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Cariogenic Antibacterial Potential of *Stevia rebaudiana Bertoni* Leaves Extract Against *Lactobacillus acidophilus*

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ABSTRACT

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Dental caries is a chronic infectious disease in hard tooth tissues with various etiologies. The prevalence of dental caries in Indonesia is relatively high. One of the factors of dental caries etiology is cariogenic bacteria known as *Lactobacillus acidophilus*. These type of bacteria is acidogenic, aciduric, and can adhere to tooth enamel by synthesizing sticky glucan from sucrose to form dental biofilms. The existence of these bacteria in saliva proved to be contributing to the frequency and activity of dental caries, therefore it is important to inhibit their growth to prevent caries. *Stevia rebaudiana Bertoni* leaves extract is now being developed as an alternative to overcome the problems that are still encountered with the chlorhexidine, as the gold standard for controlling dental biofilms. The review aims to explain the cariogenic antibacterial potential of *Stevia rebaudiana Bertoni* leaves extract against *Lactobacillus acidophilus*. Literature search was carried out with sources from the EBSCO, PubMed, and ResearchGate database from the year 2012 to 2022 employing relevant keywords akin to the article titles. Antibacterial activity of *Stevia rebaudiana Bertoni* leaves extract in various solvents has been shown to be effective against *Lactobacillus acidophilus*. In this case, it may be due to the content of bioactive components of *Stevia rebaudiana Bertoni* leaves extract, such as flavonoids, alkaloids, saponins, sterols, and tannins. *Stevia rebaudiana Bertoni* leaves extract has the potential to inhibit the growth of *Lactobacillus acidophilus* hence, can be used as a natural alternative antibacterial agent to prevent dental caries.

Keywords: *Stevia rebaudiana Bertoni*, *Lactobacillus acidophilus*, cariogenic bacteria, antibacterial activity

⁷⁰ Introduction

Dental caries is a chronic infectious disease in hard tooth tissues that may be caused by various factors (Rathee & Sapra, 2020). Factors involved in the process of occurrence of dental caries include the attachment of bacteria to the surface of the teeth, the formation of biofilms (plaque) of the teeth, and the local demineralization of the tooth surface by acids produced by bacteria from the fermentation of carbohydrates consumed (Ajagannanavar et al., 2014; Rathee & Sapra, 2020). The process of dental caries generally occurs slowly influenced by the ecological balance between dental minerals and dental biofilms (Yadav, 2016). Dental caries that cause tooth crown damage until the tooth root is exposed, can be experienced by all age groups, both in deciduous and permanent teeth (Pitt et al., 2017).

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Dental caries is still a problem today around the world. Based on research data from the Global Burden of Disease (2019), oral diseases are experienced by approximately 3.5 billion people worldwide with caries in permanent teeth as the most suffered oral disease. An estimated 2 million people worldwide suffer from caries in permanent teeth and 520 million children suffer from caries in the firstborn teeth (WHO, 2022). Riskesdas (2018), stated that the proportion of tooth decay (dental caries)

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problems in Indonesia reaches 45.3%, which indicates that dental caries are still an important problem in Indonesia (Health & Indonesia, 2018).

Streptococcus mutans, *Streptococcus sobrinus*, *Lactobacillus acidophilus*, *Actinomyces viscus*, and *Bifidobacterium dentium* are pathogenic bacteria that generally cause of dental caries from a number of 200 species of bacteria isolated from dental biofilms (Cura et al., 2012; Samaranayake L, 2018). Lactic acid from the fermentation of carbohydrates mainly produced by *Lactobacillus acidophilus* as a cariogenic bacterium of the genus *Lactobacilli*, along with the bacteria *Streptococcus mutans*, is responsible for the demineralization of tooth enamel that triggers dental caries (Cura et al., 2012; Samaranayake L, 2018). The bacterium *Lactobacillus acidophilus* is often associated with dental caries in humans because it is able to tolerate acidic environments. This bacteria is also the main flora of the oral cavity involved in the development of caries particularly in dentin (Yadav & Prakash, 2017). The number of *Lactobacilli* bacteria in saliva has also been used to predict dental caries activity in an individual (Samaranayake, 2018).

