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Original Article

Enhancement of Annexin V in response to combination of epigallocatechin gallate and quercetin as a potent arrest the cell cycle of colorectal cancer

Aumento da anexina V em resposta à combinação de galato de epigalocatequina e quercetina como uma potente parada do ciclo celular do câncer colorretal

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Abstract

Colorectal cancer (CRC) is one of the most common cancers leading to comorbidities and mortalities globally. The rational of current study was to evaluate the combined epigallocatechin gallate and quercetin as a potent antitumor agent as commentary agent for therapeutic protocol. The present study investigated the effect of epigallocatechin Gallate (EGCG) (150mg) and quercetin (200mg) at different proportions on proliferation and induction of apoptosis in human colon cancer cells (HCT-116). Cell growth, colonogenic, Annexin V in addition cell cycle were detected in response to phytomolecules. Data obtained showed that, the colony formation was inhibited significantly in CRC starting from the lowest concentration tested of 10 µg/mL resulting in no colonies as visualized by a phase-contrast microscope. Data showed a significant elevation in the annexin V at 100 µg/mL EGCG(25.85%) and 150 µg/mL quercetin (48.35%). Moreover, cell cycle analysis showed that this combination caused cell cycle arrest at the G_1 phase at concentration of 100 µg/mL (72.7%) and 150 µg/mL (75.25%). The combined effect of epigallocatechin Gallate and quercetin exert antiproliferative activity against CRC, it is promising in alternative conventional chemotherapeutic agent.

Keywords: colorectal cancer, epigallocatechin gallate, quercetin, apoptosis.

Resumo

O câncer colorretal (CCR) é um dos cânceres mais comuns, levando a comorbidades e mortalidade em todo o mundo. O racional do presente estudo foi avaliar a combinação de galato de epigalocatequina e quercetina como um agente antitumoral potente como agente de comentário para protocolo terapêutico. O presente estudo investigou o efeito de galato de epigalocatequina (EGCG) (150 mg) e quercetina (200 mg) em diferentes proporções na proliferação e indução de apoptose em células de câncer de cólon humano (HCT-116). O crescimento celular, colonogênico, anexina V, além do ciclo celular foram detectados em resposta a fitomoléculas. Os dados obtidos mostraram que a formação de colônias foi inibida significativamente no CRC a partir da concentração mais baixa testada de 10 µg/mL, resultando em nenhuma colônia conforme visualizado por um microscópio de contraste de fase. Os dados mostraram uma elevação significativa na anexina V a 100 µg/mL de EGCG (25,85%) e 150 µg/mL de quercetina (48,35%). Além disso, a análise do ciclo celular mostrou que essa combinação causou parada do ciclo celular na fase G1 na concentração de 100 µg/mL (72,7%) e 150 µg/mL (75,25%). O efeito combinado da epigalocatequina galato e quercetina exerce atividade antiproliferativa contra o CCR, é promissor como agente quimioterápico alternativo convencional.

Palavras-chave: câncer colorretal, galato de epigalocatequina, quercetina, apoptose.

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1. Introduction

Colorectal cancer (CRC) is one of the five top most common types of cancers in the world that affects both men and women. In 2018, it was estimated that around 1.1 million new cases of colon cancer were diagnosed with an estimation of 500,000 colon cancer-related deaths worldwide (Bray et al., 2018). More than half of the cases (60%) have been reported in developed countries. CRC adenocarcinoma is formed either in the tissues of the colon, the mucosal colonic polyps, or the rectum (Cappell, 2005). It is believed that the development of CRC is influenced by both genetic (inherited) and environmental (lifestyle) factors (Amersi et al., 2005). CRC could occur in patients at all ages, but the risk increases with age gradually. Patient's survival is directly linked to the metastasis of primary colorectal tumors and accounts for 90% of patients' mortality. Nearly half of the subjects with CRC can be cured by surgery and multimodal treatment. However, therapeutic options are restricted especially for metastasized patients. This is demonstrated by 5-year-survival rates, which are higher (90%) for the early stage patients followed by 65% for patients with regional lymph node metastasis, and it is quite lower (10%) in patients with advanced metastatic disease (Koelzer et al., 2015).

