UNIVERSIDADE ESTADUAL DE MONTES CLAROS

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# Atividade de extratos de *Erythrina* spp. sobre o crescimento populacional e a formação de biofilme de *Streptococcus* do grupo *mutans*.

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências em Saúde da Universidade Estadual de Montes Claros - Unimontes, como parte das exigências para a obtenção do título de Mestre em Ciências da Saúde.

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Orientador: Profa. Dra. Ana Cristina de Carvalho Botelho

Coorientador: Prof. Dr. Sérgio Avelino Mota Nobre

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UNIVERSIDADE ESTADUAL DE MONTES CLAROS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

**MESTRANDO(A):** LUDMILLA LOUISE CERQUEIRA MAIA PRATES

**TÍTULO DO TRABALHO:** "Atividade de extratos de Erythrina spp. sobre o crescimento populacional e a formação de biofilme de Streptococcus do grupo mutans".

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# RESUMO

Biofilmes representam importante estratégia de sobrevivência de microrganismos em ambientes úmidos, como a cavidade bucal humana, resultando em processos infecciosos. Dentre as doenças bucais a cárie dentária se apresenta como uma das mais prevalentes doenças humanas, principalmente na infância. O processo carioso se configura como uma doença de caráter crônico e multifatorial que esta estreitamente relacionada com a presença de *Streptococcus* do grupo *mutans*, principalmente *S. mutans*. Aquisição de *S. mutans*, juntamente com a ingestão elevada de açúcar, estabelece um ambiente favorável para o desenvolvimento da cárie. Este microrganismo está sempre presente no biofilme dental potencialmente cariogênico. Além disso, há uma forte evidência que a produção de glucanos é essencial para a expressão da virulência de *Streptococcus* do grupo *mutans*, contribuindo para a aderência efetiva da bactéria à superfície dental, pela exposição à sacarose. Além das reconhecidas medidas de prevenção da cárie dental, como os procedimentos mecânicos de remoção do biofilme cariogênico, a adequação da dieta e o uso do flúor, tem-se investigado novas estratégias. Como terapia alternativa tem se buscado novos agentes antimicrobianos de origem natural, em especial de plantas. Em geral, estes agentes antimicrobianos fitoterápicos exibem diversas estruturas que reduzem o potencial de surgimento de populações microbianas resistentes. O objetivo deste trabalho foi avaliar a atividade de extratos da casca de *Erythrina* spp. sobre o crescimento populacional e a formação de biofilme de *Streptococcus* do grupo *mutans*. Foi utilizada coleção de bactérias deste grupo taxonômico, originária de biofilmes posicionados, distintamente, nas faces palatina e vestibular da cavidade bucal de crianças em fase pré-escolar. Ensaios de produção de biofilme foram conduzidos em vidro e hidroxiapatita (HPA), nos quais foram selecionados três isolados com níveis distintos de virulência. Para os ensaios de inibição diante dos extratos de *Erythrina* spp, foram incluídas as cepas de ampla referência ATCC 25175 (*S. mutans*) e ATCC 27392 (*S. sobrinus*) conduzidos em microplacas. A eficiência da inibição das bactérias variou com o modo de obtenção do extrato e com a virulência dos isolados. Os resultados foram promissores para as concentrações acima de 750 µg/mL, com inibições significativas do crescimento bacteriano e desestruturação dos biofilmes dos isolados em HPA.



**Palavras-Chave:** *Streptococcus* do grupo *mutans*, biofilme oral, *Erythrina* spp.

# ABSTRACT

Biofilms represent an important survival strategy for microorganisms in humid environments, such as the human oral cavity, resulting in infectious processes. Among oral diseases, dental caries presents as one of the most prevalent human diseases, especially in childhood. The carious process is characterized as a chronic and multifactorial disease which is related to the presence of *Streptococcus mutans* group, especially *S. mutans*. Acquisition of *S. mutans*, along with high intake of sugar, establishes a favorable environment for the development of caries. This microorganism is always present in the potentially cariogenic dental biofilm. In addition, there is strong evidence that glucan production is essential for expression of the *Streptococcus mutans* group virulence, contributing to the effective adherence of the bacterial to the dental surface, through exposure to sucrose. In addition to the recognized measures of prevention of dental caries, such as the mechanical procedures of cariogenic biofilm removal, the adequacy of diet, and the use of fluorine, new strategies are investigated. As alternative therapy are sought new antimicrobial agents of natural origin, especially of plants. In general, these phytotherapeutic antimicrobial agents exhibit diverse structures that reduce the potential for emergence of resistant microbial populations. The objective of this work was to evaluate the activity of extracts of *Erythrina* spp bark on population growth and biofilm formation of *Streptococcus mutans* group. A collection of bacteria from this taxonomic group, derived from biofilms positioned, was used distinctly on the palatal and vestibular surfaces of the oral cavity of pre-school age children. Biofilm production assays were conducted on glass and hydroxyapatite (HPA), in which three isolates with different levels of virulence were selected. In the inhibition assay with Erythrina spp. extracts, reference strains ATCC 25175 (*S. mutans*) and ATCC 27392 (*S. sobrinus*) were included, conducted on microplates. The efficiency of inhibition of the bacteria varied with the mode of extraction of the extract and with the virulence of the isolates. The results were promising for concentrations above 750μg.mL$^{-1}$, with significant inhibitions of bacterial growth and disruption of the biofilms of HPA isolates.



**Key words**: *Streptococcus mutans* group, oral biofilm, *Erythrina* spp.

SUMÁRIO

# 1 INTRODUÇÃO

## Cárie

A cárie dentária é uma doença multifatorial de dieta dependente, caracterizada pela desmineralização localizada do tecido dentário (esmalte, dentina e cemento) (1). Apesar dos avanços na redução de incidência, continua sendo um processo com elevado índice de prevalência em seres humanos (2,3). O processo de formação de cárie dentária tem sido intensivamente estudado (4,5), vários modelos foram propostos para tentar explicar esse processo de formação. O primeiro é essencialmente ecológico, no qual a carie é caracterizada pela interação de três fatores determinantes: hospedeiro, dieta e microbiota (6). O segundo modelo proposto acrescentou o fator tempo nessa interação, no entanto ambos os modelos não foram capazes de explicar de forma efetiva a ocorrência da doença na população humana (7).

A doença cárie é muito mais complexa e com um caráter comportamental, dessa maneira podendo ser influenciada por fatores modificadores ou extrínsecos. Com relação à suscetibilidade à cárie, o indivíduo pode apresentar fatores extrínsecos relacionados à estrutura sociocultural (classe social, renda, conhecimento, atitude e escolaridade) e fatores intrínsecos como fluxo, composição e capacidade tampão da saliva, aspectos hereditários e imunológicos difíceis de serem controlados. Sendo o fator suscetibilidade de grande importância para as estratégias de prevenção (8-10).

As lesões cariosas podem ocorrer sobre todas as superfícies do dente onde o biofilme se possa formar, sendo mais comumente encontrada em fissuras, sulcos e áreas proximais (11). Apesar de ser uma doença de maior incidência na infância, também acomete indivíduos com idade avançada, nos quais é possível observar cárie na superfície radicular por exposição da raiz do dente devido à regressão gengival (12). Clinicamente, a cárie apresenta-se como uma cavidade ou fissura na superfície do dente, sendo inicialmente assintomática. Com a progressão da infecção, os pacientes podem apresentar sensibilidade dentária com dor leve ou grave, dependendo da severidade da lesão (13). O controle e até mesmos a reversão da cárie é possível caso seja diagnosticada em estágio inicial, mancha branca no esmalte do dente, sem presença de cavidades ou fissuras. No caso de cavidades dentárias, há a necessidade de

tratamento curativo e preventivo, a fim de impedir a evolução da doença que pode causar grande destruição dos dentes, até mesmo a perda, resultando em complicações locais, sistêmicas, psicológicas e sociais (14).

O processo cariogênico ocorre quando bactérias acidogênicas (capazes de produzirem ácidos a partir da fermentação de carboidratos da dieta) e acidúricas (sobrevivem em meio ácido) que interagem com outros microrganismos no biofilme sobre a superfície do dente ocasionam a erosão ou à desmineralização do tecido dentário mais externo através de ácidos, em especial o lático (15-17).

Os principais grupos de bactérias que produzem ácido lático são *Streptococcus* do grupo *mutans* e *Lactobacillus*. Esses grupos de bactérias, atuando em conjunto ou isoladamente, são os principais causadores da cárie dentária (18,19). Os microrganismos com mais destaque no processo cariogênico são os *Streptococcus* do grupo *mutans*, totalizando são cerca de 60% dos microrganismos que compõem o biofilme dentário (20). No entanto também são encontrados *Actinomyces viscosus* que esta relacionado com início da lesão cariosa (21).

## *Streptococcus* do grupo *mutans*

*Streptococcus* do grupo *mutans* (SGM) foram isolados no início do século XX, e descritos como "mutantes" por sua morfologia celular ser mais achatada do que outros estreptococos (22). Relacionam-se as espécies: *S. mutans*, *S. sobrinus*, *S. salivarius*, *S. mitis*, *S. gordonii*, *S. sanguinis*, *S. rattus*, *S. downei*, *S. macacae*, *S. feru* (23). Sendo as espécies *S. mutans* e *S. sobrinus* mais frequentes em humano, com maior prevalência da espécie *S. mutans* (5). Os *Streptococcus* do grupo *mutans* são classificados dentro de 8 sorotipos (*a*-*h*), sendo os encontrados em humanos *c*, *e* e *f* (*S. mutans*) e *d* e *g* (*S. sobrinus*) (9).