Prevention of dental caries can be achieved in various ways including control of dental biofilms (plaque) by regular tooth brushing every day using toothpaste containing fluoride (Ajagannanavar et al., 2014; Yadav & Prakash, 2017). This method should also be combined with the use of chemical antimicrobial agents or chemoprophylactic agents such as chlorhexidine, to more effectively reduce cariogenic bacteria and inhibit the formation of dental biofilms of the oral cavity (Ajagannanavar et al., 2014). Chlorhexidine, is a broad-spectrum antiseptic, so it is used as a gold standard to chemically control dental biofilms. However, the long-term use of chlorhexidine mouthwash, can cause stains (staining) on the teeth and tongue, changes in taste and have a detrimental impact on the oral mucosa, and can cause bacterial resistance (Ajagannanavar et al., 2014; James et al., 2017). Consequently, researchers are now trying to find and apply natural antimicrobial ingredients from plants as therapeutic agents, given the low risk of side effects (Ajagannanavar et al., 2014; Moselhy et al., 2016).

Plant extracts are known to have many components of new compounds with extensive pharmacological activity (Ajagannanavar et al., 2014). *Stevia rebaudiana* Bertoni is a subtropical perennial shrub of the sunflower family (Asteraceae) native to Paraguay and Brazil (Brambilla et al., 2014; Lemus-Mondaca et al., 2012). This plant is known as sugar leaf or sweet leaf (Lemus-Mondaca et al., 2012; Shinde & Winnier, 2020b). *Stevia* leaves contain glycoside diterpenes such as stevioside, isosteviol, steviolbioside, rebaudioside A-F and dulcoside (Ibrahim et al., 2020; Lemus-Mondaca et al., 2012). Rebaudioside A and stevioside have a sweet taste 200-300 times sweeter than sucrose (Ajagannanavar et al., 2014; Das, 2013). *Stevia* is a noncalorical natural sweetener that is sweeter than sucrose with no adverse side effects on health. *Stevia rebaudiana* Bertoni has been proven to show various benefits to the systemic health of the body, especially for the health of the oral cavity (Contreras, 2013). The leaves of *Stevia rebaudiana* Bertoni also contain various bioactive components or secondary metabolites that contribute to its ability to treat diseases (Chughtai et al., 2020; Lemus-Mondaca et al., 2012). Several studies have reported the antimicrobial activity of *Stevia rebaudiana* Bertoni leaves extract against fungi and various gram-positive and gram-negative bacteria (Ibrahim et al., 2020; Siddique et al., 2014). In vitro studies have shown that *Stevia* leaves extract has antibacterial activity against *Streptococcus mutans*, but few studies have evaluated the antibacterial activity of *Stevia rebaudiana* Bertoni leaves extract against *Lactobacillus acidophilus* as a bacteria involved in dental caries (Mohammadi-Sichani, 2012; Sreekur & Hegde, 2018; Usha et al., 2017). This literature review was carried out to explain the antibacterial potential of *Stevia rebaudiana* Bertoni leaves extract against *Lactobacillus acidophilus*.

Material and Methods

The literature search from this study was carried out using the EBSCO, PubMed and ResearchGate databases from the year 2012 to 2022 using keywords related to the article title: "*Stevia rebaudiana* Bertoni", "*Lactobacillus acidophilus*", "cariogenic bacteria" and "antibacterial activity". Article selection

is carried out by reading the title, abstract and analyzing the overall content of each article. Articles that did not meet the inclusion criteria will be excluded. Article were withdrawn due to several reasons : not published between 2012 and 2022, they were not relevant to the topic, they were duplicated and were not written in English

Results and Discussion

Result

Lactobacillus acidophilus is a cariogenic bacteria that can ferment carbohydrates to form acids (acidogenic) and can live in an acidic environment (aciduric). *Lactobacillus acidophilus* is gram-positive bacteria, generally rod-shaped, α - or non-haemolytic and is facultative anaerobic. *Lactobacilli* are often isolated from deep dentin caries lesions with acidic pH conditions (Samaranayake, 2018). *Lactobacilli*, is a lactic acid bacterium capable of synthesizing extracellular polysaccharides such as glucans through extracellular enzymes, as one of the components that play a role in the formation of the matrix of extracellular biofilms, which cause dental caries (Jurášková et al., 2022). Efforts to inhibit the growth of these bacteria are necessary for the framework of preventing dental caries.

