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Evaluation of antimicrobial activity and cell viability of *Aloe vera* sponges

Sávio Morato de Lacerda Gontijo[1] · Alinne Damásia Martins Gomes[2] · Alfonso Gala-García[3] · Rubén Dario Sinisterra[2] · Maria Esperanza Cortés ✉ **[3]**

[1]Universidade Federal de Minas Gerais, Institute of Biological Sciences, Belo Horizonte, Minas Gerais, Brazil
[2]Universidade Federal de Minas Gerais, Department of Chemistry, Belo Horizonte, Minas Gerais, Brazil
[3]Universidade Federal de Minas Gerais, Department of Restorative Dentistry, Belo Horizonte, Minas Gerais, Brazil

✉ Corresponding author: mecortes@ufmg.br
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Abstract

Background: *Aloe vera* L., member of the Liliaceae family, has been shown to stimulate cell proliferation and contribute to healing and angiogenesis, has anti-bacterial, anti-fungal and anti-inflammatory activity. In addition, *Aloe vera* can be used as a support for drug transport. Our objective is to evaluate antimicrobial activity and cytotoxicity of sponges of *Aloe vera* L. for use as a carrying support of drugs.
Results: In this work, sponge of free *Aloe vera* (AV) loaded with amoxicillin (AMX) or nystatin (NYS) at 1% w/w, were prepared and physico-chemically characterized via X-ray diffraction, Fourier Transform Infrared Spectroscopy and thermal analysis. Antimicrobial potency of AV sponge alone, loaded with AMX or NYS, against strains of *Streptococcus mutans*, *Staphylococcus aureus*, *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis* and *Candida albicans* was determined. Osteoblasts and human gingival fibroblasts were cultivated on AV, *Aloe vera* loaded with amoxicillin (AV/AMX) and *Aloe vera* loaded with nystatin (AV/NYS) and cellular viability was assessed. The physico-chemical characterization performed suggested that the loaded drugs were dispersed in the sponge and those interactions between the AV sponge and the loaded drugs were weak. Furthermore, AV loaded with AMX or NYS demonstrated antimicrobial potency and osteoblasts and fibroblasts were viable after 24 hrs on free AV, and AV loaded with AMX or NYS.
Conclusions: Our results indicate that sponges of free AV, loaded with AMX or NYS, are biocompatible and exhibit antimicrobial activity.

Keywords: amoxicillin, cytotoxicity, drug transport, nystatin.

INTRODUCTION

Aloe vera L. (Liliaceae), a biomaterial of vegetable origin, is a xerophyte, which are adapted to living in areas of low water availability and are characterized by an abundance of water storage tissue and elongated pointed leaves. Each leaf consists of two parts, an outer green rind and an inner clear pulp. The pulp, which is clear and mucilaginous and is the most widely therapeutically used portion of the plant, is also referred to as reserve parenchyma and makes up the majority of the leaf by volume (Ni et al. 2004; Chen et al. 2012).

The reserve parenchyma has been shown to stimulate cell proliferation and contribute to healing and angiogenesis, as well as act as an anesthetic, anti-bacterial, anti-fungal, anti-viral, and anti-inflammatory agent (Grindlay and Reynolds, 1986; Davis et al. 1994; Heggers et al. 1996; Reynolds

and Dweck, 1999; Yao et al. 2009). In addition, *Aloe vera* (AV) can be used as a matrix for drug transport (Gala-García et al. 2010; Chen et al. 2012). Natural products have promise for use as a carrier of antibiotics during infections for local drug delivery and reduce the systemic toxicity and potential side effects of parenteral administration.

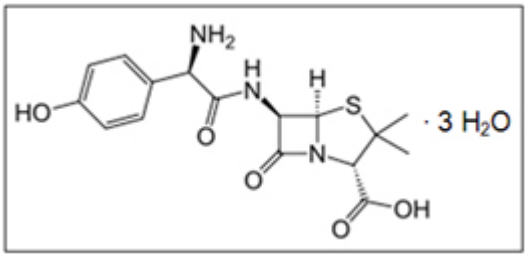

Fig. 1 Chemical structure of amoxicillin.

