United Journal of Agricultural Science and Research

Research Article

Volume 1 Issue 3

Comparison of Tumoral Inhibition Abilities between Two Colorectal Cancer Cell Lines after Supercritical Fluid Extracted-Papaya Seed Extract Treatments

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Received: 15 Mar 2021
Accepted: 03 Apr 2021
Published: 10 Apr 2021

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Citation:

Lin YH. Comparison of Tumoral Inhibition Abilities between Two Colorectal Cancer Cell Lines after Supercritical Fluid Extracted-Papaya Seed Extract Treatments. United J Agri Sci Research. 2021; V1(3): 1-5.

Keywords:

Benzyl isothiocyanate; Cell viability; Colorectal cancer; *In vitro*; Migration; Murine model; Papaya black seeds; Supercritical fluid CO_2 extraction

1. Abstract

Papaya is a rich-nutrition fruit. Benzyl Isothiocyanate (BITC) is its bioactive substance that expresses in the papaya seeds. In addition, BITC has applied in many bio-medical areas. In this research, we want to understand the comparison of anti-colorectal cancer cell viability and migration effects after papaya black seed extracts via supercritical fluid CO_2 extraction (SFCE). Two colorectal cancer cell lines, CT-26 and HT-29, were used in this study. Under the sample preparation, the black seeds from yellow (ripe) papaya were collected and then extracted by SFCE. The analysis of extract contents as fatty acids, BITC, and other components was performed by using comprehensive two-dimensional gas chromatography coupled with time of flight mass spectrometry. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay and wound-healing assay were applied to evaluate cell viability and migration, respectively. According to the results, papaya seed extracts possessed abilities to decrease cell viability of CT-26 and HT-29 colorectal cancer cell lines. Comparison of CT-26 and HT-29 cell lines was on the cell viability after papaya seed extracts treatment, CT-26 cell line was better than HT-29 cell line on the inhibition of cell viability after treatment of papaya seed extracts respectively contained 5, 10, and 2.5 μM BITC. On the other hand, according to the results, papaya seed extracts also possessed abilities to decrease cell migration of CT-26 and HT-29 colorectal cancer cell lines. After treatment of papaya seed extracts respectively contained 1.25 and 2.5 μM BITC for 48 and 72 hours, papaya seed extracts significantly suppressed CT-26 cell migration. After treatment of papaya seed extracts respectively contained 0.313, 0.625 and 1.25 μM BITC for 48 and 72 hours, papaya seed extracts significantly suppressed HT-29 cell migration. Comparison of CT-26 and HT-29 cell lines was on the cell migration after papaya seed extracts treatment, HT-29 cell line was better than CT-26 cell line on the inhibition of cell migration after treatment of papaya seed extracts respectively contained 0.625 and 1.25 μM BITC. Taken

these results together, papaya black seed extracts may have a potential for inhibiting the growth and migration of colorectal cancer cells.

2. Introduction

Cancer is a major public health problem worldwide. The top 10 cancer types for estimated deaths in Taiwan in 2019 were in order as lung cancer, liver cancer, colorectal cancer, breast cancer (female), oral cavity cancer, prostate cancer, pancreatic cancer, stomach cancer, esophageal cancer, and ovarian cancer. Therefore, the research R&D of novel anti-tumor drugs and the establishment of the suitable tumor-bearing animal models and the ideal therapeutic strategies are urgently needed [1-3].

Papaya's fruit, leaves, seed, root, bark, juice and latex possess many exceptional nutritional, medicinal and other properties. Base on the researches, Such as isothiocyanate extracted from papaya seeds possesses anti-cancer activities for many cancer cell lines of the colon cancer, breast cancer, lung cancer, leukemia, and prostate cancer have been verified via in vitro studies [4-6].

