RENAN APARECIDO FERNANDES

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Orientadora: Profª. Ass. Drª. Debora de Barros Barbosa

Co-Orientadora: Drª Andresa Aparecida Berretta e Silva

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"A ciência nunca resolve um problema sem criar pelo menos outros dez". (George Bernard Shaw)

Fernandes RA. **Fitossíntese de nanopartículas de prata e desenvolvimento de formulação para tratamento de feridas: avaliação antimicrobiana, citotóxica e potencial cicatrizante em um modelo in vivo** [Tese]. Araçatuba: Universidade Estadual Paulista; 2017.

RESUMO GERAL

O objetivo deste estudo foi avaliar a capacidade de produção de nanopartículas de prata através do extrato da casca de romã, e produzir formulações contendo estas nanopartículas para uso em feridas. Elas foram testadas quanto à ação antimicrobiana, citotóxica e potencial cicatrizante. Para a produção das nanopartículas de prata propôs-se uma síntese utilizando-se como base duas metodologias já estabelecidas na literatura, utilizando-se para reação carboximetilcelulose, propilenoglicol, nitrato de prata, água e extrato da casca de romã como agente redutor. O extrato da casca de romã foi caracterizado em parâmetros como pH, massa seca e quantidade de taninos bioativos (ácido elágico e totais fenólicos expressos em ácido gálico). Os totais fenólicos do extrato foram também dosados após seu aquecimento nas diferentes condições de tempo e temperatura propostos para as sínteses das nanopartículas de prata (12 minutos, 1 hora e 2 horas, à 50ºC e 100ºC). As nanopartículas de prata produzidas foram, então, adicionadas a uma solução contendo compostos para produção de formulações para serem utilizadas no tratamento de feridas. Elas foram caracterizadas através de espectroscopia UV-Visível, microscopia eletrônica de varredura (MEV), potencial zeta e dosagem de íons remanescentes após as reações. A atividade antimicrobiana tanto das nanopartículas como de suas formulações contra *Candida albicans* SC 5314 e *Staphylococcus aureus* ATCC 25923 foi avaliada por meio do método da microdiluição. Na avaliação dos extratos, apesar de ocorrer um aumento na concentração de totais fenólicos com o aumento da temperatura, a concentração inibitória mínima (CIM) manteve-se estável com valores de 391 µg/ml e 781 µg/ml para *S. aureus* e *C. albicans*. A formação das nanopartículas de prata foi confirmada com a formação de picos característicos na espectroscopia UV-Visível e pelas imagens de MEV, onde verificou-se que a síntese com tempo de reação de 12 minutos e aquecimento a

50ºC gerou nanopartículas mais uniformes e melhor distribuídas na formulação. As sínteses propostas promoveram a redução iônica da prata de aproximadamente 100%, independente do tempo temperatura utilizados na reação. Valores de CIM para as nanopartículas de prata foram de 67,50 µg/ml e 68,75 µg/ml respectivamente para *S. aureus* e *C. albicans* independente das variações das condições de síntese. Após a seleção da síntese das nanopartículas de prata por 12 min à 50ºC, estas partículas foram também caracterizadas por difração de raios-X e microscopia eletrônica de transmissão (TEM), e as respectivas formulações por meio de espalhamento de luz dinâmica, dosagem de íons livres, potencial zeta, MEV e TEM. Para essas formulações foi também realizado um teste de estabilidade variando-se umidade e temperatura. Além da atividade antimicrobiana contra *Candida albicans* SC 5314 e *Staphylococcus aureus* ATCC 25923, a citotoxicidade em fibroblastos (L929) das nanopartículas de prata e das formulações destas partículas e do extrato da casca de romã foram também avaliadas. Para os extratos foram observados valores de 3,13, 86,39±0,96% m/m, 3,64±0,03 mg/g, 392,0±9 mg/g respectivamente para pH, massa seca, ácido elágico e totais fenólicos. Como controle, foram produzidas nanopartículas de prata sintetizadas convencionalmente (AgNP química), e observou-se potencial redutor de 99,89% e 99,51% para as sínteses utilizando-se extrato de romã (AgNP *green*) e um agente redutor químico convencional (AgNP química). Verificou-se a formação de partículas com tamanhos médios de 89 e 19 nm para nanopartículas *green* e química. A formulação contendo as nanopartículas de prata apresentaram um potencial antimicrobiano expressivamente maior do que o princípio ativo isolado, sendo 255 e 4 vezes mais efetiva contra *S. aureus* e *C. albicans*, respectivamente. Os valores de citotoxicidade foram consideravelmente menores para as nanopartículas de prata sintetizadas pelo extrato de romã quando comparadas as produzidas convencionalmente. De acordo com os valores de CIM e da citotoxicidade, a concentração das formulações foi ajustada, gerando assim três formulações: i) AgNP *green*, ii) AgNP química e iii) extrato de romã, nas concentrações de 337,5 µg/ml, 5,55 µg/ml e 94 µg/ml respectivamente. Para o estudo in vivo, utilizou-se como controle um medicamento comercial contendo prata indicado para tratamento de feridas (Sulfadiazina de prata). Foram

selecionados noventa ratos Wistar machos com peso médio de 180 gramas. Foi induzida diabetes nos ratos, e, em seguida, realizou-se duas incisões no dorso dos animais e as lesões foram imediatamente infectadas com *S. aureus* (ATCC 25923) e *C. albicans* (SC 5314). Após 24 h, os animais foram divididos em grupos de acordo com as formulações propostas em cada tratamento, seguindo-se um protocolo de duas vezes ao dia por 2, 7 e 14 dias. Após o período de tratamento os animais foram eutanasiados e verificado o potencial reparador através do índice de fechamento de ferida, avaliação do infiltrado inflamatório, angiogenese, mieloperoxidase e hidroxiprolina. Ainda foi verificado o potencial antimicrobiano das formulações através da contagem de células viáveis de cada microrganismo infectado nas feridas. De forma geral, as formulações contendo nanopartículas de prata mostraram os melhores resultados para o fechamento das feridas, apresentando ainda uma atividade anti-inflamatória maior que a do extrato de casca de romã, o qual apresentou atividade pró inflamatória. Todos os tratamentos não foram capazes de reduzir de forma significativa o número de células de *C. albicans,* enquanto para *S. aureus* todos os tratamentos apresentaram redução significativa após quatorze dias de tratamento. Independente das nanopartículas de prata serem produzidas quimicamente ou por meio do extrato da casca de romã, ambas apresentaram considerável potencial de reparo de feridas infectadas em modelos in vivo com ratos. Os achados das presentes pesquisas reforçam e estimulam o uso potencial das nanopartículas de prata no tratamento de feridas, com destaque para a síntese *green* por gerar menos danos ao meio ambiente e às pessoas envolvidas tanto em sua produção como aos pacientes, e por apresentar, ainda, custo inferior quando comparada às sínteses utilizando reagentes químicos e processos convencionais.

Palavras-chave: Biofilmes**,** *Candida albicans*, *Staphylococcus aureus*, prata, nanotecnologia.

Fernandes RA. **Phytosynthesis of silver nanoparticles using pomegranate peel extract and development of formulation for wound healing: antimicrobial and cytotoxicity evaluation and repair potential in a in vivo model.** [thesis]. Araçatuba: UNESP - São Paulo State University; 2017.

GENERAL ABSTRACT

The aim of this study was to investigate the production of silver nanoparticles through peel extract of pomegranate, and produce formulations containing these particles to be used in wound healings. Its antimicrobial action, cytotoxicity and healing potential were tested. The synthesis of silver nanoparticles were based on two methods proposed in the literature with some modifications, which were used carboxymethylcellulose, propylene glycol, silver nitrate, water and peel extract of pomegranate as reducing agent. The peel extract was characterized by pH, dry mass and bioactive tannins (elagic acid and total phenols expressed as galic acid). The total phenols were also quantified after being heated at 50ºC and 100ºC for 12 minutes, 1 hour and 2 hours. Then, silver nanoparticles were added in a solution containing products to develop a formulation to be tested in wound healing. They were characterized by UV-Vis spectroscopy, scanning electron microscopy (SEM), zeta potential and the quantification of remaining silver ions after the synthesis reaction. The antimicrobial activity of the nanoparticles and formulations were tested against *Candida albicans* (SC 5314) e *Staphylococcus aureus* (ATCC 25923) by microdilution method. After submitting the peel extract to different conditions of temperature and times (50ºC and 100ºC for 12 minutes, 1 hour and 2 hours), it was noted that the values of the minimum inhibitory concentration was not affected and were 391 µg/ml and 781 µg/ml for *S. aureus* and *C. albicans*. The formation of silver nanoparticles was confirmed through the formation of characteristic peaks in the UV-Vis spectroscopy and SEM images, and it was observed that the reaction at 50ºC for 12 min produced silver nanoparticles with regular forms and better dispersed in the formulation. The synthesis proposed promoted the reduction of silver ions at about 100%, regardless of the time and temperature used in the reaction, which also did not

interfere in antimicrobial activity against *C. albicans* (68,75 µg/ml) and *S. aureus* (67,50 µg/ml). After selecting the reaction at 50ºC for 12 min, the silver nanoparticles produced ere also characterized by X-ray diffraction and transmission electron microscopy (TEM), and the respective formulations through dynamic light scattering, free ion dosage, zeta potential, SEM and TEM. The stability test varying humidity and temperature was also performed for those formulations. Besides antimicrobial assays, the cytotoxicity (L929 fibroblasts) of the silver nanoparticles and the formulations of these particles and of the pomegranate peel extract were evaluated. It was observed in the peel extract the values of 3,13, 86,39±0,96% m/m, 3,64±0,03 mg/g and 392,0±9 mg/g respectively for pH, dry mass, elagic acid and total phenols concentrations. Silver nanoparticles produced by conventional chemical method was prepared and used as controls, and it was noted the reduction potential of 99,89% and 99,51% for the synthesis using pomegranate peel extract (AgNP green) and chemical reducing agent (AgNP chemical). The averages sizes of green and chemical AgNP were 89 and 19 nm. The formulation containing silver nanoparticles presented an antimicrobial potential expressively higher than the active input, being 254 and 5-fold more effective against *S. aureus* and *C. albicans*. Also, the cytotoxicity was notable reduced when silver nanoparticles were produced using pomegranate peel extract. Based on the MIC values and the cytotoxicity findings, the concentration of the formulations were determined: i) AgNP green at 337.5 µg/ml, ii) AgNP chemical at 5.55 µg/ml, and iii) pomegranate peel extract at 94 µg/ml. In the in vivo study, a commercial form of silver (Sulfadizsine) to the wound healing was used as control. Ninety Wistar male rats were selected, and, after inducing diabetes, two incisions on the dorsum of the animals were made and followed infected with *S. aureus* (ATCC 25923) and *C. albicans* (SC 5314). After the infection, the animals were treated with the formulations twice a day for 2, 7 and 14 days. Then, the animals were euthanized and the repair potential was verified through wound closure index, inflammatory infiltrate evaluation, angiogenesis, myeloperoxidase and hydroxyproline. It was also determined the antimicrobial potential by counting the viable cells of each microorganism used to infect the wounds. In general, the formulations containing silver nanoparticles promoted a

better closure of the wounds, and a higher anti-inflamatory activity than the peel extract formulation which otherwise presented a pro-inflamatory effect. All formulations could not significantly reduce the viable cells of *C. albicans*, while for *S. aureus* they reduced significantly the cells after 14 days of treatment. Silver nanoparticles produced by both green and conventional chemical process present notable potential in repairing infected wounds in in vivo rat model. The findings of the present research strengthen and stimulate the potential application of silver nanoparticles in wound healings, highlighting the green production of these particles which apart from being lower costly, it is ecofriendly and less detrimental to people involved in its production and use.

Keywords: Biofilms, *Candida albicans*, *Staphylococcus aureus*, silver, virulence nanotechnology.

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INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

Muitas das doenças que acometem os seres vivos são caracterizadas por infecções que estão intimamente relacionadas com a capacidade de bactérias e/ou fungos em formar biofilmes. Os biofilmes são comunidades organizadas de microrganismos aderidos a uma superfície podendo ser biótica ou abiótica, rodeado por uma matriz extracelular em um ambiente aquoso (Costerton, Stewart et al 1999). Eles são geralmente compostos por mais de uma espécie de microrganismos, formando assim biofilmes mistos. No entanto biofilmes simples são encontrados em infecções especificas como nos casos de infecções de dispositivos e equipamentos médicos, recebendo destaque na literatura para os biofilmes de *Pseudomonas aeuraginosa*, *Pseudomonas fluorescens*, *Escherichia coli*, *Vibrio cholerae* e *Staphylococcus aureus* (O'Toole, Kaplan et al 2000). Os biofilmes além de serem capazes de colonizar as superfícies abióticas como mencionado anteriormente são capazes e se desenvolverem em diversos tecidos dos seres humanos como no trato gênito urinário, pele, aparelho respiratório, trato gastrintestinal dentre outros (De, Raj et al. 2017).

Feridas crônicas são encontradas devido à presença de biofilmes persistentes e que podem ser localizados em diversos locais, como por exemplo, em bolsas periodontais na boca, úlceras venosas, infecções em dispositivos como implantes e infecções adquiridas em âmbito hospitalar (Ammons and Copie 2013). Assim estes biofilmes são caracterizados como um problema nos casos de úlceras, pois as feridas crônicas não cicatrizam se devido à persistência de infecções e inflamações (Chen and Wen 2011).

Diversos microrganismos estão relacionados ás úlceras crônicas, e dois em específico necessitam receber atenção especial neste processo, pois são abundantemente encontrados em diversas regiões do corpo humano, além de estarem presentes em biofilmes de úlceras crônicas, entre eles a bactéria Gram positiva *Staphylococcus aureus* e o fungo *Candida albicans*. Os biofilmes envolvendo *Candida albicans* e *Staphylococcus aureus* estão diretamente relacionados com patologias como a candidíase e queilite angular (Na and Gijzen 2016). O fungo *Candida albicans* é um microrganismo pleomórfico, oportunista, que pode apresentar diferentes formas de acordo com o meio e o estímulo recebido (Shepherd. 1990). As leveduras do gênero *Candida* constituem uma menor parte da flora microbiana oral (Kulak, Arikan et al. 1997) além de colonizar a pele, órgãos genitais e trato gastrintestinal, são consideradas comensais em indivíduos adultos saudáveis (Thein, Samaranayake et al. 2006). As espécies de *Candida* produzem enzimas hidrolíticas (exemplo: fosfolipases e proteinases) que colaboram para a invasão dos tecidos hospedeiros através da digestão ou destruição das membranas celulares (Ghannoum 2000); (Naglik, Challacombe et al. 2003) (Schaller, Borelli et al. 2005), este processo inicia se quando o sistema imunológico do hospedeiro encontra se debilitado (Bassetti, Merelli et al. 2013). O fungo *C. albicans* necessita de muita atenção, pois possui a capacidade de aderir-se firmemente em tecidos e dispositivos médicos (Ramage and Lopez-Ribot 2005), além de células em biofilmes mostrarem-se resistentes a maioria dos antifúngicos convencionais como os azoles e a anfotericina B que podem causar diversos efeitos colaterais no hospedeiro (Laniado-Laborin and Cabrales-Vargas 2009).

