

**Phenolic compounds in *Physalis peruviana* Linneus) extracts and action of the extracts on the phytopathogen *Botrytis cinerea* Pers****Compostos fenólicos em extratos *Physalis peruviana* Linneus) e ação dos extratos sobre o fitopatógeno *Botrytis cinerea* Pers**

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#### **ABSTRACT**

Phenolic acids and flavonoids naturally protect plants against phytopathogenic fungi and, therefore, plant extracts containing phenolic compounds are considered a natural alternative to conventional fungicides. Goldenberry extract was evaluated for its ability to inhibit the growth of the fungus *Botrytis cinerea* Pers. in vitro and in strawberries, cultivar Albion. Caffeic, chlorogenic and ferulic acids and flavonoid quercetin were identified in the goldenberry extract at different concentrations. The different concentrations of the extract tested in vitro resulted in variations in the percentage inhibition of fungal mycelial development. The fungicidal effect was observed when the 5 mL volume of the extract at 20% (v/v) concentration was tested in vitro. The phenolic compounds present in goldenberry extract represents a preventive natural method of control of *B. cinerea* in vitro and it can be alternative method of control for postharvest strawberries of cultivar Albion.

**Keywords:** *Fragaria X ananassa* Duch., *Physalis peruviana* L., gray mold, phenolic compounds

#### **ABSTRACT**

Phenolic acids and flavonoids naturally protect plants against phytopathogenic fungi and, therefore, plant extracts containing phenolic compounds are considered a natural alternative to conventional fungicides. Goldenberry extract was evaluated for its ability to inhibit the growth of the fungus *Botrytis cinerea* Pers. in vitro and in strawberries, cultivar Albion. Caffeic, chlorogenic and ferulic acids and flavonoid quercetin were identified in the goldenberry extract at different concentrations. The different concentrations of the extract tested in vitro resulted in variations in the percentage inhibition of fungal mycelial development. The fungicidal effect was observed when the 5 mL volume of the extract at 20% (v/v) concentration was tested in vitro. The phenolic compounds present in goldenberry extract represents a preventive natural method of control of *B. cinerea* in vitro and it can be alternative method of control for postharvest strawberries of cultivar Albion.

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**1 INTRODUCTION**

Strawberry (*Fragaria X ananassa* Duch.) is characterized for producing the perishable fruit of short lifespan (Junkes; Groff, 2020) with a chemical composition, which makes it an ideal substrate for the growth of microorganisms (Petrasch et al., 2019). The main fungal disease which attacks strawberry culture is the gray mold reducing the total harvesting of the fruit by up to 50% (Jeon et al., 2017), which is caused by the fungus *Botrytis cinerea* Pers., able to infect fruit at different maturation stages and remain in green fruit to manifest only during the post-crop period, including fruits already packaged, which results in considerable economic losses for both the production and commercial sectors (Dean et al., 2012), this occur because the source inoculum of packed strawberry was derived from the cultivation fields (Jeon et al., 2017).

Factors such as the presence of chemical residues from of fungicides used to control *B. cinerea* in strawberries (Lopes et al., 2017), as the low effectiveness of some fungicides in inhibiting the germination and the conidial production of *B. cinerea*, due inappropriate use of fungicides leads to resistance of *B. cinerea* and environmental concerns (Kim et al., 2016), have contributed to an increasing interest in the development of new control methods, also called alternative methods (Brasil, 2012, Frac, 2014, Aqueveque et al., 2016, Jin et al., 2017, Li et al., 2017, Forges et al., 2018, Frac, 2018).

Plant have different antifungal mechanisms due the different secondary metabolites with antifungal potential against plant pathogens (Lagrouh; Dakka; Bakri, 2017). The effects are attributed to secondary metabolites naturally present in the different parts of plant, including in the fruit, such as the class of phenolic compounds (Aqueveque et al., 2016; Zacchino et al., 2017). The *B. cinerea* has been described in the literature with a growth inhibited by different phenolic acids and flavonoids, the example the chlorogenic and ferulic acid, what're fungicide active against *B. cinerea* (Martínez et al., 2017; Patzke; Scheieber, 2018). The phenolic acids and flavonoids caused hyphae deformation, suppressed and inhibited conidial germination and reduction of mycelial growth of *B. cinerea* (Mendonza et al., 2013, Martínez et al., 2017; Xu et al., 2018).

