Tumor suppression effect of Solanum nigrum polysaccharide fraction on Breast cancer via immunomodulation

Faizan Naeem Razali, Saravana Kumar Sinniah, Huzlinda Hussin, Nurhayati Zainal Abidin, Adawiyah Suriza Shuib

A poly saccharide fraction from Solanum nigrum, SN-ppF3 was shown previously to have an immunomodulatory activity where it could possibly be used to enhance the host immune response in fighting cancer. The non-toxic SN-ppF3 was fed orally to breast tumor bearing-mice with concentrations of 250 and 500 mg/kg for 10 days. During the treatment period, size of the tumor and weight of the mice were monitored. At the end of the treatment, blood, tumor, spleen and thymus were harvested for physiological and immunological analyses. After the treatment, the tumor volume and tumor weight were significantly inhibited by 65% and 40%, respectively. Based on the histological observation, the treatment of SN-ppF3 resulted in the disruption of tumor cells morphology. The increase in infiltrating T cells, NK cells and macrophages were observed in tumor tissues of the treated mice, which partly explained the higher apoptosis tumor cells observed in the treated mice. Moreover, the level of TNF-α, IFN-γ and IL-4 were elevated, while the level of IL-6 was decreased significantly, in serum of the treated mice. These results suggested that tumor suppression mechanisms observed in SN-ppF3-treated mice were most probably due through enhancing the host immune response.

1. Introduction

In the current decade, cancer has still remained the top listed death-causing disease despite of developments in tools of diagnosis, treatment and prevention. Among these, breast cancer is the most common-death causing cancer. According to Seigel et al. in the year of 2015, it was estimated that breast cancer contributed approximately 29% of all new female cancers in the United State [1]. Although the major modern therapies and techniques have become possible in treating this cancer, the treatments still have some drawbacks such as unavoidable toxic due to nonspecific action towards cancer cells which would affect the quality of patients’ lives [2]. Moreover, many evidences have demonstrated that the anti-tumor activities of many chemotherapeutic agents have resulted in the toxicity of normal cells and organ damages as well [3]. Thus the identification of novel drugs with a better effectiveness and lower toxicity is necessary. Numerous studies on breast cancer therapies are still ongoing, and one of the possible strategies to encounter this type of cancer is through the improvement of patient or host immune responses.

Evasion of the immune surveillance of the host by tumor cells by modifying the host’s immune response is one of the main reasons for the rapid progression of cancer [4]. One of the approaches in overcoming this problem is to modulate the immune response within the host, making the host’s own immune system more capable in eliminating cancer cells [5]. There are many mechanisms to induce the immune system and one of it is by using plant products with immunomodulatory properties.

Some plants polysaccharides are proven to have the immunomodulator characteristic and they have the potential as useful candidates in alternative, effective and non-toxic treatment which may provide exciting novel therapeutic prospects.
Unlike microbial polysaccharides, polysaccharides that were derived from higher plants are mainly non-toxic and may not cause side effects [7].

*Solanum nigrum*, an herbal plant which grows all around the world, covering both temperate and tropical regions [8]. The health benefits of this plant were recorded in several reports, thus strengthening the traditional belief that this plant possessed multiple medicinal values including curing cancer [9]. The crude extract from *S. nigrum* was shown to induce necrosis in SC-M1 stomach cancer cells [10], able to suppress radical-mediated DNA damage [11], possess an antineoplastic effect against several cancer cells [12] and directly inhibit 12-O-tetradecanoic acid-induced tumor production in MCF-7 breast cancer cell [13]. Moreover, a documented study has proven that the polysaccharide extracted from *S. nigrum* Linne had an anti-tumor activity by enhancing CD4+/CD8+ ratio of T-lymphocyte subpopulation significantly [14]. In our recent finding, a fraction of semi purified polysaccharide from *S. nigrum* L. *SN-ppF3* showed its ability in activating RAW 264.7 macrophage cell line in vitro. The activated macrophage released NO and cytokines and had a higher phagocytic activity [15]. These features not only indicated the cells’ ability in fighting diseases, but also in inducing other immune responses. To further investigate the anti-cancer potential of *S. nigrum* towards mammary carcinoma cells, in vivo analysis was carried out on 4T1 tumor-bearing mice treated with polysaccharide fraction SN-ppF3.

