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Research Article

Supplementation of *Spirulina* (= Arthrospira) Platensis Induces Immunosuppression through Increasing T-regulatory Cells in a Syngeneic Mouse Model of Breast Cancer

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Abstract

Spirulina (= Arthrospira) platensis has been reported to have several health-enhancing activities. To date, the anticancer and immunomodulatory effects of *Spirulina platensis* against breast cancer (BC) in an experimental model have not been fully explored. The aim of this study was to assess the anticancer and immunomodulatory effects of *Spirulina* (= Arthrospira) *platensis* against BC in a syngeneic mouse model of BC using two approaches (1) simultaneous treatment and (2) early treatment models. The simultaneous treatment model evaluated the effect of feeding *Spirulina* at the time of tumor induction to investigate if this can inhibit tumor growth and metastasis. The aim of the early treatment model was to study if feeding the mice with *Spirulina* daily for two weeks prior to tumor induction can prevent the onset, growth and spread of the BC. In both models, the total duration of *Spirulina* supplementation was 28 days. There were no differences in body weight ($p > 0.05$) and tumor volume ($p > 0.05$) between the simultaneous and early treatments. However, a marked increase ($p < 0.05$) in the T-regulatory (Treg) population was observed in mice from the simultaneous treatment groups compared to control animals. The findings suggest that *Spirulina* feeding may induce an immunosuppressive environment in the tumor-induced animal, which may work through increasing their Treg populations, thereby suppressing the host's immune response to the tumor.

Keywords: Breast Cancer; *Spirulina*, Immunomodulatory; T-regulatory (Treg) Cells; Metastasis

Introduction

Breast cancer (BC) is the most common malignancy in women. It is a genetically and clinically heterogeneous disease [1,2] that can be broadly categorized into invasive and *in situ* carcinoma. Breast carcinoma *in situ* can be further sub-classified as ductal or lobular-based on growth patterns and cytological features. Ductal

carcinoma *in situ* (DCIS) is more common than lobular carcinoma *in situ*, and it encompasses a heterogeneous group of BCs [3]. In Malaysia, 7,593 new BC cases were reported in 2018 alone [4]. Some risk factors associated with BC include early age at menarche, nulliparity, late age at first birth, gender, low parity, genetics and late menopause [5]. Metastasis begins with local invasion of surrounding host tissue by cancer cells that originate from the primary tumor. This process continues until the tumor cells invade the blood or lymphatic vessels [6,7]. The tumor cells disseminate through the blood or lymphatic vessels to distant organs [6], and the majority of death from BC has been reported to be due to metastasis to other organs in the body [8]. The most common sites of metastasis for BC are bones, lungs and liver [9]. Some tumors are able to continue surviving by evading immune surveillance by the host immune system as well as by overcoming apoptotic signals and undergoing the metastatic process [6,10]. Once the tumor cells initiate metastasis, the process can be repeated to produce secondary metastases [7,11].

Initiation and progression of tumor cells elicit strong inflammatory responses, which involve the innate and adaptive arms of the host immune system. In some cases, the host immune system may inadvertently promote tumor growth by allowing the cancer cells to evade immune surveillance. The main immune cells involved in the pathogenesis of cancer include T-helper (Th) cells, which express CD4 glycoprotein on the cell surface; cytotoxic T-lymphocytes (CTL), which express CD8 glycoprotein on the cell surface and natural killer (NK) cells. Other leukocytes producing anticancer responses include dendritic cells (DC), macrophages and B-lymphocytes [12].

Treatments for BC include surgery, chemotherapy, radiation therapy, targeted therapy and hormonal therapy. In addition, these therapies have been reported to improve the survival of BC patients depending on the subtype and severity of the disease. However, some of the treatment approaches are costly and also pose a number of side effects or adverse effects, which can be detrimental. For instance, some of the side effects of chemotherapy include fatigue [13], early menopause [14], thromboembolic complications [15], weight gain [16] and cardiac dysfunction [17].

Nutraceuticals have gained much attention as alternative therapeutic interventions. Furthermore, several plant extracts used to treat and prevent cancer have been found to exhibit lower adverse effects. Amongst the nutraceuticals explored for anti-cancer potential is *Spirulina*, a cyanobacterium (blue-green algae), which is widely available in the market [18]. *Spirulina* is reported to have high nutritional value [19,20] and has been used as food without any significant side effects for a long time [21]. Some of the nutritional value of *Spirulina* include its high-quality proteins, vitamins, minerals, essential fatty acids and β-carotene [22]. In addition, *Spirulina* has been shown to have several health-enhancing effects such as immunomodulatory [23], anticancer [24,25], anti-viral [26], antioxidant [27] and neuroprotective activities [28].