We highlight that the goldenberry (*Physalis peruviana* L.) is considered a rich source of phenolic acids and flavonoids (Olivares-Tenório et al., 2016). Based on these premises, here we chemically characterize natural fruits of goldenberry and strawberry and phenolically goldenberry extract. In addition, we investigated the preventive action of the goldenberry extract to control the fungus *B. cinerea in vitro* and in strawberries. We hypothesized that the preventive action of the goldenberry extract to control the fungus *B. cinerea in vitro* and in strawberries it's due the

chemically composition of goldenberry extract and that the chemical composition of strawberries, cultivar Albion may influence the action exerted by the extract.

## 2 MATERIAL AND METHODS

### 2.1 PLANT MATERIAL, IDENTIFICATION AND ISOLATION OF *B. CINEREA*

Goldenberry fruits were purchased from ItalBraz<sup>®</sup>, located in Vacaria (28° 30' 44" S, 50° 56' 02" W), RS, Brazil. Immediately after collection, the fruits were visually classified according to their degree of ripeness by staining the calyx according to NTC 4580 (Icontec, 1998). The fruits were then packed in plastic containers, wrapped in polyvinyl chloride (PVC) plastic and identified. The fruits remained frozen with the goblet (-18 °C) until the study development.

All strawberries used in our study, Albion cultivar, came from an organic production system. The fruits were purchased from a producer in the municipality of Barão de Cotegipe (27° 37' 15" S, 52° 22' 47" W), RS, Brazil.

The *B. cinerea* used in this study was identified and isolated from strawberries according to Forges et al., (2018) with some adaptations. After identification of *B. cinerea* under an optical microscope, we made its isolation in laminar flow chamber. The confirmation of the species was conducted by company Neoprosperta Microbiome Technologies through methodologies of large-scale DNA sequencing of specific molecular markers associated with the analysis of bioinformatics. Thus, we demonstrated that the isolated fungus belonged to kingdom Fungi, phylum Ascomycota, class Letiomycetes, order Helotiales, family Sclerotiniaceae, genre *Botrytis* and specie *B. cinerea*.

In this study, we performed three experiments. The first was related to the chemical composition of natural fruits of goldenberry and strawberry and phenolic characterization of goldenberry extract. The second experiment corresponded to the *in vitro* activity of goldenberry extracts on *B. cinerea*. Finally, in the third experiment we tested the *in vivo* action of these extracts on strawberries. The research was conducted in laboratories at the Universidade de Passo Fundo (28° 15' 46" S, 52° 24' 24" W) and Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Sul (27° 58' 47" S, 52° 15' 35" W), Rio Grande do Sul (RS), Brazil, in the period from 2016 to 2018.

### 2.2 EXPERIMENTAL DESIGN

In experiment I we chemically characterized natural fruits of goldenberry and strawberry and goldenberry extracts. Thus, we do not use an experimental design.

In experiment II the treatments were four extracts of physalis (extract 20%, extract 10%, extract 5% and extract 0.5%), one commercial fungicide registered for control of *B. cinerea* in strawberry, used as positive control (Mythos<sup>®</sup> 0.4%) and one negative control (sterile water 20%), arranged in a completely randomized design with five repetitions. The active ingredient of Mythos<sup>®</sup> corresponds to pyrimethanil, which belongs to the chemical group anilinopyrimidine.

In experiment III the treatments, outlined in a bifactorial scheme, were three controls of *B. cinerea* (goldenberry extract, Mythos<sup>®</sup> and sterile water) and two storage temperatures of strawberries (4 °C and 25 °C), arranged in a completely randomized design with ten replications.

### 2.3 CHEMICAL COMPOSITION OF NATURAL FRUITS OF GOLDENBERRY AND STRAWBERRY AND PHENOLIC CHARACTERIZATION OF GOLDENBERRY EXTRACT

The goldenberry fruits were analyzed by the following attributes: potential of hydrogen (pH), total titratable acidity (TTA) in citric acid, total soluble solids (TSS) through refractometry, reducing glycosides in glucose and vitamin C through titulometry with potassium iodate (Lutz, 2008).