Staphylococcus aureus é mundialmente conhecido por causar doenças na pele, tecidos moles, pneumonia, abcessos e osteomielite. (Chowers, Paitan et al. 2009). O *Staphylococcus aureus* é uma bactéria gran positiva encontrada em diversos locais do corpo humano e também em outros animais, relacionado a grande taxa de morbidade e mortalidade mundial (Van Hal et al. 2012). Relatos de pacientes portadores da Síndrome da Imunoficiencia Adquirida (AIDS) e infectados com este microrganismo chegaram ir a óbito, além de contribuir para o desenvolvimento da tuberculose, enfatizando assim a necessidade de se estudar tal microrganismo (van Hal, Jensen et al. 2012). Este também é conhecido por diversas infecções nasocomiais, além de algumas cepas mostrarem-se capazes de infectar pacientes saudáveis e ser responsável desde infecções brandas na pele, infecções de tecido mole até doenças fatais como a pneumonia necrotizante, osteomielite e septicemia (Saavedra-Lozano, J et al. 2015). Ainda há relatos da presença de *Staphylococcus aureus* em úlceras e osteomielite sendo frequente a presença de cepas resistentes a metacilina (MRSA) (Martini et al. 2015). Eles tem se tornado resistentes à maioria dos agentes antimicrobianos convencionais devido ao uso indiscriminado e incorreto de antibióticos pela população (Thurlow, L.R et al. 2012). Foi verificado também que a presença de *Staphylococcus aureus* em úlceras venosas é mais prevalente do que outras espécies de microrganismos (Ortiz et al. 2014).

Para cicatrização de feridas o organismo precisa prevenir ou combater estes microrganismos além de passar por um processo de reconstrução de tecidos lesionados e estruturas celulares. Este processo normalmente está dividido em três passos, incluindo a inflamação, proliferação das células e remodelação tecidual

(Gurtner et al. 2008). A fim de melhorar a qualidade do tecido reparado e para aumentar a eficácia de materiais utilizados na cicatrização de feridas, medicamentos e dispositivos com diferentes tipos de nanopartículas têm sido desenvolvidos. Alternativas estudadas incluem algodões com nanopartículas de prata, que foram eficazes contra *Escherichia coli*, *Staphylococcus aureus* e *Candida albicans* em concentração de 250 ppm (Hebeish et al. 2014), também celulose bacteriana com deposição de nanopartículas de prata foi desenvolvida, apresentando atividade antibacteriana contra *Staphylococcus aureus* (Wu et al. 2014), assim como o desenvolvimento de nanopartículas encapsuladas com curcumina que também inibiram o crescimento de *S. auereus* MRSA e *Pseudomonas aeruginosa* (Krausz et al. 2015). Apesar das alternativas apresentadas até o presente momento, a busca de soluções efetivas para a cura de feridas infectadas ainda tem sido constante.

Neste sentido é necessária a busca por alternativas de tratamentos que visem o combate de microrganismos patogênicos, além de auxiliar o organismo a controlar seu potencial inflamatório e estimular o reparo da lesão. A nanotecnologia associada a compostos naturais tem recebido intensa atenção na literatura atual. Dentre os compostos naturais, a romã (*Punica granatum*) se destaca por ser uma fonte rica de elagitaninos bioativos, e tem sido utilizada como vermífugo, adstringente, antibiótico, anti-inflamatório, em feridas, úlceras, vaginite e infecções do trato respiratório e urinário (Harde et al. 1970, Willianson et al. 2002; Duke, 4 Codwin & Cillier, 2002). O extrato da romã é composto principalmente de alcalóides e polifenóis. Ele tem demonstrado uma variedade de ações benéficas, incluindo efeito antioxidante e atividade antiviral. Dentre as

moléculas antioxidantes presentes nos extratos, existem uma grande quantidade de flavonoides, alcaloides, ácidos orgânicos, compostos polifenólicos, dentre outros. (Orgil et al. 2014). Atualmente, estudos tem focado na capacidade antioxidante e anti-inflamatória de extratos naturais, como investigado por Omar et al. 2015, onde o extrato de *Punica granatum* (romã) exerceu um grande potencial anti-inflamatório e antioxidante em ratos. A atividade antimicrobiana de extratos dessa planta tem sido relatada principalmente contra *Staphylococcus aureus* e *Escherichia coli* (Machado et al. 2003). Devido às suas propriedades biológicas, a casca da romã tem recebido atenção por parte de pesquisadores e consumidores (Reddy et al. 2007). O interesse de uso da romã tem se voltado também para o campo da nanotecnologia, onde já se tem comprovado a redução da prata iônica por meio de diferentes partes da romã (Barbosa et al, 2014) caracterizando a redução da prata iônica a nanopartículas de prata. Por se utilizar de plantas, a síntese de nanopartículas tem sido considerada sustentável ou "green". Estudos prévios (não publicado ainda) dos mesmos autores indicam uma efetividade antifúngica semelhante às nanopartículas de prata (NPAg) obtidas convencionalmente por rota química (Monteiro et al 2014a; Monteiro et al 2014b).

Assim, com a crescente tolerância dos microrganismos frente aos antimicrobianos convencionais e consequente aumento em sua dosagem para melhorar sua eficácia, há a necessidade pela busca de novas drogas que sejam efetivas não somente contra biofilmes patogênicos, mas também menos tóxicas as células do organismo hospedeiro auxiliando assim no processo anti-inflamatório e cicatrizante. Portanto, o objetivo geral do presente trabalho foi avaliar a

capacidade de produção de nanopartículas de prata através do extrato da casca de romã, e produzir formulações contendo estas nanopartículas para uso em feridas. Elas foram testadas quanto à ação antimicrobiana, citotóxica e potencial cicatrizante em um modelo in vivo em rato.

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CAPÍTULO 1

Silver nanoparticles: an in vitro investigation of phytosynthesis process and insights into antimicrobial formulations.

[*]Artigo enviadopara o periódico Biofouling

Silver nanoparticles: an in vitro investigation of phytosynthesis process and insights into antimicrobial formulations.

2.1. Abstract

In the present study, silver nanoparticles (AgNP) mediated by pomegranate peel extract were prepared under different times and temperatures of reaction, and the effectiveness of each synthesis process was determined by the quantification of silver ions remaining. It was also prepared spray formulations containing those AgNP, and the antimicrobial activity of the AgNP and AgNP formulations was tested against *Candida albicans* and *Staphylococcus aureus*. The phytosynthesis were carried out at 50ºC for 12 minutes (I) or 50ºC for 2 hours or 100ºC for 2 hours (III), and the samples were physicochemical characterized by spectroscopy Uv-Visible and scanning electron microscopy. The silver ions concentration was verified by a specific electrode and the rate of ion reduction was calculated. Microdilution method was used to determine the minimum inhibitory concentration (MIC) of the AgNP and AgNP formulations against the microorganisms. The AgNP formation was confirmed by Uv-Vis at wave-leght peaks ranging from 375 to 445 nm. SEM images showed regular forms and well-dispersed AgNP specially phytosynthesized at 50ºC for 12 minutes. The rate of reduction of the synthesis was I (99.89%) > II (96%) < II (98.6%). Time and temperature did not interfere in the antimicrobial activity of the AgNP samples, and surprisingly AgNP in the spray formulations increased 244-and 5-fold the effectiveness against *S. aureus* and *C. albicans*. The phytosynthesis of AgNP using pomegranate peel extract besides being quite effective and eco-friendly, it may be suitable for prophylaxis and treatment of infected wounds.

Keywords: Biosynthesis, silver, nanoparticles, *Punica granatum*, heating.

2.2. Introduction

Due to its optical, magnetic, antimicrobial and electronic properties metallic nanoparticles has received a special attention in recent years. One of the most exploited metals in this sense is silver, more specifically the silver nanoparticles, being used in several areas such as biomedical, pharmaceutical, water treatment, energy, among others (Abbasi, Milani et al. 2016), (Liu and Astruc 2017), (Sai Saraswathi, Kamarudheen et al. 2017).

For the production of silver nanoparticles several methodologies can be employed as by means of chemical reductions (Gurusamy 2017), electrochemical (Elemike, Fayemi et al. 2017), photochemical (Gabriel, Gonzaga et al. 2017) , sonochemical (Elsupikhe, Shameli et al. 2015), microwave (Francis, Joseph et al. 2017), γ-radiation (Mansour, Eid et al. 2017) and laser ablation (Delmee, Mertz et al. 2017).

One of the most used techniques for obtaining silver nanoparticles is the chemical reduction, however the use of chemical reagents causes the production to become expensive, besides producing unwanted effects such as the production of toxic nanoparticles to human cells (Gurusamy 2017) and to the environment. Even so that the aggregation of the silver nanoparticles does not occur in stabilizing agents are added, which can thus generate even more unwanted effects (Khodashenas 2015), (Ren 2015). In this sense there is a need to search for cheaper alternatives, eco-friendly and biocompatible with the human organism.

Nowadays the use of green chemistry has been approached as an alternative to the previously reported techniques. Different natural processes are used to obtain metal nanoparticles such as bacteria, fungi (Wypij, Golinska et al. 2017), honey (Philip 2010) and plant extract (Francis, Joseph et al. 2017). Thinking of the benefits offered by plant extracts such as the possibility of availability in different regions of the world, low cost, possibility of large scale production, safe for the environment, besides the possible synergism of properties like anti-inflammatory, antioxidant and antimicrobial, this technique shows very promising.

For synthesis of silver nanoparticles several plants have been used, time and temperatures of varied reactions. Therefore, the aim of this study was to produce silver nanoparticles mediated by pomegranate peel extract, and evaluate its potential of reduction according to different times and temperatures of reaction.

2.3. Materials and methods

Collecting fruit and obtaining extracts

The fruits were collected in May of 2015 from a farm in the city of Mirandópolis, São Paulo, Brazil. The peels were withdrawals from the pomegranates, dried at 50ºC (approximately 48 hours), grinding and siving, tracked by the extraction by maceration followed by percolation process (de Oliveira, de Castro et al. 2013).

Phytosynthesis of silver nanoparticles

For the development of this synthesis it was based on two methodologies proposed by Gorup et al. (2011) and Das Kumar et al. (2015) with modifications.

The base of the reaction was fixed in propylene glycol (PG) (Labsynth, Diadema, Brazil) (20%), carboxymethylcellulose (CMC) (Labsynth, Diadema, Brazil) (3.5%), and silver nitrate (100 mM), pomegranate peel extract at 30 mg/ml (based on previously data) was used as reducing agent and water to make up 100% of the samples. The variables were time and temperature of reaction.

I- Synthesis at 50ºC for 12 minutes.

II- Synthesis at 50ºC for 1 hours.

III- Synthesis at 100ºC for 2 hours.

Development of formulations

For the development of formulations, it was used reagents compatible with the synthesis of silver nanoparticles, carboxymethylcellulose (Labsynth, Diadema, Brazil), propylene glycol (Labsynth, Diadema, Brazil) and methylparaben (Labsynth, Diadema, Brazil) in a proportion of 0.1%, 7% and 0.1%, respectively, and the active input (green silver nanoparticles synthesized under different conditions at 1 mg/ml).

Determination of total phenolic

Total phenolic contents in the extract submitted to different conditions were expressed based on a previously analytical curve of gallic acid (Sigma-Aldrich Chemical Co, St Louis, USA). The samples were homogenized in water and submitted to ultrasonic bath for 30 minutes. An aliquot was mixed with Folin-Denis reagent (Qhemis - High Purity, Hexis, São Paulo, Brazil) and sodium carbonate at 29% (Cinética, São Paulo, Brazil). The next step was the light

isolation for 30 minutes followed by the measurement at 760 nm (Zielinski and Kozlowska 2000). All samples were analyzed in triplicate.

Determination of antimicrobial activity

Evaluation of antimicrobial activity of silver nanoparticles and the extract prepared in the same conditions of each synthesis were verified by minimal inhibitory concentration (MIC) according to the Clinical Laboratory Standards Institute. The microorganisms selected were *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Candida albicans* SC 5314 (wild type) (Gillum, Tsay et al. 1984) (*C. albicans*). The assay was performed in triplicate.

Characterization of silver nanoparticles and formulations

UV/Visible spectroscopy and rate of ion reduction

The qualitative verification of the silver nanoparticle formation was verified through the UV/Visible spectroscopy with wavelength ranging from 300 to 800 nm (Spectrophotometer Shimadzu MultSpec-1501, Shimadzu Corporation, Tokyo, Japan). For determination of ion reduction dosages of free silver ions were done. The content of ions was determined using a specific electrode 9616 BNWP (Thermo Scientific, Beverly, MA, USA) coupled to an ion analyzer (Orion 720 A+, Thermo Scientific, Beverly, MA, USA). A previously curve was prepared at 1000 µg Ag/ml, and the samples diluted in water. A silver ionic strength adjuster solution (ISA, Cat. No. 940011) which provides a constant background ionic strength was used (1 ml of each sample/standard: 0.02 ml ISA).

SEM analyzes

The morphology of silver nanoparticles was verified and characterized by scanning electron microscopy on a S-360 microscope, Leo, Cambridge, USA.

Statistical analyses

GraphPad Prism software (GraphPad Software, Inc, La Jolla, USA) was employed for the statistical analysis with a confidence level of 95 %. Parametric statistical analyses were conducted with one-way ANOVA followed by Tukey's Multiple Comparison test for total phenolic.

2.4. Results

Determination of total phenolic

The content of total phenolic are exposed on table 1 where interesting results were obtained for heating and time of reaction increasing the content of total phenolic in higher temperature and time. Moreover, the antimicrobial activity for the extract of peel pomegranate did not change with the altering time and temperature.

UV/Visible analyzes and rate of ion reduction

Figure 1 shows the qualitative formation of silver nanoparticles for all reactions developed. The rate of ion reduction are expressed in table 2 where it’s possible to note that the great reduction occurred in the reaction performed at 50ºC for 12 min, with a rate of reduction of 99.89%. Moreover, synthesis without pomegranate peel extract did not produced silver nanoparticles.

SEM analyzes

SEM images (fig.2) shows different morphology for all synthesis, the synthesis performed at 50ºC for 12 minutes produced more regular and dispersed silver nanoparticles.

Determination antimicrobial activity

MIC values of silver nanoparticles obtained for *S. aureus* was 67.5 µg/ml and for *C. albicans* 68.75 µg/ml and interestingly the values did not change against the different conditions of synthesis. The MIC values obtained for pomegranate peel extract was 391 µg/ml for *S.aureus* and 781 µg/ml for *C. albicans*, even temperature and time of reaction did not interfere in this values.

2.5. Discussion

Nanomaterials has been applied in several areas such as drug delivery, environment, biomedical, food packaging, among others. In this sense, the green synthesis of nanoparticles has been largely studied (Ahmed S 2016). Studies have reported that parameters for synthesis are very abstract and not very standardized for green synthesis. Factors such as pH, extract volume and ion concentration alter the final production of silver nanoparticles (Velu, Lee et al. 2017), so our research provides interesting results of the variation of parameters such as temperature and reaction time in the production of silver nanoparticles phytosynthesized using pomegranate peel extract.