The beneficial immunomodulatory effects of *Spirulina* have been demonstrated in healthy human subjects

[29] as well as animal models [30]. For instance, immulina, a high-molecular-weight polysaccharide from *Spirulina*, had been tested on human subjects for its effect on adaptive immune responses [29]. In another study, human peripheral blood mononuclear cells (PBMC) exposed to *Spirulina* extracts induced cytokine production [23]. Furthermore, daily supplementation with *Spirulina* was found to enhance the primary immune response to tetanus toxoid vaccination in a mouse model [30]. Many of the previous studies on the immunomodulatory effects of *Spirulina* have been conducted on healthy human subjects [23,29] or animals [30]. The anticancer activity of *Spirulina* against BC has been demonstrated in some cell-based and experimental models. For instance, biosynthesized biogenic silver nanoparticles AgNPs (bio-AgNPs) using *Arthrospira platensis* have been reported to induce reactive oxygen species (ROS), which arrested the treated cells in the G_0/G_1 and sub G_0 phases [31]. Furthermore, selenium-containing phycocyanin was reported to inhibit the proliferation of breast adenocarcinoma cells through induction of apoptosis, accumulation of sub-G_1 cell populations, increased deoxyribonucleic acid (DNA) fragmentation and nuclear condensation [32].

To date, the anticancer and immunomodulatory effects of *Spirulina platensis* against breast cancer (BC) in an experimental model have not been fully explored. This study was undertaken to investigate anticancer effects of *Spirulina* supplementation using a syngeneic mouse model of breast cancer, where two feeding approaches (simultaneous treatment and early treatment) were used to elucidate its' immunomodulatory activities.

Materials and Methods

Spirulina

*Spirulina platensis (*hereafter referred to as *Spirulina),* is also known as *Spirulina* (*Arthrospira) platensis* species. *Spirulina* is a multicellular filamentous cyanobacterium (blue-green microalgae) [18] with phycocyanin as its primary photosynthetic pigment. The *Spirulina* used in this study was a food-grade powder obtained as a kind gift from Earthrise Natural, U.S.A. According to the product sheet provided, the major constituents of the *Spirulina* powder were total carotenoids (≥ 370 [mg%]), β-carotene (≥ 120 [mg%]), phycocyanin (≥ 10%), crude protein (≥ 55%) and chlorophyll (≥ 0.9%). According to the ConPhyMP statement; the *Spirulina* powder was categorized as extract Type B [33]. A copy of the *Spirulina* product sheet is provided as an Appendix.

The food-grade *Spirulina* powder was prepared in soy oil (50 µL), which was the vehicle for the experiment.

Breast cancer cell line

The 4T1 mouse mammary cancer cell line was purchased from the American Type Culture Collection (ATCC) (catalogue number CRL-2539). The cells were cultured in medium recommended by the ATCC [RPMI-1640 Medium (ATCC 30-2001) supplemented with 10% fetal bovine serum (FBS) at 37^{0}C in an incubator with 5% CO_2 atmosphere.

Animals

Female BALB/c mice (five-weeks-old) were purchased from a local supplier. Animals were housed at the animal holding facility at the International Medical University (IMU). The animals were acclimatized for 7-days before the experimental procedures. All experiments with animals were performed in accordance with the international animal use guidelines and was approved by the Joint Committee on Research and Ethics, International Medical University (IMU 258/2012).

Experimental design

Prior to the experiment, all animals appeared healthy and exhibited normal eating patterns. The mice were fed with *Spirulina* using two regimes i.e. simultaneous or early treatment protocols. Food-grade Spirulina powder was prepared in soy oil (50 µL) which is the vehicle.

In the simultaneous treatment approach, the mice were randomly assigned into the vehicle-fed (n = 6) or Spirulina-fed (n = 6) groups after the acclimatization period. The mice were fed with 50 µL of vehicle (control group) or *Spirulina* (50 mg/kg body weight) (Spirulina-fed group) from day 0 to day 28 (Figure 1). The aim was to evaluate if supplementation with Spirulina can inhibit any further growth or metastasis of tumor. Feeding with *Spirulina* or vehicle was started on the same day the mice were induced with BC (Figure 1a) until the mice were sacrificed on day 28.

In the early treatment group, the mice were randomly assigned into control (n = 6) or *Spirulina* fed (n = 6) groups after the acclimatization period. Animals were fed group with 50 µL food-grade *Spirulina* powder (50 mg/kg body weight) mixed in vehicle or vehicle for 14 days. On day 14, the mice were induced with BC and supplementation with *Spirulina* or vehicle were continued until they were sacrificed on day 28 (Figure 1b).

Sample size on six mice per group were based on published studies (Radhakrishnan., *et al*. 2021; Abdul Hafid., *et al*. 2013).

