ARTÍCULO ORIGINAL

Protective effects against FeCl₃-Induced oxidative stress in Vero cells related to the polyphenol content of the hydrophilic fractions from Halimeda spp seaweed

Efecto protector de fracciones hidrofílicas de algas marinas del género Halimeda contra el estrés oxidativo en células Vero insultadas con FeCl₃ en relación con su contenido de polifenoles

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ABSTRACT
Seaweeds are a source of natural antioxidants having potential application in oxidative stress related diseases. And then, the aim of this paper was to evaluate the antioxidant properties of the seaweeds Halimeda monile, Halimeda opuntia and Halimeda incrassata, in an in vitro alternative model of FeCl₃-Induced oxidative stress in Vero cells and their relation to the content of polyphenolic compounds. The cytotoxicity of an aqueous extract from Halimeda spp was determined by MTT assays. The antioxidant properties from Halimeda spp were investigated through an in vitro alternative model of FeCl₃-Induced oxidative stress in Vero cells and the protection was determined by the cell’s survival. The phenolic compounds were estimated by using the Folin-Ciocalteu Method. The total polyphenolic content of the aqueous extracts of Halimeda monile, Halimeda opuntia and Halimeda incrassata were 5.07 ± 0.06, 4.39 ± 0.10 and 9.11± 0.11 mg of Gallic Acid Equivalent/g dry seaweed, respectively. The cytotoxicity of the genus Halimeda seaweed for Vero cells resulted in a very low reading. On the other hand, the Halimeda aqueous extracts showed a protective effect against FeCl₃-Induced oxidative stress in Vero cells to the order of 2 mg for H. incrassata and 4 mg for H. opuntia and H. monile. In this work, it was conclusively shown that the Halimeda spp seaweed was able to protect the Vero cells from oxidative stress induced by FeCl₃, together with associated effects, at least in part, by their content of polyphenolic compounds.

Keywords: Antioxidant activity, Vero cells, seaweeds, phenolics

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RESUMEN

En la actualidad las algas marinas se consideran como una fuente de antioxidantes naturales con significativo potencial en el estrés oxidativo y en enfermedades relacionadas. El objetivo de esta investigación fue evaluar las propiedades antioxidantes de las algas marinas Halimeda monile, Halimeda opuntia y Halimeda incrassata en un modelo alternativo in vitro de estrés oxidativo inducido por FeCl₃ en células Vero y su relación con el contenido de compuestos polifenólicos. La citotoxicidad de los extractos acuosos de Halimeda spp se determinaron mediante el ensayo de MTT y las propiedades antioxidantes de las Halimeda spp se investigaron a través del modelo alternativo in vitro de estrés oxidativo inducido por FeCl₃ en células Vero y la protección se determinó en función de la supervivencia celular frente al insulto de FeCl₃. El contenido de compuestos fenólicos se determinó por el método de Folin-Ciocalteu. El contenido total de compuestos polifenólicos de los extractos acuosos de Halimeda monile, Halimeda opuntia y Halimeda incrassata fue 5,07 ± 0,06, 4,39 ± 0,10 y 9,11± 0,11 mg de GAE/g alga seca, respectivamente. La citotoxicidad de las algas del genero Halimeda para las células Vero resultó muy baja. Por otra parte los extractos acuosos de las algas Halimeda demostraron una efecto citoprotector contra el daño oxidativo producido por el FeCl₃ en células Vero en cantidades de 2 mg para H. incrassata y 4 mg para H. opuntia y H. monile. En este trabajo se demostró que las algas marinas del genero Halimeda son capaces de proteger a las células Vero contra el estrés oxidativo producido por el FeCl₃ asociado este efecto, al menos parcialmente, al contenido de compuestos polifenólicos.

Palabras clave: Actividad antioxidante, células Vero, algas marinas, polifenoles.

INTRODUCCIÓN

Over the last few years, there has been an increased interest in the search for natural antioxidants, generally consisting of mixtures of compounds with a high molecular diversity and biological functionality (Aruoma, 2003).

Seaweed is to be seen as a traditional food in the diet of different people. According to their requirements, the composition and the intake of their preferred diet are low calorie foods with high concentrations of minerals, vitamins, and protein, and are rich in fiber (Mac Artain et al., 2003). Additionally, some species of seaweed have been used since ancient times against different pathologies (Proksch et al., 2003).