2. Materials and methods

2.1. Reagents and chemicals

DMEM and CTX anti-cancer drug were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethanol and petroleum ether were purchased from Merck (Darmstadt, Germany). Fetal bovine serum, penicillin/streptomycin, and amphotericin B brought were from PAA Laboratories (Colbe, Germany). The TNF-α, IFN-γ, IL-6 and IL-4 mouse ELISA kit were obtained from Abcam (Cambridge, UK). Diethyliaminoethyl cellulose pre-swollen ion exchanger was purchased from Whatman (Maidstone, England).

2.2. Sample preparation and characterization

Fresh whole *S. nigrum* L. *nigrum* plants were purchased from a local market located at Lembah Pantai, Kuala Lumpur, in February 2012 and a voucher specimen was deposited at the herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia (herbarium number: KLU 47872). *S. nigrum* polysaccharides were extracted according to the previous described method [15]. Briefly, polysaccharide was extracted from dried, ground stems of *S. nigrum* by refluxing the sample with 2 L of petroleum ether (60°C–80°C), followed by 2 L of 80% ethanol. The residue was then boiled in 2 L of 95°C water for 5 h. The polysaccharide in the filtrate of the boiled mixture was precipitated out with equal volume of 70% ethanol, overnight at 4°C. After the precipitate was collected and dried, the sample was subjected to purification by a diethyliaminoethyl cellulose column (ø20 mm × 250 mm), where polysaccharides were eluted with a linear gradient of 0–1.5 M sodium chloride in 5 mM of sodium phosphate buffer. Fraction SN-ppF3 was collected, dialyzed and freeze-dried before it was stored at −20°C. The molecular weight of SN-ppF3 was determined by subjecting SN-ppF3 to Sepharose CL-6B gel filtration column. To identify the structural features of SN-ppF3, it was mixed with potassium bromide (1:10), pressed into approximately 1 mm pellet disc and subjected to Fourier Transform Infrared (FT-IR) spectroscopy analysis. Spectrum was recorded at the absorbance mode between 4000–400 cm−1 on Perkin Elmer Spectrum RX-1 FT-IR spectrophotometer (Waltham, Massachusetts, USA).

2.3. Cell lines and animals

Mouse mammary carcinoma cell line, 4T1, was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum, 2% of 100 U penicillin/streptomycin and 1% of 100 × amphotericin B. The cells were cultured in a 5% CO2 and 100% humidified incubator (CellCulture CO2, Esco Technologies, Hatboro, PA, USA) at 37°C, and the culture was passed every 2 or 3 days. Female BALB/c mice (7–8 weeks old) weighing 18–22 g was obtained from Monash University, Sunway Campus, Malaysia and were acclimatized for 14 days before the experiment was carried out. The mice were housed under the controlled environmental conditions of temperature 25 ± 1°C and 12/12 light/dark cycle and supplied with standard food pellets and tap water *ad libitum*. Animal care, research and animal sacrificed protocols were in accordance to the principles and guidelines approved by the University of Malaya Institutional Animal Care and Use Committee (ISB/29/06/2014/FNR-R).

2.4. In vitro cytotoxicity evaluation

To demonstrate if there is a direct effect of SN-ppF3 on 4T1 tumor cell line, an MTT cell proliferation assay was carried out according to Mosmann (1983) with a slight modification [16]. Briefly, 15 × 10⁴ cells/mL of 4T1 cells were seeded in a 96-wells culture plate (Orange Scientific) and incubated for 24 h. The cells were then treated with SN-ppF3 at 0, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μg/mL for 72 h. The cell viability was observed by adding 20 μL of 5 mg/mL MTT solution to each well and incubated for another 4 h. The developed purple formazan was diluted with 200 mL of dimethyl sulfoxide and the absorbance was measured at 570 and 630 nm by using Multiskan Go microplate spectrophotometer. The IC₅₀ value, with a sample concentration that causes 50% cell inhibition or cell death [17], of SN-ppF3 toward 4T1 cell line was determined based on the response curve.

