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Antitumor Activity of C-phycocyanin from *Arthronema africanum* (Cyanophyceae)

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ABSTRACT

Pure C-phycocyanin (C-PC) was isolated from Arthronema africanum *to evaluate its potential antitumor effects in vivo and in vitro. Experimental myeloid Graffi tumor in hamsters was used as a model. The cell proliferation assay showed that C-PC treatment, at concentration of 100 µg mL^{-1} for 24 h, significantly inhibited the growth of Graffi tumor cells (51.4% viability). Agarose gel electrophoresis of the genomic DNA of treated cells displayed time- and concentration-dependent fragmentation pattern, typical for apoptosis. Apoptotic process was related to the increase in cellular manganese and copper/zinc superoxide dismutases and glutathione reductase activities, coupled with a low catalase activity. In vivo C-PC administration (5.0 mg kg^{-1} body weight) suppressed the tumor transplantability and growth, while the mean survival time of the tumor-bearing hamsters was increased. The results revealed promising antitumor activities of* A. africanum *C-PC and suggested the potential of this natural biliprotein pigment for future pharmacological and medical applications. The study provided new data on the mechanism of the C-PC-induced apoptosis in which the imbalance of antioxidant enzymes that favoured hydrogen peroxide accumulation might play a leading role.*

Key words: Antitumor activity, *Arthronema africanum*, *In vivo*, *In vitro*, C-pycocyanin, Myeloid Graffi tumor

INTRODUCTION

In the last few decades, cyanobacteria have gained much attention as a rich source of bioactive compounds (Singh et al. 2005). One of the fascinating discoveries regarding biological activity is the anticancer property demonstrated among cyanobacterial genera such as *Nostoc*, *Fhormidium*, *Gloeocapsa*, *Anabaena*, *Spirulina*, *Synechocystis* and *Synechococcus* (Surakka et al. 2005; Martins et al. 2008; Gigova et al. 2011; Oh et al. 2011). Cyanobacteria produce a wide range of cytotoxic compounds, many of which are regarded as promising candidates for drug discovery, with applications in pharmacy, because of their natural origin, uniqueness and structural diversity (Singh et al. 2005; Tan 2007; Gademann and Portmann 2008). Recently, the attention of researchers was focused on the phycobiliproteins, and especially on C-phycocyanins (Walter et al. 2011). Phycobiliproteins (phycoerythrins, phycocyanins and allophycocyanins) are light-harvesting protein pigments, characteristic for cyanoprokaryotes and two algal phyla (Rhodophyta and Cryptophyta). C-phycocyanins were established to have various biological activities and pharmacological properties. These water soluble biliproteins have shown

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antibacterial (Sarada et al. 2011; Najdenski et al. 2013), antifungal, antiviral (Murugan and Radhamadhavan 2011), and anticancer (Li et al. 2006; Roy et al. 2007; Li et al. 2010) activities as well as anti-inflammatory, fibrinolytic (Chung et al. 2010), antidiabetic (Ou et al. 2013), anti-oxidant and free radical scavenging properties (Bhat and Madyastha 2000). The investigations on antitumor activity have been focused on the C-PC from *Spirulina platensis*, while the other rich sources of this valuable pigment remain unexplored, except for *Limnothrix* sp. (Gantar et al. 2012), *Porphyra yezoensis* (Zhang et al. 2011) and *Porphyra haitanensis* (Liu et al. 2000a). Remarkably, the tested phycocyanins differed in their potential and mechanisms of action, which could be due to their different molecular weights and the specificity of the used experimental models.

Arthronema africanum strain Lukavský 1980/01 (*A. africanum*) has been assessed as a prospective phycocyanin-producing strain (Chaneva et al. 2007). The aim of this work was to examine the *in vivo* and *in vitro* antitumor effects of a highly purified C-PC from *A. africanum* using experimental myeloid Graffi tumor in hamsters as a model.