The strawberry fruits were analyzed by the following attributes: potential of hydrogen (pH), total soluble solids (TSS) through refractometry and water activity ( $w_a$ ) (Lutz, 2008).

Regarding the characterization of goldenberry extracts, the extract was obtained from in nature goldenberries macerated using a mortar and added to the solution of water and ethanol 50% (v/v). The extract remained at ultrasound bath, without heating, for two hours. After the extraction time, we proceeded with filtration and transference of the solution to the rotary evaporator at 40 °C for 2 hours (Filippi, 2015).

The following phenolic acids were determined through high-performance liquid chromatography equipped with UV-VIS detector (HPLC-UV) at reverse phase with a mobile phase flow of 1 mL.min<sup>-1</sup> and injection volume of 20 µL: caffeic acid, ferulic acid, vanillic acid, coumaric acid and chlorogenic acid in addition to the following flavonoids: quercetin, kaempferol, chrysin, myricetin and hesperidin. The determination of phenolic acids used as mobile phase acetonitrile:water (10:90 v/v), at pH 3.0, adjusted with phosphoric acid and wavelength of 280 nm. Flavonoids were determined with a mobile phase A: 0.3% formic acid in water, mobile phase B: 0.3% of formic acid in methanol and wavelength of 360 nm.

### 2.4 IN VITRO ACTIVITY

The lowest concentration of goldenberry extract capable to inhibit the mycelial growth of *B. cinerea* in a preventive way was determined using the Poisoned Food method (Balouiri et al., 2016).

The treatments were incorporated to the culture medium previously prepared in Petri dish and subsequently added with a mycelium disk of *B. cinerea* of 7 mm at the center of each disk obtained from the culture previously replicated, remaining incubated at 25 °C during 15 days, over a photoperiod of 12 hours. Having verified the total filling of plates with the *B. cinerea*, two perpendicular measurements were performed with the aid of a caliper of the mycelial growth diameters, expressed as mean and standard deviation. The percentage of antifungal activity was calculated according to Eq. 1.

$$\text{Antifungal activity (\%)} = \frac{DC-DS}{DC} \times 100 \quad (1)$$

where: “DC” is the diameter of mycelial growth in the control plates and, “DS” is the diameter of mycelial growth on the plates containing the antifungal agent tested.

## 2.5 IN VIVO ACTIVITY

The third experiment we verified the preventive action of the goldenberry extract in strawberries harvested at the stage of green maturation, under two storage temperatures, 4°C ± 1°C and 25°C ± 1°C, with the inoculation of the suspension of conidia of *B. cinerea*, at the concentration of 10<sup>5</sup> conidia.mL<sup>-1</sup> of suspension for a period of 15 days.

Were used ten strawberries for each treatment. The treatments were: sterile water (negative control), Mythos<sup>®</sup> (pyrimethanil, positive control) and goldenberry extract, consisted of three sprays on the peduncle of each strawberry (to the point of runoff). The strawberries were packed in gerbox plastic box and stored in a refrigerator at 4 °C and in a chamber at 25 °C with 12 hours photoperiod for a period of 24 hours. After incubation time, a portion of the strawberries were sprayed, totaling 3 sprays on the peduncle of each fruit, with the suspension of conidia of *B. cinerea* at the concentration of 10<sup>5</sup> conidia per mL of suspension, being were again stored at their respective temperatures where they remained for a period of 15 days.

At the end of the incubation period, the absence or presence of fungal development on strawberries stored in different temperatures was observed.

## 2.6 STATISTICAL ANALYSIS

In the experiment I the data analysis was descriptive, as we chemically characterized natural fruits of goldenberry and strawberry and goldenberry extracts.

In the experiment II the data were submitted to variance analysis and the means of the treatments were compared by Tukey test, at 5% error probability, using the Costat<sup>®</sup> software.

In the experiment III we did not perform inferential statistical analysis due to the non-opportuneness of the number of samples.

