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AVALIAÇÃO DO POTENCIAL ANTIMICROBIANO DE COMPOSTOS NATURAIS OBTIDOS DE PLANTAS DA FLORA BRASILEIRA PARA TRATAMENTO E PREVENÇÃO DE CÁRIES

Governador Valadares – MG

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Trabalho de Conclusão de Curso, apresentado no formato de artigo ao Departamento de Farmácia da Universidade Federal de Juiz de Fora – Campus Avançado Governador Valadares, como requisito parcial para obtenção do título de Bacharel em Farmácia.

Orientador: Prof. Dr. Fábio Alessandro Pieri

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Evaluation of the antimicrobial potential of natural compounds obtained from plants of the Brazilian flora for treatment and prevention of caries

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Abstract

The main bacteria for the initial formation of dental plaque are of the genus Streptococcus and the Streptococcus mutans species is the main etiological agent of caries. The antimicrobial agent most commonly used to combat these conditions is chlorhexidine digluconate, but it has adverse effects that restrict its prolonged use. Thus, the search for alternative antimicrobial drugs with lower side effects is increasing. The aim of this work was to evaluate the antimicrobial potential of the benzophenones from *Garcinia braziliensis* Guttiferone-A, 7-epiclusianone, 2,2',4-trihydroxybenzophenone, and the diterpenoic acids copalic, 3-acetoxy-copalic and 3-hydroxycopalic, from *Copaifera* sp., as well as their associations against oral bacteria. The minimum inhibitory concentration of the compounds was determined by the microdilution broth in microplates, followed by the addition of resazurin and a simplified test of the interaction of the compounds by microdilution. As a result, the main antimicrobial compounds, against *S. mutans,* were copalic acid and 7-epiclusianone isolated (MICs between 62.5µg/mL and 30.45µg/mL, respectively) and in combination, where they had an additive effect, reducing their active concentrations in combination to 32.25µg/mL and 15.22µg/mL, respectively. Thus, it is concluded that the combination between copalic acid and 7-epiclusianone presents as promising for the construction of pharmaceutical formulations to combat dental plaque and caries.

Keywords: *Copaifera*; *Garcinia*; dental plaque; *Streptococcus*

INTRODUCTION

Dental caries is a multifactorial disease related to genetic differences, lifestyle and socioeconomic conditions. Despite several preventive measures, it still remains a chronic global prevalent diseaset mainly in children in developing countries (PITTIS et al, 2017). Caries occurs due to the formation of dental plaque which is due to the excess of acidogenic microorganisms located in hard and soft tissues structured in colonies (PEPPENNEY et al., 2011).

Any acidogenic bacteria are able to ferment sugars like glucose and fructose producing acids so that the average pH is changed. This acidification leads to the demineralization of dental enamel which contributes to the formation of caries (KURAMITSU, 1993). The primary bacterium for human dental plaque formation is *Streptococcus*, with *Streptococcus mutans* being the main etiological agent of caries (MARTINS et al., 2018). *S. mutans*, in addition to supporting lower pH environments, shows higher acid release when compared to other bacteria of the genus *Streptococcus* (LI, 2010).

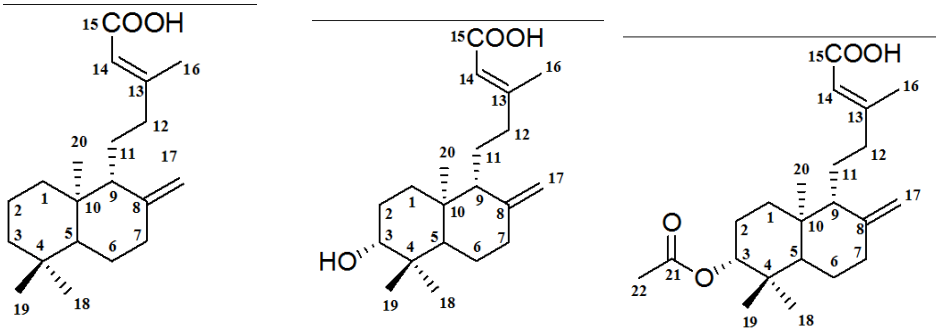
Mechanical removal of dental plaque such as brushing and flossing are the most effective procedures to prevent tooth decay, but most people can not get enough control by these means. For this reason, the use of some products containing antimicrobials becomes important for the effective control of the plaque. Chlorhexidine digluconate is the major antiseptic approved by the American Council of Dental Dentistry, however there is a limitation to its prolonged use because of its adverse effects (MARTINS et al, 2018). Changes in taste, darkening of tooth structure and ulcerations of the jugal mucosa are some of the effects observed with the use of chlorhexidine digluconate. Thus, the search for

alternative substances for the development of effective drugs with less undesirable effects has been increasing in recent years.