MATERIALS AND METHODS

Isolation and purification of C-phycocyanin

A. africanum strain Lukavský 1980/01 obtained from the CCALA (Culture Collection of Autotrophic Organisms, Třeboň, Czech Republic), was grown photoautotrophically in the nutrient medium of Allen and Arnon (1955) at 32°C and continuous lateral illumination with cool-white fluorescent lamps at a photon flux density of 250 $\mu E\ m^{-2}\ s^{-1}$ for seven days. A carbon source was provided by bubbling 1% (v/v) CO_2 in 10 L h^{-1} air through the cultures. Pure C-phycocyanin from *A. africanum* was obtained by a modified rivanol-sulfate method (Minkova et al. 2007). The equations of Siegelman and Kycia (1978) were used to estimate the phycobiliprotein content. The purity of C-PC was evaluated by the absorbance ratio A_{620}/A_{280} and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). SDS-PAGE was carried out according to Okajima et al. (1993), using a 12% polyacrylamide slab gel. The proteins in the gel were visualized by Coomassie blue staining.

IN VIVO EXPERIMENTS

Experimental Animals and Ethical Aspects

Golden Syrian hamsters, 2–4 months old, weighing approximately 100 g were purchased from a breeding base of the Oncology Center, Sofia, Bulgaria. The animals were kept under standard conditions in individual plastic cages with free access to food and water. All studies were performed in accordance with the Guide for Care and Use of Laboratory Animals, as proposed by the Committee on Care of Laboratory Animal Resources, Commission on Life Sciences and National Research Council, and a work permit № 11130006.

Experimental Graffi Myeloid Tumor in Hamsters

Experimental Graffi myeloid tumor was created and maintained monthly *in vivo* by subcutaneous implantation of live tumor cells (2×10^6 mL^{-1} in phosphate buffered saline, PBS) in the interscapular area of hamsters (Toshkova et al. 2008). Between the 10th and the 15th day after tumor transplantation on the back of the hamster, a solid subcutaneous tumor appeared, which progressively increased in size and after about 30 days, caused death of the experimental animals. Spontaneous regression in this experimental tumor model was not observed.

Phycocyanin Treatments

A stock solution of 1.0 mg mL^{-1} C-PC was freshly prepared in PBS for each experiment. Healthy animals were divided into four experimental groups of eight hamsters: Gr.1 - hamsters treated intraperitoneally (i.p.) with C-PC at a dose of 5.0 mg kg^{-1} body weight, 2 h before transplantation of 5×10^4 viable tumor cells; Gr.2 - hamsters treated twice i.p. with 5.0 mg kg^{-1} C-PC, 2 h before and 24 h after transplantation of 5×10^4 viable tumor cells; Gr.3 and Gr.4, control groups – hamsters transplanted with 5×10^4 viable tumor cells and subjected to analogous treatments with the diluent (PBS) alone. Transplantability (T%) was calculated as the number of animals that developed tumors from all the transplanted hamsters in each experimental group. Tumor growth was followed by measuring the length and width of tumors on odd numbered days after the challenge. Mortality percent and mean survival time were also determined.

IN VITRO EXPERIMENTS

Isolation of Primary Graffi Tumor Cells

Graffi tumor cells were isolated from the tumor tissue under aseptic conditions and cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco BRL), 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Lonza, Basel, Switzerland). Cultures (1×10^6 mL^{-1} cells) were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cell viability was determined by the trypan blue dye exclusion method (Bennett et al. 1976). Average viability was higher than 95%.

Isolation of Bone Marrow Cells

Hamsters were sacrificed by cervical dislocation under deep anesthesia. Femurs were dissected out, freed of connective tissue and muscle, cut off with scissors and the marrow was flushed out with Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) using a syringe and a 26G needle. The cell suspension was washed three times with the medium, followed by centrifugation at 200×*g* for 10 min (Goud 1999). The viability of bone marrow cells (BMC) evaluated by the trypan blue exclusion test was 100%.

