RESEARCH ARTICLE

Editorial Process: Submission:10/15/2022 Acceptance:07/05/2023

Favorable Antitumor Activity of *Citrus microcarpa* B. on Human Colon Adenocarcinoma Tumor Xenografted in Immunosupressed Mice

Michael Ples, Michael Santos, Rodel Jonathan S Vitor II*

Abstract

Background: The antitumor activity of Citrus microcarpa B. on HT29 human colon adenocarcinoma tumors xenografted in immunosuppressed mice was determined in this study. **Objective:** The objective of the study was to determine if the crude extract of *C. microcarpa* B. exhibited antitumor activity against HT29 human colon adenocarcinoma tumors xenografted in immunosuppressed mice. **Methods:** Cyclosporine-induced immunosuppressed mice were injected subcutaneously with 106 HT29 cells in the caudo-dorsal area of the back near the base of the tail to induce tumor growth. Tumors were grown for 9 days, and the mice were then administered with *C. microcarpa* B. (160 and 630 mg/kg) (Group A; n = 4 and B; n = 4) and normal saline solution (Group C; n = 4) intraperitoneally. Tumor volume was measured to assess the change in tumor volume after 24, 48, and 72-hour post-treatment administration. Tumors were then excised and analyzed histopathologically to evaluate the ratio of necrotic area to viable cancer cells in the tumors. **Results:** Treatment of *C. microcarpa* B. with a dose of 160 mg/kg (P=0.002) and 630 mg/kg produced a significant decrease in tumor volume with the significance only observed at 72 hours post-treatment. Histopathological analysis showed a considerable decrease in the area of necrosis against viable tumor cells in the treatment of *C. microcarpa* B. with a dose of 630 mg/kg. **Conclusion:** It can thus be said that *C. microcarpa* B. is effective in reducing tumor volume, specifically at a dose of 630 mg/kg 72-hours post-treatment.

Keywords: Calamansi- anti-cancer- HT29- xenograft

Asian Pac J Cancer Prev, **24 (7)**, 2253-2258

Introduction

Cancer is considered as the common term for all malignant tumors. There are internal and external factors that cause cancer. Internal factors include hormones, immune condition, and mutations while external factors include tobacco, chemicals, radiation, and infectious agents. Such factors may act together or in tandem to aggravate carcinogenesis (Kumar et al., 2020). According to the American Cancer Society, cancer caused approximately 556,000 deaths in 2003 which corresponds to 1,500 deaths due to cancer per day, accounting for approximately 23% of all deaths in the United States (American Cancer Society, 2022). In the Philippines, the 2021 vital statistics report by the Philippine Statistics Authority reported that cancer is the fourth leading cause of death after ischemic heart disease, cerebrovascular diseases, and COVID-19 (Philippine Statistics Authority, 2022). Specifically, in a previous study, they were able to find that 75% of cancers occur after 50 years of age, while around 3% occurs in adolescents and children 14 years old and below (Ngelangel and Wang, 2002). Various approaches in the prevention, management and treatment of cancer are being considered, but most of which are expensive for an average Filipino.

An abundance of nutrients and fibers essential for preventing cancer are mostly found in fruits, vegetables, seafood, root crops, unrefined cereals and other natural foods (Heinerman, 2009). Given that the Philippines has a vast herbal resource that is often easily available, this field of herbal medicine should continuously be explored. Several plants have been observed to have preventive or curative potentials for cancer, among them is the citrus family which has been shown to have anti-cancer activity (Cirmi et al., 2017). Currently, limited studies are available regarding the antitumor potential of Citrus microcarpa B. (calamansi), a member of the citrus family of which is native to the Philippines, albeit being one of the cheapest citrus fruits in the Philippines. Several substances are found in citrus fruits, such as flavonoids (Kandaswami et al., 2005), limonoids (Shi et al., 2020), and pectins (Hayashi et al., 2000), have been shown to have antioxidant and antitumor activity. However, most of these studies have focused mainly on citrus fruits that are not widely cultivated in the Philippines.