It is asserted that the phenolic compounds present in the extracts perform the reduction of the silver nanoparticles (El-Kassas and Ghobrial 2017). Thus, the

temperature ratio check and reaction time in the concentration of phenols and the activities of the extract as the antimicrobial activity are important. It can be observed that with increasing temperature and reaction time the total phenolic concentration increased significantly, however the antimicrobial activity remained stable, thus being able to attribute the increase concentration in relation to water evaporation, especially because the technique used to determine total phenols is colorimetric. However, we must take into account that the active compounds present in the extract with antimicrobial potential were not degraded, since MIC values were not altered.

Profiles obtained in the UV/Visible analysis as the same way determined by S Agnithori et al. (2014) showed that the increase of time and temperature occurs the formation of two peaks and larger bands, suggesting the formation of particles of varied sizes. This fact can be confirmed by analyzing the SEM images obtained, observing that with the passage of time and increasing the temperature irregular and agglomerated silver nanoparticles are formed, thus confirming the spectral profiles obtained. Moreover, the silver nanoparticles produced by lower temperature and reaction time generated more homogeneous formulation.

Regarding to antimicrobial activity values founded for *S.aureus* and *C. albicans* were very similarly. The values did not change for the different synthesis. One possible explanation would be the formation of different sizes of silver nanoparticles in the different conditions. As it is known different sizes and shapes of nanoparticles exert specific activities (Pal, Tak et al. 2007), thus given the variety of form occurred in the syntheses with higher temperature and time they also presented particles similar to the occurred synthesis in a shorter time and

temperature. According to the presented results, it can be concluded that the high reaction temperature and time can negatively favor the production of silver nanoparticles principally in the morphology and aggregation of nanoparticles. It is also worth noting that an average temperature of 50ºC and a short time of twelve minutes showed the highest efficiency both in the reduction of ions and in the production of regular and dispersed silver nanoparticles. As well as the preparation of formulations with possible uses in medical field.

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Table 1. Values of total phenols and minimum inhibitory concentration (MIC) for pomegranate peel extract submitted under different conditions.

Compounds	**Total phenols Mean ± SD (mg / g)**	**MIC values**	
		S. aureus	***C. albicans***
CMC + PG	-1.605 ± 0.1834	Absent	Absent
Extract at room temperature	10.628 ± 0.354	0.391 mg/ml	0.781 mg/ml
Extract at 50°C/12 min	9.310 ± 0.7345	0.391 mg/ml	0.781 mg/ml
Extract at50°C/2 h	9.915 ± 0.4777	0.391 mg/ml	0.781 mg/ml
Extract at 100°C/2 h	13.451 ± 0.4805	0.391 mg/ml	0.781 mg/ml

*CMC= Carboximetilcelulose; PG= Propilenoglicol.

Table 2. Values of ion reduction and minimum inhibitory concentration (MIC) for pomegranate peel extract submitted under different conditions.

Samples	µg Ag^+/mL	% of reduction	MIC (µg/ml)	
			S. aureus	***C. albicans***
*Control at 50ºC/12 min	10303.26	4.59	4.13	0.53
*Control at 50ºC/2 h	9470.56	12	4.13	0.53
*Control at 100ºC/2 h	8447.06	22.14	4.13	0.53
Pomegranate peel extract	-	-	391	781
Phytosynthesis at 50ºC/12 min (I)	10.89	99.89	67.50	68.75
Phytosynthesis at 50ºC/2 h (II)	453.05	96	67.50	68.75
Phytosynthesis at 100ºC/2 h (III)	149.49	98.61	67.50	68.75
Formulation I	-	-	0.26	16.87
Formulation II	-	-	0.26	16.87
Formulation III	-	-	0.26	16.87

*Control= Carboximetilcelulose, propilenoglicol, nitrato de prata.

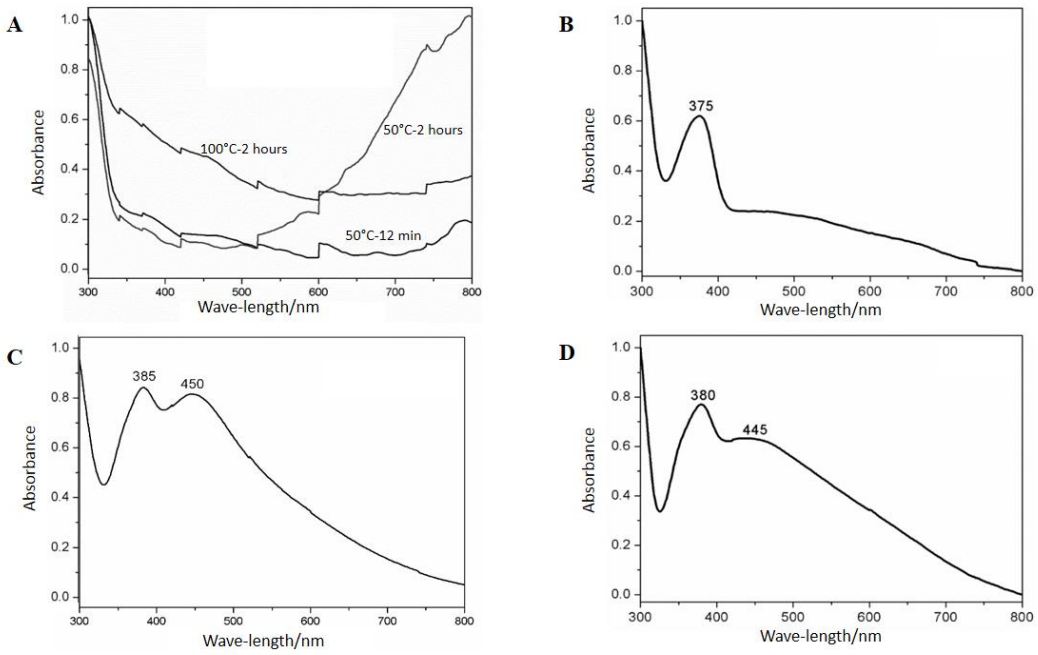

Figure 1. Spectral UV/Visible of silver nanoparticles produced under different conditions, controls without reducing agent (pomegranate peel extract) (A), 50°C for 12 min (B), 50ºC for 1 h (C), 100ºC for 2 h (D).

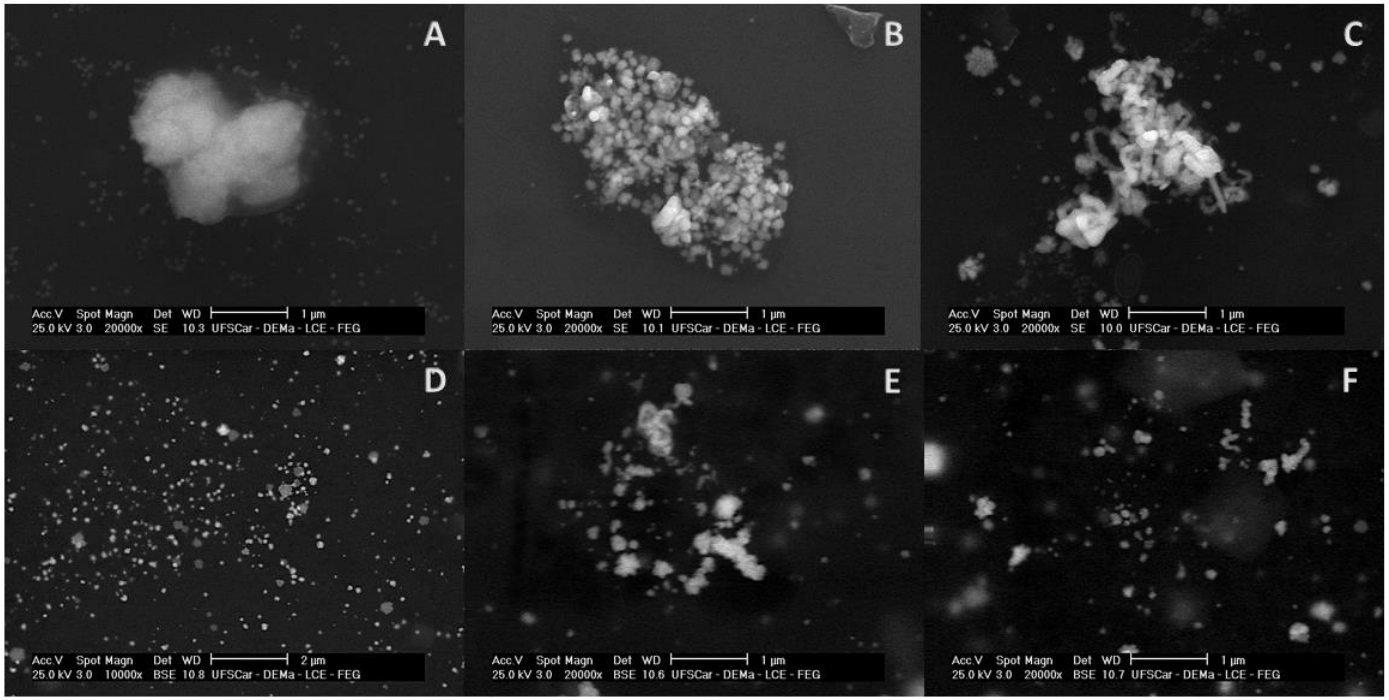

Figure 2. Images of scanning electron microscopy of silver nanoparticles produced under different conditions, 50°C for 12 min (A), 50ºC for 1 h (B), 100ºC for 2 h (C) and formulations prepared from them (D,E and H).

CAPÍTULO 2

Antimicrobial potential and toxicity of silver nanoparticles phytosynthesized by pomegranate peel extract *

Artigo nas normas do periódico Biofouling.

Antimicrobial potential and citoxicity of silver nanoparticles phytosynthesized by pomegranate peel extract

3.1. Abstract

Antimicrobial activity of colloidal and spray formulation of silver nanoparticles (AgNP) synthesized by pomegranate peel extract against *Candida albicans* and *Staphylococcus aureus*, and cytotoxicity in mammalian cells were tested. Dry mass, pH, total phenolics and ellagic acid in the extract were determined. Then AgNP were phytosynthesized and characterized by X-ray diffraction, electron scanning and transmission microscopies, dynamic light scattering, zeta potential, and Ag^+ dosage. Spray formulations and respective chemical-AgNP controls were prepared and tested. Peel extract reduced more than 99% of Ag^+, and produced nanoparticles with irregular forms and 89 nm mean size. All AgNP presented antimicrobial activity, and spray formulation of green-AgNP increased 255 and 4 times the effectiveness against *S. aureus* and *C. albicans* respectively. Cytotoxicity of colloidal and spray green-AgNP was expressively lower than the respective chemical controls. Pomegranate peel extract produced stable AgNP with antimicrobial action and low cytotoxicity, stimulating its use in the biomedical field.

Keywords: Silver; nanoparticles, *Candida albicans*, *Staphylococcus aureus*, herbal medicine, *Punicaceae*.

3.2. Introduction

Recently a state of alert on a topic that affects people all over the world, the antimicrobial resistance has received attention. This fact leads to the death of more than 700000 people a year around the world and this number has risen every year (Raut and Adhikari 2017). It is estimated that a world population will have a reduction of 11-444 million people in 2050 if the antimicrobial resistance was not bypassed (Raut and Adhikari 2017).

As a contour form and an alternative to antimicrobial resistance, an approach of inorganic particles at nanoscale has become strong. The most prominent metals in the group of inorganic nanoparticles are copper, zinc, titanium, magnesium, gold and silver (Guzman, Dille et al. 2012, Jankun, Landeta et al. 2014, He, Qiao et al. 2017). In this context, silver nanoparticles are the most exploited because it have a wide range of toxicity against several microorganisms, such as *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and others (Hebeish, El-Rafie et al. 2014).

The incorporation and use of silver nanoparticles is observed in sundry sectors, for instance in the food industry as an attempt to produce packages with antimicrobial activity (Kuorwel, Cran et al. 2011). The area of cosmetics has also received prominence, using in housecleaning, antiseptics, sunscreens, soap and shampoo (Benn, Cavanagh et al. 2010, Bansod, Bawaskar et al. 2015, Tulve, Stefaniak et al. 2015), as well as in textile manufacturing (Velazquez-Velazquez, Santos-Flores et al. 2015).

Considering the synthesis of silver nanoparticles many routes have been presented, such as electrochemical (Treshchalov, Erikson et al. 2017), by radiation (Malkar, Mukherjee et al. 2014), photochemistry (Lombardo, Poli et al. 2016), and by biological methods (Rafique, Sadaf et al. 2016). Phytochemical synthesis has been noteworthy since the use of chemical compounds may result in undesirable toxic effects not only for the human organism as well as the environment. Quite effectiveness in the production of silver nanoparticles has been demonstrated when compounds of different plants are used in the ions reduction, being characterized as rapid, low cost and nature friendly synthesis (Roy, Gaur et al. 2013). Furthermore, green-silver nanoparticles are usually less cytotoxic when compared to those reduced by conventional chemical agents (Das and Brar 2013).

Important aspects in green synthesis should be taken into account, including the choice of plant to be used, being the plants which grow in different regions of the world more eligible for (Das and Brar 2013). The previously known potential of the plant, including antioxidant, anti-inflammatory and antimicrobial, such as the case of *Punica granatum* (pomegranate), should be considered (Lansky and Newman 2007, Pande and Akoh 2009, Edison and Sethuraman 2013). Some studies have been used *Punica granatum* to reduce silver ions to silver nanoparticles (Naik and Chand 2003, Ahmad 2012, Edison and Sethuraman 2013).

Thus, taking together the benefits of pomegranate and the antimicrobial applicability of silver nanoparticles, the present study aimed to synthesize silver nanoparticles using pomegranate peel extract, and produce spray formulations

containing the previously synthesized green-silver nanoparticles. Characterize and teste against strains of *Staphylococcus aureus* and *Candida albicans*, as well as their cytotoxicity against mammalian cells.

2. Materials and methods

Plant material and preparation of pomegranate peel extract

Pomegranate samples were collected from a plantation cultivated in Eixo (21º 08' 01" S, 51º 06' 06" W), Mirandópolis, São Paulo, Brazil, during May 2015. Pomegranate peels were separated and stove-dried at 50°C, ground and sieved to a granulometry lower than 2 mm. Peels were submitted to alcohol extraction using ethanol 70% by maceration followed by percolation process (de Oliveira, de Castro et al. 2013). The extract was characterized in relation of pH, dry mass, and total phenolics expressed as gallic acid. The chemical marker of pomegranate, ellagic acid, was also identified and quantified.

Determination of total phenolic, pH and dry mass

For determination of total phenolic an analytical curve of gallic acid (Sigma-Aldrich Chemical Co, St Louis, USA) was carried out (Waterman PG 1994). All extracts obtained and the plant drug were prepared in 50 ml volumetric flasks using water as solvent. The samples were homogenized and, the flasks were brought to the ultrasonic bath for 30 minutes. A 0.5 mL aliquot was transferred to another 50 mL flask where 2.5 mL of Folin-Denis reagent (Qhemis - High Purity, Hexis, São Paulo, Brasil) and 5.0 mL of 29% sodium carbonate (Cinética, São Paulo, Brasil) were added. The samples were protected from the light and the

readings were performed after 30 minutes in a UV-Vis spectrophotometer at 760 nm (Zielinski and Kozlowska 2000). The pH was measured direct of solution of 1% extract, using a kit of pH (Merck KGaA, Darmstadt, Germany) and dry mass was calculated after the sample stove drying at 105°C and it was expressed in percentage m/m. All data were analyzed in triplicate.

