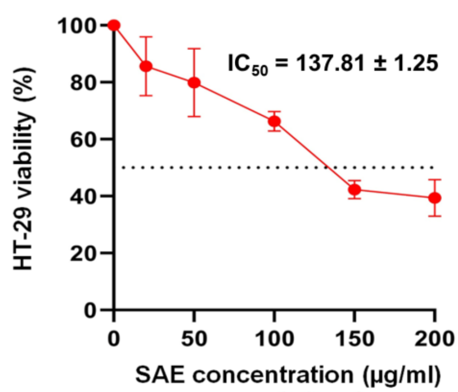

Figure-2. Viability of HT-29 cells (%) treated with *Syzygium aromaticum* extract (SAE), after 48h that showed the inhibitory concentration that kills 50% of HT-29 cells (IC_{50}). IC_{50} = 137.81 µg/ml. Data were mean ± SD of *n*=3, each measured in triplicate technical wells. The significance threshold set at $P < 0.05$.

Effect of SAE treatment on colony formation of HT-29 cells

The anticancer potential of SAE therapy on HT-29 cells was further examined using colony formation assays. Comparing the extract-treated cells to the vehicle (DMSO) group, the clonogenic assay showed that the extract greatly suppressed the colony formation of HT-29 cells. Following treatment, the colonial/population density in each plate was measured and plotted using Gel Doc. When compared to the DMSO-control HT-29 cells, the density measurement showed that the treatment with SAE at 100µg/ml (below the dose of IC_{50}) led to a significant decrease ($P < 0.05$) in colony growth by 34.42%, while the treatment with SAE at 200µg/ml (a dose higher than IC_{50}) resulted in a significant decrease ($P < 0.05$) in colony formation by 60.13% (Figure 3).

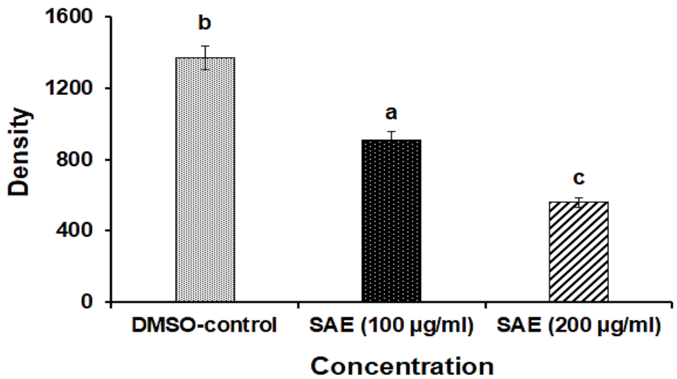

Figure-3. Inhibitory effect of SAE on the colony formation of HT-29 cells at the indicted concentrations compared to the control group represented by the density of colonies. Data were mean ± SD of *n*=3, each measured in triplicate technical wells. The significance threshold set at $P < 0.05$. Means that do not share a letter showed significant.

Effects of SAE treatment on the percentages of necrotic and apoptotic HT-29 cells

After being treated with SAE, the HT-29 cells' distribution as determined by their Annexin/PI staining changed (137.81 ± 1.25µg/ml). The viable colorectal cancer cells decreased significantly ($P < 0.05$) after SAE treatment compared to the untreated HT-29 cells (60% versus 89%). However, compared to the untreated HT-29 cells (1.9%), the percentage of necrotic HT-29 cells after SAE treatment significantly increases to 2.5%. In addition, the percentage of early and late apoptotic cells (9.8% and 27.8%, respectively) increased significantly ($P < 0.05$) in HT-29 cells treated with the estimated IC_{50} of SAE in comparison to the control HT-29 cells (2.9% and 6.2%, respectively) (Figure 4).

