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Antimicrobial activity and antibiotic modulating effect of the bark extract of *Dahlstedtia araripensis* (Benth) Fabaceae

Atividade antimicrobiana e efeito modulador antibiótico do extrato da casca de *Dahlstedtia araripensis* (Benth) Fabaceae

Actividad antimicrobiana y efecto modulador antibiótico del extracto de corteza de *Dahlstedtia araripensis* (Benth) Fabaceae

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Abstract
There is a recurring concern about the increase in infections caused by fungi and bacteria in the hospital environment. Considering the increase in resistant microorganisms and with that the need for more aggressive treatments for the treatment of these infections, research with natural products has been an effective alternative in the search for new bioactive substances that fight different species of microorganisms. therefore, the preliminary studies with plant extracts are the basis for further work in this area. *Dahlstedtia araripensis* Benth (Fabaceae), popularly known as "angelim", is an endemic species from northeastern Brazil, with little research developed. This study aims to trace the

preliminary chemical profile and evaluate the antimicrobial and antibiotic modulatory activity of the bark extract of the species *D. araripensis* (Benth). The secondary metabolite classes were identified from qualitative chemical prospecting. For the antimicrobial assays, 7 standard bacterial strains and 1 multidrug-resistant, and 3 fungal strains belonging to the genus Candida were used. Using the microdilution method the MIC for each strain was determined and the modulating potential of extract was evaluated. The chemical tests identified the presence of tannins and flavonoids. The antimicrobial assays showed good results against the Gram-Positive bacteria *Streptococcus mutans* and *Enterococcus faecalis* with MIC of 256 μg/mL, and a potentiation of the extract in the antibiotic benzylpenicillin action against *E. faecalis*. The extract did not show antifungal activity. Other works are essential for chemical characterization and bactericidal analysis by other more specific methods.
Keywords: *Dahlstedtia araripensis*; *Enterococcus faecalis*; Benzylpenicillin; *Streptococcus mutans*.

Resumo

Existe uma preocupação recorrente com o aumento de infecções causadas por fungos e bactérias no ambiente hospitalar. Considerando, o aumento de microrganismos resistentes e com isso a necessidade de tratamentos mais agressivos para o tratamento dessas infecções, a pesquisa com produtos naturais tem sido uma alternativa eficaz na busca de novas substâncias bioativas que combatam diferentes espécies de microrganismos. Portanto, os estudos preliminares com extratos vegetais são a base para futuros trabalhos nesta área. *Dahlstedtia araripensis* Benth (Fabaceae), popularmente conhecida como "angelim", é uma espécie endêmica do Nordeste brasileiro, com poucas pesquisas desenvolvidas. Este estudo tem como objetivo traçar o perfil químico preliminar e avaliar a atividade antimicrobiana e moduladora de antibióticos do extrato da casca da espécie *D. araripensis* (Benth). As classes de metabólitos secundários foram identificadas a partir de prospecção química qualitativa. Para os ensaios antimicrobianos, foram utilizadas 7 cepas bacterianas padrão e 1 multirresistente e 3 cepas fúngicas pertencentes ao gênero Candida. Pelo método de microdiluição foi determinada a CIM de cada linhagem e avaliado o potencial modulador do extrato. Os testes químicos identificaram a presença de taninos e flavonóides. Os ensaios antimicrobianos mostraram bons resultados contra as bactérias Gram-positivas *Streptococcus mutans* e *Enterococcus faecalis* com CIM de 256 μg/mL, e uma potencialização do extrato na ação do antibiótico benzilpenicilina contra *E. faecalis*. O extrato não apresentou atividade antifúngica. Outros trabalhos são essenciais para caracterização química e análise bactericida por outros métodos mais específicos.
Palavras-chave: *Dahlstedtia araripensis*; *Enterococcus faecalis*; Benzilpenicilina; *Streptococcus mutans*.