Determination of the ellagic acid content

A Shimadzu liquid chromatograph and a Shimpack ODS C18 (Shimadzu Corporation, Kyoto, Japan) reverse phase column (100 mm x 2.6 mm) were used to determine the ellagic acid content by high performance liquid chromatography (HPLC). Analytical conditions were optimized based on de Sousa et al 2007 (de Sousa, Bueno et al. 2007) with modifications. As the mobile phase, HPLC grade methanol and a 2% aqueous acetic acid solution with gradient elution (0-7 min, 20-72.5% v/v methanol, 7-7.5 min, 72.5-95 % v/v methanol, 7.5-8.5 min 95% v/v methanol, 8.5-9 min 95-20% v/v methanol, 9-10 min 20% v/v methanol) were used. The flow rate was 1.0 mL/min, and the separation was achieved at 25 °C. The injection volume was 5 µL and the wavelength used was 254 nm. Peaks were determined by comparison with authenticated ellagic acid standard. Briefly, the sample for the plant drug was transferred to a 20 mL volumetric flask which was quenched with HPLC grade methanol. Extraction was done using a vortex for 5 minutes and ultrasonic bath for 1 hour. For the extracts, samples were transferred to volumetric flasks of 10 mL, using methanol HPLC as solvent. All samples were vortexed for 5 minutes and sonicated for 30 minutes. Samples were filtered through 0.45 µm filter. All samples were prepared in triplicate.

Synthesis of green-silver nanoparticles

The protocols described by Gorup et al. (2011) and Das K et al. (2015) with modifications were used to produce silver nanoparticles. Briefly, 3.5% of carboxymethylcellulose (CMC) (Labsynth, Diadema, Brazil), 20% of propylene glycol (PG) (Labsynth, Diadema, Brazil), 100 mM of silver nitrate (SN) (Merck KGaA, Darmstadt, Germany), pomegranate peel extract at 30 mg/ml, and water to make up 100% of the samples were used. The reaction was carried out at 50ºC for 12 minutes, and it was selected based on previous results (data not yet published).

Synthesis of chemical-silver nanoparticles

Chemical-silver nanoparticles were produced according to Gorup et al. (2011). $AgNO_3$ (Merck KGaA, Darmstadt, Hesse, Germany) was dissolved in water, and brought to boiling at 90ºC. After 2 min of boiling an aqueous solution of sodium citrate ($Na_3C_6H_5O_7$)(Merck KGaA, Darmstadt, Hesse, Germany) was added, and kept boiling for more 6 min until the solution reaches yellow amber color. The stoichiometric ratio was 1:3, respectively for $AgNO_3$ and $Na_3C_6H_5O_7$.

Preparation of formulations

The reagents used were CMC (Labsynth, Diadema, Brazil), PG (Labsynth, Diadema, Brazil), methylparaben (Labsynth, Diadema, Brazil) in a proportion of 0.1%, 7% and 0.1%, respectively. The active inputs (green- or chemical-silver nanoparticles and pomegranate peel extract) concentrations were based on the minimum inhibitory concentration and cytotoxicity. Therefore, the final

concentrations of active inputs in the spray formulations were: 337.5 µg/ml of green-silver nanoparticles, 5.55 µg/ml chemical-silver nanoparticles, and 94 µg/ml of crude peel extract dry mass.

Characterization of silver nanoparticles and formulations

X-ray diffraction (XRD), Dynamic Light Scattering (DLS) and Zeta Potential analysis

Shimadzu XRD diffractometer with a Cu Kα radiation operating at 30 kV and 30 mA and 2θ range from 35 to 85° with step scan of 0.02° and scan speed 0.2°.min-1 was used to perform XRD analysis. To collect silver nanoparticles patterns, the nanoparticles were deposited on the surface of a silicon substrate (Si) by dripping the aqueous colloidal dispersion on the substrate at a room temperature, and the solvent was evaporated.

DLS experiments were performed at room temperature and at a fixed angle of 173° on a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, United Kingdom) equipped with 50 mW 533 nm laser and a digital auto correlator. The number-average values obtained were compared to the size distributions of silver nanoparticles. For Zeta Potential test a Zetasizer (Malvern instruments, Malvern, United Kingdom) with a MPT-2 titrator was used. Aliquots from each test suspension were obtained to conduct zeta potential, and mean values were obtained from three independent measurements.

SEM and TEM analyzes

The nanocompounds morphology was characterized by scanning electron microscopy (SEM) on a S-360 microscope (Leo, Cambridge, USA), and Jeol JEM-100 CXII (JEOL USA Inc. Peabody, USA) microscope equipped with Hamamatsu ORCA-HR digital camera was used to obtain TEM images.

Silver ions dosage

The dosages of free silver ions (Ag^{+}) present in the compounds and spray formulations were performed to observe if the total amount of Ag added in the synthesis reaction was successfully reduced. A specific electrode 9616 BNWP (Thermo Scientific, Beverly, MA, USA) coupled to an ion analyzer (Orion 720 A+, Thermo Scientific, Beverly, MA, USA) was used. A 1000 µg Ag/ml standard was prepared adding 1.57 g of $AgNO_3$ in 1 L of deionized water. The combined electrode was calibrated with standards containing 6.25 to 100 µg Ag/ml to achieve equivalent silver concentrations in the compounds. A silver ionic strength adjuster solution (ISA, Cat. No. 940011) which provides a constant background ionic strength was used (1 ml of each sample/standard: 0.02 ml ISA).

Stability test of the formulations

The formulations were submitted to a stability test with controlled conditions of temperature and time. This test was based on Anvisa protocols (Cosmetics stability guide (ISBN 85-88233-15-0; Copyright© Anvisa, 2005) and guide to stability studies (Ordinance No. 593 of August 25, 2000). Briefly, samples of each spray formulations were submitted to alternating cycles of temperature daily ranging from 40 to -5°C for 28 days. The tests selected to evaluate the stability of

the samples were ion dosage, total phenolic content, zeta potential and microdilution broth. All tests were done in the same conditions as described before, and were carried out at 0, 7, 14 and 28 days.

Antimicrobial activity of silver nanoparticles and formulations

For determination of the minimal inhibitory concentration (MIC) the instructions of the *Clinical Laboratory Standards Institute* were followed with modifications. The samples were first diluted in water and subsequently in culture medium specific for each microorganism, Mueller Hinton broth (BD Difco, USA) for *Staphylococcus aureus* (ATCC 25923) and RPMI (Sigma-Aldrich, St Louis, USA) for *Candida albicans* SC 5314 (wild type) (Gillum, Tsay et al. 1984). The microorganisms were adjusted to 5 x 10^5 cells/ml for *S. aureus* and 5 x 10^3 cells/ml for *C. albicans*, and the plates were incubated for 24 hours and 48 hours in aerobiosis at 37°C respectively for *S. aureus* and *C. albicans*. After the incubation, the plates were visually read. The assays were performed in triplicate.

Evaluation of cytotoxicity

For evaluation of cytotoxicity fibroblast cells L929 lineage were used. Cells were cultured in DMEM culture supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml) (Gibco®), streptomycin (100 µg/ml), amphotericin B (25 µg/ml) and incubated in stove at 37 °C with 5% CO_2. Cells were subcultured (5-7 days), using 0.9% saline to wash them and 0.25% trypsin to disintegrate them from the vial. After disruption, these cells were centrifuged at 1000 rpm for 10

minutes at 10 °C, resuspended in complete DMEM medium (supplemented with FBS), and cell counted in a Neubauer's chamber.

The sub-cultured 3rd to 8th passage fibroblasts were inoculated into 96-well microplates at a density of $0.5x10^5$ cells/well. They were then incubated at 37 ° C with 5% CO2. After 24 hours, 20 µl of different dilutions of each samples were added to the wells of the plate containing the cells in medium not supplemented with SBF (incomplete medium) and incubated. 24 hours post-treatment the medium was withdrawn, cells were washed with saline and 20 µl of resazurin (Sigma-Aldrich) 0.01% w/v in deionized H_2O was added to each well containing 180 µl of DMEM medium supplemented with 10% Of SFB. The plates were then incubated for 4 hours at 37 ºC and fluorescence was measured at 540 and 590 nm for excitation and emission, respectively (Kuete, Wiench et al. 2013). Cell viability was expressed as percentage of viable cells compared to the control group without treatment.

Statistical analysis

GraphPad Prism software (GraphPad Software, Inc, La Jolla, USA) was employed for the statistical analysis with a confidence level of 95 %. Parametric statistical analyses were conducted with one-way ANOVA followed by Tukey's multiple comparison test for total phenols and zeta potential. For ion test the statistical analyses was Dunn's multiple comparison test.

3. Results

Characterization of peel extract, silver nanoparticles and formulations

The pH and the dry mass of the peel extract obtained by maceration followed by percolation were 3.13 and 86.39 (±0.96) % m/m, and the total phenolic expressed in gallic acid and the ellagic acid were 392.0 (±9) and 3.64 (±0.03) mg/g respectively.

The formation of silver nanoparticles was confirmed by comparing the XRD patterns and the corresponding standard patterns of cubic of silver nanoparticles (Fig. 1), according to the diffraction standard (JCPDS file No. 04-0783). The reflection peak (2 2 2) is a characteristic of the substrate (Si), where silver particles were deposited as a thin film. MEV and TEM images (Fig 2) show different forms and sizes of silver nanoparticles fabricated by green and conventional chemical routes as well as in their respective formulations. In general, green synthesis produced particles with larger size than those obtained by conventional synthesis. DLS analyses of the formulations prepared with green or conventional silver nanoparticles demonstrated different sizes of the particles, being the mean values of 89 ± 21 and 19 ± 4 nm for green and conventional formulation respectively. The values of zeta potential of green- and conventional-silver nanoparticles were lower than -30 mV (-46.2 ± 6.06 mV green, and -67.5 ± 3.69 mV conventional), indicating the stability of both colloidal silver nanoparticles.

Almost 100% of the Ag^+ ions coming from $AgNO_3$ were reduced by pomegranate peel extract (99.89%) and sodium citrate (99.51%). However, in the spray formulation containing chemical-silver nanoparticles, the percentage of reduction was diminished to 68.18%. Although it has happen, that formulation maintained stable regarding Ag^+ ions concentration for 28 days (Table 2). Zeta

potential data confirmed the stability of spray formulations regardless of the method used to obtain the silver nanoparticles (Table 1). Total phenols in the spray formulations with or without silver nanoparticles were quantified at 0, 7, 14 and 28 days after having been prepared (Fig. 3), and it were significantly reduced in the green-silver nanoparticles formulation after 14 days with values ranging from 0.405 to 0.295 mg/g.

Antimicrobial activity

The antimicrobial activity expressed as MIC values (µg/ml) (Table 1) was, in general, considerably lower for spray formulations than active inputs regardless of the microorganisms tested. MIC values against *C. albicans* for active inputs and spray formulations were 781 and 0.18 for peel extract, 68.75 and 16.87 for green-, and 0.25 and 1.12 for chemical-silver nanoparticles. While for *S. aureus* the values were 391 and 0.37, 67.5 and 0.26, and 0.5 and 0.56, respectively for pomegranate peel extract, green- and chemical-silver nanoparticles in active inputs and in spray formualtions. In addition, different conditions of humidity and temperature did not affect the effectiveness of the spray formulations against both microorganisms.

Cytotoxicity

Figure 4 shows the fibroblast L929 cells viability in view of different concentrations of silver nanoparticles (green and conventional route). Green-silver nanoparticles presented lower cytotoxicity than conventional-ones. It was necessary a dosage of 50 µg/ml to initiate the toxicity, but the cell viability was

nearly 80%, while conventional-silver nanoparticles were quite toxic at very low concentration (6.25 µg/ml) and it was similar with the negative control (DMSO) with viability lower then 20%. Also, the addition of the reagents to prepare the formulations did not interfere in the toxicity of the conventional-silver nanoparticles, whereas the cytotoxicity for the green-silver nanoparticles formulation as well as for the extract formulation were considerable increased.

4. Discussion

For future reproducibility of the experiment, the extract obtained by maceration followed by percolation was duly characterized in relation to dry mass, total phenols content, ellagic acid and pH. The correct selection of the plant and the standardization of the methods to obtain the extracts to be used as reducing or capping agent in nanosynthesis of metal particles should be preponderant when green process is elected the produce products in large scale. Also, a plethora of plants used in the phytosynthesis of metal nanoparticles (Ovais, Khalil et al. 2016, Soman and Ray 2016, Elemike, Onwudiwe et al. 2017) and the lack of information of the extraction techniques used in the articles hinder the comparison of the present results with those found in the literature. For instance, different values and methods of total phenol quantification can be observed in the literature as described by Kalaycioglu and Erim (2017). Similarly, other factors interfere in the quantification of the bioactive compounds in the extracts as the chemical and genotypic composition of the plant, the variety and the soil type, the place of the plant origin, the harvest season, maturation method, among others (Li, Yang et al. 2016).

MEV and TEM images showed the smallest particles obtained by conventional chemical synthesis (Fig 2). DLS data confirmed these findings, with mean sizes of 89 and 19 nm respectively for green and chemical nanoparticles. The fission of colloidal particles of different sizes and shapes may be related to additives (salts, polymers), solvent properties (boiling temperature, affinity with created surfaces), addition of nucleation, among others (Martins 2012). The reagents used in the chemical synthesis would produce particles with more predictable characteristics than the several substances and compounds present in the plant extract and used in the phytosynthesis route. It would interfere on the size and form of the nanoparticles and make the phytosynthesis a challenge in controlling the reaction process and the morphological aspects of the particles. Moreover, the presence of different bioactive substances in the extract would reduce only a fraction of the silver ions present in the solution. The remaining silver ions would form other nuclei and further the growth of the silver nanoparticles previously formed (Agnihotri 2014). This process is called Ostwald Ripening, where the largest particles consume the smaller ones and grow larger, where the dissolution of the smaller ones and deposition of ions on the surface of larger ones occur (Houk, Challa et al. 2009).

Almost 100% of ions reduction was observed for both route of synthesis. However, there was an increase of about 30% in the Ag^{+} concentration after adding the conventional-silver nanoparticles in the formulation. This fact could be due to the presence of the components as carboxymethylcellulose and propylene glycol in the spray formulation which possibly favored the silver ions dissociation into the system (Prema, Thangapandiyan et al. 2017). This fact was not observed

when green synthesis was carried out. It could be related to the several compounds present in the extract which would readily react with the released silver ions, or even the encapsulation of the silver nanoparticles promoted by those phytocompounds may have avoided the silver ions dissociation from the silver nanoparticles and its release to the solution.

Zeta potential test demonstrated the stability of the silver nanoparticles, notedly in the spray formulations. Electrical charges on the surface of the nanoparticles prevent agglomeration, and thus afford the stability of the nanoparticles (Sadowski 2008, Salem 2011). Indeed, silver nanoparticles and spray formulations presented a mean of 70 mv, which indicates their high stability of silver nanoparticles (Leite 2012).