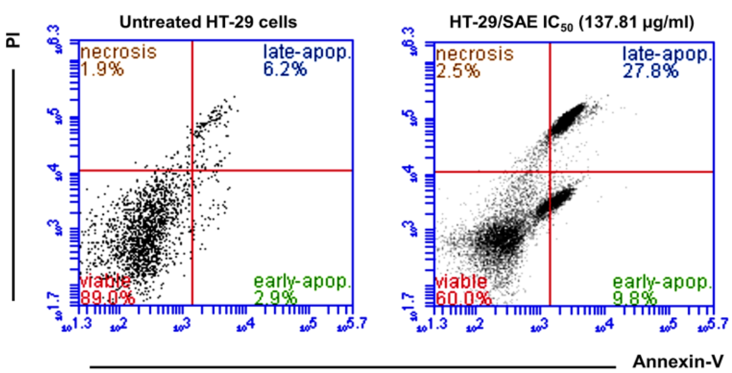

Figure-4. Annexin V expression in HT-29 colorectal cancer cells following SAE therapy is demonstrated by flow cytometric analysis. An apoptosis kit including propidium iodide (PI) and Annexin-V was used to measure apoptosis. Data were mean ± SD of n=3, each measured in triplicate technical wells. The significance threshold set at $P < 0.05$.

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Effects of SAE treatment on the cell cycle analysis of HT-29 cells

According to the data, the number of HT-29 cells in the G0/1 (sub G1) phase increased significantly (P < 0.05) to 17.8% after SAE treatment, compared to 8.6% for untreated HT-29 cells. Following SAE treatment, the proportion of HT-29 cells in the G1 phase rose in comparison to control cells (77.4% versus 67.6%). Additionally, when HT-29 cells were treated with SAE, their percentage in the S-phase increased to 5.3% from 2.8% for the untreated cells. After HT-29 cells were treated with SAE, their G2/M phase decreased significantly (P < 0.05) to 5.5% in comparison to the untreated HT-29 cells (21.8%) (Figure 5).

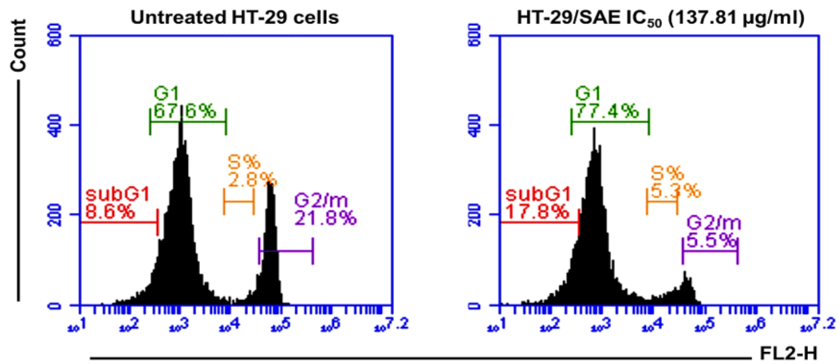

Figure-5. Cell cycle analysis of HT-29 colorectal cancer cells following SAE treatments. Following fixation and Dye Cycle Violet staining, the cells were analyzed using flow cytometry. There were three duplicates of the experiment. Data were mean ± SD of n=3, each measured in triplicate technical wells. The significance threshold set at P < 0.05.

Effects of SAE treatment on apoptotic protein levels in HT-29 cells

The levels of the Bcl-2, Bax, and caspase-3 proteins were assessed in colorectal cancer cell lines both before and during SAE therapy. Following treatment with the IC_{50} of SAE, the Bcl-2 protein levels in HT-29 cells were found to be significantly lower (P < 0.05) at 9.47 ± 1.41ng/ml as compared to 16.99 ± 1.78ng/ml in the untreated HT-29 cells. The SAE-treated HT-29 cells, on the other hand, had considerably higher levels of Bax, and caspase-3 (598.19 ± 13.12pg/ml and 13.85 ± 1.17ng/ml, respectively) than the untreated HT-29 cells (392.58 ± 12.7pg/ml and 7.56 ± 0.86ng/ml, respectively). In comparison to the untreated HT-29 cells (10.95 ± 0.87ng/ml), the PARP protein level was considerably (P < 0.05) reduced after HT-29 cells were treated with SAE, reaching 6.34 ± 0.67ng/ml (Table 4).

