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CELLULAR AND MOLECULAR BIOLOGY

Effect of combining *Zingiber officinale* and *Juglans regia* extracts on *Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis*: antibiofilm action and low toxicity

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Abstract: Objective was evaluated the therapeutic effect of *Juglans regia* (J) and *Zingiber officinale* (Z) extracts, alone or associated (Z75% + J25%, Z50% + J50% and Z25% + J75%) applied on planktonic cultures and biofilms of *Propionibacterium acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus*, as well as analyzing the cytotoxic effects of plant extracts on mouse macrophages (Raw 264-7). Broth microdilution assay was performed (M7-A6 - CLSI). Anti-biofilm activities and cytotoxicity on Raw 264-7 were studied using MTT assay and scanning electron microscopy. ANOVA with post-hoc Tukey HSD applied for parametric data and Kruskal-Wallis with Conover-Iman test, for non-parametric ($p<0.05$). On *P. acnes* biofilm, Z50% + J50% reduced 46.9% in 5 min and Z25% + J75% reduced 74.1% in 24hs. On *S. aureus*, Z75% + J25% reduced 23.1% in 5 min Z25% +J75% reduced 79.4% in 24hs. On *S. epidermidis*, Z75% + J25% reduced 74.6% in 5 min and 82.05% in 24 h. The treatments on macrophages for 24 h promoted a maximum reduction by 14,5% for groups of extracts associations. On multispecies biofilm, Z75%+J25% reduced 84.3% in 24 h. In conclusion association of glycolic extracts provided therapeutic effect, demonstrated antimicrobial activity and low cytotoxicity.

Key words: *Zingiber officinale*, *Juglans regia*, anti-infective agents, dermatologic agents, Acne Vulgaris, toxicity.

INTRODUCTION

Skin is the outermost layer of the human body, it contains abundant and diverse population of microbial organisms making this organ more susceptible to skin-environment interactions (Gallo 2017). Thus, it suffers some diseases like acne Vulgaris which is a chronic inflammation of the pilosebaceous unit (Bershad 2001).

Acne is an obstructive disease considered as one of the most prevalent and common skin conditions as it affects 80-85% of teenagers globally. Some acne lesions develop as a result of a major inflammatory process and usually require medical treatment. It has multifactorial etiology (Williams et al. 2012, Leheste et al. 2017). However, four main components contribute to its pathogenesis including: increased sebum, keratinization of the middle part of the infundibulum (Infra-infundibulum), bacterial colonization of the follicle, and inflammation sebaceous follicle channel founded in the sebaceous glands (Aydemir 2014).

The process of colonization of the follicles occurs from the human skin microbiota,

where the species that inhabit the epithelial site become part of the interior of these cavities. Three Gram-positive bacterias make part of the normal microbiota are involved in the development of acne, including *P. acnes* that produces several involved enzymes in the process of follicular rupture and dermal inflammation, producing chemotaxis factors stimulating macrophages to produce IL8, IL1β and TNFα (Liu et al. 2015). *P. acnes* uses sebum secreted by sebaceous glands as a source of nutrition and developes toxic metabolites for the epidermis. These bacteria contain lipases that degrade the sebum triglycerides, which trigger an intense inflammatory response, associated with the bacterial toxins themselves (Perry & Lambert 2006, Fitz-Gibbon et al. 2013, Dréno et al. 2018).

Another species isolated from the epithelial site is Staphylococcus aureus, which causes pathologies such as pneumonia, meningitis, endocarditis, in addition to being responsible for most hospital infections. Although its interaction with the development of acne has not yet been completely elucidated, it is known that the species elevates the local inflammatory process due to its repertoire of lithic enzymes against epithelial tissue. (Tomida et al. 2013, Totté et al. 2016). Finally, *Staphylococcus epidermidis* colonizers of the skin and mucosa, the responsible of majority of nosocomial infections, requiring a predisposed host, ranging from a normal inhabitant to an opportunistic agent (Liu et al. 2015).

Ginger is a widely used anti-inflammatory and antioxidant. It has secondary metabolites that can inhibit the growth of pathogens like *Esherichia coli, Bacillus subtilis* and *Staphylococcus* sp. (Afzal et al. 2001, Mahyari et al. 2016).

