Efficacy of *Spirulina platensis* on immune functions in cancer mice with systemic candidiasis

Hojjatollah Shokri[1*], Alireza Khosravi[2], Mehdi Taghavi[2]

[1]Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran

[2]Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

* Corresponding Author: Email: hshokri@umz.ac.ir, Tel: + 98 121 2271057

Abstract:

The aim of this study was to investigate the immunomodulatory effects of *Spirulina platensis* (*S. platensis*) by measuring the levels of serum interleukin (IL)-4, IL-10, IL-17, tumor necrosis factor (TNF)-α and interferon (IFN)-γ in mice suffered from systemic candidiasis and breast cancer. The aqueous extract of *S. platensis* was selected for this study. Balb/C female mice were inoculated with *Candida albicans* (*C. albicans*) and spontaneous mouse mammary tumor (SMMT). Five days after *Candida* inoculation, the serum levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) were assessed by Enzyme- linked immunosorbent assay (ELISA). The animals were treated daily with *S. platensis* solution (800 mg/kg, 0.2 ml, orally) for 3 days before IV challenge with *C. albicans*, and SC challenge with SMMT and continued for 10 days. The survival rate and tumor size of understudy animals were determined, as well. ELISA determined the levels of TNF-α, IFN-γ IFN T γ-4, IL-10 and IL-17 cytokines in supernatants. The results demonstrated that *S. platensis* decreased the secretion of IL-4 (45.1 pg/ml) and IL-10 (208.4 pg/ml) in tumor-bearing mice infected with *C. albicans*, whereas the levels of IL-17, TNF-α and IFN-γ increased to nearly 93.4 p/ml, 316.2 pg/ml and 137.1 pg/ml in this group, respectively. These findings clearly suggest that *S. platensis* has a remarkable immunomodulatory effect, which provides a scientific validation for the popular use of this natural substance, and assisted in the additional investigation of their complete mechanism of action.

Keywords: cancer, cytokine, disseminated candidiasis, *Spirulina platensis*.

Introduction

Candida albicans (*C. albicans*) is a common commensal organism in humans, and its importance as an opportunistic pathogen, particularly in immunocompromised patients, has continued to increase over the last two decades. The ability of *C. albicans* to establish a disseminated infection, and to persist in the infected tissues or to behave as a commensal may involve primarily downregulation of host cell-mediated adaptive immunity (Puccetti et al., 1995).

Resistance to disseminated candidiasis requires the coordinated action of innate and adaptive immune defenses. Neutrophils and macrophages can clear the yeast via phagocytosis, and macrophage activation leads to the release of several key mediators, which are important for protecting the host against disseminated candidiasis. Administration of interferon (IFN)-γ is reported to be associated with the improved survival of mice after a lethal dose of *C. albicans*, which is linked to the anticandidal activity of peritoneal macrophages (Kulberg et al., 1993; Redmond et al., 1993). In vivo administration of interleukin (IL)-12, which has been reported to prime naive T cells for high IFN-γ expression and skew cytokine production toward a Th1-type response, did not modify the course of disseminated candidiasis (Trinchieri and Gerosa, 1996). In contrast, Th2-type cytokines IL-4 and IL-

10 is reported to exacerbate the infection and neutralization of IL-4 by the specific antibody or soluble IL-4 receptor resulted in an enhanced production of Th1 cytokines, associated with increased resistance to systemic murine candidiasis (Tonetti et al., 1995). Among the different known interleukins, IL-17 is a 20- to 30-kDa homodimeric variably glycosylated polypeptide secreted by CD4^{+} activated memory (CD45^{+}RO^{+}) of the T cells. This cytokine exhibits a biologic activity with a variety of cell types, including induction of the proinflammatory cytokines IL-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8 and PGE2 (Yao et al., 1995a; Yao et al., 1995b; Fossiez et al., 1996). To date, there have been no reports alluding to a role for IL-17 in terms of macrophage activation in disseminated candidiasis. Cancer is a chronic disease with multiple immunosuppressive effects, and importantly, causes a decrease in some Th1 cytokines. Previous studies have demonstrated that the *Aspergillus* infection was caused by tumor development (Sohrabi et al., 2010). Fungal spores or yeast cells can bind to the extracellular matrix protein. The critical step for tumor invasion and metastasis in destruction of the extracellular matrix, which is mainly catalyzed by the matrix metalloproteinases (MMPs) (Hojilla et al., 2003).

