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EVALUATION OF ANTI-OXIDATIVE, AND ANTIGENOTOXIC POTENCY OF SPIRULLINA SP IN ISOLATED HUMAN LYMPHOCYTE IN VITRO STUDY

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ABSTRACT

Spirulina is a commercial alga well known to contain various antioxidants, especially phycocyanin. Most of the previous reports on antioxidant activity of Spirulina were based on chemical rather than cell-based assays. The primary objective of this study was to assess the antioxidant activity of aqueous extract from Spirulina based on its protective effect against genotoxicity induced by doxirubcin (DOX) or valproic acid (VAP).

The antioxidant and genoprotective activity of Spirulina platensis (SP) water extract was assessed using both chemical and cell-based genotoxic assays. In the cell-based genotoxic assay Isolated human peripheral leukocytes were treated with varying concentrations of SP (10.0, or 20.0 µM) alone or in combination with doxirubcin(DXR) (0.15 µg/mL) or valproic acid (VAP), Comet assays and apoptotic cell studies were performed to evaluate the effect of SP. Spectrophotometric assays were also used to assess the antioxidant activity of the extract.

Spirulina (SP) extract did not cause cytotoxic effect on human leukocytes within the range of concentrations tested (10 - 20 µM). The extract reduced significantly ($p<0.05$) apoptotic cell

death and DNA damage due to DOX and VAP. Based on the antioxidants assay, the extract showed higher total antioxidant capacity in both water-soluble and lipid-soluble antioxidants
The results showed that aqueous extract of Spirulina has a protective effect against apoptotic cell death and DNA damage induced by either DOX or VAP due to free radicals. The potential application of incorporating Spirulina into food products and beverages to enhance their antioxidant capacity and cytoprotective is worth exploring.

Keywords: Spirulina (SP), Doxirubcin (DXR), Valproic acid (VAP), Genoprotective, Comet test

INTRODUCTION

Spirulina (SP) is a photosynthetic, multicellular blue- green micro alga. Spirulina is considered as an excellent food, lacking toxicity and and have anticancer, antiviral, and immunological properties. Also it is playing an important part in stimulation of the erythropoiesis. Aqueous extract of Spirulina, has a protective effect against apoptotic cell death due to free radicals **[1]**.

Genotoxicity refers to the capability of substances to damage DNA and/or cellular components regulating the Sincerity of the genome **[2]**. Previous studies have demonstrated that Spirulina exhibits antioxidant property in various oxidative conditions that cause tissue injury **[3, 4]**. Spirulina treatment reduced the Cyclophosphamide induced testicular spermatogenic cell damage due to genotoxic effect, thereby showing its protective effect of germ cells **[5]**.

SP significantly reduced the level of DNA damage induced by Mitomycin C (MMC) which is a commonly used drug to fight several human malignancies **[6]**. Doxorubicin is a potent antitumor agent used against many cancers. It has genotoxic effect on human non-tumor (normal) cells. Hence, it is very important to reduce its toxicity to the normal cells **[7]**.

Valproic acid (VPA) is a commonly used antiepileptic drug for chronic therapy and may have adverse effects on gastrointestinal, hematologic and nervous system. Potential mutagenic effects of sodium valproate have been reported, but this effect of valproic acid has still not been clarified yet **[8]**.

To the best our knowledge, there are no published data on genoprotective activity of Spirulina on valproic acid genotoxicity, therefore the aim of the present work was to investigate the potential protective effect of Spirullina (SP) on genotoxic effect of doxorubicin (DOX) and valproic acid (VAP) on isolated human lymphocyte, a different approach for evaluation the possible genoprotective effect of SP on DNA damage. For this purpose, the comet assay was used for measuring DNA damage.

MATERIALS AND METHODS

Cells and Medium

Human peripheral blood was collected using heparinized vials from six healthy donors aged 20-30 years. Leukocyte were isolated, and cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 20% fetal calf serum (Cultilab, Campinas, SP, Brazil), streptomycin (10µg/mL) penicillin (5µg/mL), and 2% phytohemagglutinin (Life Technologies, Grand Island, NY). Cells were cultured at 37°C in culture flasks containing 5 ml of complete medium.