Currently, supercritical fluid carbon dioxide (CO_2) extraction (SFCE) is the process of one component extraction. Ethanol or methanol co-solvent are sometimes participated into supercritical fluid extraction, however, CO_2 is the most used supercritical fluid for supercritical fluid extraction. Due to CO_2 can be recycled and a cleaning and disinfect agent, SFCE is more environmentally friendly compared to other extraction methods. Therefore, safety of CO_2 has been labeled by United States Federal Drug Administration (USFDA) for the industrial extractions. Furthermore, the extraction yield of SFCE is higher than other extraction methods by regulating the physical properties as temperature and pressure. Currently, the application of SFCE is appealing to the food, beverage and medical industries because there is no residual solvent present on the extracts [7-8]. Therefore, we hypothesize that papaya seed extracts via SFCE can be effective in inhibiting CT-26 and HT-29 colorectal cancer cell viability and migration *in vitro*. In addition, we also want to compare the tumoral inhibition abilities between CT-26 and HT-29 colorectal cancer cell lines after supercritical fluid CO_2 extracted-papaya seed extract treatments.

3. Materials and Methods

3.1. Cell Lines and Culture Condition

CT-26 cells (ATCC® CRL-2638™) and HT-29 (ATCC® HTB-38™) were purchased from ATCC (Manassas, VA 20110). McCoy's 5a medium, RPMI-1640 medium, fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from Sigma-Aldrich. CT-26 cells were cultured in RPMI-1640 medium and HT-29 cells were cultured in McCoy's 5a medium. Both McCoy's 5a medium and RPMI-1640 medium were supplemented with 10% FBS and 1% penicillin and streptomycin. The cells were incubated at 37°C with 5% CO_2. Cells were sub-cultured to replace flesh media per 2-3 days when they became confluent.

3.2. Source of Papaya

Papaya was collected from Bei-Hui Papaya milk Co., Taiwan.

3.3. Chemical and Reagents

Cell viability assay kit [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MTT] was purchased from abcam®.

3.4. Preparation of Papaya Seed Extracts

Papaya seeds were washed with 3 times with distilled water and were dried in a chemical hood until a constant weight was obtained. The dried seeds were then grounded and pestle with liquid nitrogen to a fine powder. The dried fine powder was stored at -80°C until used.

3.5. Supercritical Fluid CO_2 Extraction

All supercritical fluid CO_2 extraction (SFCE) were in according to the standard of procedure [9]. Extractions were carried out using a supercritical fluid pilot-plant (Metal Industries Research and Development Centre, Taiwan, model SFE-400S-2000R) comprising a 2 L cylinder extraction cell. The temperature and pressure were held constant at 40^0C, 350 bar. Extract samples were collected every 30 mins.

3.6. Determination of Benzyl Isothiocyanate

The extracts of papaya seeds were detected Benzyl isothiocyanate (BITC) as described previously [4].

3.7. Cell Viability Assay

CT-26 and HT-29 cells (5×10^4/mL) were initially incubated for 24 h in a 96 well plate, respectively. The papaya seed extracts were respectively diluted with culture media. The BITC concentrations in these diluted papaya seed extracts were 0, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320 and 640 μM BITC, respectively. After 24 h-experimental point, cell viability was detected by MTT cell viability assay kit. The reduced purple dye intensity of color was estimated by reading at optical density 570 nm in a spectrophotometer.

3.8. *In Vitro* Scratch Wound-Healing Assay

CT-26 and HT-29 cells (5×10^5 cells/mL) were respectively seeded into 6 well plates until reaching a confluent monolayer. Plates were extensively washed with PBS for 2 times and then were scratched using a sterile 200 μL pipette tip. Later, culture medium was replaced with serum-free culture medium. CT-26 and HT-29 cells in each well were incubated with papaya seed extracts (involve 0, 0.625, 1.25, and 2.5 μM BITC for CT-26 cell culture; 0, 0.313, 0.625 and 1.25 μM BITC for HT-29 cell culture) at 5% CO_2, 37°C incubation for 24 h and photographed under phase contrast microscopy.