The effects of the natural product on cytokine profiles and their inhibitory action on some cancers have been reported (Huang et al., 2006). Despite the availability of potent antifungal agents, acute disseminated candidiasis remains a life-threatening disease. Therefore, further investigation in an effort to develop more effective and safer antifungal agents, and a new therapeutic approach to augment the antifungal capacity of the host's immune system. *Spirulina platensis* (*S. platensis*) (a blue-green algae) has proven to be a rich source of bioactive compounds of potential medicinal interest. Various algae extracts have received increased attention, due to their potent pharmacological effects, particularly in vivo antimicrobial (Soltani et al., 2012), immunological (Saker et al., 2004) and antitumoral activities (Lee et al., 2003). Therefore, in this study investigation of tumor size and serum TIMP-1 levels in the tumor-bearing mice with systemic candidacies and. the immunomodulatory effects of *S. platensis* were studied by measuring the levels of IL-4, IL-10, IL-17, and TNF-α and IFN-γ in a culture of supernatants.

Materials and Methods

Mice: Balb/C female mice, 4-6 weeks of age, were acquired from Razi Institute (Karaj, Iran). The animals were housed at the Mycology Research Center, University of Tehran, Tehran, Iran, at 20-25°C with a 12 h/12 h light/dark cycle for a week prior to resuming the process. All assays involving mice were approved and conducted in accordance with the guidelines of the Animal Care Committee at the University of Tehran, Iran.

Yeast strain: *C. albicans* standard strain (ATCC 10231) was cultured on Sabouraud dextrose agar (SDA, Merck Co., Darmstadt, Germany) at 35°C for 20 h. Yeast colonies were washed twice with phosphate-buffered saline (PBS) and adjusted to 2×10^5 cells/ml by hemacytometer slide.

Preparation of *S. platensis* solution: *S. platensis* powder was purchased from the Aquatic Research Center, Tehran, Iran and suspended in physiological saline solution (0.85% NaCl).

Tumor transplantation and measurement of the tumor volume: Spontaneous mouse mammary tumor (SMMT), spontaneously developed in female BALB/c mice. SMMT is an invasive ductal carcinoma (Hassan et al., 2003). The tumor tissue from the breast cancer-bearing BALB/c mice was separated and cut into pieces of less than 0.5cm^3 with forceps and scalpel. Each piece was transplanted subcutaneously to the syngeneic female BALB/c mice. Two weeks following the transplant, upon appearance of the tumor tissue, the mice were divided into 5 groups, 12 mice per each group. According to the results obtained from delayed-type hypersensitivity (DTH) test, the optimum dose of SLO was selected. The first test-group was inoculated with 10 mg/kg/day SLO intraperitoneally for 20

continuous days. The second and the third groups were inoculated daily with Tyrode and sunflower oil, respectively, 10 mg/kg/day for 20 continuous days. The tumor volume was measured by digimatic caliper (Mitutoyo, Japan), starting from the day 1 up to the end of the experiment. Tumor size was calculated by the following formula:

$$V = 1/2 \times LW^2$$

where V is the volume, L is the length and W is the width. one week following the initial transplant procedure, when the tumor diameter appeared to be 4-6 mm, the prepared groups of mice were infected with *C. albicans* (2×10^5 cells/ml, 0.1 ml), while the remainder received a normal saline solution When the tumor's diameter reached approximately 12 mm, the mice were prepared for immunological studies.

Survival analysis: The mice were divided into 5 groups, (12 mice per each group), including group-1 (mice engrafted with tumor), group-2 (mice infected with *C. albicans*), group-3 (tumor-bearing mice infected with *C. albicans*), group-4 (tumor-bearing mice infected with *C. albicans* and treated with *S. platensis*) and group-5 (tumor-bearing mice treated with *S. platensis*). The animals were observed and monitored for 30 days and evaluated by their survival rate (%).