J. regia (or walnut) is a medicinal plant, has the ability to cure various diseases, such as helminthiasis, diarrhea, sinusitis, stomach pain, arthritis, asthma, eczema, skin diseases, diabetes mellitus, anorexia, dysfunction thyroid, cancer and infectious diseases. It has antimicrobial activity against Gram-positive bacteria (S. *aureus* and *Streptococcus mutans*), Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans*) (Lagha et al. 2019, Taha 2011, Qadan et al. 2005).

Acne Vulgaris has been treated with some topical and systemic retinoids and some antibiotics that promote the growth of antibiotic-resistant bacterial strains and present side effects. Thus, new alternative medications like plant extracts may be used with a few side effects and promising results (Simonart 2012, Jin & Lee 2018, Kiliç et al. 2019). This study aimed to analyze the therapeutic effect of the association of glycolic extracts of *Z. officinale* and *J. regia* on planktonic cultures and biofilms of *S. aureus*, *P. acnes* and *S. epidermidis*, besides evaluating the cytotoxicity of extracts on mouse macrophages (RAW 264.7).

MATERIALS AND METHODS

Glycolic extracts

Z. officinale (Z) glycolic extracts were obtained of plant rhizome and *J. regia* (J) extract made by tree bark (Mapric - Brasil/SP) were prepared at a concentration of 20% eluted in propylene glycol (200mg/mL), with the reports attesting the veracity of the plant species.

The polyherbic associations was prepared from different proportions of each base extract, where the group Z75% + J25% were created with a proportion of 75% by extract of *Z. officinalis* and 25% of the extract of *J. regia*; Z50 % + J50% was prepared with 50% of each extract and Z25% + J75% was composed of 25% of the *Z. officinalis*

extract and 75% of the *J. regia* extract, all the groups had a final concentration by 200 mg/mL.

Strains

Microbial strains used in this study were *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228) and *P. acnes* (ATCC 6919), obtained from the Microbiology and Immunology Laboratory of the Dentistry Course of ICT - UNESP.

Determination of minimum inhibitory concentration (MIC) and minimum microbicide concentration (MMC)

The microdilution broth method was performed, according to Clinical and Laboratory Standards Institute (CLSI), protocol M7-A6 (NCCLS 2006). Standardized bacterial inoculum was prepared of culture seeded on BHI "Brain Heart Infusion" agar (Himedia, Mumbai, India) after 24 h of incubation (at 37°C, with anaerobiosis for *P. acnes*) and standardized at spectrophotometer in a concentration of 10^6 CFU/mL.

The test was performed on 96-well plates (TPP, Zollstrasse, Switzerland) in which 50-well were filled with 100 µL/well of Mueller Hinton broth (Himedia, Mumbai - India) (n=10 for five groups). Then, 100 µL of each extract including five groups Z100% (*Z. officinale* only); J100% (*J. regia* only); Z75% + J25% (Polyherbal association); Z50%+J50% (Polyherbal association) and Z25%+J75% (Polyherbal association), at concentration of 200 mg/mL, were added to the first well of each group line in the 96-well plates. Then, a serial dilution was carried out and 100µL of the bacterial inoculum was added into each well. Lastly, the plate was incubated at 37°C (with anaerobiosis for *P. acnes*).

The lowest concentration extract well with no turbidity was used to determine the MIC. To determine the MMC, aliquots were removed from the plate wells, inoculated on BHI agar and incubated for 48h at 37°C (with anaerobiosis for *P. acnes*). The lowest seeded concentration extract that did not show growth on solid medium was determined as MMC.

Antimicrobial screening on monotypic and heterotypic biofilms

Three microbial suspension of *S. aureus*, *S. epidermidis* and *P. acnes* were prepared and standardized in a spectrophotometer at a concentration of 10^7 CFU/mL. To prepare monotypic biofilms, 100 µL/well of each microbial suspension was added 96-well plates (n= 8 for each experimental group). Then 100 µL/well of BHI broth was added for microbial nutrition. The heterotypic biofilms were prepared by adding 33.3 µL/well of each microbial suspension, followed by the addition of 100 µL/well of the culture broth. The 96-well plates were incubated for 72 h at 37°C (with anaerobiosis for *P. acnes*) with the broth being changed every 24 h

Extracts application

After biofilms formation, the extracts were applied according to each experimental group for a contact time of 5 min. and 24 h, using different concentrations of glycolic extracts. For the 5 min treatment, the concentrations of 200 mg/mL and 100 mg/mL by Z100%; J100%; Z75%+J25%; Z50%+J50% and Z25%+J75% were used. For the 24 h treatment, the concentrations of 50 mg/mL and 100 mg/mL were used.

