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Original Article

FREE RADICAL SCAVENGING AND ANTIOXIDANT PROPERTIES OF MARINE RED ALGAE *HYPNEA MUSCIFORMIS*

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ABSTRACT

Objective: The study focused on determining the antioxidant properties of the *Hypnea musciformis* methanol crude extracts, from the red algae.

Methods: The evaluation of antioxidant properties was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis 3ethylbenzthiazoline-6-sulphonic acid (ABTS), ferric reducing antioxidant power (FRAP), nitric oxide scavenging assay (NO), reducing power, superoxide radical scavenging (SOD), hydroxyl and hydrogen peroxide radical assay, the metal chelating activity as well as phosphomolypdenum assay.

Results: Among the tested red algae, the maximum antioxidant activity was recorded in the methanol extract of *Hypnea musciformis*. Whereas methanolic crude extract of red algae and diatom showed good antioxidant potential.

Conclusion: This study suggests that methanolic crude extracts contain different potential antioxidant compounds capable to scavenge different types of free radicals.

Keywords: *Hypnea musciformis*, Free radical scavenging, Antioxidant activity.

INTRODUCTION

It is known that algae are part of the diet of Asian countries such as China and Japan since ancient times [1]. In these locations, there is evidence that the incidence of some types of cancer, such as breast and prostate cancers, is lower due to regular consumption of seaweeds, the main reason being their richness in bioactive compounds that may protect against those diseases [2, 3]. Natural antioxidants with multifunctional potential area of high interest as alternatives for synthetic antioxidants to prevent oxidation in complex food systems like muscle food. Aquatic plants are also gaining interest as a potential source of antioxidants. Algae are grouped into two main categories; the green algae, found in both benthic and littoral habitats and also throughout the ocean waters as phytoplankton, and the green algae or seaweeds, which occupy the littoral zone, and can be classified as red (Rhodophyta), brown (Phaeophyta) or green (Chlorophyta), depending on their nutrient and chemical composition [4, 5].

Many synthetic antioxidants, such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and tert-butyl hydroxy quinone (TBHQ) have been widely used in different food products. However, because of the potential health hazards, their use as food additives is under strict regulation in many countries. Marine algal extracts has also been demonstrated to have strong antioxidant properties [6, 7]. The Phaeophyta (brown seaweeds) shows comparatively higher antioxidant activity than green and red algae [8].

There are 18 species of red seaweeds belonging to 13 families and 6 orders. Family hypneaceae contains a species the *Hypnea musciformis*, whose plants are bushy, spreading, cylindrical, 10-30 cm high, purplish green in color, cartilaginous, much branched, branches irregular, giving a bushy look to the plant [9]. Preliminary pharmacological investigation of the algae belonging to the genus Dictyota revealed its content of considerable antibacterial, antifungal, antiviral [10], antimicrobial, antineoplastic, antifungal and cytotoxic activities [11, 12]. There are reports of antispasmodic an activity of *Hypnea musciformis* [13], antibacterial [14], anti genotoxic and anti-oxidative capacity [15], Anti inflammatory [16]. Therefore, the present investigation was attempting to study the antioxidant properties of methanolic crude extracts of marine diatom green algae the *Hypnea musciformis*.

MATERIALS AND METHODS

Chemicals

DPPH, ABTs and FRAP were obtained from Sigma Aldrich (Steinheim, Germany). Methanol was of HPLC grade (Lab-Scan, Dublin, Ireland). All the other reagents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Algal materials

Hypnea musciformis red algae were collected from the Rameswaram area on January 5th, 2015. The freshly collected seaweeds were washed with clean seawater to remove salt, epiphytes and sand attached to the surfaces of the samples and transported to the laboratory. The samples were carefully rinsed with tap water, wiped with paper towel. For *Hypnea musciformis* the steps and heaters were removed and the new and old parts of the blades were separated. The samples were lyophilized for 72 h, pulverized into powder and stored at 80 °C prior to extraction.

Preparation of sample extract

5 g of *Hypnea musciformis* powdered seaweed was extracted overnight with 100 ml methanol at room temperature and centrifuged at 2800 rpm for 10 min. The supernatant was collected in a separate bottle after passing through a filter paper and the residue was re-extracted three times under the same conditions as mentioned above. The combined extracts were frozen, dried. These extracts were kept at 80 °C until analysis. The freeze dried extracts were re dissolved in methanol and used for the analysis.