One reason that the materials used for regeneration and repair of bone defects often fail is that the injured tissue often becomes infected. Amoxicillin (AMX) has been considered the antibiotic of first choice for many types of bone infections, including periodontal disease, osteomyelitis and alveolitis (Palmer et al. 2000). AMX (Figure 1), an antibiotic of the penicillin group, acts to inhibit the bacterial cell wall of Gram-positive and Gram-negative bacteria. It is active against bacterial strains of *Streptococcus mutans* (Castillo et al. 2006), *Staphylococcus aureus*, *Enterococcus faecalis* and *Aggregatibacter actinomycetemcomitans* (Hoelscher et al. 2006).

Nystatin (NYS) (Figure 2) is an antifungal agent extracted from cultures of *Streptomyces*, and belongs to the group of polyenes that interact with ergosterol, a sterol present in the plasma membranes of fungal cells, causing a functional disruption due to the formation of transmembrane channels. These changes lead to a loss of selective permeability of the fungal cells, culminating in damage and cell death (Silva et al. 2006). NYS is effective against most infections caused by *Candida* spp., which may be linked to osteomyelitis, however it is used only for prophylaxis and treatment of superficial candidiasis of the skin and external mucous membranes (Arias et al. 2004; Ship et al. 2007).

AV is used as a natural sponge for drug transport. The work proposed here seeks to physico-chemically characterize, evaluate the antimicrobial activity and the biocompatibility, of *Aloe vera* sponge alone, loaded with AMX or NYS. To evaluate these sponges, we used X-ray diffraction (XRD), thermal analysis, Fourier Transform Infrared Spectroscopy (FTIR), viability testing with osteoblasts and fibroblasts, as well as agar diffusion tests to antimicrobial activity.

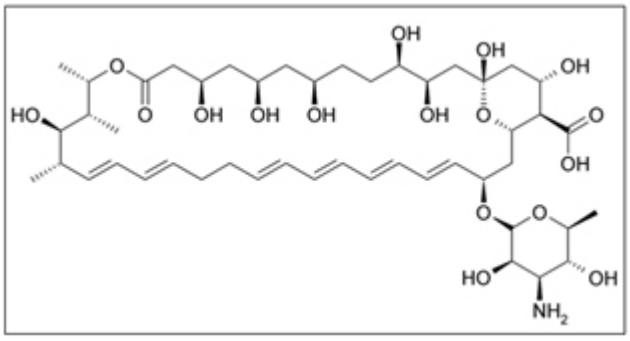

Fig. 2 Chemical structure of nystatin.

MATERIALS AND METHODS

Materials

Dulbecco's phosphate buffered saline, trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) kit were purchased from Gibco (NY, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT kit was purchased from Invitrogen Corporation, USA. Sodium dodecyl sulfate (SDS) 10% HCl was purchased from American Bioanalytical (Natik, USA). Dulbecco's Modified Eagle Medium (DMEM) for cell culture was purchased from Sigma (St. Louis, USA). Amoxicillin (AMX), nystatin (NYS), Mueller Hinton agar, and brain heart infusion broth were obtained from Himedia laboratories PVT limited, India. Disk AMX-clavulanic acid (30 µg) and disk of NYS 100 international unit (IU) were purchased from Cecon, Brazil.