Resumen

Existe una preocupación recurrente por el aumento de infecciones causadas por hongos y bacterias en el ambiente hospitalario. Considerando el aumento de microorganismos resistentes y con ello la necesidad de tratamientos más agresivos para el tratamiento de estas infecciones, la investigación con productos naturales ha sido una alternativa eficaz en la búsqueda de nuevas sustancias bioactivas que combatan diferentes especies de microorganismos. Por lo tanto, los estudios preliminares con extractos de plantas son la base para trabajos posteriores en esta área. Dahlstedtia araripensis Benth (Fabaceae), conocida popularmente como "angelim", es una especie endémica del Noreste de Brasil, con poca investigación desarrollada. Este estudio tiene como objetivo rastrear el perfil químico preliminar y evaluar la actividad moduladora antimicrobiana y antibiótica del extracto de corteza de la especie D. araripensis (Benth). Las clases de metabolitos secundarios se identificaron a partir de la prospección química cualitativa. Para los ensayos antimicrobianos se utilizaron 7 cepas bacterianas estándar y 1 multirresistente y 3 cepas fúngicas pertenecientes al género Candida. Mediante el método de microdilución se determinó la CMI para cada cepa y se evaluó el potencial modulador del extracto. Las pruebas químicas identificaron la presencia de taninos y flavonoides. Los ensayos antimicrobianos mostraron buenos resultados frente a las bacterias Gram-Positivas Streptococcus mutans y Enterococcus faecalis con CIM de 256 μg/mL, y una potenciación del extracto en la acción del antibiótico bencilpenicilina frente a E. faecalis. El extracto no mostró actividad antifúngica. Otros trabajos son imprescindibles para la caracterización química y el análisis bactericida por otros métodos más específicos.
Palabras clave: *Dahlstedtia araripensis*; *Enterococcus faecalis*; Bencilpenicilina; *Streptococcus mutans*.

1. Introduction

The worsening of the health status of patients due to hospital infections has been a public health problem in the world. In addition to the worsening of the health condition, hospital infections can in many cases lead to the death of the patient. Among the microorganisms involved in the contamination, the bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* can be mentioned, as well as fungi of the genus *Candida* (Michelin & Fonseca, 2018).

Associated with nosocomial infections, the increase in antimicrobial resistance has been an obstacle to the treatment of diseases, requiring the development of new products that can combat resistant microorganisms more effectively (Rocha et al. 2017). Resistance has been associated with the use of non-selective drugs, making it necessary to constantly search for

products with greater specificity to guarantee the success of the treatment and maintenance of the system (Felix et al. 2018).

Taking into account the resistance of microorganisms associated with the number of nosocomial infections, there is great interest in conducting research involving natural products that can overcome the challenges offered by these pathogens, either by interfering with the growth of these microorganisms or by modulating the effectiveness of antibiotics already in use. (Bohneberger et al. 2019).

Dahlstedtia araripensis, popularly known as "angelim", belongs to the Fabaceae family, and it is endemic to the Northeast region of Brazil, occurring mainly in areas of Cerrado and Caatinga; It is a tree that can reach up to 12 m in height, and can be found in sandy soils, low, wet, rocky plains, and plateaus (Silva et al. 2019).

Considering the lack of studies on this species, as well as the need to find new safe alternatives for the treatment and combat of microbial resistance, the objective of this work was to trace a chemical profile and evaluate the antimicrobial effect and antibiotic modulation capacity of the bark extract. of *D. araripensis* against bacterial and fungal strains.

2. Methodology

2.1 Collecting and obtaining extract from *Dahlstedtia araripensis*

Dahlstedtia araripensis bark (Benth) (Fabaceae) was collected at Serra do Boqueirão located in the municipality of Lavras da Mangabeira, mesoregion of South-Central Ceará in Northeast Brazil, under the following geographical coordinates: 06º 72' 2432" S and 38º 97' 7396" W. The botanical identification was done at the Herbário Caririense Dárdano de Andrade Lima - HCDAL of the Universidade Regional do Cariri - URCA, with registration number 13.693.

The material obtained was previously selected and dried in an oven, at 60 ºC. The extract was prepared with 260 g of the bark, by maceration in ethanol and water (1:1) for 72 h. After this period, the liquid was filtered and distilled in a rotary evaporator under reduced pressure (40 xg at 60 ºC). The resulting product was transferred to a sterile container and frozen in a conventional freezer at -18 ºC and subsequently submitted to freeze-drying. A total of 6.49 g of freeze-dried extract (HEDa) was obtained, corresponding to a yield of 2.49 % (m/m).