In recent years, research on the possible therapeutic properties of seaweed has claimed a marked importance, driven to some extent by their content of bioactive metabolites (Lordan et al., 2011). Different in vitro studies and animal models and epidemiological investigations have shown a direct relationship between the consumption of algae and the incidence of some pathologies (Gomez-Gutierrez et al., 2011).

Most seaweed is located in the photic zone, with a notable exposure to solar radiation, which promotes the generation of free radicals, and this in turn, could determine the biosynthesis of secondary metabolites with antioxidant properties. It has been suggested that a lack of oxidative damage to the structural components of the seaweed and their stability against certain adverse conditions is due to the presence of effective antioxidants (Sampath-Wiley et al., 2008). The absence of oxidative damage in their structural and physiological components suggests that these organisms present an efficient system of antioxidant defenses, and consequently, different authors have demonstrated a possible application of the seaweeds as being a source of natural antioxidants (Cornish and Garbary, 2010).

Some authors have shown that seaweed extracts have an antioxidant activity that is explained by the presence of dissimilar types of compounds such as mycosporine-like amino acids, terpenoids, chlorophylls, polysaccharides, and polyphenolic compounds (Dutra Rocha et al., 2007). This diversity of chemical compounds that are present in seaweeds explains the several mechanisms that they possess and are realized in antioxidant seaweed extracts. In the last year, due to safety issues, together with industrial requirements, and population demand, there has been a considerable interest in replacing synthetic antioxidants with natural compounds. Therefore, an increasing number of vegetal extracts have been researched for their use in food applications and or for their use in phytodrugs (Embuscado, 2015).
**Halimeda** spp seaweed grows at a shallow depth, it is easy to collect, it is not toxic, and it is widely distributed. All of these aspects support their potential as natural sources for bioactive compounds. **Halimeda** spp seaweed has been shown to possess some therapeutic properties, such as an antifungal, an inducer of apoptosis, an anti-trichomonal, an antibiotic, and to be anti-inflammatory and antiatherogenic (Dzeha et al., 2003; Huang et al., 2005; Moo et al., 2008; Nor Afifah et al., 2010; Boonchum et al., 2011; Costa-Mugica et al., 2013).

In a previous study, we have shown that **Halimeda** spp seaweed was one of the most active specimens when presenting an antioxidant activity in an initial screening study using a lipoperoxidation model (Rivera et al., 2003). The antioxidant activity of aqueous extracts has been investigated in different *in vitro* assays and has been observed *in vivo* in models of oxidative stress (Vidal et al., 2009; Batista-Gonzalez et al., 2012). **Halimeda incrassata** has also shown antioxidant activity in the mouse hypothalamic cell lines and has exerted neuroprotection against toxicity induced by methyl-mercury (Fallafero et al., 2003; Linares et al., 2004). Additionally, it has been demonstrated that different species of **Halimeda** have hepatoprotective properties in animal models (Mancini-Filho et al., 2009; de O. e Silva et al., 2012).

The antioxidant activity from **Halimeda** spp has been associated with the polyphenol content. Vidal et al., (2009) identified approximately one third of the total polyphenol content from the hydrophilic fractions of **H. opuntia** and **H. monile** as having phenolic acids and compounds that were detected at higher rates of salicylic, p-cinnamic, caffeic, and pyrogallic acids. Additionally, Vidal et al., (2011) identified phenolic acids in **H. incrassata**, being 32% constituted as salicylic ferulic acid. Yoshie et al., (2002) also identified teen flavonoids and eight polyphenolic compounds, including caffeic acid and catechol, in **H. macroloba** and **H. opuntia**, and additionally, they demonstrated different compositions by species.

In view of these considerations, the aim of this paper was to evaluate the antioxidant properties of three species of seaweeds of the genus **Halimeda**, by an *in vitro* alternative model of FeCl3-Induced oxidative stress in Vero cells and their relation to the content of polyphenolic compounds.