Antimicrobial results are promising for the silver nanoparticles as well as the pomegranate extract obtained. The formulations notable showed better results compared with the input active only. This fact could be explained for the proper dissolution of the actives inputs (silver nanoparticles and pomegranate peel extract) in the spray formulation. Also, a synergistic effect could be occurred between those active inputs and the metylparaben present in the formulation. In the literature studies with antimicrobial effect of pomegranate extract were conducted against *Staphylococcus aureus*, *Enterobacter aerogene*, *Salmonella typhi*, *Klebsiella pneumonia* (Malviya, Arvind et al. 2014). MIC values obtained in this study for pomegranate extract is in accordance with Bakkiyaraj et al. (2013) for both microorganisms studied, a difference was observed in *C. albicans*, but this fact may be explained by de difference between the *C. albicans* strains used in the studies.

Chemical-silver nanoparticles produced MIC values against *S. aureus* similar to those found by Prema et al.(2017) (60 µg/ml), whom also produced silver nanoparticles stabilized with CMC. Indeed, the antimicrobial activity of chemical silver nanoparticles was also determined by Monteiro et al. (2011) with MIC values for *C. albicans* (0.5 µg/ml) in accordance with this present study.

Noteworthy is the difference found in the present study in respect of cytotoxicity between the chemical and green routes to obtain silver nanoparticles. Studies have shown that silver nanoparticles produced with *Protium serratum* and *Nyctanthes arbortristis* extract were biocompatible when tested in L929 fibroblasts (Gogoi, Babu et al. 2015, Mohanta, Panda et al. 2017). It is believed that what makes the silver nanoparticle toxic to human cells is the type of reducing agent used, such as sodium citrate or sodium borohydride (Asharani, Lian Wu et al. 2008). Even in the conventional syntheses of silver nanoparticles it is used reagents that prevent the aggregation of these nanoparticles (Ren 2015), which may further favor their cytotoxicity.

In the case of phytosynthesis of metal nanoparticles, plant extracts besides acting as reducing agent it would act stabilizing the particles, and hence reducing the toxicity of the silver nanoparticles solution. Furthermore, it is possible that some compounds in the extracts may have a synergistic effect with the silver nanoparticles (Gengan, Anand et al. 2013), becoming them less toxic to human cells. Also, extracts of *Punica granatum* exhibits antioxidant (Delgado, Rouver et al. 2016) and anti-inflammatory (Houston, Bugert et al. 2017) activity, and it may have contributed in reducing the cytotoxicity of green- in comparison with chemical-silver nanoparticles.

In general, the stability assay (silver ions dosage, zeta potential and antimicrobial activity) showed a high stabilizing capacity of the formulations. However, the spray formulations of green silver nanoparticles and pomegranate peel extract showed a significant reduction in the content of total phenols in 14 and 28 days. The decrease in the content of phenolic total may have occurred due to the temperature variations inherent of stability test, as occurred in the study of (Mgaya-Kilima et al. (2015) which the temperature affected the total phenolic content in the roselle-mango juice blends. Moreover, in formulations containing green-silver nanoparticles, the components of the extract may have been degraded or associated with the nanoparticles, explaining the faster decrease of the total phenol content compared to the pomegranate extract formulation. Interestingly, ion dosage, zeta potential and antimicrobial activity were not affected by different conditions of temperature, time and humidity of the stability test.

In view of the results obtained and the limitations of present study, it is concluded that the use of pomegranate peel extract shows as an efficient reducing agent for the production of silver nanoparticles. Moreover, the antimicrobial potential and the low cytotoxicity demonstrated by green-silver nanoparticles stimulate the search for improvements in the bio-nanotecnhology field. Also, anti-inflammatory and antioxidant properties of pomegranate encourage further researches to use nanosystems with future application in prophylaxis or treatment of biofilm-dependent diseases.

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Table 1. Ion concentration and values of minimum inhibitory concentration (MIC) for S. aureus and C. albicans.

Samples	µg Ag^+/mL	% of reduction	MIC (µg/ml)	
			S. aureus	***C. albicans***
Control	10303.26	4.59	4.13	4.59
Pomegranate peel extract (I)	-	-	391	781
Silver nanoparticles green (II)	10.89	99.89	67.50	68.75
Silver nanoparticles chemical (III)	130.40	99.51	0.50	0.25
Formulation I	-	-	0.37	0.18
Formulation II	0.249	99.93	0.26	16.87
Formulation III	1.769	68.15	0.56	1.12

*Control= Carboximetilcelulose, propilenoglicol, nitrato de prata.

Table 2. Values of silver ionic reduction and zeta potential for green and chemical spray formulations in different periods.

	Green-spray formulation			***Chemical-spray formulation***		
Time	**µgAg^{+}/mL**	**% of reduction**	**Zeta potential**	**µgAg^{+}/mL**	**% of reduction**	**Zeta potential**
T0	0.249	99.93%	-73.7 ± 6.49	1.769	68.15%	-78.2 ± 3.06
T7	0.178	99.95%	-68.3 ± 4.92	1.927	65.31%	-72.9 ± 3.10
T14	0.220	99.94%	-72.8 ± 6.49	1.543	72.22%	-85.5 ± 3.36
T28	0.186	99.95%	-68.6 ± 5.62	1.846	66.77%	-76.5 ± 4.05

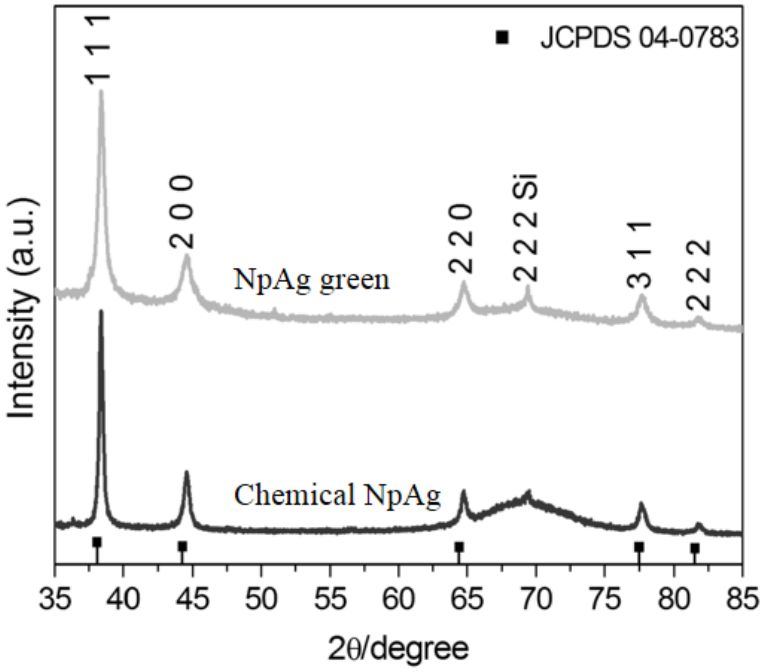

Figure 1. XDR of green and chemical silver nanoparticles.

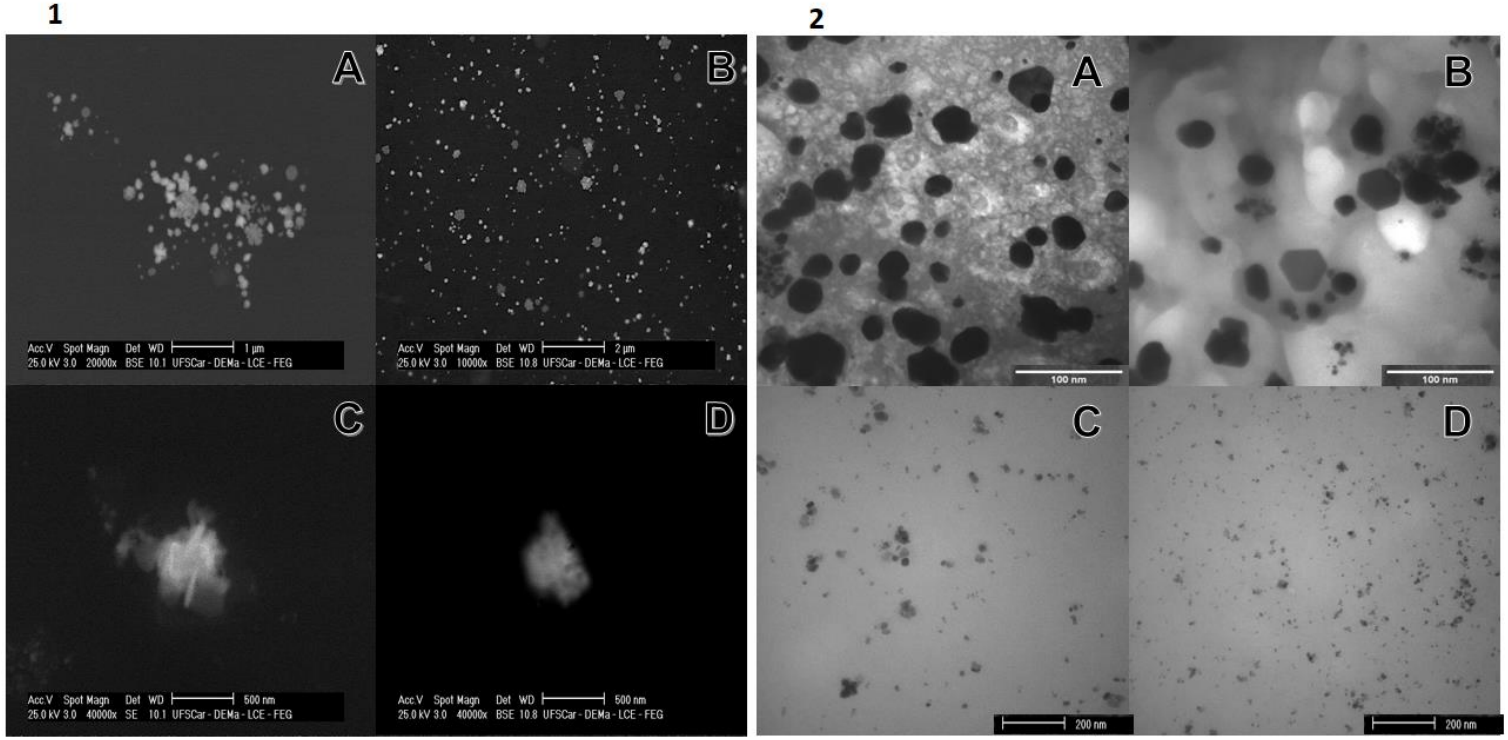

Figure 2. Images of scanning electron microscopy (MEV) (1) and transmission electronic microscopy (TEM) (2). A- Green silver nanoparticles, B- Green sprat formulation, C- Chemical silver nanoparticles and D- Chemical spray formulation.

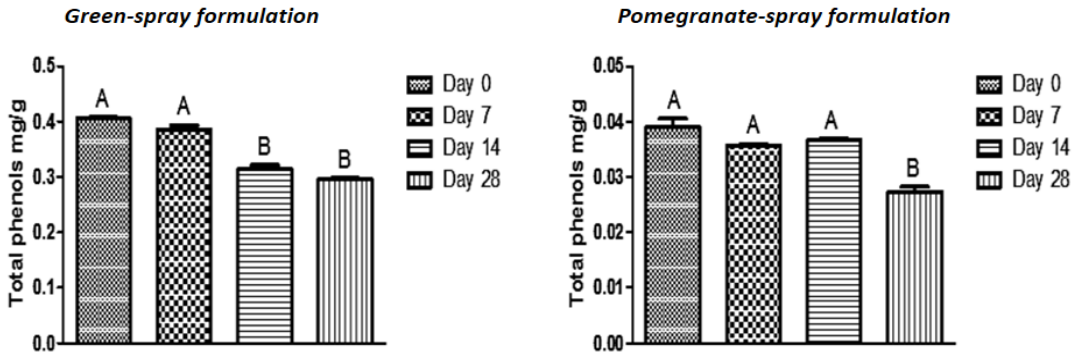

Figure 3. Total phenols concentration for green-spray-formulation and pomegranate-spray formulation in different periods. Different capital letters denote significant difference ($p < 0.05$; one-way ANOVA followed by Tukey´s multiple comparison test) among the groups.

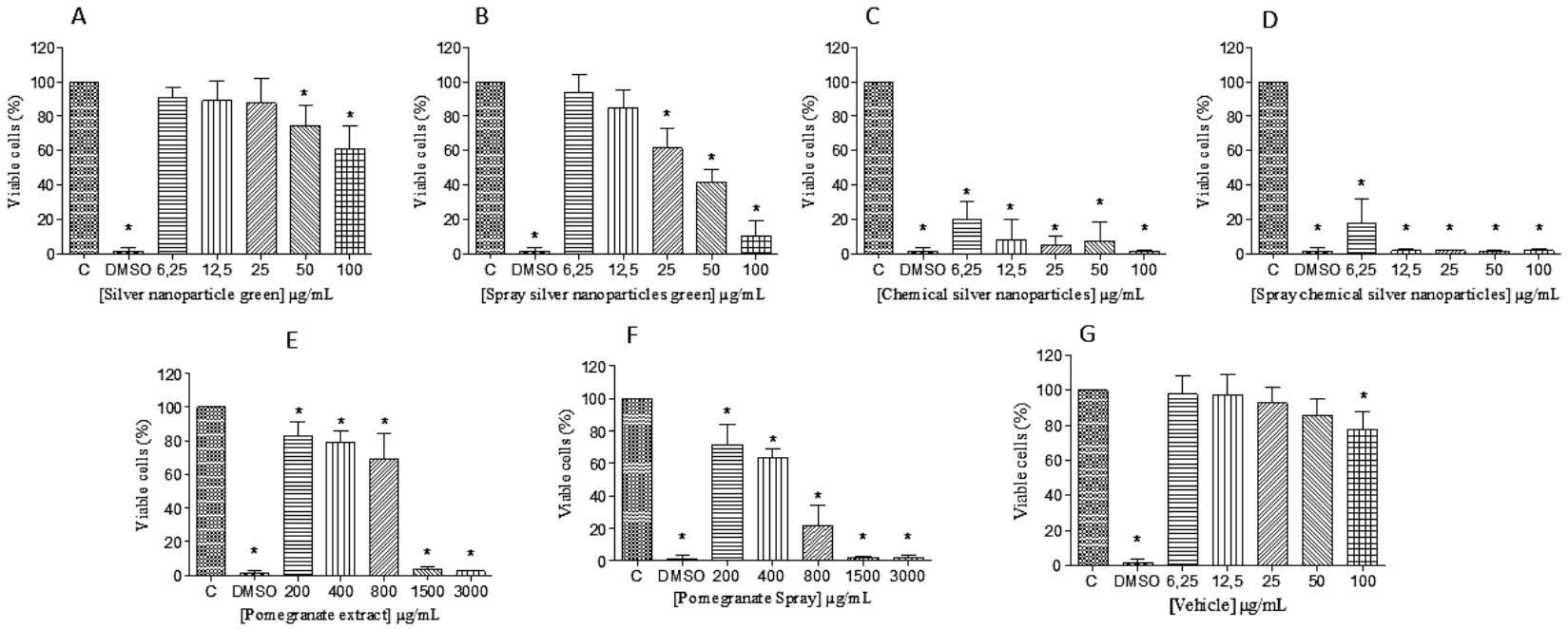

Figure 4. Cytotoxicity evaluation of respective active input and their formulations.