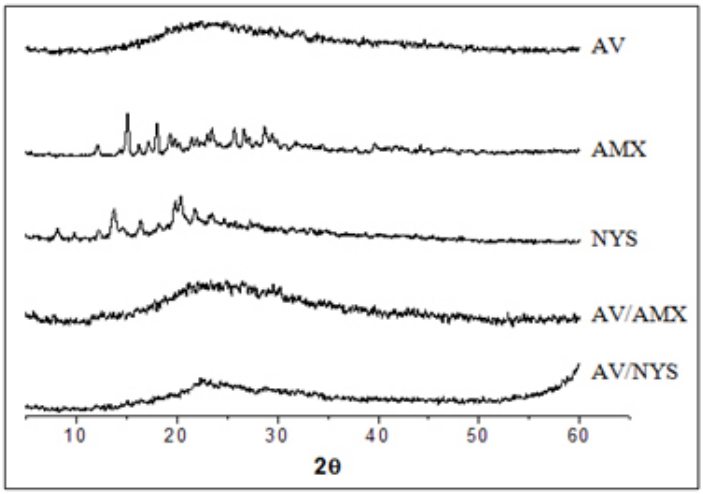

Fig. 3 X-ray powder pattern diffraction of free *Aloe vera* (AV), amoxicillin (AMX), nystatin (NYS), *Aloe vera* loaded with amoxicillin (AV/AMX) and *Aloe vera* loaded with nystatin (AV/NYS).

Plant materials

Based on criteria derived from the literature (Villalobos and Salazar, 2001), a healthy *Aloe vera* L. plant of approximately 4 years old was selected from the University Federal of Minas Gerais (UFMG) Botanical Garden collection (Belo Horizonte, Brazil).

Leaves of *Aloe vera* were collected, left to rest in distilled water for 8 hrs to eliminate aloine, and cut into pieces. Afterwards, pulp fragments were liquefied, sieved, filtered with negative pressure to obtain the juice, and freeze-dried. After that, they were sterilized by ethylene oxide and stored at 4ºC (Gala-García et al. 2008; Chakraborty et al. 2011).

Microorganisms

Four bacterial and one fungal microorganism were used in these studies: *Streptococcus mutans* (*S.m*) (ATCC 25175), *Staphylococcus aureus* (*S.a*) (ATCC 27664), *Aggregatibacter actinomycetemcomitans* (*A.a*) (Y4FDC), *Enterococcus faecalis* (*E.f*) (ATCC 14508) and *Candida albicans* (*C.a*) (ATCC 18804).

Preparation of samples

The freeze-dried *Aloe vera* L. assumed a sponge-like structure, of which for each 99 mg weighed was mixed with 1 mg of either AMX or NYS dissolved in 50 mL of distilled water. This mixture was

incubated at room temperature for 4 hrs, and then freeze-dried. The result was sponge of *Aloe vera* L. loaded with AMX or NYS at 1% w/w.

Characterization studies

Samples and free substances were characterized by FTIR, XRD and thermogravimetric analysis. Absorption spectra in the infrared range of 4000-400 cm^{-1} were obtained from KBr pellets and recorded on a Perkin Elmer Espectrum GX spectrophotometer. The X-ray powder diffraction patterns of the samples were obtained in a Rigaku Geigerflex 2037 diffractometer using copper pipe and CuK_{α} (λ = 1.54051) radiation at 2θ angles ranging from 4 to 60 degrees and a scan rate of 40 min^{-1}, the voltage and current per step were 30 kV and 5 mA, respectively. Thermal analyses were performed using a TA-Instruments Q600 thermogravimetric analysis system. Samples were heated from ambient (~25ºC) to 700ºC at a β value of 10ºC min^{-1}, in nitrogen gas with a flow rate of 100 mL/min.

