

Full Length Research Paper

Analysis of the antimicrobial action of copaiba oil and endodontic substances against anaerobic bacteria

Amaro de Mendonça Cavalcante^{1*}, Joanna Rodrigues da Silva¹, Lisiâne Cristina Lopes Oliveira¹, Marcos Aurelio Bonfim da Silva¹, Karlos Antônio Lisboa Ribeiro Junior^{2,3} and Antônio Euzébio Goulart de Santana^{2,3}

¹Faculty of Dentistry, Federal University of Alagoas, (FOUFAL-UFAL), Maceió, AL, Brazil.

²Center of Agricultural Sciences, Federal University of Alagoas, (CECA-UFAL) Rio Largo, AL, Brazil.

³Institute of Chemistry and Biotechnology (IQB-UFAL), Maceió, AL, Brazil.

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Endodontic infections are polymicrobial and predominantly caused by anaerobic bacteria and some facultative bacteria. The list of microorganisms involved in endodontic infections keeps expanding and has the potential to become increasingly more accurate during the next few years. Copaiba oil is an important Amazonian herbal medicine commercialized worldwide. In this study, we evaluated the antimicrobial activity and minimum inhibitory concentration (MIC) of copaiba oil and substances used in the treatment of endodontic infections against anaerobic microorganisms such as *Prevotella melaninogenica*; *Prevotella intermedia*; and *Clostridium acetobutylicum*. The MIC was determined by thioglycollate broth dilution. The data were statistically analyzed by Tukey's parametric and non-parametric methods of Cochran and Kruskal-Wallis test with a confidence level of 99%. The analysis of the antimicrobial activity showed that the samples of Copáiba oil, Sodium Hypochlorite, Otosporin, Tricesol formalin, Chlorhexidine and PMCC showed high antimicrobial activity ($p < 0.01$). However, different copaiba samples presented different activities. The results reveal Copáiba I sample was the most effective against anaerobic bacteria.

Key words: Essential oil, endodontic therapy, anaerobic microorganisms.

INTRODUCTION

Periapical lesions are diseases resulting from microbial contamination, necrosis of the pulp tissue, and infection progression toward the periodontal ligament and alveolar bone (Lucisano et al., 2014). The microorganisms, in particular Gram-negative, predominantly anaerobic, bacteria, that are able to initially colonize the dental pulp tissue cause primary infection, whereas those present in

the treated root canal system are the etiological agents responsible for secondary infections (Arias et al., 2016; Pourhajibagher et al., 2017). Among the manoeuvres of endodontic therapy, only mechanical instrumentation is not able to effectively and/or permanently reduce a load of bacteria present in the root canal system. Therefore, antibacterial agents for irrigation and medication have

*Corresponding author. E-mail: am_cavalcante@outlook.com. amc@ccen.ufal.br. Tel: 005582999838009.

been used to aid in the reduction of these microorganisms. However, there are studies demonstrating that despite the use of such agents, there may still be bacterial resistance (Al-Ahmad et al., 2014; Cavalcante et al., 2017). Because of this, much has been investigated on the antimicrobial action of substances used in endodontics.

These infections occur due to the presence of opportunistic microorganisms or resistance to conventional treatment, with a predominance of Gram-negative bacteria and strict anaerobes (Pan et al., 2014; Armalytē et al., 2019). The presence of strict anaerobes belonging to the genus *Prevotella spp* was investigated and evidenced in bacterial communities in infected root canals (Gomes and Herrera, 2018). There is a study showing of 43% of *prevotella* in orofacial infections (Chundurin et al., 2012). It is known that microorganisms can gain resistance against disinfecting agents and endodontic medicaments, increasing the challenge to completely eliminate them during root canal treatment (Chavez de Paz et al., 2010, Tennert et al., 2014). The use of medicinal plants has been investigated in search of new sources of pharmacologically active principles more effective, in order to scientifically validate popular empirical knowledge (Leitão et al., 2010; Cavalcante et al., 2016). Effective medicinal plant use has contributed to disseminating information about their therapeutic importance and medicinal effects, validating therapeutic knowledge that has been accumulated for centuries (Vieira et al., 2018).