*Department of Biology, College of Science, De La Salle University, Philippines. *For Correspondence: rodel.vitor@dlsu.edu.ph*

In line with this, the study aimed to determine if the crude extract of *C. microcarpa* B. exhibited antitumor activity against HT29 human colon adenocarcinoma tumors xenografted in immunosuppressed mice. This study also aimed to compare the percent decrease of tumor volume at various doses of crude extracts of *C. microcarpa* B. within different time periods and determine the percent necrotic area in the HT29 human colon adenocarcinoma tumor xenografted in immunosuppressed mice after treatment with *C. microcarpa* B.

Materials and Methods

Animal Subjects

Six- to eight-week-old male BALB/c mice were procured from the National Institutes of Health, University of the Philippines Manila and were randomly allocated to the different groups to be used for the different procedures. Four mice per group (5 groups) were used for the determination of approximate lethal dose (ALD) for a total of 20 mice. Four mice per group (3 groups) were also used for determination of tumor volume in cyclosporine-induced immunosuppression of mice with three-different time points for a total of 36 mice.

Four mice were housed in cages with sterile wood-chip bedding and are provided with chow pellets and distilled water ad libitum. The caudo- dorsal surface of the mice was kept hairless prior to inoculation of the HT29 cancer cells in order to facilitate injection of the cancer cells, for detection of tumor growth, and for measuring changes in tumor volume. Depilation was continuously carried out by spreading depilatory lotion over the body surface. All procedures described below were approved by the Department of Biology Animal Care and Use Committee, De La Salle University (Reference # 2014-003).

Source and Maintenance of Cancer Cell Line

HT29 human colon adenocarcinoma cells were purchased from the Research and Biotechnology Division (RBD) of St. Luke's Medical Center, Quezon City, Philippines. Cells were maintained in a 25-cm2 culture flask containing 10 mL of culture medium (pH 7.0) consisting of 88% RPMI (Sigma-Aldrich, Missouri, US) supplemented with 10% FBS, Pen-Strep and NaHCO3, incubated in 5% CO_2 at 37°C. When the culture medium was changed, the RPMI in the flask was initially removed using serological pipettes, then approximately 10 mL of PBS was used to wash the cancer cells twice. Another 10 mL of the culture medium was added to the culture flask and the culture flask was again incubated.

Crude Plant Extraction

Authenticated fresh fruits of *C. microcarpa* B. (calamansi) were collected from the Bureau of Plant Industry, Department of Agriculture, Philippines. Crude extract was prepared using approximately 20 kg of the fruit. After washing and air-drying, the fruits were squeezed. The extract was filtered through a cheesecloth and allowed to stand overnight in a refrigerator. The percolate was decanted, centrifuged and filtered through Whatman (No. 1) filter paper (Whatman PLC, Maidstone, UK). The crude extract was collected, and the total volume was noted. The extract was then submitted for lyophilization at the Chemistry Laboratory, De La Salle University.

Determination of Approximate Lethal Dose

Determination of approximate lethal dose (ALD) of the crude extract was carried out using the procedures as described by the OECD for acute toxicity studies (OECD, 2001). An initial dose of 40 mg/kg body weight was arbitrarily chosen. Succeeding doses were increased logarithmically at 0.6 log interval and were calculated to be 160, 630 and 2500 mg/kg. These doses were administered to randomly selected mice by intraperitoneal (IP) administration. Normal saline solution (10 mL/kg) was used as negative control and likewise administered IP. The number of mice that died in one-hour duration or with observed adverse effects of the extract were recorded.

Cyclosporine-induced Immunosuppression of Mice

Cyclosporine diluted with NSS was administered to each mouse IP using a 29-gauge needle at a dose of 60 mg/kg of body weight once daily. Injections were made 5 days before cancer cell inoculation and until tumor excision (Zhou et al., 2018).