Current dental caries prevention strategies mainly include removing dental biofilms or killing oral cavity bacteria with antibacterial components (Ajagannavar et al., 2014). The inhibition of growth or selective killing of cariogenic bacteria in pathogenic dental biofilms will allow the formation of commensal bacterial communities that act as an effective barrier to prevent the colonization of cariogenic bacteria on the surface of the teeth thus supporting anti-caries therapy (Ajagannavar et al., 2014). Chlorhexidine as a bis-biguanide kills bacteria (bactericidal) and kills fungi (fungicidal). Chlorhexidine as an antibacterial agent with this broad spectrum is effective against gram-positive and gram-negative bacteria (Nagappan & John, 2012). The use of chlorhexidine as an antimicrobial agent with this broad-spectrum can damage the balance of bacteria inside the oral cavity, so it is necessary to control the dose only for patients with a high risk of caries for a limited period (Usha et al., 2017).

Natural products such as herbal extracts have been proven to be biocompatible with body tissues compared to synthetic chemicals (Usha et al., 2017). Herbal remedies of plant origin, have been used in dentistry to inhibit microorganisms, reduce inflammation, relieve irritation, and relieve pain (Gupta et al., 2015). *Stevia rebaudiana Bertoni* is a plant that has received a lot of attention recently. The two main components isolated from *Stevia* leaves, stevioside and rebaudioside, have a sweetness level 200-300 times sweeter than sucrose (Ajagannavar et al., 2014; Das, 2013). *Stevia* as a natural sweetener has good stability, does not contain calories, and have medicinal benefit as well (Ajagannavar et al., 2014; Rezaei-Soufi et al., 2016). *Stevia rebaudiana Bertoni* leaves also contain a number of bioactive components or secondary metabolites that contribute to treatment, such as phenolics, flavonoids, tannins, saponins, sterols (Chughtai et al., 2020; Lemus-Mondaca et al., 2012). *Stevia* leaves extract also contains water-soluble chlorophylls, xanthophylls, hydroxycinnamic acids (caffeine, chlorogenic), water-soluble oligosaccharides, amino acids, fats, essential oils, minerals, vitamins, and fiber (Khiraoui et al., 2017; Lemus-Mondaca et al., 2012; Shinde & Winnier, 2020a). *Stevia* leaves extract has antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anticancer, antidiabetic, antihypertensive and antimutagenesis activities (Chughtai et al., 2020; Elashtokhy & Taha, 2017; Gupta et al., 2013; Shinde & Winnier, 2020a; Yildiz-Ozturk et al., 2015). *Stevia* can grow up to 1 m or more on moist soils as well as suitable drainage systems (Lemus-Mondaca et al., 2012). *Stevia* has an extensive root system and a weak stem. *Stevia* leaves do not have petioles, spatula-shaped with a blunt leaves tip and jagged leaves edge from the middle to the tip of the leaves with a leaves length of 3-4 cm. *Stevia* plant has small flowers of white color. The temperature required for optimal growth is 20-24° C (Ajagannavar et al., 2014; Rezaei-Soufi et al., 2016). *Stevia*'s natural sweetener has been known to be noncariogenic (Gamboa & Chaves, 2012). *Stevia rebaudiana Bertoni* leaves extract has been shown to have a property to prevent caries because it contains antibacterial properties and can reduce carbohydrate intake that can be fermented by bacteria (Brambilla et al., 2014).

Research by Usha et al. (2017) was conducted to determine the effect of mouthwash *Stevia rebaudiana Bertoni* leaves extract 0.5% (aqueous leaves extract) in patients with a high risk of caries. The study concluded that *Stevia rebaudiana Bertoni* 0.5% leaves extract was able to improve the pH and buffering capacity of saliva in patients with high risk of caries and effectively reduced cariogenic bacteria in saliva in a period of 1 week after rinsing the mouth 2 times a day for 7 days. The chlorhexidine mouthwash group of 0.12% and the *Stevia rebaudiana Bertoni* leaves extract mouthwash 0.5% showed a reduction in the number of *Streptococcus mutans* and *Lactobacilli* bacteria up to 10⁵ colony forming units (CFUs)/ml in all patients after stimulated saliva from both groups of mouthwash treatments were cultured and incubated for 48 hours.