DPPH radical scavenging activity

Various concentrations of *Hypnea musciformis* of the sample (4.0 ml) were mixed with 1.0 ml of methanol solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM [17]. The mixture was shaken vigorously and left to stand for 30 minutes and the absorbance was measured at 517 nm. BHT was used as a control. The percentage of DPPH decolorization of the sample was calculated according to the equation

DPPH radical scavenging activity (%) = $[(A\ control - A_{test})/A_{control}] \times 100$

IC_{50} values (mg extract/ml) were the inhibitory concentration at which DPPH radicals were scavenged by 50%. BHT was used for comparison.

$ABTS^{+}$scavenging activity

Samples were diluted to produce 5-50 µg/ml. The reaction was initiated by the addition of 1.0 ml of diluted $ABTS^{+}$to 10 ml of different concentrations of *Hypnea musciformis* of the sample or 10 m l methanol as control [18]. The absorbance was read at 734 nm and the percentage inhibition was calculated. The inhibition was calculated according to the equation

$$I = A_{test}/A_{control} \times 100$$

Where A is the absorbance of control reaction and absorbance of test compound

Ferric-reducing antioxidant power assay (FRAP)

A stock solution of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL, 20Mm $FeCl_3.6H_2O$ and 0.3M acetate buffer (pH 3.6) was prepared [19]. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution, and 25 ml acetate buffer. It was freshly prepared and warmed to 37°C. FRAP reagent (900 ml) was mixed with 90 ml water and 30 ml *Hypnea musciformis* of the sample and standard antioxidant solution. The reaction mixture was then incubated at 37 °C for 30 minutes and the absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex was reduced to ferrous (Fe^{2+}) form. The absorption at 540 nm was recorded

Nitric oxide radical activity

Nitric oxide radical generated from sodium nitroprusside was measured [20]. Briefly, the reaction mixture (5.0 ml) containing sodium nitroprusside (5 mM) in phosphate-buffered saline (pH 7.3), with *Hypnea musciformis* sample at different concentration was incubated at 25 °C for 3 hours. The nitric oxide radical thus generated interacted with oxygen to produce the nitrite ion which was assayed at 30 minute intervals by mixing 1.0 ml of the incubation mixture with an equal amount of Griess reagent. The absorbance of the chromophore (purple azo dye) formed during the digitization of nitrite ions with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 546 nm.

Reducing power assay

The reducing power was determined as described by Yen and Chen [21]. Briefly, 0.13 ml of *Hypnea musciformis* different concentration (5-50 µg/ml) in phosphate buffer (0.2 M, pH 6.6) were mixed with 0.125 ml of potassium ferricyanide (1%, w/v) and incubated at 50 °C for 20 min. Afterwards, 0.125 ml of TCA (10%, w/v) was added to the mixture to terminate the reaction. Then, the solution was mixed with 1.5 ml ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm.

Hydroxy radical activity

The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate, and varying concentrations of *Hypnea musciformis* sample [22]. After incubation for 1 hour at 37 °C, the absence of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as Scavenging activity

$$= [1-(A_1-A_2)/A_0] \times 100$$

Where A_0 was the observance of the control (without extract), A_1 was the observance in the presence of the extract, and A_2 was the observance without sodium salicylate.

Superoxide anion radical scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of nicotinamide adenine dinucleotide (NADH) and phenazine methosulfate (PMS) under aerobic condition [23]. The 3 ml reaction mixture contained 50 ml of 1M NBT, 150 ml of 1M NADH with or without sample, and Tris buffer (0.02M, pH 8.0). The reaction was started by adding 15 ml of 1M PMS to the mixture and the absorbance change was recorded at 560 nm after 2 minutes. Percent inhibition was calculated against a control without the extract

Hydrogen peroxide radical

Hypnea musciformis against H_2O_2 was measured according to the method [24]. A solution of 40 Mm H_2O_2 was prepared in phosphate buffer (p^H-7.4). Next, 1.4 ml of different concentrations (5-50 µg/ml) of the *Hypnea musciformis* was added to 0.6 ml of the H_2O_2 solution. The assay mixture was allowed to stand for 10 minutes at 25 °C and the absorbance measured against a blank solution at λ max =230 nm. The *Hypnea musciformis* on the H_2O_2 scavenging capacity index was calculated as follows:

$$= \frac{A_{Blank} - A_{Test} \times 100}{A_{Blank}}$$

Hypnea musciformis was expressed as IC_{50}, which is defined as the concentration (mg/ml) of the *Hypnea musciformis* required to scavenge 50 % of H_2O_2. BHT was used as a control.

