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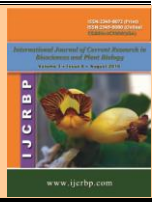

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Antioxidant Activity of Phycobiliproteins from *Dermonema virens* (J.Agardh) Pedroche & Ávila Ortiz (Rhodophyta: Liagoraceae) on *Lactuca sativa* L. Seedlings

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Abstract

Live beings have shields against oxidative damage from reactive oxygen (ROS). These shields are the antioxidant systems and can be enzymatic or non-enzymatic. The phycobiliproteins are part of the non-enzymatic systems and they are water soluble photosynthetic pigments found in cyanobacteria and red algae. They can scavenge free radicals. Phycobiliprotein extract from seaweed *Dermonema virens* was performed macerating it in liquid N_2, then the biomass was suspended in water and centrifuged, and finally the supernatant spectrophotometrically characterized. *Lactuca sativa* seeds were exposed to three different media to evaluate germination and elongation of the radicle and hypocotyl. A high concentration of phycoerythrin was found, with an absorption peak at 550 nm. In trials with water, 4-6 cm growth in radicle and hypocotyl was obtained, while the ROS caused necrosis in the radicle and reached just 10% of germination. Testing the extract in the presence of ROS, seed germination was at 90% and radicle from 1 to 3 cm. It is concluded that the extract has antioxidant activity that protects lettuce seeds from oxidative damage from ROS.

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Introduction

Reactive Oxygen Species (ROS) are reactive molecules and free radicals derived from molecular oxygen. ROS include free radicals such as superoxide anion, hydroxyl radical and non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen. All of them are very harmful to cells at high concentrations. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of "oxidative stress". ROS can damage cells in several ways: by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and leading them to death (Sharma et al., 2012). But at low to moderate concentrations, they function in vital physiological cell processes (Birben et al., 2012; Delgado Olivares, 2010; Fina, 2009). To counterbalance the effect of oxidants the cell is equipped with a variety of antioxidants, these can be divided into 2 categories: enzymatic and non-enzymatic (Birben et al., 2012; Gallardo, 2010). The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutahione (AsA-GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). Whereas ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, Q coenzyme, reduced glutation and phycobiliproteins serve as potent non-enzymic antioxidants within the cell (Bermejo et al.,

2002; Mates et al., 1999; Ramírez-Moreno and Olvera-Ramírez, 2006; Sharma et al., 2012; Valko et al., 2006; Warner et al., 2004).

Phycobiliproteins are pigments of the photosynthetic light-harvesting antenna complexes of cyanobacteria and red algae, which are isolated like trimers or hexamers (α, β and γ) covalently attached linear tetrapyrrole (Gantt, 1981). Three phycobiliproteins R-phycoerythrin, B-phycoerythrin, and allophycocyanin serve as valuable fluorescent tags with numerous applications in flow cytometry, fluorescence activated cell sorting, histochemistry and, to a limited degree, in immunoassay and detection of reactive oxygen species (Básaca-Loya, et al., 2009; Chamorro, 2002; Glazer, 1994).

Oxidative stress contributes to many pathological conditions and diseases, including cancer, hypertension, diabetes, and even death. Antioxidants can reduce and prevent damage from oxidative stress (Halliwell, 2015). Therefore, the aim of the present investigation was to study the antioxidant activity of phycobiliproteins from *Dermonema virens* (Rhodophyceae: Liagoraceae) on bioassays with lettuce seeds (*Lactuca sativa*).

Materials and methods

Collection of macrophyte

Dermonema virens (J. Agardh) Pedroche & Ávila Ortíz were collected from field populations in Playa Aragón, Puerto Ángel, Oaxaca. Plants were deposited inside sterile plastic bags and transported in ice to the laboratory, washed with distilled water to eliminate the sand adhered and keep them freezed.