Another study by Gamboa and Chaves (2012) has explained the antibacterial potential of *Stevia* leaves extract in various types of solvents against sixteen groups of the bacterial genus *Streptococcus* and *Lactobacillus* related to the development of caries. The antibacterial activity of *Stevia* leaves extract was tested using the method of diffusion of Agar wells (well diffusion) with vancomycin and azithromycin as the positive control group and each type of solvent as a negative control. The minimal inhibitory concentration (MIC) values of *Stevia* leaves extract in hexane, methanol, ethanol, ethyl acetate, and chloroform solvents against 16 bacterial strains of the genus *Streptococcus* and *Lactobacillus* were 30 mg/ml, 120 mg/ml, 120 mg/ml, 60 mg/ml, and 60 mg/ml, respectively. The bacterial inhibition zone of *Stevia* leaves extract in 5 types of such solvents varies from 9 mm to 17.3 mm. The bacterial inhibition zone of *Stevia* leaves extract in the 5 types of solvents was slightly larger against 4 strains of *Lactobacillus* compared to 12 strains of *Streptococcus*, with the smallest value of 12.3 mm and the largest of 17.3 mm. The inhibition zone of *Stevia* leaves extract against *Lactobacillus acidophilus* bacteria ATCC 4365 in solvents hexane, methanol, ethanol, ethyl acetate, and chloroform is 13.6 mm, 13.0 mm, 14.0 mm, 15.0 mm, and 17.3 mm, respectively. The positive control group (vancomycin 180 g/ml and azithromycin 150 g/ml) had bacterial growth inhibition activity varying from the 16 strains tested, with inhibition zone values of 18 mm to 25 mm. The results of this study showed that all *Stevia* leaves extracts antibacterial activity on MICs between 30 mg/ml to 120 mg/ml. In *Stevia* leaves extract MICs 60 mg/ml ethyl acetate and 60 mg/ml chloroform, the bacterial inhibition zone against four *Lactobacillus* species, appeared slightly larger.

Research by Tiwari et al. (2018) has been carried out to compare the efficacy of *Stevia* leaves extract in 1% aqueous suspension, in 5% alcohol, and 6% both combinations against *Streptococcus mutans* and *Lactobacillus acidophilus*. Tests were carried out to determine the average minimum inhibitory concentration (MIC) through the broth dilution method and the average minimum bactericidal concentration (MBC) through the agar plate sub culture streaking method at various concentrations for the three types of *Stevia* leaves extract. The average MIC value of *Stevia* leaves extract in 1% aqueous suspension against *Streptococcus mutans* and *Lactobacillus acidophilus* appeared to be significantly better ($p < 0.05$) which was 0.83 g/200 ml and 0.66 g/200 ml, respectively, compared to the MIC value of *Stevia* leaves extract in 5% alcohol which was 4.16 g/200 ml and 3.3 g/200 ml and compared to the MIC value 6% combination of the two extracts, which was 5 µg/200 ml and 5 µg/200 ml, sequentially. This study concluded that the inhibitory effect of *Stevia* leaves extract in 1% aqueous suspension against *Streptococcus mutans* and *Lactobacillus acidophilus* was better than *Stevia* leaves extract in 5% alcohol and 6% combination of both extracts. However, there is still a lag from the results of this study because MBC values cannot be observed in *Stevia* leaves extract in 1% aqueous suspension and it is likely to need to increase concentrations to be able to observe the MBC ratio.