Metal ion chelating activity

The reaction mixture contained 1.0 ml of various concentrations of the *Hypnea musciformis* sample, 0.1 ml of 2 mM $FeCl_2$, and 3.7 ml methanol [25]. The control contained all the reaction reagents except the sample. The reaction was initiated by the addition of 2.0 ml of 5 mM frozen.

After 10 minutes at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher iron chelating ability. The capacity to collate the ferrous ion was calculated by

$$\% \text{ chelation} = [1-(ABS_{sample}/ABS_{control})] \times 100.$$

Phosphomolybdenum assay

The phosphomolybdenum assay used for determining the antioxidant capacity is based on the reduction of M_0 (VI)–M_0 (V) by the antioxidants and subsequent formation of a green phosphate/M_0 (V) complex at acid pH. 0.3 ml of *Hypnea musciformis* sample is taken in a tube and mixed with 3 ml of reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and incubated at 95 °C for 90 min. BHT is utilized as a reference standard. The absorbance of the mixture is then measured at 695 nm with methanol blank. The antioxidant activity is expressed as the number of gram equivalents of BHT [26].

Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean±standard deviation. The results were analyzed using one-way analysis of variance and the group means were compared using Duncan's multiple range tests using SPSS version 16.

RESULTS AND DISCUSSION

DPPH have been used extensively as a free radical to evaluate reducing substances [27]. This purple color generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colorless/bleached product (i.e., DPPH or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm. Hence, the more rapidly the absorbance decreases, the more potent antioxidant activity of the extract.

The DPPH radical assay is a suitable model for estimating radical scavenging activities of antioxidants. *Hypnea musciformis* exhibited a significant dose dependent inhibition of DPPH activity. This had a lesser activity than the standard of butylated hydroxy toluene. The results are presented in Fig.1 the IC_{50} value of butylated hydroxy toluene and *Hypnea musciformis* was 35.58 µg/ml, 39.31 µg/ml, respectively.

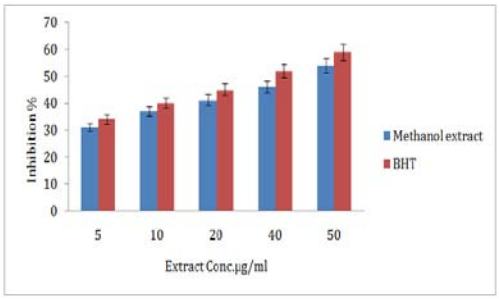

Fig. 1: DPPH radical scavenging activity of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3)

ABTS assay is a simple indirect method for determining the activity of natural antioxidants. In the absence of phenolics, ABTS radical is rather stable, but it reacts energetically with an H-atom donor, such as phenolics, been converted into a non-colored form of ABTS [28].

The ABTS radical cation-scavenging assay performed showed that the antioxidant activity increases with an increase in the concentration. The inhibition capacity of the radical $ABTS^{+}$and the BHT% values, expressed in *Hypnea musciformis*, for the different concentration was presented in fig. 2. The results show that *Hypnea musciformis* presented the highest BHT% behavior, with values of 42.26µg/ml and 35.58 µg/ml, respectively.

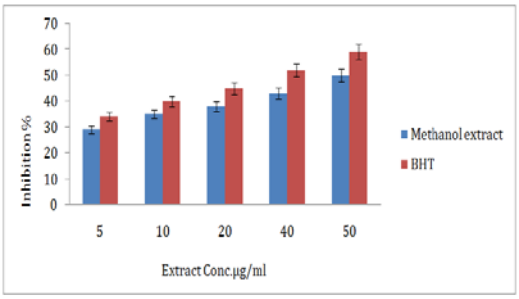

Fig. 2: ABTS radical scavenging ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3).

Ferric-reducing power is an important indicator of the antioxidant potential of a compound or an extract [29]. The ability to reduce ferric ions indicates that the antioxidant compounds are electron donors and could reduce the oxidized intermediate of lipid peroxidation processes, thus acting as primary and secondary antioxidants [30,31]. The antioxidant activity of the methanolic crude extract determined by FRAP assay varied as seen in Fig.3. The reducing power was found to be higher in methanolic extract. At a concentration of 37.23 µg/ml of *Hypnea musciformis* 50% of FRAP generated by incubation was scavenged. The IC_{50} value of BHT was 35.58 µg/ml.