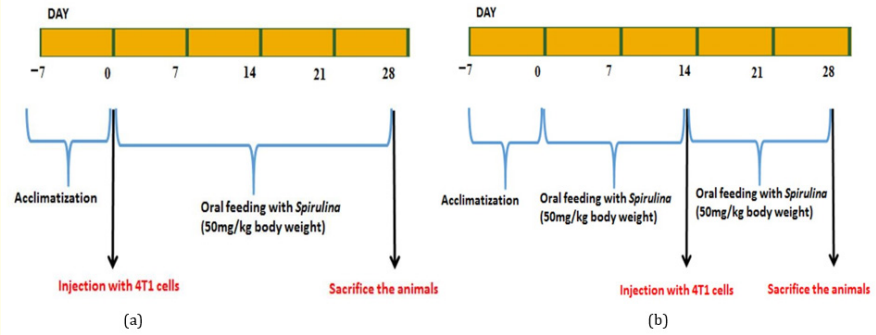


Figure 1: Schematic diagram showing the experimental design for the study on the effects of 173 *Spirulina* feeding on mice induced with breast cancer: (a) simultaneous and (b) early treatment models.

Tumor induction

Tumor induction was carried out as described previously [34]. Briefly, the mice were induced with BC by injecting 100 µL of a cell suspension containing $1x10^5$ cells/mL of 4T1 cells into the mammary fat pad of female BALB/c mice. The animals were monitored daily for tumor growth signs of distress. When tumor was palpable on day 11-12, tumor size was measure using a digital caliper once every 3 days until the animals were sacrificed. In both models (simultaneous or early treatment), the animals were sacrificed on day 28 as the animals showed restricted mobility due to their large tumors.

Body weight and tumor volume

The body weight and tumor volume were recorded once every three days until the animals were sacrificed. Tumor volume was calculated using the following formula: V = 0.52 x $width^2$ x length, where V = tumor volume (mm^3), width = shorter diameter (mm) and length = longer diameter (mm) [35]. Prior to culling, the animals were lightly anesthetized and cardiac puncture was performed to withdraw peripheral blood, which was collected into heparinized tube. The blood was used for immunophenotyping and biochemical analysis. At autopsy, tumor, lung, liver, kidney and heart were harvested and preserved in 10% formalin solution for histopathology studies.

Immunophenotyping

The expression of several cell surface markers (e.g. CD4, CD8, CD25, CD127 and CD73) on the peripheral blood leucocytes were analyzed using flow cytometry. Briefly, 500 µL of blood was transferred into appropriately labelled tubes. Then, 2 mL of red blood cell (RBC) lysis solution (eBioscience, USA) was added to each tube. The tubes were gently vortexed and incubated in the dark at room temperature for 3-10 min. The lysis activity was stopped by adding 2 mL 1x cold phosphate-buffered saline (PBS). The leucocytes were recovered by centrifugation (350 g for 5 min) after the supernatant discarded. The remaining pellet was re-suspended in 200 µL sheath fluid (eBioscience, USA). Then, 1.0 µL of appropriate fluorochrome-conjugated monoclonal antibodies was added to the sample and the tube was incubated in the dark at room temperature for 20-40 minutes. Following this, the samples were washed with 300 µL of wash buffer (PBS) and the cells were recovered by centrifugation (350 g for 5 minutes). The supernatant was discarded and 500 µL of sheath fluid was added to each tube and the samples were analyzed using a flow cytometer (FACS Calibur, Becton-Dickinson, USA]. Data from 10,000 cells were acquired from each sample for analysis. The data collected was analyzed using the Cell-Quest software. Dot-plots for the respective fluorochromes were obtained from the gated population for each sample.

Histopathological analysis

The primary tumor, lung, liver, heart and kidneys stored in 10% formalin were processed for histopathological studies. Briefly, the organs were transferred into appropriately labelled small cassettes. The cassettes were placed in an automatic tissue processer (Leica TP1020 Automatic Tissue Processor, Leica, Germany) and processed for histopathological studies. The paraffin impregnated tissues were embedded into paraffin embedding media and casted into tissue blocks using a tissue embedding machine (Leica Tissue Embedding Machine, Germany). Tissue sections were deparaffinized by placing the slides in the xylene solution and rehydrated through descending concentrations of ethanol to water prior staining with hematoxylin and eosin (H&E) stains. After removal of excess stains, the tissue sections were dehydrated via ascending concentrations of ethanol and cleared in xylene substitute-X solution. The slides were then mounted with distyrene plasticizer xylene (DPX) and covered with cover slips. The slides were examined using a bright field microscope (Nikon Eclipse 80ί (CF160), Japan) with a 12.0megapixel resolution camera at various magnifications (100X, 200X and 400X) and the relevant images were captured.

Statistical analysis

Data were analyzed using SPSS Statistics version 20. All the values were presented as mean ± SEM of six mice per group. The data were analyzed using an independent T-test. A P-value less than 0.5 ($p < 0.05$) is considered significant (95% confidence interval) compared to the control group.