### 3 RESULTS

#### 3.1 CHEMICAL COMPOSITION OF NATURAL FRUITS OF GOLDENBERRY AND STRAWBERRY AND PHENOLIC CHARACTERIZATION OF GOLDENBERRY EXTRACT

The Table 1 presents the chemical characterization that we performed in natural goldenberry fruits and natural strawberry fruits and shows the total phenolic content and individual phenolic composition (phenolic acids and flavonoid) of the goldenberry extract obtained by HPLC analysis. We observed that the natural goldenberry fruits are sweeter and less acidic than the natural strawberry fruits (Table 1). Regarding the phenolic composition, we verified the presence of a flavonoid (quercetin) and three phenolic acids (caffeic, chlorogenic and ferulic) in the goldenberry extract (Table 1).

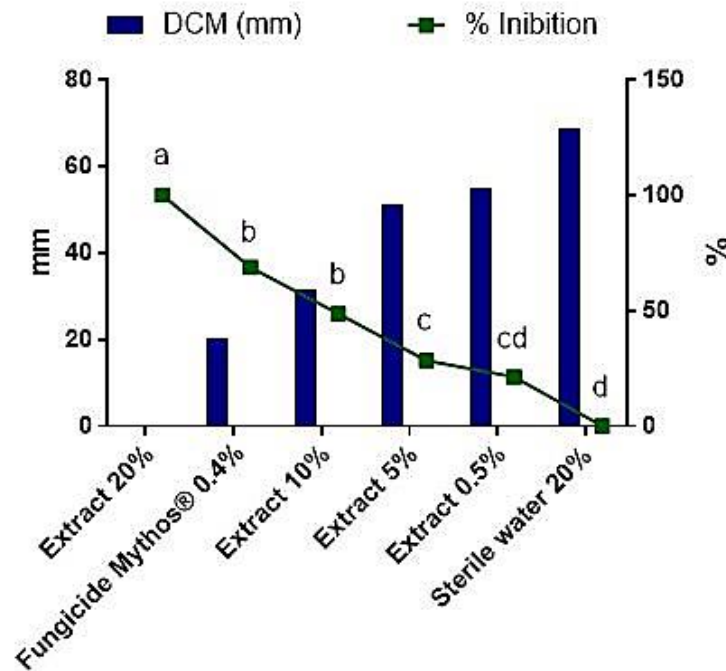
Table 1. Chemical attributes identified in the goldenberry and in the strawberries fruits and phenolic acids and flavonoid identified in the extract of goldenberry.

Atributes evaluated in the goldenberry fruits	Result
pH	4.98 ± 0.09
ATT in citric acid (g.100 g <sup>-1</sup> )	0.68 ± 0.01
SST (°brix)	14.17 ± 0.06
Reducing sugars (g.100 g <sup>-1</sup> )	1.50 ± 0.30
Ascorbic acid (mg.100 g <sup>-1</sup> )	34.67 ± 0.13
Atributes evaluated in the Albion strawberries	Result
pH	3.33 ± 0.05
SST (°brix)	6.00 ± 0.03
Water activity (w <sub>a</sub> )	0.95 ± 0.05
Composition of the goldenberry extract by HPLC	Concentration (µg.mL <sup>-1</sup> )
Chlorogenic acid	148.80
Caffeic acid	14.42
Ferulic acid	2.44
Quercetin	190.00
Total content of the phenolic compounds	355.66

### 3.2 IN VITRO ACTIVITY

The Figure 1 presents the results of diameter of mycelial growth (DCM) and percentage of inhibition of the treatment systems studied. It is possible to verify that the mycelial growth of *B. cinerea* was inhibited in 100% with a volume of 5 mL of the extract incorporated to the culture medium Potato Dextrose Agar, which resulted in a final concentration of 20% (v/v). Lower extract volumes, corresponding to the concentrations of 10%, 5%, and 0.5%, presented an inhibition capacity below 50% (48.63%, 28.45%, and 21.26%, respectively). The percentage of inhibition presented by the volume of 5 mL of extract was above the percentage of inhibition of 68.81% presented by Mythos<sup>®</sup> 0.4% (pirimetanil).

Figure 1. Diameter of mycelial growth (DCM) of *B. cinerea* and inhibition (%) presented by the goldenberry extracts. Data presented as mean. Means followed by the same letter in the column do not differ significantly by the Tukey test ( $p \leq 0.05$ ).



