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HYDROXY APATITE - ALGINATE DENTAL COMPOSITE LOADED WITH *MYRISTICA FRAGRANS* AND *SYZYGIUM AROMATICUM* EXTRACTS FOR THE MANAGEMENT OF DENTAL CARIES

C.SMITHA AND R.USHA*

Department of Microbiology, Karpagam Academy of Higher Education, Coimbatore, TamilNadu, India.

ABSTRACT

Dental caries is one of the most common chronic diseases of childhood. It affects more than 80% of the child population and a vast majority of adults. These are bacterial born infection which will ultimately end up in tooth decay and associated complications. The current approaches for the management of dental caries involves the lowering the number bacteria or reduction of cariogenic activity of bacteria in teeth by mechanical or chemical agents followed by a fluoride treatment to increase the resistance of teeth. The limitations of endodontic irrigants such as chlorhexidine in caries etiology along with the development of multiple drug resistance put forth the need for a more efficient antibacterial agent. Sustained release of phytochemicals can be a potent alternative to this issue owing to the fact that phytochemicals can address the multiple drug resistance by different mechanism of action with broad spectrum of activity. In the present study a complex system of hydroxyl apatite (HA) and alginate loaded with *Myristica fragrans* and *Syzygium aromaticum* were synthesized by *in situ* incorporation and characterized by FTIR analysis. *Myristica fragrans* and *Syzygium aromaticum* possess a potent anti-microbial activity against dental pathogens and inclusion of HA can enhance re-mineralization and dental tissues. Nutmeg loaded HA-clove loaded alginate (NMHA-CA) exhibited potent anti-bacterial and antibiofilm activities against *Streptococcus mutans* and *Lactobacillus* spp. . NMHA-CA showed superior effects against oral pathogens and forms a potent candidate for the management of dental caries.

KEY WORDS: **Cariogenic activity, dental caries, *Streptococcus mutans*, and biofilm**

R.USHA
Department of Microbiology, Karpagam Academy of Higher Education, Coimbatore, TamilNadu, India.

INTRODUCTON

Dental caries, biofilm-mediated disease, affect millions of people of all age groups throughout the globe. The composition of the biofilm associated with caries initiation and progression varies from person to person. The cariogenic *Streptococci mutan* are the main etiology of development of dental plaque. *Lactobacillus* species and *Actinomyces* species were also reported to be responsible for dental decay including root caries and other periodontal diseases[1] *Candida* sp. is another important fungi which frequently inhabit as oral commensal, these yeasts exist predominantly within biofilms, which are spatially organized heterogeneous communities of fungal cells encased in a matrix of extra cellular polymeric substances but is variably effected by saliva and dietary sugars[2] . *Streptococcus mutans* one of the initiators of dental caries, are acidogenic bacteria which colonize the oral cavity[3]. Glucans synthesized by these microbes help in the adhesion of the bacterium to form the plaque matrix[4]. The acidic pH imparted due to the bacterial action will activate the dissolution of the hydroxyl apatite (HA) to release calcium and phosphate to the surrounding medium. As HA forms the basic structural unit of bone and teeth, the pH induced calcium and phosphate release alter the normal structure of the tooth. The diffusion of these ions out of the tooth leads to the formation of the caries lesions[5] . The management strategies of dental caries include fluoride therapy, restorative materials like dental amalgam, composite resins, porcelain, gold, etc. The toxic effect of these components, especially mercury - a potent neurotoxin that constitute about 50% of the amalgam fillings, on human health and environment was very well established[6]. The phytoremediation of dental caries has been practiced even from prehistoric periods. A wide variety of plants and plant parts either in raw form or processed form have proven to be efficient in exhibiting anti-cariogenic properties. *Myristicafragrans* (Family: Myristicaceae) commonly called nutmeg plant has been used for various dental applications. Their anti-cariogenic properties were reported to be the presence of active components like myristicin, elemicin, safrole, terpenes, alpha-pinene, beta-pinene, myristic acid, trimyristin, neolignan (myrislignan), macelignan and so on[7,8] . *Syzygium aromaticum* (Clove) of family Myrtaceae is an indigenous plant found throughout India. Clove oil has a long history in dentistry as dressing material, analgesic, anti-carcinogenic agent, disinfectant as well as general hygiene[9] . It was reported that the anti-carcinogenic activities were due to its antioxidant properties[10] . It was proven to be safe up to the dosage of 3.752 g/Kg body weight[11, 12]. The sustenance of these herbal extracts is a challenge due to the flushing actions of the tongue and washing effects of saliva. In order to enhance the sustained availability we synthesized immobilized extracts of nutmeg (NM) and clove (CL) on HA and alginate respectively to form a biopolymer based dental composite implant for the management of dental caries and associated complications.