2.5. Effect of SN-ppF3 treatment on tumor growth

Twenty four female BALB/c mice (8 weeks old) were divided into four groups (n = 6) and maintained in well-ventilated cages with normal food pellets and tap water *ad libitum*. The mice in each group were subcutaneously inoculated on their left breast line with 1 × 10⁶ 4T1 mouse mammary carcinoma cells and monitored daily for tumor growth. After 14 days of tumor induction, the tumor-bearing mice were orally administered with SN-ppF3 (250 and 500 mg/kg), normal saline and CTX (25 mg/kg) for 10 days, respectively. Tumor progression and regression were monitored by measuring two perpendicular dimensions (long and short) using a caliper and integrated in the formula \( V = \frac{(a + b)^2}{2} \), where \( a \) is the larger and \( b \) is the smaller tumor dimension [18]. In addition, tumor, spleen and thymus were collected and weighted after the mice were sacrificed. The spleen and thymus indices were calculated according to the formula [19]:

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\text{Spleen and thymus index (mg/g)} = \frac{\text{weight of the spleen or thymus}}{\text{Body weight}}
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2.6. Histology

Tumors from the tumor-bearing mice were collected and fixed with at least 20 mL of 10% formalin solution between 16–24 h fixation periods at room temperature. Then, the fixed tumor tissues
were sampled into cassettes and processed. The processed tissues were embedded into a paraffin block, sectioned into 5 μm in thickness, fixed on poly-L-lysine slides and stained with H&E stain solution. The stained slides were observed and photographed using a light microscope attached with camera (Olympus, Shinjuku, Tokyo, Japan). The slides of the control and treated tumor tissues were observed for any cellular changes, particularly on the apoptosis features.

2.7. Detection of infiltrating immune cells by immunofluorescent staining

The tissue samples were embedded into a paraffin block, sectioned into 3 μm in thickness and then fixed on poly-L-lysine slides. The immunostaining procedures were followed according to Robertson et al. with a slight modification [20]. Briefly, the slides were deparaffinized and incubated in 95 °C and room temperature with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 20 and 10 min, respectively. Then, the tissues were blocked with PBS supplemented with 1% bovine serum albumin and 2% fetal bovine serum in room temperature for 30 min and washed 3 times with PBS for 2 min each. The slides were then incubated with 0.2 μg/mL anti-CD69 NK cell, anti-CD8+ and anti-F4/80 macrophage antibodies conjugated with Cy5® phycocerythrin and FITC fluorescent dyes, respectively. (Abcam, Cambridge, UK) in the dark for 60 min. Then the slides were washed again 3 times with PBS for 2 min each. The slides were slowly blotted to remove excess buffer, observed with a constant exposure of blue light laser and photographed using a fluorescent microscope (×200) equipped with camera (Leica, Wetzlar, Germany).

2.8. Apoptosis detection of tumor tissue by TUNEL staining

The tissues samples were embedded in paraffin blocks, sectioned into 3 μm in thickness and then fixed on poly-L-lysine slides. The next procedures were followed according to In Situ Cell Death Detection Kit Fluorescin protocol (Roche, Basel, Switzerland). After the deparaffinized and dehydration processes, slides were washed twice with 1 × PBS and incubated in a freshly prepared 0.1% Triton-X in 0.1% sodium citrate solution for 8 min at room temperature. Slides were then washed twice with 1 × PBS. In the staining process, 50 μL of TUNEL solution was slowly dropped onto the tissue sections and layered with a cover slip to avoid evaporation during incubation. Slides were then incubated in a humidified atmosphere for 60 min at 37 °C in the dark. Slides were then washed three times with 1 × PBS, slowly blotted and directly analyzed under a fluorescent microscope (×200) using excitation wavelength in the range of 450–500 nm and detection wavelength in a range of 500–516 nm, with a constant exposure of blue light laser. Images were photographed using an equipped camera (Leica, Wetzlar, Germany).
2.9. Cytokines level

The serum collected from the sacrificed mice was assayed for the concentration of TNF-α, IFN-γ, IL-4 and IL-6 by using commercial ELISA kits (Abcam, Cambridge, UK). The assay was carried out according to the procedure provided by the manufacturer.