**MATERIALS Y METHODS**

**Seaweed collection and preparation of aqueous extract**

Specimens of the seaweed **Halimeda incrassata** (J.Ellis) J.V. Lamouroux, 1816, **Halimeda monile** (J. Ellis & Solander) J.V. Lamouroux, 1816 and **Halimeda opuntia** (Linnneas) J.V.Lamouroux, 1816 were collected in December 2014 in the Bajo de Santa Ana, La Habana, Cuba. Voucher specimens were authenticated at the Seaweeds Laboratory of the Marine Research Center of the University of Havana, Cuba.

Freshly collected specimens were washed with distilled water and dried at room temperature (24–27°C) for 10 days. The dried seaweed was then ground into a powder and sieved. The dried seaweed (1 g) was then washed three times with distilled water (50 mL) for 5 h at room temperature under magnetic stirring and centrifuged at 800 g (4°C) for 20 min. The supernatants were then collected, lyophilized and kept at -20°C.

**Determination of the total polyphenolic compounds**

The total of phenolics was determined as stated by Vidal et al., (2009) and expressed as mg of gallic acid/g of seaweed.

**Vero cell culture**

Vero cells, a line of fibroblasts derived from the African green monkey’s kidney were purchased from the Institute Butantan (Sao Paulo, Brazil) and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin -streptomycin, in monolayers, at 37°C in a humidified atmosphere of 5% CO2.

**Cytotoxicity of the seaweed’s aqueous extract**

The cell viability assay was assessed by the MTT reduction. Live cells can reduce a yellow water soluble MTT into a water-insoluble purple formazan product (Plumb et al., 1989). Briefly, the cells were seeded in 96-well microtter plates at a concentration of 1x10⁴ cells per well in 200 µL of final volume. After 24 h, the cells were treated with a seaweed aqueous extract (1-10 µg of polyphenols dissolved in water) for 24 h at 37°C under a humidified 5% CO2 atmosphere. The MTT solution (50 µL, 2mg/mL) was added to each well and the cells were incubated for 4h in the dark at 37°C under a humidified 5% CO2 atmosphere.
RESULTS

Determination of total phenol

The yield of the final lyophilized aqueous extract (LAE) in terms of the starting dry seaweed was 3.46%, 3.03%, and 7.08%, for *H. monile*, *H. opuntia* and *H. incrassata*, respectively.

The total polyphenolic content of the aqueous extract of *Halimeda monile*, *Halimeda opuntia* and *Halimeda incrassata* are 5.07 ± 0.06, 4.39 ± 0.10 and 9.11± 0.11 mg of GAE/g dry seaweed. The seaweeds *H. monile* and *H. opuntia* have similar values of polyphenolic compounds; however, *H. incrassata* had twice the amount of polyphenolic compounds.

Cell viability assay

Initially, a study was conducted to determine the cytotoxicity of FeCl$_3$ in the Vero cells, in order to define a toxic dose of FeCl$_3$; the results can be seen in figure 1.

The viability decreased in a dose-dependent manner with increasing concentrations of FeCl$_3$. As shown, FeCl$_3$ exerts no toxic effects at doses of 0.5 and 1 mM; however, at 3 mM, the cell viability decreases to values of 48%, whereas with 6 mM, the cell viability is approximately 5-10%.

Protective effect of aqueous extract in FeCl$_3$-induced oxidative stress model

The oxidative stress of the cells was induced by FeCl$_3$. The Vero cells were seeded in 96-well microtiter plates at a concentration of 1x10$^4$ cells per well in 200 µL of final volume. After 24 h, the cells were treated with a seaweed aqueous extract (1-10 µg of polyphenols) for 6 h at 37º C under a humidified 5% CO$_2$ atmosphere. FeCl$_3$ (3 mmol/L) was then added and the cells were incubated for additional 2 h under the same conditions. The compounds were added to the cells (powdered extracts, positive controls, and toxic agents) that had been dissolved previously and were sterilized by filtering according to the protocols as detailed above.

All of the exposures lasted for 3 hours and were carried out in the dark at room temperature. Finally, the medium was removed and the formazan crystals were dissolved in 200 µL of DMSO. The absorbance was measured at 570 nm with a reference wavelength of 630 nm. The absorbance was proportional to the number of viable cells. The evaluation of the dead cells was also performed by microscopic observation. Ascorbic acid (2 µg) was used as positive control.