Cell Proliferation Assay

Cell proliferation was determined using the colorimetric assay as referred by Mosmann (1983). Graffi tumor cells and bone marrow cells (2×10^4 $well^{-1}$) were seeded into 96-well culture plate in the presence of 100 µg mL^{-1} C-PC (at 100 µL final volume) for 24 h. Cells cultured only in the nutrient medium were used as negative controls. The antineoplastic drug doxorubicin hydrochloride (DOX, Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 10 µg mL^{-1} was used as a positive control. After the treatment, the medium was aspirated and 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) working solution (5.0 mg mL^{-1} in PBS) was added to each well, followed by further incubation at 37°C for 3 h. Formed formazan crystals were dissolved in 100 µL of the lysing solution (DMSO/EtOH, 1:1, v/v). The absorbance was recorded using an ELISA reader (TECAN, SunriseTM, Grödig/Salzburg, Austria) at a wavelength of 570 nm. The percentage of viable cells was calculated as follows: Cell viability = $[(A_t - A_b)/(A_c - A_b)] \times 100$, where A_t, A_b, and A_c are the absorbance value of the test sample, blank and negative control, respectively.

Agarose Gel Analysis of DNA Fragmentation

After a careful titration, concentrations of 50 and 100 µg mL^{-1} C-PC were selected for these *in vitro* experiments. Graffi tumor cells were treated with C-PC for 4, 12 and 24 h. DNA isolation and agarose gel electrophoresis were carried out following the protocol of the Morimoto laboratory [Internet]. Briefly, treated and untreated Graffi tumor cells were lysed in 10 mM Tris (pH 8), 20 mM EDTA, 200 mM NaCl, 0.2% Triton X-100, and 100 µg mL^{-1} Proteinase K (Sigma-Aldrich, St. Louis, MO, USA). DNA was precipitated with isopropanol (1:1, v/v) and treated with 250 µg mL^{-1} RNase A (Sigma-Aldrich, St. Louis, MO, USA). DNA fragments (10 µg of total DNA) were separated by electrophoresis in 1.2% agarose gel at 80 V for 90 min in TBE buffer and visualized using ethidium bromide (1 µg mL^{-1}) staining and a UV transilluminator.

Preparation of Cell Extracts and In-gel Enzyme Activity Staining

Whole-cell extracts from Graffi cells were prepared following Bravard et al. (1999). Protein concentration in the extracts was determined by the method of Bradford (1976). Equal amounts (20 µg) of protein from C-PC-treated and C-PC-untreated tumor cells were subjected to PAGE essentially as described by Okajima et al. (1993), except that the SDS was omitted. Electrophoretic separation was performed on 10% polyacrylamide gels for 3–4 h with a constant current of 35 mA per gel. The in-gel activity staining of superoxide dismutase (SOD), glutathione reductase (GR), and catalase (CAT) was performed on separate gels, following the methods of Azevedo et al. (1998), Anderson et al. (1995), and Chandlee and Scandalios (1983), respectively. The different metaloforms of SOD were identified by the incubation of resolved parallel gels for 30 min either in 50 mM potassium phosphate buffer (pH 7.8), or in the buffer containing 2 mM KCN prior to staining. The Cu/ZnSOD is sensitive to KCN, while MnSOD is resistant (Azevedo et al. 1998). All the reagents used for enzyme activity staining were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gel patterns were recorded immediately after the staining using the UVItec

gel documentation system (Cambridge, UK). Image analysis of the gels was performed on a PC using Gel-Pro32 Analyzer software (Media Cybernetics Inc., USA). The activity (intensity) of each isoenzyme (band) was recorded as integrated optical density (IOD) in arbitrary units.

Statistical Analysis

All data are expressed as the means ± standard deviation. The statistical significance between the treatments was evaluated by one-way ANOVA and a Bonferroni post hoc test using InStat (GraphPAD Software Inc., La Jolla, CA, USA). Values of $P<0.001$ were considered significant.