All the plates were incubated at 37°C (with anaerobiosis for the monotypic and heterotypic biofilms of *P. acnes*). BHI broth was used as a negative control and chlorhexidine digluconate (0.12% for 5 min and 0.06% for 24 h) was used as a positive control. Subsequently, the extracts were discarded and the biofilm was washed with sterile saline solution three times, to remove the dead cells. To check the viability of bacterial cells, the MTT test was used.

Biofilm viability by MTT test

After the treatments, 100 µL of the MTT solution (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Missouri - USA) was added in the plate wells and incubated for 1 hour at 37ºC (with anaerobiosis for the monotypic and heterotypic biofilms of *P. acnes*). Then, the MTT solution was removed, followed by the addition of 100 µL/well of Dimethylsulfoxide (DMSO), the plates were incubated again at 37ºC for 10 min. and then shaken on constant agitation for 10 min. Optical densities of the wells were measured by microplate reader at a wavelength of 570 nm, and the values obtained were converted to cell viability percentage.

Antimicrobial analysis by scanning electron microscopy

This analysis was carried-out over heterotypic biofilm. The microbial suspension was applied over polystyrene discs measuring 1 cm in radius and 0.5 cm in thickness, deposited within 24 well plates. The microbial suspensions of *S. aureus*, *S. epidermidis* and *P. acnes* were standardized at a concentration of 10^7 CFU/mL using a 333.33 µL/well of each microbial suspension together with 1000 µL/well of BHI broth (n = 2 for each experimental group). The plates were placed in anaerobic incubator (Whitley DG250 Workstation, UK) at 37ºC for 72 h. Then, treatments were applied using concentrations of 50 and 100 mg/mL of Z100%; J100%; Z75%+J25%; Z50%+J50% and Z25%+J75%. BHI broth was used as a positive control and chlorhexidine digluconate (0.06%) was used as a negative control. After 24 h, the extracts were discarded and the biofilm was washed with sterile saline solution (0.9%) with subsequent fixation with methanol for 1 hour. Then, samples were gradually dehydrated with ethanol 10%, 25%, 50%, 75% and 100% concentrations, and incubated at 37°C for 24 h. The polystyrene discs were placed in aluminum stubs and covered with gold for 120 seconds at 40 mA (EMITECH - SC7620, Quorum Technologies - Kent, United Kingdom), then analyzed and photographed by the scanning electron microscope (FEI - Inspect S50, Oregon, USA).

Toxicity on mouse macrophages (RAW 264.7)

Mouse macrophages (RAW 264.7) (Rio de Janeiro Cell Bank -APABCAM – RJ, Brazil) were grown in cell culture flasks (TPP, Switzerland) containing Dulbecco's modified Eagle medium (DMEM - LGC Biotecnologia, Cotia, Brazil) and supplemented with 10% fetal bovine serum (FBS) (Invitrogen, New York, USA) at 37°C and 5% CO_2 with atmospheric humidity. Viable cells were quantified by Trypan blue (0.4%, Sigma-Aldrich) and 4 x 10^4 cells/mL were transferred to the 96-well plate and cultivated with DMEM + 10% FBS and incubated for 24 h for cell adhesion.

The same extracts Z100%; J100%; Z75%+J25%; Z50%+J50% and Z25%+J75% were diluted in DMEM at concentrations of 100 mg/mL and 200 mg/mL (for 5 min application) and at concentrations of 50 mg/mL and 100 mg/mL (for 24 h application) (n=8 per group). DMEM was used as negative control (0 mg/mL). Lastly, the cells were washed with sterile 0.9% saline and sent to the MTT test.

