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Extraction and determination of protein content and antioxidant properties of ten algae from Persian Gulf

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Abstract: There are a variety of algal species in Iran and little research has been carried out on their antioxidant activity. We tested the antioxidant properties of ten algae of Persian Gulf by determine the reducing power, NO radical scavenging, phenolic contents and inhibition lipid peroxidation. In order to evaluate the nutritional compounds of the algae, we determined the protein content of some Persian Gulf algae**.** *Cladophoropsis* sp*.* extract showed the highest reducing power (concentration 1.48±0.04 mg/ml with absorbance 0.5). *Gracilariopsis longssima* showed the highest NO radical scavenging ($p < 0.01$). Among the species studied, *Gracilaria salicornia* had the highest phenolic content (2.7±0.13 mg/g). After ten days the lipid peroxidation inhibition of *Cystoseira myrica*, *Cladophoropsis* sp. and *Colpomenia sinousa* extracts were 79.29%, 79.29% and 79.25% respectively, which higher than the antioxidant standards BHT (66.86%) and Vit C (64.06%). In present study, *Cladophoropsis* sp*.* had the highest protein content (2.49±0.113 g/100g). More studies should be carried out on its potential as a source of food.

Key Words: Algae, antioxidant properties, lipid peroxidation inhibition, protein

Introduction

Reactive oxygen species (ROS) like hydrogen peroxide, hydroxyl radicals and superoxide anions can be produced in living organisms by different metabolic reactions. ROS react with different oxidizable macromolecules compounds, such as proteins, lipids and

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lipoproteins (Lee *et al*., 2010) and induce damage to these macromolecules, which can lead to cancer, diabetes and inflammation. Antioxidant compounds can prevent the effects of free radicals and diseases induced by them. The human consumption of synthetic antioxidants such as butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA) has decreased because of their suspected role in carcinogenesis (Miranda *et al*., 1998). In Japan, algae are widely used as natural antioxidant. Also in France, brown seaweeds are allowed for human usage.

In Italy, specific algal species are consumed in diets. Some studies have shown that some algal species can prevent the damage of oxidant compounds and thus prevent cancer (Rastian *et al*., 2007). Since, marine algae possesses abundant of polysaccharides, proteins, vitamins and minerals, proof of algal antioxidant properties increases their importance in human diet (Nahas *et al*., 2007).

In Persian Gulf, there are many different species of algae but few studies have focused on their biological activities. Thus, our research investigated the antioxidant potentials of ten algae using different methods, such as NO radical scavenging activity, reducing power, determination of phenolic compounds and inhibition of lipid peroxidation. Also, in this study, the protein content of ten algae was detected, including *Botryocladia leptopoda*, *Cladophoropsis* sp., *Colpomenia sinousa*, *Cystoseria myrica*, *Gracilaria folifera*, *Gracilaria salicornia*, *Gracillariopsis longssima*, *Hypnea flagelliformis*, *Iyengaria stellata* and *Laurencia papillosa*.

Materials and methods

Ten algae were collected from different parts of the Persian Gulf: Qeshm Island, Bandar Abbas, Bandar Bushehr and Bandar Guatr in Oman Gulf (Tab. 1). Some reagents were purchased from Merck Chem. Co.

The algae were collected, identified and authenticated by Rabiei and Sohrabipour. The vouchers deposited in Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences.

Algae were washed to remove salts; these samples were frozen and lyophilized. Samples were extracted in ethanol 90% then concentrated using a rotary evaporator (30-40 ^{o}C). After the reduction of solvent, the reminder was evacuated by speed vacuum. In this condition, vacuum and fast rotation of the samples helped the evaporation of the solvent in the room temperature.

The reducing power of the algal extracts was evaluated according to the method of Moein *et al*. (2007). One ml of the extract was suspended in distilled water with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), to which 1% $K_3Fe(CN)_6$ was added. The mixture was incubated at 50 ^{o}C for 20 min and then 2.5 ml of trichloroacetic acid (TCA, 10%) was added. The

Tab. 1: Sampling information: Taxonomic status, gathering location, herbarium number of algae.