Statistical Analysis

All of the experiments were carried out at least in triplicate and the results were expressed as mean values ± standard deviation. The antioxidant activity measurements were compared by the One-Way Analysis of Variance (ANOVA) and the Tukey Post-Test, with a significance value of (p< 0.05).
An aqueous extract from *H. monile*, *H. opuntia*, and *H. incrassata*, were tested in a cell culture to evaluate their cytotoxic activity. Figure 2 shows the percentage of Vero cell viability after 6 h of incubation in the presence of aqueous extracts of seaweed when compared to the controls.

As shown, *H. opuntia* had no change of values of cell viability in the concentration range studied, while the *H. monile* seaweed was more toxic from 6 mg. Our results have clearly shown that an aqueous extract from *Halimeda incrassata* induced a cell mortality of Vero cells in a concentration-dependent manner.

**Protective effect of aqueous extract in FeCl₃-induced oxidative stress model**

Figure 3 shows the cytoprotection of an aqueous extract from *H. monile*, *H. opuntia*, and *H. incrassata*, on the FeCl₃-induced oxidative stress in Vero cells.

For all of the seaweeds, the protector effect increased in a dose-dependent manner with increasing concentrations of aqueous extract. From 4 µg, the *H. monile* and *H. opuntia* extracts significantly protected the Vero cells from the oxidative stress caused by the FeCl₃, while this effect was observed in *H incrassata* from 2 µg.

The extracts of *H. opuntia* and *H. monile* showed a similar behavior to the positive controls, with an ascorbic acid and a concentration of 2 µg; however, *H incrassata* showed this behavior with a concentration of 1 µg.

**DISCUSSION**

**Determination of total phenol**

Different researchers have demonstrated the presence of phenolic compounds in seaweed and their relationship with antioxidant properties (Dutra Rocha et al., 2007). According to Kuda & Ikemori (2009), the polyphenol content of a seaweed species varies considerably, even within the same genus, from very low levels to high values.

The polyphenol content from the seaweed *H. incrassata* was similar for an aqueous extract of this species as that reported by Costa-Mugica et al., (2013) although in a previous work, these authors reported a lower value (Costa-Mugica et al., 2012). In this study, the polyphenol totals for *H. opuntia* and *H. monile* were higher than those obtained by de O. e Silva et al., (2012) and Batista-Gonzalez et al., (2012) when working with the aqueous extracts of these algae.
Figure 3. Antioxidant activity against FeCl₃-induced oxidative stress in Vero cells, determined by the % cellular viability with an MTT assay, of the aqueous extract from *H. monile*, *H. opuntia* and *H. incrassata*. Expressed as µg of polyphenolic compounds (1-10 µg). p< 0.001 versus control (n=6). Ascorbic acid (2 µg) was used as positive control.

However, Zubia *et al.* (2007) reported a similar amount of polyphenols for the alga *H. monile*. Additionally, Yoshie *et al.* (2002) found a similar value of the total polyphenols (13.46 mg / g) for *H. opuntia*. Vidal *et al.* (2009) reported in a study on the antioxidant activity of seaweed, stressing higher values of total polyphenols for *H. opuntia* and for *H. monile*.

In seaweed extracts, we have identified phenolic acid as one of the most abundant polyphenolic compounds. In previous studies by our group, the phenolic acids of these seaweeds have been identified and quantified by gas-liquid chromatography. Vidal *et al.*, (2009) identified salicylic, cinnamic, gallic, pirogalic, and caffeic acids, as being the principal polyphenolic compounds in hydrophilic extracts from *H. opuntia* and *H. monile*. While with *H. incrassata*, they identified major polyphenolic compounds of salicylic and ferulic acids and they suggested that their levels were related to the antioxidant activity of the seaweed (Vidal *et al.*, 2011).

Additionally, Yoshie *et al.*, (2002) found differences in the composition of polyphenolic compounds and related phenolic compounds between the seaweeds *Halimeda macroloba* and *Halimeda opuntia*. Interestingly to note that different seaweed species of the genus *Halimeda* have phenolic acids as major polyphenolic compounds; however, it is possible to appreciate the differences that are quantitative and qualitative in these compounds. This fact could explain the differences in the antioxidant activity between the different species from the genus *Halimeda*.