METHODOLOGY

Preparation of Myristica fragrans and Syzygium aromaticum extracts

Nutmeg (NM) and clove (CL) buds were collected from Trivandrum located in Kerala, India and dried under shade. The dried NM and CL were then chopped to form a fine powder. 10g of each were soaked in absolute methanol for 3 days. The extracts were then concentrated by evaporating the methanol under a hood. The viscous extracts so formed were preserved aseptically in a refrigerator.

Synthesis of NM and CL loaded HA-alginate composites (NMHA-CA)

Hydroxyl apatite (HA) was synthesized by the reaction between calcium chloride and Sodium phosphate. 2% (w/v) of NM extract was added along with the mixture and stirred at room temperature for 1 day to form HANM complex. The unreacted extracts were then removed by washing in distilled water. Similarly 2% CL extracts were stirred with 2% alginate solution for 1 day at 60^{o}C to form CL-Alginate solution (CA). CA (1ml) was then mixed with 1.5g HANM and cured at 37^{o}C to form the final composite NMHA-CA. A composite of HA and alginate (HA-Alg) without NM and CL were also maintained as control.

Evaluation of surface functionalization by ATR-FTIR spectroscopy

The NM and CL loaded HA-alginate composites were analysed for recording the spectra. ATR spectrum of NMHA-CA was recorded by using Nicolet 5700 FTIR Spectrometer and identified the functional groups of the composite.

Determination of cytotoxicity of HA on L929 fibroblast cells

We investigated the *in vitro* cytotoxic effects of HA. To a sub confluent monolayer of L929 fibroblast cells, different concentrations of HA were added and incubated at 37˚C for 24 h in a CO_2 incubator. Then the culture was washed with PBS and then 200 µl MTT (MTT 5 mg/ml) solution was added and kept at room temperature for 30min to get a homogenous color. The OD was read at 540nm and percentage of viable cells were calculated. The fate of L929 fibroblasts grown on contact with HA was determined by live-dead assay using ethidium bromide (EtBr) and acridine orange (AO)[13] . Around 2 x 10^5 cells were seeded and allowed to grow for 5days in DMEM supplemented with 10% FBS and 100 µg HA was added and maintained for 48 h and washed twice in PBS. Then 0.2 ml ethidium bromide / acrydine orange (EtBr /AO) mixture (1:1) was added and incubated for 10min. The excess dye was washed with PBS immediately and observed under a fluorescent microscope.

Antibacterial effects of NMHA-CA

Bactericidal effects

The anti-bacterial effects of NMHA-CA were evaluated against the cariogenic organisms *Streptococcus mutans*

and *Lactobacillus* sps by the agar well diffusion method. The turbidity of the inoculum for the assay was adjusted with respect to Macfarlands standard. The organisms were swabbed on Muller Hinton agar plates and varying concentrations of NMHA-CA (25, 50 and 100 µg) was then added to the wells. Gentamicin was used as positive control. The plates were incubated for 24 h at 37°C and the zone of inhibition was measured[14]. [14].

Inhibitory effects of NMHA-CA against biofilm formation

The inhibition offered by the NMHA-CA to the biofilm formation by *Streptococcus mutans* and *Lactobacillus* sps was determined based on the ability of the bacteria to form biofilm on solid surfaces. Crystal violet (CV) was used to stain the biofilm. The cells were allowed to grow in nutrient broth in a 24 well plate for 6 days. NMHA-CA was added and the incubation was continued for another 48 h. Then the media was removed and the plate was washed with phosphate-buffered solution (PBS, pH 7.2). Then 1% crystal violet was added to each well and kept for 30min. Again the unreacted crystal violet was removed by extensive washing with PBS and allowed to dry. The crystal violet in the biofilm was extracted with a bleaching solution (ethanol/glacial acetone in the ratio 70:30). The colour intensity was read at 595nm using a microplate reader [15].