RESULTS

Isolation and Purification of C-phycocyanin

C-phycocyanin of *A. africanum* was isolated and purified by a simple, non-chromatographic, rivanol-sulfate method, developed in the laboratory as an alternative to existing multistage procedures. The C-PC obtained by this rapid, inexpensive and reliable method corresponded to the basic criteria for purity of the pigment. C-phycocyanin had absorption and fluorescence maxima at 620 and 650 nm, respectively (Fig. 1A and B) and purity ratio A_{620}/A_{280} of 4.3. Homogeneity of the protein pigment was further confirmed by the SDS-PAGE where only its α and β subunits were visible as the bands of molecular weights of about 17.6 and 22.4 kDa, respectively (Fig. 1C, lane 1). For comparison, the purified C-PC of *Spirulina platensis* was resolved on the same gel (Fig. 1C, lane 2), where the bands of molecular weights of 18.5 and 21.5 kDa corresponded to its α and ß subunits.

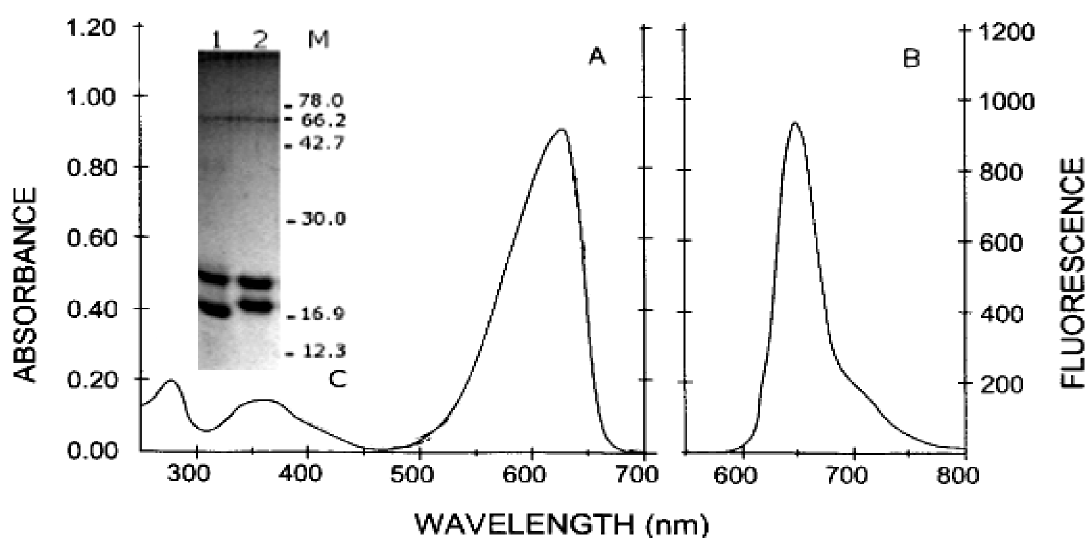

Figure 1 – Purity analysis of C-phycocyanin from *Arthronema africanum*. **A**, Absorbtion spectrum of C-PC. **B**, Fluorescence spectrum of C-PC. **C**, SDS-PAGE of C-PC. Lane 1, pure C-PC of *A. africanum* - its α and ß subunits are visible as bands of molecular weights of 17.6 and 22.4 kDa, respectively; lane 2, purified C-PC of *Spirulina platensis*, shown for comparison – the bands of molecular weights of 18.5 and 21.5 kDa correspond to its α and ß subunits, respectively; M, positions of the protein markers (Merck, Darmstadt, Germany), indicated in kDa.