Harvesting and Counting of Cancer Cells

Ten mL of PBS was used to wash the cancer cells twice. Five mL of trypsin-EDTA solution was introduced into the flask to dislodge the cells which was expected to adhere at the bottom of the flask. The flask was immediately returned to the CO2 incubator for at least 5 minutes after which the trypsin-EDTA solution containing the dislodged HT29 cells was resuspended in 10 mL RPMI, transferred into a 50-mL conical tube, and centrifuged at 800 rpm for 5 minutes. The supernatant was discarded and the remaining pellet containing the harvested cancer cells was resuspended in 10 mL RPMI. The harvested cells were counted using the trypan blue exclusion method. In brief, four hundred μL of trypan blue stain was used to dilute 100 μL of the cancer cell solution. Then, a sample of the dilution preparation was transferred into a hemocytometer slide and the viable cells which remained colorless were counted under the microscope.

Treatment and Inoculation of Cancer Cells

Tumors were xenografted into the test animals through the injection of at least 106 HT29 cells suspended in RPMI subcutaneously (SC) into the caudo-dorsal area of the back of each mouse following 5 days of immunosuppression (Day 6) with cyclosporine which continued until tumor excision. The tumors were allowed to grow for 9 days after which they were injected with the different treatments (Day 15 to 17).

Measurement of Tumor Volume and Histopathological Analysis

Administration of 160 and 630 mg/kg of the crude extract, determined to be non-lethal, and NSS (10 mL/kg) were done IP. Measurements of two perpendicular dimensions using vernier caliper of

each tumor was performed at 24-, 48- and 72-hr after administration of treatments to determine the tumor volume (TV) by measuring the longest and shortest distances on the dorsal side of the mice. The TV is then calculated as longest dimension x (shortest dimension)2 x 0.5 (Fu et al., 2004). The percent (%) change in TV was computed using the following formula (Nishino et al., 2013):

$$\%\ change\ in\ TV = \left(\frac{TV_{final} - TV_{initial}}{TV_{initial}}\right) \times 100$$

The mice were sacrificed at 24-, 48- and 72-hr after administration of treatments and the tumors were excised and preserved in 10% buffered formalin for histopathological analysis. The tumors were then processed for routine hematoxylin-eosin (H&E) staining for histopathological analysis subjected to analysis by a pathologist blinded to the study. The ratio of the area of necrosis to the area of viable cancer cells were determined and was graded using the standard set by Blakey et al., (2002) (Table 1).

Statistical Analysis

The differences in the percent decrease in tumor volume were analyzed using repeated measures ANOVA after satisfying the assumptions of homogeneity and normality and the differences within treatment and between treatment per time period were compared using Bonferroni's test with $P < 0.05$. All statistical analyses were performed using STATA v. 15.1.

Results

Approximate Lethal Dose of Citrus microcarpa B

No toxicity was observed in 40, 160, and 630 mg/kg body weight of the crude extract of *C. microcarpa* B. However, at 2,500 mg/kg body weight, 3 mortalities out of 4 were observed after a 1-hour period, and slight abrasion of the skin at the site of injection was also seen. The ALD of was determined to be between the dose range of > 630–2,500 mg/kg body weight (Table 2).

Table 1. Tumor Grading based on Percentage of Necrotic Area (Blakey et al., 2002).

Necrotic Area Percentage (%)	Tumor Grade
0-10	1
20-Nov	2
21-30	3
31-40	4
41-50	5
51-60	6
61-70	7
71-80	8
81-90	9
91-100	10

Effect of C. microcarpa B. on Tumor Volume at Varying Dosages

Figure 1 shows the results of the repeated measures ANOVA where significance was only observed for the group effects ($P<0.0001$) while the time effect ($P=0.1454$) and interaction between group and time effects ($P=0.3087$) were not significant. Analysis of the differences between time points showed that differences were only observed for 160 mg/kg ($P=0.002$) and 630 mg/kg ($P<0.001$) as compared to normal saline at 72 hours after administration. There are no significant differences between 160 mg/kg and 630 mg/kg at all time points.