Phycobiliprotein extract procedure

The phicobiliprotein extracts were obtained as follows: 3g of fresh *D. virens* biomass were homogenized in mortar and pestle with liquid N_2 until obtaining a soft plant mass. Distilled sterile water was added to this biomass (10 mL) and 0.5g streptomicin), mixed by inversion and left the stand for 24 hrs at -20ºC. The plant suspension was centrifuge at 3,000 xg at 10°C for 30 minutes; the supernatant was recovered and kept protected from light at 4ºC for the lettuce bioassays. This was performed with five samples and was mixed to use in the assays.

Spectroscopic analyses were carried out using a Shimadzu™ Spectrophotometer UV/Vis 2000 UV), reading at 650, 620 and 565 nm. Also, continuous spectra of absorbance (400–700 nm) were collected using a 1 cm quartz cuvette. The emission intensity signal for the extraction buffer was subtracted from the values obtained from each sample. Emission spectra were obtained for every 5 nm of the extraction buffer was used as reference for phycobilins. The amount of C-PC, C-PE and C-APC in the sample was calculated using simultaneous equations and the extinction coefficients as follows (Soni et al., 2006):

PC (phycocyanin) = {A620nm– 0.7(A650nm)}/7.38
AP (allophycocyanin) = {A650nm– 0.19(A620nm)}/5.65
PE (phycoerythrin) = {A565nm– 2.8 (PC) – 1.34(AP)}/12.7

ROS media bioactivity assay

Twenty commercial acquired seeds of *Lactuca sativa* L. were surface-sterilized with 10% sodium hypochlorite and then thoroughly rinsed with sterile distilled water and placed in Petri dishes with sterile Whatman Nº 3 paper, then 3mL of one of the concentrations of ROS media was added in each plate (Table 1). Petri dishes were sealed with plastic film to prevent water loss and incubated in dark at room temperature (22°C) for five days. All the experiments were performed by octuplicate and after incubation, the percent germination and length of radicles (primary root) and hypocotyl were recorded.

Table 1. Ascorbic acid and $CuSO_4$ in ROS media bioactivity assay.

Plate	Ascorbic acid (100mm)	$CuSO_4$ (10mm)	Water	Final volume
1	0.0 mL	0.0 mL	3.0 mL	3 mL
2	0.05 mL	0.2 mL	2.75 mL	3 mL
3	0.1 mL	0.2 mL	2.7 mL	3 mL
4	0.2 mL	0.2 mL	2.8 mL	3 mL
5	0.4 mL	0.2 mL	2.6 mL	3 mL
6	0.6 mL	0.2 mL	2.4 mL	3 mL
7	0.8 mL	0.2 mL	2.2 mL	3 mL
8	1.0 mL	0.2 mL	2.0 mL	3 mL
9	1.2 mL	0.2 mL	1.8 mL	3 mL
10	1.4 mL	0.2 mL	1.4 mL	3 mL
11	1.6 mL	0.2 mL	1.2 mL	3 mL
12	1.8 mL	0.2 mL	1.0 mL	3 mL

Antioxidant bioactivity of the phycobiliprotein extract

Twenty commercial acquired seeds of *Lactuca sativa* L. were surface-sterilized with 10% sodium hypochlorite and then thoroughly rinsed with sterile distilled water and placed in Petri dishes with sterile Whatman Nº 3

paper, then 3mL of distilled water (T-), other with 3mL ROS media like plate Nº 12 (Table 1) (T+), the third with 1.5mL of phycobiliprotein extract and 1.5 mL ROS media like plate Nº 12, finally the fourth with 3mL of phycobiliprotein extract. Petri dishes were sealed with plastic film to prevent water loss and incubated in dark at room temperature (22°C) for five days. All the experiments were performed by octuplicate and after incubation; the percent germination and length of radicles (primary root) and hypocotyls were recorded. The inhibition was assessed as follows:

$$Inhibition\% = 100[1-(ALP/ALC)]$$

Where, ALP is the average length of radicle or hypocotyl seedlings in extract (problem) and ALC is the average length of radicle or hypocotyl of seedlings in the control (cm).

All data obtained was analyzed by two-way analysis of variance using the statistics program Graph Pad Instat Ver. 2.03.

Results

Phycobiliprotein extract

Spectrophotometric data and concentration of each phycobiliprotein are shown in Table 2 and 3. The absorbance spectra exhibits one maximum at 495 nm, one at 557 NM and one at 615 (Fig. 1).

