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Original Article

Evaluation of antimicrobial effect of azadirachtin plant extract (Soluneem™) on commonly found root canal pathogenic microorganisms (viz. *Enterococcus faecalis*) in primary teeth: A microbiological study

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ABSTRACT

Aim: The aim of this study is to evaluate the antimicrobial activity of Soluneem™ when used as an irrigating solution along with other commonly used irrigating solution sodium hypochlorite (NaOCl) against *Enterococcus faecalis*. **Materials and Methods:** Microorganism used in this study was *E. faecalis* (Microbial Type Culture Collection 439). Test substance used was Soluneem™, which was obtained from Vittal Mallya Scientific Research Foundation (VMSRF), Bengaluru. This study was conducted in a microbiology laboratory (Biocare Research India Pvt., Ltd. Laboratory, Ahmedabad, Gujarat) to evaluate the antimicrobial effect of Soluneem™ (Azadirachtin) on *E. faecalis*. Antimicrobial activity testing was performed using the macrobroth dilution method according to the Clinical Laboratory Standards Institute guidelines. All determinations were performed thrice. **Results:** Minimum bactericidal concentration (MBC) was seen as 2.6% for Soluneem™ while the same was seen at 0.1% for NaOCl. Independent sample *t*-test was carried out to compare the MBC of Soluneem™ and NaOCl, which showed that there was no statistically significant difference between them, i.e., 2.6% Soluneem™ was as effective as 0.1% NaOCl. **Conclusion:** Soluneem™ showed antimicrobial activity against *E. faecalis* at various concentrations. It was also found that the efficacy of Soluneem™ at 2.6% concentration and above was relatively similar to that of gold standard irrigating solution (NaOCl) on inhibition of *E. faecalis*.

KEYWORDS: Antimicrobial efficacy, *Enterococcus faecalis*, macrobroth dilution, sodium hypochlorite, Soluneem™

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Introduction

Complete debridement and effective disinfection of the root canal space is an important prerequisite for achieving long-term success of nonsurgical endodontics.[1] Microorganisms and their by-products are considered

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to be the primary etiologic agents in endodontic diseases. Failure, during and after endodontic treatment, is linked to the presence of bacteria in the root canal.[2]

Enterococcus faecalis and *Candida albicans* are known to be important resistant species in infected root canals, and they may cause treatment failures.[2]

E. faecalis, a Gram-positive cocci, is associated with different forms of periradicular diseases, including primary endodontic infections and persistent infections.[3]

Numerous irrigants have been recommended for use in the treatment of root canal infections. Sodium hypochlorite (NaOCl) has been widely used as an irrigant since its introduction in endodontics by Walker in 1936.[2] NaOCl is extremely toxic to periapical tissues if injected beyond apex.[3]

Hence, there is a need to find a substance which can be used to reduce or eradicate *Streptococcus mutans* and *E. faecalis* with least amount of side effects.

A predominant trend in modern dentistry has been to search for biocompatible agents, especially those to be used in direct contact with tissues. In this context, phytotherapy has evolved as a science, and there has been growing interest in evaluating plant extracts with a potential therapeutic application in dentistry.[4]

The antibacterial activity of neem has been evaluated and known from ancient times. It has been considered to have various activities such as astringent, antiseptic, insecticidal, anti ulcer and for cleaning the teeth in pyorrhea and other dental diseases. Apart from this the leaf extract of neem showed superior antiviral and antihyperglycemic activity *in vitro* and *in vivo* on animals. It had showed good *in vitro* broad range antibacterial activity.[5]

Azadiractin is a secondary metabolite present in neem tree seeds.

Soluneem™ is a unique, world's first water-soluble antimicrobial agent consisting of various limonoids, primarily azadirachtin – A, derived from neem seed kernel. It is produced by a novel patented technology that enhances thermostability and bioavailability, and has a greater shelf life. It is highly stable even at 110°C (thermostable) whereas technical Neem Seed Kernel Extracts are thermolabile. Soluneem™ is available as off-white, amorphous powder, which could be stored for more than 2 years without loss in bioactivity. It instantly dissolves in water to give a clear solution that is ready for use.[6]

Aim

The aim of this study was to evaluate the antimicrobial activity of Soluneem™ when used as an irrigating solution along with other commonly used irrigating solution (NaOCl) against *E. faecalis*.