3.9. Statistical Analysis

The data were expressed as mean ± SD. All comparisons were made by one-way ANOVA and all significant differences are re-

ported at $^{*/\#}p < 0.05$, $^{**/\#\#}p < 0.01$, $^{***/\$\$\$}p < 0.001$, and $^{***/\#\#\#\#/\$\$\$\$}p < 0.0001$.

4. Results

4.1. Concentration of Benzyl Isothiocyanate in the Extracts of Papaya Seeds via Supercritical Fluid CO_2 Extraction

The extracts of papaya seeds were detected the concentration of Benzyl isothiocyanate (BITC). Data showed that the recovery rate of SFCE can reach 23% (40°C, 350 bars). BITC concentration in the papaya seed extracts was detected as 405 mg/kg papaya seed extracts.

4.2. Suppression of Colorectal Cancer Cell Viability after Treatments of Papaya Seed Extracts

Various BITC concentrations of papaya seed extracts were used to evaluate the effects of cell viability on CT-26 and HT-29 colorectal cancer cells. Data showed that 1.25-640 μM BITC in papaya seed extracts significantly possessed anti-CT-26 cancer cell viability ability (Table 1). 0.625-640 μM BITC in papaya seed extracts significantly possessed anti-HT-29 cancer cell viability ability (Table 1). Comparison of CT-26 and HT-29 cell lines was on the cell viability after papaya seed extracts treatment, CT-26 cell line was better than HT-29 cell line on the inhibition of cell viability after treatment of papaya seed extracts respectively contained 5, 10 and 2.5 μM BITC (Figure 1).

4.3. Suppression of Colorectal Cancer Cell Migration after Treatments of Papaya Seed Extracts

Various concentrations of papaya seed extracts were used to evaluate the effects of cell migration on the colorectal cancer cells. Data showed that non-cytotoxic and lower cytotoxic concentrations of BITC in papaya seed extracts were also used to evaluate anti-cancer cell migration ability. Data presented that 1.25 and 2.5 μM BITC in papaya seed extracts possessed anti-CT-26 cancer cell migration ability (Figure 2A). 0.313, 0.625 and 1.25 μM BITC in papaya seed extracts possessed anti-HT-29 cancer cell migration ability (Figure 2B). Comparison of CT-26 and HT-29 cell lines was on the cell migration after papaya seed extracts treatment, HT-29 cell line was better than CT-26 cell line on the inhibition of cell migration after treatment of papaya seed extracts respectively contained 0.625 and 1.25 μM BITC (Figure 3).

Table 1: Inhibition cell viability of papaya seed extracts on CT-26 and HT-29 colorectal cancer cell lines. The data is expressed as mean ± SD for 3 replicates. All significant differences compared to 0 μM Benzyl isothiocyanate (BITC) of papaya seed extracts were reported at $^{**}p < 0.01$ and $^{***}p < 0.001$.

BITC concentration (μM)	0	0.625	1.25	2.5	5	10	20	40	80	160	320	640
CT-26	100.0±22.7	75.9±18.7	62.4±6.0**	64.5±6.0**	38.1±3.1***	26.1±4.6***	19.7±1.1***	22.5±2.0***	19.2±1.1***	23.6±5.1***	21.8±3.8***	20.1±0.8***
HT-29	100.6±2.9	88.4±4.0*	66.8±1.7***	63.6±4.9***	53.6±2.5***	62.1±2.1***	46.4±1.3***	26.0±6.4***	11.5±2.2***	6.8±0.6***	9.1±0.9***	14.4±0.9***