Cell viability by MTT test

The MTT solution (100 µL/well) was added to the 96-well plate. The plate was incubated at 37ºC for 4 h. Then, the MTT solution was removed followed by the addition of 100 µL of dimethylsulfoxide (DMSO), the plate was again incubated at 37ºC for 10 min and shaken for 10 min. The absorbance of the wells was measured by spectrophotometer (Biotek -ELX 808, Vermont, EUA) at 570 nm and data generated were converted to cell viability percentage.

Mouse macrophages analysis by scanning electron microscopy

Polystyrene discs (1 cm in radius and 0.5 cm in thickness) were placed in 24-well plates. A standardized solution in the concentration of 10^4 cells/well was applied over the discs. Macrophages (RAW 264.7) were cultured for 24 h for initial adherence and subsequently were subjected to treatments for 24 h with the groups Z100%, J100%, Z75% + J25%, Z50% + J50% and Z25% + J75%, all applied in concentration of 100 mg/mL. DMEM + 10% SFB was used a negative control and chlorhexidine 0.06% as a positive control. Subsequently, the solutions were removed and the discs were subjected to the fixation process with methanol for 1 hour and then dehydrated with 5 different concentrations of alcohol (10%, 25%, 50%, 75% and 100%) applied for 20 min. The polystyrene discs were placed in aluminum stubs and covered with gold for 120 seconds at 40 mA, then analyzed and photographed by the scanning electron microscope (FEI - Inspect S50, Oregon, USA).

Statistical analysis

The data were subjected to normality test Shapiro-Wilks and for homogeneity of variance using Bartlett's test. For normal distribution, ANOVA and post-hoc Tukey were used. For anormal distribution, Kruskal-Wallis with Conover-Iman. All tests were performed by the GraphPad Prism 5.0 and Minitab 17 at a significance level $\alpha \leq 0.05$.

RESULTS

Table I represent the results obtained of tests of normality of the residuals and homogeneity of variances.

As noticed in table II, the association of the extracts demonstrated antimicrobial action against *S. epidermidis, S. aureus and P. acnes*. Against *S. aureus*, all groups of extracts obtained MIC with a concentration of 50 mg/mL. For *S. epidermidis* the groups J100% and Z75% + J25% obtained MIC with 25 mg/mL; The groups Z50%+J50% and Z25%+J75% obtained MIC with 50 mg/mL. On *P. acnes* the inhibitory concentrations varied, where the groups Z100%, J100% and Z25% + J75% obtained MIC with 12.5 mg/mL; the groups Z75% + J25% and Z50% + J50% obtained MIC with 25 mg/mL.

The MMC was obtained against *P. acnes* with a concentration of 50 mg/mL for all groups, for *S. epidermidis* the MMC was obtained with 25 mg/mL for the groups Z100%, J100% and Z75% + J25%; The groups Z50% + J50% and Z25% + J75% obtained MMC with 50 mg/mL. None of tested groups was able to promote MMC over *S. aureus*.

Table I. Normality datas.

	Raw		*P. acnes*		*S. aureus*		*S. epidermidis*	
	5m	**24h**	**5m**	**24h**	**5m**	**24h**	**5m**	**24h**
p-value	< 0.001	0.063	0.329	< 0.001	0.010	< 0.001	< 0.001	< 0.001
H0	no	yes	yes	no	yes	no	no	no
Test	Kruskal	Anova	Anova	Kruskal	Anova	Kruskal	Kruskal	Kruskal
Comp.	Conover	Tukey	Tukey	Conover	Tukey	Conover	Conover	Conover

Legend: Summary of p-value at variances homogeneity analysis based on Bartlett's test, considering confidence level of 99% (alpha equal to 0.01).

Table II. MIC and MMC promoted *by Z. officinalis* and *J. regia* extracts.

Groups	*S. aureus*		*S. epidermidis*		*P. acnes*	
	MIC	MMC	MIC	MMC	MIC	MMC
Z100%	50 mg/mL	absent	12,5 mg/mL	25 mg/mL	12,5 mg/mL	50 mg/mL
J100%	50 mg/mL	absent	25 mg/mL	25 mg/mL	12,5 mg/mL	50 mg/mL
Z75%+J25%	50 mg/mL	absent	25 mg/mL	25 mg/mL	25 mg/mL	50 mg/mL
Z50%+J50%	50 mg/mL	absent	50 mg/mL	50 mg/mL	25 mg/mL	50 mg/mL
Z25%+J75%	50 mg/mL	absent	50 mg/mL	50 mg/mL	12,5 mg/mL	50 mg/mL