Measurement of the serum TIMP-1 level: Serum TIMP-1 was quantitated with a commercially available, Quantikine Mouse TIMP-1 Immunoassay kit (R&D Systems, Wiesbaden-Nordenstadt, Germany). Briefly, 5µl of diluted sample or serially diluted human TIMP-1 (standard) and 75µl of peroxidase-conjugated anti human TIMP-1 antibody were added to the wells of a microtiter plate. Following a 30 min incubation period at 30°C, the wells were washed with 0.05% Tween-20 in phosphate-buffered saline. The bound complex was then incubated with o-phenylenediamine containing hydrogen peroxidase for 15 min at 30°C. The reaction was stopped by adding 1M H2SO4 and the absorbance at 492 nm was measured with a microtiter plate reader. Bood samples were collected from all groups 1 through 5, from orbital veins, clotted at room temperature and left at 4°C overnight. The

serum were collected by centrifugation at 3000g for 10 min at 4°C and stored at -20°C for further use.

Cytokine assays: For cytokine analysis, mice were divided into 4 groups (10 mice per each group) as indicated below:

- Group A: tumor-bearing mice infected with *C. albicans* (IV, 2×10^5 cell/ml, 0.1 ml); one week after the initial surgery, when tumor diameter appeared to be 4-6 mm, the animals were infected with *C. albicans*.

- Group B: mice infected with *C. albicans* (IV, 2×10^5 cell/ml, 0.1 ml) (infection control mice).

- Group C: tumor-bearing mice received normal saline (NaCl 0.9%, 0.1 ml/day) (tumor control mice).

- Group D: mice only administered with normal saline (NaCl 0.9%, 0.1 ml/day) (healthy mice).

Five mice of groups A, B and C were treated daily with *S. platensis* solution (800 mg/kg, 0.2 ml, orally). Orally administration to each mouse was performed by gavage for 3 days before the intravenous (IV) challenge with *C. albicans* and SC challenge with SMMT and continued for 10 days. Following a 24 h of the respective treatment, animals were prepared for autopsy using a CO_2 inhalation chamber.

Spleen cell culture: The mice were euthanized; the spleen was removed in sterile conditions. Mononuclear cells were separated by ficoll-*Paque* reagent and centrifugation at 600g for 20 min. Cells were washed in Dulbecco's Modified Eagle Medium (DMEM) medium and centrifuged at 200g for 5 min. The cell pellet was then resuspended in 1 ml endotoxin-free DMEM medium (Gibco RBL, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% L-glutamine DMEM medium. They were counted by hemocytometer and a final concentration of 3×10^7 cell/ml was made. A suspension of 3×105 cells was cultured in flat-bottomed 96-well plates. Levels of IL-4, IL-10, IL-17, and TNF-α and IFN-γ cytokines in supernatant samples were determined by the commercially available ELISA kits (Pierce Endogen, Rockford, IL, USA) and processed according to manufacturers' recommendation.

Statistical analysis: Data was analyzed using SPSS for Windows version 15. comparisons of the data for each group were performed to test for significance (P<0.05) by one-way analyses of variance when the variance between groups was homogeneous was performed; and distribution of the data was normal or a nonparametric test (Kruskal-Wallis) was performed when the normality test or homogeneity of variance test failed.

Results

Survival rate: The survival rate of the mice was recorded in percentages, after treatments from day 1 up to day 30. The results indicated that the survival rates were 33.3%, 33.3%, 0%, 16.7% and 41.7% in groups 1 to 5, respectively. There was a significant difference between group 3 and other groups (P<0.05).

Tumor size measurement: The kinetics of tumor growth in the tumor bearing mice is illustrated in Fig. 1. Approximately 3-4 days posgt engraft, the tumor mass appeared. The mean size of athe tumors was greatly increased in the tumor-bearing mice infected with *C. albicans* (16.98 ± 0.49 mm^2), mice infected with *C. albicans* (9.64 ± 0.27 mm^2), tumor-bearing mice infected with *C. albicans* and treated with *S. platensis* (8.25 ± 0.29 mm^2) and tumor-bearing mice treated with *S. platensis* (8 ± 0.19 mm^2).