SGM são bactérias gram positivas presentes no biofilme dental potencialmente cariogênico, sendo considerados como um dos principais microrganismos associados com início e patogenia da cárie humana. (23-25). O *S. mutans* usulamente coloniza a superficie dentária, mas também pode ser encontardo na lingua, saliva e membranas da mucosas. Embora a maioria dos casos de colonização de *S. mutans* não apresentarem consequências graves, esses

microrganismos podem adaptar ao ambiente, resistir à defesa imunológica, colonizar os tecidos cardíacos e causar endocardite infecciosa. Também sendo responsável por algumas inflamações oculares de neonatos (26).

A endocardite infecciosa é uma doença sistêmica grave e muitas vezes fatal. Foi observado a partir de 2007 um aumento significativo da incidência de endocardite infecciosa causada por estreptococos (27). Essas bactérias são capazes de sintetizar polissacarídeos extracelulares insolúveis, por meio de enzimas glicosiltransferases (*GTFs*) o que promove o acúmulo e permanência dos microrganismos no dente (28,29). A capacidade de sintetizar glucanos, na presença de sacarose, representa importante fator de virulência associado SGM (5,30).

A enzima glicosiltransferase (*GTF*) secretada pelo *Streptococcus* do grupo *mutans* hidrolisa a sacarose em glicose e frutose e polimeriza as moléculas de glicose liberadas, sintetizando polissacarídeos, glucanos, que irão constituir a matriz extracelular da placa bacteriana. Diversos tipos de glucanos são produzidos, os quais variam na solubilidade em água, onde *GTF B*: responsável pela produção de glucanos insolúveis em água, *GTF C*: pela produção de glucanos solúveis e insolúveis e *GTF D*: pela produção de glucanos solúveis. Os glucanos insolúveis em água são aqueles onde prevalecem às ligações do tipo α-(1-3) entre as moléculas de glicose, e são os mais importantes na formação de uma matriz extracelular insolúvel, essencial para o acúmulo de *S.* do grupo *mutans* no biofilme dentário dental (31-36).

Os glucanos promovem o acúmulo de SGM na superfície dental e contribuem para o volume, cariogenicidade e integridade estrutural do biofilme dental e, além de ser um essencial fator de virulência dos SGM relacionado a patogênese da cárie dental (35), são reserva de energia para bactérias, reguladores da permeabilidade da placa dental controlando a acidez nos dentes (37).

## Biofilme

A maior parte da atividade bacteriana na natureza ocorre, não com as células individualizadas crescendo de maneira planctônica (livres, em suspensão), mas com as bactérias organizadas

em comunidades de diferentes graus de complexidade, associadas a superfícies diversas, geralmente compondo um biofilme. Esses biofilmes são constituídos por células aderentes a uma superfície inerte (abiótica) ou viva (biótica), embebidas numa matriz de exopolissacarídeo. A associação dos organismos em biofilmes constitui uma forma de proteção ao seu desenvolvimento, fomentando relações simbióticas e permitindo a sobrevivência em ambientes hostis (38-40).

Os biofilmes na natureza são dos mais diversos ao nível filogenético, sendo geralmente constituídos por vários tipos de microrganismos (bactérias, arqueas, fungos filamentosos, leveduras e alga unicelulares como diatomáceas), podendo os produtos do metabolismo de uma espécie auxiliar o crescimento das demais e a adesão de uma dada espécie fornecer substâncias que promovem a ligação de outras. Inversamente, a competição pelos nutrientes e a acumulação de metabólitos tóxicos produzidos pelas espécies colonizadoras poderão limitar a diversidade de espécies num biofilme (41-42).

Dos microrganismos frequentemente encontrados num biofilme, as bactérias são o grupo predominante (43). As elevadas taxas de reprodução, grande capacidade de adaptação e de produção de substâncias e estruturas extracelulares, são as principais características que as fazem organismos com grandes capacidades de produção de biofilme (29).

Os biofilmes são encontrados em diversas áreas podendo ter função benéfica ou prejudicial na atividade humana. No contexto industrial, os biofilmes são utilizados em diversos processos de base tecnológica como forma de resolver alguns problemas causados pela atividade humana, como exemplo no tratamento de resíduos e de contaminações do meio ambiente (44). No entanto, os biofilmes são também responsáveis por elevadas perdas econômicas quando estão associados a problema de corrosão, de perda de carga de fluidos em tubagens ou de contaminações em produtos alimentares (45).

No contexto clínico, os biofilmes têm tipicamente um papel prejudicial. A sua ação é fundamental quer na progressão de infecções associadas à colonização de dispositivos médicos inseridos no corpo humano, quer associadas à colonização direta de tecidos, como é o caso da fibrose cística. Entretanto, são conhecidos alguns exemplos de biofilmes com ação protetora na saúde. Por exemplo, no trato gastrointestinal, biofilmes mistos aderidos a células epiteliais podem formar uma barreira que impede o acesso de microrganismos patogênicos

(46,47). Da mesma forma, a formação de biofilmes nos dentes ou na vagina por microrganismos comensais impedem que microrganismos patogênicos possam formar eles biofilmes. Quando os biofilmes comensais são perturbados nestas situações, surgem problemas como cárie ou vaginoses bacterianas (42).

## Biofilme oral

A cavidade oral é um ambiente único caracterizado pela presença constante de água na saliva, por uma grande variação da entrada de carbono e azoto, temperatura entre 35 a 36°C, pH entre 6,75 e 7,25, e ainda pela presença de superfícies distintas, como os dentes, os sulcos gengivais, a língua, o palato duro ou mole (48).

A regulação da formação de biofilme oral apresenta vários fatores, estando dependente da capacidade de os microrganismos sobreviverem a condições adversas, da sua capacidade de obter nutrientes a partir do ambiente, de crescer rapidamente como os microrganismos vizinhos, e da sua capacidade, enquanto comunidade microbiana, de se adaptar aos fatores ambientais (37,48).

A formação do biofilme ocorre através de um processo ordenado e dinâmico onde há necessidade da fixação e proliferação de bactérias sobre as superfícies dos dentes (49), sendo formado por uma película incolor aderente e não mineralizada, composta por diversos microrganismos, sustentada por uma matriz de proteínas salivares, polissacarídeos, células, leucócitos e restos alimentares. Sendo de difícil remoção (50-53).

O biofilme oral apresenta as seguintes etapas de formação: adesão reversível e irreversível, maturação e dispersão. Na adesão bacteriana, as bactérias planctônicas, que são transportadas passivamente na saliva e no fluido gengival, sem contato direto com a superfície do dente podem estabelecer interações físico-químicas fracas entre microrganismos e a película adquirida no dente, o que facilita a adesão não específica e reversível das bactérias. Seguidamente, pode ocorrer a adesão específica e irreversível das bactérias à película adquirida. Ocorre a formação de microcolônias, fenômenos de co-adesão, onde as bactérias se ligam a células microbianas distintas que já se encontram aderidas à película adquirida do

dente. Na maturação as células além de proliferarem, com consequente aumento de biomassa, sintetizam e degradam polímeros. E a dispersão, no qual as células do biofilme se desprendem para criar um novo foco de infecção (54,55).

A disrupção de um ou mais fatores ambientais pode induzir alterações na homeostasia da comunidade do biofilme oral, induzindo um estado de doença (33). O controle mais comumente utilizado é o método mecânico através principalmente da escovação, porém este método possui algumas limitações e por este motivo associa-se a métodos químicos para a desestruturação deste biofilme cariogênico (56). Apesar da associação com método químico ser muitas vezes eficaz o desenvolvimento de resistência pelos microrganismos a antimicrobianos e compostos sintéticos usuais tem levado pesquisadores de diversas áreas a buscar alternativas em produtos naturais empregados na medicina tradicional para combater as mais diversas variedades de patologias bucais (57-59).

## Produtos naturais como inibidores do biofilme

Em decorrência da incidência de doenças orais, do aumento da resistência de bactérias aos antibióticos e dos efeitos adversos de alguns agentes antibacterianos usados na odontologia, existe a necessidade de novas alternativas de prevenção e tratamento que sejam seguras, efetivas e econômicas (60).

As plantas são uma fonte importante de produtos naturais biologicamente ativos, muitos dos quais se constituem em modelo para a síntese de um grande número de fármacos. Pesquisadores da área de produtos naturais mostram-se impressionados pelo fato desses produtos encontrados na natureza revelarem uma gama quase que inesgotável de diversidade em termos de estrutura e de propriedades físico-químicas e biológicas (60-62). Apesar do aumento de estudos nessa área, os dados disponíveis revelam que apenas 15 a 17% das plantas foram estudadas quanto ao seu potencial medicinal (59).

Diversos trabalhos relatam o uso bem sucedido de produtos farmacêuticos à base de extrato vegetal. A ausência de efeitos colaterais associada a baixo custo de produção é considerado de

fundamental importância para o seu desenvolvimento. Assim extratos ou substâncias isoladas de plantas medicinais ou de outros produtos encontrados na natureza podem ser pesquisados como alternativas terapêuticas para o controle do biofilme dental cariogênico (63,64).

