ฤทธิ์ต้านมะเร็งของสารสกัดแป้งกล้วยในเซลล์มะเร็งลำไส้ใหญ่
ANTICANCER ACTIVITY OF BANANA FLOUR EXTRACTS ON COLORECTAL CANCER CELLS

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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อตรวจสอบฤทธิ์ต้านมะเร็งของสารสกัดจากแป้งกล้วยที่สกัดด้วยตัวทำละลายต่าง ๆ ได้แก่ เอทานอล เมทานอล เฮกเซน และน้ำ ต่อเซลล์มะเร็งลำไส้ใหญ่ 2 ชนิด ได้แก่ HCT116 และ SW480 โดยตรวจสอบการมีชีวิตรอดของเซลล์และการตายของเซลล์ด้วยเทคนิค MTT และ trypan blue dye exclusion ตรวจสอบรูปแบบการตายของเซลล์แบบอะพอพโทซิสด้วยเทคนิค western blot และการย้อมสี Hoechst 33342 ผลการทดลองพบว่าสารสกัดแป้งกล้วยที่สกัดด้วยเอทานอลทำให้เกิดการตายของเซลล์ทั้งสองชนิดได้ดีที่สุด เมื่อเปรียบเทียบกับสารสกัดแป้งกล้วยที่สกัดด้วยตัวทำละลายชนิดอื่น โดยสารสกัดส่งผลต่อการยับยั้งการเจริญเติบโตของเซลล์มะเร็งลำไส้ใหญ่ทั้งสองชนิดเพิ่มขึ้นตามความเข้มข้นและเวลาที่ทดสอบ เมื่อพิจารณาจากค่า IC_{50} พบว่าสารสกัดส่งผลต่อการยับยั้งการเจริญเติบโตของเซลล์มะเร็งลำไส้ใหญ่ชนิด HCT116 ได้ดีกว่าชนิด SW480 นอกจากนี้ยังพบว่าสารสกัดแป้งกล้วยเหนี่ยวนำให้เซลล์เกิดการตายแบบอะพอพโทซิส จากการทำงานของเอนไซม์แคสเปส 3 และลักษณะการอัดแน่นของโครมาตินสรุปได้ว่าสารสกัดแป้งกล้วยที่สกัดด้วยเอทานอลนี้มีองค์ประกอบของสารออกฤทธิ์ที่สามารถยับยั้งการเจริญเติบโตในมะเร็งลำไส้ใหญ่ การศึกษานี้แสดงให้เห็นว่าแป้งกล้วยมีศักยภาพในการป้องกันและรักษามะเร็งได้

คำสำคัญ: แป้งกล้วย ฤทธิ์ต้านมะเร็ง อะพอพโทซิส มะเร็งลำไส้ใหญ่

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Abstract

This study was aimed to investigate the anticancer effect of banana flour extracted with different solvents (ethanol, methanol, hexane and water) against colorectal cancer HCT116 and SW480 cell lines. Cell viability and cell death were determined by MTT and trypan blue dye exclusion assay. Apoptosis was investigated by western blot analysis and Hoechst 33342 staining. The results found that ethanolic banana flour extract (eBFE) inhibited cell viability of HCT116 and SW480 greater than other solvent extracts. The eBFE was able to significantly inhibit cell proliferation by dose and time dependent manners. According to the IC_{50} values, eBFE had a greater anti-proliferative effect on HCT116 than SW480 cells. In addition, eBFE induced cell death and apoptosis by proteolytic activation of caspase-3 and the appearance of chromatin condensation. Collectively, the crude extract of eBFE contains active components that exert growth inhibition in colorectal cancer. This study suggested that banana flour had the potential for cancer prevention and therapy.

Keywords: Banana flour, Anticancer activity, Apoptosis, Colorectal cancer

Introduction

Colorectal cancer (CRC) is a leading health problem, being the second most prevalent cancer in women and the third in men globally, with increase the prevalence to 60% in 2030 (Rawla et al., 2019). The CRC incidence are reported to increase by environmental factors, in particular lifestyle and diet alterations (Rattray et al., 2017). Although, there are many therapeutic approaches to treating CRC relied on the toxic compounds. Unfortunately, the cytotoxicity properties of most chemotherapy drugs are nonspecific and had a wide range of side effects (Blagosklonny et al., 2005). However, several experimental and epidemiological studies have shown the link between higher fruit consumption and a decreased incidence of cancers (Galisteo et al., 2008). Therefore, the search for chemoprevention or chemotherapy from the fruit is of great promising for CRC.