Materials and Methods

Microorganism used in this study was *E. faecalis*. The strain was purchased from Microbial Type Culture Collection (MTCC 439), Institute of Microbial Technology (IM-TECH), Chandigarh, India. Soluneem™ was obtained from Vittal Mallya Research Institute, Bengaluru. The study was conducted in a microbiology laboratory (Biocare Research India Pvt., Ltd. Laboratory, Ahmedabad, Gujarat) to evaluate the antimicrobial effect of Soluneem™ (Azadirachtin) on *E. faecalis*, which is responsible for root canal failure.

The substances used in the study were divided into following groups:

- Group 1: Soluneem™
- Group 2: NaOCl
- Group 3: Normal saline (negative control).

Antimicrobial activity testing was performed using the macrobroth dilution method according to the Clinical Laboratory Standards Institute guidelines. By adjusting density standards (Densimate Nephelometer, Densimat Biomerieux France), the microorganism suspensions were prepared to equal the density of a 0.5 McFarland standard (1.5×10^8 CFU/ml for bacteria). Cultures of *E. faecalis* were maintained on brain heart infusion (BHI) broth and agar at 37°C.

Stock solutions were used as follows:

- Group 1: Soluneem (20 mg/100 ml of distilled water, i.e., 20%)
- Group 2: NaOCl (3%)
- Group 3: Normal saline.

Microorganisms were then inoculated as 1.5×10^5 CFU/ml for *E. faecalis*, in the final concentrations. *E. faecalis* was incubated at 37°C for 24 h. All determinations were performed thrice. This was done to find out the inhibitory effect of various test substances against *E. faecalis*.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was defined as the lowest concentration that did not result in any visible growth of the microorganisms compared with the growth in the control tubes.

Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined by spreading samples from each tube with a concentration equal, or higher than the MIC (i.e., tubes that showed no visible growth) onto the surface of BHI agar plates for *E. faecalis*, and incubating it at 37°C for 24 h. The MBC was determined to be the lowest concentration that precluded bacterial growth on the BHI agar plate [Figure 1].

Results

The results obtained were statistically analyzed using one-way ANOVA followed by Tukey honest significant difference (HSD) test.

Normal saline was included as the negative control in the study and it did not have any inhibitory effect against *E. faecalis*.

About 0.1-10% concentration of Soluneem™ was tested for their inhibitory effect on *E. faecalis*. Concentration below 2% showed no inhibitory effect against *E. faecalis*, whereas at 2% first inhibitory effect was observed [Table 1 and Graph 1].

One-way ANOVA done for Soluneem™ showed no statistically significant difference between samples of different concentrations but showed no statistically significant difference between samples of same concentrations [Table 2].

Multiple comparisons using Tukey HSD test showed statistically significant difference between 2% concentration and concentrations 3% and above ($P < 0.05$, Table 3). No statistically significant difference was observed between concentration 3% and above (i.e., $P > 0.05$, Table 3).

Hence to find out at which particular concentration complete inhibition was obtained, further test was carried out using concentration between 2.5% and 3% [Table 4]. Data were then subjected to one-way ANOVA followed by Tukey HSD test and it was found that maximum inhibition of *E. faecalis* was at 2.6% concentration and all the concentrations above that were similar [Table 3 and Graph 2]. The 0.1-3% concentration of NaOCl was tested for their inhibitory effect on *E. faecalis*. Concentration of 0.1% and above showed an inhibitory effect against *E. faecalis* [Table 5, Graphs 3 and 4].

One-way ANOVA done for NaOCl showed no statistically significant difference between samples of same concentrations and also there was no statistically significant difference seen between various concentrations [Table 6]. This was followed by Tukey HSD test which showed that there was no statistically significant difference between concentrations 0.1% and above (i.e., $P > 0.05$, Table 7), which means that 0.1% concentration was as effective as concentrations higher than that.

MBC was seen as 2.6% for Soluneem™ while the same was seen at 0.1% for NaOCl.