In vivo effects of *A. africanum* C-phycocyanin on Graffi myeloid tumor

The effect of C-PC on the transplantability of Graffi tumor in hamsters was monitored at 7, 10, 15 and 20th day after implantation of viable tumor cells. No appearance of tumors was observed (0% transplantability) at the 7th and 10th day of the study in both the groups treated with C-PC, while in the control groups, 40 and 60% of the hamsters, respectively developed tumors (Fig. 2A). The

transplantability in the untreated hamsters reached 100% at the 15th day. At the same day of observation, tumor appeared in about 80% of the hamsters in C-PC-treated groups. The transplantability was 100% in all the hamsters (C-PC-treated and untreated) at the 20th day after implantation (Fig. 2A). After subcutaneous tumor formation, inhibition of the tumor growth was observed in C-PC-treated experimental animals. The average size of tumors in the hamsters treated once, or two times with C-PC was significantly lower than in untreated tumor-bearing hamsters (TBH) at days 15, 20 and 25 (Fig. 2B). The most significant inhibition of tumor development was observed in group 1 - hamsters treated with a single dose of C-PC, where the mean values of tumor size were 0.3 ± 0.12, 1.5 ± 0.5 and 2.86 ± 0.7 mm at the 15th, 20th and 25th day, respectively, while in the controls, these were 1.44 ± 0.46, 1.9 ± 0.7 and 4.08 ± 0.5 mm, respectively (Fig. 2B). The C-phycocyanin of *A. africanum* was able also to substantially reduce the mortality rate of TBH at all the stages of the study. None of the hamsters died in the groups 1 and 2 (treated with C-PC), till the 30th and 35th day of the study, respectively, while in the control groups, the mortality reached 100% on the 30th day. The lowest rate of mortality was observed in group 1, treated with a single dose of C-PC, and 20% of the hamsters survived more than 45 days (Fig. 2C). In accordance with these findings, the mean survival time (MST) in both C-PC-treated experimental groups was markedly longer than in untreated TBH (control). As seen in Figure 2D, mean survival time in the PBS-treated controls was 28.2 ± 1.9 days, while in the C-PC-treated groups 1 and 2, the MST reached 41.3 ± 5.2 and 39.2 ± 2.9 days, respectively.