CAPÍTULO 3

Green and chemical silver nanoparticles and pomegranate formulations as potential wound healing and antimicrobial medicines[*]

Artigo nas normas do periódico Biofouling.

Green and chemical silver nanoparticles and pomegranate formulations as potential wound healing and antimicrobial medicines

3.1. Abstract

Infected cutaneous ulcers from diabetics rats with *C. albicans* and *S. aureus* were treated with three different spray preparations. Formulations presented different actives substances, chemical and green silver nanoparticles or pomegranate peel extract, and all were compared to negative (without treatment) and positive groups (silver sulphadiazine). After the development and infections of wounds, the treatments were performed during 14 days and applied twice/day. After the treatments periods of 2, 7 and 14 days, analyses for wound healing activity, determination of UFCs, inflammatory infiltrate, angiogenesis, fibroplasia, myeloperoxidase determination and the evaluation of collagen production (indirectly) were done. An expressive improvement in wound healing activity could be noted for treatment with both silver nanoparticles with 7 days of treatment. In the end of the study, all treatments showed superiority when compared to negative and positive groups. All treatments reduced *S. aureus* after 14 days of treatment. Chemical silver nanoparticles showed better anti-inflammatory results, while pomegranate spray showed a pro inflammatory activity, these results were corroborated by myeloperoxidase assay. Both silver nanoparticles exhibited a bigger number of fibroblasts when compared with other treatments and the results of collagen were corroborated with this finds. Although the limitations inherent to the techniques, formulations containing silver nanoparticles regardless of the route of synthesis being chemical or green, have shown a considerable potential for the treatment of wounds and the same is true

for pomegranate formulation, however wound healing were reach by completely different mechanisms of action. Even taking into consideration the ease reproducibility of the green synthesis, low cost, eco-friendly besides other properties that it could present, these proposals needs to be more explored as a wound healing medicines.

Keywords: Biosynthesis, silver, nanoparticles, *Punica granatum*, Wound Healing, *Staphylococcus aureus*, *Candida albicans*.

3.2. Introduction

Cutaneous ulcers are prevalent and recurrent in diabetic patients, and this fact need to receive a special attention mainly because statistics show that by the year 2030 about 350 million people will be affected by diabetes (Gottrup 2004), (Wild, Roglic et al. 2004). Maintenance of open ulcers is mainly due to the persistence of inflammation and infections that are directly attributed to biofilms (Chen and Wen 2011). Biofilms are responsible for the development and / or maintenance of about 2% of the American population present chronic wounds that result in amputations provided they do not receive special care (Gottrup 2004), this factor is related to diabetic people (about 24%) (James, Swogger et al. 2008), (Gottrup 2004). In addition to this factor the resistance of microorganisms to conventional antimicrobial agents is of concern to the scientific community, thus necessitating the immediate search for new alternatives that control the formation or progression of biofilms (Smith, M'Ikanatha N et al. 2015).

The availability of medicines to combat this worrisome word wild problem is so limited. The search for drugs that perform activities against microorganisms in both their planquitonic state and biofilm, besides presenting anti-inflammatory effect need to be explored (Chen and Wen 2011). In this sense, studies on the fight against biofilms and ulcers treatment have been tackled, such as the use of nanotechnology (silver nanoparticles) (Monteiro, Gorup et al. 2009) and phytotherapy (Berretta, Nascimento et al. 2012).

The use of silver nanoparticles has been widely studied and applied nowadays, such as the incorporation in shampoos, soaps, detergents, cosmetics, medical devices and other applications. Due to this wide use of silver

nanoparticles as an antimicrobial agent has also been growing, as in the textile industry, in the production of wound dressing among others (Eby, Luckarift et al. 2009), (Mohite and Patil 2016), (Hebeish, El-Rafie et al. 2014) .In the field of phytotherapy, several plant extracts have been studied for the treatment of ulcers, such as Chrozophora tinctoria (Maurya, Semwal et al. 2016), Hibiscus rosa-sinensis L (Shen, Chen et al. 2017), Pongamia Pinnata (Dwivedi, Dwivedi et al. 2017), Chamaemelum nobile L. (Kazemian, Ghafourian et al. 2016), Punica granatum (Mo, Panichayupakaranant et al. 2014), (Nayak, Rodrigues et al. 2013) , (Hayouni, Miled et al. 2011), (Murthy, Reddy et al. 2004). Plants that are cultivated all over the world and that have demonstrated properties as anti-inflammatory, antimicrobial, antioxidant, among others, should be preferred in order to be used with batch-to-batch reproducibility, as is the case of Punica granatum (Das and Brar 2013).

It is known that conventional silver nanoparticles have some undesired effects, such as their toxicity to mammalian cells, thus the proposal for the production of silver nanoparticles through the reduction by natural compounds as vegetable extracts (Roy, Gaur et al. 2013) are interesting. So, the present study aimed to evaluate the antibiofilm and repair capacity of cutaneous ulcers produced in rats of three different spray preparations containing silver nanoparticles synthesized by a chemical or green route, and Punica granatum peel extract, previously obtained and characterized by Fernandes Ra 2017.

3.3. Materials and methods

Spray formulations

Briefly, after obtaining the peel pomegranate extract and the synthesis of silver nanoparticles by the Turkvich methodology and by the green route using pomegranate peels extract, the spray formulations were prepared considering the MIC values of each active ingredient (Fernandes RA 2017), taking account the results of cellular toxicity (unpublished data). Thus the final formulations contained carboxymethylcellulose (Labsynth, Diadema, Brazil), propylene glycol (Labsynth, Diadema, Brazil), methylparaben (Labsynth, Diadema, Brazil) in a proportion of 0.1%, 7% and 0.1%, respectively, and the active ingredients in concentrations of 337.5 µg/ml and 5.554 µg/ml for green and chemical silver nanoparticles, respectively and 94 µg/ml for *Punica granatum* extract. These values correspond to silver for green or chemical nanoparticles and pomegranate dry matter for the vegetal extract.

In vivo models for wound healing activity

Ninety male Wistar rats (180-200g) were obtained from the Central Biotherm of the Medical School of Ribeirão Preto, University of São Paulo, Brazil. Previously, animal experimental protocol was approved by the Ethics Committee for Animal of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil, (process nº 140/2016). The animals were kept in isolation conditions in individual cages, with water and feed (ad libitum) and alternating cycles of luminosity every 12 hours.

Animals received streptozotocin (50 mg / kg) intravenously via caudal artery. After 24 hours the blood glucose concentration was checked for diabetes induction (Shukla, Rasik et al. 1999). After confirmation of the induction of diabetes the rats were weighed, and anesthetized via intraperitoneal with ketamine 70mg / kg and Xilazine 12mg / kg. Later, the dorsum of the rat was trichotomized and performed two surgical excisions with a punch of 1.5 cm in diameter, thus leaving the dermo-epidermal region affected. After this procedure, the microorganisms were adjusted to 10^4 CFU/ml for each, *Staphylococcus aureus* and *Candida albicans*, and so, they were pipetted (100 µl) directly onto the lesion. The ulcerated skin was collected to be used as control (day 0), non-ulcerated skin. After surgery, oral dipyrone, 50 mg / kg body weight, diluted in saline solution every 12 hours for the first 48 hours, was administered orally according to the behavioral changes of the animals regarding to the pain.

The animals were divided into 5 groups containing 6 animals each and the analysis was performed at three different times 2, 7 and 14 days after treatments. Each animal was treated twice a day topically, at the beginning of the morning and at the end of the afternoon. The groups were identified as: SHAM, where the animals received no treatment (C), spray green, formulation containing green silver nanoparticles (SG), chemical spray, spray formulation with chemical silver nanoparticles (SQ), pomegranate spray (PS) and commercial silver sulfadiazine (Sulf).

Wound healing activity

After each treatment period the animals were euthanized, the lesions photographed with a Sony DSC-P100 digital camera coupled to a base containing a millimeter ruler, with a standard distance of 30 cm, being perpendicular to the ulcers. After obtaining the images, the lesion areas were measured with Image J software. The following step was the determination of the ulcer healing index, which is equivalent to the quotient of the difference between: Initial and Final Areas divided by the Initial Area [ICU = (Ai-Af) / Ai] (Robson, Hill et al. 2000).

Collection of material for study

After the photos procedure the animals had all the skin trimmed around the ulcers and through the 1.5 cm diameter punch that were used for the following purposes:

- Fragment 1: packed in 4% formaldehyde in Sorensen phosphate buffer 0.1 M, pH 7.3 for histological study (inflammatory infiltrate, angiogenesis and fibroplasia) (hematoxylin-eosin).
- Fragment 2: frozen in freezer -70 to -80 °C in specific buffer for myeloperoxidase (MPO) dosing and counting of CFUs and hydroxyproline (HYP).

Determination of CFUs from ulcers

The tissues were thawed, packed into falcon tubes (50ml) containing sterile cell strains (BD), 3 ml of PBS was added and vortexed under constant stirring for 1 minute. After this step, the contents were diluted in serial dilutions and plated in culture medium specific for each microorganism, Candida chromagar (Becton,

Dickinson and Company, Heidelberg/Germany) for *C. albicans* and Mannitol salt agar (Becton, Dickinson and Company, Heidelberg/Germany) for *S. aureus*. The plates were incubated at 37 ° C for 24 hours for *S. aureus* and 48 for *C. albicans*, and colony count was determined by tissue weight.

Quantitative image evaluation for inflammatory infiltrate, angiogenesis and fibroplasia

Hematoxylin-eosin-stained histological slides were visualized on the LEICA® DM 4000B optical microscope with LEICA® DFC 280 camera with LAS® software - Leica Application Suite for image capture. 4-5 fields per blade were obtained at 200x and 400x magnification. The "CellCounter" Plugin of the ImageJ 1.48 software was used to count the cells of the inflammatory infiltrate, fibroblasts and blood vessels in each of the 5 individually quantified images of each animal. The differentiation of each cell / structure was made by the observer in the image of the lamina increasing by 400x, whereas the blood vessels were quantified in increase of 200x.

This protocol was applied to the animals of each treatment and each time, and for the final result, the mean number of inflammatory infiltrates, fibroblasts and vessels found in the 4-5 fields of each animal was considered. The graphs were plotted with the mean obtained in each group, along with the presentation of the standard deviation.

Dosage of the enzyme myeloperoxidase (MPO)

Neutrophil dosage in the lesion was performed by the myeloperoxidase assay (Souza, Cassali et al. 2001). After washing the tissues with PBS for CFU determination, the tissues were subjected to MPO determination procedures. Validation assays were done previously, in order to demonstrate that washing tissue with PBS for CFU count prior to MPO dosing, did not interfere with the result. Biopsies were then thawed and buffered in 0.1 M NaCl, 0.02 M NaPO 4, 0.015 M NaEDTA, pH 4.7 at -70 ° C. After buffering the fragments, they were triturated in POLYTRONÂ® PT 3100 at 13000 rpm. In the next step they were centrifuged and resuspended in NaPO4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). 96 well plates then received 25 µL of the supernatant 25 µL of TMB ("3, 3 ', 5, 5'-tetramethylbenzidine") and then 100 µL of H_2O_2, then the reaction is quenched with 4 M sulfuric acid and read Plate reader (ELISA detector) at 450 nm. The results were expressed as optical density / mg tissue.

Evaluation of collagen

Collagen was assessed by the measurement of hydroxyproline in tissues, following the methodology described in (Reddy and Enwemeka 1996) and (Dwivedi, Dwivedi et al. 2017) with some modifications.

Tissues frozen at -70 ° C were thawed and incubated in an oven at 60 ° C in uncapped microtubes for 15 hours. After the incubation period, the samples were weighed and homogenized in POLYTRON® PT 3100 at 13000 rpm with 6N hydrochloric acid (HCl) and transferred to capped glass test tubes. HCL ¨N was

added in a proportion of 100 µL per 1 mg of dry tissue, homogenized and subjected to acid hydrolysis, thus incubating the samples in HCL at 130 ° C for 4 hours.

After the incubation time, the pH of the samples was adjusted to 7,0 with 2M sodium hydroxide (NaOH). Subsequently 10 µL of each sample along with 90 µL of 0.056M T-Chloramine solution were pipetted into 96-well plates incubated in the dark for 25 minutes. After the time, 100 µL of Ehrlich reagent (Woessner 1961) was added for the formation of the chromophore. Thus, the plates were incubated at 60 ° C for 20 minutes as by performing the absorbance reading at 550 nm.

Statistical analyses

GraphPad Prism software (GraphPad Software, Inc, La Jolla, USA) was employed for the statistical analysis with a confidence level of 95 %. Parametric statistical analyses were conducted with one-way ANOVA followed by Turkey's Multiple Comparison test. Except for ion dosage the statistical analyses used was Dunn's Multiple Comparison test.

Results

Wound healing activity

Figure 1 showed that a significant wound healing activity could be noted for treatments with silver nanoparticles in both chemical and green synthesis, from seven days of treatment. The treatment with pomegranate spray had a significant increase in wound healing with fourteen days of treatment, as well as all others treatments, but silver nanoparticles green and chemical as well as pomegranate

spray showed better result when compared with control group and silver sulfadiazine treatment ($p<0.05$). Statistical analysis could be clearly corroborated by the photographs presented.

Determination of CFUs from ulcers

Considering microbiological results, it is possible to observe in figure 2 (a) that *Candida albicans*, independently of the treatment used, maintained the same number of UFCs during all time. But for *S. aureus* all treatments used (green or chemical silver nanoparticles, pomegranate spray or silver sulphadiazine) reduced the UFCs (mean of 1.35) after fourteen days of treatment, demonstrating that all formulations presented were effective against this pathogenic gram-positive microorganism.

Quantitative image evaluation for inflammatory infiltrate, angiogenesis and fibroplasia

The results of inflammatory infiltrate can be showed in figure 3, as well as fibroplasia. Inflammatory infiltrate graphics were obtained after the histological results analysis and this data can be an compared with MPO results, i.e., the quantitatively chemically data for inflammation in the tissue (figure 5), Chemical silver nanoparticles showed a reduction in inflammatory infiltrate with seven days of treatment, however, this data couldn't be linked with MPO results because of the high standard deviation of the last one. Curiously, pomegranate spray showed higher inflammatory filtrate with 2 and 7 days in both measures, but only for 7 days of treatment this data were statistically different from the other treatments,

suggesting that wound healing induced for this natural product was a consequence of a pro-inflammatory response. The inflammation was similar for all groups with 14 days.

Silver nanoparticles chemical and green resulted in a bigger number of fibroblasts compared to the other treatments ($p<0.05$) in the beginning of the treatment (2 days). It is probable that this higher amount of fibroblasts for these two treatments in this moment, was responsible for the better closer of the wounds for these two groups with 7 days (figure 1). After this period, all treatments showed no statistically difference among the groups. Although some could expected that the wound healing in diabetics could be improved by the treatments done as a consequence of increase in blood vessels, this was not the case in the present work, since the number of blood vessels (Fig.4) weren't affected for the treatments done in all period of treatment. Usually diabetic patients present bad oxygenation in the lesions and usually this is one factor that compromises de wound healing.