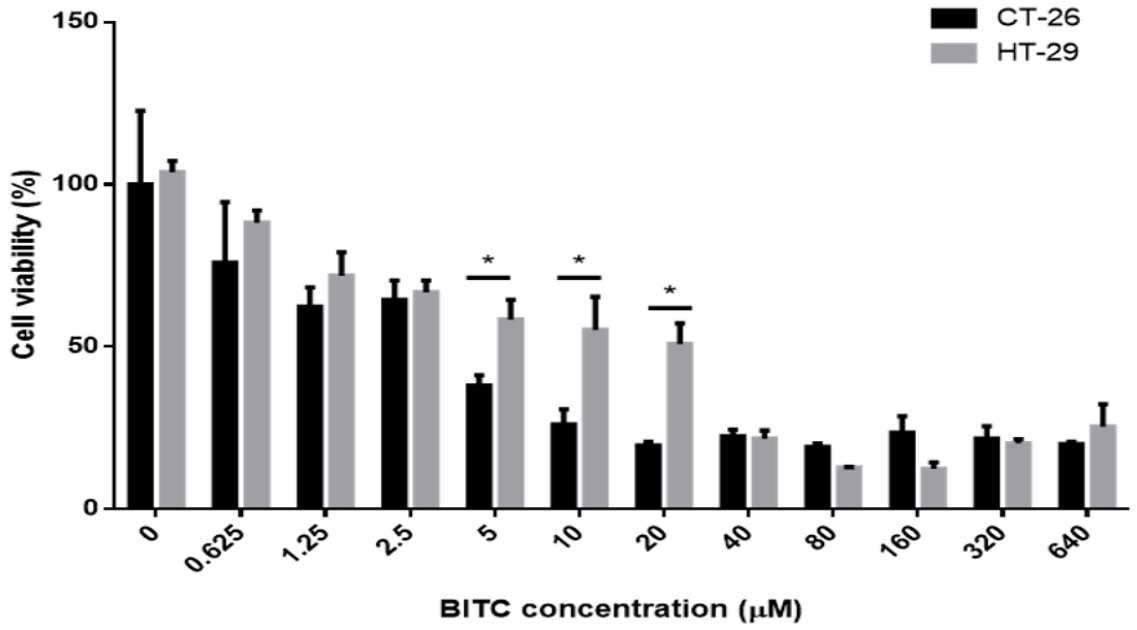

Figure 1: Inhibition cell viability abilities of colorectal cancer cell lines (CT-26 and HT-29) after papaya black seed extract treatment (24 hours). All data are expressed as mean ± SD for three replicates. All significant differences compared to each other after papaya black seed extract treatment were reported at $^{*}p < 0.05$.