*Erythrima* spp.

O gênero *Erythrina* pertence à família Fabaceae contêm cerca de 130 espécies com distribuição em regiões tropicais e subtropicais dos hemisférios norte e sul. O nome genérico *Erythrina* vem do grego “erythros” que significa vermelho, em decorrência da cor das flores das espécies do gênero (65).

Espécies de *Erythrina* são comumente utilizadas para vários tipos de doenças humanas na medicina popular (66). Estudos realizados anteriormente nesse gênero mostram uma rica fonte de alcalóides bioativos e flavonóides, em especial, isoflavonas e flavononas (67).

No Brasil são encontradas cerca de onze espécies deste gênero. São elas: *E. verna* (*E. mulungu*), *E. falcata, E. domuinguezii, E. amazonica, E. velutina, E. crista-galli, E. fucas, E. poeppigiana, E. speciosa, E. similis, E. ulei* (68). As plantas deste gênero possuem diferentes ações farmacológicas como: *Erythrina verna* antidepressiva, sedativa, ansiolítica, antibacteriana e tônico hepático (69); *Erythrina caffra* é usada para tratar dor de ouvido, entorses, feridas, tuberculose, infecções respiratórias, abscessos, artrite e dor de dente (66,67); *Erythrina sigmoidea* como antídoto (picadas venenosas, picadas, etc.), diurético, febril e tratamento de artrite, reumatismo, problemas pulmonares, problemas de estômago, doenças infecciosas e doenças renais (70); *Erythrina velutina* como sedativo, calmante, sudorífica, emoliente, anestésico (71,72).

# 2 OBJETIVOS

## Objetivo Geral

Avaliar a atividade de extratos de *Erythrina* spp. sobre o crescimento populacional e a formação de biofilme de *Streptococcus* do grupo *mutans*.

## Objetivos Específicos

- Estimar a concentração inibitória mínima (CIM) de extratos da casca de *Erythrina* spp. sobre *Streptococcus* do grupo *mutans*.
- Estimar a concentração bactericida mínima (CBM) de extratos da casca de *Erythrina* spp. sobre *Streptococcus* do grupo *mutans*.
- Mensurar o efeito *in vitro* dos extratos em diferentes concentrações sobre a produção biofilme por *Streptococcus* do grupo *mutans*.
- Avaliar a presença de compostos secundários dos extratos de *Erythrina* spp.

# 3 PRODUTO CIENTÍFICO

Produto 1: Activity of extracts of *Erythrina* spp on population growth and biofilm formation of *Streptococcus mutans* group, formatado segundo as normas para publicação do periódico Brazilian Journal of Microbiology.

# Activity of extracts of *Erythrina* spp on population growth and biofilm formation of *Streptococcus mutans* group.

## Phytotherapeutic control of dental biofilm

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## ABSTRACT

Biofilms represent important strategy for the survival of microorganisms in humid environments, as the human oral cavity, resulting in infectious processes. Among oral diseases, dental caries is one of the most prevalent, especially in childhood, being of a chronic and multifactorial character that is associated with the presence of *Streptococcus mutans* group. This bacteria is always present in the potentially cariogenic dental biofilm. In addition to the recognized measures of prevention of this disease, such as mechanical procedures to remove biofilms, dietary adequacy and use of fluoride, new strategies are investigated. As alternative therapy are sought new antimicrobial agents of natural origin, especially of plants. In general, these phytotherapeutic antimicrobial agents exhibit diverse structures that reduce the potential for emergence of resistant microbial populations. The objective of this work was to evaluate the activity of extracts of *Erythrina* spp bark on population growth and biofilm formation of *Streptococcus mutans* group. A collection of bacteria from this taxonomic group, derived from biofilms positioned, was used distinctly on the palatal and vestibular surfaces of the oral cavity of pre-school age children. Biofilm production assays were conducted on glass and hydroxyapatite (HPA), in which three isolates with different levels of virulence were selected. In the inhibition assay with Erythrina spp. extracts, reference strains ATCC 25175 (*S. mutans*) and ATCC 27392 (*S. sobrinus*) were included, conducted on microplates. The efficiency of inhibition of the bacteria varied with the mode of extraction of the extract and with the virulence of the isolates. The results were promising for concentrations above 750μg.mL$^{-1}$, with significant inhibitions of bacterial growth and disruption of the biofilms of HPA isolates.

**Key words**: *Streptococcus mutans* group, oral biofilm, *Erythrina* spp.

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## Introduction

Dental carie although presenting a significant decline in some populations, continues to be an important public health problem[1]. It is a multifactorial, transmissible and diet dependent disease caused by specific types of acid producing bacteria that cause demineralization and destruction of the teeth being induced by the decrease of the pH of the dental plaque.[2-5]

The microbial nature of dental caries involves taxonomically organized bacteria such as *Streptococcus mutans* group (SMG).[6,7] It is a polyphyletic group, with expressive genetic and physiological variability, being distinguished by serotyping.

The following species are related: *S. mutans*, *S. sobrinus*, *S. salivarius*, *S. mitis*, *S. gordonii*, *S. sanguinis*, *S. rattus*, *S. downei*, *S. macacae*, *S. ferus*.[8] The species *S. mutans* and *S. sobrinus* are exclusive to humans, with a higher prevalence of the *S. mutans* species.[9] The *Streptococcus mutans* group are classified into 8 serotypes (*a-h*), being found in humans *c*, *e* and *f* (*S. mutans*) and *d* and *g* (*S. sobrinus*)[10]. In addition to dental caries *Streptococcus mutans* is also important cause of infective endocarditis. In a recent study, it was observed that from 2007 the incidence of infective endocarditis caused by streptococci increased significantly, being this species isolated from 8-10% of the patients with endocardial disease and 14.2% of the patients with cardiovascular diseases caused by streptococci[11].

These bacteria have spherical shapes and positive response for Gram staining (GPC), colonizing the surface of the teeth and participating in the formation of oral biofilm[12].This biofilm consists of a complex and structured community of microorganisms adhered to a biotic or abiotic surface immersed in a matrix of extracellular polymers under the continuous action of a flow.[12,13] The formation of the biofilm occurs through an orderly and dynamic process where there is a need for the fixing and proliferation of bacteria on the surfaces of the teeth (reversible and irreversible bacterial adhesion), maturation and dispersion[14].The most commonly used control is the mechanical method principally through brushing, but this method has some limitations and for this reason it is associated to chemical methods for restructuring of this cariogenic biofilm.[15,16]

Although the association with chemical method is often effective the development of resistance by the microorganisms the usual antimicrobial and synthetic compounds has led researchers from several areas to seek alternatives in natural products used in traditional medicine to combat the most diverse varieties of oral pathologies.[17-20]

Plants are important sources of natural therapeutic products, many of which are a model for the synthesis of a large number of drugs. Studies report that plant extracts and phytochemicals compound have been used more frequently for medicinal aim.[21-26] The World Health Organization estimates that 65-80% of the population in developing countries depend on medicinal plants as the only form of

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basic health care.[28-29] It is estimated that Latin American countries has part of the world's biodiversity, with Brazil accounting for approximately 22% of all plant and microbial species on the planet.[29] Essential oils, flavonoids, alkaloids, tannins, quinones among other substances are isolated from plants, are described in the literature because they present antibacterial activity.[30-32]

The genus *Erythrina* belongs to the *Fabaceae* family distributed in tropical and subtropical regions of the northern and southern hemispheres. The generic name *Erythrina* comes from the greek "*erythros*" which means red, because of the color of the flowers of the species of the genus. In Brazil, about 11 species of this genus are found, being *E. verna* (*E. mulungu*), *E. falcata, E. domuinguezii, E. amazonica, E. velutina, E. crista-galli, E. fucas, E. poeppigiana, E. speciosa, E. similis, E. ulei*.[33] *Erythrina verna* antidepressant, sedative, anxiolytic, antibacterial and hepatic tonic[34]; *Erythrina caffra* is used to treat earache, sprains, wounds, tuberculosis, respiratory infections, abscesses, arthritis and toothache[35-36]; *Erythrina sigmoidea* as antidote (poisonous stings, stings, etc.), diuretic, febrile and treatment of arthritis, rheumatism, lung problems, stomach problems, infectious diseases and renal diseases[37]; *Erythrina velutina* as sedative, soothing, sudorific, emollient, anesthetic[38,39].

The objective of this work was to investigate the inhibitory activity of extracts of *Erythrina* spp., considering isolates of *Streptococcus mutans* group, obtained from the oral cavity of pre-school children, selected and categorized as to adhesion capacity and biofilm formation.