Dosage of the enzyme myeloperoxidase (MPO)

Myeloperoxidase is a biochemically measure of the inflammatory process. The results showed in figure 5 demonstrated that with 2 days, no one of the products were able the significantly reduce this process. After 7 days of treatment, only for pomegranate spray the inflammatory process remained the same as it was with 2 days, suggesting that this natural compound could stimulate wound closure by pro-inflammatory process (result corroborated with histological analysis). And

finally, with 14 days of treatment, all groups demonstrated an absence of inflammatory response.

Evaluation of collagen

Figure 6 exhibits indirectly the production of collagen by the measure of hydroxy proline, with interesting results for treatment with silver nanoparticles chemical and green, with two days of treatment. These results corroborate with fibroblast number showing that these both sprays clearly accelerates the collagen production, probably been responsible for the better closure observed in figure 1. After this time of treatment, all groups had similar effects, i.e, the same collagen production.

Discussion

The emergence of multidrug-resistant microorganisms is resulted of use of conventional antimicrobial therapy constantly and unduly, in parallel the growing of systemic diseases as arterial hypertension and diabetes encourage the search for new biomaterials that may be used as medicinal products. Thinking about the emergence of diabetes, added to antimicrobial resistance, the major question in this study was to evaluate the capacity of repairing infected cutaneous ulcers treated with formulations containing silver nanoparticles produced by two distinct routes and pomegranate peel extract. All treatments were compared with the negative control (without of treatment) and a positive one, silver sulphadiazine treatment, a reference standard treatment.

It has been observed an increase in the wound healing activity for treatments with silver nanoparticles formulations, especially with seven days of treatment. Result that was corroborated with fibroblasts cells and hydroxyproline, all parameters linked to tissue reparation. These results were supported by Adibhesami et al. (2017), where its believe that silver nanoparticles have the capacity in reducing time required for hyperactive cells (myofibroblasts), responsible for contract of wounds. Moreover, silver nanoparticles may improve the anti-inflammatory activity in wounds, increasing the repair process (Karim, Adnan et al. 2012) (Liu, Lee et al. 2010), however, in the presented data, this was only observed for chemical silver nanoparticle after 7 days of treatment.

Results of fibroplasia (figure 3) corroborate with the finds related above, where the number of fibroblasts increased significantly in two days of treatment with both silver nanoparticles. These results are confirmed again (figure 6) for collagen determination where the results for silver nanoparticles are increased in two days of treatment. Furthermore, the results of fibroplasia showed the better results for chemical silver nanoparticles, decreasing the inflammatory cells in seven days of treatment, as opposed to the pomegranate spray, which exhibited a pro inflammatory activity. The pro inflammatory activity of pomegranate spray was confirmed in the myeloperoxidase assay (fig 5).

The explanation for the better results founded for silver nanoparticles could be because the silver nanoparticles promote the differentiation and maturation of keratinocytes through the stimulation of skin stem cells (Cha and Falanga 2007) (Cotsarelis 2006) (Tiede, Kloepper et al. 2007). Added to the

AgNPs can indeed stimulate cell maturation and hence promote the differentiation of fibroblasts into myofibroblasts during healing (Liu, Lee et al. 2010).

For pomegranate spray, the pro inflammatory activity could be based in the literature, where some studies shows the production of pro inflammatory cytokines. (Mix, Mengshol et al. 2001) (Larrosa, Gonzalez-Sarrias et al. 2010) (Hayden and Ghosh 2004). These studies reported that pomegranate inhibits the p38-mitogen-activated protein kinase (p38- MAPK) pathway and transcription factor, NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells). Thus, when the p38-MAPK and NF-kB are activated increase the gene expression of COX-2, TNF-α, MCP1, IL-1β and iNOS which are inflammatory mediators (Hayden and Ghosh 2004).

Regarding to antibofilm activity, all treatments were able to reduce the number of UFCs for *S. aureus* after 14 days of treatment, similarly to Adibhesami M et al. (2017) where all treatments used by authors had effect against *S. aureus* as of 14 days of treatment. While for *C. albicans* it was not possible to reduce the number of UFCs. Although the results of the antibiofilm action were not so expressive for *C. albicans*, factors such as inter-kingdom interaction must be taken into account, since as reported by Kean et al 2017, the formation of biofilms of *C. Albicans* and *S. aureus* favored the strengthening of both increasing the resistance to fluconazole (Kean, Rajendran et al. 2017). It can still be attributed to the greater susceptibility to *S. aureus* against the studied drugs from 14 days, for a possible production of quorun sensing molecules by *C. albicans* such as farnesol, a molecule that regulates several functions in the biofilm, including population control (Wongsuk, Pumeesat et al. 2016).

In view of the presented results and the inherent limitations of the techniques used it can be emphasized that formulations containing silver nanoparticles, regardless of the route of synthesis being chemical or green, have shown a considerable potential for the treatment of wounds, especially showing a better wound closure than the conventional therapy, with only 7 days of treatment. It is also interesting to consider that pomegranate spray showed a better result than the positive control group, i.e. the reference standard used, silver sulphadiazine. For both silver nanoparticles, the mechanism of action were focused on the re-epithelization stimulation and for the phytomedicine evaluated, the wound closure was mediated for a pro-inflammatory stimulation, similar behavior to other natural compounds evaluated in the same model.

Even taking into consideration the ease reproducibility of the green synthesis, low cost, eco-friendly besides other properties that it could present, and the same benefits for pomegranate spray, these proposals needs to be more explored as a potential antimicrobial and wound healing medicines.

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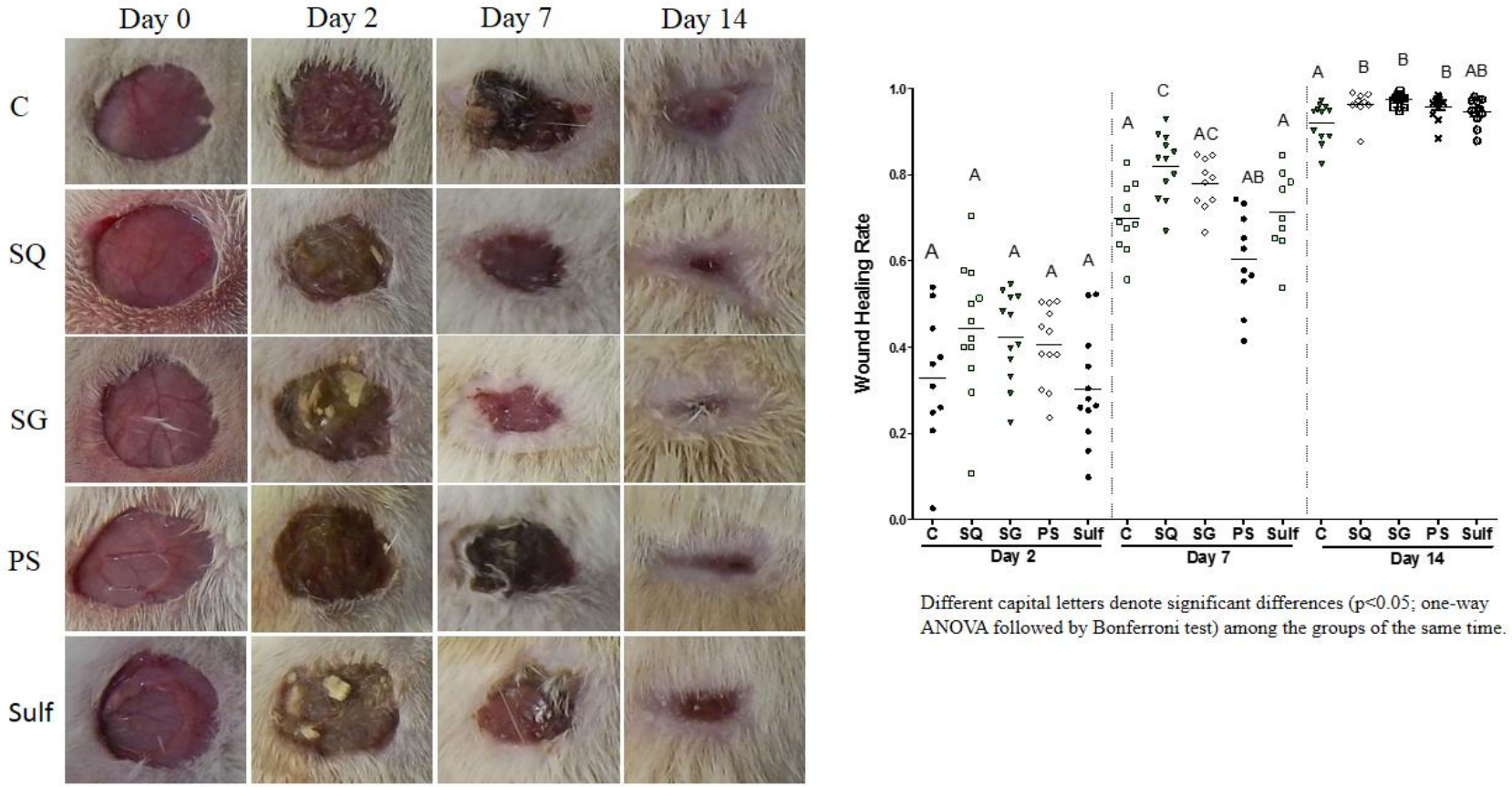


Different capital letters denote significant differences ($p<0.05$; one-way ANOVA followed by Bonferroni test) among the groups of the same time.

Figure 1. Images and wound healing rate of different treatments for 14 days. C: control (without treatment); SQ: chemical spray; SG: green spray; Ps: spray of pomegranate peel extract; Sulf: silver sulfadiazine.

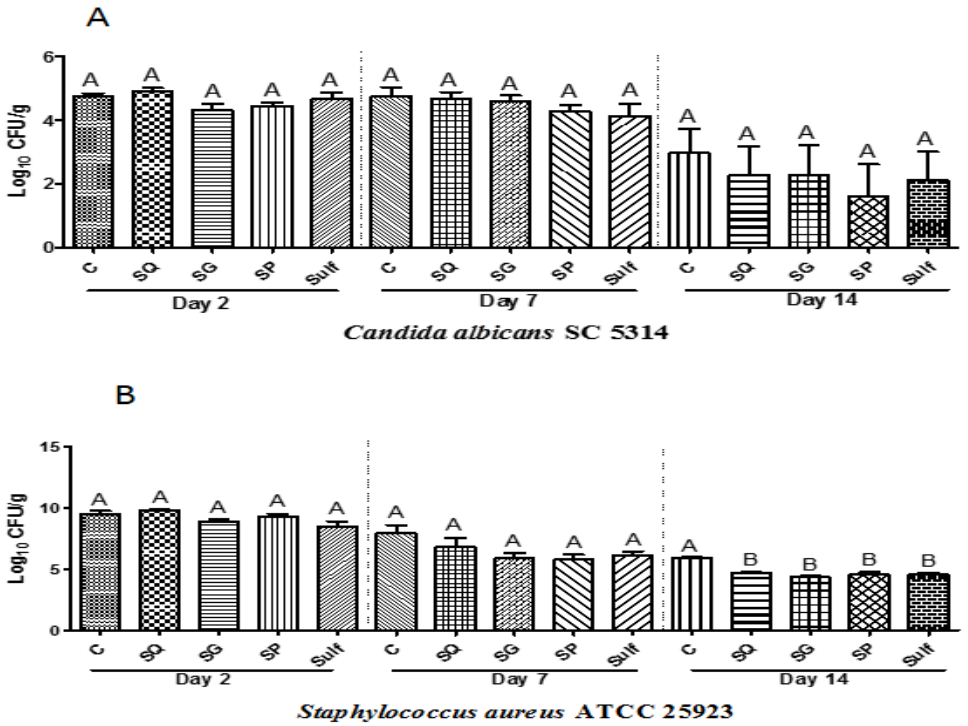


Figure 2. Mean values of the logarithm of colony forming units per gram (log_{10} CFU/g) for mixed *C. albicans* and *S. aureus* biofilms treated for different treatments for 14 days. C: control (without treatment); SQ: chemical spray; SG: green spray; Ps: spray of pomegranate peel extract; Sulf: silver sulfadiazine.

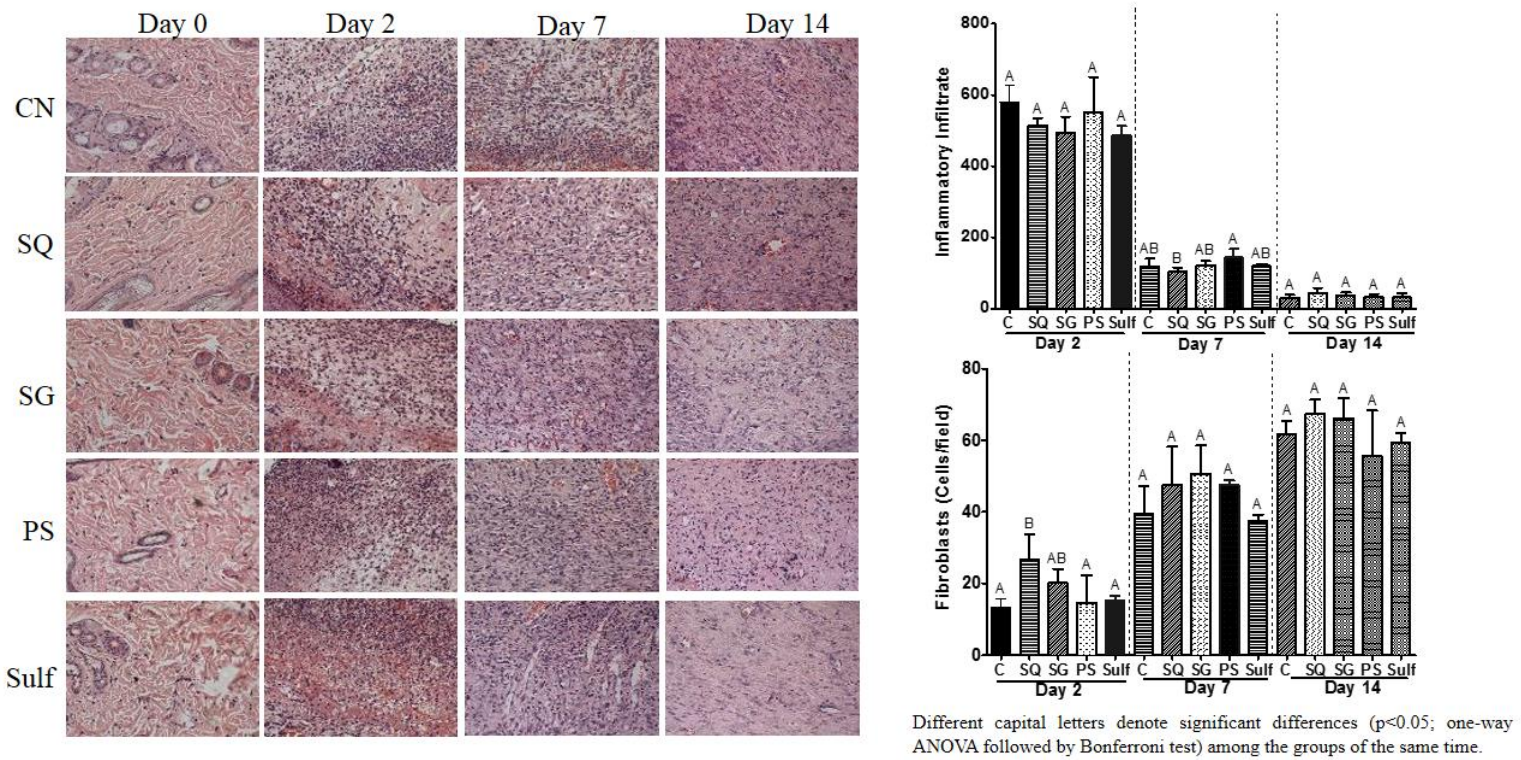


Figure 3. Results of inflammatory infiltrate and fibroblasts cells for 14 days of treatment. C: control (without treatment); SQ: chemical spray; SG: green spray; Ps: spray of pomegranate peel extract; Sulf: silver sulfadiazine.