Phylum	Family	Scientific name	Date	Locality	Herb. number
Rhodophyta	Rhodymeniaceae	*Botryocladia leptopoda* (J.Agardh) Kylin	3.10.2008	Chahbahar-Guatr	556
Rhodophyta	Rhodomelaceae	*Laurencia papillosa* or *Palisada Perforata* (Bory de Saint-Vincent) K.WW. Nam	9.4.2008	Bandar Bushehr	558
Rhodophyta	Gracilariaceae	*Gracilaria foliifera* (Forsskal) Borgesen	5.3.2008	Bandar Abbaas	559
Rhodophyta	Gracilariaceae	*Gracilaria salicornia* (C.A gardh) E.Y. Dawson	9.4.2008	Qeshm	554
Rhodophyta	Gracilariaceae	*Gracilariopsis longssima* or *Gracilariopsis persica* A.M. Bellorin, J Sohrabipour. & E.C. oliveria	8.4.2008	Bandar Abbas	2217
Rhodophyta	Hypneaceae	*Hypnea flagelliformis* Greville ex J.Agardh	8.4.2008	Qeshm	2212
Phaeophyta	Scytosiphonaceae	*Iyengaria stellata* (Borgesen) Borgesen	10.3.2008	Bandar Moalam	552
Phaeophyta	Scytosiphonaceae	*Colpomenia sinousa* (Mertens ex Roth) Derbes & Solier	5.3.2008	Bandar Lengeh	555
Phaeophyta	Sargassaceae	*Cystoseria myrica* (S.G. Gmelin) Draima, Ballesteros, F. Rousseau & T. Thibaut	9.4.2008	Qeshm	579
Chlorophyta	Boodleaceae	*Cladophoropsis* sp.	9.4.2008	Qeshm	553

mixture was centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of 0.1% $FeCl_3$. Then the absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer (T80 plus, PG Instrument, UK). The higher the absorbance of the reaction mixture indicates more reducing power (Tab. 2).

For determination of NO radical scavenging, 50 µl of nitroprosside (10 mM nitroprosside in 20 mM phosphate buffer, PH = 7.4) was added to 50 µl of different concentrations of alga extract (12.5-3200 µg/ml) and the mixture was incubated for 150 min at 27°C. Then 100 µl Greiss solution was added and the absorbance was read at 542 nm. The blank contained algal extract and no reagent. The control contained 50 µl of nitroprosside, 50 µl methanol and 100 µl Greiss solution. The inhibition of NO radical was calculated as follow (Moein and Moein, 2010):

Tab. 2: Reducing power, phenolic contents and NO radical scavenging of algae.

Samples	Extract concentration with absorption 0.5 (µg/ml)	Total Phenolic content (mg/g)	NO radical scavenging (%)
Gallic acid	28.15±1.9 [a]	-	ND
BHT	0.21±0.001	-	ND
Botryocladia leptopoda	13.8±2.18[e]	0.11±0.001[g]	ND
Cladophoropsis sp	1.48±0.04[b]	2.19± 0.08[b]	19.5±3.3[a]
Colpomenia sinousa	3.07±0.09[cb]	0.07±0.01[e]	48.1±0.63[c]
Cystoseria myrica	3.46±0.8[cb]	1.42±0.05[c]	-1.26±0.3[d]
Gracilaria foliifera	5.07±0.6[cb]	0.56±0.03[fe]	6.05±0.45[e]
Gracilaria salicornia	3.45±1.5[cb]	2.7± 0.13[a]	32.95± 1[bf]
Gracilariopsis longssimas	6.05±1.04[cb]	0.43±0.003[f]	55.55±4.05[g]
Hypnea flagelliformis	7±0.8[dc]	1.08±0.03[d]	23.2±2.2[ab]
Iyengaria stellata	10.8±3.4[d]	0.13±0.003[g]	29.2±0.5[b]
Laurencia papillosa	21.19±3.4[d]	0.47±0.004[f]	16.7±1.7[a]

Values represent means of three replicates ± SD.

[a]The same alphabetic letters implied there are not any statistical differences P> 0.05 and different letters represented statistical differences P< 0.05. ND: non determined.