Natural products have been a source for the development of new biologically active drugs. The Copaiba tree (*Copaifera* sp.) of the botanical classification belonging to the Leguminosae (Fabaceae) family, Caesalpinoideae subfamily, *Copaifera* genus has shown relevance for the exploration of its constituents (PIERI, 2012). Copaiba oil obtained from the tree trunk has been widely used in the cosmetic and pharmaceutical industries due to its main constituents sesquiterpenes and diterpenes (ZHENGYUAN XIN et al., 2016). Among its all classes of plant metabolites, diterpenes such as copalic acid show prominences due to a broad spectrum of biological activities, including antimicrobial activity (SOUZA et al., 2011). The structures of three diterpenes frequently obtained from *Copaifera* oils are shown in Figure 1.

Figure 1 - Structures of diterpene acids obtained from Copaiba oil

Copalic acid 3-acetoxy-copalic acid 3-hydroxy-copalic acid

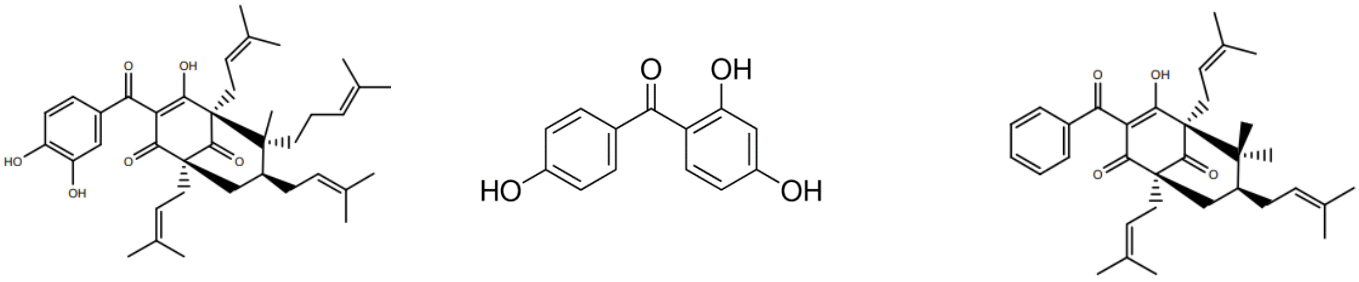


Source: Laboratório de Química de Produtos Naturais e Sintéticos da Universidade Federal de Juiz de Fora, *Campus* Governador Valadares (UFJF/GV).

Another genus that is being highlighted is *Garcinia*, also denominated previously as *Rheedia*, which is present in the Amazon, Paraguay and northern Argentina. Guttiferone-A and 7-epiclusianone are polyisoprenylated derivatives of benzophenone, and are two of the most abundant bioactive substances isolated from fruits of *Garcinia brasiliensis*, and has presenting several pharmacological properties, such as antimicrobial action on *Sthaphylococcus aureus* and *Bacillus cereus*, anti-HIV, trypanocidal, antiplasmodic, antioxidant, protease inhibitor and antineoplasic (DIAS, KST et al., 2012; GUSTAFSON; MCKEE, 1992). 2,2',4-trihydroxybenzophenone is a synthetic benzophenone and, like other members of its class, is expected to have several pharmacological properties, as previously reported including antimicrobial (NALDONI et al., 2009; SANTOS, et al., 1999). The structures of three benzophenones obtained from extracts of the genus *Garcinia* are shown in Figure 2.

Figure 2 - Benzofenones isolated from fruits of *Garcinia brasiliensis*

Guttiferone-A 2,2',4 trihydroxybenzophenone 7-epiclusianone



Fonte: DIAS, 2011; Sigma 2019.

Considering the relevance of the construction of antimicrobial drugs with lower undesirable effects and observing the antimicrobial potential presented by compounds

obtained from *Copaifera* sp. and fruits of *Garcinia brasiliensis*, the aim of this work was to evaluate the antimicrobial potential of compounds originating from these plants alone or in combinations for treatment and prevention of caries.