The protocol of the experiments was approved by College Research Ethics Committee and also prior to joining the study, a written consent was obtained from each blood donor.

Chemicals

All chemicals and reagents used in the present study except Doxorubicin (DOX) were of the highest analytical grade from Sigma- Aldrich (St. Louis, MO, USA). DOX was purchased as a 10 mg vial ready for infusion (Adriamycin® - Pharmacia), with a concentration of 2mg/ml.

Extraction

Food-grade Spirulina microalgae powder was obtained from Bluebio (Yantai) Biopharmaceutical Co., Ltd. (Sichuan, China).The dry powdered Spirulina (SP) microalgae (20g) was extracted with 200 ml of 96% ethanol for 24 h by using Soxhlet equipment **[9]**. The extract was filtered by using syringe filter (0.22µm), the liquid extract was cooled and concentrated using rotary evaporator at 30-45°C. The extract was stored in labeled sterile screw-capped bottles at -20°C until used.

Determination of Lipid- Soluble Antioxidants

Samples were homogenized with hexane and shaken for 1h at 4^{0}C in the dark. After centrifugation at 6000g for 10 min; the supernatant was transferred to new tubes. Samples of hexanic extracts (200 µl) were placed in eppendorf tubes, dried out and re-dissolved in the same volume of ethanol. These ethanolic solutions were supplemented with 1ml phosphomolybdenum reagent (32mM sodium phosphate, 4mM amonium molybdate, 0.6M sulfuric acids) and were incubated at 95°C for 90min. Finally the absorbance at 695 nm was measured. Lipid soluble antioxidant capacity is expressed as equivalents of α-tocopherol **[10]**.

Determination of Water- Soluble Antioxidants

Samples of SP water extracts (200µl) were supplemented with 1ml phosphomolybdenum reagent and incubated at 95^{o}C for 90 min. Finally the absorbance at 695nm was measured. Water- soluble antioxidant capacity is expressed as equivalents of L-ascorbic acid **[10]**.

Determination of Carotene, Chlorophyll a (Chl a) and Carotene Contents

Chl a contents of SP were determined in accordance with the Jeffrey and Humprey method [11] with 90% acetone as a solvent. Carotene was determined according to the Lichtenthaler and Wellburn **[12]** method with 90% acetone.

Total Phenolic Content

Phenolic contents of SP extract were measured using Folin Ciocalteu's method as described by Taga *et al*., **[13]**. One hundred microlitre aliquot of sample was mixed with 2ml of 2% Na2CO3 and allowed to stand for 2min at room temperature. After incubation, 100 ll of 50% Folin Ciocalteu's phenol reagent was added, and the reaction mixture was mixed thoroughly and allowed to stand for 30 min at room temperature in the dark. The absorbance was measured at 720 nm and the total phenolic content was calculated with a Gallic acid standard and expressed as Gallic acid equivalent per gram.

Culture Treatments

As negative and positive controls, distilled water, DXR (0.15 µg/mL) or VPA (0.25 µg/mL) were used, respectively. The cells were treated DXR (0.15 µg/mL) or VPA (0.25 µg/mL) and diluted sterile extract of spirulina (SP) (10, and 20 µM).

Different concentrations of spirulina (SP) (10, and 20 µM) tested together with DXR or VPA, 24 h after initiation of incubation, the cultures were treated with DXR or VPA and treatment with SP was performed simultaneously. After each respective treatment, drugs remained in the cultures until harvesting. After 24 h of treatment, cells were harvested and then assessed for cell viability, apoptosis (apoptotic assay) and DNA damage of individual cells (comet assay).