Table-4. Protein levels of Bcl-2, Bax, caspase-3, and PARP in the untreated HT-29 cells and SAE-treated HT-29 cells.

Treatment/proteins	Bcl-2 (ng/ml)	Bax (pg/ml)	Caspase-3 (ng/ml)	PARP (ng/ml)
Untreated HT-29 cells	16.99 ± 1.78 [a]	392.58 ± 12.79	7.56 ± 0.86	10.95 ± 0.87
HT-29 cells/SAE (IC_{50})	9.47 ± 1.41 [c]	598.19 ± 13.12	13.85 ± 1.17	6.34 ± 0.67

SAE: *Syzygium aromaticum* extract; Bcl-2: B cell lymphoma-2; Bax: Bcl-2-Associated X Protein; PARP: Poly (ADP-ribose) polymerase. Data were mean ± SD of n=3, each measured in triplicate technical wells. The significance threshold set at P < 0.05. Means that do not share a letter showed significant difference.

Effects of SAE treatment on cilia signal transduction pathways in HT-29 cells

The expression of *Hh, Wnt-4,* and *PDGFR-β* genes was detected in the colorectal cell lines (HT-29) after the treatment with SAE, to investigate whether SAE treatment influenced the cilia signaling cascade. The *Hh, Wnt-4,* and *PDGFR-β* gene expressions were significantly downregulated ($P < 0.01$) in SAE-treated HT-29 colorectal cells after normalization using the GAPDH gene by 1.80, 1.62, and 1.67 folds, respectively (Figure 6).

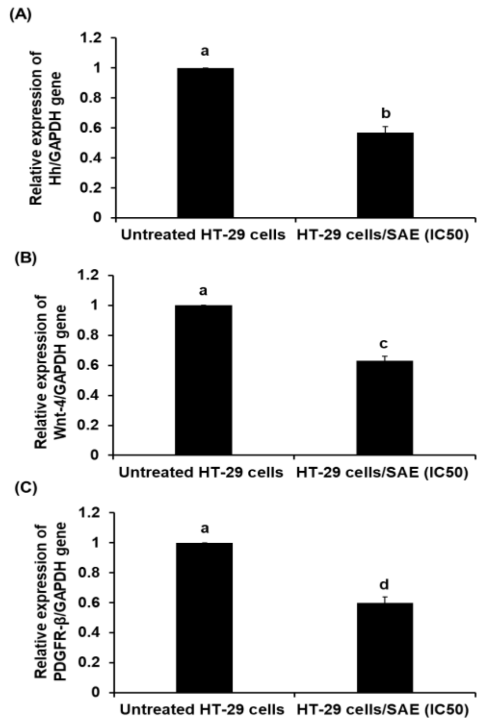

Figure-6. The HT-29 colorectal cancer cell lines' relative mRNA expression levels of the Hh (A), Wnt-4 (B), and PDGFR-β (C) genes following SAE treatment. The values were mean ± standard deviation. Three duplicates of the experiment were conducted. Significant differences ($P < 0.01$) were observed between means that do not share a letter.

Discussion

Due to their molecular heterogeneity, CRC can be further classified into clinically significant subtypes that are linked to therapy response and patient prognosis. Chemotherapy, radiation therapy, and/or surgical resection are all part of the current treatment plan (Berg et al., 2017). Conventional chemotherapy generates severe side effects because it causes damage to normal cells, even though current treatment procedures are modified frequently to boost patient survival rates. Additionally, because antineoplastic medications have weak pharmacokinetic qualities, it is crucial to create new anticancer agents with optimal pharmacological qualities (Munteanu et al., 2024). Therapeutic regimens for CRC are often linked to severe systemic toxicities including myelosuppression, mucositis, cardiotoxicity, and neuropathy. These side effects limit the therapeutic window. In contrast, phytochemicals derived from medicinal plants, such as cloves, have been recognized for their potential anticancer activity, targeting cancer cells with relatively reduced systemic toxicity. Several studies suggest that herbal extracts exert anticancer effects by modulating molecular signaling cascades without causing extensive collateral damage to healthy tissues, making them promising complementary agents (Jenča et al., 2024). The most important forerunner of contemporary medicine is the utilization of natural products to cure a variety of diseases. Given the vast array of naturally occurring molecules produced from natural goods currently utilized in