Fluorouracil chemotherapy has been the only approved treatment for the management of metastatic colorectal cancer (mCRC) since 1950s (Petrelli et al., 1989). Subsequently, oxaliplatin, irinotecan, and leucovorin along with fluorouracil-based chemotherapeutical agents have been proven to be well-tolerated with increased response rate, time of progression and survival in patients with mCRC, and are used as a reference first-line therapy against mCRC (Zhang et al., 2015). During the last decade, novel targeted therapies, as a single agent or combinations, have been used for the treatment of mCRC. Bevacizumab (Avastin) is the most common one that has been used to treat mCRC patients (Zhang et al., 2015). However, there is an urgent need to develop alternative or complementary treatment regimens to counteract the resistance to chemotherapy, concentration-limiting toxicities, off-target effects and relapse.

Previous reports stated that, the use of natural products in the treatment of CRC has attracted a lot of interest (Katz and Baltz, 2016; Siddiqui et al., 2014). The protective effect of natural compounds may be attributed to the presence of numerous phytochemicals such as resveratrol, quercetin, rutin, catechin, myricetin, kaempferol, and Epigallocatechin gallate (EGCG). EGCG has been reported to modulate multiple key elements in signal transduction pathways affecting cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis and metastasis in CRC (Araujo et al., 2011; Kuerban et al., 2017; Soengas Fernández et al., 2011; Moselhy et al., 2018). Initial studies have revealed that these compounds are able to affect cell proliferation, cell cycle regulation, and usually participate in multiple signaling pathways which are often disrupted in tumor initiation, proliferation and propagation (Kotecha et al., 2016). Generally, phytochemicals do not interfere with chemotherapy; instead, they enhance the effects of the widely used chemotherapeutic agent in a concentration-dependent manner (Iqbal et al., 2017; Simone II et al., 2007).

Many previous studies have reported the use of a combination of, two or often more, phytochemicals to evaluate their anti-carcinogenic potential, both *in vitro* and *in vivo* settings against different human cancer cell lines as well as in laboratory animals (Araújo et al., 2011; Fantini et al., 2015; Ramos 2008; Scalbert et al., 2005). The aim of the current study was to investigate the effect of phytobiological (PB) on proliferation and induction of apoptosis against HCT116 human colon cancer cells. The chemical composition of the PB contained. Previous studies have shown the anti-proliferative and anti-cancerous potential of PB in breast, sarcoma, skin and head and neck cancer cell lines (Alqarni et al., 2017; Niedzwiecki et al., 2016; Roomi et al., 2011, 2015).

Many previous studies have reported the antiproliferative and anticancer effect of some polyphenols against colon cancer using *in vitro* models (Araujo et al., 2011; Fantini et al., 2015; Ramos, 2008; Scalbert et al., 2005). However, the *in vitro* studies with a mixture of all these polyphenols are quite limited and the mechanism of action is still unclear. Therefore, building upon the previous studies, we evaluated the effect of different concentrations of combined effect of quercetin and EGCG on HCT116 CRC cells line. To the best of our knowledge, it is the first time that PB was used to evaluate its *in vitro* anticancer effect on CRC cells. The effect of individual phytochemical was previously studied, for that the rational of this study investigated the combined effect EGCG and quercetin in cell cycle arrest CRC cell lines as a target for antitumor action.

2. Materials and Methods

2.1. Preparation of stock solution

The EGCG and quercetin (Santa Clara, California, USA) used in this study was a generous gift from Dr. Steve Harakeh. The Stock solutions of the EGCG and quercetin were prepared with a concentration of 100mg/mL in pure DMSO (Thermo Fisher, USA) and were stored in aliquots at -20 °C. Routine working standard solutions were prepared with diluting 100 ul of stock solution in 9,900 µL FBS-free DMEM. Different concentrations of the drug were prepared and used for all the experiments (10, 25, 50, 75, 100, 150 µg/mL). For each experiment, fresh dilution of the drug was used in the treatment and for all the experiments where DMSO was used as a solvent, the final concentration of DMSO was maintained at 0.1-0.5%. The doses given according to previous study.