Comet Assay

An aliquot of 300µL from each culture was taken after 48 hours of incubation to test for cell viability by trypan blue exclusion and for the alkaline version of the Comet assay as described by Singh *et al*., **[14]**. Briefly, 300µL of the cell suspension was centrifuged for 5 minutes (500 rpm) in a refrigerated microcentrifuge. The resulting pellet was homogenized with 80 µL of a low melting point agarose (0.5%), spread onto microscope slides pre-coated with a normal melting point agarose (1.5%), and covered with a cover slip. After 5 min at 4°C, the cover slip was removed and the slides were immersed in cold lysis solution (2.4 M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton X-100, pH 10) for 24 h. After lysis, the slides were placed in an electrophoresis chamber and covered with electrophoresis buffer (300 mM NaOH per 1 mM EDTA, pH>13), for 20 min to allow the unwinding of DNA. The electrophoresis proceeded for 20 min (25 V and 300 mA). Afterwards, the slides were submerged for 15 min in a neutralization buffer then dried at room temperature and fixed in 100% ethanol for 5 min. Immediately before analyzing slide staining was performed using ethidium bromide (20µg/mL). Slides were prepared in duplicate and 100 cells were screened per sample (50 cells from each slide) in a fluorescent microscope (ZEISS, Germany). According to the migration of the fragments ,the nucleus was classified visually into : class 0 (no damage); class 1 (little damage with a short tail length smaller than the diameter of the nucleus); class 2 (medium damage with a tail length one or two times the diameter of the nucleus); class 3 (significant damage with a tail length between two and a half to three times the diameter of the nucleus); class 4 (significant damage with a long tail of damage greater than three times the diameter of the nucleus) **Figure 1**.

Morphological characterization of normal, apoptotic and necrotic cells

We determined the frequencies of normal, apoptotic and necrotic cells, using 2µL of staining solution [fluorescein diacetate dissolved in dimethyl-sulfoxide (15µg/mL), propidium iodide (5µg/mL) and Hoechtst 33342 (2µg/mL) all from Sigma] mixed with 100µL of cell suspension. For the three independent experiments, Five hundred cells were analyzed per treatment, using an epi-fluorescence microscope. An intact and blue nucleus was considered normal, fragmented and a blue nucleus was considered apoptotic while a red nucleus was considered necrotic.

Statistical Analysis

Statistical analysis was performed using the Kruskal–Wallis One-way Method of Variance [six independent experiments were analyzed] and followed by Student–Newman–Keuls considering a confidence interval of 95%.

RESULTS

Figure 2 shows the results of the determination of total water-soluble and lipid-soluble antioxidant capacity in water extract of spirulina samples. Determined bioactive compounds are chlorophylls a, Total phenol and carotenes are also determined. In general, all tested bioactive molecule contents of SP were relatively higher, also, lipid- and water-soluble antioxidant capacities.

The results of genotoxicity/antigenotoxicity tests are summarized in **Tables 1 and Figure 1, 3** shows the comet assay results and the DNA damage % findings for human leukocytes treated with DXR (0.15µg/mL), VPA (0.25µg/mL), and different concentrations of SP (10, and 20 µM).

As shown in Table 1, SP treatment (10, and 20 µM) significantly reduced the number of apoptotic cells induced by DXR (0.15 µg/mL) or VPA (0.25 µg/mL), and consequently increased the number of viable cells. The protective effect of SP was not dose-dependent.

Results of the visual scoring of total DNA damage are illustrated in **Figure 1, 2** there are a significant increase ($p < 0.05$) of the total DNA damage in human leukocytes treated with either DOX or VPA compared with the control group. Combined treatment of DOX or VPA with Sp (10, and 20 µM) was significantly reduced comet tail length, % of DNA damage and tail moment compared with DOX or VPA treated group **(Figure 3)**.