Inhibition: $A_0 - A / A_0 \times 100$

A_0*: absorbance of control*

A: absorbance of test - absorbance of blank

Determination of total phenolic content was determined according to the method of Moein *et al.* (2010). Briefly; 0.5 ml of the algal extract (10g/l) was mixed with 2.5 ml of Folin - Ciocalteu (Sigma, ST. Louis, MO) (diluted ten - fold) and 2 ml (75 g/l) sodium carbonate. The mixture was incubated for 1 h at room temperature. The absorbance was measured at 760 nm (T80 plus, PG Instrument, UK) and converted to phenolic contents (Tab. 3) according to the calibration curve of gallic acid (Sigma, ST. Louis, MO).

The ferric thiocyanate (FTC) assay (Natrah *et al.*, 2007) was used to measure the amount of peroxides formed during lipid oxidation. Methanol extracts of algae (4 mg) were dissolved in 4 ml 99.5% methanol. Distilled water (3.9 ml) was mixed with 8 ml phosphate buffer solution and 4.1 ml (2.5%) linoleic acid.

The samples were kept in screw-capped vials and placed in an oven at 40 ^{o}C in the dark. Starting from first day to the final stage of the assay, 0.1 ml of 30% aqueous ammonium thiocyanate were added. After that, 0.1 ml of 0.02 M ferrous chloride ($FeCl_2$, $4H_2O$) in 3.5 % hydrochloric acid was added to the reaction mixture. Precisely, 3 min after the addition of hypochloric acid, the absorbance was measured at 500 nm. The measurement was taken every

Tab. 3: Amounts of algae, extraction efficiency and protein contents of algae.

Samples	Amounts of algae	(%) extraction efficiency	Protein contents ± SD g/100g
Botryocladia leptopoda	100	28.3	Not detected
Cladophoropsis sp.	50	3.76	2.5±0.113[a]
Colpomenia sinousa	50	8.04	2.05±0.113[b]
Cystoseira myrica	100	3	1.02±0.04[dc]
Gracilaria foliifera	100	10.23	0.055±0.043[e]
Gracilaria salicornia	100	2.07	0.053±0.002[e]
Gracilariopsis longssima	100	10.7	0.19±0.012[e]
Hypnea flagelliformis	100	5.02	1.92±0.13[b]
Iyengaria stellate	100	13.38	1.27±0.15[cd]
Laurencia papillosa	100	8.67	Not detected

Values represent means of three replicates ± SD.

[a]The same alphabetic letters implied there are not any statistical differences P> 0.05 and different letters represented statistical differences P< 0.05.

24 h for 10 days; the maximum absorbance of the tests was recorded.

For protein extraction, 50 mg of algal extract was mixed with 4 ml of distilled water and kept in a refrigerator for 15 h, at 4 ^{o}C. Then we homogenized it for 5 min, and added 4 ml of distilled water. The sample was centrifuged for 30 min at 4 ^{o}C and the supernatant was collected. For precipitation, 1 ml of 0.1 N NaOH was added and mixed it for 1 h at room temperature (25 ^{o}C).

The mixture was centrifuged for 20 min at room temperature (15000 rpm). The supernatant was collected and added to the previous supernatant. In this way, 9 ml of algal extract for protein determination was collected (Marrion *et al*., 2005; Barbarino and Laurenco, 2005).

Precipitation of proteins was done as follows: trichloroacetic acid 25% (TCA: homogenate, 2.5:1 V/V, at 4 ^{o}C) was added to the algal extract. The sample was kept on ice for 30 min then centrifuged for 20 min in 15000 rpm at 4^{o}C. The supernatant was removed. To wash the precipitate, 5 ml of TCA 10 % was added and centrifuged for 2 min in 15000 rpm. The supernatant was removed and the precipitated protein was suspended by 5 % TCA. The sample was centrifuged for 20 min in 15000 rpm at 20 ^{o}C and the supernatant was removed. The total protein content of the precipitate was determined (Marrion *et al*., 2005; Barbarino and Laurenco, 2005): two ml of sodium hydroxide 0.1 N was added to the precipitate and shaker. To one ml of this mixture, 5 ml of reagent C (1 ml reagent A+ 50 ml reagent B) was added. Reagent A contains (Na_2CO_3 2%+ NaOH 0.1 N) and reagent B

contains ($CuSO_4$ 5%, $5H_2O$+ 1% $C_4H_4Na\ KO_6$, $4H_2O$). Then 0.5 ml of diluted (1 :2) Folin - Ciocaltu was added. After 30 min, the absorbance was read at 750 nm in a spectrophotometer (T80 plus, PG Instrument, UK).