Bananas are one of the interesting plants which have been widely cultivated in tropical countries (Singh et al., 2016). Traditionally, bananas have been used as food and medicine. Banana fruit are rich sources of valuable phytochemicals, including polyphenols, fatty acids, phytosterols, flavonoids, carotenoids, steroids and biogenic

amines (Ajijolakewu et al., 2021; Qamar & Shaikh, 2018; Someya et al., 2002; Vilela et al., 2014). Recently, bananas possessed pharmacological activities, such as antioxidant, immunomodulatory, antimicrobial, anticancer, antiulcerogenic, hypolipidemic, hypoglycemic, leishmanicidal, and anthelmintic properties (Mathew & Negi, 2017). Previous findings showed that bananas extracts exhibited cancer preventive and anticancer activities in breast, cervical, esophageal, hepatic, oral, prostate, skin, and colorectal cancers (Mondal et al., 2021). The effect utilized various mechanisms, which include cytotoxicity, cell cycle arrest, apoptosis of cancer cells, antioxidant, and anti-inflammatory effects (Mondal et al., 2021). Moreover, the role of banana and its phytoconstituents on colorectal cancer has been studied extensively in both *in vitro* and *in vivo* (Jeong et al., 2013; Barroso et al., 2019; Dahham et al., 2015; Navarro et al., 2015). However, the phytoconstituent compositions of bananas are differ quantitatively due to spices, parts, forms (Unripe/ripe) cultivated area, extraction methods (Falcomer et al., 2019).

In Thailand, the banana (*Musa* ABB cv. "Kluai Namwa") exports are wildly popular and increase growing market in Sukhothai area. However, there are a lot of the small unwanted banana become a waste of the factory leading us an idea to improve valuable of unripe banana fruit waste to unripe banana flour (UBF). Previously, we demonstrated that the presence of the phytochemical composition of UBF depends on the extractant types (ethanol, methanol, hexane and distilled water). All extracted fractions contained sugar (glucose and sucrose) and organic acid (citric acid, malic acid, lactic acid, succinic acid). The phenolic compounds such as gallic acid, glutaric acid, 2-hydroxyvaleric acid, protocatechuic acid, 1,4-Ipomeadiol, 5,6-dimethoxyflavone were found in methanolic and ethanolic extracts. The hexane fraction found the short chain hydrocarbons, fatty acids and its derivative including caproic acid, 1-(2-Thienyl)-1-heptanone, 2-tetradecanone, azacridone A, 5-aminopentanoic acid, piperine and phytosphingosine. Finally, various organic acids (gluconic acid, succinic acid, 3-hydroxy-cis, cis-muconic acid, trans, cis-aconitic acid dibutyl succinic acid) were found in water extract (Jannoey et al., 2021). Interestingly, the methanolic and ethanolic extracts exhibit the great antioxidant activity. However, the biological activities of UBF were not yet determined. The aim of this study was to further investigate the anticancer activities of banana flour extracts on two types of colorectal cancer cell lines (HCT116 and

SW480) by; (i) compare the cytotoxicity among four banana extracts; (ii) determine the anti-proliferative effect of banana extracts and (iii) determine their ability to induce apoptosis.

Material and methods

Cell lines

Human colorectal cancer HCT116 and SW480 cell lines were purchased from American Type Culture Collection (ATCC). Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL of penicillin and 100 g/mL of streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

Chemical reagents

Four banana flour extracts (BFE) were extracted briefly the unripe banana fruit waste (*Musa* ABB cv. "Kluai Namwa") which was obtained from the Looktung banana frying factory, Nongtoom, Sukhothai, Thailand was peeled, sliced, dried, and milled to obtain the banana flour. Then, the banana flour was separately mixed with methanol, ethanol, hexane and water followed by filtration and evaporation as previously described (Jannoey et al., 2021). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen, Molecular Probes products. Trypan blue solution was purchased from Gibco. The primary antibodies, anti-cleaved caspase-3, anti-procaspase-3 and anti-β-actin were purchased from Cell Signaling Technology. Hoechst 33342 was purchased from Sigma-Aldrich.