Histopathological Analysis of Tumors

Successful xenografting of HT29 cells in the immunosuppressed BALB/c mice was confirmed by the growth of tumor in the area of injection. On gross examination, the tumors that were grown subcutaneously took on the color of the skin. The tumors were solid, smooth and well-demarcated. The presence of viable nests of poorly differentiated adenocarcinoma cells in the excised tumors was confirmed by histopathological examination.

Tumor treated with 160 mg/kg extract that was excised

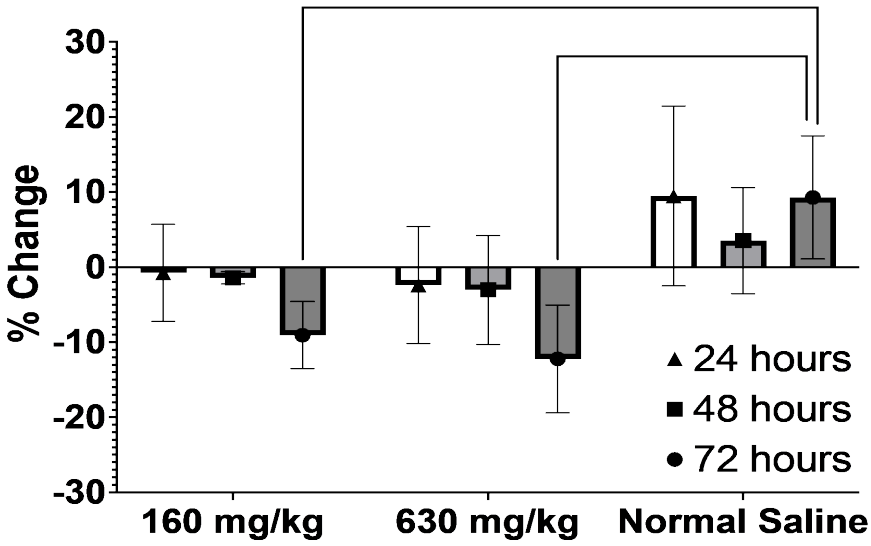

Figure 1. Mean % Change in the Tumor Volume in Different Treatment Groups at Different Time Points.

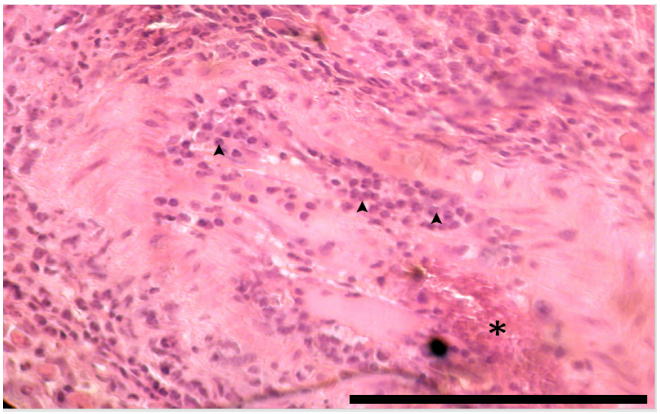

Figure 2. Tumor Treated with *C. microcarpa* B. (160 mg/kg) excised 72-hr Post-Treatment Showing Necrotic Debris Mixed with Blood (*) Seen within the Tumor which is Haphazardly Admixed with Inflammatory Cells (Arrowhead) (Tumor Grade 1). Bar scale = 200 um.

Table 2. The Approximate Lethal Dose (ALD) Range of Intraperitoneal (IP) Administration of the Crude Extract of *C. microcarpa* B.

Treatment Group	Dose (mg/kg)	Number of Mice	Dead: Alive Ratio
A	40	4	0:04
B	160	4	0:04
C	630	4	0:04
D	2500	4	3:01
NSS	10	4	0:04

72-hr post-treatment showed presence of necrosis which is haphazardly admixed with inflammatory cells. Others contained only small foci of tumor infiltrates without visible necrosis (Figure 2). Tumor treated with 630 mg/kg extract that was also excised 72-hr post-treatment contained 40% area of necrosis with the viable cancer cells occupying the remaining 60% (Figure 3). Tumor treated with normal saline shows foci of tumor infiltrates with inflammation notably present (Figure 4).