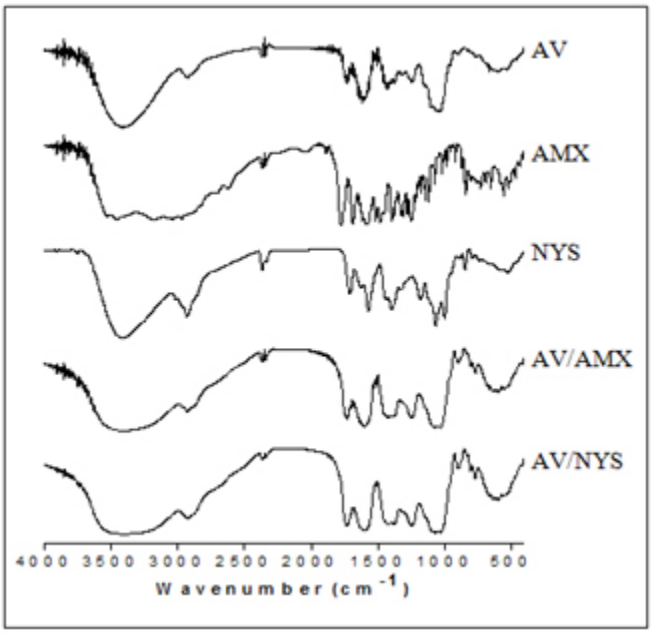

Fig. 4 IR spectra of free *Aloe vera* (AV), amoxicillin (AMX), nystatin (NYS), *Aloe vera* loaded with amoxicillin (AV/AMX) and *Aloe vera* loaded with nystatin (AV/NYS).

Antimicrobial test by agar diffusion

To evaluate the antimicrobial potential of the sponges, antimicrobial assays were performed in triplicate using diffusion of a blank disk of 5.0 mm diameter in Mueller-Hinton agar. The inoculum was obtained from recent cultures of microorganisms (between 20-24 hrs at 35ºC for *Candida albicans* and 37ºC for other strains) by preparing standardized suspensions at 0.5 MacFarland (10^5 colonies forming units - (CFU)). Agar cavities were then performed with 50 µL tips for insertion of the freeze-dried samples. The experimental groups (sponge of free *Aloe vera*, and loaded with AMX or NYS at 1% w/w) were inserted into the wells made. The control group was treated with disk AMX-clavulanic acid (30 µg) or disk of NYS (100 IU). Cultures with disks were incubated at 35ºC for *Candida albicans* and 37ºC for the other strains for 24 hrs. Results were obtained by measuring the diameter of inhibition zones formed around the disks with the aid of a halometer and expressed in millimeters and analyzed by ANOVA and Tukey ($p < 0.05$).

Cultivation of human gingival fibroblasts

Primary cultures of immortalized human gingival fibroblasts (HGF) were generously donated from the University of São Paulo (USP-Brazil). These cells were used for this experiment according to the

experimental protocol approved by the committee of USP, Brazil (047/06). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% streptomycin/amphotericin in an incubator containing 5% CO_2 at 37ºC. After confluence the cells were used for experiments on the 7th passage and seeded at a density of 10^5 cells/mL in 96 wells plates (Yao et al. 2009).

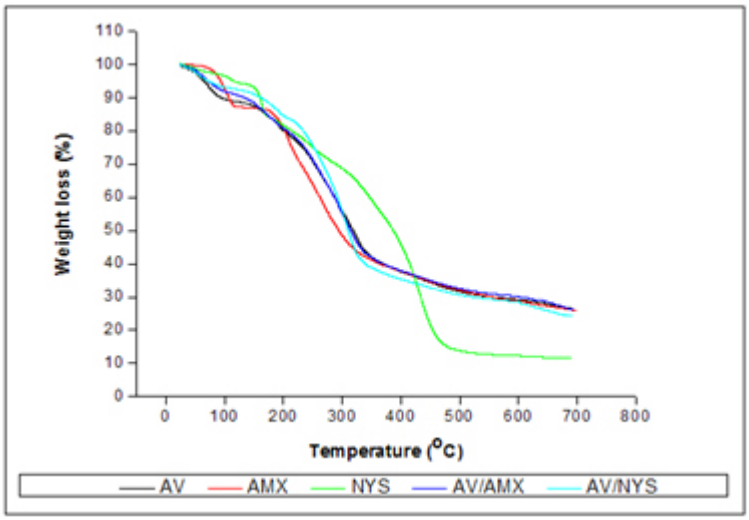

Fig. 5 TG curves: Free *Aloe vera* (AV), amoxicillin (AMX), nystatin (NYS), *Aloe vera* loaded with amoxicillin (AV/AMX) and *Aloe vera* loaded with nystatin (AV/NYS).