Results

Body weight

In the simultaneous treatment approach, there were no difference in the body weights ($p > 0.05$) of animals fed with *Spirulina* or vehicle (Figure 2a). At the start of the study, the body

weight of animals from the simultaneous treatment was 17.6 (± 0.15) g, which increased to 20.1 (± 0.61) g and 20.2 (± 0.25) g, in the control and *Spirulina*-fed groups (Figure 2a), respectively. A similar observation was recorded in the early treatment groups, where the initial weight was 17.6 (± 0.16) g and their weight at the end of study were 21.0 (mean ± 0.34) g and 20.4 (± 0.25) g, for control and *Spirulina*-fed groups (Figure 2b), respectively.

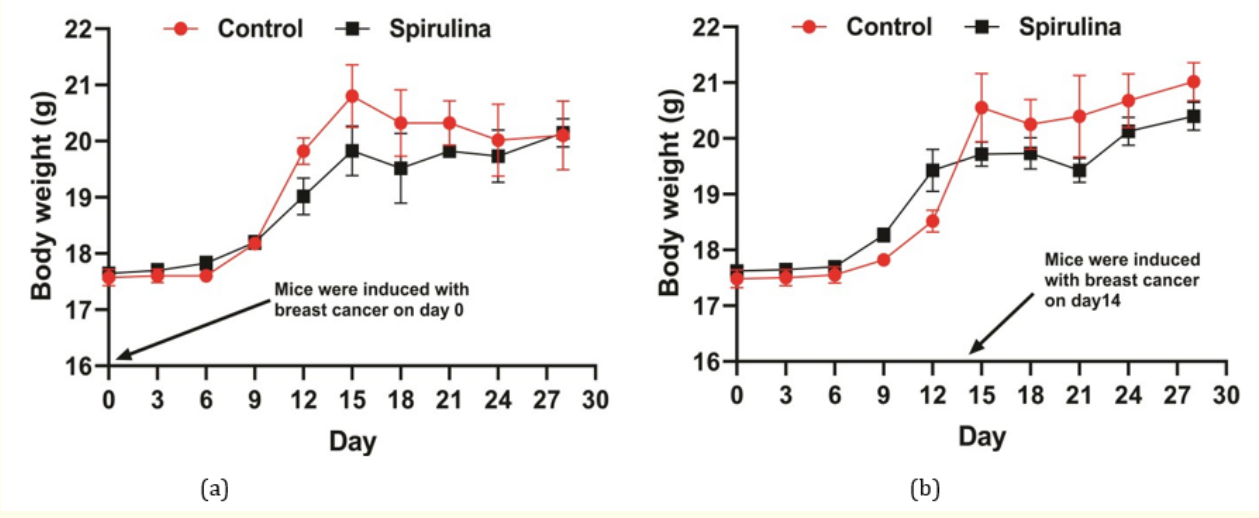


Figure 2: Changes in body weight in *Spirulina* or vehicle-fed mice from the (a) simultaneous and (b) early treatment models. Body weight was recorded every three days from day 0 to 28. Data expressed as mean ± standard error mean (SEM) of six mice per group (n = 6). There were no significant differences ($p > 0.05$) in body weight of animals between the control and *Spirulina* fed group.

Tumor volume

There was no significant difference ($p > 0.05$) in the tumor volumes between mice fed with *Spirulina* (Figure 3a) or vehicle (Figure 3b) for both treatment approaches (simultaneous or early treatment]. In the simultaneously-treated group, the tumor was palpable 12 days post-inoculation of the 4T1 murine BC cells (Figure 3a); whilst in the early treatment group, tumor was palpable after 7 days post-induction with BC cells (Figure 2b). The highest tumor volume recorded was on day 28, which was less than 100 mm^3 in the early treatment group compared to 200 mm^3 in the simultaneously treated group.

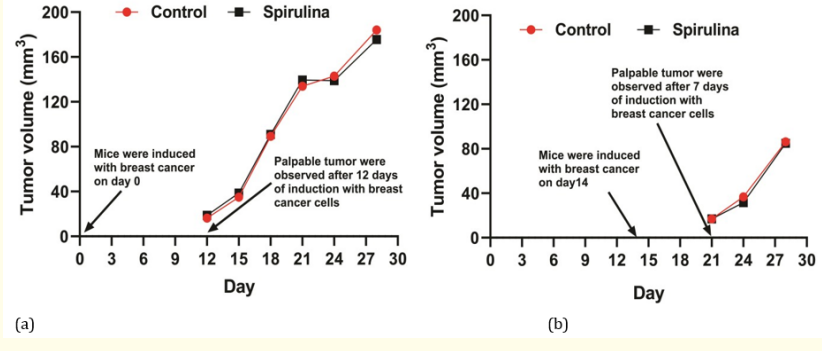


Figure 3: Tumor volumes of *Spirulina* or vehicle-fed mice in the (a) simultaneous and (b) early treatment models. Diameter of tumor volume was measured every three days. Data expressed as mean ± standard error mean (SEM) of six mice per group (n = 6); SEM values range from 0.86 to 3.75 mm^3. There were no significant differences ($p > 0.05$) between tumor volume of animals from the control and *Spirulina* fed group.