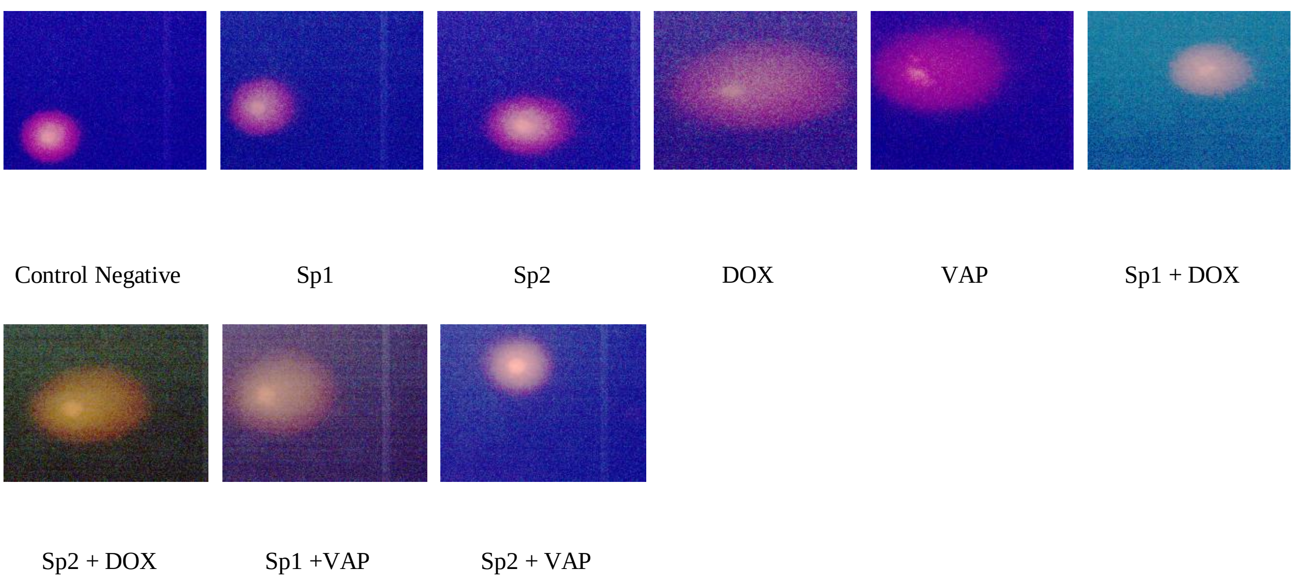

Figure 1: Representative Comet images of isolated human leukocyte in different groups

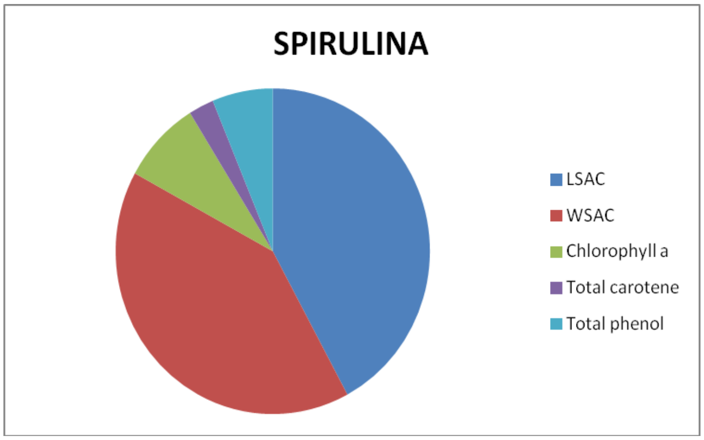

Figure 2: Antioxidant capacity of Spirulina
NOTE: LSAC ***lipid-soluble antioxidant capacity (lmol a tocopherol/g),*** **WSAC** ***water-soluble antioxidant capacity (lmol L-ascorbic acid/g);*** **Chlorophyll a** ***(mg/100g);*** **Total carotene** ***(mg/100g);*** **Total phenol** ***(mg/100g)***

