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# Chitosan/gelatin/copaiba oil emulsion formulation and its potential on controlling the growth of pathogenic bacteria

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ABSTRACT

This work report the development of an emulsion formulation combining chitosan and copaiba oil. The antimicrobial activity and cytotoxic effect of three different copaiba oils (CO-A, CO-B and CO-C), chitosan/gelatin gel (CG), and chitosan/gelatin/copaiba oil (CGCO) emulsions against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were evaluated. Susceptibility tests were performed by the minimum inhibitory concentration (MIC) using the micro-broth dilution technique. The cytotoxicity effect of compounds on Vero cell line was also evaluated using the MTT assay. CG inhibited *S. aureus*, *E. coli*, and *P. aeruginosa* growth, showing MIC of 31.2, 62.5, and 31.2 $\mu g\,mL^{-1}$, respectively. Copaiba oils inhibited *S. aureus* with MIC of $2.0 \times 10^3$ $\mu g\,mL^{-1}$ for CO-A, 500 $\mu g\,mL^{-1}$ for CO-B, and 62.5 $\mu g\,mL^{-1}$ for CO-C. The results observed for CGCO-C emulsion against *S. aureus* suggest synergism. The CO-C and CG gel demonstrate bacteriostatic and bactericidal activity against *S. aureus*, respectively, whereas CGCO-C emulsion was bactericidal at a lower concentration and at short time interval than the individual compounds. CO-A and CO-B showed toxicity to Vero cells whereas, for CO-C, CG gel and all the emulsions, no cytotoxic effect was observed. The results of this study demonstrated that emulsions formulated by the combination of chitosan/gelatin/copaiba oils have potential as a new selective agent to control *S. aureus*.


## 1. Introduction

The introduction of antibiotics offered a number of benefits to public health however, this intervention in human-microorganism relationships, without considering microbial ecology and evolution, contributed to the spread of microbial resistance (Baker, 2015; Howard et al., 2014). Bacterial infections in hospital environments are of great concern, since they contribute to the widespread resistance to known antibiotics and to the emergence of multidrug-resistant bacteria, reinforcing the constant search for new molecules (Baker, 2015; Howard et al., 2014). The development of sustainable drugs that involve natural products has arisen as an alternative to solve these problems.

Chitin is a structural polysaccharide that occurs as two allomorphs, depending on the source, named α and β forms. α-chitin is present in fungal mycelium and yeast cell walls; in krill, lobster, and crab tendons and shells; in shrimp shells; in insect cuticles; and in crustaceans. β-chitin is found in squid pens associated with proteins (Gavhane et al., 2013). Chitosan is a linear copolymer composed of β-(1–4)-linked N-acetyl-D-glucosamine obtained by partial chitin deacetylation. There are currently a variety of applications for chitosan in the biomedical, food, and chemical industries owing to its interesting properties, such as biodegradability, biocompatibility, low toxicity, and biological activity (Anitha et al., 2014; Gavhane et al., 2013). Chitosan has demonstrated good potential as an antimicrobial agent, although a few studies has been done using β-chitin (Chen and Zhao, 2012; Jeon et al., 2014; Park et al., 2015) once α-chitin is more abundant and easy available. Nevertheless, chitosan obtained from squid pens (β-chitin) has interesting properties in comparison to chitosan extracted from shrimp or crab (α-chitin), such as higher molecular weight, higher intrinsic viscosity, higher solubility, and more efficient deacetylation yields than α-chitin (Rinaudo, 2006). The solubility and degree of deacetylation are some of factors that affect chitosan-bacteria interactions, since they promote better accessibility of amine groups of chitosan to the bacteria (Kong et al., 2010; Younes et al., 2014).

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Gelatin is linear protein obtained by denaturation of collagen by acid hydrolysis (type A, pI = pH 7–9) or alkaline treatment (type B, pI = pH 4.8–5.2) consisting of amino acids organized in soft blocks, with triads containing always a glycine in the position of the third amino acid; and rigid blocks, composed by a sequence of hydroxyproline, proline and glycine, presenting a narrow distribution of molar mass. Furthermore, gelatine form gels by cooling prewarmed solutions and is a biocompatible, biodegradable, non-toxic and low cost natural polymer (Wang et al., 2016; Gavhane et al., 2013; Pereda et al., 2011).

Chitosan and gelatin are versatile biopolymers that have been utilized in different formulations as gels, membranes, films, sponges, emulsions, and nanoparticles (Wang et al., 2016; Gavhane et al., 2013; Pereda et al., 2011).

Copaiba oil is produced by exudation from the trunks of trees belonging to the genus *Copaifera*, which is abundant in the Brazilian flora, mainly in Amazon region. The oleoresin contains mixtures of sesquiterpenes and diterpenes that differ between and among species (Veiga et al., 2001). Several studies have confirmed its anti-inflammatory properties (Veiga Junior et al., 2007), anti-tumor properties (Lima et al., 2003), analgesic properties (Gomes et al., 2007), and healing and antibiotic activity (Morelli et al., 2015; Santos et al., 2008; Veiga et al., 2001). Copaiba oil is widely used in folk medicine to treat many diseases and combat various pathogens, and comprises a good alternative for targeting antibiotic resistant microorganisms (Santos et al., 2008; Veiga et al., 2001). However, use of the oleoresin in the clinical practice is limited due to certain drawbacks, such as its intense aroma, potential toxicity, and volatile nature, leading to poor lasting effects (Dias et al., 2014). In addition, the water-insoluble nature of copaiba oil may cause an unpleasant feeling when used topically. To reduce these undesirable effects an interesting approach is to combine copaiba oil with chitosan/gelatin in emulsion form. Some advantages on such emulsion formulations is the presence of positive charges, resulting in cationic droplets that makes emulsions less prone to destabilization by multivalent cations (e.g., $Ca^{2+}$) and increased bioactivity. In addition, they can form networks promoting stabilitiy against coalescence, and improve the stabilization of certain drugs comparatively to other systems, resulting in a strategy to treat locally inflamed skin and bacterial infections (Wang and Heuzey, 2016; Dias et al., 2014).