Table 2. Absorbance of phycobiliprotein extracts.

Extract	Absorbance		
	650nm	620nm	565nm
1	0.5231	0.7993	1.771
2	0.0802	0.1214	0.2936
3	0.1526	0.2728	0.7261
4	0.2126	0.279	0.488
5	0.3139	0.4051	0.6279

Table 3. Pigment concentration of extracts (mg/mL).

Extract	PE	PC	AP
1	1.83	0.749683	0.496221
3	0.3141	0.113793	0.076118
2	0.7603	0.258326	0.143426
4	0.5364	0.258835	0.143426
5	0.6917	0.375326	0.300277

ROS media bioactivity assay

The effects of ROS are shown in Table 4, in 12 plate the seeds germinated (the expansion growth of the embryo culminated in rupture of the covering layers and emergence of the radicle) but neither radicle or hypocotyl grew more than 0.5 cm, and they were too thin (Fig. 2).

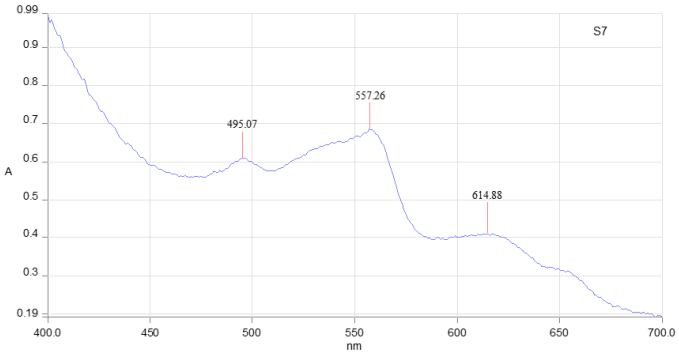

Fig. 1: Phycobiliprotein extract 5 spectrum absorption. Maximum at 557nm almost A=0.7.

Fig. 2: Seedlings in plate 12 (ROS media bioactivity assay results).

Antioxidant bioactivity of the phycobiliprotein extract assay

Fig. 3 shows four plates where the seeds were tested in water (A), oxidant conditions (B), phycobiliproteins extract plus oxidant conditions (C) and just phycobiliproteins extract (D). Seedlings in A show an average of 3.2 cm for radicles and 2.2 cm for hypocotyls. B seedlings show radicles and hypocotyles that didn't grow more than 0.5 cm, besides they were too thin and some showed signs of necrosis, curlings and malformations. C plates get an average of 1.32 cm for radicles and 1.73 cm for hypocotyls and seedlings in D showed an average of 1.5 cm for radicles and 1.6 cm for hypocotyls. Fig. 4 showed average size of radicle and hypocotyl for each condition (Table 5, Fig. 4).

Table 4. Average extension of radicle and hypocotyl (ROS media bioactivity assay results).

PLATE	Radicle average extension (cm)	Hypocotyl average extension (cm)	Radicle extension inhibition rate (%)	Hypocotyl extension inhibition rate (%)
1	2.2	1.6	31.25	27.27
2	1	1.21	67.81	45.00
3	1.11	1.17	65.31	46.82
4	1.28	1.34	60.00	39.09
5	1.21	1.38	62.19	37.27
6	0.88	1.32	72.50	40.00
7	0.95	1.01	70.31	54.09
8	0.88	1.05	72.50	52.27
9	0.72	0.97	77.50	55.91
10	0.5	0.6	84.38	72.73
11	0.5	0.6	84.38	72.73
12	0.35	0.0	89.06	100.00

Table 5. Average radicle and hypocotyl size (Antioxidant bioactivity of the phycobiliprotein extract assay).