2.2 Qualitative chemical prospection

The tests for the identification of the secondary metabolites classes were performed according to the methodology of Matos (1997), by observing the color change or precipitate formation after the addition of specific reagents.

2.3 Culture medium and strains

The following ATCC and INCQS collection strains were used: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15442), *Klebsiella pneumoniae* (ATCC 10031), *Staphylococcus aureus* (ATCC 12624), *Streptococcus mutans* (ATCC 0446), *Bacillus cereus* (ATCC 33018), *Enterococcus faecalis* (ATCC 4083), and the multidrug-resistant *Escherichia coli* 05 strain. The fungi were *Candida albicans* INCQS 40006, *Candida krusei* INCQS 40095, and *Candida tropicalis* INCQS 40042 obtained from the Oswaldo Cruz Foundation. These strains were inoculated on Sabouraud Dextrose Agar (SBD) (SDA - KASVI) for the fungal strains and on Brain Heart Infusion (BHI) for the bacterial strains, and incubated at 37 ºC for 24 h. The concentration of the inoculum was standardized according to the McFarland scale, by comparing the turbidity of the inoculum with the 0.5 standard of the scale. The prepared inocula were used in the Minimum Inhibitory Concentration (MIC) tests, and in the test performed to verify the potential of the extract to modulate antibiotics by direct contact.

2.4 Assessment of minimum inhibitory concentration (MIC) in fungal strains

The assay was performed in 96-well plates by the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2008). To determine the MIC, 1350 µL of medium SDB and 150 µL of saline solution (10% inoculum) were diverted to the eppendorfs. Then, the plate was filled by adding 100 µL of this solution to each well (96-well plates), and then a serial dilution of the extract was carried out where they ranged from 4 µg / ml to 4096 µg / ml. The last well was used as a control for the growth of microorganisms. Dilutions of extracts (using saline solution instead of inoculum) and media sterility controls were also performed. All tests were performed in triplicate. Plates were incubated at 37 °C for 24 h. After incubation, absorbance was measured at 630 nm using a microplate reader (ELISA) to determine IC50 values. The MIC value was defined as the lowest concentration at which no growth was observed.

2.5 Assessment of minimal inhibitory concentration (MIC) in bacterial strains

For the MIC assay, 900 µL of 10% medium liquid bhi and 100 µL of the inoculum (corresponding to 10% of the total solution) were added to a tube (NCCLS, 2003). From this solution, 100 µL was transferred to each well of a 96-well plate and then serial dilution was performed by adding 100 µL of the extract at concentrations ranging from 512 to 8 µg/mL. The plates were placed in an oven at 35 ± 2 °C for 24 h, after which the MIC of the substances was determined. For this, sodium resazurin (20 µg) was added to each well. The interpretation of results was performed by ocular analysis of the color change of resazurin after 1h of reaction (Coutinho et al. 2008; Mann and Markham, 1998). No treatments were added to positive controls and negative controls were not used for bacterial inoculation. The tests were performed in triplicate.

2.6 Modulation of the activity of antimicrobials

In order to evaluate the capacity of the extract as a modulator of antimicrobial, the MICs of aminoglycosides (amikacin and gentamicin) and beta-lactams (cephalothin and benzylpenicillin) were determined against bacterial strains, while MIC of fluconazole was determined against fungal strains. The testes were performed by microdilution according to the methodology used by Coutinho et al. (2008). Antimicrobials were serially diluted into the wells in a volume of 100 µl containing 10% specific culture medium, the suspension of the strain and the extract in a subinhibitory concentration (MIC/8 for bacteria and MIC/16 for yeast). The final concentration of the antibiotics ranged from 0.5 to 512 µg/l and for fluconazole from 4 to 4096 µg/ml.

2.7 Statistical analysis

The data obtained for each sample and concentration were checked for their normal distribution and then analyzed by one-way ANOVA with Tukey's post hoc test. The IC_{50} values were obtained by nonlinear regression for the purpose of interpolating values from standard curves (using the software Graphpad Prism, v.5.0) of the % growth values plotted against concentration and IC_{50} values are expressed as µg/mL.