Legend: MIC and MMC values of the associations of *Zingiber officinale* and *Juglans regia*. Legend: Z100% - Glycolic extract of *Z. officialis* (200 mg / mL); J100% - Glycolic extract of J. regia (200 mg / mL); Z75% + 25% - association of the glycolic extracts of *Z. officialis* (150 mg / mL) and *J. regia* (50 mg / mL); Z50% + 50% - association of the glycolic extracts of *Z. officialis* (100 mg / mL) and *J. regia* (100 mg / mL); Z25% + 75% - association of the glycolic extracts of *Z. officialis* (50 mg / mL) and *J. regi*a (150 mg / mL).

Biofilm viability by MTT test

The microbial reduction of *P. acnes* biofilm reached 46.9% and 43.9% with the group J50% + Z50% of 200 mg/mL and 100 mg/mL respectively. It also reached 45.2% and 37.7% of reduction with the group Z75%+J25% of 100 mg/mL and 200 mg/mL respectively applied for 5 min. The chlorhexidine solution promoted a 50.5% reduction in *P. acnes* biofilm, without a significant difference with the groups of Z75%+J25% of 200 and 100 mg/mL; Z50%+J50% in concentrations 200 and 100 mg/mL; J100% in concentrations 200 and 100 mg/mL; and Z25%+J75% in concentrations 100 and 50 mg/mL (figure 1).

The treatments applied for 24 h resulted in reductions of 72.9% and 74.1% with the group Z25% + J75% of 100mg/mL and 50mg/mL as shown in figure 1. The group Z75% + J25 % promoted reduction of 59.7% and 51.5% with the same concentrations. Chlorhexidine resulted in a 66.2% reduction in monotypic biofilm. All groups of extract association showed to be more effective in controlling biofilm by presenting a greater percentage reduction (figure 1).

The microbial reduction of *S. aureus* biofilm treated for 5 min. reached 23.1% with the group Z75% + J25% of 200 mg/mL, without a statistical difference ($p < 0.05$) with the chlorhexidine group. The other groups did not show a statistically significant reduction. However, the treatments applied for 24 h resulted in reduction of 79.4% with the group Z25% + J75% of 50mg/mL and a reduction of 71.8% with the group Z75% + J25% of 100 mg/mL. The group Z25% + J75% of 50 mg/mL was statistically similar ($p < 0.05$) to the chlorhexidine that exhibited the reduction and 79.4% (figure 1).

The microbial reduction of *S. epidermidis* biofilm treated for 5 min. reached 66.2% and 64.6% with the group Z100% of 200 and 100 mg/mL respectively and reached 74.6% and 66.4% with the group Z75% + J25% of 200 and 100 mg/mL respectively. Treatment with chlorhexidine promoted reductions of 74.9%, statistically similar ($p<0.05$) to the group Z75% + J25% of 200 mg/mL. However, the treatments applied for 24 h resulted in reduction of 78.6% and 61.5% with the group Z50% + J50% of 100 and 50 mg/mL respectively and a reduction of 82.0% and 80.6% with the group Z75% + J25% of 100 and mg/mL (figure 2).

The microbial reduction of the heterotypic biofilm treated for 24 h reached 85.7% and 84.3% with the group Z75% + J25% of 100 and 50 mg/mL respectively. Both concentrations were statistically equal to chlorhexidine group. The group Z100% of 100 mg/mL and 50 mg/mL promoted reduction of 87.3% and 88.4% respectively when applied for 24 h (figure 2).

