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CT-26 Colorectal Cancer Inhibition after Administration of Supercritical Fluid Extracted-Papaya Seed Extracts *In vivo*

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Abstract: *Cancer is a major public health problem worldwide. Colorectal cancer is the top 3 for the estimated cancer-caused deaths in Taiwan in 2019. Therefore, Research and development (R&D) of novel anti-colorectal cancer drugs and the ideal therapeutic strategies are urgently needed and important. This study was investigated the inhibition efficacy of CT-26 colorectal cancer via orally administered with the supercritical fluid extracted-papaya seed extracts for 6 weeks in an ectopic allograft CT-26 colorectal cancer-bearing mouse model. The results showed (1) the body weight (BW) of mice in each group (the negative control group, BITC 2.5 mg/kg group, BITC 5.0 mg/kg group, and BITC 10.0 mg/kg group) was decrease from 2nd week after tumor induction. (2) The survival rate of mice in the negative control group, BITC 2.5 mg/kg group, and BITC 5.0 mg/kg group were 100%. However, the survival rate of mice in BITC 10.0 mg/kg group was 0% and the dead mice died between 5-7 days administration with the supercritical fluid extracted-papaya seed extracts (10.0 mg BITC/kg). (3) The tumor weight of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group ($p < 0.05$) and BITC 5.0 mg/kg group ($p < 0.001$). (4) The tumor volume of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group ($p < 0.01$) and BITC 5.0 mg/kg group ($p < 0.001$) after 6 weeks administration of the supercritical fluid extracted-papaya seed extract. Additionally, the tumor volume of the BITC 2.5 mg/kg group was significantly higher than BITC 5.0 mg/kg group ($p < 0.05$) after 6 weeks administration of the supercritical fluid extracted-papaya seed extract. Based on the above test results, although the results of BW, tumor weight, and tumor volume, BITC 2.5 mg/kg group and BITC 5.0 mg/kg group were better than those of BITC 10.0 mg/kg group. However, BITC 10.0 mg/kg group was administrated with 10.0 mg BITC/kg and caused severe side effects to cause all mice to die between 5-7 days administration with the supercritical fluid extracted-papaya seed extracts (10.0 mg BITC/kg). On the other hand, the side effects of BITC 2.5 mg/kg group and BITC 5.0 mg/kg group were better than BITC 10.0 mg/kg group. The overall efficacy and safety data showed that the supercritical fluid extracted-papaya seed extracts (2.5 and 5.0 mg BITC/kg) were significantly better in the anti-cancer choice than the supercritical fluid extracted-papaya seed extracts (10.0 mg BITC/kg). According to these results, the supercritical fluid extracted-papaya seed extracts (2.5 and 5.0 mg BITC/kg) had safety and the positive effect on the inhibition of CT-26 colorectal cancer in mice.*

Keywords: *Colorectal cancer; Inhibitory efficacy; In vivo; Papaya seed; Supercritical fluid extraction*

1. INTRODUCTION

At present, the supercritical fluid extraction is the process of one component extraction. Although ethanol or methanol are sometimes participated as co-solvent into the supercritical fluid extraction, however, CO_2 is the most used supercritical fluid for supercritical fluid extraction. CO_2 safety has be labeled by United States Federal Drug Administration (USFDA) for the industrial extractions. CO_2

can be also recycled and a cleaning and disinfect agent, therefore, the supercritical fluid CO_2 extraction is more environmentally friendly. Additionally, the extraction yield of the supercritical fluid CO_2 extraction is higher than other extraction methods with the regulation of the physical properties as temperature and pressure. Currently, the supercritical fluid CO_2 extraction has been applied to the food, beverage and medical industries [1-2].

Cancer is the world's major public health problem [3-6]. There were 836,150 and 852,630 new cancer cases in the United States in 2017. The top 3 cancers in men in the United States are prostate cancer (19% incidence), followed by lung and bronchial cancer (14% incidence), and colorectal cancer (9% incidence). The top 3 cancers in women in the United States are breast cancer (30% incidence), followed by lung and bronchial cancer (12% incidence), and colorectal cancer (8% incidence) [3-4]. The top 3 cancer in Taiwan in 2019 are lung cancer, followed by liver and intrahepatic cholangiocarcinoma, and colorectal and anal cancer. According to these informations, colorectal cancer is a very important cancer in the United States and Taiwan [7].