Independent sample *t*-test was carried out to compare the MBC of Soluneem™ and NaOCl, which showed that there is no statistically significant difference between them. Hence, 2.6% Soluneem™ is as effective as 0.1% NaOCl [Table 8].

Discussion

Root canal irrigation plays a key role in the success of endodontic treatment, as it helps in progressive removal of the smear layer and neutralizes the root canal microbial flora.[7]

Total cleaning of root canal system using mechanical instrumentation is ineffective due to extremely complex root canal anatomy. Irrigants serve several purposes including tissue solvent, disinfection, flushing of gross debris, and lubrication. Proper irrigation of root canal system during endodontic treatment is essential for successful treatment.[8]

Table 1: Correlation between various concentrations of Soluneem™ solution and microbial load encountered in respective samples (using one-way ANOVA test)

Concentration	*n*	Mean	SD
2.00	3	50.00	8.000
3.00	6	0.50	0.837
4.00	3	0.00	0.000
5.00	3	0.00	0.000
6.00	3	0.00	0.000
7.00	3	0.00	0.000
8.00	3	0.00	0.000
9.00	3	0.00	0.000
10.00	3	0.00	0.000
Total	45		

**n* = Number of samples of a particular concentration, SD = Standard deviation

Table 2: Statistical analysis through one-way ANOVA test of the data collected for seeing the inhibitory effect of Soluneem™ solution on *Enterococcus faecalis*

Samples studied	Sum of squares	Mean square	ANOVA (*P*)
Between different concentrations	7187.078	552.852	<0.0001
Between different samples of same concentration	166.167	5.360	
Total	7353.244		

Figure 1: Subculturing for minimum bactericidal concentration

Table 3: Multiple comparison between various concentrations of Soluneem™ by Tukey honest significant difference (HSD) test

Concentrations compared		Mean difference (I – J)	SE	P
Concentration in percentage (I)	Concentration in percentage (J)			
2	2.6	49.33	1.89	<0.0001
2	2.7	49.33	1.89	<0.0001
2	2.8	49.33	1.89	<0.0001
2	2.9	49.67	1.89	<0.0001
2	3	49.5	1.637	<0.0001
2	4	50	1.89	<0.0001
2	5	50	1.89	<0.0001
2	6	50	1.89	<0.0001
2	7	50	1.89	<0.0001
2	8	50	1.89	<0.0001
2	9	50	1.89	<0.0001
2	10	50	1.89	<0.0001
2.5	2.6	13.33	1.89	<0.0001
2.5	2.7	13.33	1.89	<0.0001
2.5	2.8	13.33	1.89	<0.0001
2.5	2.9	13.67	1.89	<0.0001
2.5	3	13.5	1.637	<0.0001
2.5	4	14	1.89	<0.0001
2.5	5	14	1.89	<0.0001
2.5	6	14	1.89	<0.0001
2.5	7	14	1.89	<0.0001
2.5	8	14	1.89	<0.0001
2.5	9	14	1.89	<0.0001
2.5	10	14	1.89	<0.0001
2.6	2.8	0	1.89	1.0000
2.6	2.9	0.33	1.89	1.0000
2.6	3	0.17	1.637	1.0000
2.6	4	0.67	1.89	1.0000
2.6	5	0.67	1.89	1.0000
2.6	6	0.67	1.89	1.0000
2.6	7	0.67	1.89	1.0000
2.6	8	0.67	1.89	1.0000
2.6	9	0.67	1.89	1.0000
2.6	10	0.67	1.89	1.0000
2.7	2.8	0.67	1.89	1.0000
2.7	2.9	0.33	1.89	1.0000
2.7	3	0.17	1.637	1.0000
2.7	4	0.67	1.89	1.0000
2.7	5	0.67	1.89	1.0000
2.7	6	0.67	1.89	1.0000
2.7	7	0.67	1.89	1.0000
2.7	8	0.67	1.89	1.0000
2.7	9	0.67	1.89	1.0000
2.7	10	0.67	1.89	1.0000
2.8	2.9	0.33	1.89	1.0000
2.8	3	0.17	1.637	1.0000
2.8	4	0.67	1.89	1.0000
2.8	5	0.67	1.89	1.0000
2.8	6	0.67	1.89	1.0000
2.8	7	0.67	1.89	1.0000
2.8	8	0.67	1.89	1.0000
2.8	9	0.67	1.89	1.0000