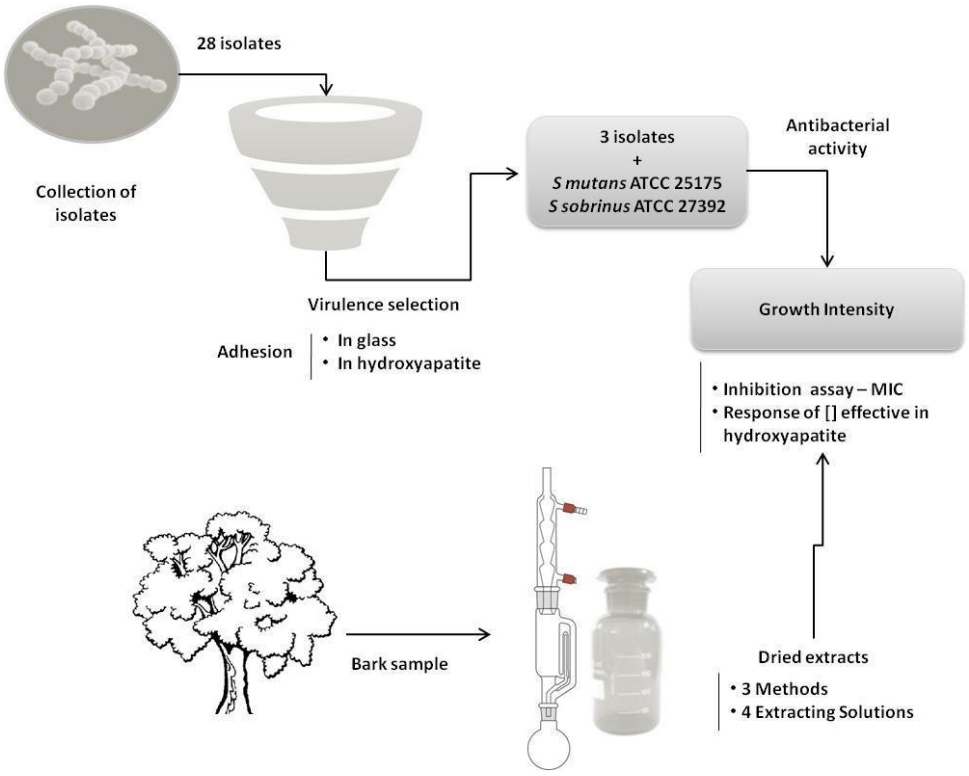


**Fig. 1 – Organogram with steps and procedures used during the evaluation process of extracts of *Erythrina* spp. on population growth and biofilm formation of *Streptococcus mutans* group.**

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## Materials and methods

*Origin of isolates*

A collection of 28 isolates belonging to the Laboratory of Epidemiology and Biocontrol of Microorganism of the State University of Montes Claros was used. The isolates originated from dental biofilms of pre-school children, positioned distinctly in the oral cavity: (I) palatine face and (ii) vestibular face, being coded by the letters P and V respectively. Strains of the American Type Culture Collection, ATCC 25175 (*S. mutans*), and ATCC 27392 (*S. sobrinus*) were also used.

*Selection of isolates of Streptococcus mutans group*

The process of selection of isolates *Streptococcus mutans* group (SMG) was done from two adhesion assays, being the first on glass surface and second on hydroxyapatite crystals (HPA). The glass surface assay was adapted from Kim et al. (2011) [40] using Broth Heart Infusion (BHI-Oxoid England) added with 1% sucrose (BHIS). The inoculum was adjusted on the McFarland 0.5 scale and the cell concentration was approximately $10^8$cfu.mL $^{-1}$. Aliquots of 10mL of the broth, previously inoculated, were distributed in glass tubes, and incubated in an inclined position (30°) for 24h at 37°C in anaerobiosis. To evaluate the bacterial cells adhered to the wall of the tube, the supernatant was initially discarded and then the surfaces of the biofilm were washed with SDW (sterile distilled water). This procedure was repeated twice for removal of cells that did not adhere efficiently. Five milliliter of potassium phosphate buffer (0.05M, pH 6.8) containing 0.02% sodium azide was added and sequence was performed: vortex shaking (1-Ika); detachment of the adhered bacterial cells with the aid of glass sitck and more vortex shaking. Subsequently, serial decimal dilution ($10^{-1}$ to $10^{-6}$) of the suspension of suspension buffer-biofilm cells and 100µL aliquots were spread, in triplicate on BHI agar. The plates were incubated for 24h at 37°C, in anaerobiosis, followed by direct counting of the colonies and the results recorded (ufc.mL$^{-1}$). The adhesion assay on hydroxyapatite (HPA) crystals was adapted from the methodology of Fukushima *et* al. (1992).[41] Three hundred micrograms of HPA crystals (diameter, 80µm, Bio-Rad, Hercules, CA, USA) were immersed in human saliva previously sterilized by centrifugation (300rpm for 5 minutes) and clarification. The system was left standing for 1 hour at room temperature. Saliva-coated HPA crystals were washed three times with potassium phosphate buffer and immersed in 2mL of bacterial suspension ($10^8$cfu.mL$^{-1}$). After incubation, accompanied by slight shaking of the HPA crystals with the bacteria for 24h at 37°C, the crystals were washed with SDW and potassium phosphate buffer added. The cells of the Streptococcus mutans group adhered to the HPA were detached using the vortex apparatus, diluted and spread on plates containing Agar Mitis Salivarius (AMS - HIMEDA), the latter process performed in triplicate. After incubation for 24h at 37ºC in anaerobiosis, the colony forming units (ufc.mL$^{-1}$) were counted. Three isolates of SMG were

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selected, being one of each class of virulence: high, medium and low. The quantitative definition of these levels was based on analyzes of the values obtained in the above mentioned assays.

*Vegetable species and extraction of extracts*

The species *Erythrina* spp. was collected in the locality called Vale do Arapuim, city of Varzelândia with coordinates 15.76107°S and 043.85611°W. The botanical material was identified by a specialist and the voucher was deposited in the herbarium of the State University of Montes Claros voucher number 5802. The collected plant material (bark) went through the process of asepsis, then dried in a forced-circulation oven (FANEM, 502) at 50°C, crushed and pulverized in industrial blender (VITALEX) and knife mill (TECNAL) with subsequent sieving.[41] To prepare the cold extracts were weighed 50g of the powdered vegetable material and transferred to an amber bottle, where 500 ml of the solvent (hexane, dichloromethane, ethyl acetate and butanol (VETEC)) were added. The vials were stored for five days, with daily agitation and homogenization. After this time the extracts were filtered to retain the larger fragments. Thereafter vacuum filtration of the liquid extracts was carried out. Soxhlet extraction was also extracted by dragging, where 50g of the plant material was used for 500mL of the solvent (hexane, dichloromethane, ethyl acetate and butanol). The other extraction process was conducted in Soxhlet, using the same proportions mass:solvent, however without fractionation. The extracts were dried in a forced circulation oven at 50°C.

*Quality control and interference of the diluent in the extract*

The minimum inhibitory concentration (MIC) of dimethylsulfoxide (DMSO), to be used as dilution of the dry extracts (DE) was determined in a pilot assay. Five SMG isolates were used (three selected in the adherence assays and two of wide reference: ATCC 27392 and ATCC 25175). The concentration used for the diluent was 6.25% (Fig. 2).

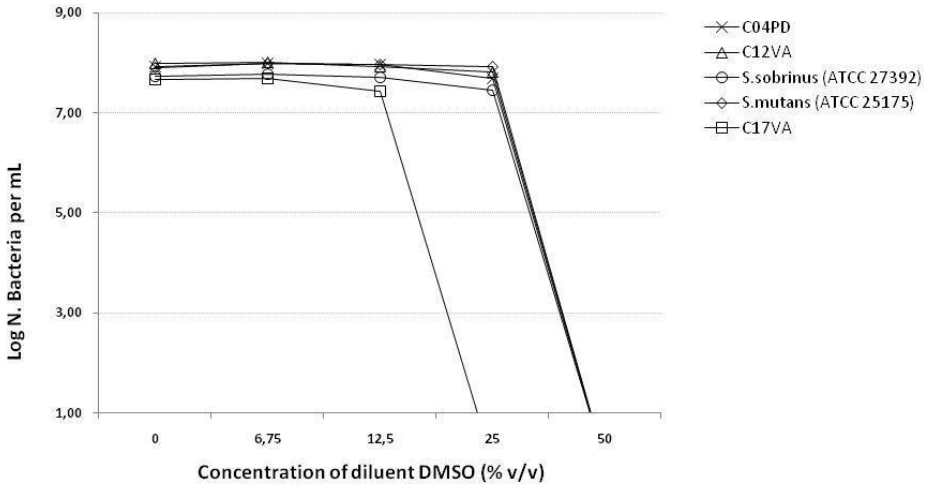


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**Fig. 2 - Inhibition curves of the *Streptococcus mutans* group, as a function of DMSO concentrations, for use as diluent of plant extracts of *Erythrina* spp.**

*Phytochemical analysis of dry extracts*

The DE were investigated for the presence of secondary compounds. The classes of substances considered were: alkaloids, tannins, flavonoids, saponin, steroids and triterpenes. The analyzes were performed according to Matos.[42]