The fruit, leaves, seed, root, bark, juice, and latex of papaya (*Carica papaya* L.) have many exceptional nutritional, medicinal, and other properties. Benzyl isothiocyanate (BITC) extracted from papaya seeds that possesses anti-cancer activities have been verified *in vitro* for many cancer cell lines as colorectal cancer, breast cancer, lung cancer, leukemia, and prostate cancer [1-2]. Therefore, we hypothesize that the extracts of papaya seed via the supercritical fluid CO_2 extraction can be effective in inhibiting CT-26 colorectal cancer in an allograft mouse model. We wish that the extracts of papaya seed via the supercritical fluid CO_2 extraction will be a potential role on the growth inhibition of colorectal cancer.

2. Material And Methods

2.1 Reagent

Zoletil 50 (50 mg/mL, Virbac Laboratories, France), TrypLE™ Express (Sigma-Aldrich), Fetal bovine serum (FBS; Gibco®, USA), Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco®), penicillin-streptomycin (Sigma-Aldrich), and the supercritical fluid extracted-papaya seed extracts were used in this study.

2.2 Cell Lines and Culture Condition

CT-26 *Mus musculus* colon carcinoma cell line (ATCC® CRL- 2638™) was purchased from ATCC (Manassas, VA 20110). RPMI-1640 medium was supplemented with 10% FBS and 1% penicillin and streptomycin. The cells (1×10^5/100 μL) were incubated at 37°C with 5% CO_2. Cells were sub-cultured with TrypLE™ Express to replace flesh media per 2-3 days when they became confluent.

2.3 Animal Care

All animal experiments and animal care were approved by the Institutional Animal Care and

Utilization Committee (IACUC) of Agricultural Technology Research Institute (ATRI), Taiwan. The approval number of IACUC, ATRI was No. 108088. Four weeks old male C57BL/6 mice (n = 20; the average of body weight was 16-18 g) were ordered from BioLASCO Taiwan Co. Ltd and were freely fed a standard laboratory diet and the sterile drinking water and kept on a 12-h light/dark cycle at 24-27°C and 60-70% humidity using an automatic control system in the GLP Animal Laboratory, Animal Technology Research Center, ATRI, Taiwan.

2.4 Experimental Design

All mice (n = 20) were divided into four groups as the negative control group (Negative control) (n = 5), BITC 2.5 mg/kg group [n = 5; administration with 2.5 mg BITC/kg body weight (BW)], BITC 5.0 mg/kg group (n = 5; administration with 5.0 mg BITC/kg BW), and BITC 10.0 mg/kg group (n = 5; administration with 10.0 mg BITC/kg BW). The experimental time is 6 weeks. The evaluation of CT-26 colorectal cancer inhibitory efficacy of the supercritical fluid extracted-papaya seed extracts in mice.

2.5 Establishment of an Ectopic allograft CT-26 Colorectal Cancer Model in C57BL/6 Mice

In the CT-26 colorectal cancer-bearing mouse model, CT-26 cells (1×10^7/mouse in 100 μL 0.9% saline) were subcutaneously injected into the right abdomen frank of male C57BL/6 mice (n = 5/group). Later, the mice in the negative control group (Negative control) was free intake with normal drinking water (sterilized water) and fed the normal standard feeds. Mice in the BITC 2.5 mg/kg group, BITC 5.0 mg/kg group, and BITC 10.0 mg/kg group were respectively orally administrated with 2.5, 5.0, and 10.0 mg BITC/kg BW and were also free intake with normal drinking water (sterilized water) and fed the normal standard feeds. At the end of the experiment (W6), all mice were sacrificed and dissected. The tumor masses of mice were collected, weighted, and calculated the tumor volume. The survival rate and body weight of CT-26 colorectal cancer-bearing mice in each group was evaluated.