2.10. Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA), with the significant difference between the means was determined by Duncan’s multiple range test 95% significant different (p < 0.05), using SPSS 17.0 Statistic software (IBM Corporation, Endicott, NY, USA). The graphs representing the results of animal body weight, tumor volume, ELISA, cytotoxicity response curve as well as all standard curves was constructed using GraphPad Prism 5 software (GraphPad software, Inc, CA, USA).

3. Results and discussions

3.1. Sample characterization

The biological activity of an immunomodulatory polysaccharide depends on many factors including their chemical composition, glycosidic linkages, branching, molecular weight and solubility. Therefore, some characterizations were carried out on SN-ppF3 in order to understand how the biological activity was induced. Based on the size exclusion chromatography (data not shown), the size of the polysaccharide was estimated around 109.42 kDa. Various sizes of immunomodulatory polysaccharides have been reported, ranging from about 1–7000 kDa [21]. A study carried out on partially cellulose digested Aloe polysaccharide revealed that polysaccharide fractions within the size of 5–400 kDa had the most potent anticancer activity, indicating size of polysaccharide may contribute to its biological activity [22].

Previously, we have reported that among the neutral sugar residues detected in SN-ppF3 were glucose, galactose and rhamnose [15]. To further characterize the structure of this polysaccharide, FT-IR analysis was carried out. The FT-IR spectrum of SN-ppF3 (Fig. 1) displayed a typical absorption peak of polysaccharides [23,24]. The broadly stretched intense peak at 3424 cm⁻¹ was designated to hydroxyl (–OH) stretching vibration, followed by a weak C–H peak at 2932 cm⁻¹ [25]. The relatively strong asymmetric peak at around 1656 cm⁻¹ indicated the characteristic of deprotonated carboxyl group (COO⁻) [26], suggesting the presence of uronic acid in this polysaccharide [27]. A weak absorption peak at 1409 cm⁻¹ corresponded to the vibration of CH₂ and C–OH groups [23,28]. The absorption peaks at range 1078–1034 cm⁻¹ indicated as aldehyde (C–O=H) and ketone (C–O–C) groups [28]. The absorption peak at about 775 cm⁻¹ suggested the rings vibration due to α-configuration of rhamnose units [29]. Based on the FT-IR data, it was suggested that this heteropolysaccharide possessed unique binding features [30] that allow the recognition by macrophages or the other immune cells, and subsequently stimulates immune responses [31,32].

3.2. In vitro cytotoxicity evaluation

To confirm whether SN-ppF3 has a direct cytotoxicity effect toward 4T1 tumor cells, MTT assay was carried out. By referring to the cytotoxicity dose response curve (Fig. 2), the IC₅₀ value of SN-ppF3 could not be determined (IC₅₀ > 100 µg/mL). According to the U.S National Cancer Institute plant screening program, the plant crude extract with IC₅₀ value of 20 µg/mL or less was considered to have cytotoxicity effect on tested cancer cells upon 48–72 h of incubation [33]. Thus, it could be concluded that SN-ppF3 has no direct cytotoxicity effect on 4T1 cells after a 72-h incubation period. Although many reports have shown direct cytotoxicity effect of purified polysaccharides towards cancer cell lines, indirect cytotoxicity is not uncommon. Polysaccharides purified from Innotus obliquus [34], Panax ginseng [35], Schisandra chinensis [36] and many others did not show significant cytotoxicity on cell lines tested, but significantly inhibit the tumor growth in vivo. The anti-cancer activity of these polysaccharides observed in vivo was most probably due to their ability to modulate the immune system, which in turn eliminates the cancer cells.