Culture of osteoblasts

Osteoblasts (OB) were isolated from the calvaria of 1-4 day old neonatal male Wistar rats obtained from the bioterium of the Institute of Biological Science, UFMG. These cells were used for this experiment according to the experimental protocol approved by the animal experiment committee of UFMG, Brazil (167/07). The calvaria was dissected and freed from the soft tissue, cut into small pieces and rinsed in sterile phosphate-buffered saline without calcium and magnesium. The calvaria pieces were incubated with 1% trypsin-EDTA for 5 min, followed by four sequential incubations with 2% collagenase at 37ºC for 40 min each. The supernatant of the first collagenase incubation, which contains a high proportion of periosteal fibroblasts, was discarded. Subsequent digestions produced a suspension of cells with a high proportion of osteoblasts. After centrifugation at 208 g, each pellet was resuspended in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic. Cells were grown in a controlled humidified incubator at 37ºC and 5% CO_2. After confluence, cells were used for experiments on the 3rd passage and seeded at a density of 10^5 cells/mL in 96 wells plates.

Cellular viability

Fresh DMEM solution consisting of free *Aloe vera*, and *Aloe vera* loaded with AMX or NYS at 1% w/w, was left shaking for 24 hrs, followed by centrifugation at 10621 g. The supernatant was then collected and filtered through a membrane of 0.22 µm. Cells were then treated with different concentrations of free *Aloe vera*, loaded with AMX or NYS (100, 500 and 1000 µg/mL) for 24 hrs, followed by a determination of cell viability.

After 24 hrs of incubation with various treatments, osteoblast and fibroblast viability was evaluated by MTT assay based on the reduction of tetrazolium salt to formazan crystals in living cells. About 10 µL of MTT (5 mg/mL) was added to each well. Four hours later, cell morphology was analyzed by inverted optical microscopy and formazan crystals were dissolved with SDS 10% HCl. After incubation for 14 hrs, optical density measurements were performed at 570 nm. All quantitative results were obtained from hexaplicate samples. Data were expressed as mean ± SD. Statistical analysis was carried out using ANOVA and Bonferroni's post-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Physico-chemical characterization

The XRD pattern of AMX (Figure 3) was semi-crystalline, with a halo of the amorphous region at 35-60º 2θ. The presence of diffractogram peaks of crystallinity were found at 12.27º, 15.47º, 17.24º, 18.13º, 19.56º, 23.11º, 30.05º, 23.64º, 25.78º, 26.84º and 28.98º 2θ. The XRD pattern of AMX was found to be in accordance with those reported by Reverchon et al. (2000), in which they were found to be polycrystalline, possibly due to a polymorphism presented by the drug.

The XRD pattern of NYS (Figure 3) exhibited a semi-crystalline diffraction profile, with a halo of the amorphous region at 30-60º 2θ. Diffractogram peaks of crystallinity were found at 8.12º, 13.75º, 16.25º, 20º, 20.62º, 22.04º and 23.64º 2θ (Llabot et al. 2007a).

The XRD pattern of free *Aloe vera*, and that loaded with AMX or NYS 1% w/w (Figure 3), exhibited amorphous profiles. On the basis of these results, we postulate that NYS and AMX are dispersed in the *Aloe vera* sponge, resulting in an amorphous solid mixture of sponge and drugs (Llabot et al. 2007b).