It is important to consider that aqueous extracts of seaweed are included in different types of antioxidant molecules, such as terpenoids, phlorotannins, mycosporine-like amino-acids, and polyphenolic compounds, which could all be involved in the possible changes related to the antioxidant mechanisms of action for the different types of compounds present (Sahayaraj *et al.*, 2014).
Vero cell viability assay

Cell cultures may actually be considered to be an important model to evaluate antioxidant compounds and their mechanisms of action against oxidative stress; so this study may be useful before animal model studies and human clinical trials are investigated. Several methods are used to assess the cytotoxicity induced by xenobiotics, including cell death, the inhibition of cellular growth, an evaluation of the capacity of the cells to synthesize the cellular macromolecules needed for the physiological functions as being a replication, and the capacity of this xenobiotic to induce oxidative stress (López-Alarcon & Denicola, 2013).

In this paper, we observed cytotoxicity values of the aqueous extracts of Halimeda genus seaweeds similar to those obtained by Rani et al., (2013), whom also investigated the cytotoxicity of the methanolic extracts from Gracilaria edulis and Padina tetrastromatica in Vero cells. Lakmal et al., (2014) also evaluated the cytotoxicity and antioxidant properties of the methanolic extracts of red algae (Chondrophycus ceylanicus, Gelidiella acerosa and Gracilaria corticata), two species of green algae (Chaetomorpha crassa and Caulerpa racemosa), and one species of brown algae (Sargassum cassifolium). The MTT assay confirmed that all of these extracts were not cytotoxic at 50 and 100 µg/mL concentrations in the Vero cells.

Vidal et al., (2012) when studying the cytotoxicity of B. triquetrum extracts in Caco-2 cells, observed this effect at high concentrations, which may be explained by their content of cinnamic acids, which reduces the Caco-2 cell proliferation. As can be seen, all of the extracts had similar cell viability values at doses of 6 mg, and they exerted a significant cytotoxic activity only at concentrations of polyphenolic compounds higher than 8 µg.

The cytotoxicity of the xenobiotics in Vero cells, as polyphenols, could be explained by altering the organelle functions and the lysosomal functions, and that they could act differently, depending upon the cell type, the concentration, and the type of assay performed (Resende et al., 2012; Cariddi et al., 2015). The aqueous extracts from Halimeda seaweeds contained appreciable amounts of polyphenolic compounds and it is logical to presume that their cytotoxic effects could be due to these molecules. In this study, we found that in the aqueous extracts of Halimeda spp, the cell viability decreases to values near 60% with amounts of 2-6 µg polyphenols, while their cellular viability value decreased to 55% when increasing the amounts of polyphenolic compounds (8-10 µg), thus showing that these results for an evaluation of the cytotoxicity of these extracts as being weak.

Da Silva Campelo et al., (2013) demonstrated that polyphenolic-rich extracts from juçara (Euterpe edulis) exhibit a strong antioxidant activity and an antiproliferative activity against the Vero cells. The extracts of juçara contained phenolic acids and flavonols as the principal phenolic compound constituents, which may significantly contribute to any antioxidant activity, and it also may occur similarly in Halimeda spp extracts.

In previous studies with GT1-7 neuronal cells, the aqueous extracts of Halimeda incrassata were not cytotoxic for exposures of 48 hours and it was established that these extracts exerted a neuroprotection at much lower concentrations; in doing so, these authors considered these extracts to be of low cytotoxicity (Fallerero et al., 2003). In summary, the cytotoxic effects of the aqueous extracts of Halimeda spp occurred in Vero cells at concentrations greater than those which act as antioxidants.

Namvar et al., (2013) evaluated the antioxidant, antiproliferative, and antiangiogenesis effects of the polyphenol-rich seaweed Sargassum muticum. The total phenolic contents were 78.95 ± 4.33 mg gallic acid equivalents per 100 g dried seaweed. The values were much higher than those found for the Halimeda spp algae and the polyphenol-rich seaweed had no cytotoxic concentrations until 200 µg/mL were added to the normal Vero cell line.