3.3. The effect of SN-ppF3 treatment on tumor growth

Before the in vivo anti-tumor assay was carried out, a daily oral toxicity study was conducted using 500 mg/kg of SN-ppF3 and the polysaccharide was proven to be non-toxic to the healthy mice since all of the mice remained alive, with no abnormal behavior and side effects commonly associated with toxicity after 14 days of treatment session (data not shown). The potential of SN-ppF3 to inhibit tumor progression in tumor-bearing mice was investigated by evaluating the tumor progression before and after 10 days of treatment with doses of 250 and 500 mg/kg. A rapid development of tumor in the tumor-bearing mice control group could be observed as early as on the third day of the assay and the tumor volume was significantly higher compared to the treated groups starting on day 7 (Fig. 3). As for the treated mice, whether with CTX, low dose or high dose of SN-ppF3, it seemed that the volume of the tumors was slightly increased, but the values were not statistically significant. When compared to the control group after 10 days of treatment, almost 85, 54 and 65% of the tumor progression was inhibited for CTX, 250 and 500 mg/kg SN-ppF3 treated groups, respectively. A similar study has been done by Li et al. where U14 cervical cancer cell line was used in the analysis [37]. The group reported 58% and 49% inhibition when 180 mg/kg and 360 mg/kg of the polysaccharide were used in the treatment, respectively. The difference in inhibition percentage at the end of the assay as compared to ours might be due to different polysaccharide fraction and different cancer cell line was used.

At the end of the treatments, the tumor, spleen, thymus and blood of the mice were sampled. Referring to Table 1, the oral administration of SN-ppF3 daily could significantly (p < 0.05) inhibit the tumor weight with an inhibitory rate of about 38% for 250 mg/kg and 40% for 500 mg/kg treatment. The tumor inhibition rates by SN-ppF3 were comparable to 200 mg/kg of neutral polysaccharide.
fraction extracted from Gynostemma pentaphyllum, which was able to suppress about 50% of tumor progression in H22 tumor-bearing mice after 10 days of treatment [38]. The spleen weight indexes for control and the treated groups were significantly (p < 0.05) higher as compared to normal mice, while the thymus weight indexes for the treated groups were significantly (p < 0.05) lower compared to the normal and control mice. No significant difference was observed between CTX-treated and SN-ppF3-treated groups. It has been reported that the spleen weight of mice with hepatoma was much higher than normal mice. In most scenarios, spleen index of drug-treated tumor-bearing mice were significantly decreased as compared to the control group [14,39]. Moreover, H22 tumor-bearing mice showed a significant increase in spleen size compared to normal mice, which correlate with the increase in cell number in this organ [40]. On the other hand, the thymus weight of the tumor-bearing group was significantly lower than that of the normal group [41,42]. Furthermore, a closely similar pattern in the thymus indexes were also been observed when 5-fluorouracil and G. pentaphyllum polysaccharide were used to treat H22 hepatocarcinoma-bearing mice [38], which comparable to the current finding.

### 3.4. Cytokines concentration

Cytokine production in the blood serum is often used as a parameter to elucidate the immunomodulatory effect, since cytokines play a prominent role in the regulation of host immune responses. For this study, focus was given to the level of TNF-α, IFN-γ, IL-4 and IL-6 in the serum of the tumor-bearing mice. TNF-α plays a pivotal role in the host defense system by inducing the expression of other immunoregulatory and inflammatory mediators to battle against infection [43], and depending on the cellular context, TNF-α may play a role in eliminating tumor cells directly [44]. Since TNF-α is produced by immune cells such as macrophage and T helper cells, the elevation observed (about 14% as compared to the control) indicated the induction of immune response upon treatment (Fig. 4,

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**Table 1**

Effect of SN-ppF3 treatment on tumor weight, body weight and organ indices toward tumor-bearing mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Tumor weight (g)</th>
<th>Body weight (g)</th>
<th>Spleen weight index (mg/g)</th>
<th>Thymus weight index (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>–</td>
<td>–</td>
<td>18.75 ± 0.64</td>
<td>4.39 ± 1.40a</td>
<td>2.80 ± 0.94a</td>
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<tr>
<td>Control</td>
<td>–</td>
<td>1.788 ± 0.63a</td>
<td>20.16 ± 1.90</td>
<td>48.49 ± 5.72c</td>
<td>1.01 ± 0.11c</td>
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<tr>
<td>SN-ppF3</td>
<td>25</td>
<td>0.602 ± 0.32b</td>
<td>18.62 ± 1.00</td>
<td>38.89 ± 15.65d</td>
<td>0.43 ± 0.21d</td>
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<tr>
<td></td>
<td>250</td>
<td>1.117 ± 0.09b</td>
<td>20.88 ± 0.84</td>
<td>50.03 ± 11.28f</td>
<td>0.67 ± 0.19f</td>
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<tr>
<td></td>
<td>500</td>
<td>1.074 ± 0.32b</td>
<td>22.00 ± 1.55</td>
<td>55.78 ± 10.43f</td>
<td>0.62 ± 0.30f</td>
</tr>
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</table>

Data presented were the mean ± SD (n=6). Different superscripted letters in each column indicate significant differences at p < 0.05.