3. Results and Discussion

3.1 Chemical prospection

Chemical prospecting showed important classes of secondary metabolites, such as flavonoids and tannins (Table 1). Numerous biological activities have been described for these metabolites, notably anti-inflammatory, antimicrobial, antiallergic, enzyme inhibitor and antioxidant activities (Cushnie & Lamb, 2005).

Table 1: Identification of the main chemical classes of the freeze-dried hydroethanolic extract of the bark of *D. Araripensis*

HEDa	Secondary Metabolites													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	-	-	+	+	+	-	-	-	+	+	-	-	+	-

1: Phenols; 2: Pyrogallic tannins; 3: Flobabenic tannins; 4: Anthocyanins; 5: Anthocyanidins; 6: Flavones; 7: Flavonols; 8: Xanthones; 9: Chalcones; 10: Aurones; 11: Flavonones; 12: Leucoanthocyanidins; 13: Catechins; 14: Alkaloids. (+) presence; (-) absence. Source: Authors.

The chemical composition of the genus Dahlstedtia is scarcely studied. Different studies with species of this genus show the presence of compounds belonging to the class of flavonoids, such as chalcones, flavanones, flavones, flavonols, rotenoid and pterocarpan, all compounds identified in extracts of roots of D. pinnata, D. pentaphylla, D. glaziovii and D. grandiflora species (Garcez et al. 1988; Canzi, 2014; Nepel, 2015).

The presence of phenolic compounds in the bark of the studied species may be related to the plant's ability to produce metabolites in its different parts due to the influence of natural factors such as solar radiation, seasons, presence of pollutants, and others; these conditions may or may not favor the production of these metabolites (Flambó 2013).

3.2 Minimum Inhibitory Concentration (MIC)

3.2.1 Antifungal assay

Tests of Minimum Inhibitory Concentration (MIC) demonstrated that HEDa did not show inhibitory effect against the growth of *Candida* fungal strains (Table 2). The IC_{50} values were higher than fluconazole with values of ≥ 512 µg/ml, for *Candida albicans* strain and ≥ 1024 µg/ml for *C. krusei* and *C. tropicalis* strains.

Table 2: IC_{50} of hydroalcoholic extract of *D. araripensis* against different strains of *Candida*.

IC_{50} µg/mL			
Product	**CA INCQS 40006**	**CK INCQS 40095**	**CT INCQS 40042**
Fluconazole (FCZ)	30.38 ± 0.031	51.73 ± 0.027	12.19 ± 0.014
HEDa	≥ 512 ± 0.026	≥ 1024 ± 0.018	≥ 1024 ± 0.045

IC_{50} values with statistically significant difference ($p < 0.01$) when compared to the commercial antifungal, fluconazole (FCZ); CA: *Candida albicans*; CK: *Candida krusei*; CT: *Candida tropicalis;* HEDa: Extrato hidroalcoólico de *Dahlstedtia araripensis*. Source: Authors

3.2.2 Antibacterial assay

The antibacterial analysis revealed low action of the extract as an inhibitor of bacterial growth, with inhibitory concentrations starting at 512 µg/ml for most microorganisms. Best results were shown for Gram-positive bacteria *S. mutans* and *E. faecalis* with MIC of 256 µg/ml. These bacteria are oral cavity colonizers, and they are considered the main etiological agents in the formation of dental caries and biofilms (Franco et al. 2007). According to Corrêa (2007), plant extracts not only can control microbial proliferation, but are also associated with the removal of dental biofilms.

Streptococcus are the main cause of dental plaque; particularly, bacteria *S. mutans* and *S. sobrinus*, which participate in the first caries generation stage. Several studies have shown that tea-based treatments, which are rich in polyphenols, are effective in preventing oral infections caused by these microorganisms, thus suggesting that the use of extracts has potential clinical use in treating these infections (Li et al. 2019).

There are few literature reports on the antimicrobial activity of species of the genus *Dahlstedtia*. Regarding *D. araripensis* species, this is the first work that reports the action of the hydroalcoholic extract against different bacterial strains. Canzi et al. (2014) tested different extracts of *D. glaziovii*, in which there was no significant activity against strains of *E. coli*, *P. aeruginosa* and *S. Aureus*. The difference between the results may be associated with factors such as the method used, virulence of the microorganisms, and the chemical composition of the extract.