2.6 Statistical Analysis

The data is expressed as mean ± SD or mean ± SEM. All comparisons were made by one-way ANOVA using Graphpad Prism 6 statistical analysis software. All significant differences are reported at $^{*}p < 0.05$, $^{**/\#\#}p < 0.01$, $^{***}p < 0.001$.

3. Results

In this experiment, we implanted the mice with the colorectal cancer line CT-26 by subcutaneously injection into the right abdomen flank of BALB/c mice (n =20) to induce the ectopic allograft colorectal cancer. During the experimental period, we measured the mice' body weight and observated the survival rate every week. At the end of the experiment (W6), the lung weight of mice was measured and the number of lung tumor masses was counted to evaluate the inhibitory effect of the supercritical fluid

extracted-papaya seed extract on colorectal cancer.

3.1 Mice' Survival Rate in Each Group

Between the experimental periods (W1-W6), the number of survival mice in each group was observed daily. The results showed the survival rate of the BITC 10.0 mg/kg group (administration with 10.0 mg BITC/kg) was 0% (0/5) and all mice were dead between 3 to 5 days administration of the supercritical fluid extracted-papaya seed extract. On the other hand, the survival rates of the negative control group (Negative control), BITC 2.5 mg/kg group (administration with 2.5 mg BITC/kg), and BITC 5.0 mg/kg group (administration with 5.0 mg BITC/kg) were 100% (5/5) until the end of the experiment. Comparing the survival rate changes between 4 groups, the survival rate of BITC 10.0 mg/kg group was the lowest than the negative control group (Negative control), BITC 2.5 mg/kg group, and BITC 5.0 mg/kg group ($^{*}p < 0.05$) (Table 1).

Group	Number	Number of survival mice	Survival rate
Negative control	5	5	100%
BITC 2.5 mg/kg	5	5	100%
BITC 5.0 mg/kg	5	5	100%
BITC 10.0 mg/kg	5	0	0%*

Table 1: The survival rate of each group at the end of the experiment. The survival rate of BITC 10.0 mg/kg group was the lowest than the negative control group (Negative control), BITC 2.5 mg/kg group, and BITC 5.0 mg/kg group ($^{*}p < 0.05$).

3.2 Mice' BW in Each Group

After the beginning of the experiment (W1-W6), BW of the mice was measured every week. After 5 and 6 weeks administration of the supercritical fluid extracted-papaya seed extract, the mice' body weight of BITC 5.0 mg/kg group (n = 5) was higher than the negative control group (Negative control) (n = 5) ($^{***}p < 0.001$). Additionally, after 6 weeks administration of the supercritical fluid extracted-papaya seed extract, the mice' body weight of BITC 2.5 mg/kg group (n = 5) was higher than the negative control group (Negative control, n = 5) ($^{\#\#}p < 0.01$) (Fig. 1).