FeCl₃-Induced Oxidative Stress in Vero Cells

Different authors have investigated oxidative stress using culture cells and, in particular, Vero cells, as an in vitro alternative model that would be useful for studying antioxidants and action mechanisms (Rosa et al., 2007, 2008; Ouanes-Ben Othmen et al., 2008; Ayed et al., 2011; El Golli-Bennour et al., 2012; Da Silva Campelo et al., 2013). On the other hand, it has been properly established that transition metals, such as free iron, enhance oxidative stress, by directly participating in free radical formation and by catalyzing the conversion of hydrogen peroxide (H₂O₂) to the hydroxyl radical (OH⁻) via the Haber-Weiss/Fenton reaction (Halliwell & Cross, 1994).
Additionally, some researchers have reported interesting results on the evaluation of antioxidants with FeCl$_3$-induced oxidative stress in Vero cells (Garcia-Alfonso et al., 1996; Rosa et al., 2005).

In this work, it has been convincingly demonstrated that there is a protective effect of the extracts of *Halimeda* spp algae in an alternative model of FeCl$_3$-induced oxidative stress in Vero cells. The results of the cell viability assay of Vero cells in these experiments were in agreement with those obtained by Garcia-Alfonso et al., (1996) and Rosa et al., (2005) when they investigated the FeCl$_3$ compound as being the cause of inducing oxidative stress in Vero cell cultures.

An aqueous extract from *Halimeda* spp also showed a significant protective effect against oxidative stress induced in Vero cells. The data obtained at non-cytotoxic concentrations (at 24 h of incubation) confirmed this potent action to be a radical scavenger in FeCl$_3$-induced damage. In this experimental model, the oxidative damage was defined by cell death, and that this may be due to diverse cellular dysfunctions and different biochemical damage in response to FeCl$_3$, especially as an induced oxidative process. The *Halimeda* spp extract treatment would then lead to a significant decrement of this cell mortality. *Halimeda* spp aqueous extracts have similar antioxidant activities with a dose-dependent relationship with respect to the amount of polyphenols.

The antioxidant activity of polyphenolic compounds depends on the presence of hydrogen-donating bonds (like phenolic hydroxyls), and the ability of the polyphenols to chelate the redox-active metals, and thus prevent a catalytic breakdown of hydrogen peroxide through the Fenton reaction, and consequently, by reducing the amount of ROS (Rice-Evans et al., 1996).

In the last few years, it has been proposed to be an antioxidant action mechanism for polyphenolic compounds, the modulation of cell signaling, and the over-expression of antioxidant enzymes (Bahia et al., 2012). Within this context, in a previous work with an animal model, it was reported that *Halimeda* spp could act as an antioxidant via the mechanism of over-expression of the antioxidant enzymes of catalase and superoxide dismutase (Mancini-Filho et al., 2009; de O. e Silva et al., 2012).

Moreover, the antioxidant properties of aqueous extracts from *Halimeda* spp have been investigated in different *in vitro* assays of oxidative stress and we may assume that the ability of the scavengers ROS to be an important mechanism in the action of these seaweeds. However, Lakmal et al., (2014) evaluated the antioxidant effects of selected Sri Lankan marine algae and they considered that the total phenolic content of the extracts did not give supportive facts that correlated with the determined antioxidant activity. It is believed that a different kind of secondary metabolite could be involved in the antioxidant activity.

The antioxidant properties of *Halimeda* spp have also been studied in cultured cells. Fallarero et al., (2003) evaluated the effect of an aqueous extract from the seaweed *Halimeda incrassata* against the oxidative stress induced by hydrogen peroxide on the GT1-7 mouse hypothalamic cells and it increased cell viability and reduced the ROS production. Linares et al., (2004) also investigated the antioxidant properties of an aqueous extract from *Halimeda incrassata* against the oxidative stress induced by methyl-mercury on the GT1-7 cells and the extract reduced the ROS production. Considering these circumstances, it may be safe to assume that the cytoprotective properties of the *Halimeda* spp aqueous extract in Vero cells with FeCl$_3$ are insulted and they could be an ROS scavenger.

In this work, it has been conclusively shown that the seaweeds *Halimeda* spp are able to protect Vero cells from an oxidative stress induced by FeCl$_3$, and an associated effect, at least in part, of their content of polyphenolic compounds. Thus, these polyphenol-rich green marine *Halimeda* spp algae might be a potential and an abundant source of complementary and alternative functional food for the prevention and treatment of pathologies related to oxidative stress.

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