An FTIR study was performed to determine the interactions between *Aloe vera* and AMX or NYS. The spectrum obtained (Figure 4) indicates the presence of a band in all samples (free *Aloe vera*, and *Aloe vera* loaded with AMX or NYS) at a frequency of about 3500 cm^{-1}, corresponding to the vibrations of the hydroxyl group stretch. The band present at a frequency of 1850-1700 cm^{-1} corresponds to the stretching vibrations of a carbonyl group of carboxylic acid that is present in all samples and drugs. The band of the wave number in the range of 1300-1150 cm^{-1} and 1150-900 cm^{-1} corresponds to stretching vibrations of aliphatic ester and ether respectively, which is not present in the amoxicillin trihydrate (Di Stefano et al. 2002; Saibuatong and Phisalaphong, 2010; Chang et al. 2011; Krokida et al. 2011). This analysis of the spectrum suggests that there is not a strong interaction between the array of *Aloe vera* and the drugs used, due to the fact that the combination of *Aloe vera* and drugs did not result in a new band or the displacement of an existing band.

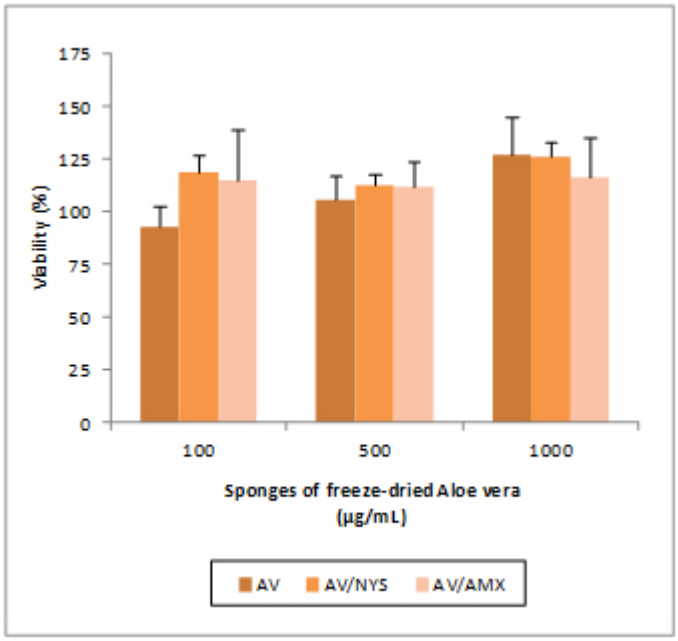

Fig. 6 Effect of different concentrations (µg/mL) of free *Aloe vera* (AV), and *Aloe vera* loaded with nystatin (AV/NYS) or amoxicillin (AV/AMX) on human gingival fibroblasts (HGF) viability *in vitro*. Values represent means ± standard error (n = 6).

Thermogravimetry (TG) curves for AMX (Figure 5) show an initial loss of 13% mass in the region of 40-115ºC, equivalent to the loss of water molecules in the hydration of raw material. Following this there is a plateau up to about 175ºC. From this region up to 700ºC there are events of mass loss until complete decomposition with formation of a residue of 20% (Di Stefano et al. 2002). The TG curves for free *Aloe vera* (Figure 5) show a succession of events of mass loss in the region of 50-700ºC, with the first event of a loss of 10% in the region of 50-100ºC due moisture loss. Thus, after complete decomposition, there was a formation of residue equivalent to 25%. This pattern of thermograms can be explained by the complexity of the sponge, which contains a large number of substances (Maensiri et al. 2008; Xing and Li, 2009). TG curves for NYS (Figure 5) show sequence mass loss events in the region of 25-700ºC, which may indicate a polymorphism of the drug. Thus, after complete decomposition, there was a formation of residue equivalent to 12%. TG curves of *Aloe vera* loaded with AMX or NYS (Figure 5) maintained a thermogravimetric pattern similar to that of free *Aloe vera*, which can be explained by the weak interactions between the sponge and the antimicrobial drugs.