In this test, Bovine Serum Albumin (BSA) was used as standard in 0.5-2 mg/ml. The curve standard for BSA was plotted and algal protein content was determined on the BSA standard curve (Lowry *et al.*, 1951). All the experiments were repeated three times.

Data were expressed as mean± SD. The concentration of the algal extract required to scavenge 50% of the free radicals (IC_{50}) was calculated by using Curve Graph pad instat 3. The data were analyzed for statistical significance using one way ANOVA followed by Tukey post test. P value less than 0.05 was considered significant.

Results

The scientific names, gathering location, herbarium number, phyla and families of algae are shown in Table 1.

The amount of algae, extraction efficiency and protein content of algae are presented in Table 3. The highest amount of protein was found in *Cladophoropsis* sp. (2.5±0.113 g/100g, P < 0.001).

The concentrations of algal extracts in 12.5-3200 µg/ml were evaluated for reducing power. For comparison, the reducing power of the extracts, the concentration which showed absorption of 0.5 was determined (Tab. 2). The decrease in reducing power as follow: Gallic acid > *Cladophoropsis* sp > *Colpomenia sinousa* > *Cystoseria myrica* > *Gracilaria salicornia* > *Gracilaria folifera* > *Gracillariopsis longssima* > *Hypnea flagelliformis* > *Iyengaria stellata* > *Botryocladia leptopoda* > *Laurencia papillosa*. NO radical scavenging of algal extracts is shown in Table 2. The greatest percentage of NO radical scavenging was found in *Gracillariopsis longssima* (55.55±4.05%, P< 0.001) extract. *Gracilaria salicornia* possessed the highest phenolic compounds (2.7±0.13 mg/g, P < 0.05).

The reducing power of algae is elucidated in Figure 1. The relationship between reducing power and phenolic compounds is shown in Figure 2.

The decrease in phenolic compounds as follow: *Gracilaria salicornia* > *Cladophoropsis* sp > *Cystoseria myrica* > *Hypnea flagelliformis* > *Colpomenia sinousa* > *Gracilaria folifera* > *Laurencia papillosa* > *Gracillariopsis longssima* > *Botryocladia leptopoda* ≥ *Iyengaria stellate*.

Lipid peroxidation inhibition of *Cystoseria myrica*, *Cladophoropsis* sp. and *Colpomenia sinousa* extracts were higher than the antioxidant standards and other algae (Fig. 3). However, there were no significant differences in inhibition of lipid peroxidation, between *Cystoseria myrica*, *Cladophoropsis* sp. and *Colpomenia sinousa* extracts (P > 0.05).

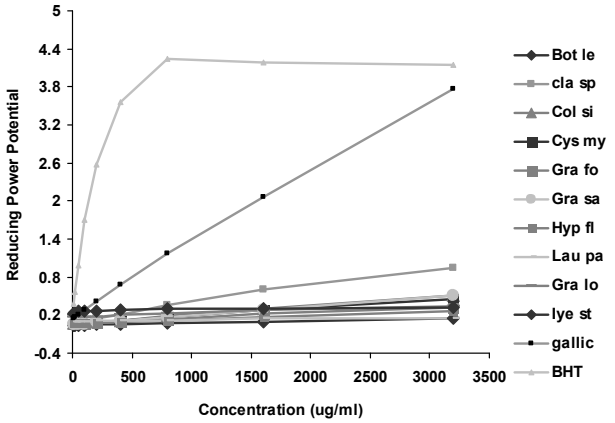

Fig. 1: Reducing power of algal extracts compared with standards gallic acid and BHT standards. Gallic acid was diluted 1:10.