*Minimum Inhibitory Concentration (MIC) of the extracts on the growth of SMG.*

The minimum inhibitory concentration (MIC) of each plant extract was evaluated in microdilutions, according to CLSI (Clinical and Laboratory Standards Institute), using 96-well sterile acrylic plates[43]. The volumes for each well were: 80µL BHI, 100µL of the extract specific concentration and 20µL of the inoculum of the SMG isolate. The evaluated concentrations of the extracts were: 3000, 1500, 750, 375, 187.5 and 93.75µg.mL$^{-1}$. Three previously selected SMG isolates were used, in addition to two broad reference strains (ATCC 27392 and ATCC 25175). Chlorhexidine (CLX - 0.12% v/v) was used as the reference chemotherapeutic treatment. A control treatment of culture broth sterility (CBS), extract diluent (CDS), of each extract (CES) and a control treatment of the number of bacteria inoculated for each SMG used (CI). Each treatment was performed in triplicate, being each well a repeat. The microplate was incubated at 37°C for 24h in anaerobiosis. After the incubation period, 20 µL of 0.01% aqueous resazurin solution (RES) was added to each well. The RES was pre-sterilized by filtration on a 0.22µm hydrophilic filter membrane (Millex, Millipore ®). The microplates were incubated for an additional 120 minutes and then the visual evaluation was performed, where the blue color was interpreted as absence of microbial growth and red to pink color, as the presence of metabolically active SMG. MIC was defined as the lowest concentration of the extract whose color remained blue. Aliquots of 10µL were removed from each well in blue and the first in a pink color and scattered on BHI Agar surface for subsequent direct counting of the colonies. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of the extract capable of causing 99.9% death of SMG cells, in relation to the control of the number of inoculated bacteria for each SMG used (CI). The plates were incubated at 37°C for 24h in anaerobiosis, and subsequently the number of colonies was measured in each replicate. Absence of bacterial growth after 24h of incubation determined MBC. Calculations were done according to ISO 7218.[44]

$$N = \frac{\sum c}{V \times 1{,}1 \times d}$$

Where:

N= Number of microorganisms present in the test sample as a weighted mean from two successive dilutions;

$\sum c$ = Sum of the colonies counted in the two plates retained from two successive dilutions, at least one of

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which contains a minimum of 10 colonies;

V = Volume of inoculum placed in each plate, in millilitres;

d = Dilution corresponding to the first dilution retained.

175

*176 Effect of the extracts in biofilm formation on hydroxyapatite crystals*

177 The procedures applied in the stage of colonization of the crystals by the selected SMG, followed the
178 methodology described previously, in the HPA adhesion assay. The procedures for the application of
179 extracts and measurement of biofilms are discussed below: (i) application of the extracts - the crystals
180 were lightly washed twice with SDW and subsequently immersed in the extracts, at concentrations of
181 1500µg.mL$^{-1}$ to 350µg.mL$^{-1}$. The system was incubated again under conditions already described; (ii)
182 biofilm measurement - The HPA crystals were washed lightly with SDW, followed by the addition of
183 potassium phosphate buffer. The apparatus was vortexed, followed by serial decimal dilution with
184 subsequent withdrawal of aliquots for direct counting of the disintegrated cells of the biofilm on MSA.
185 The same procedure described above was performed in another assay to determine the preventive
186 effect, where the HPA crystals were pretreated with the extracts and then inoculated with SMG. To
187 better understand the effects, the inhibition index of each MS was calculated to each SMG isolate,
188 considering the control treatment (Absence of inhibitory factor).

$$Inb_{n-z}(\%) = \left[\frac{(CEM_nSGM_z - CEM_{n=})}{CSGM_n}\right] \times 100$$

Where:

$Inb_{n-z}(\%)$ = Intensity of inhibition of the *EM-n*, on isolated z-Streptococcus spp

$CEM_nSGM_i$ = Counting in treatment *EM-n* vs isolate *z-Streptococcus* spp

$CEM_{n=}$ Counting in treatment *EM-n*

$CSGM_z$= Observed count for isolate *z-Streptococcus* spp**.** in the absence of *EM-n*

189

*190 Data analysis*

191 The obtained data were evaluated regarding the mathematical assumptions for parametric analysis.
192 The analysis of variance (ANOVA) and means tests for the qualitative variables and regression
193 analysis were performed for the quantitative variables, with significance level $\alpha = 0.05$.
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## Results and discussion

*Selection of isolates of Streptococcus mutans group*

All the isolates presented adherence in glass and HPA, however the selection for the next step was by the HPA assay. Fig. 3 expresses the intensity of Streptococcus spp. cells recovered from biofilms, developed by each HPA isolate, evidencing the existence of 4 statistically similar mean sets ($P \leq 0.05$), distinguished by Student's *t*-test.

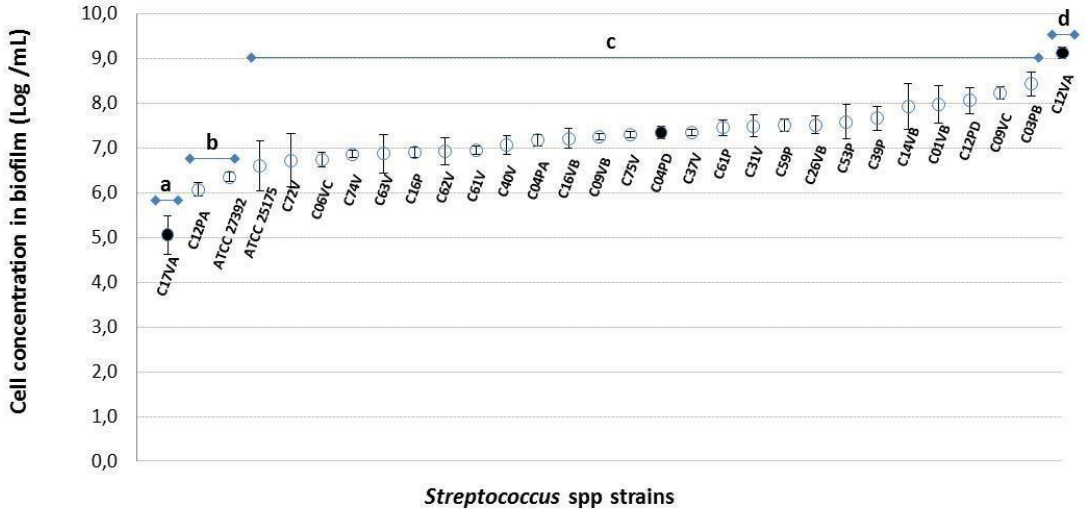


**Fig. 3 - Intensity of bacterial cells, present in biofilms produced on crystals of HPA, in function of *Streptococcus* spp. strains obtained from the oral cavity of pre-school children. Bars = confidence interval ($\alpha = 0.05$).**

The C12VA isolate was selected for the category of high virulence, as of medium virulence the C04PD and as of low virulence the C17VA. The choice of the C04PD isolate was partially arbitrated by the presence of median density of adhered cells, combined with lower variance and the fact that it originates in the palatine face, since the other selected ones came from the vestibular face. The virulence properties of SGM are acid tolerance, the production of acids and the proteolytic activity, which allow the survival of the microorganism in the biofilm adapting to the environment, by means of adhesins, glycosyltransferases, extracellular polysaccharides and degradation of collagen of the substrate[45]. As the isolates used had a difference in the capacity of biofilm production, one of the factors that may explain this variation may be the effectiveness of the glycosyltransferase (GTF) synthesized for each isolate. Once SGM isolates can secrete three types of glycosyltransferase, being that *GTF B* produces water insoluble glucans[40], to water-soluble *GTF D*[46] and soluble and water insoluble *GTF C* glucans[40]. As the HPA crystals were washed in water before counts, the isolates that had as products of *GTF C* and *D*, therefore they would have released glucans soluble in water, may

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have had entrained cells during the washing process, giving smaller counts. Venkitaraman et al. (1995)[47], concluded that enzymes the *GTF B, C* and *D* influence the stability of the biofilm and that the nature of the glucan produced can influence the adhesion of SMG in an experimental film. Bacteria involved in periodontal diseases live in communities functionally organized and structured in biofilm. The oral cavity presents several complex and continuously changing microenvironments, in which microorganisms must rapidly adapt to survive[48]. The biofilm growth promotes a gradual decrease of oxygen concentration over time, favoring anaerobic and microaerophilic bacteria[49]. Strategies of formation of dental biofilm by Streptococcus mutans group has ecological determinant function for the survival of these microorganisms, which may incite diseases such as dental caries and infectious endocarditis[50-51].

*Physical and chemical attributes of crude extracts obtained*

Table 1 expresses the yield in function of the extraction method (EM) and the solvent used. The highest yields were obtained in the cold extraction modes (4.2% with Ethyl Acetate) and by drag using only solvents (5.56% with Butanol), without fractionation. Drag extraction (hot) with only and fractional solvent were realized in the soxhlet, but in different ways. In the first the vegetal material is renewed with each input of a solvent; thus, the processing makes possible a highly efficient extraction, while in the fractionated with only cartridge of the vegetal material is used for the passage of the four solvents (hexane, dichloromethane, ethyl acetate and butanol), in this way the exhaustion of the vegetal material occurs, consequently the least drag of substance in the latter solvents. In cold extraction, the process occurs at ambient temperature for a long period (days), under occasional shaking and without renewal of the liquid extractor (static process). Therefore it does not lead to the exhaustion of vegetal material, but occurs at the saturation of the liquid extractor or the establishment of a balance between the extraction medium and the interior of the plant cell[52]. Soxhlet extraction has demonstrated the best solvent extraction efficiency, results similar to other studies[53-54]. The results of this work demonstrate that the extraction method, the solvent used and the temperature directly influence the total yield of the extract, however it is necessary to take into account that many substances are thermolabile and others may undergo irreversible structural changes at high temperatures[54].