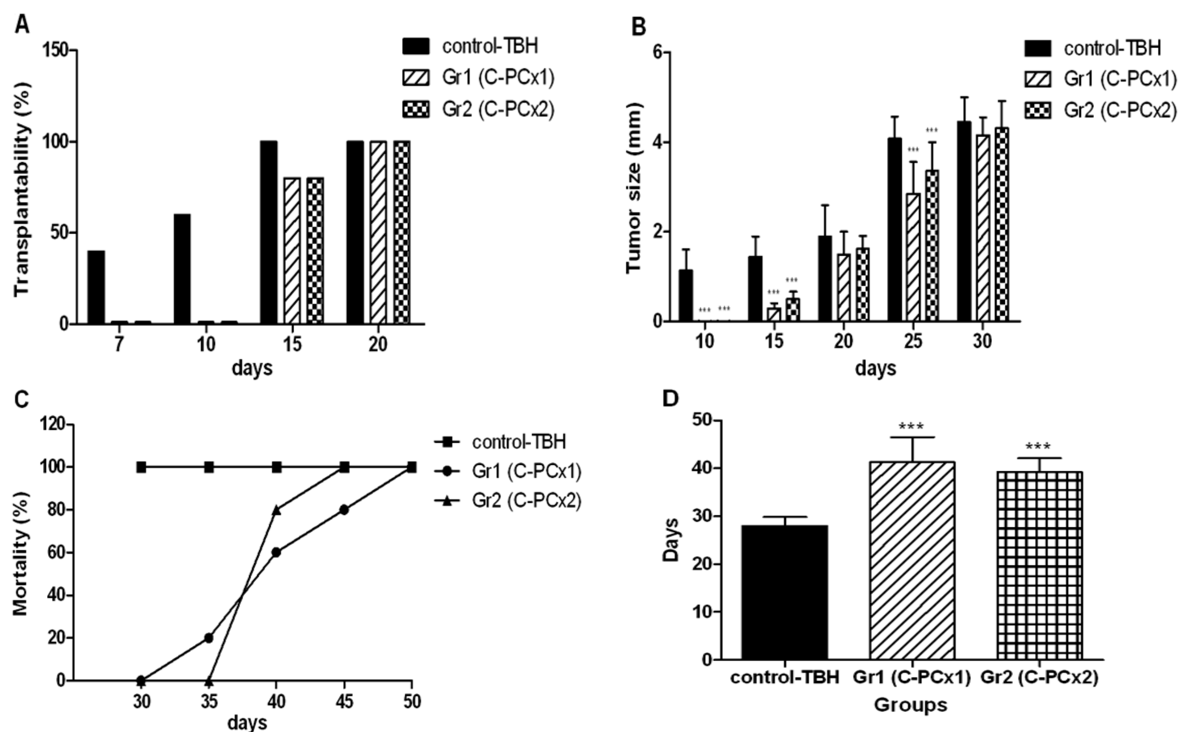

Figure 2 – *In vivo* antitumor effects of C-PC. **A**, Transplantability (%) of Graffi tumor. **B**, Tumor size (mm). **C**, Mortality (%) of tumor-bearing hamsters (TBH). **D**, Mean survival time (days) of tumor-bearing hamsters. ***$P<0.001$ indicate significant differences from the control (C-PC-untreated TBH) values.

***In vitro* effect of *A. africanum* C-phycocyanin on the viability of tumor and bone marrow cells**

The treatment of Graffi tumor cells with 100 µg mL^{-1} C-PC caused a decrease in the number of viable cells to 51.4 ± 4.0% (P<0.001), while the same amount of C-PC did not exhibit any toxicity to bone marrow cells (BMC), isolated from the femurs of Graffi tumor-bearing hamsters. The biliprotein significantly (P<0.001) stimulated BMC proliferation, and cell viability reached to

174.1 ± 2.9%. The antineoplastic drug DOX (10 µg mL^{-1}) used as a positive control in these experiments decreased tumor cell viability only to 64.15 ± 1.33%. The viability of the untreated tumor cells and BMC (negative controls) was considered 100%.

DNA fragmentation

Cells freshly isolated from the Graffi tumor were cultured in the absence (negative control), or presence of 50 and 100 µg mL^{-1} C-PC for 4, 12 and 24 h and then subjected to DNA-fragmentation tests. Agarose electrophoresis of genomic DNA isolated from the cells treated with C-PC showed fragmentation pattern (DNA ladder of 180-200 bp oligomers), typical for apoptotic cells, which was not observed in the untreated (control) cells (Fig. 3). Internucleosomal DNA cleavage in phycocyanin-treated Graffi cells occurred in a time- and concentration-dependent manner. The DNA fragmentation was most pronounced after the treatment with 100 µg mL^{-1} C-PC for 24 h (Fig. 3).

Effect of C-phycocyanin on the activity of SOD and GR in Graffi tumor cells

Two superoxide dismutase (SOD) metaloforms – manganese (MnSOD) and copper/zinc superoxide dismutase (Cu/ZnSOD) (Fig. 4A) and two glutathione reductase (GR) isoenzymes designated GR1 and GR2 (Fig. 4B) were visualized in the Graffi tumor cells. The isoenzyme patterns were not affected by the C-PC treatments and did not show any change. However, the relative total activity of both the enzymes clearly increased in the C-PC-treated cells in a concentration- and time-dependent manner. Each the SOD metaloform responded differentially to the treatments. MnSOD activity was enhanced after the application of 50 and 100 µg mL^{-1} C-PC for 4, 12, and 24 h by 30 and 60%, 41 and 103%, and 214 and 268%, respectively (Fig. 4A). The activity of Cu/ZnSOD was slightly lower in the treated than in untreated cells at the 4th h, whereas at the extended duration of the C-PC action, a significant increase in Cu/ZnSOD activity was registered. The activities reached 215 and 254% at a dose of 50 and 100 µg mL^{-1} C-PC, respectively after 12 h of treatment, and 288 and 335%, respectively after 24 h of treatment (Fig. 4A). In contrast to SOD, the activity of GR was very low in both the treated and control cells at the 4th and 12th h (not shown). A concentration-dependent effect of C-PC was observed after prolonged treatment (24 h, Fig. 4B). GR2 was more active than GR1 but both the isoenzymes were responsive to C-PC. GR1 activity was increased approximately 1.3 and 2.1 times at a dose of 50 and 100 µg mL^{-1} C-PC, respectively and the activity of GR2 was about 1.6 and 2.5 times higher, respectively compared to the C-PC-untreated controls (Fig. 4B, lane 1). Catalase activity was not detected in the Graffi tumor cells in the current study (not shown).