The economic and ecological relevance of the species belonging to the genus *Copaifera* has aroused researchers' interest (Mattos et al., 2010; Masson et al., 2013; Dias et al., 2015). Thus, *in vivo* and *in vitro* evaluation has demonstrated that oils obtained from various *Copaifera* species have anti-inflammatory, healing, anti-edematogenic, antitumor, trypanocidal, and bactericidal activities (Mendonça and Onofre, 2009; Abrão et al., 2015). Because of that the therapeutic properties of *Copaifera* oil could be beneficial in odontological products such as intracanal pastes used for direct dental pulp capping as bacterial resistance. Their presence in inaccessible areas of the pulp canal may enable their presence even after rigorous endodontic chemical-mechanical preparation (Alvares and Junior, 2009). Investigating natural products is clearly essential to the search for new molecules with antibacterial activity. In this sense, this work shall contribute to research into the potential use of *Copaiba* oil and endodontic substances against bacterial strains involved in pulp and periapical diseases.

MATERIALS AND METHODS

Plant material

The choice of *Copaifera spp.* (*Copaíba*) is due to its antimicrobial action in previous studies that used plants of the same genus (Faria

et al., 2017; Vieira et al., 2018). Three samples of *Copaíba* oil, commercially available were obtained: *Copaíba* 1 from Santarém (2°30'17.19"S, 54°56'52.249"W); *Copaíba* 2 from Monte Alegre (1°45'27.018"S 55°51'42.89"W) and *Copaíba* 3 from Oriximiná (1°45'27.018"S, 55°51'42.89"W), all from the municipalities in the state of Pará. In the selection of endodontic substances, commercial formulations of topical chemical substances were used in endodontic therapy, besides the standard antimicrobials Metronidazole and Clindamycin.

Microbiological methods

Prevotella intermedia (ATCC 00463), *Prevotella melaninogenica* (ATCC 25845) and bacterial cultures were obtained from the Oswaldo Cruz Foundation's (National Institute of Quality and Health Control -INCQS) and *Clostridium acetobutylicum* (ATCC 4259) cultures were obtained from the Biochemistry and Physiology of microorganisms Laboratory (Department of Antibiotics), Federal University of Pernambuco (UFPE), Recife (PE), Brazil.

Bacterial strains were plated on Sodium Thioglycolate (BBL) broth supplemented with Hemin + Vitamin K (5µg / ml) (LABORCLIN, Brazil). The plates containing bacterial strains were incubated overnight at 37°C and subsequently stored at 4°C. The test cultures were prepared by inoculating 5 ml of BBL broth supplemented with Hemin (5 µg/ml) + Vitamin K (5 µg / ml). The tubes were incubated under anaerobiosis for 48 h at 37°C.

Sample preparation

Samples were prepared by solubilizing 200 µg of Metronidazole in 2 ml of distilled water to obtain a solution with a concentration of 0.1 mg / ml. Clindamycin disks commercially obtained (LABORCLIN, Pinhais - PR, Brazil) with a standard concentration of 2 µg/mL were used. For Calcium Hydroxide [Ca(OH)₂] with and without PMCC, 2.4 mg of the calcium hydroxide slurry (CALEN®- SSWhite São Cristovão - RJ) was diluted in 2 ml of distilled water to give a concentration of 1.2 mg/ml solution in each aliquot. The concentration used in this study for the different *Copaíba* oils was 20µg / mL. The other substances used in endodontic therapy were used according to their commercial formulations: Parammonochlorophenol camphorated® (Biodynamics Chemicals and Pharmaceuticals LTDA®, PR, Brazil), Tricesol formalin (Biodynamics Chemicals & Pharmaceuticals LTDA®, PR, Brazil), Sodium Hypochlorite 2.5% (Q-Boa®, Indústrias Anhembi S/A, Osasco – SP), Otosporin® (Farmoquímica S.A. Rio de Janeiro, RJ, Brazil) and 2% Chlorhexidine (Chlorhexidine®-Maquira Indústria de Produtos Odontológicos S.A.Maringá, PR/ Brazil). These substances had their antimicrobial activity tested in this study by the broth diffusion method, a methodology recommended by Konemam (1997).