Table 3: Continued

Concentrations compared		Mean difference (I – J)	SE	P
Concentration in percentage (I)	Concentration in percentage (J)			
2.8	10	0.67	1.89	1.0000
2.9	3	−0.17	1.637	1.0000
2.9	4	0.33	1.89	1.0000
2.9	5	0.33	1.89	1.0000
2.9	6	0.33	1.89	1.0000
2.9	7	0.33	1.89	1.0000
2.9	8	0.33	1.89	1.0000
2.9	9	0.33	1.89	1.0000
2.9	10	0.33	1.89	1.0000
3	4	0.5	1.637	1.0000
3	5	0.5	1.637	1.0000
3	6	0.5	1.637	1.0000
3	7	0.5	1.637	1.0000
3	8	0.5	1.637	1.0000

SE = Standard error

Table 4: Correlation between 2.6% and 3% concentrations of Soluneem™ solution and microbial load encountered in respective samples (using one-way ANOVA test)

Concentration	n	Mean	SD
2.50	3	14.00	4.000
2.60	3	0.67	0.577
2.70	3	0.67	0.577
2.80	3	0.67	0.577
2.90	3	0.33	0.577

SD = Standard deviation

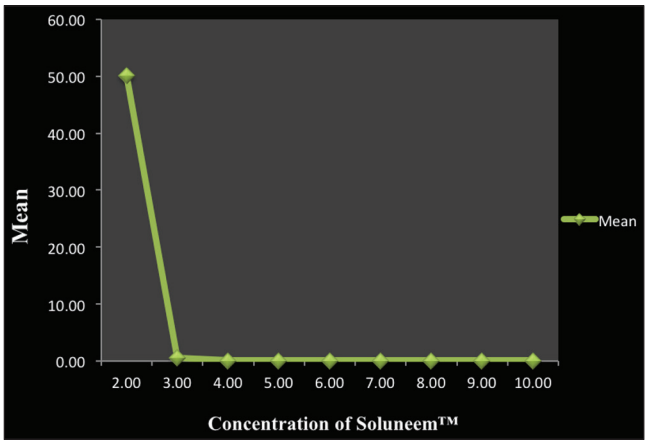
Table 5: Correlation between various concentrations of sodium hypochlorite solution and microbial load encountered in respective samples (using one-way ANOVA test)

Concentration	N	Mean	SD
0.10	3	0.67	0.577
0.20	3	0.67	0.577
0.30	3	0.67	0.577
0.40	3	0.33	0.577
0.50	3	0.00	0.000
1.00	3	0.00	0.000
2.00	3	0.00	0.000
3.00	3	0.00	0.000
Total	24	0.29	0.464

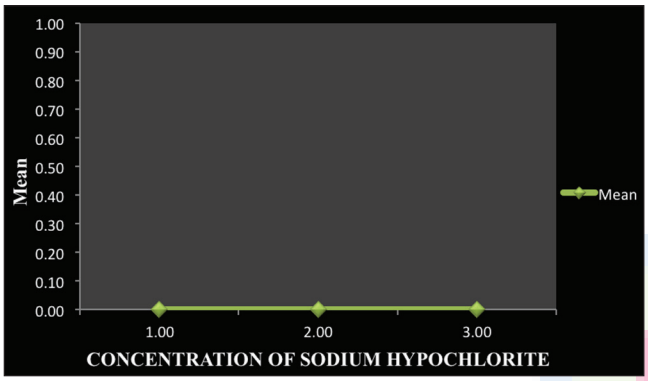
*n = Number of samples of a particular concentration, SD = Standard deviation

Various irrigating solutions have been used in root canal treatment. Among them the most commonly used are NaOCl and chlorhexidine, and this can be attributed to their antimicrobial action.[4]

This *in vitro* study was designed to assess the antibacterial effects of most commonly used root canal irrigant (i.e., NaOCl) along with a newer herbal extract of neem (Soluneem™) against *E. faecalis*.