In this work, we report the antimicrobial activity and cytotoxicity of a new emulsion formulation combining chitosan/gelatin and copaiba oil.

## 2. Materials and methods

### 2.1. Materials

Chitosan was obtained from squid pens (*Loligo* sp.) by deproteinization and deacetylation process as previously described (Horn et al., 2009). Chitosan molecular weight (MW) and degree of acetylation (DA) were determined by viscosimetry and condutimetric titration, respectively, as described by Raymond et al. (1993). DA calculated value was 9.7% ± 1.2 and MW was $1.5 \times 10^5$ g mol$^{-1}$. Gelatin (type A) derivated from porcine skin, with average molecular weight of 50,000–100,000 was purchased from Sigma®. A 2% chitosan gel was prepared in 1% acetic acid. A gelatin gel (2%) was obtained by dissolving commercial gelatin in water and heating at 60 °C for 30 min. Copaiba oils (CO) utilized in this study were acquired in São Paulo (CO-A) and from a public market in Belém, in the state of Pará, Brazil (CO-B and CO-C).

### 2.2. Characterization of copaiba oils

Gas chromatography coupled with mass spectrometry (GC/MS) was performed on a Shimadzu QP2010 (Shimadzu Corporation, Kyoto, Japan) system consisting of an AOC −20 auto-sampler and gas chromatograph interfaced to a mass spectrometer (GC–MS QP2010 Plus) with a J&W Scientific DB-5MS (Folsom, CA, USA) (5% phenylmethylpolysiloxane) fused-silica capillary column (30 cm x 0.25 mm i.d., 0.25 μm film thickness). The procedure was carried out in electron impact mode at 70 eV. Helium (99.99%) was used as a carrier gas at a constant flow of 0.99 mL min$^{-1}$ and an injection volume of 1 μL was employed (split ratio of 1:10). The injector temperature was 250 °C and the ion-source temperature was 250 °C. The oven temperature was programmed for 50 °C (isothermal for 1 min) with an increase of 5 °C min$^{-1}$ until reaching 300 °C, ending with a 10 min isothermal period at 300 °C. Mass spectra were taken at 70 eV with a scan interval of 0.3 s and fragments of 40–500 Da.

Methyl esters were identified by computerized matching of the acquired mass spectra with those stored in the WILEY8, NIST107, NIST05, NIST05s, and NIST21 mass spectral libraries of the GC–MS data system. For terpene analysis, a mixture of hydrocarbons ($C_9H_{20}$–$C_{18}H_{38}$) was injected under the conditions described above, and the constituents were also identified by computerized matching and comparison of the spectra obtained with those from the databank, considering the relative retention index (RRI) (Adams, 2007).

### 2.3. Preparation of emulsions

Chitosan/gelatin gel was prepared by mixing chitosan and gelatin gels at the ratio of 2:1 (w/w) under mechanical constant stirring ($2.0 \times 10^3$ rev min$^{-1}$) for 30 min at 25 °C. The final mixture was separated in two fractions. The first was adjusted to pH 5.0 with 0.5 mol L$^{-1}$ $NaHCO_3$ and named CG. Emulsions were prepared using the second fraction of the gel by mixing with copaiba oils. First, copaiba oils were diluted in 1% dimethyl sulfoxide (DMSO) to a final concentration of $8.0 \times 10^3$ μg mL$^{-1}$, $2.0 \times 10^3$ μg mL$^{-1}$, and 250 μg mL$^{-1}$ of CO-A, CO-B, and CO-C, respectively. Each solution of copaiba oil was gently dropped to 125 μg mL$^{-1}$ of chitosan/gelatin gel under mechanical constant stirring ($2.0 \times 10^3$ rev min$^{-1}$) for 30 min at 25 °C. Chitosan/gelatin/copaiba oil (CGCO) emulsions were named according to the type of oil used: CGCO-A, CGCO-B, and CGCO-C. CGCO emulsions were formulated using 4 x the minimum inhibitory concentrations (MIC) found for CG gel and copaiba oils individually. Table 1 shows the composition of the formulations.

**Table 1**
Composition of chitosan/gelatin/copaiba oil emulsions (CGCO).

| Emulsions | Concentration (μg mL$^{-1}$)[b] | | | |
|---|---|---|---|---|
| | Chitosan/gelatin gel (CG)[a] | Copaiba oil-A (CO-A) | Copaiba oil-B (CO-B) | Copaiba oil-C (CO-C) |
| CGCO-A | 125 | $8.0 \times 10^3$ | – | – |
| CGCO-B | 125 | – | $2.0 \times 10^3$ | – |
| CGCO-C | 125 | – | – | 250 |

[a] Chitosan/gelatin gel was prepared at the ratio of 2:1 (12.0 g/6.0 g) as described in experimental section.
[b] The emulsions were prepared using 4 x MIC found for CG and CO individually.