MTT assay

HCT116 or SW480 cells were seeded at density of $1x10^4$ cells/well in 96-well plates and cultured for 24 h. The cells were treated with different concentrations of BFE or control (0.1% dimethyl sulfoxide: DMSO) for desired incubation times. After the incubation, MTT was added and incubated for 4 h at 37°C. The formazan crystal was dissolved by DMSO and the absorbance at 540nm was measured with a microplate reader. The half-maximal inhibitory concentration (IC_{50}) values for each extract were calculated using GraphPad Prism 8.0 software.

Trypan blue dye exclusion assay

HCT116 or SW480 cells were seeded at a density of 2.5 x10^4 cells/well in 6-well plate and cultured for 24 h. Following treatment with different concentrations (1-5 mg/ml) of BFE for 48 h, the cultured cells were harvested and resuspended in completed media. The cell suspension was thoroughly mixed with an equal volume of 0.4 % trypan blue solution. The numbers of viable and dead cells were counted using a hemocytometer, and the cell death percentage was determined as % cell death = (number of cell death/ number of total cell) x 100.

Western blot analysis

HCT116 or SW480 cells were seeded at a density of 1.5x10^5 cells/well in 6-well plate and incubated for 24 h. After treatment, cells were collected and lysed with RIPA buffer on ice. Then, cell lysates were centrifuged at 12,000 rpm for 20 min at 4℃. The supernatants were collected and determined protein concentration by Bradford assay. Equal amounts of protein from the lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were probed with primary antibodies diluted in TBST at 4 ℃ overnight and further incubated for 1 h with horseradish peroxidase conjugated secondary antibodies. Chemiluminescent signals were then developed and detected by the gel documentation system. β-actin was used as an internal control.

Hoechst 33342 staining

HCT116 cells were seeded at a density of 1.5x10^3 cells/well in 8-well chamber slide and incubated for 24 h. Cells were treated with eBFE for 48 h. After incubation, cells were fixed with 4% formaldehyde, and stained with 0.01 mg/ml Hoechst 33342 for 10 min. The condensed signals were observed under fluorescent microscope at 400x magnification.

Statistical analysis

All results were expressed as mean ± standard error of mean (SEM). Statistical comparison with the control group was carried out using one-way ANOVA with Dunnett's post hoc test. A *p*-value of less than 0.05 was considered significant. The experiments were repeated three times under the same condition.

Results

Effects of BFEs on cell viability in colorectal cancer cells

To compare the anti-viability efficacy of BFEs extracted from different solvents (methanol, ethanol, hexane and water), HCT116 and SW480 cells were screened with 0-800 μg/ml BFEs for 48 h and assessed the cell viability using MTT assay. It was found that banana flour extracted with methanol, hexane and water did not affect the viability of HCT116 and SW480 colorectal cancer cells while banana flour extracted with ethanol significantly exhibited an anti-viability effect on both cell lines. In HCT116, the cell viability was decreased about 10% and 40% after treatment with ethanolic BFE (eBFE) at concentration of 400 and 800 μg/ml respectively. (Figure 1A). In addition, eBFE at concentration 800 μg/ml reduced the cell viability of SW480 cells about 20% (Figure 1B). These results indicated that the ethanolic BFE (eBFE) was more potent in inhibiting cell viability of colorectal cell lines than other extractants.

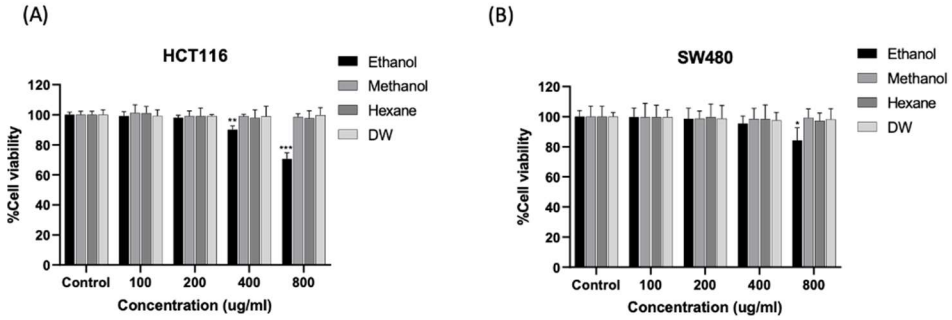

Figure 1 The effects of BFEs on cell viability of (A) HCT116 and (B) SW480 colorectal cancer cells after treatment for 48 h. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