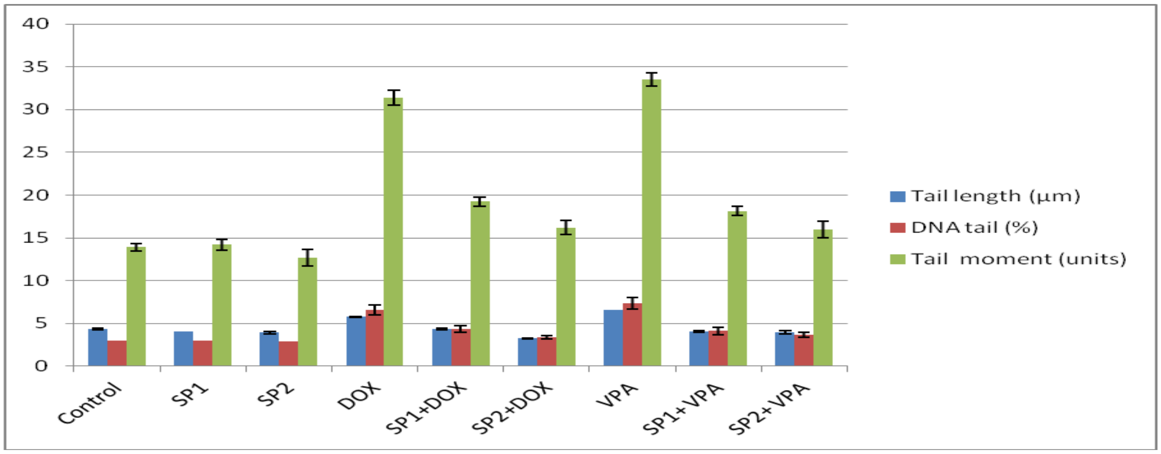

Figure 3: Percentage of DNA damage in isolated human lymphocyte in different treated groups (means ± S.E)
NOTE: Means within column carrying different superscripts are significant at (P <0.05)

Table 1: Percentage of apoptotic cells observed in isolated human leukocytes following different treatments with SP and/ VPA or DXR

Treatment (%)	Scored cells	Viable cells (%)	Apoptotic cells (%)	Necrotic cells (%)
Single drug treatment				
Control	1500	95.9	2	2.1
DXR(0.15 μg/mL)	1500	69	27.6[a]	3.4
VPA(0.25 μg/mL)	1500	73.2	24.8[a]	2
10 μM SP	1500	93.6	4.9[a]	1.5
20 μM SP	1500	92.6	6.3[a]	1.1
Concurrent treatment (SP + DXR)				
10 μM SP + DXR	1500	86.6	11.8[b]	1.6
20 μM SP + DXR	1500	84.2	14.2[b]	1.6
10 μM SP + VPA	1500	82.6	13.8[b]	3.6
20 μM SP + VPA	1500	83.8	12.8[b]	3.4

NOTE: A total of 1500 cells were scored in each one of the three replicates;; $^{a}p < 0.05$ compared with negative control (vehicle-treated); $^{b}p < 0.01$ compared with positive control (DXR)

DISCUSSION

Genotoxicity refers to the capability of chemotherapeutic agents to damage DNA or cellular components regulating the accuracy of the genome. The main problem of chronic drug therapy namely anticancer and antiepileptic drugs targeting DNA as mechanism of action, that they causing the same damage to both abnormal and normal cells **[15]**.

The Genotoxicity is believed to be an important mechanism in the development of DOX or VPA toxicity, so the protection of normal tissues against anticancer drug (DOX) or antiepileptic drug (VPA) genotoxicity is of clinical interest. In the present study we evaluated the possible anti-genotoxic mechanism of SP against DOX or VPA induced DNA damage in isolated human leukocyte. DNA damage of both drugs (DOX or VPA) was evaluated by means of the comet assay, which is widely used in genotoxicity testing *in vitro* and also becoming an important tool for evaluating the genotoxic potential and mutagenicity of many chemicals and natural compounds *in vivo* where as it play important roles in the determination of DNA damage level **[16]**.