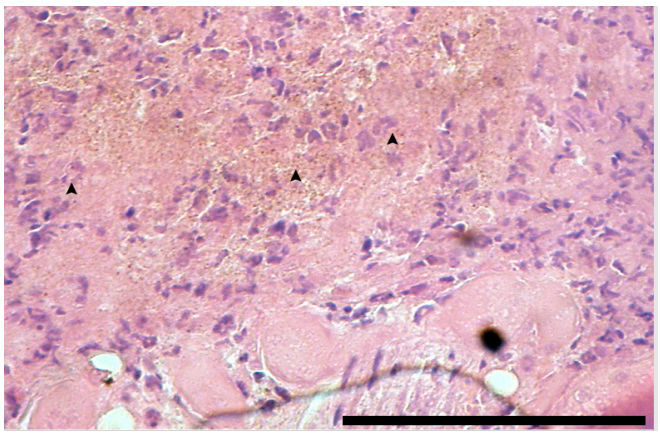

Figure 3. Tumor Treated with *C. microcarpa* B. (630 mg/kg) Excised 72-hr Post-Treatment Containing Areas of Necrosis (Arrowhead) (Tumor grade 4). Bar scale = 200 um.

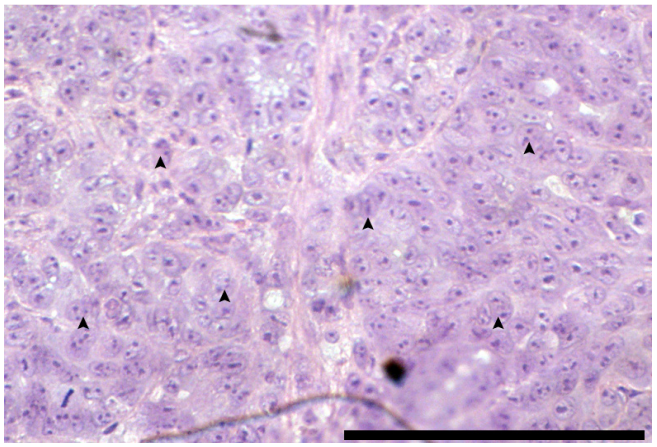

Figure 4. Tumor Treated with Normal Saline Excised 72-hr Post-Treatment Showing Foci of Tumor Infiltrates (Arrowhead) without any Visible Area of Necrosis (Tumor Grade 1). Bar scale = 200 um.

Discussion

The present study demonstrated the antitumor activity of the crude extract of *C. microcarpa* B. on HT29 human colon adenocarcinoma tumors xenografted in immunosupressed mice. Two possible mechanisms of decreasing the tumor volume that were exhibited by some members of the Citrus family may also apply to *C. microcarpa* B. either through the induction of apoptosis or by eliciting an immune response that may eventually lead to the destruction or necrosis of viable tumor cells (Cirmi et al., 2017).

A number of studies support the first mechanism by identifying biochemical components present in citrus fruits that are involved in antitumor activity (Alshatwi et al., 2011). Quercetin is a well-known flavonoid occurring in citrus fruits known to have anti-tumorigenic activity. The antitumor mechanism of quercetin includes lymphocyte proliferation, neutrophilia, free radical scavenging, anti-angiogenesis, downregulation of the mitotic cycle in tumor cells, gene expression alteration, and induction of apoptosis (Hayashi et al., 2000). Quercetin has also been shown to downregulate the expression of the mutant p53 gene in cancer cell lines, cause G1 phase arrest in several cancer cell lines, and inhibit tyrosine kinase responsible for tumor growth (Avila et al., 1994). Limonoid glucosides belong to a class of water-soluble and tasteless limonoids which were found to have anti-neoplastic activity against chemically-induced cancers of the colon (Shi et al., 2020). Purified limonoid glucosides are both cytostatic and cytotoxic to undifferentiated human cancer cells in culture. One proposed mechanism of induction of apoptosis and the regulation of the apoptotic signaling pathway is through calcium which is strongly implicated in the activation of 2 proteases: μ-calpain and caspase-12 (Poulose et al., 2005). Apoptosis induction can also be explained through the action of pectin. In a study by Avivi-Green et al. (2000), pectin-enriched diet was shown to increase the apoptotic index of highly proliferative cells in colon crypts of rats. Upregulation of active caspase-1 subunit (20 kDa) and caspase-3 precursor, causing higher caspase-3 activity of the luminal colonocytes were reported. A number of mechanisms of limonene action have been suggested including induction of carcinogen metabolizing enzymes, growth factor/growth factor receptor expression, and inhibition of 3-hydroxy-3-methylglutaryl CoA reductase and inhibition of Ras protein farnesylation (Crowell, 1999).