Body weight of mice at the end of the treatment.
Panel A). This increase was comparable to that of CTX treatment, which is known to induce immune response at low dosage [45]. In the host defense system, TNF-α is also necessary as a primary regulator to boost other cytokines production, which may explain the increment of IFN-γ (~22%) and IL-4 (~12%) in the serum of treated mice as compared to the control group (Fig. 4, Panel B and C), after 10 days of oral administration with a high dose of SN-ppF3. The presence of IFN-γ and IL-4 will help to prolong inflammation and responsible to promote the activation of macrophage against infection [46]. IFN-γ has many functions and plays a crucial role in the activation of Th1 immune response. Apart from that, IFN-γ was also known to play an important role in eliminating cancer cells by increasing the expression of MHC class I which may cause cancer elimination by cytotoxic T lymphocytes. Interestingly, a study

**Fig. 5.** H&E staining of tumor tissues. The untreated tumor tissue as control (A), CTX drug (B), 250 mg/kg SN-ppF3 (C) and 500 mg/kg SN-ppF3 (D) treated groups. Magnification: (×400).

**Fig. 6.** Images of NK cell (left panel), CD8+ T-cell (middle panel) and macrophage cell (right panel) detection by immunofluorescent stain. Control (A1–A3), CTX treated (B1–B3), 250 mg/kg of SN-ppF3 treated (C1–C3) and 500 mg/kg of SN-ppF3 treated (D1–D3) groups. Scale showed 1 cm = 100 μm.
by Criychley-Thorne et al. has shown that the impairment of IFN-γ signaling could be associated with breast cancer, indicating the importance of this cytokine on the T and B cells’ anti-tumor activity [47]. As for IL-4, although it is known as an important cytokine in activating Th2 immune response and as an antagonist for IFN-γ, several evidences have shown IL-4 plays a role in the production of IFN-γ in NK and dendritic cells [48]. Furthermore, IL-4 is needed for the proliferation and differentiation of B cells. Thus, the increase of cytokine observed in this study supported the previously reported B cells activation by Li et al. [14]. Intriguingly, the level of IL-6 for CTX and a high dose of SN-ppF3 treated group were significant (p < 0.05) lower as compared to the control group by ~58% and ~49%, respectively. IL-6 is also needed for B cell proliferation but it is commonly elevated in patients with cancer. It was previously reported that there was an elevation of IL-6 concentration (~48%) in patient serum which was unresponsive to any therapy agent. The results suggested that the elevated IL-6 level in the serum may reflect to the poorer prognostic predictor in metastatic breast cancer [49], which also proved the high capability of 4T1 cancer cell line to metastasize [50]. Thus, the high level of IL-6 observed in this study may indicate the malignancy of the tumor, rather than the decrease in immune response.

3.5. Histology

The H&E slides of the controlled and treated tumor tissues (Fig. 5) were microscopically analyzed on the architecture and morphology of the tumor cells. The nuclei were stained blue by hematoxylin dye and cytoplasms were stained pink by eosin dye. Panel A in Fig. 5 showed the normal cellular morphology of 4T1 cancer cell line. However, the treated tumor cells for tissues in panel B–D showed features of apoptosis where the cells shrinkage, the condensation of nuclear chromatin and the formation of apoptotic bodies were clearly observed. These findings suggested that cellular
3.6. Detection of infiltrating immune components by immunofluorescent staining