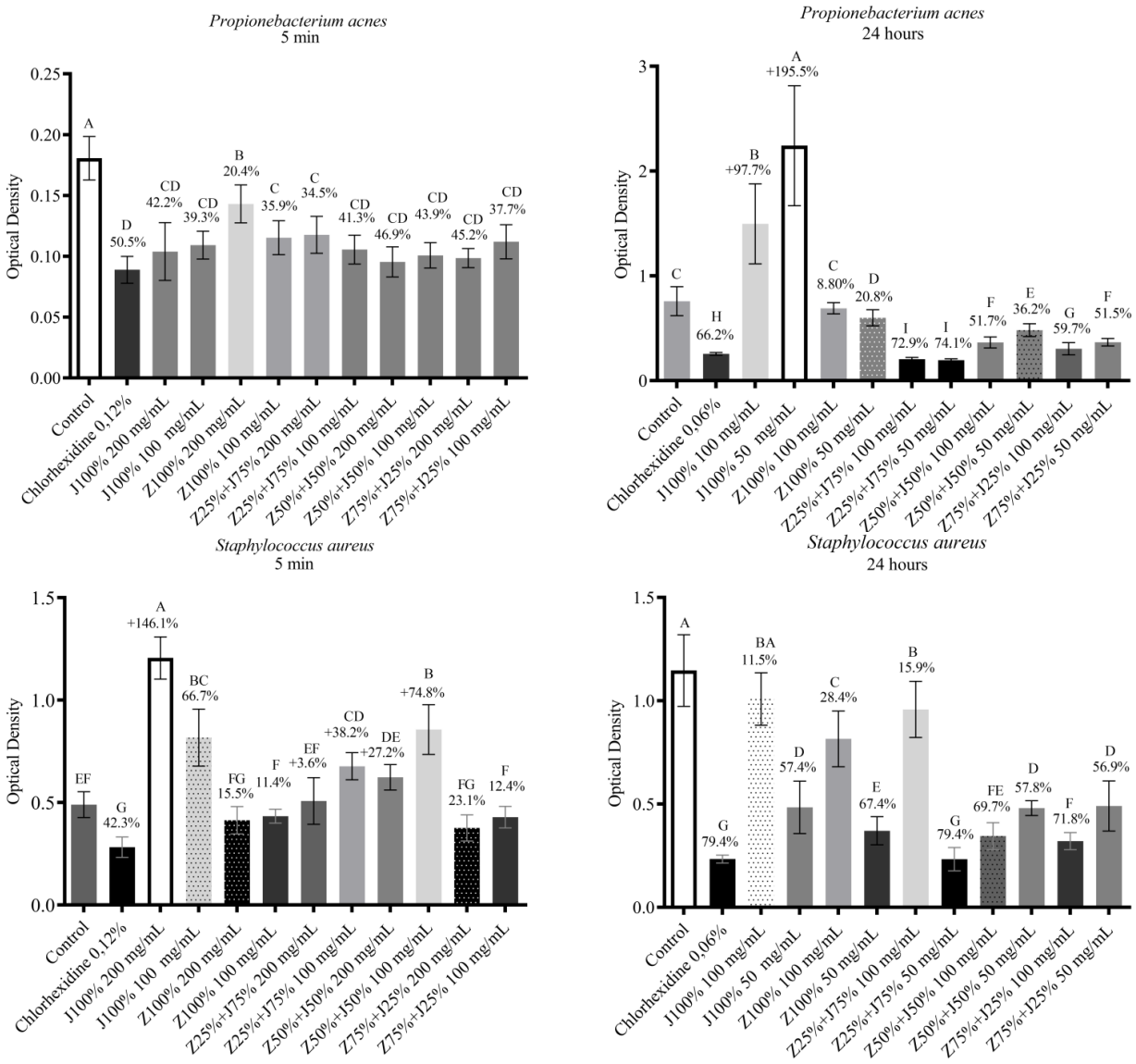

Figure 1. Anti-biofilm action of association by *Z. officinale* and *J. regia* extracts on monotypic biofilm of *P. acnes* and *S. aureus*.

SEM analysis

It was noticed that the application of the group Z50% + J50% of 100 mg/mL promoted a visual reduction in the heterotypic biofilm. However, almost a completely removal of polymicrobial biofilm was noticed with the application of the group Z75% + J25% of 100mg/mL (figure 3).