MATERIAL AND METHODS

Bacterial inoculum

Cariogenic and/or plaque forming strains of *Streptococcus mutans* NCCT 10449, Streptococcus mitis NCTC 12261, *Streptococcus salivarius* NCTC 8618 and *Streptococcus sanguinis* NCTC 7863 from the Reference Bacteria Collection in Sanitary Surveillance of the National Institute of Quality Control in Health - Oswaldo Cruz Foundation (FIOCRUZ), *Streptococcus mutans* ATCC UA159 and *Streptococcus sanguinis* ATCC-SK36 from the UNICAMP microbiology laboratory, which were assigned to the Laboratory of Microbiology of UFJF/GV.

Cultures were reactivated with Muller Hinton broth (MH) at 37ºC and incubated for 24 hours. Bacterial suspensions were prepared in test tubes containing 1mL of sterile saline until reaching the 0.5 McFarland scale, corresponding to the concentration of $1.0x10^8$ CFU/mL. Subsequently, each bacterial suspension was diluted to $1{,}0\times10^5$ UFC/mL, thus obtaining the standardized inoculum for the use of the tests (CLSI, 2012).

Solubilization of substances

The diterpenoic acids were transferred from the Laboratório de Química de Produtos Naturais e Sintéticos da Universidade Federal de Juiz de Fora, Campus Governador Valadares (UFJF/GV). The synthetic substance 2,2',4-trihydroxybenzophenone and the

substances obtained from the fruits of *Garcinia brasiliensis*, Gutiferona-A and 7-epiclusianone were available from the Laboratório de Síntese de Agroquímicos da Universidade Federal de Viçosa (UFV).

Diterpenoic acid substances were solubilized in 1mg/mL of DMSO (dimethylsulfoxide) and serial dilutions were made in MH broth so that the concentrations of the copalic acid samples ranged from 241.76µg/mL to 0.47µg/mL, 3-acetoxy-copalic acid from 290µg/mL to 0.56µg/mL and 3-hydroxy-copalic acid from 256.4µg/mL to 0.5µg/mL. These values varied because of consideration of the molar mass of the substances, so that the concentrations of all the compounds ranged from 800µM to 3.125µM.

For 2,2',4-trihydroxybenzophenone and 7-epiclusianone, 1mg of lyophilized powder was diluted by the addition of 50µL of DMSO and 950µL of PBS (Phosphate Buffered Saline) pH 7.6. For Guttiferone-A, 1mg was diluted in 500µl of DMSO, heated in a water bath and after 500µl of PBS pH 7.6 were added. Thus, the working solutions are obtained at a concentration of 1mg/mL. Subsequently serial dilutions were made in MH broth so that the sample concentrations ranged from 500µg/mL to 0.97µg/mL.

As reference for comparison (control of treatment), chlorhexidine (CL) was used, which was solubilized in sterile distilled water at a concentration of 1mg/mL and serial dilutions were then made in MH broth so that the concentrations of the samples varied from 358.4µg/mL to 0.7µg/mL.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined by broth microdilution method (CLSI, 2012). In the first column the positive growth control consisted of 50µL of the bacterial inoculum with 50µL of MH. In the last column the negative control (sterility) was prepared with 100µL MH. To each test orifice were added with 50µL of bacterial inoculum and 50µL of the substances.

The plates were incubated for 48 hours at 37°C in anaerobic jars. The reading was performed visually initially by turbidity of the medium in the orifice and later confirmed by the addition of 50µL of 0.01% resazurin solution per orifice to verify the presence of cell viability. All experiments were performed in duplicate, considering MIC at the lowest concentration that inhibited bacterial growth. Results were expressed as µg/mL.

Compounds interaction test

The combination of the compounds was made from the 4xMIC value of each of the substances until 0,0078125xMIC. In the first column of a microtiter plate the positive growth control was performed consisting of 50µL of the bacterial inoculum with 50µL of MH broth. In the last column the negative control was prepared with 100µL MH. The other orifices were filled with 50µL of the solutions and 50µL of the bacterial inoculum. After incubation at 37°C for 48 hours, 50µL of 0.01% resazurin solution was used for reading, as described for MIC.

Source: personal archieve.