Plasma level of TIMP-1: For evaluation of the serum, on the seventh day after *Candida* infection, the ELISA methods were used to measure the levels of TIMP-1. For this purpose, five mice from each group were sampled as representative ones. As illustrated in Fig. 2, the level of TIMP-1 was significantly elevated in tumor-bearing mice infected with *C. albicans* (345 ± 32.6 ng/ml) (P<0.05). The levels were not significantly different in mice infected with *C. albicans* (127.64 ± 15.42 ng/ml), tumor-bearing mice (197 ± 8.27 ng/ml), tumor-bearing mice infected with *C. albicans* and treated with *S. platensis* (104.21 ± 14.3 ng/ml) and tumor-bearing mice treated with *S. platensis* (119.67 ± 8.22 ng/ml).
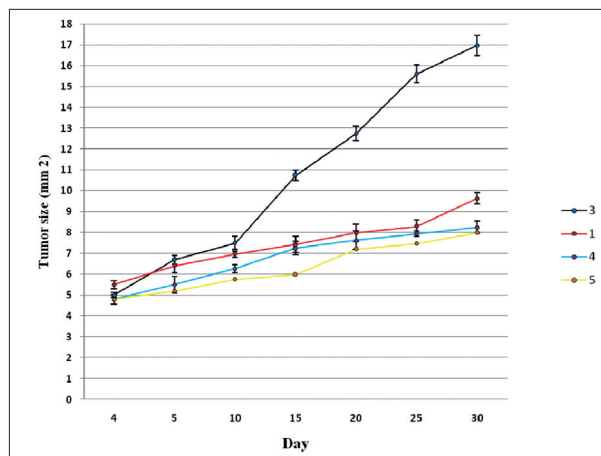

Fig. 1. Effect of *Spirulina platensis* on tumor growth. - Group 1: mice engrafted with tumor; group 2 (deleted); group 3: tumor-bearing mice infected with *Candida albicans*; group 4: tumor-bearing mice infected with *C. albicans* and treated with *S. platensis*; group 5: tumor-bearing mice treated with *S. platensis*.
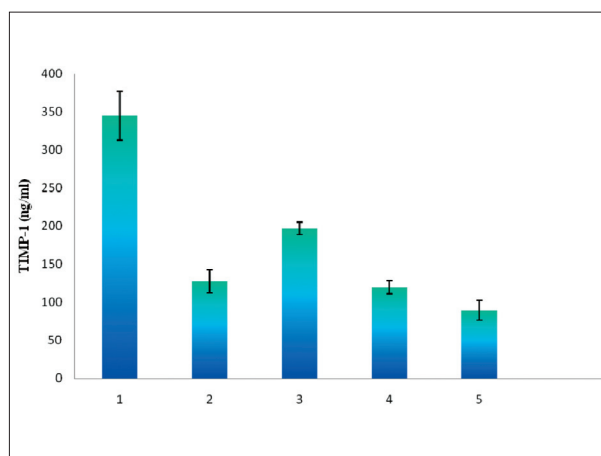

Fig. 2. Effect of *Spirulina platensis* on serum levels of TIMP-1 in different mice groups. - Group 1: mice engrafted with tumor; group 2: mice infected with *Candida albicans*; group 3: tumor-bearing mice infected with *C. albicans*; group 4: tumor-bearing mice infected with *C. albicans* and treated with *S. platensis*; group 5: tumor-bearing mice treated with *S. platensis*.