Minimal inhibitory concentration (MIC) determination

This assay consists in the determination of chemical agent spectrum of action, according to resistance of studied microorganisms. The minimum inhibitory concentration (MIC) for every chemical agent was evaluated through the classic method of successive dilution. MIC was performed using the liquid dilution method recommended (Punjabi et al., 2018). Once the disks were prepared, the subsequent step was to set up the experiment by introducing one disk of each drug into each tube containing thioglycollate broth inoculated with the respective microorganism. These tubes were hermetically sealed and incubated at 37°C for 24 h under anaerobic conditions. After the incubation period, the reading was performed by checking the presence of growth of the

Table 1. Antibacterial activity of substances and drugs against microorganisms tested ($p < 0.01$).

Sample	Microorganisms		
	<i>P. intermedia</i>	<i>C. acetobutylicum</i>	<i>P. melaninogenica</i>
Calen® with PMCC	+	+	+
Calen®	+	+	-
Metronidazole	+	+	-
Clindamycin	+	+	-
Chlorhexidine 2%	-	+	-
Copaíba 1	-	-	-
Copaíba 2	-	+	-
Copaíba 3	-	+	+
Otosporin®	-	-	+
PMCC	-	+	-
Tricresol formalin	-	-	+
Sodium hypochlorite	-	+	+

+ : (There was bacterial growth), - : (There was no bacterial growth).

microorganism, visualized with the naked eye through the turbidity of the medium. This assay was performed on 03 sterile 96-well microtiter plates, 11 wells from each row of one plate being used for 11 substances and the other wells of its last column and the first column of the other plate for the culture medium with and without microbial inoculation. Microbial inoculums were standardized to 10^4 CFU of the Mc Fallard scale. A volume of 200 $\mu\text{l}/\text{ml}$ of the substance was placed in the 11 wells of the first top line and 100 $\mu\text{l}/\text{ml}$ of microbial inoculum diluted in the 11 wells of the remaining lines of a plate. Then, 100 μl of the first-line substance was withdrawn and placed in the corresponding well of the second line, successively being repeated until reaching the last line of the plate, obtaining a serial dilution ranging from 200 $\mu\text{l}/\text{ml}$ to 0.78 $\mu\text{l}/\text{ml}$ for each substance. Afterward, all microplates were incubated at 37°C for 24 h in a stove under anaerobic atmosphere. After this time, 20 μl of a 5% aqueous solution of Triphenyl Tetrazolium (VETEC® Rio de Janeiro, Brazil) was added to each well and the plates were again incubated for 24 h under the same conditions.

Analysis of data

Statistical results were previously evaluated by normality tests and afterward analysed by Tukey's parametric and non-parametric methods of Cochran and Kruskal-Wallis, considering 95% and 99.99% confidence intervals and a level of significance (p) ranging from 0.05 to 0.01. The analysis of the results was performed by the Tukey parametric and non-parametric statistical tests of Cochran and Kruskal-Wallis.

RESULTS

The antimicrobial activity of the studied samples of copaiba oils and endodontic substances tested are shown in Table 1. Minimum Inhibitory Concentration (MIC) values are shown in Table 2. In terms of sensitivity and resistance of microorganisms, the results obtained for growth or not in the culture medium were transformed

into percent and statistically evaluated and expressed in Figure 2.

In this study, Calen® with PMCC was not effective against any tested strain and Calen® without PMCC only showed action against *P. melaninogenica*. The Chlorhexidine 2% solution showed a better efficiency against *P. intermedia* when compared to the antimicrobial action of the 10% solution of calcium hydroxide and PMCC. The experiments were conducted in triplicate (Figure 1). In the current research, three different samples of Copaíba oils were used, which obtained varied performances in the biological activity test. Sample 1 of Copaiba oil was effective against all microorganisms tested. Nevertheless, samples 2 and 3 showed no effectiveness against *C. acetobutylicum* and Gram-positive organisms. Clindamycin and Metronidazole are drugs indicated for the control of endodontic infections, however, in the present study, such medications did not show effectiveness against *P. intermedia*. This can be explained by the various resistance mechanisms of Gram-negative bacteria, including the presence of the Lipopolysaccharide molecule (LPS) present in its outer membrane according to Vianna in 2005. The experiments were performed in triplicate. Figure 2 shows information on the resistance of the strains used. *P. intermedia* showed the most sensitive microorganism in relation to the tested active substances, presenting a MIC value of 0.78 $\mu\text{l}/\text{ml}$ for 75% of the substances tested.