2.2. Cell culture

The human colorectal carcinoma from a cell line (HCT-116) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in a humidified incubator with 5% CO_2 at 37 °C. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (UFC Biotech, KSA) supplemented with 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (UFC Biotech, KSA) antibiotics (100 units/mL).

2.3. Cell growth assay

The cell growth effect of the EGCG and quercetin on CRC cells was evaluated through a rapid colorimetric cell proliferation assay using WST-1 reagent (Abcam, USA). The cells were seeded in a 96-well plate at a density of $8x10^3$ cells/well in 100 uL of culture medium and incubated for 24 hours. The cells were treated with different concentrations of EGCG and quercetin at different concentrations and incubated for 24, 48 and 72h. After incubation for different time periods, WST-1 solution (10 µL) was added to each well and incubated for 2h at 37 °C. Finally, the absorbance was recorded at 450 nm with an ELx800™ microplate ELISA reader (Biotek, USA) and the results were analyzed by the Gen5 software (Biotek, USA). The percentage of cell growth was calculated relatively to the control (untreated).

2.4. Clonogenic assay

The cells were seeded in a 6-well plate at a density of 1000 cells/well in 3 mL of culture medium and incubated for 24 h. The culture medium was changed and the cells were treated with different concentrations of EGCG and quercetin. The cells were incubated at 37 °C in a CO_2 incubator for colony formation for 10 days. The media was gently removed from each well by aspiration and washed with 2 mL DPBS. Then, colonies were fixed with 1.5 mL of 4% formaldehyde for 30 minutes, washed with 2 mL of DPBS and then 1 mL of 0.5% of crystal violet was added and incubated for 30 mins. Then the stain was removed by washing with 2 mL DPBS and the plates were allowed to dry. The wells with colonies with >50 cells were counted under a phase-contrast microscope.

2.5. Annexin V/PI assay

The APC Annexin V kit (BD Bioscience, USA) was used for apoptotic assay according to the manufacturer's recommendations. In brief, the cells were seeded in 6-well plate at a density of $3x10^5$ cells/well in 2 mL of culture medium and incubated for 24 h. The culture medium was changed, and cells were treated with different concentrations of EGCG and quercetin (10, 25, 50, 100, 150 µg/mL). Cells were trypsinized with (0.05%) trypsin-EDTA (500 µL) and incubated for 7 min and then 2 mL of culture medium was added and centrifuged at 1200 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended twice in ice-cold PBS and then the cells were resuspended in FACS tubes ($1x10^5$ cells) in 100 µL 1x binding buffer. APC Annexin V (3 µL) and PI (5 µL) were added, gently vortexed and incubated for 15 min at room temperature in the dark. Finally, 300 µL of 1x Binding Buffer was added to each tube and analyzed by flow cytometer (BD Biosciences, USA).

2.6. Cell cycle analysis

The cell cycle analysis was done using a commercially available BD Cycletest™ Plus DNA kit (BD Bioscience, USA) as per the manufacturer's protocol. The cells were seeded in 6-well plate at a density of $3x10^5$ cells/well in 2 mL of culture medium and incubated for 24 h. The culture medium was changed, and cells were treated with different concentrations of EGCG and quercetin (10, 25, 50, 100, 150 µg/mL). Cells were trypsinized with (0.05%) trypsin-EDTA (500 µL), incubated for 7 min and then 2 mL of culture medium was added and centrifuged at 1200 rpm for 5 min. The supernatant was discarded, pellet was resuspended twice in ice-cold PBS and then into the FACS tubes. The staining procedure for DNA ploidy analysis requires a test sample of 5×10^5 cells. The reagents were added as per the manufacturer's instructions and finally, the tubes were incubated for 10 min in the dark. The samples were then analyzed on the flow cytometer.

2.7. Statistical analysis

Statistical analyses (student t test or two-way ANOVA) was performed using GraphPad Prism 6 software (GraphPad, San Diego, USA). Results are presented as Mean ± SEM of triplicates in the same experiment or three independent experiments. The significant statistical differences have been designated as $*p<0.05, **p<0.01$ and $***p<0.001$.