Further evaluation was done on the control tumor tissues and treated tissues to detect the infiltration of immune cells such as NK cell, CD8+ T-cell and macrophage into the solid tumor. In this experiment, the paraffin blocks of the control and treated tumor tissues were sectioned and fixed on slides and then stained with anti-69 NK cell, anti-CD8+ and anti- F4/80 macrophage antibodies conjugated with fluorescent dye and observed (×200) under a fluorescent microscope. In the left panel of Fig. 6, 500 mg/kg of SN-ppF3 treated group (D1) showed the most significant intensity of NK cell population in the tumor tissues followed by CTX drug (B1) and 250 mg/kg SN-ppF3 (C1) treated groups as compared to the control tumor tissue (A1). In the middle panel, tumor tissues from the CTX drug (B2), 500 mg/kg SN-ppF3 (D2) and 250 mg/kg SN-ppF3 (C2) treated groups showed a significant intensity of CD8+ T-cell population as compared to the control tumor tissue (A2). The presence of macrophages was also detected as high FITC intensity in tumor tissue from 500 mg/kg SN-ppF3 treated group (D3), followed by the CTX treated group (B3). However, density intensity of macrophages was observed in tumor tissue treated with 250 mg/kg SN-ppF3 (C3) as compared to control (A3) group. The NK cells, CD8+ T-cells and macrophages possessed a potent anti-cancer ability by invading several types of solid tumor [51], producing related chemokines in inducing inflammatory responses [52], exerting direct damages to tissues [53] and releasing cancer-related cytokines as well as phagocytosed apoptotic particles [54], respectively. Interestingly, it seemed that the intensity for NK cells was the highest in the tissue of mice treated with 500 mg/kg SN-ppF3. Many studies have shown the importance of NK cells in eliminating cancer where without it, cancer will become more aggressive and metastasize [55]. Although NK cells is inefficient in infiltrating tumor, treatment with cytokines such as IL-2 and IFNs can markedly increase the infiltration [56]. As for CD8+ T-cells and macrophage, the intensity was the highest in samples from CTX treated mice. CD8+ T-cells are able to infiltrate into many solid tumors [53]. However, unlike NK cells, they require specific activation by antigen presenting cells. As for macrophage, further analysis has to be carried out to identify the subset of this cell since alternatively activated macrophages were known to cause poor prognosis of tumor as compared to classically activated macrophages which can cause tumor destruction [57].

3.7. Apoptosis detection of tumor tissue by TUNEL staining

Apoptosis detection is a typical biological hallmark for tumor suppression in tumor tissues, which is commonly confirmed by TUNEL assay. In apoptosis, the cellular morphology undergoes changes as described in the histology section. The nuclear changes were due to the extensive damage to the chromatin and led to DNA cleavage and fragmentation [58]. The DNA cleavage yielded double-stranded DNA fragments and can be specifically identified by labeling the free 3’-OH termini with modified nucleotides conjugated with fluorescent dye in an enzymatic reaction [59]. Fig. 7 showed a highly significant fluorescein intensity (E) in the tumor tissue of CTX (B) followed by 500 mg/kg of SN-ppF3 (D) and 250 mg/kg of SN-ppF3 (C) treated groups as compared to the control tissue (A). These findings strongly suggested that the treatment of SN-ppF3 was able to suppress tumor progression via the mechanism of apoptosis, which supports the H&E analysis. Furthermore, it may reflect the tumor elimination mechanism by the infiltrating immune cells. A study done by Zhao et al. also showed that the oral treatment of 50 and 200 mg/kg polysaccharide isolated from Trametes robiniaphila toward U-2 OS-bearing nude Balb/c mice for 30 days were able to increase the apoptosis percentage up to ~40 and ~50% respectively [25], as compared to the control group by TUNEL analysis.

4. Conclusion

This present study have successfully elucidated the anti-tumor properties of S. nigrum polysaccharide fraction, SN-ppF3 toward 4T1 tumor-bearing mice. The tumor volume and tumor weight were obviously inhibited after 10 days of oral administration of SN-ppF3 to tumor-bearing mice. Moreover, SN-ppF3 exhibited an immunomodulatory effect on the tumor-bearing mice as it was able to elevate the cytokines (TNF-α, IFN-γ and IL-4) level, which may contribute to the disruption of tumor cell morphology through necrosis and apoptosis. The enhanced in infiltration of immune cell into the solid tumor was also observed in the treated mice. These results suggested the in vivo tumor suppression mechanism of SN-ppF3 was most probably by improving the host immune response, thus it has potential to be developed as a novel anti-cancer agent.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2016.06.079.

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