Effects of eBFE on anti-proliferation in colorectal cancer cells

To further assessed the anti-proliferative effect of eBFE on colorectal cancer cells, HCT116 and SW480 were treated with eBFE at a concentration of 0-5 mg/ml for 24, 48 and 72 h and analyzed by MTT assay. The results showed that the eBFE was able to reduce the proliferative rate of both types of cells by dose and time dependent manners as shown the cell density under microscope and MTT results in Figure 2 and 3. Cells were detached and correlated with cell viability rate. According to the IC_{50} values obtained for eBFE in two colorectal cell lines, eBFE was found to possess the greatest anti-proliferative potential in HCT116 compared with SW480 as described in Table 1.

The IC_{50} values at 24 h for eBFE were 3.31±0.96 and 5.92±0.91 mg/ml in HCT and SW480 cells respectively. The IC_{50} values at 48 h for eBFE were 1.98±0.94 and 3.32±0.97 mg/ml in HCT and SW480 cells, respectively. Finally, the IC_{50} values at 72 h for eBFE were 1.39±0.97 and 2.87±0.96 mg/ml in HCT and SW480 cells, respectively. These results indicated that eBEF exerts the growth inhibition of colorectal cancer cells.

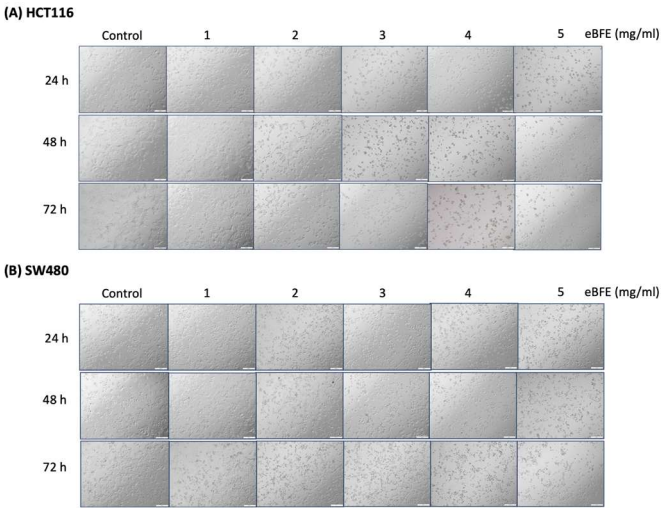

Figure 2 The effects of eBFE treatment for 24, 48 and 72 h on cell density of (A) HCT116 and (B) SW480 colorectal cancer cell lines under inverted microscope at 100x magnification.

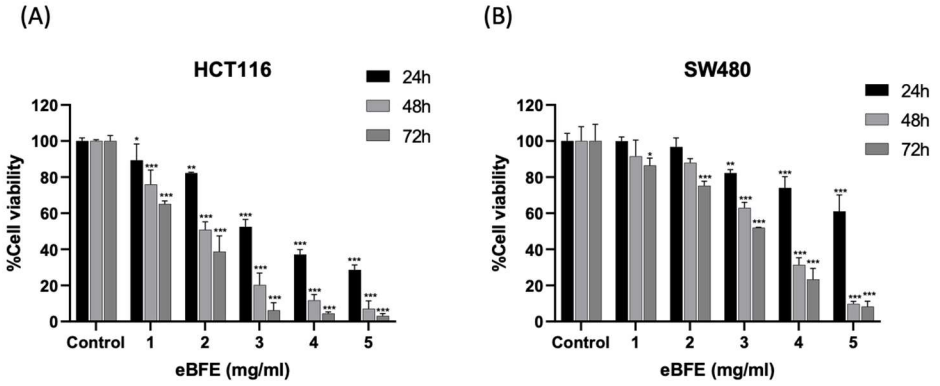

Figure 3 The effects of eBFE on cell proliferation of (A) HCT116 and (B) SW480 colorectal cancer cell lines. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

Table 1 The IC_{50} values of eBFE in HCT116 and SW480

Cell lines	IC50 (mg/ml)		
	24 h	48 h	72 h
HCT116	3.31 ± 0.96[a]	1.98 ± 0.94	1.39 ± 0.97
SW480	5.92 ± 0.91	3.32 ± 0.97	2.87 ± 0.96

Remark [a] $p<0.05$ versus SW480.