3. Results

EGCG and quercetin **treatment inhibits the cell growth of HCT116 cells in a concentration-dependent manner**

Initially, we evaluated the antiproliferative effect of EGCG and quercetin on HCT116 cells using WST-1 staining (Figure 1). Treatment of HCT116 cells for 24 h with EGCG and quercetin exerted a significant decrease in the cell growth in a concentration-dependent manner. At 10 µg/mL, there was a significant decrease in the cell growth to 68% compared to the control untreated cells. Similarly, a significant decrease was observed to 69%, 69%, 53%, and 48% at different concentrations (25, 50, 100 and 150 µg/mL, respectively) (Figure 1A). Moreover, it was observed that after 48 h treatment, the percentage of viable cells at 10, 25, 50, 100 and 150 ug/mL was 74%, 69%, 72% 66% and 58%, respectively, compared to untreated control cells (Figure 1B). Furthermore, when we treated the cells for 72 h the cell viabilities at 10, 25, 50, 100 and 150 µg/mL were 96%, 88%, 86%, 71% and 64%, respectively (Figure 1C). The IC50 values of EGCG and quercetin at 24, 48 and 72 h were calculated as 145 ± 3, 167 ± 1, 248 ± 1.7 µg/mL, respectively.

EGCG and quercetin suppresses the formation of colonies in HCT116 cells

To further verify the inhibitory effect of PB against HCT116 cells, the colony formation assay was performed using different concentrations of EGCG and quercetin (25, 50, 100 and 150 µg/mL). As shown in Figure 2, EGCG and quercetin exerted a concentration-dependent inhibitory effect on the colony formation of HCT116 cells. EGCG and quercetin at a concentration of 10 ug/mL significantly decreased the number of colonies of HCT116 cells by 46.92% compared to the control cells. However, at concentrations of 25 and 50 ug/mL, there were individual cells present in the medium, but colony formation was not observed even after 7 days. Interestingly, at higher concentrations of 100 and 150 ug/mL, neither cells nor colonies were visualized.