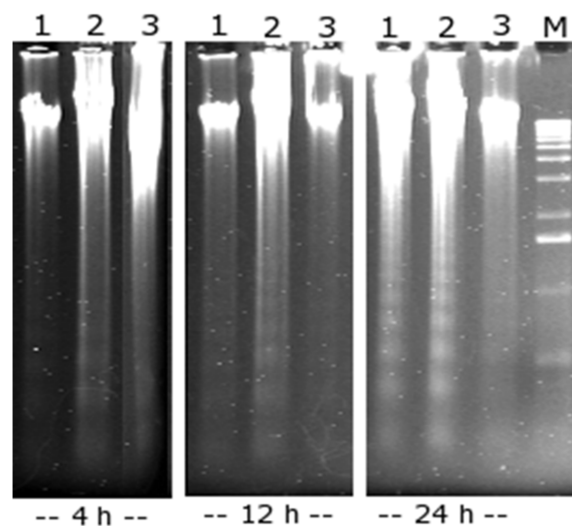

Figure 3 – Effect of C-PC on DNA integrity of Graffi tumor cells. Lane 1, DNA from cells treated with 50 µg mL^{-1} C-PC; lane 2, DNA from cells treated with 100 µg mL^{-1} C-PC; lane 3, DNA from control cells. Lane M, 1kb DNA Ladder, range 250 to 10 000 bp (Fisher Scientific, UK). DNA integrity was evaluated after treatment with PBS (control) or C-PC for 4, 12, and 24 h. Equal amounts of DNA (10 µg) were loaded in each lane and subjected to agarose gel electrophoresis. Results are representative of three independent experiments.

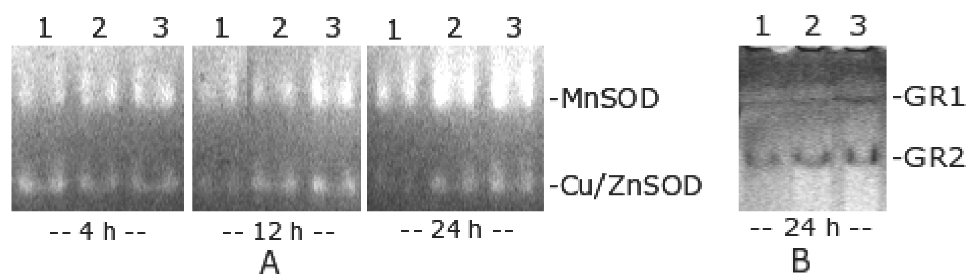

Figure 4 - Effect of C-PC on the antioxidant enzyme activities of Graffi tumor cells. **A**, Changes in SOD activity. **B**, Changes in GR activity. Lane 1, PBS-treated control cells; lane 2, cells treated with 50 µg mL^{-1} C-PC; lane 3, cells treated with 100 µg mL^{-1} C-PC for 4, 12, and 24 h (A) and for 24 h (B). Equal amounts of protein (20 µg) were loaded in each lane, subjected to native polyacrylamide gel electrophoresis and the respective in-gel activity staining. Results are representative of three independent experiments.