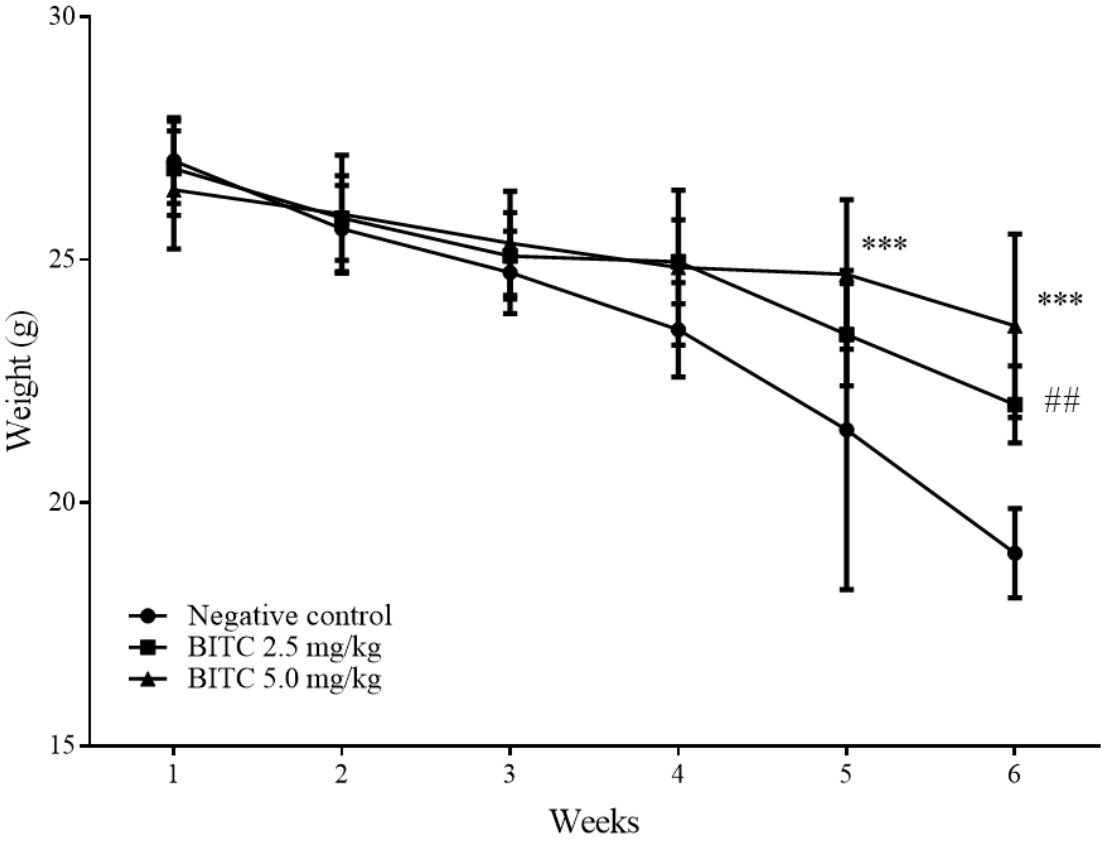

Figure 1: The change of mice' body weight in each group. After 5 and 6 weeks administration of the supercritical fluid extracted-papaya seed extract, the mice' body weight of BITC 5.0 mg/kg group was higher than the negative control group (Negative control) ($^{***}p < 0.001$). Additionally, after 6 weeks administration of the supercritical fluid extracted-papaya seed extract, the mice' body weight of BITC 2.5 mg/kg group was higher than the negative control group (Negative control) ($^{\#\#}p < 0.01$). The data showed mean ± SD. All significant differences were reported as $^{\#\#}p < 0.01$ and $^{***}p < 0.001$.

3.3. Mice' Tumor Weight and Tumor Volume in Each Group

At the end of the experiment (W6), all mice were sacrificed. The tumor masses of the mice were collected to weigh. The results were presented that the tumor weight of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group ($^{*}p < 0.05$) and BITC 5.0 mg/kg group ($^{***}p < 0.001$) (Fig. 2). The tumor volume of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group ($^{**}p < 0.01$) and BITC 5.0 mg/kg group ($^{***}p < 0.001$) after 6 weeks administration of the supercritical fluid extracted-papaya seed extract. Additionally, the tumor volume of the BITC 2.5 mg/kg group was significantly higher than BITC 5.0 mg/kg group ($^{*}p < 0.05$) after 6 weeks administration of the supercritical fluid extracted-papaya seed extract (Fig. 3).