Data presented as mean  $\pm$  standard deviation. Means followed by the same letter in the column do not differ significantly by the Tukey test ( $p \leq 0.05$ ).

### 3.3 IN VIVO ACTIVITY

The extract efficiency as a function of strawberry maturation and storage temperature is shown in Table 2. The assessment of strawberries showed that the extract acts in a preventive way regarding the *B. cinerea* when applied in green strawberries stored at 25 °C.



Table 2. Efficiency of the goldenberry extract as a function of strawberry storage temperature on fungal growth control (%)<sup>1</sup>.

Treatment	Strawberries without <i>B. cinerea</i> conidia <sup>2</sup>		Strawberries with <i>B. cinerea</i> conidia <sup>3</sup>	
	4 °C	25 °C	4 °C	25 °C
Sterile water (negative control)	100	40	100	40
Pyrimethanil (positive control)	100	80	100	50
Goldenberry extract	100	80	100	50

<sup>1</sup> In each treatment were used ten strawberries. The ten fruits represent 100%. The results given in percentage correspond to the number of fruits without the fungal development.

<sup>2</sup> Strawberries without application of the suspension of *B. cinerea* conidia by spraying.

<sup>3</sup> Strawberries with application of the suspension of *B. cinerea* conidia ( $10^5$  conidia.mL<sup>-1</sup> suspension) by spraying.

#### 4 DISCUSSION

The goldenberry fruit do not normally have a pH above six even during the final period of maturation (Licodiedoff et al., 2013). The acid pH of the plant may be related to the pH of the vacuole of the plant cell, which normally ranges from 4.0 and 5.5, but in some citric fruit can reach 2.0. The acid nature of the goldenberry fruit is also highlighted by the percentage of total titratable acidity, expressed in citric acid, which is the main organic acid present in fruit of the species *P. peruviana* (Curi et al., 2018).

The content of total soluble acids identified in the goldenberry fruit demonstrates the content of sugar contained around of 5% (m/m) of total sugars present in the fruit correspond to reducing sugars (Yildiz et al., 2015), in addition glucose and galactose (Corrales-Bernal et al., 2015; Curi et al., 2018).

Different parts of goldenberry contain different phenolic acid and flavonoid and distinct contents of the same compounds (Olivarez-Tenório et al., 2016, Ertürk et al., 2017). Seed and fruit are the most effective parts of the *P. peruviana* in terms of their antimicrobial activity (Ertürk et al., 2017). Phenolic compounds play an important role influenced by responses linked to the host-pathogen interaction on host resistance in infected tissues (Mikulic-Petkovsek et al., 2013).

Therefore, the production of the extract from the whole fruit (skin, pulp and seeds) contributed to the presence of different phenolic compounds identified. Likewise, the varied concentration of phenolic compounds in the goldenberry fruit may be influenced by factors like variety, ecotype, maturation stages, analysis method, extraction solvent, reaction time and extraction conditions (Olivarez-Tenório et al., 2016).

Based on the results (Figure 1), it is possible to infer that the fungicide action found from 5 mL of extract probably occurred due to the presence of phenolic compounds identified in the extract,

which in total correspond to  $1778.30 \mu\text{g.mL}^{-1}$  ( $744 \mu\text{g.mL}^{-1}$  chlorogenic acid,  $72.1 \mu\text{g.mL}^{-1}$  caffeic acid,  $12.2 \mu\text{g.mL}^{-1}$  ferulic acid and  $950 \mu\text{g.mL}^{-1}$  quercetin). Antifungal activity can occur by synergistic action, considering that the concentration of different compounds present in an extract is majorly not sufficient for a compound to exclusively exert an antifungal action (Patzke; Schieber, 2018). Moreover, the intensity of the antifungal action may also vary according to factors such as the extraction method used to reach target compounds, in addition to solvent, fungal species, time and incubation temperature (Zabka, Pavela, 2013, Gyawali, Ibrahim, 2014).

Phenolic compounds extracted from different parts of the goldenberry plant have antimicrobial action regarding different species of microorganisms, such as *Escherichia coli*, *Bacillus megaterium*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Trichophyton* sp. and *Epidermaphyton* sp., whose growth is inhibited in different intensity rates (Goztok, Zengin, 2013). Studies evaluating the action of phenolic acids and flavonoids extracted from goldenberry fruits to control *B. cinerea* are still scarce in the literature. However, some of the phenolic compounds identified in the goldenberry extract have been described in the literature for their antifungal ability to control *B. cinerea*.