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**Table 1 - Yield (% m/v) of the extracts obtained in each type of extraction of the bark of *Erythrina* spp.**

| Solvent | Yield (% m/v) | | |
|---|---|---|---|
| | Without heating extraction | With fractionated heating extraction | With heating solvent extraction only |
| **Hexane** | 1,10 | 1,02 | 1,84 |
| **Dichloromethane** | 2,60 | 2,00 | 3,94 |
| **Ethyl acetate** | 4,20 | 1,84 | 4,32 |
| **Butanol** | 4,00 | 1,24 | 5,56 |

The results obtained in the phytochemical prospection of the 12 extracts used, they are represented in table 2. In the phytochemical analysis of the extracts of the bark of *Erythrina* spp the presence of tannin, flavonoid, resin and tripterpenos / steroids was verified. In studies with species of Erythrina spp. some authors corroborate the results found. The phytol substance of the class of terpenes was found in methanolic extract of dried flowers of the same species[55]. Already flavonoids (alpinumioso and derrona) in hydroalcoholic extracts of the stalk[56]. The negative result for alkaloids differ from results obtained by Almeida[57]. Some authors report that tannins have inhibitory effects on fungi and bacteria.[57-58] There are three hypotheses for the mechanism of antimicrobial action. The first hypothesis presupposes the inhibition of bacterial and fungal enzymes and / or the complexation of the substrates of these enzymes; the second would be the action of tannins on the cell membranes of microorganisms, modifying their metabolism. Finally, the third hypothesis mentions the complexation of tannins with metal ions, thus reducing the availability of these essential elements for the metabolism of microorganisms. The results show that each type of solvent dragged the metabolite by affinity for polarity. Hexane steroids and tripterpenes, dichloromethane flavonoids and steroid / tripterpenes, ethyl acetate flavonoids, tannins and tripterpenes and butanol flavonoids and tannins. Reports such as those obtained herein are reported in the literature.[59]

**Table 2 - Secondary compounds diagnosed in extracts of Erythrina spp bark, depending on the extraction method and solvent used.**

| Solvent extractor | Without heating extraction | With fractionated heating extraction | With heating solvent extraction only |
|---|---|---|---|
| Hexane | te | te | te |
| Dichloromethane | f; r; te | f; r; te | f; r; te |
| Ethyl acetate | f; r; t; te | f; r; t; te | f; r; t; te |
| Butanol | f; t | f; t | f; t |

t-tannin; f-flavonoid; r-resin; te-tripterpene /steroid.

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*Inhibitory concentrations of extracts on Streptococcus spp.*

Table 3 expresses the viable cell count values, recovered after submission to MS of *Erythrina* spp., Considering the concentrations capable of inhibiting the growth and causing the death of the SGM population, in proportion ≥90%.

The selection strategy of SGM isolates, based on adherence-virulence in HPA, was shown to be useful, but evidenced the existence of biochemical components not considered in our study model. Thus, it was observed that, for extracts EM-2, EM-3, EM-6 and EM-10, the isolate selected as less virulent (C17VA) was inhibited with lower extracts concentration than the more virulent (C12VA). The opposite occurred for the extracts EM-1, EM-5 and EM-8, where this logic was reversed. Suggesting that some of these EM act efficiently on the destabilization of the biofilm, without, however, being lethal bacterial cells.

Cold extraction with butanol presented higher efficiency in the inhibition of the selected isolates. Already in relation to the mode of extraction by drag, the use of the only solvent provided better results than the sequential use of the solvents in the same sample. Although the presence of the same substances in the phytochemical analysis was confirmed (Table 2) the levels of these substances were distinct for the two methods in question. Regarding the extractive solvents, ethyl acetate, dichloromethane and butanol were more efficient than hexane. The results demonstrated that Soxhlet extraction using only solvent is the one with the highest yield, but the yield is not directly related to the increase in total bioactive. Study conducted by Herzie et al. (2013), demonstrates significant differences in the nature of the components And the amount thereof with respect to the extraction method.[60] Numerous studies were carried out with the objective of determining the antimicrobial and anti-adherent activity of various types of plant extracts against *Streptococcus mutans* group.[61-63] For the purpose of inhibit or reduce the formation of the dental biofilm, bacterial growth, and consequently the adhesion of microorganisms on the surface of the tooth.[64-68] However, studies on antimicrobial and anti-adherent activity of *Erythrina* extract are scarce in the national and international literature. A study related to Erythrina spp. has the purpose of evaluate its anxiolytic and antimicrobial effect, with other microorganisms.[68-69] In the study, the presence of antimicrobial activity of the bark of *Erythrina* spp. on the C17VA, C04PD and C12VA isolates and the broad reference strains: ATCC 25175 and ATCC 27392. The results showed that all isolates tested were sensitive to at least one of *Erythrina* spp. (Table 3). There is a lot of divergence between the authors regarding the concentration of the extract for prospecting of drugs.[70-71] Considering rigorous criteria for the evaluation of the potential of EM for pharmaceutical purposes, we have chosen those suggested by Aligiannis and collaborators (2001)[71], in which an extract is considered a strong inhibitor MIC ≤ 500μg.mL$^{-1}$, moderate inhibitor ≥500μg.mL$^{-1}$ MIC ≤1500μg.mL$^{-1}$and weak inhibitor: MIC ≤1500μg.mL$^{-1}$. The bactericidal concentrations (Suppression ≥ 90% population) indicate that the best results were for the

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317 inhibition of strain ATCC25175 (*S. mutans*) EM4, strain ATCC 27392 (*S. sobrinus*) EM2, EM4, EM6,
318 EM10 and EM11, for the isolate C17VA the extracts EM2, EM3, EM6 and EM10, none of the EM
319 was satisfactory in inhibiting the C04PDe C12VA isolates. However, the results showed that EM was
320 generally effective in reducing the population growth of SGM isolates at concentrations $\geq 750 \mu g.mL^{-1}$.

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**Table 3 – Minimum Inhibitory and Bactericidal Concentrations capable to reducing *Streptococcus mutans* group populations to levels less or equal than 90% in function of different preparations of extracts of *Erythrina* spp.**

| Extract Method[1] | *Streptococcus mutans* group Strains | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ATCC 25175 | | ATCC 27392 | | C17VA | | C04PD | | C12VA | |
| | MIC[2] | $MBC_{90}$[3] | MIC[2] | $MBC_{90}$[3] | MIC[2] | $MBC_{90}$[3] | MIC[2] | $MBC_{90}$[3] | MIC[2] | $MBC_{90}$[3] |
| EM-1 | ≥750 | ≥3000 | ≥375 | ≥750 | ≥750 | ≥1500 | WI | WI | ≥1500 | ≥1500 |
| EM-2 | ≥1500 | ≥3000 | ≥187,5 | ≥187,5 | ≥187,5 | ≥187,5 | WI | WI | ≥187,5 | ≥750 |
| EM-3 | ≥1500 | ≥3000 | ≥375 | ≥750 | ≥375 | ≥375 | ≥750 | ≥1500 | ≥187,5 | ≥750 |
| EM-4 | ≥187,5 | ≥375 | ≥187,5 | ≥375 | ≥750 | ≥750 | ≥750 | ≥750 | ≥375 | ≥750 |
| EM-5 | WI | WI | ≥1500 | ≥1500 | ≥3000 | ≥3000 | WI | WI | ≥1500 | ≥1500 |
| EM-6 | ≥350 | ≥750 | ≥375 | ≥375 | ≥187,5 | ≥375 | ≥187,5 | ≥750 | ≥187,5 | ≥750 |
| EM-7 | ≥750 | ≥1500 | ≥375 | ≥750 | ≥375 | ≥1500 | ≥375 | ≥1500 | ≥750 | ≥1500 |
| EM-8 | WI | WI | ≥1500 | ≥3000 | ≥3000 | ≥3000 | ≥3000 | ≥3000 | ≥1500 | ≥1500 |
| EM-9 | WI | WI | ≥3000 | ≥3000 | ≥3000 | ≥3000 | WI | WI | ≥3000 | ≥3000 |
| EM-10 | ≥1500 | ≥3000 | ≥187,5 | ≥375 | ≥93,75 | ≥375 | ≥375 | ≥750 | ≥187,5 | ≥750 |
| EM-11 | ≥750 | ≥1500 | ≥375 | ≥375 | ≥375 | ≥750 | ≥375 | ≥750 | ≥187,5 | ≥750 |
| EM-12 | ≥750 | ≥3000 | ≥375 | ≥750 | ≥750 | ≥1500 | ≥750 | ≥1500 | ≥375 | ≥1500 |

[1]EM-1: Hexane and without heating extraction; EM-2: Dichloromethane and without heating extraction; EM-3: Ethyl acetate and without heating extraction; EM-4: Butanol and without heating extraction; EM-5: Hexane and with fractionated heating extraction; EM-6: Dichloromethane and with fractionated heating extraction; EM-7: Ethyl acetate and with fractionated heating extraction; EM-8: Butanol and with fractionated heating extraction; EM-9: Hexane and with heating solvent extraction only; EM-10: Dichloromethane and with heating solvent extraction only; EM-11: Ethyl acetate and with heating solvent extraction only; EM-9: Butanol and with heating solvent extraction only.