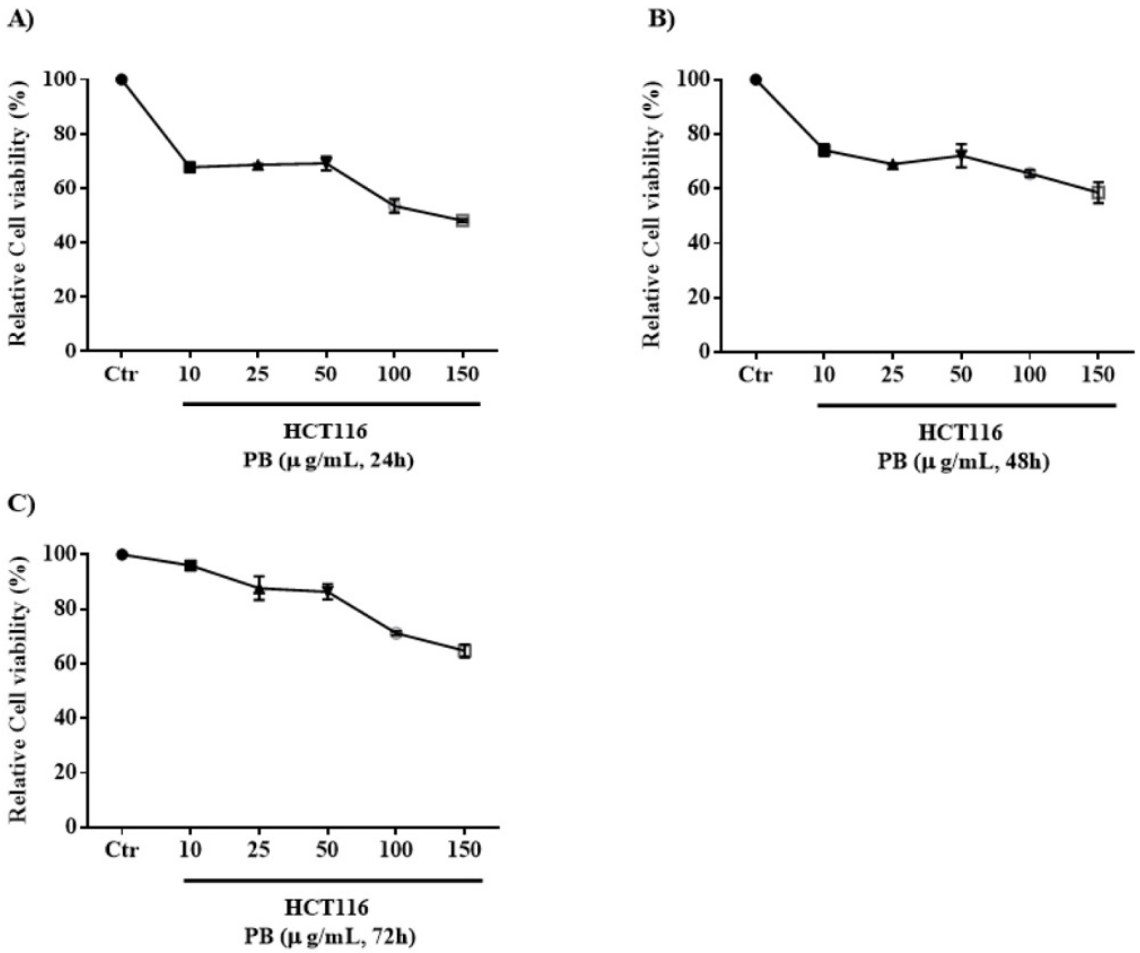

Figure 1. Effect of Phytobiological mixture (PB) on HCT116 cell growth. To evaluate the antiproliferative effect of PB, HCT116 Cells were exposed to increasing concentrations of PB for 24 h (A), 48 h (B) and 72 h (C). Cell growth rate was assessed by WST-1 assay as indicated in the methods and materials. The data are representative of three different experiments. Values are shown as mean ± S.E.M. (n = 5) versus respective control.

EGCG and quercetin **exerts a pro-apoptotic effect on HCT116 cells**

To study the hypothesis that EGCG and quercetin could have the potential to induce apoptosis in HCT116 cells, we performed a concentration-dependent Annexin V and PI double staining to detect the apoptosis stages. The cells were treated with EGCG and quercetin at different concentrations (25, 50,100 and 150 µg/mL) for 24 h. As shown in Figure 3, the percentage of annexin V positive cells significantly increased from a concentration of 50 ug/mL (6.15%) to 100 µg/mL (25.85%) compared to untreated cells. At the highest concentration of 150 µg/mL, the percentage of annexin V positive cells stood at 48.35%. This could suggest that the pro-apoptotic effect could have been initiated in the cells at this stage as a result of the compromised plasma membrane and subsequent exposure of phosphatidylserine which needs to be investigated further.

EGCG and quercetin **treatment induces G1 arrest in HCT116 cells**

To explore the effect of EGCG and quercetin on cell cycle progression of HCT116 cells, the cells were exposed to the indicated concentrations of PB for 24 h. As shown in Figure 4, EGCG and quercetin -treatment induced a significant G1 phase arrest as compared to the control untreated cells. As shown in Figure 4, the percentage of cells in G1 phase were 71, 70.06, 72.7, 81.7%; in 'S' phase were 19.1, 26.2, 25.8, 17.2% and finally in 'G2' phase the percentage of cells were 9.83, 11.4, 3.7, 1.9%; in control, 50, 100 and 150 µg/mL, respectively.

4. Discussion

Globally, colon cancer is one of the most common cancers affecting men and women with a significant number of annual mortalities. The chemotherapeutic regimens used against this cancer acquire resistance over a prolonged period in addition to exerting off-target toxicities (Cappell, 2005; Koelzer et al., 2015; Zhang et al., 2015).