To support the cell-mediated immune response as the possible mechanism for anti-cancer activity, other biochemical compounds were also identified. In the study of Tanaka et al., (1999), auraptene has been shown to enhance macrophage and lymphocyte function in mice. This produces a variety of cytokines to induce defense systems. It has also been shown to enhance the Th1 cell production of IL-2 and IFN which increases cell-mediated immunity; but no effect on Th2 cell production of IL-4, which downregulates cell-mediated immunity. The antitumor effect is mediated primarily through this enhancement of immune function. Murakami et al., (2000) reported that nobiletin, a polymethoxyflavonoids occurring exclusively in citrus fruit, was found to successfully suppress leukocyte infiltration which is a critical step in the priming stage. This is mediated through an increase in the release of IL-1 which is an important factor released by keratinocytes. Due to its function involving cell proliferation and hyperplasia via autocrine signaling, it has been especially studied in skin cancer models. Nobiletin was also found to inhibit the expression of COX-2 which is an inducible and rate-limiting enzyme for PG synthesis. Nobiletin inhibited the release of PGE2 from RAW 264.7 cells and from rabbit synovial cells. The suppression of NO, VEGF, and PGE2 production may be responsible for edema formation and the reduction of epidermal thickness in certain cancer cell lines (Murakami et al., 2000)

Anti-angiogenesis is another aspect that could explain the antitumor activity of the citrus fruits. In a study by Schindler and Mentlein, flavonoids have been shown to interfere with the process of neoangiogenesis which is required for the tumor to develop and progress. Vascular endothelial growth factor (VEGF) release by solid tumors was inhibited by naringin, a class of flavonoid present in citrus. Auraptene, a coumarin flavonoid also present in citrus may also explain some of its anticarcinogenic action (Schindler and Mentlein, 2006).

The crude extract of *C. microcarpa* B. exhibited antitumor activity against HT29 human colon adenocarcinoma tumors xenografted in immunosuppressed mice. Treatment of *C. microcarpa* B. with a dose of 160 mg/kg and 630 mg/kg produced a significant decrease in tumor volume. The greatest decrease in tumor volume were observed at 72-hours post-treatment. Histopathological analysis showed considerable area of necrosis (20% and 40%) against viable tumor cells in the treatment of *C. microcarpa* B. with a dose of 630 mg/kg. To further elucidate the effect of C. macrocarpa on cancer inhibition, phytochemical analysis of the crude extract of *C. microcarpa* B. for analysis of its components and their respective modes of action for its antitumor activity is warranted. Further, the use athymic nude mice in the place of cyclosporine immunosuppressed female BALB/c mice as hosts for inoculation of human cancer cells may be used as well as the use of other human cancer cell lines to induce tumor growth in mice.

Author Contribution Statement

All authors have contributed equally to the conceptualization, data gathering, data analysis, and editing of the manuscript..

Acknowledgements

Ethical Statement

All procedures described below were approved by the Institutional Animal Care and Use Committee, De La Salle University.

Ethical Approval

This study was approved by the De La Salle University Department of Biology Animal Use and Care Committee

(Reference # 2014-003) as part of the Master's Thesis of Michael Santos.

Conflict of Interest

All authors declared that there are no conflicts of interest.