The result was evaluated algebraically by the fraction inhibitory concentration index (FICi) for the identification of the interaction between the concentrations of the compounds according to the following equation:

FICi = FIC $_{ac}$ + FIC $_{7\text{-}ep}$

FIC $_{ac}$ = MIC $_{ac}$ in combination / MIC $_{ac}$ alone

FIC $_{7\text{-}epi}$ = MIC $_{7\text{-}ep}$ in combination / MIC $_{7\text{-}ep}$alone

Where: MIC_{ac} = MIC of copalic acid; MIC $_{7\text{-}epi}$ = MIC 7-epiclusianone

For interpretation of the FICi values, according to Fratini et al. (2017), interaction of compounds occurs as follows: synergism when FICi<1; additive effect when FIC=1; indifferent when 1<FIC<2; and antagonist when F IC> 2.

RESULTS AND DISCUSSION

Minimum inhibitory concentration

The antimicrobial evaluation of diterpene acids obtained from *Copaifera s*p. and substances obtained from the *Garcinia brasiliensis* against dental plaque bacteria are listed in Table 1, represented in μg/mL.

Table 1 - Minimum values of inhibitory concentration of benzophenones in μg / mL.

Genus/ Species	Isolate	Guttiferone-A	2,2',4-trihydroxybenzophenone	7-epiclusianone
Streptococcus mutans	ATCC UA159	62,5	125	62,5
Streptococcus sanguinis	ATCC SK36	15,62	>500	15,62

Table 2 - Minimum values of inhibitory concentration of diterpenes in μg / mL.

Genus/ Species		Copalic acid	3-acetoxy-copalic acid	3-hidroxy-copalic acid	Chlorexidine
Streptococcus mutans	CCT 3440	30,45	290	>256,4	5,6
Streptococcus mitis	NCTC 12261	30,45	>290	>256,4	11,22
Streptococcus salivarius	NCTC 8618	30,45	290	256,4	11,22
Streptococcus sanguinis	NCTC 7863	30,45	>290	>256,4	5,6

The MIC values of the copalic acid were 30,45μg/mL for all the isolates, which was been highlighted in relation to the other diterpene acids. The 3-acetoxy-copalic showed activity at 290μg/mL only against *S. mutans* and in the other strains there was no inhibition within the values tested. The 3-hydroxy-copalic acid presented the MIC of 256.4μg/mL just against *S. salivarius*. Based on the results presented, the copalic acid was been highlighted when compared to the other substances and its values were near from the MIC obtained for the gold standard drug, chlorhexidine digluconate, that were between 5.6μg/mL and 11.22

µg/mL, witch corroborated a previous study of its activity aganst dental bacteria (SOUZA et. al., 2011), that obtained values ranging from 12.5 to 172.1µg/mL.

In view of the chemical structure of 3-acetoxy-copalic acid and 3-hydroxy-copalic acid (figure 1), it is notable that the presence of a hydrophilic functional group in the three-position promoted a decrease in their antimicrobial activity. The same occurs with penicillin, when there is a hydrophilic side chain in its structure, the antimicrobial activity has reduced against Gram-positive bacteria, and the presence of a hydrophobic side chain causes an increase in its antimicrobial activity (PATRICK, 2009).

The substances Gutiferone-A, 2,2',4-trihydroxybenzophenone and 7-epiclusianone were tested only on strains *S. mutans* ATTCC UA169 and *S. sanguinis* ATTCC SK36 due to the availability of the substances purchased and the next step of this work. Against *S. mutans*, Guttiferone-A and 7-epiclusianone showed MIC values of 62.5µg/mL, and for 2,2',4-trihydroxybenzophenone, a result of 125µg/mL was obtained. The same substances were observed when tested in *S. sanguinis*, so that Guttiferone-A and 7-epiclusianone presented values of 15.62µg/mL and 2,2',4-trihydroxybenzophenone did not obtain a result at the concentrations tested.