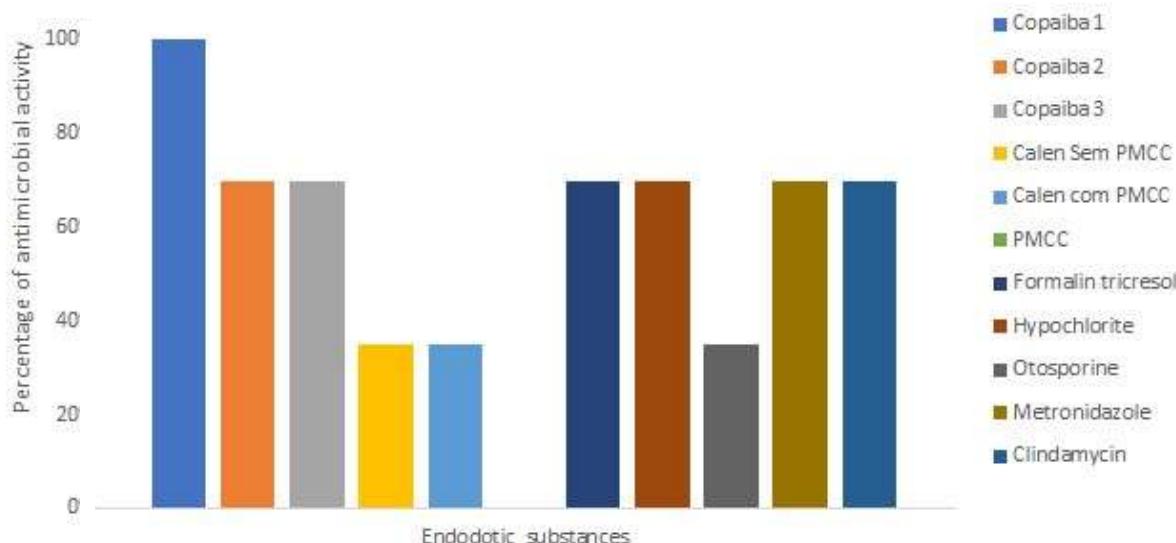
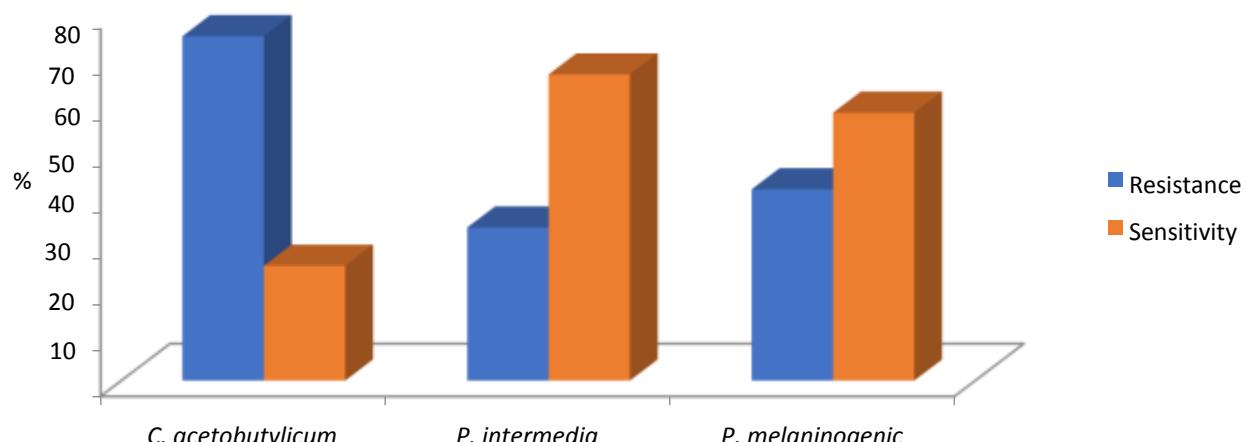
DISCUSSION

The aim of endodontic treatment is the elimination of infection; therefore, the mechanical instrumentation associated with antimicrobial chemical substances is

Table 2. Minimal inhibitory concentration (MIC) ($p < 0.01$).