DISCUSSION

The antitumor potential of largely studied C-PC isolated from *Spirulina platensis* is well known. Various mechanisms of its antitumor activity studied in different tumor cell lines have been proposed ranging from the interference of DNA synthesis in the tumor cells (Wang et al. 2001), activation of caspase-dependent programmed cell death pathways (Pardhasaradhi et al. 2003; Li et al. 2006; Roy et al. 2007; Li et al. 2010), inhibition of tumor cell growth by pathways other than apoptosis like a membrane destruction, leading to increased leakage of cell constituent (Abd El-Baky 2003), or stimulation of expression level of the proto-oncogene c-myc (Liu et al. 2000b), to the improvement of host immune functions (Hayashi et al. 2006; Li et al. 2010). In contrast to the C-PC of *S. platensis*, the C-PC from the cyanobacterium *Limnothrix* sp. exhibited low activity (Gantar et al. 2012). When combined with low dose of the anticancer drug topotecan, however, the C-PC from *Limnothrix* sp. significantly improved the topotecan efficacy. In combination, these two compounds induced apoptosis in the LNCaP (prostate cancer) cells through the generation of ROS and activation of caspases (Gantar et al. 2012). The other studied phycocyanins, those of the red algae *Porphyra haitanensis* (Liu et al. 2000a) and *P. yezoensis* (Zhang et al. 2011) significantly inhibited the growth of human leukemia HL-60 cells, and Hep-2 (laryngeal carcinoma) and A375 (malignant melanoma) cell lines, respectively by the unidentified pathways. Most of these knowledges were obtained from *in vitro* studies. There are only few reports concerning the *in vivo* effects of C-PC (Hayashi et al. 2006; Li et al. 2010).

The present study examined the *in vivo* and *in vitro* effects of a highly purified C-PC from *A. africanum* on myeloid Graffi tumor in hamsters. The results demonstrated the abilities of this C-PC to decrease significantly tumor transplantability and to delay the development of the Graffi tumors as well as to improve substantially the survival of tumor-bearing hamsters. The performed *in vitro* experiments showed that C-PC inhibited the growth of Graffi tumor cells, but enhanced the proliferation of bone marrow cells, thus shedding light on the effects observed *in vivo.* It can be assumed that apart from the negative effects on tumors, C-PC may have a positive impact on hamster's immune system, which is compromised during tumor progression. In order to investigate the mechanisms involved in the growth inhibition of tumor cells, studies on DNA integrity and antioxidant enzyme activities (SOD, GR, and CAT) were undertaken. Graffi tumor cells treated with C-PC showed a concentration- and time-dependent ladder-like DNA fragmentation pattern. Such internucleosomal DNA degradation to 180-200 bp oligomers is considered one of the hallmarks of apoptosis, a specific mode of cell death recognized by a characteristic pattern of molecular, biochemical and morphological changes (Elmore 2007). The treatment with *A. africanum* C-PC led also to increase in the activities of MnSOD, Cu/ZnSOD and GR compared to the untreated tumor cells. At the same

time, both C-PC-treated and untreated cells maintained very low activity (undetectable by in-gel assay) of CAT, a major hydrogen peroxide detoxifying enzyme. Apparently the C-PC-elevated SOD activity, coupled to a suppressed CAT activity in the Graffi tumor cells, led to the accumulation of hydrogen peroxide (H_2O_2), which initiated the apoptotic process and could negatively regulate the growth of the tumor. The involvement of H_2O_2 in the induction of apoptotic death pathways in cancer cells has already been shown (Mao et al. 2006; Cerella et al. 2009). It has been reported that H_2O_2 up-regulated the Bax (pro-apoptotic protein) but down-regulated the survival protein Bcl-2 (Mao et al. 2006), and activated some caspases (Hampton and Orrenius 1997), a known class of cysteine proteases that play a critical role during the apoptosis. The exact molecular mechanism of the *A. africanum* C-PC-induced apoptotic process in the Graffi tumor cells needs to be further studied.

CONCLUSIONS

The results revealed for the first time that the C-phycocyanin from *A. africanum* had *in vivo* and *in vitro* antitumor activities and could be a promising natural antitumor agent with a potential for future pharmacological and medical applications. The experimental model provided a new idea on the mechanism of the C-PC-induced apoptosis in which up-regulation of SOD activity and the imbalance of antioxidant enzymes that favoured H_2O_2 accumulation could play a leading role.

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