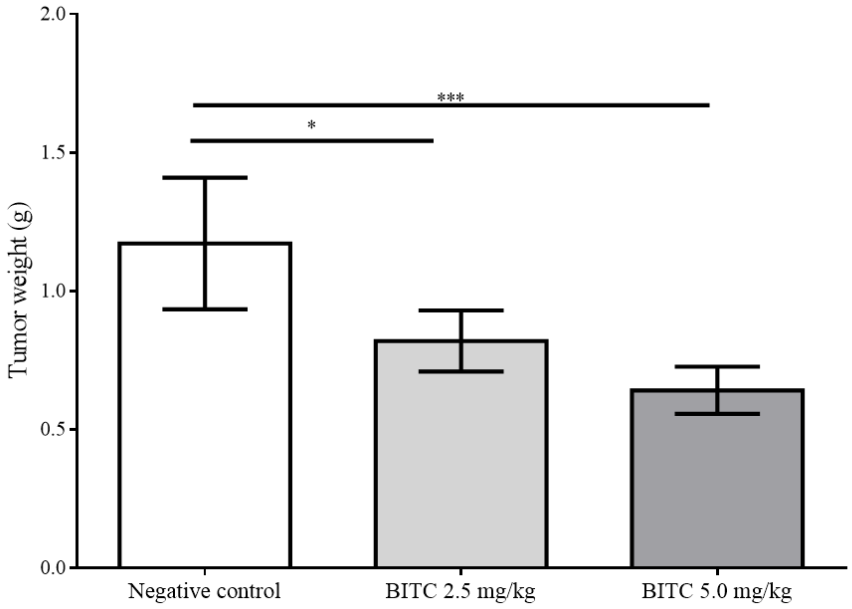

Figure 2: Analysis of tumor weight in each group. The tumor weight of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group ($^{*}p < 0.05$) and BITC 5.0 mg/kg group ($^{***}p < 0.001$). The data showed mean ± SD. All significant differences were reported as $^{*}p < 0.05$ and $^{***}p < 0.001$.

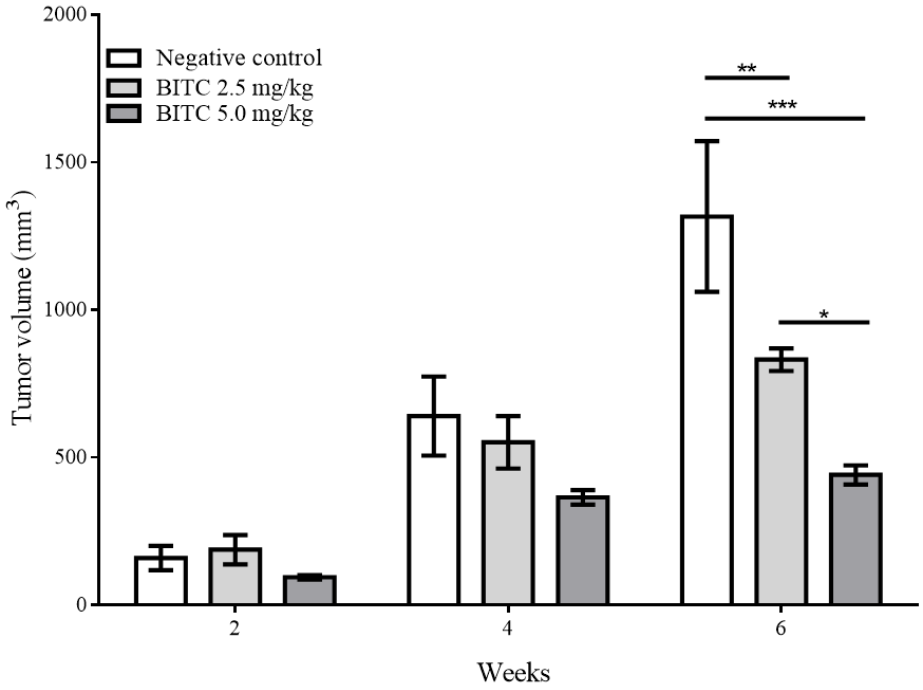

Figure 3: Analysis of the tumor volume in each group. The tumor volume of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group ($^{**}p < 0.01$) and BITC 5.0 mg/kg group ($^{***}p < 0.001$) after 6 weeks administration of the supercritical fluid extracted-papaya seed extract. Additionally, the tumor volume of the BITC 2.5 mg/kg group was significantly higher than BITC 5.0 mg/kg group ($^{*}p < 0.05$) after 6 weeks administration of the supercritical fluid extracted-papaya seed extract. The data showed mean ±