RESULTS AND DISCUSSION

NMHA was synthesized by *in situ* incorporation of NM extracts to the HA. CL was loaded on alginate (2%) by physical interactions. The final composite NMHA-CA was synthesized by the blending and subsequent cross linking of the alginate fraction of the composite with available calcium ions in the HA. The phytoextracts were designed to release on demand to the surroundings which will depend on the moisture content of the medium. It is well known that the microorganisms prefer a moist environment for their proliferation. We intend the composite for the management of dental caries as a temporary filling material. The phytoextracts from two different plant sources will be able to provide better protection against a series of invading microbes. The presence of NM and CL in the NMHA-CA composite will add extra benefits to the composite. This can be attributed to the anti-microbial activities and anti-cariogenic effects of these plants. Hydroxyapatite [$Ca10(PO_4)6(OH)_2$] based bio ceramics have been used as implants due to their close similarities with the inorganic constituent of biological hard tissues like bone and teeth and due to their excellent biocompatibility[16] . In these tissues HA provides stability and hardness. Owing to the mechanical interlocking with the hard tissue, it is widely used in dental implants. Moreover, the osteoconduction and integration with the dental tissues without evoking immune responses have added extra credits to this class of biomaterial[17] . The sea weed derived polysaccharide alginate was reported to be nontoxic and nonirritant. Due to their hydrophilicity they facilitate the making of accurate impressions in the presence of saliva or blood[18] . Since their tear strength is low they can reproduce sub gingival contours and anatomy even after tear removal[19] . Both the biomaterials HA and alginate will provide additional supports for the regeneration of the lesion area of tooth.

Evaluation of surface functionalization by ATR-IR spectroscopy

The surface functional groups of NMHA-CA were determined by ATR-IR spectral analysis (**Figure-1**). The broad peak around 3300 cm^{-1} corresponds to the hydrogen bonded –OH groups of alginate indicating a hydrophilic surface. The peaks around 2900 indicate C-H stretching of alginate and also for various phytochemicals. A sharp peak around 1700 signifies the carbonyl stretching of ester groups and the peak around 1640 corresponds to the –C=C- stretch of alkene groups. Sharp peaks around 1400 and 1200 indicate the presence of alkane functional and aliphatic amines respectively. The presence of several mild peaks around 1100 reveals the C-N stretching vibrations of aliphatic amines. The peak around 950 corresponds to O-H bending of carboxylic acids. The mild peaks from 900 to 650 show the presence of primary and secondary amines and also the presence of aromatic rings. These peaks indicated the presence of the alginate fraction and secondary metabolites of NM and CL on the surface of the composite. The mild peaks ranging from 1000-1100 and 600-560 correspond to the presence of phosphate groups of HA indicating the presence of HA as a mosaic fashion on the surface of the composite.

Cytocompatibility of HA

In order to confirm the nontoxic effects of our HA we have done MTT assay, direct contact assay and live/dead assay using ethidium bromide/acrydine orange (EtBr/AO) cock tail. The L929 cells grown in the presence of even a higher concentration of HA (500mg/ml) exhibited an increase in cell viability more than 90% (**Figure-2**). The phase contrast images of these cells revealed the absence of changes in the characteristic cell morphology upon contact with the HA particles (**Figure-3**). These results were confirmed the nontoxic effects of the HA particles. Live/dead assay using EtBr/AO displayed mostly green nucleus indicating the absence of apoptosis (**Figure-4**). This confirmed that the cells were able to maintain their healthy being even in the presence of a higher concentration of HA particles. All these studies confirmed the compatibility of the HA particles that we have synthesized. HA was already proven to be highly compatible and this bioactive inorganic material has been used for several biomedical applications including the repair and regeneration of bone and teeth. The compatibility of HA is due to the similarities in chemical composition, size, crystallinity and morphology to bone minerals[20,21] . Alginate forms the other major component of our composite. Since alginate remains as the most well-known polymer for various biomedical applications we have not gone for the cytotoxicity evaluations of alginate. Tremendous research outcomes were available in the literature dealing with the biocompatibility of alginates[22] [22]. Apart from the

nontoxic effects the excellent gelling properties especially upon contact with the divalent metal ions like calcium will facilitate the easy release of the drugs loaded on to these materials[23] [23]. With these biomaterials we have prepared a highly compatible composite for the management of end stage dental caries.