Cell viability by MTT test

For 5 min. application, it was noticed that the group J100% of 200 mg/mL promoted a reduction of 78% of the macrophage's viability and the group Z100% promoted a reduction of 68.0%. The groups of association of extracts were less cytotoxic in which the maximum reduction of cells viability was 47.3% with the group Z25% + J75% of 100 mg/mL. The chlorhexidine group promoted a reduction of 85.4% without a significant difference with the J100% group. However, the application for 24 h promoted a maximum reduction of 36.7% for isolated

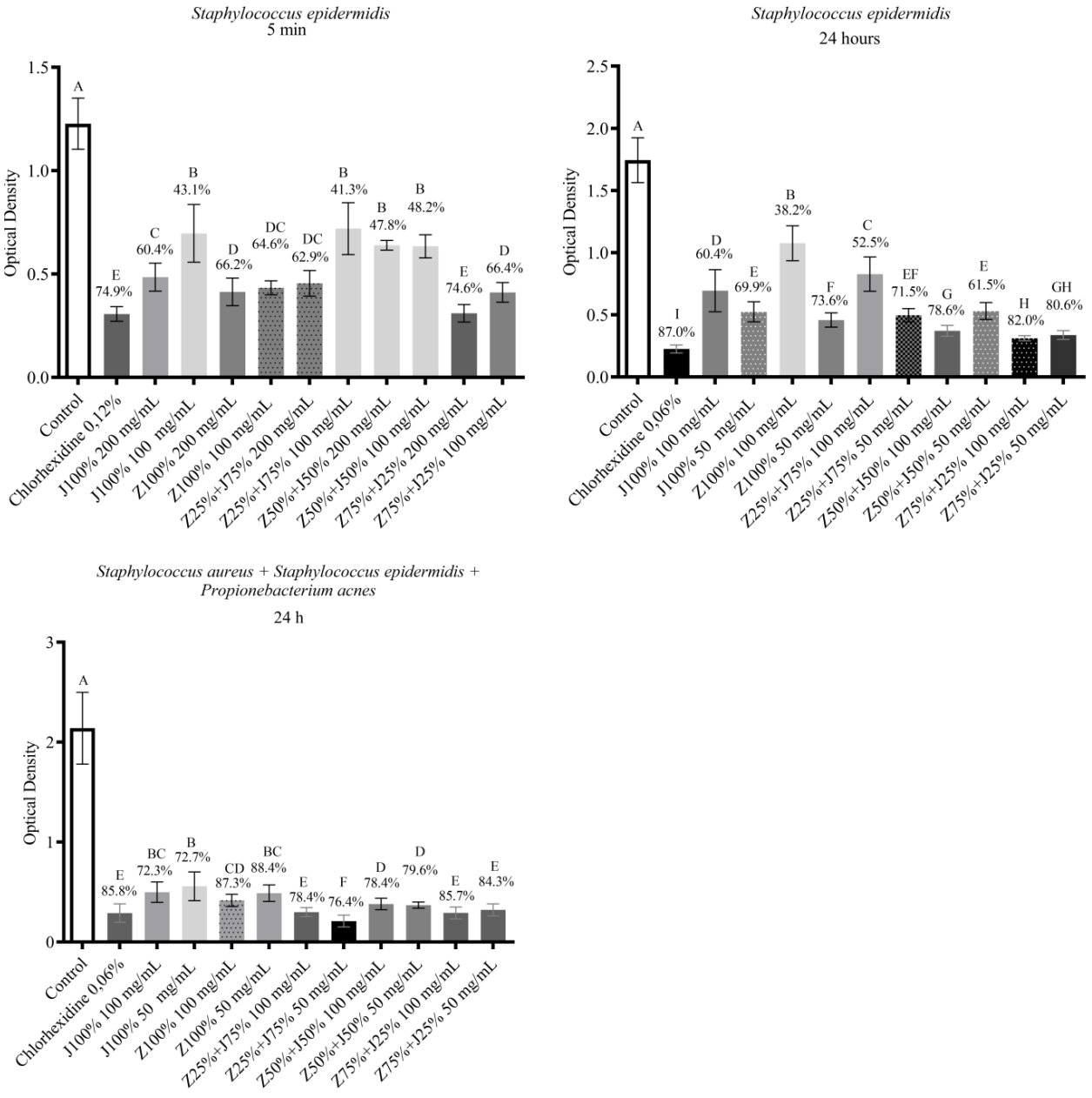

Figure 2. Antibiofilm action of association by *Z. officinale* and *J. regia* extracts on monotypic biofilm of *S. epidermidis* and heterotypic biofilm.

extracts and a maximum reduction of 14.5% for groups of associations of extracts (figure 4).