Effect of *S. platensis* on serum cytokines production in different mice groups: Tables 1 and 2 depicted the effect of *S. platensis* on the different cytokines levels in control and experimental mice with cancer and disseminated candidiasis. *S. platensis* administration to mice decreased a Th-2-type response. We demonstrated that *S. platensis* decreased the secretion of IL-4 and IL-10 to near 45.1 pg/ml. In addition to 208.4 pg/ml in tumor-bearing mice infected with *C. albicans* (group-A), 51.6 pg/ml and 192.4 pg/ml in mice infected with *C. albicans* (group-B). Moreover,

Table 1. Th1 (IL-17 and IFN-γ), Th2 (IL-4 and IL-10) and macrophage (TNF-α) cytokines basal production (pg/ml) in different mice groups without treatment with *Spirulina platensis*.* Mean ±SD: Mean±Standard deviation.- Group A: tumor-bearing mice infected with *Candida albicans*; Group B: mice infected with *C. albicans* (infection control mice); Group C: mice engrafted with tumor (tumor control mice); Group D: mice administered with normal saline (healthy mice).

	IL-4	IL-10	IL-17	TNF-α	IFN-γ
	(Mean±SD) *	(Mean±SD)	(Mean±SD)	(Mean±SD)	(Mean±SD)
Group A	92.41±14.00	298.23±21.20	69.75±14.90	311.43±12.30	81.44±39.00
Group B	82.31±31.11	339.71±13.20	52.33±21.00	287.21±31.00	79.48±30.00
Group C	87.12±32.00	593.62±21.40	46.27±24.00	67.56±14.00	272.45±53.00
Group D	57.14±29.00	128.45±19.40	24.12±14.00	169.33±49.20	70.25±33.00

Table 2. Th1 (IL-17 and IFN-γ), Th2 (IL-4 and IL-10) and macrophage (TNF-α) cytokines basal production (pg/ml) in different mice groups treated with *Spirulina platensis*. * Mean ±SD: Mean±Standard deviation. - Group A: tumor-bearing mice infected with *Candida albicans*; Group B: mice infected with *C. albicans* (infection control mice); Group C: mice engrafted with tumor (tumor control mice); Group D: mice administered with normal saline (healthy mice).

	IL-4	IL-10	IL-17	TNF-α	IFN-γ
	(Mean±SD) *	(Mean±SD)	(Mean±SD)	(Mean±SD)	(Mean±SD)
Group A	45.12±31.40	208.41±8.40	93.45±18.20	316.27±11.34	137.12±21.40
Group B	51.67±14.80	192.41±27.33	43.21±8.27	369.68±21.20	102.41±11.29
Group C	71.60±21.23	384.31±14.00	57.43±11.80	298.14±23.13	86.63±14.22
Group D	38.51±12.80	96.80±21.00	26.14±18.40	258.31±16.00	104.00±14.20

71.6 pg/ml and 384.3 pg/ml in the mice engrafted with the tumor (group-C), and 38.5 pg/ml and 96.8 pg/ml in healthy controls (group-D), respectively. A significant decline of IL-4 secretion was observed in-group A as compared to the other groups (P<0.05). In this study, a significant increment in serum IL-17 cytokine was observed in groups A (93.4 pg/ml), C (57.4 pg/ml) and D (26.1 pg/ml) as compared to group B (P<0.05). In addition, the levels of TNF-α, IFN-γ increased in mice groups A, B, C and D to nearly 316.2 pg/ml and 137.1 pg/ml, 369.6 p/ml and 102.4 pg/ml, 298.1 pg/ml and 86.6 pg/ml, and 258.3 pg/ml and 104 pg/ml, respectively.

Discussion

Cytokines appear to play a major role, acting not only as modulators of antifungal effector functions but also as key regulators in the development of the different TH subsets from precursor TH cells. Studies in mice have demonstrated that the development of protective anticandidal Th1 responses requires the concerted actions of several cytokines, such as IFN-γ and TNF-α (Yao et al., 1995b) in the relative absence of inhibitory Th2 cytokines, such as IL-4 and IL-10, which inhibit development of Th1 responses (Tonetti et al., 1995). The present study examined the effect of the aqueous extract of *S. platensis* in mice-bearing Balb/C mice with haematogenously disseminated candidiasis. The results of this study indicated that the highest mortality rate was in the tumor-bearing mice with disseminated candidiasis. Previous studies in experimental models have reported that the tumor growth was stimulated during the infection (Hojilla et al., 2003; Hornbeck et al., 2005). In fungal infections, this may be a result of the soluble factor production induced by the infection.