Antibacterial effects of NMHA-CA

The agar well diffusion assay revealed the inhibitory effect of NMHA-CA against cariogenic bacteria. From the zone of inhibition it was very vivid that the effects were concentration dependent (**Figure 5**). . *Streptococcus mutans* and *Lactobacilli* sps are the most common among the dental plaque forming bacteria. The increased risk of multiple drug resistance prevents the application of antibiotics for eliminating these bacteria from the oral cavity. The sodium hypochlorite and 2% chlorhexidine were reported to elicit side effects like inflammation, accumulation of pus inside the root canals, occlusion of dentinal tubules and carcinogenicity[24,25] . The removal of cariogens from the oral cavity with the plant extracts are highly appreciated, owing to their comparatively lesser side effects[26] .

Inhibitory effects of NMHA-CA against biofilm formation

Similarly, NMHA-CA elicited an inhibitory effect against biofilm formation against *Streptococcus mutans* and *Lactobacillus* sps. The results revealed that the NMHA-CA was more than 50% effective in removing the biofilm on comparing with the conventional irrigant, hypochlorite. Moreover NMHA-CA was comparable to that of the conventional filling material zinc oxide/euginol (**Figure 6**).

Figure 1
ATR IR spectrum of NMHA-CA

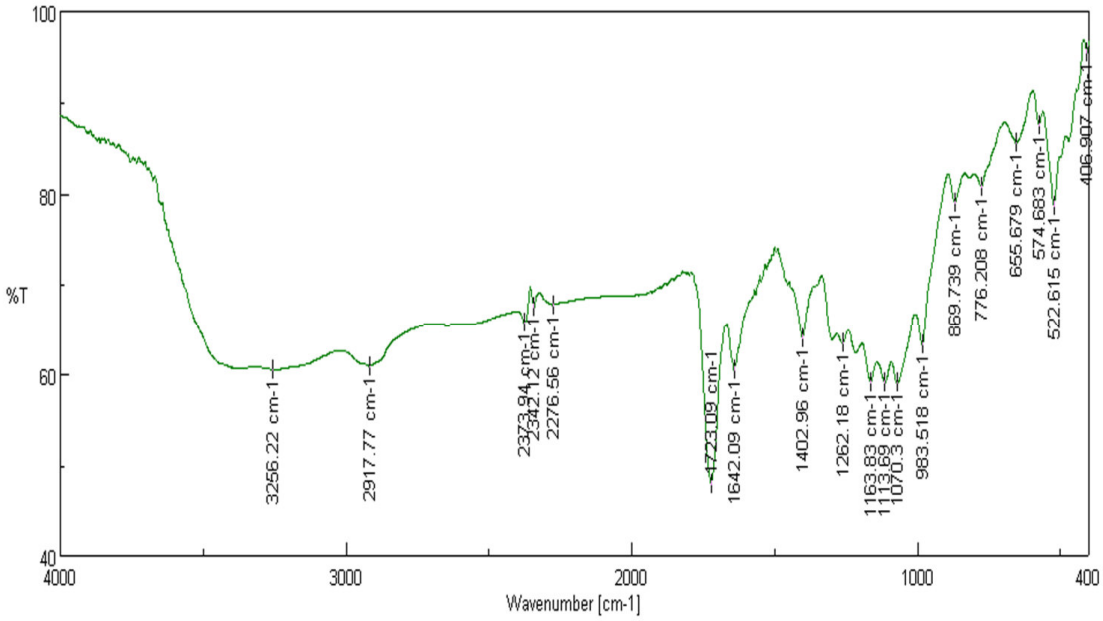


Figure 2
MTT cell viability assay to determine the cytotoxic potential of HA

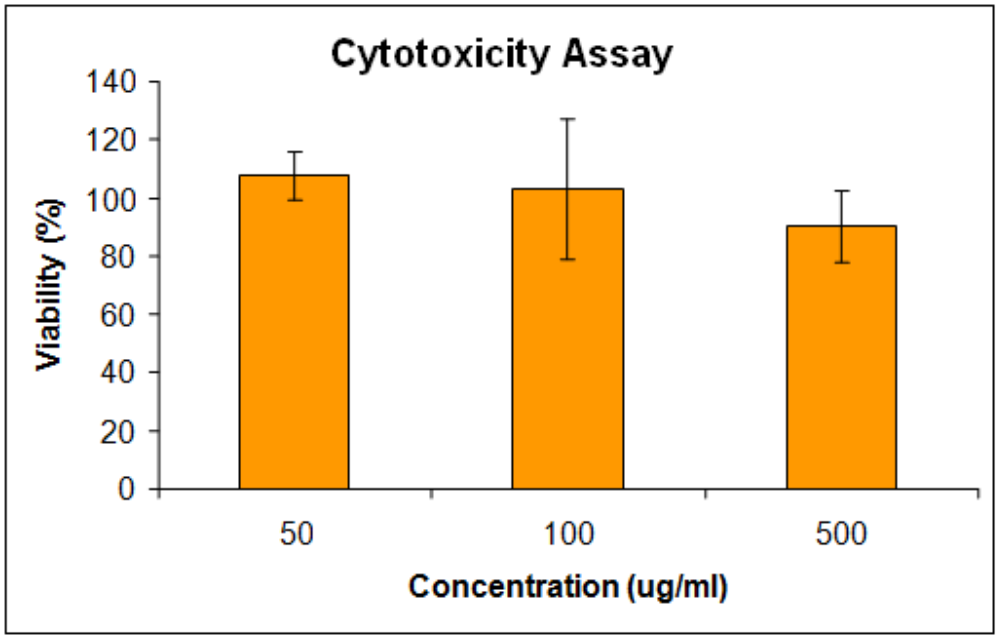


Figure 3
Phase contrast microscopy showing the absence of change in cell morphology upon contact with HA (50ug/ml) (B) with respect to control (A)

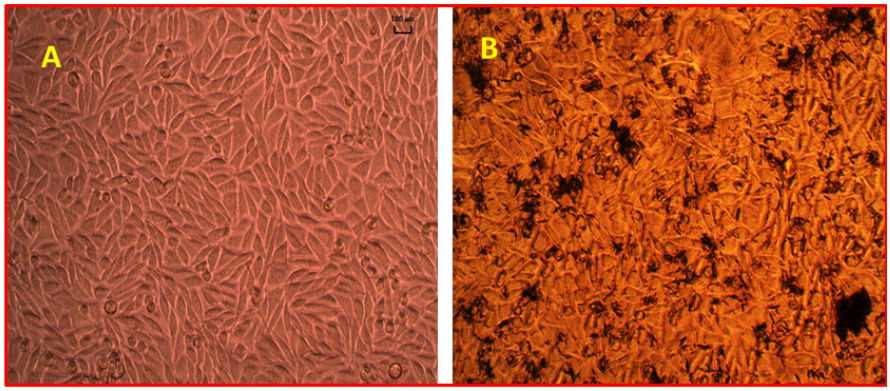


Figure 4
Live/dead showing the absence of apoptosis upon contact with HA (50ug/ml) (A) with respect to control (B)