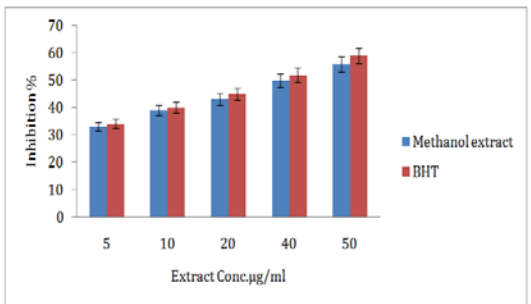

Fig. 3: FRAP radical scavenging ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3)

Seaweeds inhibit nitrite formation by competing with oxygen to react with nitric oxide directly. These compounds alter the structure and function of many cellular components. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this damage [32]. The nitric oxide radical scavenging assay was also performed with methanolic crude extract of the seaweed samples. The scavenging of nitric oxide by *Hypnea musciformis* was increased in a dose-dependent manner as illustrated in Fig.4. At a concentration of 38.11 µg/ml of *Hypnea musciformis* 50% of nitric oxide generated by incubation was scavenged. The IC_{50} value of BHT was 35.58 µg/ml.

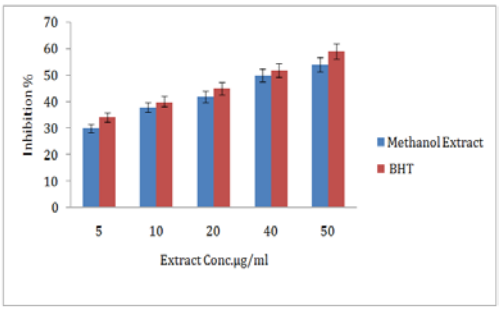

Fig. 4: Nitric oxide radical scavenging ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3)

Reducing capacity is considered as a significant indicator of potential antioxidant activity of a compound or sample [33]. The presence of reductants (i.e. Antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 655 nm, the amount of Fe^{2+}can be monitored. Higher absorbance indicated higher reducing power [34]. Fig.5 elucidated the reduced capabilities of *Hypnea musciformis* compared to BHT. The reducing power of Hypnea musciformis was increased in quantity of sample. *Hypnea musciformis* could reduce the most Fe^{3+}ions, which had a lesser reductive activity than the standard of BHT. The IC_{50} value of *Hypnea musciformis* and BHT was 35.58 µg/ml and 37.28µg/ml respectively.

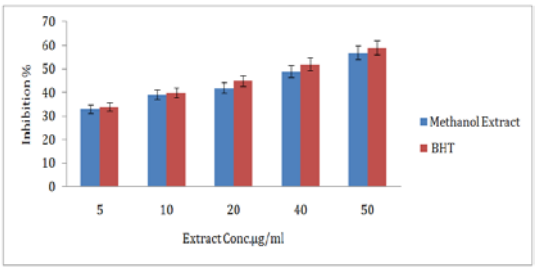

Fig. 5: Reducing power radical scavenging ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3).

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated after the oxygen is taken into living cells. Superoxide anion changes to other harmful ROS and free radicals such as hydrogen peroxide and hydroxyl radical, which induce oxidative damage [35]. The decrease the absorbance at 560 nm with the *Hypnea musciformis* thus indicates the consumption of superoxide anion in the reaction mixture. The antioxidant activity of the methanol crude extract determined by Superoxide anion radical assay varied as seen in Fig.6. The reducing power was found to be higher in methanol extract. At a concentration of 38.05 µg/ml of *Hypnea musciformis* 50% of Superoxide anion radical generated by incubation was scavenged. The IC_{50} value of BHT was 35.58 µg/ml, respectively.

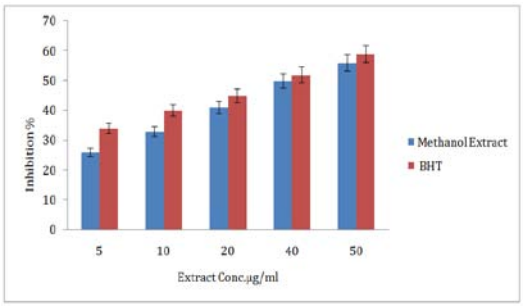

Fig. 6: Superoxide anion radical scavenging ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3).

The hydroxyl radical is the major active oxygen, causing lipid peroxidation in enormous biological damage. The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipid and protein [36, 37]. In this study, *Hypnea musciformis* were found to scavenge O2 significantly and in a dose dependent manner and may protect the DNA, protein and lipid from damage. The results for hydroxyl scavenging assay are shown in Fig.7. The concentrations for 50% inhibition was found to be 37.93 and 35.58 µg/ml for the *Hypnea musciformis* and BHT respectively.