The present study revealed that human leukocyte treated with either DOX anticancer drug or VPA antiepileptic drug significantly showed an increase of DNA damage through increased tail length, tail moment and tail DNA % comparing to control group. This result in accordance with Luo *et al* **[17]** who found that Lipid peroxidation inducing capacity of adriamycin may be related to their toxic potential as exemplified by adriamycin-induced cardiotoxicity, which occurs through free radical mediated process. DOX is a recognized topoisomerase II poison that generates reactive oxygen species (ROS) via two different routes of free radical formation. The first involves a non-enzymatic reaction of DXR with iron to form a stable complex, which reacts with oxygen to form superoxide anions, hydrogen peroxide, and hydroxyl radicals. The second involves formation of highly reactive semiquinone intermediates through redox modifications **[18]**.

Many of the antiepileptic drugs have been found to be mutagenic and teratogenic in laboratory animals **[19]**. Valproic acid (VPA), a frequently used drug for the treatment of epilepsy, has been used worldwide. VPA effects are associated with several biochemical/molecular modes of action that have been put forward to account for VPA action, such as interference with folate-methionine metabolic pathways **[20]**, activity as a histone deacetylase inhibitor **[21]**, alteration of gene expression **[22]**, altered antioxidant enzyme activities **[23]** or increase in the formation of toxic VPA metabolites **[24]** such as 2, 4-diene- VPA which can form glutathione conjugate altering glutathione homeostasis **[25]**. In addition, Tabatabaei *et al*., **[26]** indicated that the increased flux of oxygen free radicals during the VPA metabolism leads to nuclear DNA damage, resulting in ensuing cell death. Recently, Sanaa *et al*. **[27]** demonstrated that the administration of VPA to mice at a dose of 100 mg/kg daily for 3 weeks is capable of inducing marked chromosomal aberrations, alterations in mitotic index and significant chromosomal aberrations.

The present study investigated the genoprotective and antioxidant effect of SP alone and in combination with DXR or VPA. We observed that cells treated concurrently with SP and DOX or VPA exhibited genoprotective effects; with a significant decrease in the percentage of necrotic cells at 10µM SP. Furthermore, as observed with of the comet test following DXR or VPA treatment belonged to classes 2, 3, and 4, the SP-treated cultures exhibited predominantly class 1 comets. It is noteworthy that the protective effects of SP demonstrated here did not occur in a dose-dependent manner. SP equally reduced DXR or VPA-induced DNA damage at the lowest and highest concentrations tested .These results are consistent with previous reports that showed that the cytoprotective property of SP algae. They showed that aqueous extract of Spirulina has a protective effect against apoptotic cell death due to free radicals **[1, 28]**.

As shown in **Figure 2**, in the present study, we determined the carotene, chlorophyll a contents in SP. All tested bioactive molecule contents of SP are relatively higher; also, lipid- and water-soluble antioxidant capacities and types of tested vitamins of SP are relatively higher. Spirulina platensis, have a direct effect on reactive oxygen species. It also contains an important enzyme superoxide dismutase that acts indirectly by slowing down the rate of oxygen radical generating reactions **[29, 30]**. The positive correlation between polyphenolic content of algae and its antioxidant activity is well documented **[31]**. Spirulina extract is known to have higher antioxidant activity than another commercial alga, Chlorella due to its higher content of phenolic compounds **[32]**. Therefore, the content of total phenolic compounds in the extracts might explain their high antioxidant activities. Also, in this study, SP showed a remarkable antioxidant activity, this might be due to its high polic content (38 ± 6 mg/100g, **Figure 2**).

In addition, Spirulina has been shown to have protective effects against oxidative stress induced by lead acetate in the liver and kidney of rats and against nephrotoxicity induced by gentamicin **[33, 34]**.

In conclusion, from the findings, we suggest that SP may play a role in reducing genotoxicity induced by anti-neoplastic (DOX) and antiepileptic drugs VAP through repair the DNA damage and reducing the number of apoptotic cells induced by DXR or VAP. It is recommended to supplement SP in the diet concurrently with genotoxic drugs to exert its beneficial effects. Additional researches are needed to study the possible additional desired effects of SP.

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