Evaluation of antimicrobial activity

Previous results have indicated that gel *Aloe vera* has no antimicrobial activity against strains of *C.a* and *S.a* (Moody et al. 2004; Cock, 2008). However, according to Moodyet al. (2004), ethanol extract of *Aloe vera* at a concentration of 100 mg/mL has antimicrobial activity against *C.a* (15 ± 0,2) and *S.a* (20 ± 0,3). In addition, a protein of 14 kDa from the *Aloe vera* leaf gel isolated exhibited a potent anti-fungal activity against *Candida paraprilosis*, *Candida krusei* and *Candida albicans* (Das et al. 2011). In this study, sponge of *Aloe vera* was shown to inhibit the growth of *C.a* in a similar manner to controls (nystatin disk). This result is in accord with Das et al. (2011), reinforcing that the protein of 14 kDa from the *Aloe vera* leaf gel could be essential to antifungal activity. This protein probably is preserved in the freeze-dried *Aloe vera*.

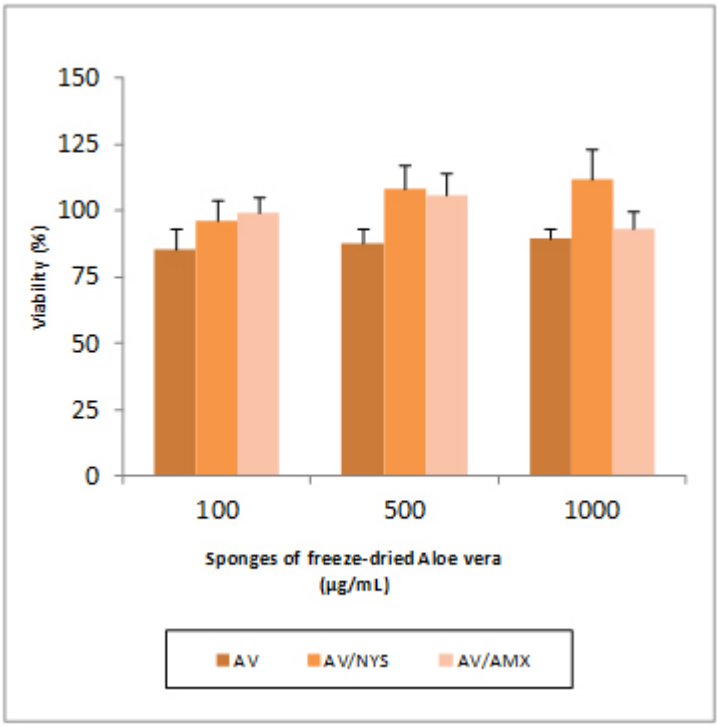

Fig. 7 Effect of different concentrations (µg/mL) of free *Aloe vera* (AV), and *Aloe vera* loaded with nystatin (AV/NYS) or amoxicillin (AV/AMX) on osteoblasts (OB) viability *in vitro*. Values represent means ± standard error (n = 6).

In relation with the antimicrobial activity, *S.a* and *S.m* strains were sensitive only to *Aloe vera* with AMX, none difference compared to control (amoxicillin-clavulanic acid 30 µg disk). *A.a* and *E.f* strains were significantly more sensitive to *Aloe vera* with AMX than to control (amoxicillin-clavulanic acid 30 µg disk) (Table 1). These results are suggesting that have a synergistic effect between the antimicrobial and *Aloe vera* sponges in both Gram-negative and Gram-positive bacteria, respectively. However, George et al. (2009) founded that gel *Aloe vera* has antimicrobial activity against *S.m* and *E.f*. In addition, Cock (2008) demonstrated that fractions isolated from parenchyma reserve have antimicrobial activity against Gram-negative strains, however the Gram-positive strains are resistant to the components of *Aloe vera*.

Study of cell viability of human gingival fibroblasts

Gingival fibroblasts and fibroblasts from the periodontal ligament are the primary cells of the periodontal tissue. Infection and inflammation of tissue that is composed of the alveolar bone, periodontal ligament and cementum, is called periodontitis. Agents and devices used to combat periodontal infections should be biocompatible and maintain or increase cell viability (Dzierżewicz et al. 2010).