References

Alshatwi AA, Shafi G, Hasan TN, et al (2011). Apoptosis-mediated inhibition of human breast cancer cell proliferation by lemon citrus extract. *Asian Pac J Cancer Prev*, **12**, 1555-9.

American Cancer Society 2022. Cancer facts & figures 2022, Atlanta, Author.

Avila MA, Velasco JA, Cansado J, et al (1994). Quercetin mediates the down-regulation of mutant p53 in the human breast cancer cell line MDA-MB468. *Cancer Res*, **54**, 2424-8.

Avivi-Green C, Madar Z, Schwartz B (2000). Pectin-enriched diet affects distribution and expression of apoptosis-cascade proteins in colonic crypts of dimethylhydrazine-treated rats. *Int J Mol Med*, **6**, 689-98.

Blakey DC, Westwood FR, Walker M, et al (2002). Antitumor activity of the novel vascular targeting agent ZD6126 in a panel of tumor models. *Clin Cancer Res*, **8**, 1974-83.

Cirmi S, Maugeri A, Ferlazzo N, et al (2017). Anticancer potential of citrus juices and their extracts: A systematic review of both preclinical and clinical studies. *Front Pharmacol*, **8**, 420.

Crowell PL (1999). Prevention and therapy of cancer by dietary monoterpenes. *J Nutr*, **129**, 775-8.

Fu SL, Wu YL, Zhang YP, et al (2004). Anti-cancer effects of COX-2 inhibitors and their correlation with angiogenesis and invasion in gastric cancer. *World J Gastroenterol*, **10**, 1971-4.

Hayashi A, Gillen AC, Lott JR (2000). Effects of daily oral administration of quercetin chalcone and modified citrus pectin on implanted colon-25 tumor growth in Balb-c mice. *Altern Med Rev*, **5**, 546-52.

Heinerman J (2009). The healing power of fruits vegetables and herbs, Birmingham, The Magni Group, Inc.

Kandaswami C, Lee LT, Lee PP, et al (2005). The antitumor activities of flavonoids. *In Vivo*, **19**, 895-909.

Kumar V, Abbas A, Aster J (2020). Robbins & Cotran pathologic basis of disease, Amsterdam, Elsevier.

Murakami A, Nakamura Y, Torikai K, et al (2000). Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res*, **60**, 5059-66.

Ngelangel CA, Wang EH (2002). Cancer and the Philippine cancer control program. *Jpn J Clin Oncol*, **32**, 52-61.

Nishino M, Dahlberg SE, Cardarella S, et al (2013). Tumor volume decrease at 8 weeks is associated with longer survival in EGFR-mutant advanced non-small-cell lung cancer patients treated with EGFR TKI. *J Thorac Oncol*, **8**, 1059-68.

OECD (2001). OECD guideline for testing of chemicals: Acute oral toxicity - acute toxic class method [Online]. Available: https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl423.pdf [Accessed October 31, 2022].

Philippine Statistics Authority (2022). Causes of deaths in the Philippines (preliminary): January to December 2021 [Online]. Available: https://psa.gov.ph/content/causes-deaths-philippines-preliminary-january-december-2021 [Accessed October 12, 2022].

Poulose SM, Harris ED, Patil BS (2005). Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. *J Nutr*, **135**, 870-7.

Schindler R, Mentlein R (2006). Flavonoids and vitamin E reduce the release of the angiogenic peptide vascular endothelial growth factor from human tumor cells. *J Nutr*, **136**, 1477-82.

Shi Y-S, Zhang Y, Li H-T, et al (2020). Limonoids from citrus: Chemistry, anti-tumor potential, and other bioactivities. *J Funct Foods*, **75**, 104213.

Tanaka T, Sugiura H, Inaba R, et al (1999). Immunomodulatory action of citrus auraptene on macrophage functions and cytokine production of lymphocytes in female BALB/c mice. *Carcinogenesis*, **20**, 1471-6.

Zhou S, Gravekamp C, Bermudes D, et al (2018). Tumour-targeting bacteria engineered to fight cancer. *Nat Rev Cancer*, **18**, 727-43.