The benzophenones are phenolic compounds with a higher hydrophobic character because of their low polarity. These substances already stand out due to its diversity of biological activities. The evaluation of the antimicrobial activity of the benzophenones Guttiferone-A and 7-epiclusianone against Gram-positive bacteria already presented satisfactory results previously (DIAS, 2011; BARROS, 2016). For the semi-synthetic substance 2,2',4-trihydroxybenzophenone, a broader spectrum of antimicrobial activity was

expected as reported by Aranda et al. (2019), which obtained activity against Gram-positive and Gram-negative bacteria. In this work the cell wall was affected with consequent greater release of proteins from the bacteria. This mechanism of action would revert in a desirable activity on the bacteria tested in the present work, Gram-positive, however the results obtained for this substance were little relevant to the others, because its action was only against *Streptococcus mutans*, in high concentrations, of 125µg/mL, and thus its activity was considered low, taking into account the parameters suggested by Ríos et al. (2005) and Gibbons (2008). According to these authors, the ideal value for the minimum inhibitory concentration of substances to be considered good antimicrobial compounds would be 100µg/mL for extracts and 10µg/mL for isolated compounds, so that the compounds analyzed would not stand out, including chlorhexidine which presented MIC values of 11.22 µg/mL against *S. salivarius* and *S. mitis*, that is the gold standard for use for combat dental plaque.

Despite of this, the source of the compounds should still be taken into account, since a compound with high biological availability could be employed in higher concentrations, since that it does not have cytotoxicity on its site of action, in the case the cells of the mouth of the patient. The present work highlights the compounds copalic acid, 7-epiclusianone and Guttiferone-A as promising for use in the prevention and control of oral diseases derived from dental plaque, as they obtained MIC results between 15.62µg/mL and 30,45µg/mL, values close to those obtained in the present work for chlorhexidine from 5.6µg/mL to 11.22µg/mL, the gold standard currently used.

Compounds interaction test

From the results obtained for MIC, *S. mutans* was selected as the major caries-causing agent in humans (MARTINS et al., 2018), to verify the type of interaction between one of the diterpenoic acids from *Copaifera*, copalic acid and a benzophenone from *Garcinia brasiliensis*, 7-epiclusianone, by simplified adaptation of the method of REUK-NGAM et al. (2014). The best molecules of each plant, regarding the antimicrobial activity were selected for the test. In the present study, the presence of 7-epiclusianone and copalic acid were the best antimicrobial agent for *S. mutans.*

The MICs of the substances alone, against *S. mutans*, were 62.5µg/mL for 7-epiclusianone and 30,45µg/mL for copalic acid. With the interaction test of the compounds the MICs of 7-epiclusianone and of the copalic acid were reduced to 31.25µg/mL and 15.23µg/mL, respectively. When evaluating the FIC value, both substances had values of 0.5 (representing 0,5xMIC). Since FICi is the sum of the FICs of the substances, the value found is 1, classified according to Fratini et al. (2017) as an additive effect.

The evaluation of the synergism of conventional antibiotics with natural plants has already been investigated and presenting satisfactory results. Test performed by Maia et al. (2018) evaluated the synergism of Guttiferone-A and 7-epiclusianone with conventional antibiotics finding satisfactory results, so that 7-epiclusianone and gentamicin tested against *S. uberis* presented synergism with FICi of 0.63. ZANG et al. (2018) highlighted the relevance of the combination as manner that would help to prevent the spread of bacterial resistance and improvement of antibiotic efficacy, so that *Buddleja albiflora* compounds

tested by them demonstrated a synergistic effect with erythromycin against *Listeria monocytogenes*.

This is the first work that investigated the combination of two natural compounds from different plants as a antimicrobial alternative, since previous work has only evaluated combinations of a natural compound with conventional antimicrobial drugs. The performance of different combinations of *Copaifera* sp diterpenoic acids with the benzophenones of the *Garcinia* genus is something that should be further investigated in future works, with the establishment of more robust experimental procedures, such as the amplification of the test carried out in the present work for the technique of Checkerboard.

CONCLUSION

It is concluded with the results obtained in the present work that some of the compounds analyzed showed promising antimicrobial activity on plaque-forming bacteria, especially on the microorganism most involved in the formation of caries, and that the combination of 7-epiclusianone and copalic acid, was found to have an additive effect and a consequent reduction of its active concentrations against cariogenic bacteria, presenting with high potential for the future formulation of antimicrobial drugs for caries treatment and prevention.

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TABLES LEGENDS

Table 1 - Minimum values of inhibitory concentration of benzophenones in μg / mL.

Table 2 - Minimum values of inhibitory concentration of diterpenes in μg / mL.

FIGURE LEGENDS

Figure 1 - Structures of diterpene acids obtained from Copaiba oil.

Figure 2 - Benzofenones isolated from fruits of *Garcinia brasiliensis.*