In this study, the administration of *S. platensis* to the mice decreased the secretion of IL-4 and IL-10 to nearly 51% and 30% in group A, 37% and 43% in group B, 18% and 35% in group C, and 33% and 25% in group D, respectively. Additionally, the levels of TNF-α and IFN-γ increased in groups A, B, C and D to nearly 2% and 41%, 22% and 22%, 9% and 22%, and 34% and 32% basal levels, respectively. In accordance with results of this study, multiple researchers have established the immunomodulatory effect of *S. platensis*. In a study conducted by Mao et al. (2000), *S. platensis*

stimulated the secretion of IL-4 and IFN-γ to nearly 3.3 and 13.6 times basal levels from human peripheral blood mononuclear cells, respectively. The preponderance of IFN-γ over IL-4 is believed to show that *S. platensis* is more effective in stimulating a Th1-type response and hence potentiates cell-mediated immunity. The moderate stimulation of the production of IL-4, which may be due to the antagonistic activity of IFN-γ on IL-4, may mean that *S. platensis* help balance in the production of Th1 and Th2 cytokines. Thus, *S. platensis* may provide strong protection against intracellular and extracellular pathogens. Soltani et al. (2012) showed that administration of *S. platensis* in mice with systemic candidiasis resulted in the upregulation of TNF-α production at 24 h from mean 8.7 pg/ml to 9.13 pg/ml and at 72 h from mean 7.44 pg/ml to 12.68 pg/ml. Additionally, IFN-γ levels at 24 h and 72 h increased in *S. platensis*-treated mice with systemic candidiasis from mean 16 pg/ml to 39.5 pg/ml and from mean 11.85 pg/ml to 20 pg/ml.

The antioxidant and immune modulation effects of *S. platensis* and their extracts are to a certain extent associated with Spirulina's cancer prevention potential. In a murine model, Lisheng et al. (1991) found that a polysaccharide extract of *S. platensis* inhibited the proliferation of ascites hepatoma cells of mice injected at a dose of 200 mg/kg. Schwartz and Shklar (1987) studied the effect of the administration of 250 μg of *S. platensis* extract and beta-carotene in squamous cell carcinoma of hamster buccal pouches. After 4 weeks of treatment, total tumor regression was found in 30% of the animals treated with extract and 20% of the beta carotene-treated animals. An interesting observation of this study is that *S. platensis* extract appears to be more effective than beta-carotene alone, suggesting a synergistic effect between the various components of *S. platensis*. According to these studies, this remarkable antitumor activity might provide a promising basis for improved therapeutic approaches to tumor invasion and metastasis.

IL-17 is a newly described, T cell-derived cytokine with ill-defined physiologic properties. A limited number of studies have shown that IL-17 is capable of inducing the production of other cytokines from stromal cell elements , such as fibroblasts, endothelial cells, and epithelial cells. *C. albicans* is a dimorphic fungus that primes CD4 T-cell Th17 differentiation after infection in mice. T cells secreting IL-17 are among the first to be activated during immune responses, suggesting IL-17 may play an important role in the early stages of candidiasis (Hovhannisyan et al., 2011). Our results demonstrated that *S. platensis* resulted in a significant increment in serum IL-17 in groups A (25%), C (20%) and D (8%) as compared to group B. To date, there have been no reports alluding to a role for IL-17 in terms of antitumor activation.

Also determined in this study, was that that TIMP-1 was significantly secreted in the serum of tumor-bearing mice infected with *C. albicans*. It was speculated that *C. albicans* can bind to the extracellular matrix protein. Furthermore, the decreased level of TIMP-1 was associated with the decreased mean tumor size. This study revealed that *S. platensis* resulted in the decreased of TIMP-1 level and tumor size in both tumor-bearing mice and tumor-bearing mice infected with *C. albicans*. In conclusion, our data suggest that *S. platensis* exerted a possible antifungal and tumoricidal actions in vivo enhancing the pro-inflammatory (TNF-α) and IFN-γ production, but further investigation is still required to evaluate the efficacy of *S. platensis* in tumor-bearing mice with disseminated candidiasis.

Acknowledgments

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