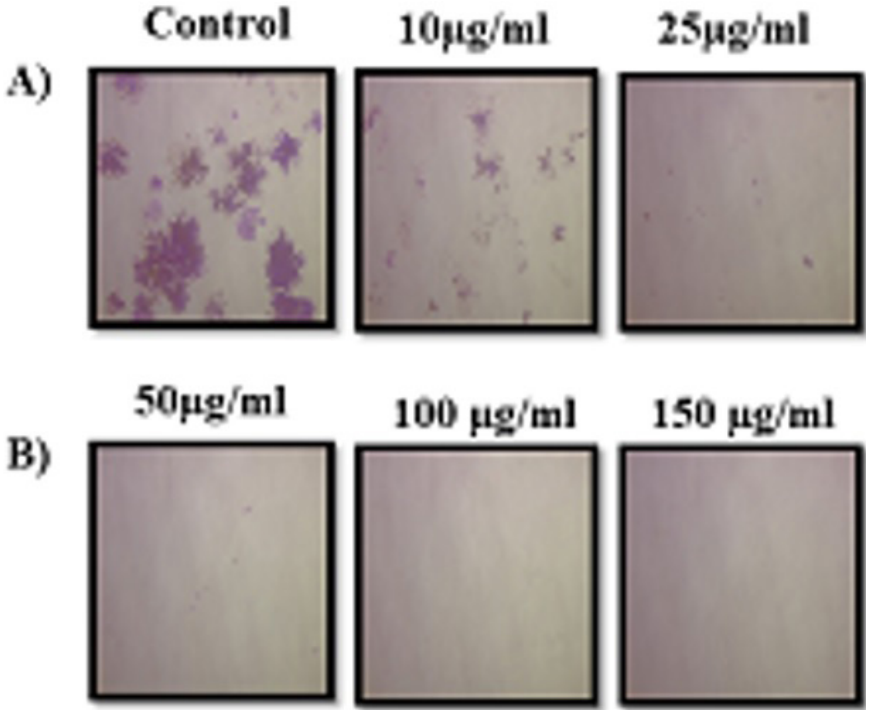

Figure 2. Clonogenic assays in colon cancer cells treated with Phytobiological mixture. Colon cancer, HCT116 were plated at very low density in six-well plates and incubated 24 h at 37 °C. Cells were left untreated ; Control or treated by addition of 10, 25, (Row A), 50, 100 and 150 µg/mL (Row B). After 10 days of treatment, cells were washed with cold PBS, fixed with paraformaldehyde, and stained with crystal violet solution.

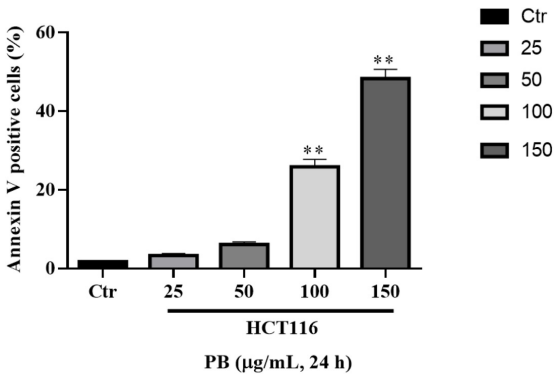

Figure 3. Effect of Phytobiological mixture (PB) on apoptosis in HCT116 cells. To evaluate the apoptotic effect of PB, cells were exposed to increasing concentrations of PB for 24 h. Apoptosis in HCT116 cells was assessed by flow cytometry using the Annexin V/7AAD staining apoptosis assay. Annexin V level was significantly increased as means ± S.E.M. (n = 5); $^{**}p < 0.01$, versus respective control.