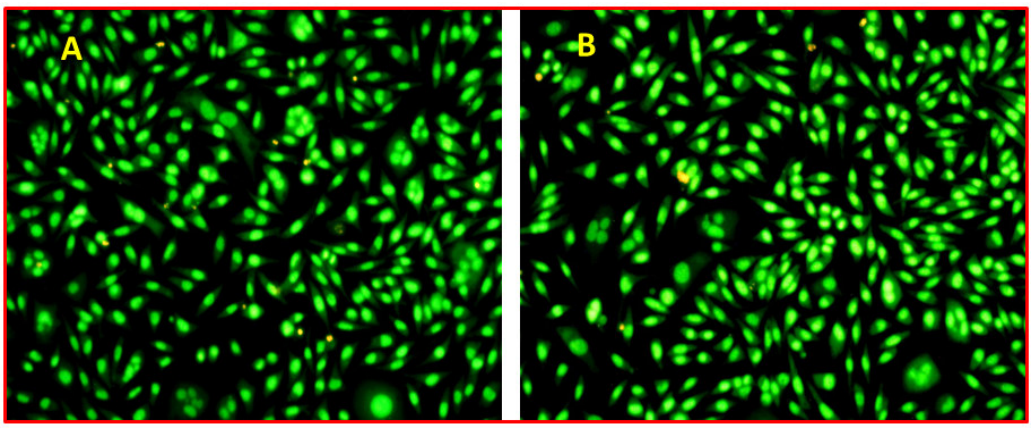


Figure 5
Bactericidal effects of NMHA-CA

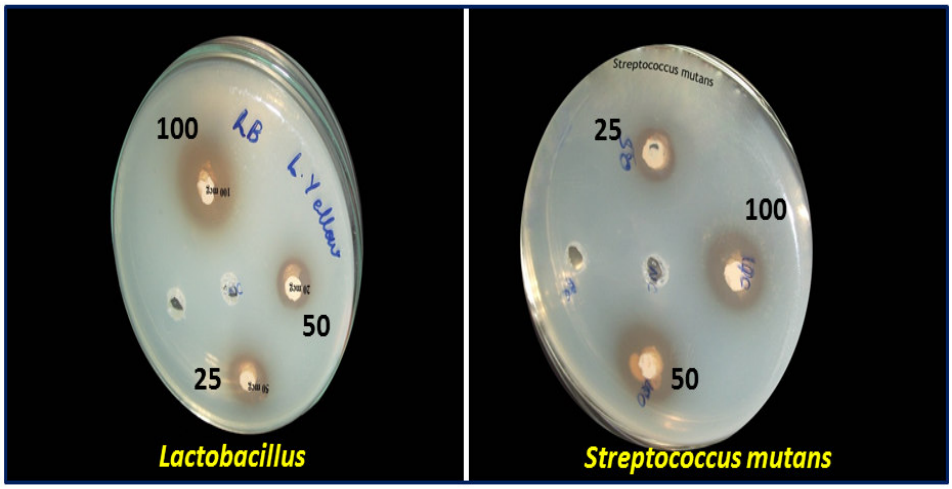
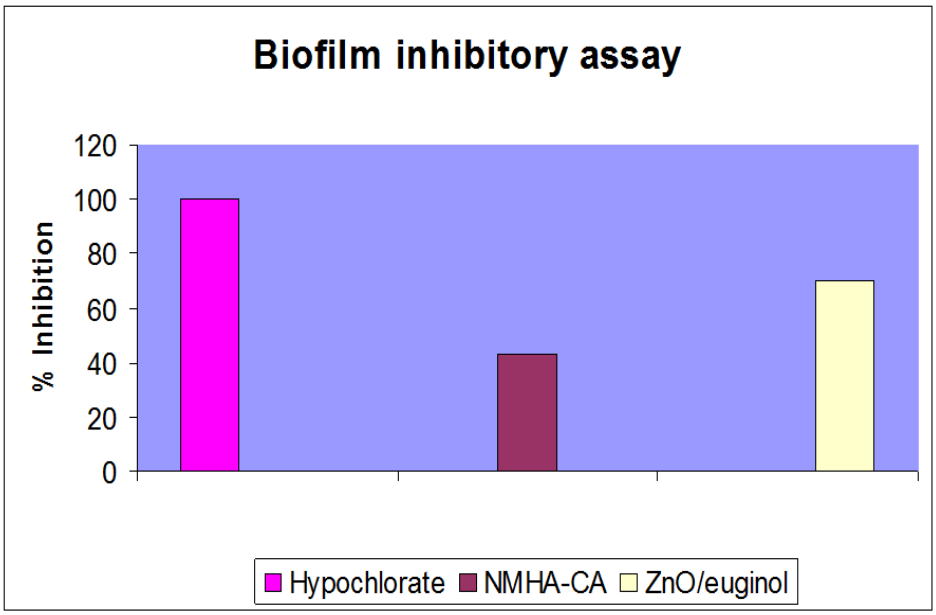


Figure 6
Biofilm inhibitory effects of NMHA-CA against Lactobacillus sps



CONCLUSION

Our results revealed that the NMHA-CA system could offer a sustained release of the loaded phytochemicals, nutmeg extract and clove extract, which was effective against the common cariogenic bacteria *Streptococcus mutans* and *Lactobacillus* sps. The prepared composite for the management of dental caries can be applied as a temporary filling material without causing any side effects. The phytoextracts from two different plant sources will be able to provide better protection against a series of invading microbes.

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