Effects of eBFE on cell death induction in colorectal cancer cells

The eBFE at 0-5 mg/ml were treated in colorectal cancer cells, HCT116 and SW480 for 48 h, and the cell death was investigated by trypan blue dye exclusion assay. The results showed that the eBFE was able to induce a higher number of dead cells in accordance with the concentration of eBFE treatment compared with the untreated control cells. The results showed the similar tendency in both cell lines (Figure 4). At concentration of 5 mg/ml eBFE, cell death was induced to 88.5% and 74% in HCT116 and SW480, respectively.

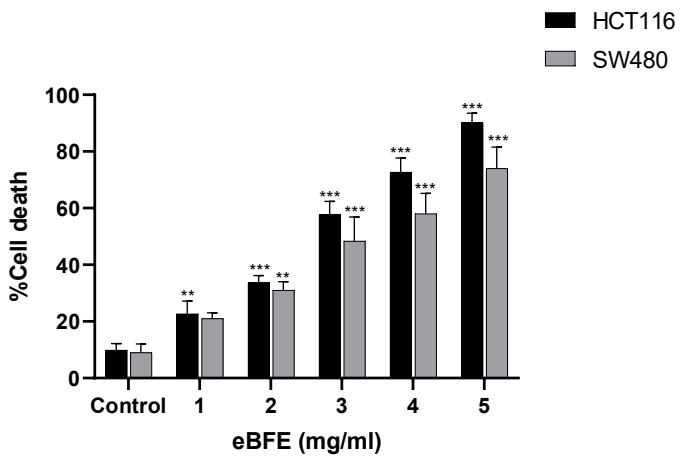

Figure 4 The effects of eBFE on percentage of cell death on HCT116 and SW480 cells from trypan blue dye exclusion assay. ** $p<0.01$ and *** $p<0.001$

Effects of eBFE on apoptotic induction in colorectal cancer cells

The eBFE at concentrations of 0-3 mg/ml were tested on colorectal cancer cells, HCT116 and SW480 for 48 h, the effect of apoptotic induction was examined by measuring the proteolytic activation of caspase-3 via western blotting. The results

showed that in eBFE treated HCT116 cells increased the expression of cleaved-caspase-3 similar to the positive control cell using hydrogen peroxide (H_2O_2) treatment. In addition, eBFE treated SW480 cells showed slightly increase the expression of cleaved-caspase-3 and decreased the expression of procaspase-3 (Figure 5A). Moreover, the results of Hoechst 33342-staining showed that DNA condensation was observed after eBFE treatment in HCT116 cells (figure 5B). These results indicated that eBFE exerted the apoptotic induction in colorectal cancer cells.

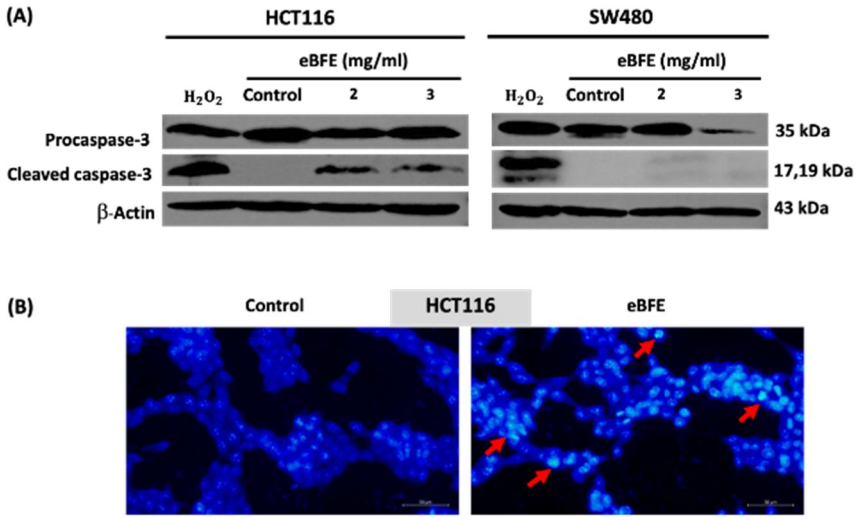

Figure 5 The effects of eBFE on (A) caspase-3 activation and (B) DNA condensation in colorectal cancer cell lines after treatment for 48 h. Red arrows in Hoechst 33342-stained cells indicate condensed chromatin.