On previous study done by Ajagannanavar et al. (2014) compared the antibacterial efficacy of *Stevia rebaudiana Bertoni* leaves extract in aqueous solvents and alcohol (ethanol) solvents versus chlorhexidine against *Streptococcus mutans* and *Lactobacillus acidophilus* at various concentrations. The test of determining the inhibitory zone of bacteria against *Streptococcus mutans* and *Lactobacillus acidophilus* was carried out using the Agar disk diffusion method and the determination of the

28 minimum inhibitory concentration (MIC) using the serial broth dilution method. The MIC value of 10 *Stevia rebaudiana Bertoni* leaves extract in an aqueous solvent against *Lactobacillus acidophilus* is 50% and 25% against *Streptococcus mutans*. The MIC value of *Stevia rebaudiana Bertoni* leaves extract in alcohol solvents (ethanol) 35 against *Lactobacillus acidophilus* was 6.25% and 12.5% against *Streptococcus mutans*. The antibacterial activity of *Stevia rebaudiana Bertoni* leaves extract in an aqueous solvent against *Lactobacillus acidophilus* is 10.8 mm, while *Stevia rebaudiana Bertoni* leaves extract in alcohol solvent (ethanol) is 12.3 mm, 47 chlorhexidine as a positive control group is 13.2 mm. The average bacterial inhibition zone of *Stevia rebaudiana Bertoni* leaves extract in water solvent against *Streptococcus mutans* was 22.8 mm, while *Stevia rebaudiana Bertoni* leaves extract in alcohol solvent (ethanol) was 24.7 mm, and chlorhexidine as a positive control group was 26.5 mm. This study concluded that the effect of inhibiting bacterial growth from *Stevia rebaudiana Bertoni* leaves extract in alcohol solvents (ethanol) against *Lactobacillus acidophilus* and *Streptococcus mutans* was better than *Stevia rebaudiana Bertoni* leaves extract in aqueous solvent but lower than chlorhexidine.

Discussion

According to the findings of a study 17 conducted by Usha et al. (2017) both 0.5% aqueous extract of *Stevia leaves* and 0.12% chlorhexidine mouthwash were effective in reducing the cariogenic microbial (*S. mutans* and *Lactobacilli*) count. The result is consistent with the previous study done by Gamboa & Chaves (2012), which evaluated the antibacterial activity of *Stevia rebaudiana Bertoni* leaves extract against cariogenic bacteria in vitro, one of which is *Lactobacillus acidophilus*. In the study done by Usha et al (2017) an aqueous extract of *Stevia leaves* was planned to be used as a mouthwash for patient safety. Although Usha et al (2017) found that 10⁵ CFU/ml of *S. mutans* and *Lactobacilli* in saliva is considered low caries index, long-term clinical studies are required to prove its efficacy, similar to that of chlorhexidine as a broad-spectrum antimicrobial agent.

In a study done by Gamboa & Chaves (2012) 30 the inhibition zone of *Stevia leaves* extract against bacteria *Lactobacillus acidophilus* ATCC 4365 39 in hexane, methanol, ethanol, ethyl acetate, and chloroform were respectively 13.6 mm, 13.0 mm, 14.0 mm, 15.0 mm, and 17.3 mm. The inhibition zones of the 5 extracts were slightly higher for the 4 *Lactobacillus* strains than for the 12 *Streptococcus* strains, primarily in the 60 mg/ml ethyl acetate and 60 mg/ml chloroform extracts, indicating that they 33 the most susceptible microorganisms. Their increased susceptibility to the various extracts could be attributed to the presence of active substances in *Stevia rebaudiana Bertoni* leaves extract or to active metabolites that are most soluble and act as antibacterial substances in these solvent systems. that can easily penetrate the bacteria and cause more damage (Gamboa & Chaves, 2012; Mohammadi-Sichani, 2012).

The reason why the mean inhibition rates were higher with alcoholic (ethanol) extract of *Stevia leaves* against *Lactobacillus acidophilus* than in the aqueous form in the study by Ajagannanavar et al. (2014) is unknown. However, the ethanolic extract of *Stevia rebaudiana Bertoni* showed better inhibitory results than the aqueous extract, which could be attributed to better dissolving capacity in alcohol, better bioavailability (thus increasing bioactivity), and the polarity of the antibacterial compounds, which allows the compounds to be extracted more easily by organic solvents. The limitation of this study was that it could not have been conducted with other group of 70% ethyl alcohol to state that it was the effect of *Stevia* alone that inhibited *Lactobacillus acidophilus* and *Streptococcus mutans* and not alcohol (Ajagannanavar et al., 2014) In contrast to this study, Tiwari et al. (2018) found that an aqueous suspension of *Stevia rebaudiana Bertoni* outperformed inhibitory result compare to its alcoholic extract and their combination, possibly due to the method of preparation of the suspension, which avoided the extraction process, that preserved the active components and anti-oxidants such as tannins, xanthine (theobromine and caffeine), and flavonoids in stevioside.