Botryocladia leptopoda (*Bot le*), *Cladophoropsis sp* (*Cla sp*), *Colpomenia sinousa* (*Col si*), *Cystoseria myrica* (*Cys my*), *Gracilaria folifera* (*Gra fo*), *Gracilaria salicornia* (*Gra sa*), *Hypnea flagelliformis* (*Hyp fl*), *Laurencia papillosa* (*Lau pa*), *Gracillariopsis longssima* (*Gra lo*), *Iyengaria stellata* (*Iye st*).

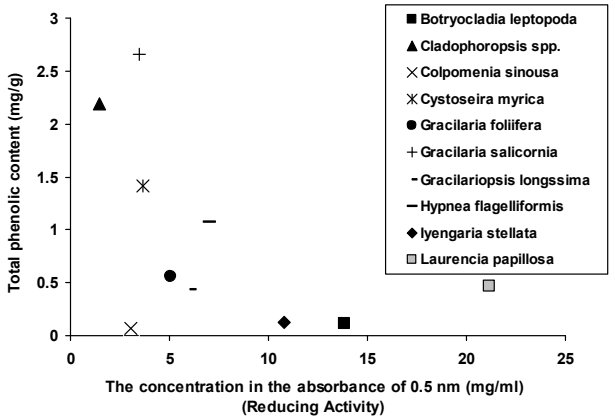

Fig. 2: Relationship between absorbance 0.5 in reducing power and total phenols of algal extracts.

Iyengaria stellata and *Botryocladia leptopoda* extracts were inactive in inhibition of lipid peroxidation. In the fourth day, the percentage of lipid peroxidation inhibition of *Gracilaria folifera, Gracilaria salicornia* and *Gracillariopsis longssima* extracts were higher than the antioxidant standards. After the fourth day, this property of these algae was decreased (Figs. 3, 4).

Discussion

Marine algae have long been used as nutrients and medicines in Asian countries like Japan, China and Korea (Fitton, 2003). Also usage brown algae reduce some inflammatory disorders, such as breast cancer and high cholesterol level (Fitton, 2003). Khanavi *et al*. (2010) reported that *Colpomenia sinousa* extract does not possess cytotoxic effects, but *Cystoseria myrica* extract possesses cytotoxic effects. In other research, the antioxidant activities of algae evaluated (Bhuvaneswari *et al*., 2013) using DPPH radical scavenging and determination phenolic compounds. Also, our research, determined the antioxidant potentials of ten algae from Persian Gulf.

The reducing potential of the extract may show antioxidant activity by measuring the reduction of Fe^{3+} to Fe^{2+} (Duan *et al*., 2006). Compounds with reducing power potential donate electrons and can reduce the oxidized intermediates in lipid peroxidation process, so that they can function as primary and secondary antioxidants (Haung *et al*., 2005).

Cladophoropsis sp. extract showed the most reducing power ability (in 1.48±0.04 mg/ml with absorbance 0.5, Tab. 3) and this property was significantly lower than the

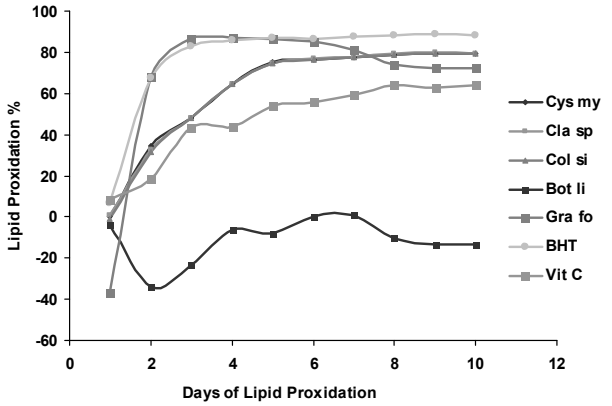

Fig. 3: Inhibition of lipid peroxidation by algal extracts for ten days in comparison with BHT and Vit C (antioxidant standards).