[2]Minimal Inhibitory Concentration ( $\mu g.mL^{-1}$)

[3] Minimal Bactericidal Concentration capable to reducing ≥90% of cell counting ($\mu g.mL^{-1}$)

WI– Inhibitory effect absent

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*Effect of the extract on biofilm production on hydroxyapatite crystals*

The application of the extracts in a curative mode, therefore after the previous colonization of HPA crystals by SGM resulted in exclusion of all the populations of the isolates under study. Table 4 expresses the resistance to biofilm disorder, formed by the different SGM isolates when submitted to contact with the EM of *Erythrina* spp preventively. Considering the inhibition indices, it is possible to state that the best results were for the inhibition of ATCC 27392 strain (S. sobrinus) extracts EM2, EM3, EM4, EM6, EM7, EM10 and EM11, for the C17VA isolate extracts EM2, EM3 EM10, EM10, EM10 and EM12, for the C04PD isolate the extracts EM4, EM6, EM7, EM10 and EM11, for the C12VA isolate the extracts EM10 and EM11, none EM was satisfactory in inhibiting strain ATCC 25175 (*S. mutans*) in a preventive mode. Confirming as previously observed that the selection of the isolates by the virulence factor was useful in the study of the antimicrobial properties of plant extracts. The concentration of $1500\mu g.mL^{-1}$ more effective in preventing biofilm formation by SGM, however, strain ATCC 27392 (*S. sobrinus*) and C17VA isolate were sensitive at concentrations lower than $1500\mu g.mL^{-1}$.

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**Table 4 –Assessment of *Streptococcus mutans* group populations in biofilm structured on hydroxyapatite (HAP), expressed as $Log_{10}$, and Inhibition Rate (IR), expressed as percentage, in function of different preparations of extracts of *Erythrina* spp. applied preventively.**

| Extract µg.mL$^{-1}$ | | *Streptococcus mutans* group Strains | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ATCC 25175 | | ATCC 27392 | | C17VA | | C04PD | | C12VA | |
| | | Log Count[2] | IR[3] (%) | Log Count[2] | IR[3] (%) | Log Count[2] | IR[3] (%) | Log Count[2] | IR[3] (%) | Log Count[2] | IR[3] (%) |
| EM-1 | 1500 | NR | - | 7,39±0,14 | 0,00±1,28 | NR | --- | NR | --- | 9,96±0,00 | 0,00±1,53 |
| | 750 | NR | - | 9,96±0,00 | 0,00±2,74 | NR | --- | NR | --- | 9,96±0,00 | 0,00±1,53 |
| | 375 | NR | - | 9,96±0,00 | 0,00±2,74 | NR | --- | NR | --- | 9,96±0,00 | 0,00±1,53 |
| EM-2 | 1500 | NR | - | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | NR | --- | 8,41±0,11 | 6,17±1,52 |
| | 750 | NR | - | 0,00±0,00 | 100±0,00 | 5,82±0,22 | 0,00±0,00 | NR | --- | 9,96±0,00 | 0,00±1,53 |
| | 375 | NR | - | 9,96±0,00 | 0,00±2,74 | 9,96±0,00 | 0,00±0,00 | NR | --- | 9,96±0,00 | 0,00±1,53 |
| EM-3 | 1500 | NR | --- | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 3,16±0,08 | 57±0,29 | 8,43±0,00 | 7,71±1,45 |
| | 750 | NR | --- | 9,96±0,00 | 0,00±2,49 | 6,27±0,13 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |
| | 375 | NR | --- | 9,96±0,00 | 0,00±2,74 | 9,96±0,00 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |
| EM-4 | 1500 | 9,96±0,00 | 0,00±2,44 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 8,10±0,11 | 11,28±0,00 |
| | 750 | 9,96±0,00 | 0,00±0,00 | 6,87±0,15 | 0,00±3,47 | 0,00±0,00 | 100±0,00 | 7,24±0,03 | 0,00±1,74 | 9,96±0,00 | 0,00±0,00 |
| | 375 | 9,96±0,00 | 0,00±1,67 | 9,96±0,00 | 0,00±2,74 | 0,00±0,00 | 100±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±0,00 |
| EM-5 | 1500 | NR | --- | 7,06±0,04 | 0,00±1,53 | NR | --- | NR | --- | 9,96±0,00 | 0,00±2,05 |
| | 750 | NR | --- | 9,96±0,00 | 0,00±1,53 | NR | --- | NR | --- | 9,96±0,00 | 0,00±1,67 |
| | 375 | NR | --- | 9,96±0,00 | 0,00±1,53 | NR | --- | NR | --- | 9,96±0,00 | 0,00±1,67 |
| EM-6 | 1500 | 7,41±0,07 | 0,00±2,05 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 8,41±0,06 | 7,91±1,27 |
| | 750 | 9,96±0,00 | 0,00±1,67 | 7,29±0,03 | 0,00±2,36 | 0,00±0,00 | 100±0,00 | 6,65±0,41 | 10,5±6,21 | 9,96±0,00 | 0,00±1,53 |
| | 375 | 9,96±0,00 | 0,00±1,67 | 9,96±0,00 | 0,00±2,74 | 9,96±0,00 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |
| EM-7 | 1500 | NR | --- | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 8,26±0,05 | 9,58±0,76 |
| | 750 | NR | --- | 9,96±0,00 | 0,00±2,74 | 6,47±0,08 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |
| | 375 | NR | --- | 9,96±0,00 | 0,00±2,74 | 9,96±0,00 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |
| EM-8 | 1500 | NR | - | NR | --- | 0,00±0,00 | - | NR | - | 8,43±0,03 | 7,69±1,04 |
| | 750 | NR | - | NR | --- | 7,14±0,15 | - | NR | - | 9,96±0,00 | 0,00±1,53 |
| | 375 | NR | - | NR | --- | 9,96±0,00 | - | NR | - | 9,96±0,00 | 0,00±1,53 |
| EM-9 | 1500 | NR | - | 7,40±0,05 | 0,00±1,42 | NR | - | NR | - | NR | - |
| | 750 | NR | - | 9,96±0,00 | 0,00±2,74 | NR | - | NR | - | NR | - |
| | 375 | NR | - | 9,96±0,00 | 0,00±2,74 | NR | - | NR | - | NR | - |
| EM-10 | 1500 | NR | - | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 |
| | 750 | NR | - | 7,17±0,06 | 0,00±2,77 | 6,48±0,22 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 8,18±0,07 | 10,39±1,78 |
| | 375 | NR | - | 9,96±0,00 | 0,00±2,74 | 9,96±0,00 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |
| EM-11 | 1500 | 6,65±0,00 | 0,00±0,88 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 | 0,00±0,00 | 100±0,00 |
| | 750 | 9,96±0,00 | 0,00±1,67 | 0,00±0,00 | 100±0,00 | 9,96±0,00 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 7,61±0,00 | 19,91±1,78 |
| | 375 | 9,96±0,00 | 0,00±1,67 | 9,96±0,00 | 0,00±2,74 | 9,96±0,00 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |
| EM-12 | 1500 | NR | - | 6,42±0,24 | 0,00±2,21 | 0,00±0,00 | 100±0,00 | 6,12±0,22 | 16,67±3,16 | 8,31±0,04 | 8,98±1,30 |
| | 750 | NR | - | 9,96±0,00 | 0,00±2,74 | 0,00±0,00 | 100±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |
| | 375 | NR | - | 9,96±0,00 | 0,00±2,74 | 7,42±0,04 | 0,00±0,00 | 9,96±0,00 | 0,00±2,55 | 9,96±0,00 | 0,00±1,53 |

EM-1: Hexane and without heating extraction; EM-2: Dichloromethane and without heating extraction; EM-3: Ethyl acetate and without heating extraction; EM-4: Butanol and without heating extraction; EM-5: Hexane and with fractionated heating extraction; EM-6: Dichloromethane and with fractionated heating extraction; EM-7: Ethyl acetate and with fractionated heating extraction; EM-8: Buatnol and with fractionated heating extraction; EM-9: Hexane and with heating solvent extraction only; EM-10: Dichloromethane and with heating solvent extraction only; EM-11: Ethyl acetate and with heating solvent extraction only;EM-9: Butanol and with heating solvent extraction only.[1]Mean to $Log_{10}$ of Bacterial Count; Bars = Confidence Interval (α=0,05; *n*=3); [2] Percentual of Inhibition (%) at control treatment; Bars = Confidence Interval (α=0,05; *n*=3); NR – Not rated.

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# 4 CONCLUSÕES

- Os extratos de *Erythrina* spp.apresentaram atividade antimicrobiana e antiaderente frente aos isolados de SGM.
- A seleção de isolados de SGM baseado no fator de virulência é eficaz para estudos relacionados à ação inibitória de extratos vegetais.
- Os extratos de *Erythrina* spp. nas concentrações superiores a 750 $\mu g.mL^{-1}$, mostraram inibição significativa ao crescimento de alguns isolados de SGM.
- Os extratos de *Erythrina* spp. na concentração 1500 $\mu g.mL^{-1}$, mostraram que são capazes de desestruturar biofilmes dos isolados de SGM.
- Extratos de *Erythrina* spp. podem ser uma fonte valiosa para a descoberta de novas moléculas bioativas empregadas para inibição do crescimento de *Streptococcus* do grupo *mutans* e consequentemente na formação de biofilme dentário.