Discussions

In the present study, we investigate the anticancer of banana flour extracted with different organic solvents including ethanol, methanol, hexane as well as water to colorectal cancer cells. We found a potential anticancer activity in ethanolic banana flour extract compared to other extractants. Similar to our finding, ethanol extract of banana (*M. paradisiaca*) exhibited anticancer activity against HeLa cervical cancer cell line (Nadumane et al., 2014). In contrast, a study to showed that the hexane extracts of *Musa sapientum* (Banana) fruit exhibited the highest anti-proliferative effects against HCT116 and MCF-7 compared to ethanol and water extracts (Dahham et al., 2015). These

data indicated that phytochemical constituents in extracts vary in banana species, parts, and extraction methods.

In this study, eBFE may contain the important substances that potentially suppress colorectal cancer cells. Our previous study revealed that eBFE contained phenolic compounds such as gallic acid, glutaric acid, 2 - hydroxyvaleric acid, protocatechuic acid, 1,4-Ipomeadiol (Jannoey et al., 2021). There are several reports about anticancer effects of these compounds. Gallic acid can exert its cytotoxic and anti-proliferative effect against via modulation of antioxidant/pro-oxidant balance in HCT115 colon cancer cells. (Subramanian et al., 2021; Kahkeshani et al., 2019). Glutaric acid was found to enhance the toxicity in breast cancer cells (Kumar et al., 2007). In addition, protocatechuic acid or Protocatechualdehyde (PCA, 3,4-dihydroxybenzaldehyde) has several evidence to show anti-cancer effects *in vitro* and *in vivo* (Abotaleb et al., 2020). PCA isolated from green cavendish bananas exhibited antiproliferative activity by triggering apoptosis in human colorectal carcinoma cells (HCT116 and SW480) in a concentration-dependent manner via histone deacetylase 2 (HDAC2)-initiated cyclin D1 suppression (Jeong et al., 2013; Lee et al., 2014). In nanomedicine and drug delivery it is associated with improvements in the therapy efficacy, reduction of side effects and prolonged bioavailability. From these data, anti-cancer effect of eBEF could be due to these phenolic compounds. However, in the present study demonstrated the anticancer effect of crude extract not from the individual compound. These data indicate that those compounds may function alone or synergistically with other compounds.

Presently, apoptosis is a therapeutic goal of cancer therapy because its targeting is more advantageous than other cell death mechanisms (Gavrilescu et al., 2003). Activations of caspases and chromatin condensation are the hallmark of apoptotic cell death. We demonstrated that eBFE induced apoptotic by increasing caspase-3 activation in HCT116 and SW480 cells. Moreover, chromatin condensation was observed after eBFE treated HCT116. Our findings are consistent with previous reports that ethanol extract of banana and its phenolic compounds can induce apoptotic cell death by activation of caspases, cleaved PARP and DNA fragmentation. (Dahham et al., 2015; Lee et al., 2014).

The results obtained from this study were performed by two different types of colorectal cancer cells, HCT116 and SW480. The extract was more potent on HCT116 cells than SW480, possibly because HCT-116 cells were considered to have less cancer aggressiveness. SW480 is a very aggressive cell due to proliferation and gene mutations of P53R273H, P53P309S and APC resulted in low sensitivity to therapeutic extracts. (Parrales et al., 2015). Our results were similar to a previous report that the inhibitory effect of PCA at 200 mM were 71% and 58% in HCT116 and SW480 cell lines (Jeong et al., 2013). Collectively, our data provided evidence that eBEF has potential for anti-cancer. However, the results of this study have some limitations, for example, the concentration used to kill cancer cells is still considered high which may need to be studied in terms of cytotoxicity to the normal cells as well. Additional animal studies are also needed to look at safety and long-term side effects. Moreover, further experiments in a purified compound or molecular mechanism of eBEF need to be more elucidated.

Conclusions

We clearly demonstrate that the banana flour extracted with ethanol has greater anti-growth effect than other extracted solvents. More importantly, the result revealed that banana flour extract showed anti-proliferative effect by inducing apoptosis in human colorectal cancer HCT116 and SW480 cells. Our finding suggested that the banana flour can serve as a good source for development of an anticancer agent against colorectal cancer and other cancer as well.

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