	Radicle average extension (cm)	Hypocotyl average extension (cm)	Radicle extension inhibition rate (%)	Hypocotyl extension inhibition rate (%)
Water (Control)	3.148777778	2.044522222	---	---
ROS	0.563472222	0.275694444	82.10	86.51
PBP + ROS	1.5890625	1.66875	49.53	18.37
PBP	1.509272727	1.598181818	52.06	21.83

Table 6. ANOVA

Source of variation	Sum of squares (SS)	Degrees of freedom (df)	Mean Square (MS)	F	*p*	Critical value
Rows	561.0524631	225	2.493566503	6.11992056	9.98897E-38	1.245800069
Columns	22.17835206	1	22.17835206	**54.4319763**	3.07234E-12	3.883119873
Error	91.676429	225	0.407450796			
Total	674.9072441	451				

Fig. 3: A) Water plate (T-), B) ROS plate, C) ROS + PBP extract plate and D) PBP extract plate (Antioxidant bioactivity of the phycobiliprotein extract assay).

The statistic analysis results are showed in Table 6, F value is 54.431, so there are significant differences.

The algae must be taken from its habitat in a specific time of year, or phycobiliproteins concentration would vary in a significant degree because of the complementary chromatic adaptation. Talarico and Maranzana (2000) reports red algae have more phycoerythrin, phycocyanin and chlorophyll *a* when are exposed to higher light intensity; so they conclude phycobiliproteins protect from excessive radiation. Furthermore, *Dermonema virens*, a tropical species, lives at high tide level, so it is subject to long time lapses in high solar (and UV) irradiance.

The ROS media bioactivity assay with ascorbic acid and $CuSO_4$ results show that an auto-oxidation reaction occurs because transition metals like copper [Cu(II)] significantly increase ROS production (Cervantes-Cervantes et al., 2005), particularly hydroxyl radical, OH

(Troncoso and Guija, 1998), known to be the most biologically active free radical. By definition, radicals possess an unpaired electron, which makes them highly reactive and thereby able to damage all macromolecules (Fina, 2009), including lipids, proteins and nucleic acids, between the best known effects, there is the peroxidation of unsaturated fatty acids present in membrane phospholipids, best known as lipid peroxidation, that leads to damage to cellular membranes (plasma, mitochondrial and endomembrane systems). The most realistic *in vivo* production of hydroxyl radical according to Fenton reaction occurs when M^{n+} is iron, copper, chromium, cobalt and certain other metals (Valko et al., 2006), like the conditions used in this work to generate ROS.

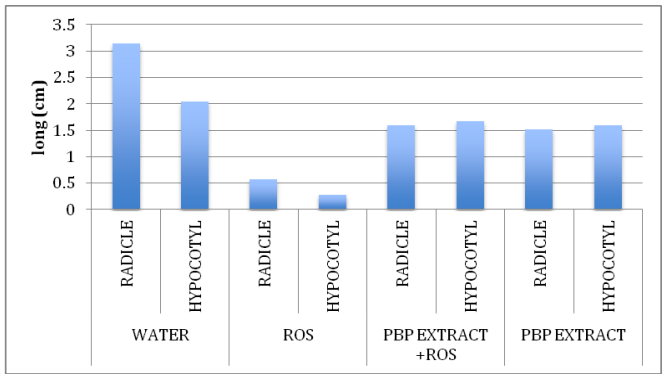

Fig. 4: Radicle and hypocotyl average extension (Antioxidant bioactivity of the phycobiliprotein extract assay).

This assay indirectly shows Fenton reaction efficiency: in oxidative conditions the germination was 21.2%, and damage of radicle was notorius (Fig. 3): less growth, necrosis, thinning, curling and malforming, like malformations in seedlings with potassium dichromate (Aportela and González, 2001).

Antioxidant bioactivity of the phycobiliprotein extract assay is accurate to evaluate effects of toxic conditions, and it is based on the height sensibility of seedlings to adverse environmental factors (Sobrero and Ronco, 2008). The results showed that phycobiliprotein extract can counteract the oxidative conditions (ascorbic acid and $CuSO_4$) of the seedlings, like Gallardo (2010) and Romay et al. (2001) found using different models (cell damage on mouse kidney and human erythrocytes, respectively.

Conclusion

Phycobiliproteins extract from *Dermonema virens* protected *Lactuca sativa* seedlings in an oxidative stress assay: germination rate and size of radicle and hypocotyl were normal, even surrounded by ROS.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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