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therapy, natural compounds are essential to the management of cancer (Asma et al., 2022). SAE exhibits anticancer activity against several cell lines due to its phytochemical constituents, which induce apoptosis in CRC cells by activating apoptotic pathways. Bioactive compounds like eugenol and β-caryophyllene present in SAE can trigger apoptosis (Kumar et al., 2014; Ali et al., 2023; Mohamed Abdoul-Latif et al., 2023).

The current study demonstrated that *S. aromaticum* flower buds (SAFB) contain adequate phenolics, flavonoid, and saponin contents. The DPPH scavenging activity was 81% ± 3.15, and its TAC was 65.89 ± 3.24mg AAE/g DW. SAE has noticeable phytochemicals, indicated by the GC-MS study. GC-MS identified eugenol (~64%) and β-caryophyllene (~18%) as the dominant volatilizable constituents of the extract. However, the present study did not test isolated eugenol or fractionated extracts; therefore, causality cannot be assigned to any single constituent based on our data. These results were consistent with earlier research on the phytochemical composition of SAE and its primary active ingredients (Batiha et al., 2020; Lone and Jain, 2022; Mostafa et al., 2023).

In the present study, SAE was evaluated as an antiproliferative agent against HT-29 cells that revealed potential antitumor activity towards colon cancer cell line. The IC_{50} of SAE for HT-29 cells was 137.81 ± 1.25µg/ml. Moreover, the clonogenic assay showed that SAE-treated HT-29 cells demonstrated significant inhibition of colony formation in HT-29 cells. The presence of several phytochemical components, primarily eugenol (more than 50%), β-caryophyllene, α-caryophyllene, and acetyl eugenol, has been linked to the anticancer properties of cloves (Elbestawy et al., 2023; Liñán-Atero et al., 2024). By causing oxidative stress, apoptosis, and genotoxic effects, eugenol demonstrated potent anticancer activity against a variety of cancer cell lines, hence preventing tumor growth (Al Wafai et al., 2017; Haro-González et al., 2021).

Cancer treatment, including chemotherapy, entails activating the cellular apoptosis signaling pathways of cancer cells. Apoptosis is crucial in controlling carcinogenesis and treatment responses (Lee et al., 2014). When HT-29 cells were treated with the IC_{50} dose of SAE, the number of early and late apoptotic cells increased significantly in comparison to the cells that were left untreated. One of the key phases in the development of cancer is the failure of the cell cycle, which controls cell growth (Williams and Stoeber, 2012). The data demonstrated that following SAE treatment, HT-29 cells in the G1 phase were higher than that of control cells. Previous studies have reported the effects of herbal extracts on CRC by targeting apoptosis and cell cycle arrest (Li et al., 2018; Lai et al., 2021; Kiernozek et al., 2022).

The Bcl-2 levels in the SAE-treated HT-29 cells were significantly lower than those in the untreated HT-29 cells, according to the current study. However, following SAE treatment, there was a substantial rise in the Bax, and caspase-3. An increase in apoptosis-related proteins was observed in HT-29 cells in a prior work that examined the apoptotic effects of *Levisticum officinale koch* extracts (Lotfian Sargazi et al., 2024). Furthermore, tetrandrine suppresses HT-29 cells through the Bcl-2/Caspase 3 pathway, according to Li et al. (2019). The PARP is a key DNA repair enzyme that allows tumor cells to survive genotoxic stress induced by chemotherapy. Inhibition of PARP not only hinders DNA repair but also sensitizes cancer cells to apoptosis when combined with cytotoxic agents. Previous studies have shown that natural compounds can enhance PARP cleavage, an indicator of apoptosis, thereby augmenting the cytotoxicity of anticancer agents. In the context of SAE, PARP cleavage observed in HT-29 cells suggests enhanced apoptotic induction. This mechanism could potentially enhance the cytotoxic impact of traditional chemotherapies, providing a synergistic benefit in colorectal cancer treatment strategies. (Sodhi et al., 2010; Yang et al., 2024). The anticancer activity of lycopene in HT-29 cells was enhanced by downregulating PARP proteins (Ataseven et al., 2023).