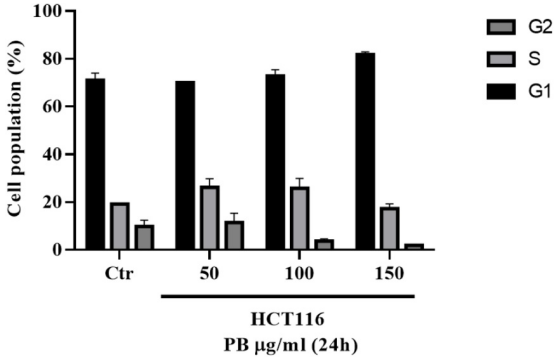

Figure 4. Effects of Phytobiological mixture (PB) on the cell cycle progression of colon cancer cells, HCT116. Cells were seeded in 6-well plate and then treated with PB at increasing concentrations for 24 h. It was observed that there was a significant G1 phase cell cycle arrest with the successive increase in the concentration. The data are representative of three different experiments. Values are shown as mean ± S.E.M. (n = 5); $^{*}p < 0.05$; $^{**}p < 0.01$ versus respective control.

Therefore, there is a persistent need to explore alternative therapeutic strategies to prolong the patient survival and reduce toxicities. In recent years, many medicinal plants have attracted the attention of researchers globally due to their substantial sources of substances with active antiproliferative and anticarcinogenic potential (Kaur et al., 2018). Natural product research has gained momentum in the circles of drug discovery leading to the novel anticancer drugs today (Rajesh et al., 2015).

We noticed that there was a significant decrease in the percentage of viable cells at 24, 48 and 72 h in a concentration-dependent manner. However, the inhibitory effect of PB was more pronounced at 24h than 48 or 72 h, which could be further tested with time-dependent

assays. A previous study has shown that PB-treatment for 72 h demonstrated a concentration-dependent toxicity to HCT116 cells *in vitro* with 27% (p=0.0003) at 75 ug/mL and 48% (p=0.0004) at 100 ug/mL, compared to the control (Roomi et al., 2015). Subsequently, we further verified the inhibitory effect of PB against HCT116 CRC cells proliferation using colony formation assay which revealed a significant inhibition of colony formation of PB-treated HCT116 CRC cells.

Apoptosis induction is a classical approach in initiating cell death characterized by biochemical and morphological hallmarks (Kumaraswamy et al., 2013; Kitazumi and Tsukahara, 2011). The annexin V/PI assay with flow cytometry showed that PB induced the pro-apoptotic effect in HCT116 cells in a concentration dependent manner at 24 h. At the highest concentration of 150 ug/mL, the percentage of annexin V positive cells stood at 28.55%. Our findings are in accordance with the previous studies that reported that the exposure of MDA-MB-231 breast cancer cells to PB resulted in the induction of apoptosis in a concentration-dependent manner (Alqarni et al., 2017).

Progression through the cell cycle is regulated carefully to avoid proliferation or mitosis when adverse conditions exist. DNA damage causes cell cycle arrest in G1, S or G2 to prevent replication of damaged DNA or to prevent aberrant mitosis (Srivastava and Singh, 2004). Dysregulation of the cell cycle is a key feature of tumour cells and therefore targeting the cell cycle of cancer cells is an important approach in cancer therapy (Goi et al., 1997). In the present study, PB induced G1 phase cell cycle arrest in HCT116 CRC cells at high concentrations of 100 and 150 ug/mL. It can be inferred that at higher concentrations, PB may have arrested the replication of DNA and hence prevented the cells to enter into the G2 phase leading to the decreased cell division and overall inhibitory effect on the cancer cell growth. Previous studies have also reported that exposure of MDA-MB-231 to concentrations of PB of 10 ug/mL and 30 ug/mL resulted in a significant cell cycle arrest at the G1/S phase with a population of 25.3 ± 1.6% and 20.5 ± 0.4%, respectively, as compared to the control untreated cells 12.4±1.5% (p<0.001) (Alqarni et al., 2017). However, further molecular studies at the protein and gene level are necessary which can enable us to arrive at a concrete decision. As we mentioned earlier that the combination of bioactive polyphenols in the treatment regimen could increase the bioavailability of the bioactive component and could be a good supportive agent for the current treatments without any detrimental effects.

5. Conclusion

The present study showed that the EGCG combined with quercetin has a potential to exert pro-apoptotic inhibitory effect accompanied by cell cycle arrest in HCT116 CRC cells. It is promising approach to identify a complementary treatment regimen for colon cancer. However, further *in vivo* evaluation and molecular investigations are recommended.

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