The in vitro mycelial development of *B. cinerea* is inhibited by chlorogenic acid, while spore germination and hyphae development are partially inhibited by this phenolic acid (Martínez et al., 2017). The growth of *B. cinerea* isolates is reduced between 54 and 70% when applied with ferulic acid enriched emulsion, which is considered a promising natural antifungal to control this phytopathogen (Patzke, Scheiber, 2018).

A preventive application of the goldenberry extract promoted a satisfactory antifungal action in green strawberries stored at  $25 \text{ }^{\circ}\text{C}$  (Table 2). Previous studies reported the relationship between the chemical composition of the fruit and the availability of nutrients for the development of *B. cinerea*. The chemical composition of strawberries belonging to the Albion cultivar at different maturation stages with an increase in the content of total soluble acids, pH, and moisture as the maturation of the fruit advanced (Orneles-Paz et al., 2013). *B. cinerea* can germinate in green fruits and eventually develop early stages of colonization and begin their growth and survive as quiescent until the strawberry fruit is fully ripe (Nagpala et al., 2016).

Strawberries stored at refrigeration temperature ( $4 \text{ }^{\circ}\text{C} \pm 0.1 \text{ }^{\circ}\text{C}$ ), inoculated or not inoculated with *B. cinerea* suspension showed an absence of gray mold development in all fruits during the 15 days of follow-up (Table 2). Temperature and humidity emerged as having the most correlation with incidence of the disease in the fields (Jeon et al., 2017). Temperature influenced the development

of *B. cinerea* regarding the different treatments assessed (Table 2). The capacity of *B. cinerea* to develop close to 0 °C is described in the literature (Gindro, Pezet, 2001). However, studies have found that it can grow at low temperatures, which separately has strong influence ( $p < 0.0001$ ) on the development of the fungus (Ahlem et al., 2007).

The growth of *B. cinerea* at different temperatures between 6 and 30 °C, for seven days, demonstrated an absence of fungal growth in strawberries stored at 5 °C during the incubation period. The remaining temperatures showed a lower rate of mycelial growth according to their re-education (Lahlali et al., 2012). The reduced mycelial growth in at a lower temperature strongly influenced when the fruits were stored at 5 °C. Temperature is also related to the factor of water activity ( $w_a$ ) in the strawberries (Table 1) emphasized by the reduced growth of *B. cinerea* with lower temperature and  $a_w (\leq 0.93)$  as well as development at 5 °C, but rates of  $w_a$  of 0.98 at 0.99 (Ahlem et al., 2007).

Considering the storage temperature, 6 °C  $\pm$  0.1 °C, and the rate of  $w_a$  (0.95) of the strawberries used in this experiment (Table 1), the absence of *B. cinerea* development for all the strawberries was due to both factors, regardless of the treatment applied. Combined with these two factors, the content of soluble solids of 6 °brix (Table 1) may have also contributed to such a result. The influence of the solutes content on the growth of *B. cinerea* corroborating that a higher saccharose content, for example, increases the development rate of the *B. cinerea* (Ahlem et al., 2007). Likewise, the chemical composition of the strawberries also proved to be related to the intensity of the action exerted by the extract. Finally, the goldenberry extract represents an alternative for a preventive control of *B. cinerea* in strawberries belonging to the Albion cultivar.

Differences in the chemical composition of fruits of different species end up in plant extracts with antifungal action at different intensities. Therefore, it is important to establish the plant-phytopathogen species in order to identify natural antifungals and use them as alternative methods of phytopathogen control.

## 5 CONCLUSION

The antifungal action exerted naturally and synergistically by caffeic, chlorogenic and ferulic acids and the flavonoid quercetin, after extraction of the plant matrix, against the fungus *B. cinerea*, varies depending on the chemical composition of the fruits of goldenberry and strawberry. The chemical composition of the plant matrix from which the extract was obtained, as well as the chemical composition of the fruit to which the extract was applied, influence the antifungal action exerted by the extract, which may be fungicidal and/or fungistatic.

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