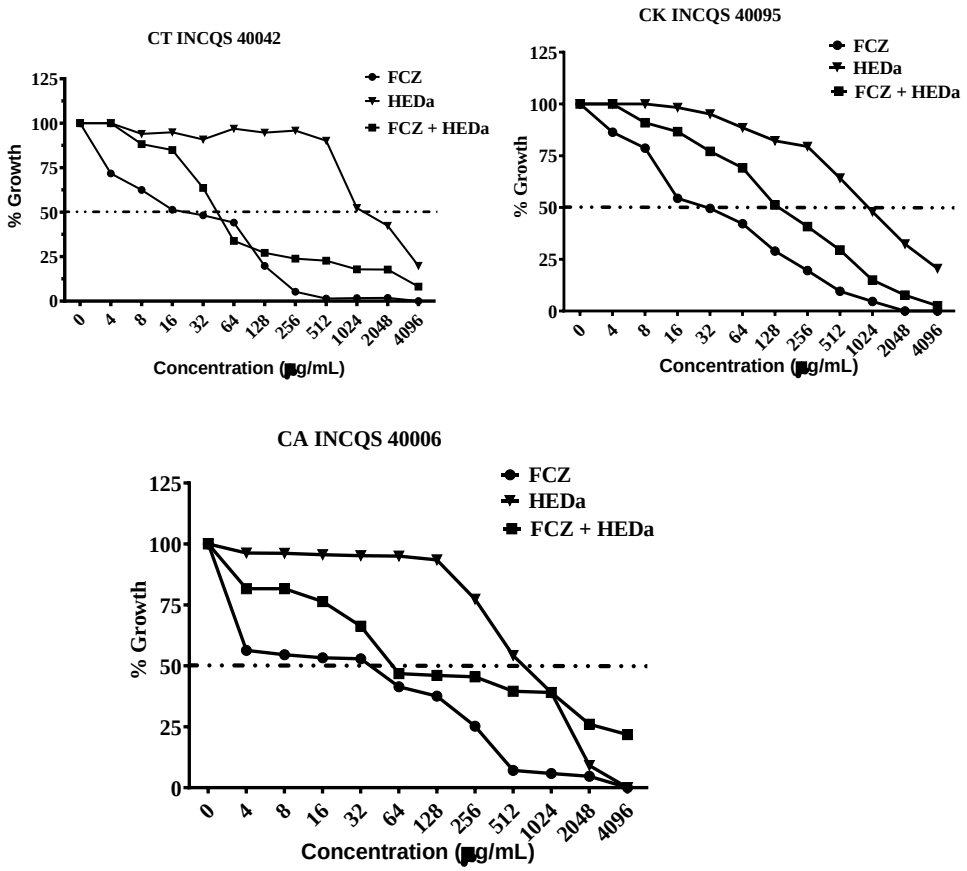
The lower MIC values observed for Gram-positive bacteria in this study are associated with morphological characteristics of these microorganisms, since Gram-negative bacteria have lipopolysaccharide layer that protect their cell wall, making the action of antimicrobials more difficult (Rabêlo et al. 2014). Phenolic compounds such as flavonoids and tannins are known for their antibacterial action, which hold a broad spectrum of action acting. The action of these compounds varies depending on the type of chemical structure, target bacterial cell, and the exposure time to the phenol-rich extract (Lima et al. 2019). Moreover, they can modify the microorganism's virulence profile through alterations in its genetic code, which can prevent functions such as the expression of efflux pumps, characterized as a resistance mechanism (Lima et al. 2019).

3.3 Antibiotics modulating effect

3.3.1 Modulatory effect on fungal strains

Through the study of the mixtures between HEDa and FCZ, it was found that the extract does not produce potentiation of the antifungal activity of the commercial drug. The subinhibitory concentrations were higher than the commercial drug tested. This can be analyzed by observing the viability curve of the fungal strains (Figure 1); therefore, the results indicate that HEDa has no antifungal activity when tested alone and antagonistic action when modulated with fluconazole.

Figure 1: Antifungal and modulatory activity of *Dahlstedtia araripensis* extract- HEDa; CT= *Candida tropicalis*; CK= *Candida krusei;* CA= *Candida albicans*; FCZ= fluconazole.