Immunophenotyping

In both the simultaneous (Figure 4a) or early (Figure 4b) treatment approaches, there were no differences ($p > 0.05$) observed in the proportion of $CD4^+$, $CD8^+$ and $CD4^+/CD127^+$ T-cell populations in peripheral blood of mice that were fed with *Spirulina* compared to vehicle groups. However, in the early treatment approach, there was a significant increase ($p < 0.05$) in the number of T-regulatory (Treg) cells ($CD4^+CD25^+$) observed in the peripheral blood of mice fed with *Spirulina* compared to vehicle-fed (Figure 3b). The Treg population appeared to have increased by four-fold in these mice, which was not observed in mice from the early treatment group (Figure 4b).

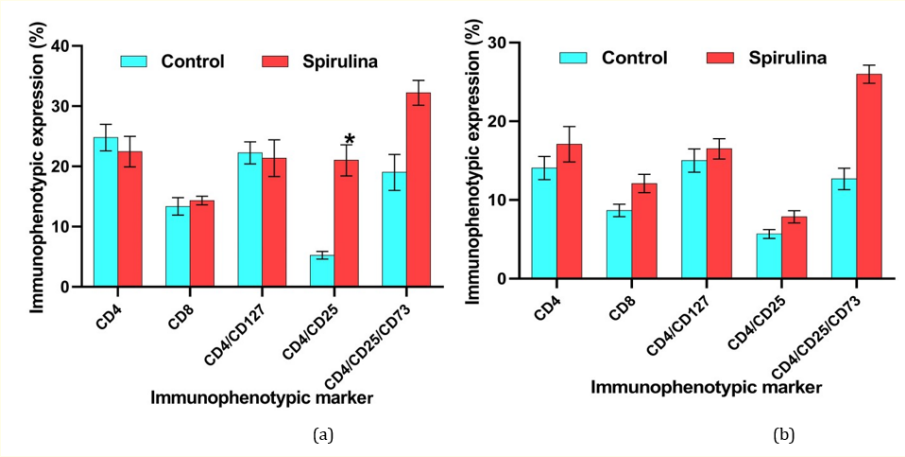


Figure 4: Levels of T helper cells (CD4+), Cytotoxic T-cells (CD8+), T helper cells that secrete IL-7 (CD4+/CD127+), T regulatory cells (CD4+/ CD25+) and T-regulatory cells which expressed CD73 enzyme (CD4+/ CD25+/CD73) in peripheral blood lymphocytes of tumor-laden mice from the (a) simultaneous and (b) early treatment models. The levels of immunophenotypic markers were determined by flow cytometry. Mice in the control group were fed with vehicle (soy oil) while those in the experimental group were supplemented with *Spirulina* (50 mg/kg body weight).

Data expressed as mean ± standard error mean (SEM) (n = 4). * denotes significant difference ($p < 0.05$) compared to the control group.

Histopathological analysis

Gross examination of breast tissues from vehicle-fed mice from simultaneous (Figure 5a) and early (Figure 5b) treatment mice showed large irregular growth attached to the chest wall. The breast tissue sections showed presence of dysregulated cells representing tumor tissues and carcinoma cells displaying features of anaplasia. In addition, the tumor cells appeared large and pleomorphic with hyperchromatic nuclei and dense cytoplasm, arranged in clusters and sheets (red arrow) (Figure 5a and 5b). There was numerous atypical mitosis observed in the breast sections. The breast sections from *Spirulina*-fed mice from the simultaneous (Figure 5c) and early (Figure 5d) treatment mice revealed primary breast adenocarcinoma, which was poorly differentiated. In addition, there were numerous atypical mitosis and abundant areas of necrosis in the central areas with peripheral active growth in these breast sections.

Tumor deposits were observed in liver tissues from vehicle-fed animals from the simultaneous (Figure 6a) and early (Figure 6b) treatment models, which suggests that metastasis to the liver had taken place in these animals. The metastasis was accompanied by inflammation and congestion of the surrounding parenchyma. In