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# ANEXO

## ANEXO A – Normas do periódico Brazilian Journal of Microbiology

ELSEVIER

BRAZILIAN JOURNAL OF MICROBIOLOGY

# GUIDELINES TO AUTHORS

## SCOPE OF THE JOURNAL

Brazilian Journal of Microbiology, published by the Brazilian Society of Microbiology, publishes original research papers and reviews, covering all aspects of Microbiology. The publication fee for this journal is **USD 300** for non-Brazilian citizens, and, **R$ 840,00** for Brazilian citizens.

The following categories of papers are acceptable for publication in Brazilian Journal of Microbiology:

- ***Research paper:*** the research paper reports results of original research, which has not been published elsewhere.
- ***Short Communication:*** a Short Communication is new and significant findings. Submit form is the same way as research paper. They receive the same review, they are not published more rapidly than research paper.
- ***Short-review:*** Review articles should deal with microbiological subjects of broad interest (ONLY BY INVITATION).
- ***Letter to the editor:*** Letters to the Editor are intended only for comments on final, typeset articles published in the journal (manuscripts posted online are not accepted) and must cite published references to support the writer's argument.

Your manuscript must be written clearly, in **comprehensible English.**

The text submitted for publication has to be English reviewed before **submission**. **To submit the manuscript, you must attach the issued certificate in supplementary files.**

## SECTIONS

### *Biotechnology and Industrial Microbiology*

**Bacterial Fermentation**

- biosynthesis and bioconversion of natural products, including antibiotics, xenobiotics, and macromolecules produced by bacteria.
- molecular aspects of bacterial biotechnology

**Fungal Fermentation**

- biosynthesis and bioconversion of natural products, including antibiotics, xenobiotics, and macromolecules produced by fungi
- molecular aspects of fungal biotechnology

### *Food Microbiology*

**Technology**

- applications of microorganisms (bacteria and fungi) for food production

**Safety and Quality**

- food borne diseases
- food spoilage
- microbial ecology in foods

### *Clinical Microbiology*

**Bacteria, Fungi, and Virus**

- Laboratory diagnosis of human infections and the role of the laboratory in both the management of infectious diseases and the elucidation of the epidemiology of infections.
- Microbial resistance and mechanisms of antimicrobial agents.

### *Environmental Microbiology*

**Microbial Ecology**

- ecology of natural microbial assemblages, microbial diversity of natural environments such as water, soil, sediments and higher organisms
- microbial interactions
- environmental aspects of public health
- biodegradation
- bioremediation

### *Bacterial and Fungal Molecular Pathogenesis*

- Genetic, biochemical, and structural basis of bacterial and fungal pathogenesis

### *Bacterial and Fungal Physiology*

- Biochemistry, biophysics, metabolism, cell structure, stress response, growth, differentiation, and other related process of bacteria and fungi

### *Veterinary Microbiology*

- control and/or treatment of animals
- animal pathogen diagnostics
- veterinary or zoonotic pathogens

## SUBMISSION OF A MANUSCRIPT

Submission of a manuscript to Brazilian Journal of Microbiology is understood to imply that it has not

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previously been published (except in an abstract form) and that it is not being considered for publication elsewhere.

Upon receipt of a manuscript all authors will receive an electronic message acknowledging the receipt.

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## PUBLICATION OF A MANUSCRIPT

Manuscripts are accepted for publication after having been critically reviewed by at least two referees, indicated by the Editors.

The suggestions and recommendations of the reviewers and Editors will be forwarded electronically to the corresponding author, who should return the reviewed manuscript to the Editors within the stipulated date, via online system. Whenever applicable, the corresponding author should explain or comment each modification introduced in the text.

The corresponding author will receive an electronic message whenever the manuscript moves from one status to the next.

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Nonmember scientists from Brazil and other countries are invited to submit papers for analysis.

## ETHICS

When the study, described in the manuscript, is related to experiments carried out with human beings and/or animals, author(s) must inform, within the text, if the research project has been approved by the Research Ethics Committee of their institution, according to the Declaration of Helsinki (http://www.fcm.unicamp.br/fcm/sites/default/files/declaracao_de_helsinque.pdf). Experimental studies involving animals should follow the guidelines established by the "Guide for the Care and Use of Laboratory Animals" (http://www.ncbi.nlm.nih.gov/books/NBK54050/) (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, D. C. 1996), and the *Princípios Éticos na Experimentação Animal do Colégio Brasileiro de Experimentação Animal* (COBEA) (Ethical Principles for Animal Experimentation of the Brazilian College of Animal Experimentation - http://www.cobea.org.br/conteudo/view?ID_CONTEUDO=65).

## PREPARATION OF A MANUSCRIPT

The manuscript should be submitted as one single WORD file. This single file should include: the whole text, figures, tables, etc. Only manuscripts written in English will be considered.

For **research papers**, the WORD file should contain:

- Title (100 characters)
- Running title (40 characters)
- Authors and Affiliations
- Abstract (up to 200 words)
- Three to five key-words
- Introduction
- Materials and Methods
- Results
- Discussion
- Acknowledgements (optional)
- References

For **short communications**, the WORD file should contain:

- Title
- Running title
- Authors and Affiliations
- Abstract (up to 50 words)
- Three to five key-words
- Text not divided in topics
- Acknowledgements (optional)
- References

For **short-review**, the WORD file should contain:

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- Running title (40 characters)
- Authors and Affiliations
- Abstract (up to 200 words)
- Three to five key-words
- Text
- Acknowledgements (optional)
- References

For **Letter to the Editor** the WORD file should contain:

- Title (100 characters)
- Running title (40 characters)
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- Text (no more than 500 words and must be typed double-spaced)
- References

Author affiliations should be presented in decreasing hierarchical order (e.g. Harvard University, Harvard Business School, Boston, USA) and should be written as established in its own language (e.g. Université Paris-Sorbonne; Harvard University, Universidade de São Paulo).

All manuscripts should be typed double-spaced with 3 cm margins and pages should be numbered sequentially. The lines in each page of the manuscript should be numbered too. The Editors recommend that a manuscript should be critically read by someone fluent in English before submission.

Manuscripts written in poor English will not be accepted.

*Research papers* and *short-review* consist of 20 pages, including references, tables and figures.

Abbreviations of terms and symbols should follow the recommendations of IUPAC-IUB Commission

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(*Comission on Biochemical Nomenclature, Amendments and Corrections*) and the units are to be used according to SI (*International Systems of Units*).

### Suggested Reviewers

Authors may submit suggestions of reviewers to evaluate the manuscripts. The following information must be provided: reviewer name, e-mail address, and the home institution.

### Use of plant extracts in microbiological experiments

Articles that present studies with plant extracts, or other complex substances, will be accepted only after identification of compounds.

## ORGANIZATION

The full **Title** of the article should be as brief as possible, not exceed 100 characters including spaces, should not contain abbreviations, and be truly indicative of the subject of the paper. Authors should suggest a **Running title** that appears in the page header which should not exceed 40 characters, including spaces.

Expressions like "Effects of", "Influence of", "Study on", etc, should be avoided. Care should be exercised in preparing the title since it is used in literature retrieval systems.

The **Abstract** should summarize the basic content of the paper. The abstract should be meaningful without reference to the text. An abstract should not contain references, tables or unusual abbreviations. Abstracts are reprinted by abstracting journals and therefore will be read by persons who do not have access to the entire paper.

The **Introduction** should provide the reader with sufficient information so that the results reported in the paper can be properly evaluated without referring to the literature. However, the introduction should not be an extensive review of the literature. The introduction should also give the rationale for and objectives of the study that is being reported.

The **Materials and Methods** section should provide enough information for other investigators to repeat the experiments.

Repetition of details of procedures which have already been published elsewhere should be avoided. If a published method is modified, such modification(s) must be described in the paper. Sources of reagents, culture media and equipment (company, city, state, country) should be mentioned in the text. Names that are registered trade marks should be so indicated. Subheading often makes this section easier to read and understand.

The **Results** section should, by means of text, tables and/or figures, give the results of the experiments, extensive interpretation of results has to be avoid but do so in the *Discussion* section. Tables and figures should be numbered using Arabic numerals. All tables and figures must be mentioned in the text.

The approximate location of tables and figures in the text should be indicated.

The **Discussion** section should discuss the results in relation to the literature cited.

**In-text citations:** Indicate references by (consecutive) superscript arabic numerals in the order in which they appear in the text. The numerals are to be used outside periods and commas, inside colons and semicolons. For further detail and examples you are referred to the AMA Manual of Style, A Guide for Authors and Editors, Ninth Edition, ISBN 0-683- 40206-4, copies of which may be ordered from Lippincott Williams & Wilkins (http://www.lww.com/index.html).

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**Reference list:** Number the references in the list in the order in which they appear in the text.

General rules from the 10th edition

- Items are listed numerically in the order they are cited in the text
- Include up to 6 authors
- For more than six, provide the names of the first three authors and then add et al

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**ACKNOWLEDGMENTS:** This section is optional. It acknowledges financial and personal assistance.

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