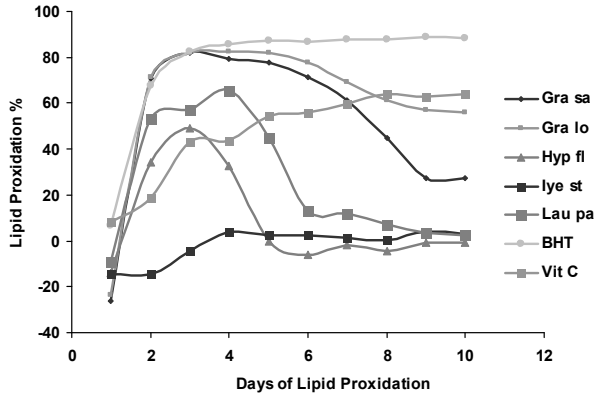

Fig. 4: Inhibition of lipid peroxidation by algal extracts for ten days in comparison with BHT and Vit C (antioxidant standards).

antioxidant standards of gallic acid and BHT ($P<0.05$). These differences in reducing potential may be induced from the variable amounts of L-ascorbate and GSH (Haung *et al*., 2005).

Phenolic compounds in plants are well known and these groups of compounds possess antioxidant activity in biological systems (Fridovich, 1995). Folin-Ciocalteu method, which is used for determination total phenols, usually measures reducing power property. Actually, some studies have shown a correlation between the total phenols assay by Folin-Ciocalteu reagent and an electron transfer-based on AOA assay (ferric ion reducing antioxidant assay, Kelman *et al*., 2010).

In our study*, Gracilaria salicornia* and *Cladophoropsis* sp extracts possessed the highest reducing power and phenolic compounds. In this case, a correlation is found between these two variables (Fig. 2). Fe^{+2} can start (Lee *et al*., 2010) lipid peroxidation by a Fenton reaction, as well as enhance peroxidetion by dissociation of lipid hydroperoxides into peroxyl and alkoxyl radicals (Sabahi *et al*., 2013)

In inhibition of lipid peroxidation *Cystoseria myrica*, *Cladophoropsis* sp. and *Colpomenia sinousa* extracts showed more antioxidant activities. The data showed that there is not any significant difference between the percentages of lipid peroxidation by these three extracts ($P > 0.05$, Fig. 3) and this property of these algae was higher than the standards Vit C and BHT ($P< 0.001$, Fig. 3). Similarly, some microalgae have been reported to possess more antioxidant activity than the synthetic antioxidant BHT (Natrah *et al*., 2007). In another study, the ethyl acetate fraction of two green microalgae, *Halochlorococcum*

porphyrae and the chloroform fraction of *Oltamann siellopsis* were similar to alpha-tocopherol in inhibition of lipid peroxidation (Lee *et al*., 2010). For determination of protein, the Lowry method which is more sensitive than the Bradford method and more precisely determines protein contents (Barbarino *et al*., 2005) was used. In this method, the protein content is determined from BSA standard curve.

The amounts of proteins in decreasing order are *Cladophoropsis* sp.> *Colpomenia sinousa* > *Hypnea flagelliformis* > *Iyengaria stellata* > *Cystoseria myrica* > *Gracillariopsis longssima* > *Gracilaria folifera* > *Gracilaria salicornia*. *Cladophoropsis* sp possessed the highest amount of protein (2.49 ± 0.11g/100g, $p<0.001$, Tab. 3). *Botryocladia leptopoda* and L. pap. llos possessed the lowest amount of proteins.

In our study, *Gracillariopsis longssima* possesses the highest NO radical scavenging (55.55± 4.05) which is more than BHT as an antioxidant standard (42± 0.79%), Table 2. Also, in other research, it is reported that the ethyl acetate fraction of *Halochlorococcum porphyrae* extract and the methanol extract of *Oltamannsiellopsis unicellularis* have more NO radical scavenging effects than antioxidant standards (Lee *et al*., 2010).

In lipid peroxidation, formation of the Ferrozine-Fe^{2+} complex is inhibited by algal extracts, which indicates significant chelating capacity. In the present study, two algal extracts (*Botryocladia leptopoda, Iyengaria stellata*) could not inhibit lipid peroxidation (Fig. 3, 4). Perhaps these two extracts possessed prooxidant properties.

In our study, *Cystoseria myrica* extract showed the highest antioxidant properties. Similarly, brown algae had the highest antioxidant activity among Hawaiian algae tested by Kelman *et al*. (2010). Some of the Persian Gulf algae tested might be useful as a substitute for synthetic antioxidants or as additives to prevent food deterioration.

Acknowledgment

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