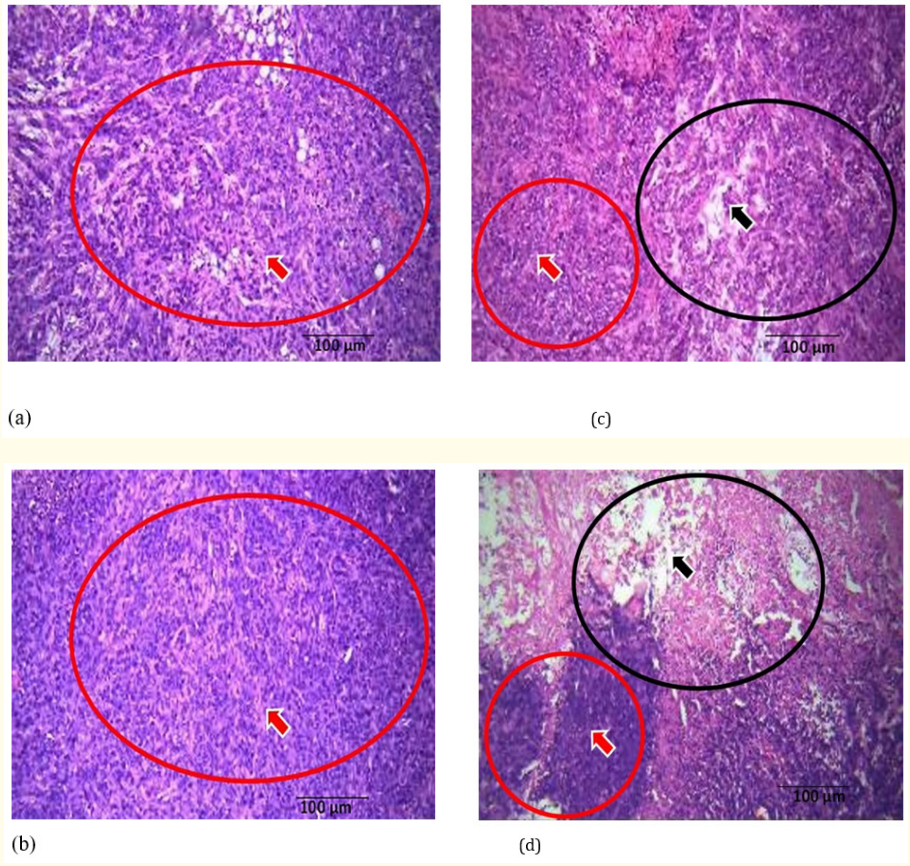
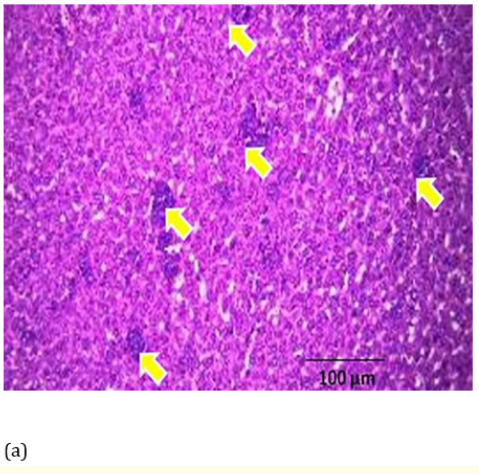
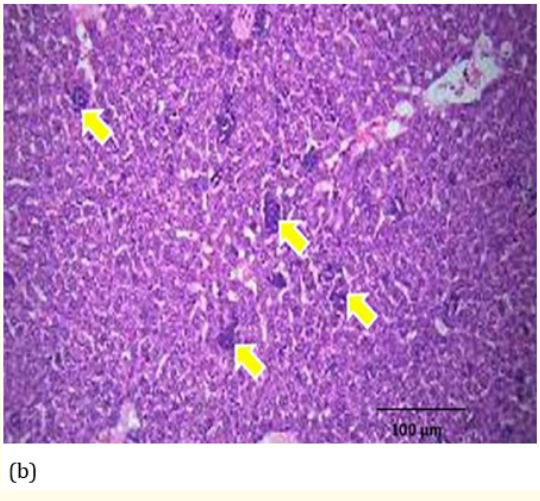
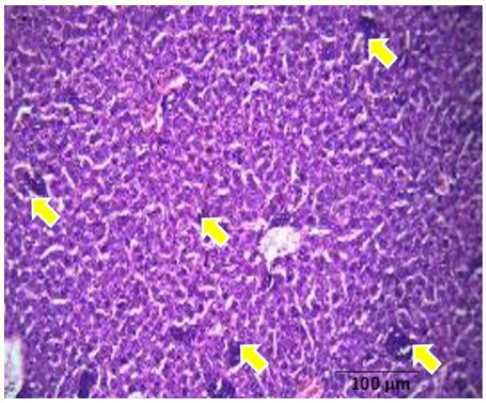


Figure 5: Photomicrographs (H&E, 200x) of breast tumor tissue of the control (non-treated) [a (simultaneous treatment), b (early treatment)] and Spirulina (50 mg/kg body weight [c 321 (simultaneous treatment), d (early treatment)] treated mice. Comparison of tumor necrosis upon Spirulina treatment. Red arrow and circle indicate viable tumor cells and black colored arrow and 323 circle indicate necrotic tumor cells.

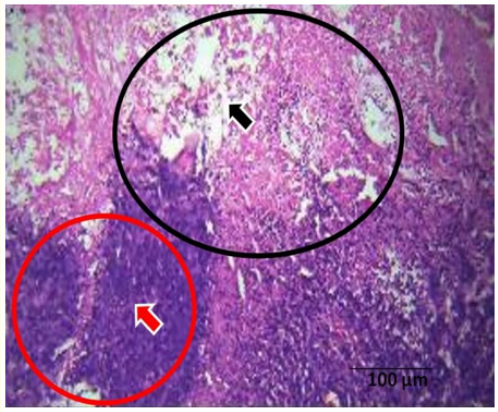
comparison, there were only scattered micro-metastases in the liver of *Spirulina*-fed mice from both models (Figure 6c and 6d).