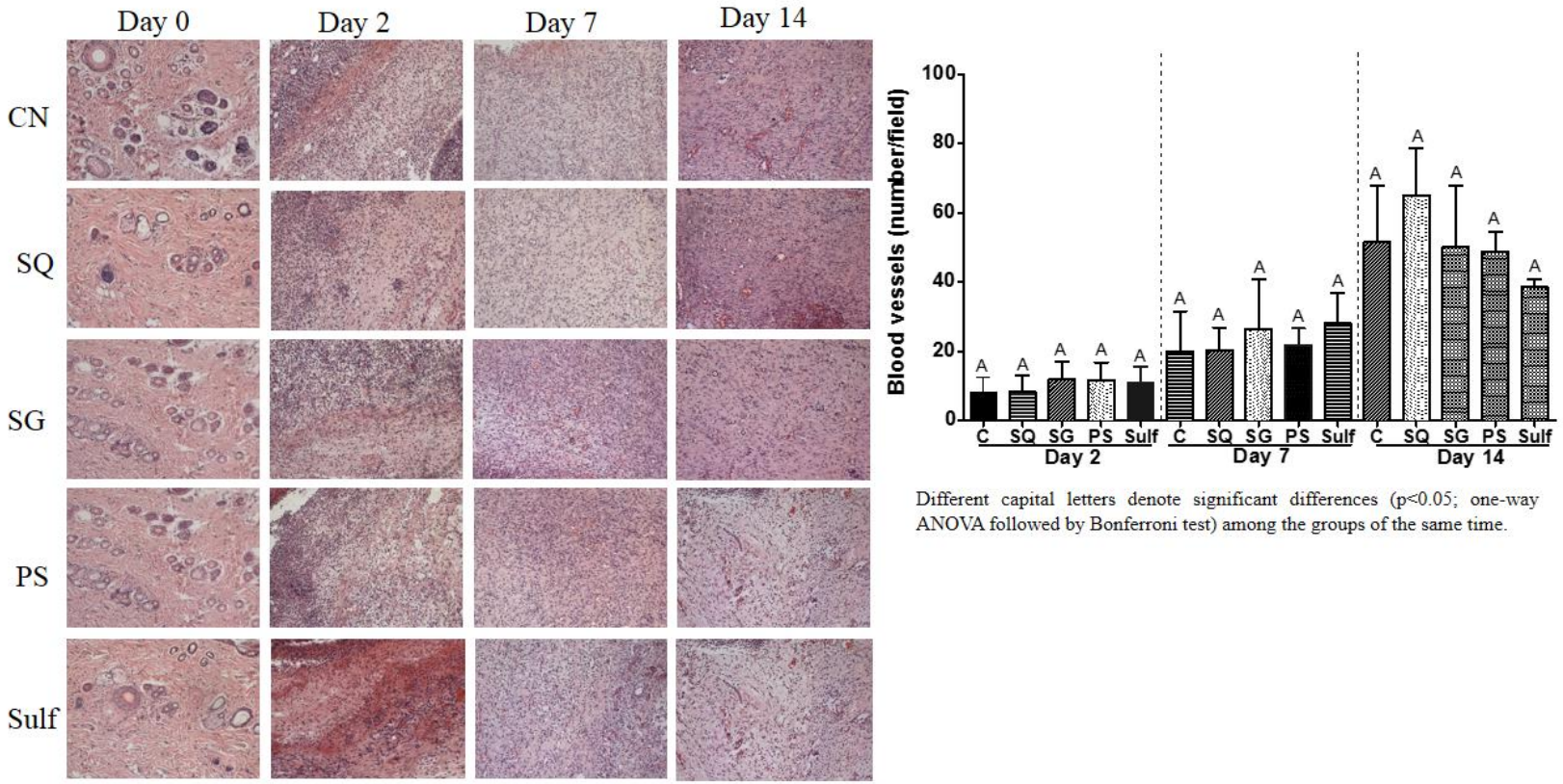


Figure 4. Results of blood vessels count for 14 days of treatment. C: control (without treatment); SQ: chemical spray; SG: green spray; Ps: spray of pomegranate peel extract; Sulf: silver sulfadiazine.

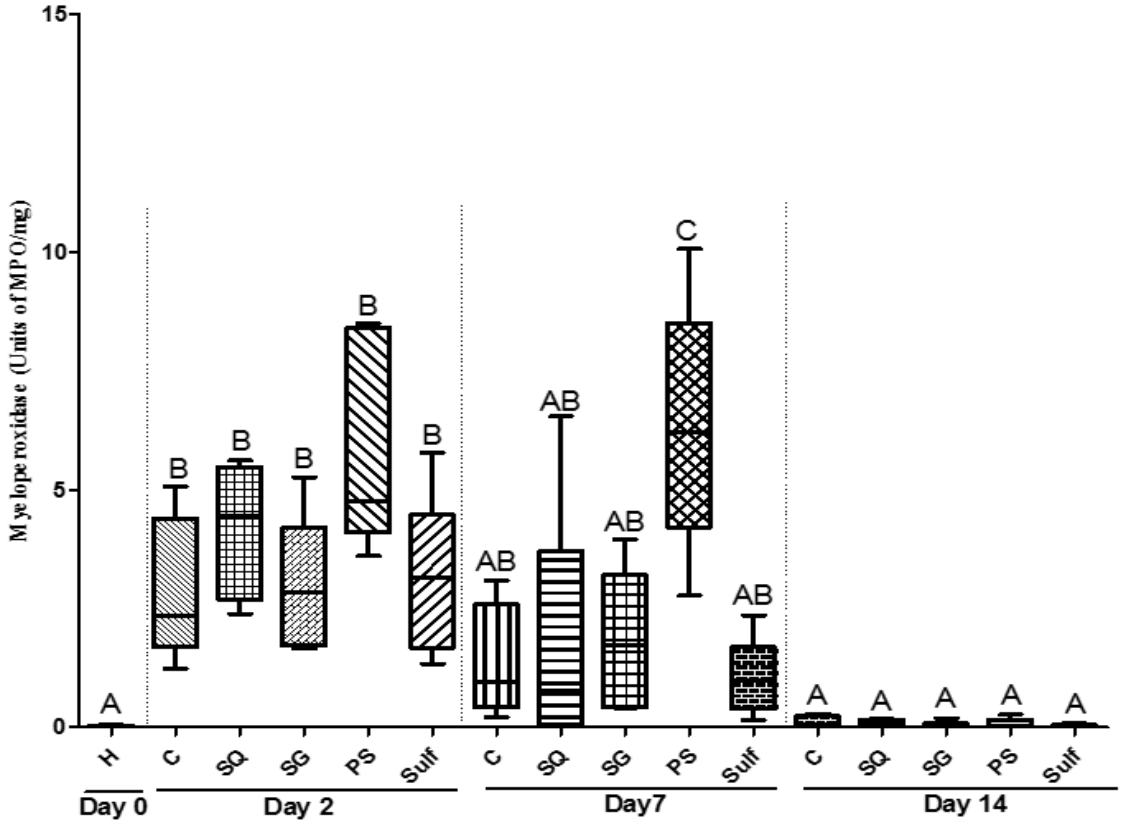


Figure 5. Results of myeloperoxidase for 14 days of treatment. C: control (without treatment); SQ: chemical spray; SG: green spray; Ps: spray of pomegranate peel extract; Sulf: silver sulfadiazine.

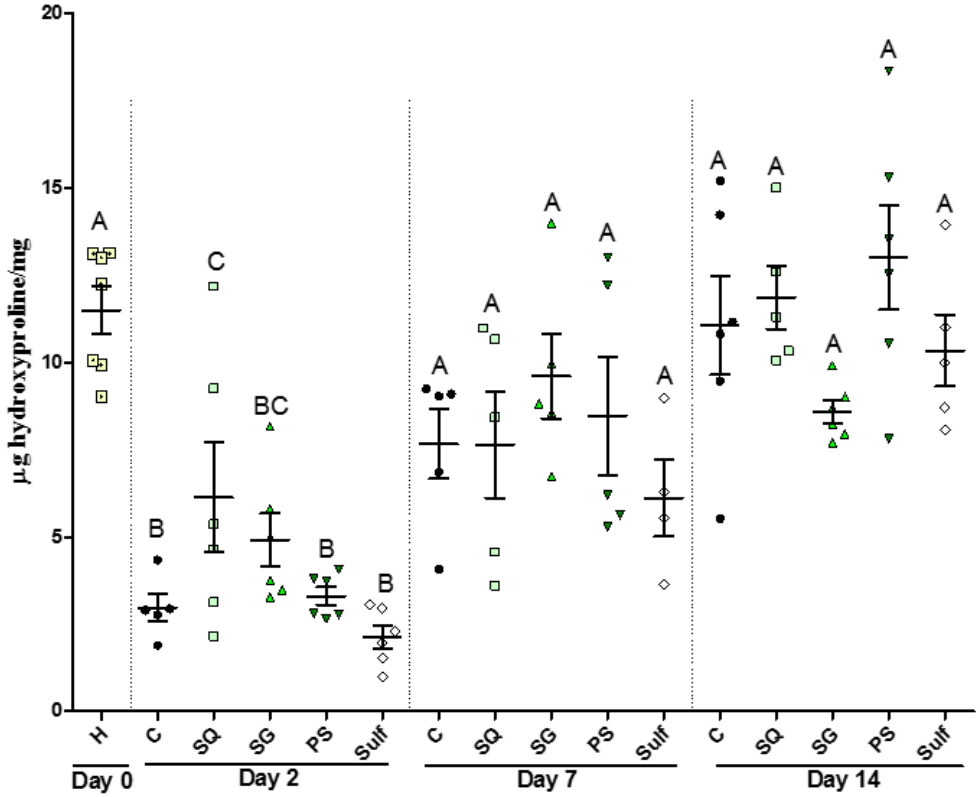
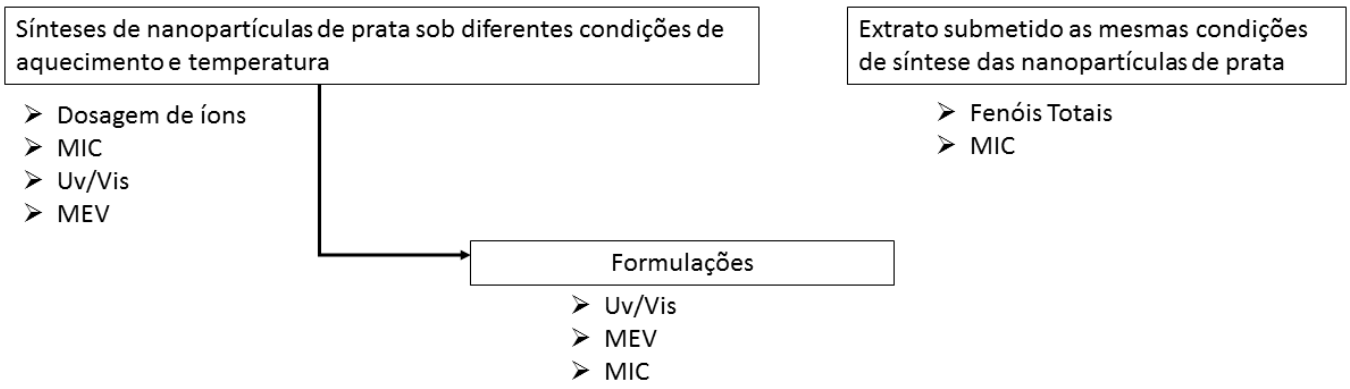


Figure 6. Results of hydroxyproline for 14 days of treatment. C: control (without treatment); SQ: chemical spray; SG: green spray; Ps: spray of pomegranate peel extract; Sulf: silver sulfadiazine.

Anexo A – Organograma capítulo 1.



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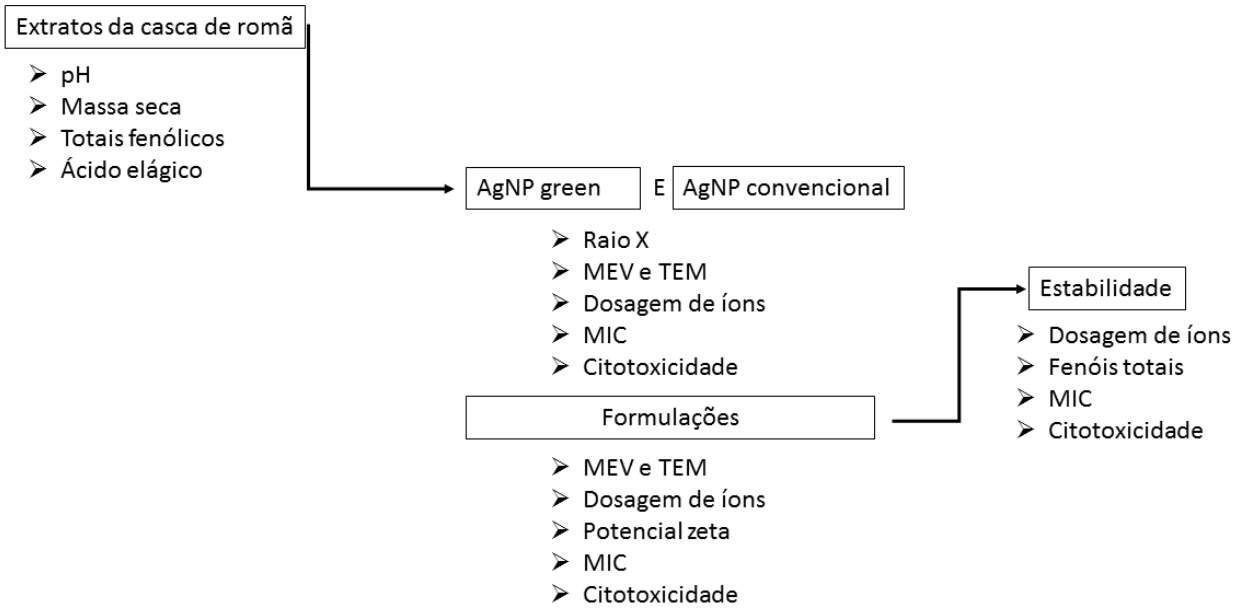

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