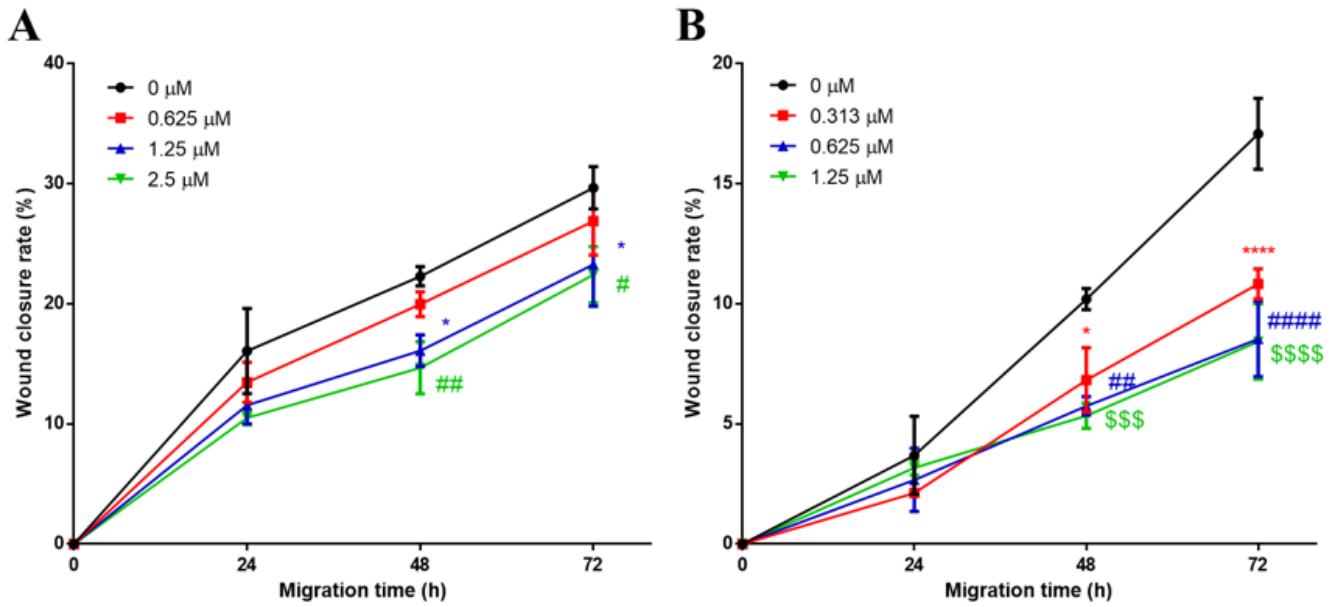

Figure 2: Inhibition cell migration abilities of colorectal cancer cell lines (CT-26 and HT-29) after papaya seed extract treatments (24-72 hours). All data are expressed as mean ± SD for three replicates. All significant differences compared to 0 µM BITC of papaya black seed extract treatment werereported at $^{*/\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\$\$\$}p < 0.01$, and $^{\#\#\#\#/\$\$\$\$/****}p < 0.0001$.

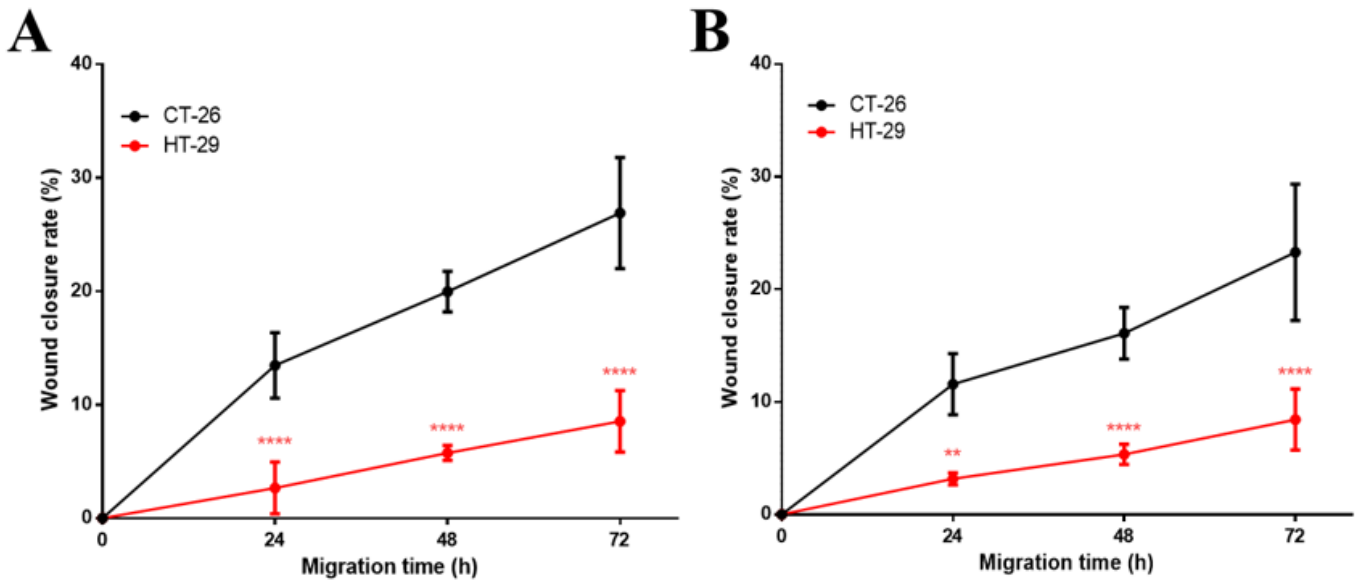

Figure 3: Inhibition cell migration abilities of colorectal cancer cell lines (CT-26 and HT-29) after papaya seed extract treatments (24-72 hours). (A) Papaya seed extract treatment (0.625 µM BITC); (B) papaya seed extract treatment (1.25 µM BITC). All data are expressed as mean ± SD for three replicates. All significant differences compared to 24-72 hours papaya black seed extract treatment were reported at $^{**}p < 0.01$ and $^{****}p < 0.0001$.