² *Stevia rebaudiana Bertonii* leaves extract has been scientifically proven to have antibacterial activity against *Lactobacillus acidophilus* in a variety of solvents. These could be attributed to bioactive compounds found in *Stevia* leaves extract. Gupta E et al. (2017) discovered the presence of phenols as the most abundant bioactive constituents or phytochemicals in methanol and ethanolic extracts of *Stevia rebaudiana Bertonii*, followed by phytosterols, tannins, saponins, glycosides, and flavonoids. The preliminary analysis of water and acetone extract conducted by ⁶¹ Moselhy et al (2016) showed that acetone extract contains more phenolic and flavonoids than aqueous extract. The total phenolic content of the aqueous and acetone extracts was 30 and 85 mg gallic/gm, respectively. While total flavonoids were discovered to be 60 mg and 80 mg/gm, respectively. It was discovered that the antibacterial activity of acetone extract differed in terms of growth inhibition against selected five bacteria species. The presence of a high flavonoid content could explain the antibacterial activity. The inhibition zone diameter (mm) of the acetone extract revealed a variable inhibitory effect on bacteria at different concentrations. The analysis of ³⁴ chromatography and mass spectroscopy in this study revealed that monoterpene and indole are ²⁴ main components of the essential oil of *Stevia* leaves extract (Moselhy et al., 2016). Preliminary phytochemical screening of different extracts ⁶⁴ *Stevia rebaudiana Bertonii* by Siddique et al. (2014) revealed that alkaloids ²⁴ and steroids were the most abundant compounds in the *Stevia rebaudiana Bertonii* leaves extract, followed by tannins, saponins, and flavonoids. ¹⁰

The presence of these bioactive constituents or phytochemicals in the extract of *Stevia rebaudiana Bertonii* leaves from those studies provides antimicrobial property. The presence of bioactive components in these extracts generally inhibits microorganism growth and metabolism and is quantified by determining the minimum bactericidal activity and minimum inhibitory concentration (Gupta et al., 2017).

These medicinally bioactive components have ⁴⁵ antimicrobial activity via various mechanisms. Tannins inhibit the synthesis of bacterial cell walls by forming irreversible complexes with proline-rich protein. Saponins can cause protein and enzyme leakage ⁴⁵ from bacterial cells. Terpenoids are responsible for the ³⁴ dissolution of microorganism cell walls by weakening the membranous tissue. Flavonoids, which have been discovered ³⁴ to be effective antimicrobial substances against a wide range of microorganisms, can complex with extracellular and soluble proteins, as well as bacterial cell walls. Steroids are also known for their bacterial activity, which is ⁸ specifically associated with membrane lipids and causes leakage from liposomes ⁸ (Mujeeb et al., 2014). Through lysis activities of the bacterial cell wall, alkaloid compounds can inhibit bacterial cell wall synthesis. Furthermore, alkaloid compounds can interfere with the formation of peptidoglycan constituents in bacterial cells, resulting in bacterial cell wall layer failure (Hastuty, 2019).

In addition, the presence of steviol glycosides in *Stevia* leaf extract may inhibit ⁶⁷ the growth of cariogenic bacteria (Wölwer-Rieck, 2012). This is consistent with the findings of an in vitro study conducted by Brambilla et al. (2014), who discovered that *Stevia rebaudiana Bertonii* leaves extract, which contained stevioside and rebaudioside A, did not support the growth of cariogenic bacteria, including *Streptococcus mutans*.

More research is required to determine the most potent bioactive component of *Stevia rebaudiana Bertonii* leaves extract was tested against the cariogenic bacterial *Lactobacillus acidophilus*, as well as its antibacterial activity against a variety of microorganisms known as a cause of dental caries.

Conclusion

Stevia rebaudiana Bertonii leaves extract has the potential to inhibit the growth of the cariogenic bacteria *Lactobacillus acidophilus*, hence can be used as a natural alternative antibacterial agent or natural remedy to prevent dental caries.

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