Graph 1: Mean microbial load after exposing *E. faecalis* to various concentrations of Soluneem™



Graph 3: Mean microbial load after exposing *E. faecalis* to various concentrations of sodium hypochlorite

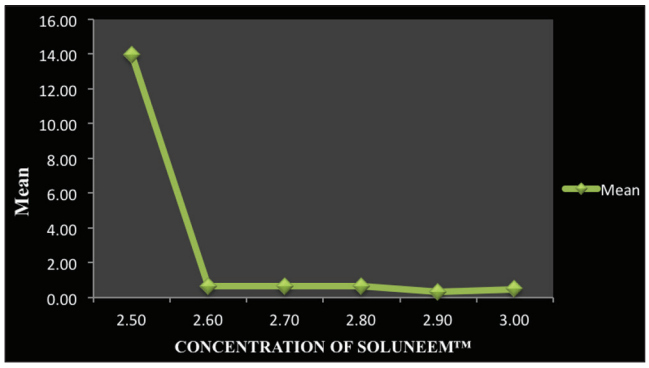
Table 6: Statistical analysis through one-way ANOVA test of the data collected for seeing the inhibitory effect of sodium hypochlorite solution on *Enterococcus faecalis*

Samples studied	Sum of squares	Mean square	ANOVA (*P*)
Between different concentrations	2.292	0.327	0.125
Between different samples of same concentration	2.667	0.167	
Total	4.958		

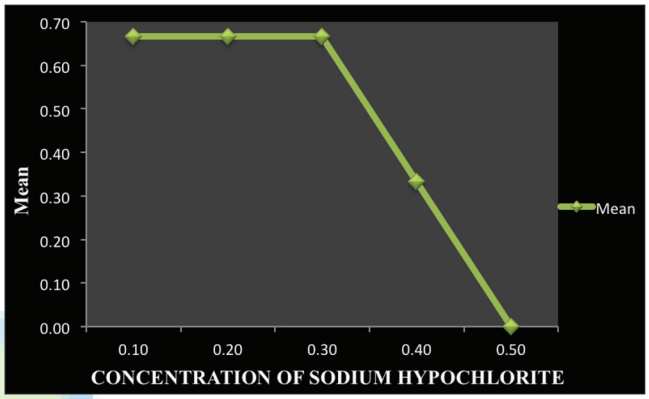
E. faecalis is a facultative anaerobic Gram-positive coccus and is implicated in persistent root canal infections[9-13] and has been used in several previous studies on the efficacy of endodontic irrigants, especially for its high level of resistance to a wide range of antimicrobial agents.[7]

In this study, MTCC strain was used because it was also utilized in previous *in vitro* studies investigating the antibacterial effects of endodontic irrigants. BHI medium was used in this study.[1,14-16]

NaOCl is a commonly used intracanal irrigating solution, and its antimicrobial properties are attributed



Graph 2: Mean microbial load after exposing *E. faecalis* to 2.5-3% concentrations of Soluneem™



Graph 4: Mean microbial load after exposing *E. faecalis* to various concentrations of sodium hypochlorite

to HOCl acid.[17] It has been used for a long time in endodontics as a root canal irrigating solution at different concentrations ranging from 0.5% to 5%. In this study, 0.05-3% NaOCl was used. Several other studies have demonstrated the antimicrobial action of NaOCl on *E. faecalis* with concentration as low as 0.1% which is in accordance with this study.[7]

Even though antibacterial effects of NaOCl are recognized, the exact mechanism of microbial killing is not well elucidated. When NaOCl is added to water, HOCl acid is formed, which contains active chlorine, a strong oxidizing agent.[18]

However, on the other hand, NaOCl presents negative properties such as corrosion of endodontic instruments, ineffectiveness for some microorganisms when used at low concentrations and not able to differentiate between necrotic and vital tissue when in contact with apical and periapical tissue,[7] it has various other disadvantages such as unpleasant taste and potential weakening of tooth structure by decreasing hardness and structural integrity of dentin within the root canal.[8]

There is a growing interest throughout the oral health-care profession in incorporating herbal products in oral health-care products for eradicating caries, decreasing plaque, and root canal irrigation.[8]

Table 7: Multiple comparisons between various concentrations of sodium hypochlorite by Tukey highly significant difference test