(c)



(d)

Figure 6: Photomicrographs (H&E, 200x) of liver tissue of the control (non-treated) [a (simultaneous treatment), b (early treatment)] and *Spirulina* (50 mg/kg body weight) [c (simultaneous treatment), d (early treatment)] treated mice. Comparison of metastasis from primary tumor site to the liver. Arrow indicates metastasized tumor cells.

Discussion

In this study, two experimental models were tested to assess the effectiveness of *Spirulina* as an anticancer agent using a syngeneic mouse model of BC**.** In the simultaneous treatment model, the aim was to evaluate if supplementation with *Spirulina* can inhibit any further growth or metastasis of tumor, while the objective of the early treatment model was to study if early *Spirulina* supplementation can prevent the onset, growth and spread of breast cancer.

Only one dose of *Spirulina* (50 mg/kg body weight) was tested in both experimental models. This concentration of *Spirulina* was chosen as a previous study reported that mice fed daily with 50 mg/kg body weight *Spirulina* did not show any adverse effects as indicated by similar weight gain of the fed mice compared with the control [30]. Other studies, which involved feeding mice with *Spirulina* had reported the weight gain observed in mice were fed with a diet containing 10% or 20% *Spirulina* were almost identical with the control [36]. As these studies have also found that feeding 50 mg/kg body weight of *Spirulina* was sufficient to elicit immune response, we maintained this concentration of *Spirulina* in the current study. Soy oil was chosen as the vehicle in this study as several immunomodulatory/anticancer studies involving a mouse model have used it and found on influence on immune components/anti-cancer components at the low volume (50 µL) used [34,37,38].

There were no significant changes in body weight between control and *Spirulina*-fed mice in both models in the current study but a previous study has found that feeding *Spirulina* was reported to overcome reduction of body weight in mice induced with BC [39]. In addition, there were no significant changes tumor volume between control and *Spirulina*-fed mice in both models. At the end of the experiment, the mice in the simultaneous treatment model showed higher tumor volume compared to those in the early treatment model. This might be due to the shorter period of tumor growth monitoring in the simultaneous treatment group tumor growth was monitored for 21 days after it was palpable. In contrast, in the early treatment group, tumor growth was monitored for 12 days once it was palpable. This means that the higher tumor volume in the mice from the simultaneous treatment model could be due to the longer exposure to the BC cells compared to animals in the early treatment model. The absence of antitumor activity of *Spirulina*, observed in this study does not conform with previous studies. For instance, *Spirulina* supplementation was shown to reduce BC formation induced by Dimethylbenzanthracene (DMBA) in mice [39]. In another study, 40 mg/kg immulina was reported to inhibit 4T1 cells-induced BC growth in mice [40]. Supplementation with *Spirulina* exerted anticancer effects against skin carcinogenesis induced by ultraviolet-B (UVB) irradiation in mice through its anti-inflammatory and antioxidant and delayed induction of skin cancer [41]. Similar findings have been reported for other cancers using mouse models of skin or stomach cancers and the authors

associated the anticancer effects observed to the ability of *Spirulina* to induce phase 1 and phase 2 metabolizing enzymes [42]. However, there are also several studies that do not support the anticancer activity of *Spirulina*. For instance, supplementation with *Spirulina* did not show any anticancer effects against Ehrlich carcinoma [43]. In another study, it was reported that *Spirulina* or selenium-enriched *Spirulina* did not exert any anticancer activity in athymic nude mice xenografted with renal carcinoma cell [44]. Hence, it could be argued that the different claims on the anticancer effects of *Spirulina* may be attributed to the experimental settings or the form of *Spirulina* used. In the present study, the mice were fed with edible *Spirulina* powder. However, there are other forms of *Spirulina* available that have been tested in animal studies, where different preparations of *Spirulina* have been used, such as ground *Spirulina* tablets [43], dried powder-enriched with purified phycocyanobilin [41] and cultured *Spirulina* [39]. It is possible that there may be variations in the amount or proportion of the active ingredients (e.g. phycocyanin) in the different *Spirulina* preparations.