Table 1. Inhibition zones (mm) ± SD against *Streptococcus mutans* (*S.m*), *Staphylococcus aureus* (*S.a*), *Aggregatibacter actinomycetemcomitans* (*A.a*), *Enterococcus faecalis* (*E.f*) and *Candida albicans* (*C.a*). n = 3.

	Strains				
	S.m	*S.a*	*A.a*	*E.f*	*C.a*
AV	-	-	-	-	17,0 ± 2,9
AV/AMX	28.0 ± 3.0	27.3 ± 1.2	29.3 ± 0.5*	29.0 ± 1.0***	n.a
AV/NYS	n.a	n.a	n.a	n.a	26.0 ± 2.9
Control	26.7 ± 1.2	25.0 ± 1.0	25.3 ± 1.9	21.7 ± 2.0	19.3 ± 1.7

AV: free *Aloe vera*; AV/AMX: *Aloe* vera loaded with amoxicillin; AV/NYS: *Aloe vera* loaded with nystatin; Control: amoxicillin-clavulanic acid 30 µg disk (*S.m*, *S.a*, *A.a* and *E.f*) or nystatin disk (*C.a*) 100 IU. * $p < 0,05$ and *** $p < 0,001$ (statistical difference compared to control for the same strain). n.a (not applicable).

Figure 6 show the effects of different concentrations of free *Aloe vera*, and *Aloe vera* loaded with AMX or NYS, on HGF cell viability as evaluated using the MTT assay (n = 6). Atiba et al. (2011) evaluated the effect of *Aloe vera* oral administration on the fibroblast proliferation in radiation-exposed rats compared with radiation-only and control rats. The fibroblast proliferation was significantly higher in the radiation plus *Aloe vera* group compared with the radiation-only group. Yao et al. (2009) evaluated the *in vitro* viability of a polysaccharide fraction isolated from fresh *Aloe vera* leaves on human fibroblasts. In this study, cells were treated with different concentrations of polysaccharide (25, 50, 100, 200 and 400 µg/mL) for 48 hrs. With increasing concentrations of polysaccharide from 25 to 400 µg/mL, cell viability increased gradually, and was significantly increased between 100-400 µg/mL. Our study showed that increasing concentrations of free *Aloe vera* resulted in increased cell viability, but these differences were not significant. This data suggests that the samples studied were not cytotoxic and maintained cell viability.

Study of cell viability of osteoblasts

OB are responsible for osteogenesis, namely the synthesis and secretion of organic matrix, type I collagen, proteoglycans and glycoproteins. The OB cytoplasm is rich in rough endoplasmic reticulum, developed Golgi apparatus and secretion granules (Nanci, 2008). When OB are in contact with various biomaterials, their behavior is altered, depending on the composition of these biomaterials. The activity of mitochondrial enzymes, as evaluated by MTT assay, is an important parameter for the analysis of the effects on bone, since it reflects osteoblastic activity (Yao et al. 2009).

Figure 7 show the effect of different concentrations of free *Aloe vera*, and *Aloe vera* loaded with AMX or NYS, on OB cell viability using the MTT assay (n = 6). Our results suggest that the cell viability of free *Aloe vera*, and *Aloe vera* loaded with AMX or NYS, are similar to control (OB untreated). From these data we conclude that the *Aloe vera* sponges were not cytotoxic and maintained cell viability.

CONCLUDING REMARKS

The physico-chemical characterization performed suggests that the interactions between the *Aloe vera* sponge and amoxicillin or nystatin are weak, and that these drugs are dispersed in the sponge. Sponges of *Aloe vera* loaded with amoxicillin or nystatin were biocompatible with HGFs and OBs, as well as showed antimicrobial activity against *Streptococcus mutans*, *Staphylococcus aureus*, *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis* and *Candida albicans*. Furthermore, OBs and HGFs were viable on free *Aloe vera*.

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