Concentrations compared		Mean difference (*I* − J)	SE	*P*
Concentration in percentage (*I*)	Concentration in percentage (*J*)			
0.1	0.2	0.00	0.333	1.000
0.1	0.3	0.00	0.333	1.000
0.1	0.4	0.33	0.333	0.968
0.1	0.5	0.67	0.333	0.511
0.1	1	0.67	0.333	0.511
0.1	2	0.67	0.333	0.511
0.1	3	0.67	0.333	0.511
0.2	0.3	0.00	0.333	1.000
0.2	0.4	0.33	0.333	0.968
0.2	0.5	0.67	0.333	0.511
0.2	1	0.67	0.333	0.511
0.2	2	0.67	0.333	0.511
0.2	3	0.67	0.333	0.511
0.3	0.4	0.33	0.333	0.968
0.3	0.5	0.67	0.333	0.511
0.3	1	0.67	0.333	0.511
0.3	2	0.67	0.333	0.511
0.3	3	0.67	0.333	0.511
0.4	0.5	0.33	0.333	0.968
0.4	1	0.33	0.333	0.968
0.4	2	0.33	0.333	0.968
0.4	3	0.33	0.333	0.968
0.5	1	0.00	0.333	1.000
0.5	2	0.00	0.333	1.000
0.5	3	0.00	0.333	1.000
1	2	0.00	0.333	1.000
1	3	0.00	0.333	1.000
2	3	0.00	0.333	1.000

SE = Standard error

Table 8: Independent sample *t*-test

Concentration	*n*	Mean	SD	*P*
2.60 (minimum bactericidal concentration of Soluneem™)	3	0.67	0.577	1.00
0.10 (minimum bactericidal concentration of sodium hypochlorite)	3	0.67	0.577	

**n* = Number of samples of a particular concentration, SD = Standard deviation

The major advantages of using herbal alternatives are easy availability, cost effectiveness, increased shelf life, low toxicity, and lack of microbial resistance reported so far.[18]

Neem is of particular interest in the field of dentistry for it has a long history treating teeth and gum problems.[3] It has been investigated due to its antimicrobial potential against oral microorganisms.

The presence of active constituents such as nimbidin, nimbin, nimbolide, gedunin, azadirachtin, mahmoodin, margolone, and cyclic trisulfide contributes to the antibacterial activity of neem. These active constituents uncouple mitochondrial oxidative phosphorylation, thus inhibiting the respiratory chain. This resulted in its anti-adherence activity by altering the bacterial adhesion and the ability of the microorganism to colonize thereby causing maximum reduction in adherence of *E. faecalis* to dentin.[18]

Besides the antimicrobial action, this group of compounds also demonstrates anti-inflammatory function (ability to prevent the production of prostaglandins, especially prostaglandin E1 and 5-hydroxytryptamine) which is a desirable characteristic of the irrigant. An immunomodulatory function has also been suggested for the neem drug that enables antigen presentation to immunocompetent cells.[19]

Use of neem as an endodontic irrigant might be advantageous because it is a biocompatible antioxidant thus not likely to cause severe injuries to patients that might occur via NaOCl accidents.[20]

This study also demonstrated the antimicrobial action of Soluneem™ on *E. faecalis* with MIC of 2% and MBC of 2.6%. This is supported by other studies done on neem extracts.[15,16,18-21]

Furthermore, in this study, NaOCl showed bactericidal effect at concentration as low as 0.1%. Soluneem™ at 2.6% concentration was equally effective as 0.1% NaOCl.

Conclusion

In this study which was done to check the antimicrobial action of Soluneem™ (Azadirachtin) on most commonly found root canal pathogen, i.e., *E. faecalis*, it was concluded that Soluneem™ showed antimicrobial activity against *E. faecalis* at various concentrations. It was also found that the efficacy of Soluneem™ at 2.6% concentration and above is relatively similar to that of Gold Standard irrigating solution (NaOCl) on inhibition of *E. faecalis*.

Limitations

1. Further *in vitro* studies with a larger sample size as well as incorporating other microorganisms responsible for root canal infection need to be carried out.
2. In addition to this, long-term *in vivo* studies also need to be done to establish the role of Soluneem™ as a root canal irrigating solution.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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