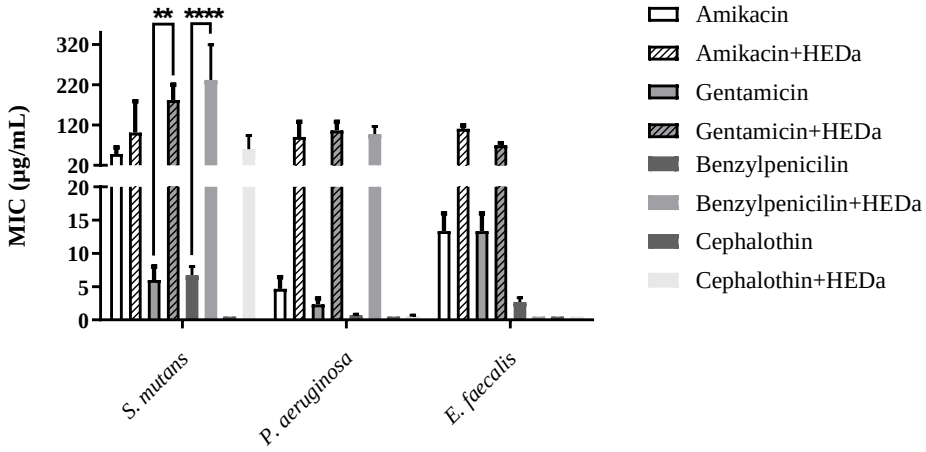
Source: Authors

Several resistance mechanisms such as biofilm formation and expression of resistance genes, especially those encoding efflux pumps have been developed by fungi against the action of specific drugs (Donlan, 2001; Braga et al. 2008). These mechanisms also suggest resistance of these microorganisms to the action of several plant extracts. This probably happened with the hydroalcoholic extract of *D. araripensis*, in which none of its concentrations inhibited the growth of the fungal strains tested; moreover, when combined with the antifungal drug fluconazole, HEDa demonstrated an antagonistic action, decreasing the efficacy of the commercial drug, and consequently interfering with clinical treatment.

3.3.2 Modulating effect on bacterial strains

The results of the modulating effect of antibiotics showed potentiation between the extract and benzylpenicillin against *E. faecalis*, decreasing the MIC value by 50 %; however, for the other bacteria, the extract decreased the efficiency of all tested antibiotics, having an antagonistic effect (Figure 2). The detection of antagonistic effects of extracts has fundamental importance in the efficiency of treatment, especially when the patient makes association of natural products, such as teas, with concomitant use of antibiotics, thus reducing the effect of the drug (Coutinho et al. 2013).

Figure 2. Antibacterial and modulating effect of the extract of *Dahlstedtia araripensis*.



Source: Authors.

Dental professionals often find themselves confronted lesions in dental canals caused by bacteria. *E. faecalis* is one of the main species which has been associated with this type of recurrent problem due to its morphological and genetic features. The resistance mechanisms of this microorganism originate from physiological factors, and changes in cell structure in response to the misuse of antimicrobials (Pinheiro et al. 2003; Endo et al. 2014). Some antibiotics, such as benzylpenicillin, are still considered effective in treating infections caused by this microorganism.

The resistance profile of 21 strains of *E. faecalis* isolated from the human oral cavity was reported in the work of Pinheiro et al. (2004), in which it was observed that the bacterial strains were sensitive to most antibiotics, resisting to azithromycin, erythromycin, tetracycline, and others. Although highly effective, benzylpenicillin is known for causing side effects associated with the nervous system, such as seizures, encephalopathy, and psychosis (Esposito et al. 2017). Consequently, the association of plant extracts, which can reduce the inhibitory concentration of this antibiotic is important to decrease its side effects, and the products generated from the plants can be sources of new antimicrobials or modulators of its activity.

4. Conclusion

The results obtained in this work indicate that the species D. araripensis has antimicrobial and antibiotic-modifying potential. These findings are probably associated with the classes of compounds identified, being necessary the continuation of the study to carry out the chemical profile of the species as well as to determine the possible mechanisms of action involved in its antimicrobial activity.

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