Substances	Microrganisms		
	<i>C. acetobutylicum</i>	<i>P. melaninogenica</i>	<i>P. intermédia</i>
Calen®	X	X	50 µl/ml
Metronidazole	X	X	200 µl/ml
Clindamycin	X	X	3,12 µl/ml
Chlorhexidine 2%	0.78 µl/ml	X	0.78 µl/ml
Copaíba 1	6.25 µl/ml	0.78 µl/ml	200 µl/ml
Copaíba 2	12.5 µl/ml	X	200 µl/ml
Copaíba 3	0.78 µl/ml	X	X
Otosporin®	0.78 µl/ml	6.25 µl/ml	X
PMCC	0.78 µl/ml	X	0.78 µl/ml
Tricresol formalin tricresol	0.78 µl/ml	0.78 µl/ml	X
Sodium hypochlorite	0.78 µl/ml	X	X

X= means any antimicrobial action.

**Figure 1.** Percentage of antimicrobial activity efficacy of tested substances and drugs ($p > 0.05$).**Figure 2.** Percentage of sensitivity and resistance of microorganisms to substances and drugs tested ($p < 0.01$).

used to eliminate bacteria that can remain viable after root canal preparation, multiplying in the period between treatments (Rahimi et al., 2014).

Gram-negative anaerobes predominate in primary endodontic infections, while facultative Gram-positive tend to become prevalent in secondary endodontic infections (Andrews, 2001). As a result, the present study tested strains of two Gram-negative anaerobes (*P. intermedia* and *P. melaninogenica*) and one Gram-positive anaerobic (*C. acetobutylicum*). Currently, substances with antimicrobial activity, such as Calcium Hydroxide with and without PMCC, Tricresol formalin, Paramonochlorophenol Camphor, Sodium Hypochlorite, Otosporin®, Chlorhexidine, Metronidazole and Clindamycin (Santos et al., 2008; Rajasekharan et al., 2018; Nopnakeepongse et al., 2019).

In this study, Calen® with PMCC was not effective against any tested strain and Calen® without PMCC only showed action against *P. melaninogenica*. Chlorhexidine 2% solution presents better efficiency against *P. intermedia* when compared to the antimicrobial activity of the Ca(OH)₂ 10% solution and of PMCC which showed high significant difference ($p < 0.001$). In the present investigation, in the 24 h interval, 2% Chlorhexidine showed activity against *P. melaninogenica* and *P. intermedia*, whereas Metronidazole was effective only against *P. melaninogenica*. Clostridium perfringens was more resistant, in agreement with the results obtained by Ferreira (2010) and Matos et al. (2010), who, when evaluating these chemical substances, observed that they had no antimicrobial action against strains of *C. perfringens* and *C. difficile*. This is in line with the studies of Nisengard and Newman (1994) that Gram positive microorganisms have greater antimicrobial resistance due to the composition of their cell wall which, despite being simple, consists of a thick layer, which has peptidoglycan responsible for its maintenance and stiffness.

The minimum inhibitory concentrations (MICs) of chlorhexidine (CHX) and Paramonochlorophenol (PMC) were investigated using solid-state dilution tests against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Candida albicans*, *P. intermedia*, *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella denticola* and *Prevotella melaninogenica* and it was detected that even at low concentrations PMC and CHX showed antimicrobial activity against several microorganisms commonly found in endodontic infections (Lima et al., 2006). In the same way, in this study, CHX presented a high action spectrum; such antimicrobial activity is explained by the interaction of its cationic molecule with the anionic cell wall, altering the surface structures and increasing the permeability of the bacterial membrane.

The antimicrobial action of camphorated Paramonochlorophenol was evaluated in another research, through the agar diffusion method, on *Staphylococcus aureus*, *Pseudomonas aeruginosa*,

Bacillus subtilis, and *C. albicans*. This confirms its effectiveness (Oliveira et al., 2010). Likewise, in the current study, PMCC also showed antimicrobial activity against *Prevotella intermedia* and *P. melaninogenica*. Also in the present study, Tricresol formalin showed activity against *P. intermedia* and *C. acetobutylicum*. In another work, when evaluating the antimicrobial activity of Tricresol formalin and Paramonochlorophenol camphorated against *F. nucleatum* and *Clostridium difficile*, authors evidenced a high level of antimicrobial activity (Panzarini et al., 2006). The main antimicrobial action of Tricresol formalin is, according to Siqueira Jr. and Lopes (2010), the formaldehyde portion of the drug, with alkylating action on proteins and nucleic acids of microorganisms.