By regulating cell cycle entry and responding to cell-extrinsic signals, cilia play a crucial role in controlling cell proliferation. According to recent research, primary cilia and the proteins they are linked to play a role in autophagy and genome stability, two processes that are crucial for oncogenesis. Oncogenesis may result from abnormal primary cilia functioning, defective cilia can, in fact, either encourage or inhibit malignancy. Thus, the role of cilia is context-dependent: in some cancers, loss of cilia supports malignancy by preventing proper signaling, whereas in others, intact cilia facilitate oncogenic signaling (Carotenuto et al., 2023). The gene expression of the *Hh, Wnt-4,* and *PDGFR-β* genes was significantly downregulated after treating HT-29 colorectal cells with SAE. Our observation that SAE interferes with ciliary signaling suggests an additional mechanism by which phytochemicals exert tumor-suppressive effects, highlighting the importance of targeting cilia-associated pathways in CRC (Collinson and Tanos, 2025). It has been reported that the Hh signaling pathway is involved in CRC tumorigenesis, and its inhibition block tumor in angiogenesis, cell proliferation, and metastasis in CRC formation. Loss of cilia potentiated signaling pathway cascades activation leading to cell death in colorectal carcinoma (Gerling et al., 2016; Chen et al., 2023; Remo et al., 2023). Taking together, our results suggest that SAE exerts anti-proliferative effects in HT-29 cells through multi-targeted mechanisms: induction of apoptosis, cell cycle arrest, PARP cleavage-mediated sensitization, and modulation of primary cilia signaling. These combined actions not only enhance the cytotoxic potential of phytochemicals but also provide a mechanistic basis for their potential use as adjuvants to conventional colorectal cancer therapy. Importantly, unlike traditional chemotherapy, the relatively lower toxicity profile of phytochemicals underscores their translational potential in improving therapeutic outcomes while minimizing adverse effects. A limitation of this study is the absence of a standard chemotherapeutic agent (such as doxorubicin or cyclophosphamide) as a comparator. While our aim was to explore the mechanistic effects of *Syzygium aromaticum* extract on apoptosis, cell cycle arrest, and cilia signaling pathways in HT-29 cells, direct comparison with established chemotherapeutics would provide important insight into its relative efficacy and potential clinical value. Future studies should therefore include such comparators to better contextualize the anticancer potential of *S. aromaticum* extract.

Conclusion

The present study demonstrates that *Syzygium aromaticum* extract (SAE), rich in eugenols, exerts potential anticancer activity against HT-29 colorectal cancer cells. SAE induced a significant increase in both early and late apoptosis, accompanied by marked suppression of cell cycle progression at the G2/M phase, confirming its ability to inhibit uncontrolled cell proliferation. At the molecular level, SAE downregulated critical components of the cilia-mediated signaling pathways, including Hedgehog (Hh), Wnt-4, and PDGFR-β, which are known to play essential roles in colorectal cancer progression. These outcomes provide mechanistic evidence that SAE exerts its antiproliferative effects through dual targeting of apoptosis and cell cycle arrest, in parallel with disruption of oncogenic ciliary signaling. Collectively, these findings position SAE as a promising natural therapeutic candidate or adjuvant agent for colorectal cancer management, with potential advantages in reducing the systemic toxicity typically associated with conventional chemotherapeutics. The limitations of our study, including the use of a single colorectal cancer cell line (HT-29) and the need for dose-optimization, pharmacokinetic studies, and *in vivo studies* to confirm translational potential are acknowledged. Although prior work shows that eugenol can reduce HT-29 viability and modulate APC/p53/KRAS expression and that β-caryophyllene induces apoptosis and inhibits angiogenesis in colorectal cancer models, our data do not assign causality to any single molecule. Dedicated fractionation and combination testing are needed to dissect contributions of major and minor constituents.

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