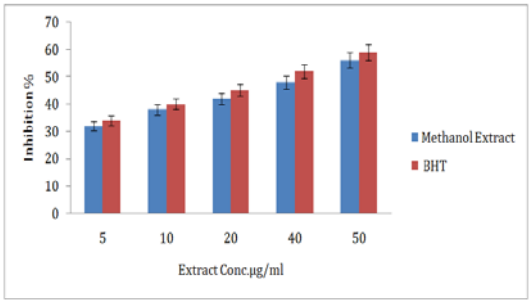

Fig. 7: Hydroxy radical scavenging ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3).

Many species of seaweed possess scavenging ability of hydrogen peroxide [38]. It can cross membranes and may slowly oxidize a number of compounds. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of the rise in the hydroxyl radicals in the cells. The H_2O_2 radical scavenging assay was also performed with the methanolic crude extract of the seaweed samples. From the results, *Hypnea musciformis* showed concentration dependent activity and the H_2O_2 scavenging effect at a concentration was 39.25µg/ml. This activity was comparable to the scavenging effect on the concentration of BHT 35.58 µg/ml fig. 8.

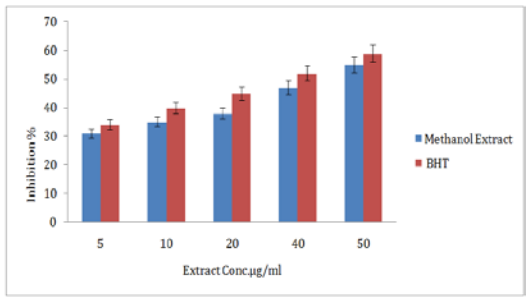

Fig. 8: Hydrogen peroxide radical scavenging ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3).

The metal iron chelating ability of seaweeds may be attributed to the presence of endogenous chelating agents, mainly phenolics because certain phenolic compounds properly orient functional groups, which can chelate metal ions [39]. Ferrozine can cognitively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. The metal chelating activity of *Hypnea musciformis* was 37.96 µg/ml. This was comparable to the scavenging effect on the concentration of BHT was 35.58 µg/ml. fig. 9.

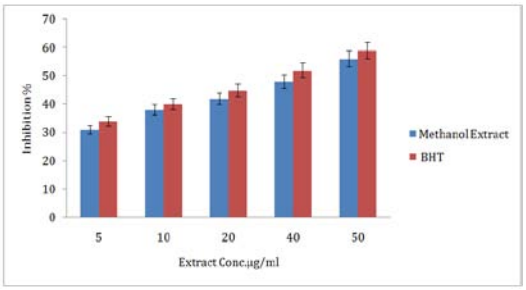

Fig. 9: Metal chelating ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3).

Phosphomolybdenum assay, which is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity), the extract demonstrated high electron-donating capacity showing its ability to act as chain terminators, transforming relative free radical species into more stable non-reactive products [40,41]. In the ranking of the antioxidant capacity obtained by this method, in Fig.10. This revealed that increased phosphomolybdenum reduction of *Hypnea musciformis* to the quantity of the sample. The IC_{50} value of Hypnea musciformis was 41.05µg/ml and 35.58 µg/ml as a standard BHT.

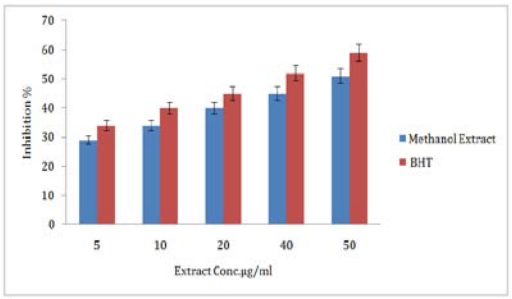

Fig. 10: Phosphomolybdenum radical scavenging ability of *Hypnea musciformis*

Each value is expressed as mean±standard deviation (n=3).

CONCLUSION

It can be concluded that seaweeds selected in the present study can be utilized as a source of natural antioxidant compounds as their methanolic crude extracts exhibits good antioxidant activity. Bioactive compounds found in seaweeds await a major breakthrough for a variety of food/medical applications as they have the potential for application as natural antioxidants in different food/pharmaceutical products.

CONFLICT OF INTERESTS

Declared None.

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