When comparing Metronidazole, Calcium Hydroxide and the association of these as a delay dressing in endodontic therapy, researchers concluded that the use of Metronidazole or its association with calcium hydroxide did not favour endodontic treatment when compared to the use of Calcium hydroxide alone (Packer and Luz, 2007). However, another study compared endodontic treatments performed with Calen® Paste without PMCC and Metronidazole in the form of a gynecological gel and verified that both medications showed good results, indicating Metronidazole as a possible alternative in endodontic therapy (Montero-Miralles and Martín González, 2018). On the other hand, this conclusion differs from the current investigation, since Metronidazole proved to be ineffective against *P. intermedia*.

Clindamycin and Metronidazole are drugs used for the control of endodontic infections (Siqueira and Lopes, 1999); however, in the present study, such medications did not show effectiveness against *P. intermedia*. This can be explained by the different mechanisms of resistance that certified strains (ATCC) have in relation to clinical isolate strains (CI), not used in the present study.

Our results showed that Otosporin® was effective against *C. acetobutylicum* and *P. intermedia*, a result equivalent to the one which showed the antimicrobial action of Otosporin® against *Enterococcus faecalis* and *Klebsiella pneumoniae*, both facultative anaerobic microorganisms (Matos et al., 2010). Otosporin® is the combination of hydrocortisone, a corticosteroid, and antibiotics, Polymyxin B Sulfate and Neomycin Sulfate. The antimicrobial spectrum of Polymyxin B encompasses only gram-negative germs (Soares et al., 2010), and Neomycin is effective against Gram-positive and particularly Gram-negative bacteria (Tortamano et al., 2008), possibly explaining the action spectrum of Otosporin® in the current research.

The antimicrobial activity of plants used in folk medicine has been analyzed on microorganisms present in root canal infections, and its antimicrobial activity has obtained satisfactory results (Arévalo-Híjar et al., 2018; Babaji et al., 2016). The oil of Copaiba has medicinal properties quite widespread among Latin American Indians since the first European explorers arrived in the

16th century (Leitão et al., 2010).

In the current research, three different samples of Copaíba oils were used, which obtained varied performances in the biological activity test. These results can be explained by the different chemical compositions that each species of *Copaifera* can present (Leitão et al., 2010). The presence of terpenoids in this mixture is also recognized, as is the endowment of antimicrobial properties (Veiga et al., 2005).

Sample 1 of Copaíba oil was effective against all microorganisms tested. There are several results in the literature when evaluating the antimicrobial activity of Copaíba oil as well as cement associated with dental use against Gram-positive and Gram-negative bacteria (Santos et al., 2008; Dias et al., 2015; Simões et al., 2016).

However, Samples 2 and 3 of Copaíba oils showed no effectiveness against *Clostridium acetobutylicum*, a Gram-positive microorganism. However, there is a report where Copaíba oil was effective only against Gram-positive microorganisms (Vianna, 2005). In the present evaluation, the resistance of *C. acetobutylicum* may be related to the composition of the samples of the tested oils, which may present distinct components with or without antimicrobial activity. How else can we explain the fact that these differences in the activity of oils assessed can be attributed to factors such as the location of the collection, seasonality, problems in the extraction and synergism.

Conclusions

In this study, 11 endodontic substances were assessed for their antibacterial activities. The results indicated that samples of Copaíba 1, Copaíba 2, PMCC, Formocresol and Otosporin have potential antibacterial effects on bacterial strains tested. This was confirmed by determination of both diameters of inhibition zones and minimal inhibitory concentrations. Copaíba I was the most effective. This indicates that these endodontic substances potentially have antibacterial properties. The Calen paste with PMCC proved to be ineffective against all microorganisms. According to the MIC obtained, *Clostridium acetobutylicum* was the most resistant of the tested bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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