SEM. All significant differences were reported as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

4. Discussion

The living habits, genes, and environment etc are the important causes of cancer. According to the statistic report in Taiwan, cancer has been the top 10 cause of death for many years. According to 2019 year statistic reports, the top 3 cancers among Taiwanese are lung cancer, followed by liver and intrahepatic cholangiocarcinoma, and colorectal and anal cancer. According to the latest global cancer incidence rate by the Organization for Economic Cooperation and Development, Taiwan's cancer incidence rate is 296.7 per 100,000 population, ranking 10th among 45 countries in the world [3, 4, 8]. WHO also lists cancer as one of top 10 threats to public health. Currently, ten million people die by cancer each year worldwide. Additionally, the global cancer cases will be increase to 60% in 2040 and 29.4 million new cases of cancer will occur each year [3, 4, 8]. Therefore, R&D of anti-tumor drugs is very need for these patients with cancer.

The diagnosis of colorectal cancer in clinic includes colonoscopy and blood tests. Colonoscopy applied a long, flexible and slender tube with a video camera and monitor to view the entire colon and rectum. If any suspicious areas are found, the clinic doctor can pass surgical tools through the tube to perform biopsies for analysis and remove polyps. If people have been diagnosed with colorectal cancer, the clinic doctor may recommend tests to determine the stage of cancer. Understand of the cancer stage will help determine what treatments are most appropriate for the patients. The evaluation methods of the cancer stage include the imaging procedures like abdominal, pelvic and chest CT scans. The stages of colorectal cancer are stage 0 to IV. The lowest stage indicates cancer that is limited to the lining of the inside of the colon. The highest stage indicates cancer is advanced and metastasis [9-14].

Cancer is a major public health problem in the world. It is currently the first leading cause of death in Taiwan. Therefore, the establishment of the suitable cancer-bearing animal models and therapeutic strategies for development of more effective treatments for inhibition, not only of proliferation, but also of cancer metastasis is urgently needed [15-21]. In this study, CT-26 murine colorectal cancer cell line was successfully applied to establish the suitable cancer-bearing allograft model. We hope this CT-26 colorectal cancer-bearing mouse model will be applied to research and develop the novel anti-cancer drug in the future. On the other hand, the supercritical fluid extracted-papaya seed extracts was tried to therapy CT-26 colorectal cancer-bearing experimental mice. After 6 weeks oral administration of the supercritical fluid extracted-papaya seed extracts in C57BL/6 mice, the results were showed that the supercritical fluid extracted-papaya seed extracts has a positive effect on inhibiting the growth of CT-26 colorectal cancer.

5. Conclusion

This study was investigated the inhibition efficacy of CT-26 colorectal cancer via orally administered with the supercritical fluid extracted-papaya seed extracts for 6 weeks in an ectopic allograft CT-26 colorectal cancer-bearing mouse model. These results in

each group were showed as BW of mice, the survival rate of mice, the average tumor weight, and the average tumor volume. Based on these results, the supercritical fluid extracted-papaya seed extracts (2.5 mg BITC/kg BW and 5.0 mg BITC/kg BW) were safety for C57BL/6 mice. However, 10.0 mg BITC/kg BW of the supercritical fluid extracted-papaya seed extracts was a toxic dose for mice; the tumor weight of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group and BITC 5.0 mg/kg group; the tumor weight of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group and BITC 5.0 mg/kg group. The tumor volume of the negative control group (Negative control) was significantly higher than BITC 2.5 mg/kg group and BITC 5.0 mg/kg group after 6 weeks administration of the supercritical fluid extracted-papaya seed extracts. Additionally, the tumor volume of the BITC 2.5 mg/kg group was significantly higher than BITC 5.0 mg/kg group after 6 weeks administration of the supercritical fluid extracted-papaya seed extracts. The overall efficacy and safety data showed that BITC 2.5 mg/kg group and BITC 5.0 mg/kg group was significantly better in against CT-26 colorectal cancer in mice.

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Conflicts of Interest

The authors declare no conflict of interest.

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