5. Discussion

CO_2 is an environmentally friendly solvent. Its benefits are no residue and low viscosity. It allows penetrating into the material that is more easily for the faster extractions. Unlike alternative extraction techniques, therefore, SFCE is a green alternative to solvent-based extraction techniques. The properties of SFCE can be altered by controlling the pressure and temperature for selective extraction [7-9]. In this study, SFCE was applied in the papaya seed extraction. Based on the pressure and temperature (40^0C, 350 bars) of SFCE, BITC concentration is 405 mg/kg papaya seed extracts. In the future, we want to apply this green extraction technology to extract non-toxic agricultural extracts for R&D of anti-cancer drugs.

Papaya is a rich-nutrition fruit for its exceptional nutritional and medicinal properties [10-12]. Different parts of papaya have been demonstrated its functional effects for live health maintain or prevention or therapy diseases. According to the literatures, papaya seed extracts have shown anti-fungal activity, anti-parasite activity and inhibition of sperm motility, increase gastro-protection, and anti-cancer activities [12-15]. Currently, the anti-cancer effects of papaya on the prevention, treatment, or improvement have been

even verified. These cancer types included as gastric cancer, lung cancer, pancreatic cancer, colon cancer, hepato-cancer, ovarian cancer, neuroblastoma, lymphoma, leukemia and other blood cancers [13]. Anti-cancer activities of papaya have been also demonstrated. Significantly, the extracts of papaya juice induced HepG2 hepato-cancer cell death. Papaya seed extracts presented significantly anti-tumor activity on HL-60 cells and PC-3 prostate cancer. Extracts of papaya pulp inhibited significantly MCF-7 breast cancer cell proliferation [13, 16-18]. This aim of study is the investigation of the effect of papaya seed extracts on anti-CT-26 and HT-29 colorectal cancer cell viability and migration. All results have been presented that papaya seed extracts via SFCE significantly decreased cell viability and inhibited migration of CT-26 and HT-29 colorectal cancer cell lines in vitro. Papaya seed extracts may have a potential to inhibit the growth and metastasis of colorectal cancer cells. We hope that papaya seed extracts will be more deeply researched and developed in the anti-colorectal cancer fields in the future.

6. Conclusion

Papaya is a rich-nutrition fruit. Benzyl isothiocyanate (BITC) is a bioactive substance of papaya. This aim of study is the investigation of the effect of papaya seed extracts on anti-colorectal cancer cell viability and migration. Taken these results together, the supercritical fluid CO_2 extracts from papaya seeds significantly decreased cell viability and migration of CT-26 and HT-29 colorectal cancer cell lines ($p < 0.05$). Papaya seed extracts may have a potential to inhibit the growth and metastasis of colorectal cancer cells. Additionally, papaya seed extracts will be demonstrated their anti-cancer ability by applying the colorectal cancer-bearing mouse allograft and xenograft models. We hope that papaya seed extracts will be more deeply researched in the R&D of new anti-colorectal cancer drug in the future.

7. Acknowledgements

Authors thank the Council of Agriculture in Taiwan (ExecutiveYuan) for supporting this study [grant number 109AS-1.1.3-ST-a1, 109AS-22.3.1-ST-a3 and 110AS-14.3.2-ST-a2]. Finally, we thank all the people who joined and helped in this study.

8. Conflicts of Interest

The authors declare no conflict of interest.

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