Modulation of the host immune system is an important therapeutic strategy to fight cancer as host immune system is the main natural defense system against cancers. To date, there are several reports on the immunomodulatory effects of *Spirulina* [30,45]. The cytotoxic T-lymphocytes (CTL) and T-helper (Th) cells are regarded as the principal weapons of immunity against cancer [46]. The present study analyzed the immunomodulatory effects of oral Spirulina on the blood leucocytes, by quantifying the immunophenotypic expression of CTL ($CD8^+$), T-helper (Th) ($CD4^+$), Th17 ($CD4^+/CD127^+$) and T-regulatory (Treg) ($CD4^+/CD25^+$) or $CD4^+/CD25^+/CD73^+$) cells in peripheral blood leukocytes. These white blood cells were chosen for analysis as these cells are the main regulators of cell-mediated immunity, which is important in fighting cancers. There was a marked increase ($P < 0.05$) in the Treg population ($CD4^+/CD25^+$) in the peripheral blood of *Spirulina*-fed mice from the simultaneous treatment model compare to the control mice. The Tregs are a sub-population of Th cells that induce an immunosuppressive environment by secreting cytokines that suppress activation of host immune responses. However, there were no increase in Treg cells in *Spirulina*-fed mice from the early treatment group. The findings from the current study appear to contradict a previous study, where we reported that mice induced with BC after 28-days of Spirulina feeding did not show any significant reduction of T-reg cells after 56 days [47]. This difference could be attributed to the longer feeding period of *Spirulina*, which may have modulated the immune response. The proportion of Treg cells that expressed the CD73 enzyme ($CD4^+/CD25^+/CD73^+$) increased by 10.7% and 13.9%, in peripheral blood of *Spirulina* fed mice from the early treatment and simultaneous treatment models, respectively. However, the difference observed was not statistically different. The CD73 is an ecto-enzyme expressed on tumor cells and Treg cells are reported to be involved in production of extracellular adenosine, which promotes tumor growth [48]. Higher numbers of Tregs in cancer has been associated with poor survival in many solid tumors including breast cancer [49], gastric cancer [50], ovarian cancer [51] and non-small-cell lung carcinoma (NSCLC) [52]. In NSCLC, increased percentage of Tregs potently inhibited T-cell proliferation, which is crucial to provide immune protection [52]. The findings from the present study suggest that supplementation with *Spirulina* may cause an increase in the proportion of Treg cells; thereby creating an immunosuppressive environment that allow tumor growth to take place. Increase in Treg numbers can suppress host immune system, which also allows tumors to grow [51].

In some cancers such as prostate cancer [53] and non-small cell lung cancer [54], increased levels of Treg cells are associated with positive diagnosis. This could be related to the ability of Tregs to suppress inflammation by suppressing host immune system; which is a critical processes in promoting tumor growth by enhancing proliferation, angiogenesis [55] and metastasis [56]. In the present study, the increase in Treg cells did not exacerbate cancer development in the *Spirulina* fed group compared to control group; based on the tumor volume and body weight data as well as the histopathological findings. Further studies are required to elucidate the mechanisms involving the different subsets of the T-cells in modulating immune responses in BC following *Spirulina* feeding. One important aspect that needs attention is the balance between Treg and other effector T-cells, which normally influences the outcome of a disease [57].

In both simultaneous treatment and early treatment models, the histological features of breast tumor, lung, liver, heart and kidney tissues from *Spirulina*-fed mice appeared to be similar to vehicle-fed mice. Breast tissue sections from *Spirulina*-fed mice showed poorly differentiated primary adenocarcinoma with

necrosis compared to vehicle-fed mice., which might indicate that *Spirulina* supplementation may cause some of the BC to die via necrosis in both simultaneous treatment and early treatment models. In *Spirulina*-fed mice, metastasis was present in the liver but not in the lung, kidney or heart tissues. It is well-documented that BC cells metastasize to the bony skeleton, lungs, liver and brain via the circulation [58]. Amongst these organs, liver is recognized as a common metastatic site for solid cancers and represents the third most common site for BC [58]. Other studies have shown that calcium spirulan (Ca-SP), a polysaccharide of *Spirulina platensis*, can inhibit tumor invasion and metastasis and reduce lung tumor colonization of B16-BL6 cells in a spontaneous lung metastasis model [59]. In comparison, in the present study, although *Spirulina* were able to kill some of the breast cancer cells, it did not prevent metastasis to the liver, which might be due to the highly invasive 4T1 cells used to induce breast cancer in this late stage model [60] compared to the use of carcinogens such as DMBA [61].

Conclusions

Supplementation with *Spirulina* did not exhibit any anticancer effects against BC in both models.

The findings suggest that supplementation with *Spirulina* may induce an immunosuppressive environment in this animal model as there was a higher proportion of Treg cells ($CD4^+/CD25^+$) in the *Spirulina*-fed mice compared to the vehicle-fed animals.

Author Contributions

Investigation, H.S.; project design and administration, W.-L.C., A.K.R., H.S.,S.C., and Y.-Y.K.; draft preparation, H.S., W.-L.C. and A.K.R.; data curation, H.S.; formal analysis, H.S., W.-L.C., A.K.R. and S.C.; writing—review and editing, H.S., W.-L.C., A.K.R., S.C. and K.R.S.; supervision, W.-L.C., A.K.R. and S.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declared no conflict of interest with respect to the research authorship, and/or publications of this article.

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