The analysis by electron microscopy showed that the polyherbic extract in the concentration of Z75% + N25% did not promote cellular alteration in the lineage of mouse macrophages, much less promoted a reduction in cell volume, showed on figure 5.

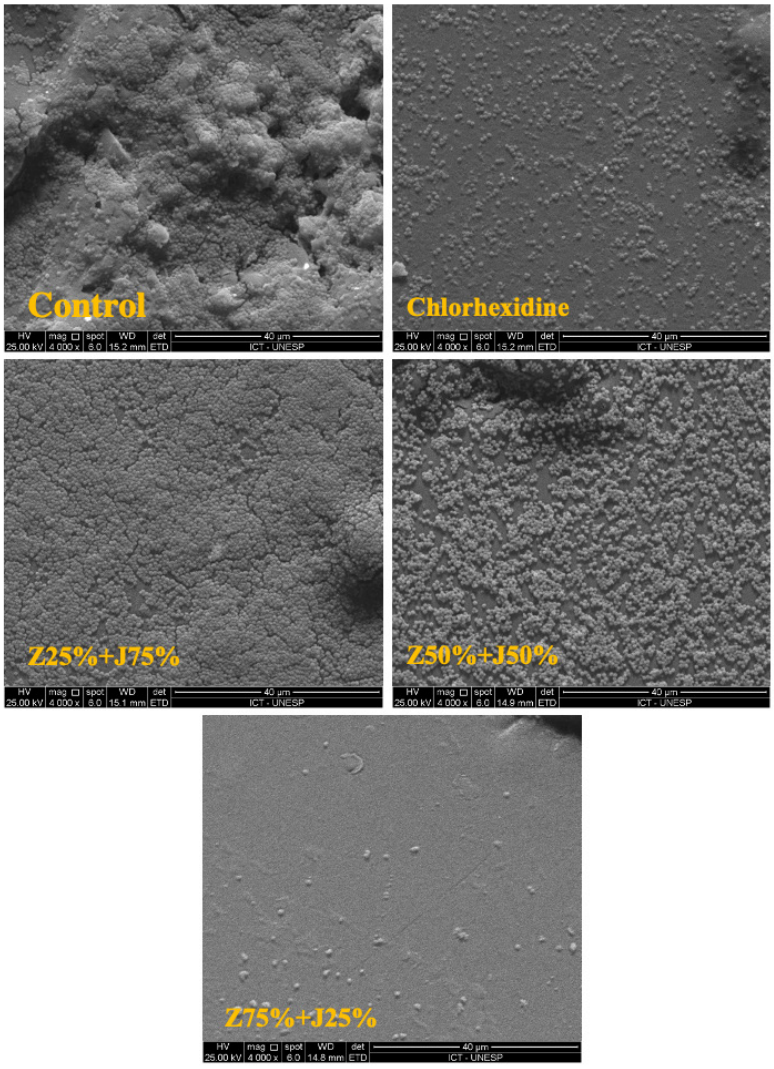

Figure 3. Antibiofilm activity of the association of extracts analyzed by scanning electron microscopy.

DISCUSSION

In the present study, the antimicrobial action of glycolic extracts of *Z. officinale* (Z), *J. regia* (J) and the associations of both of them in different concentrations on planktonic cultures and biofilms was evaluated. It was founded that these extracts have an effective antimicrobial action over *S. epidermidis, S. aureus* and *P. acnes*. In the literature, it was mentioned that the association of some extracts of *J. regia* and *Z. officinale* results in more promising outcomes (kiliç et al. 2019).

The antimicrobial action of the isolated extracts can be seen in some studies, as in the study of Qadan et al. (2005) where the alcoholic extract of *Juglans regia* (Nogueira) was applied over clinical strains of *S. epidermidis, S. aureus* and *P. acnes* to verify its antimicrobial action over planktonic cultures through the diffusion disc test. The authors tested three different concentrations of the extract (10%, 15% and 20%) where the inhibition zones were 15, 17 and 18 mm, respectively. In this study, it was founded that the extract J. regia of 100 mg/mL and 50 mg/ mL reduced the microbial load of *S. aureus* in 11.5% and 57.4%, respectively. The same extract was effective in reducing the microbial load of *S. epidermidis* to 60.4% and 69.9% at

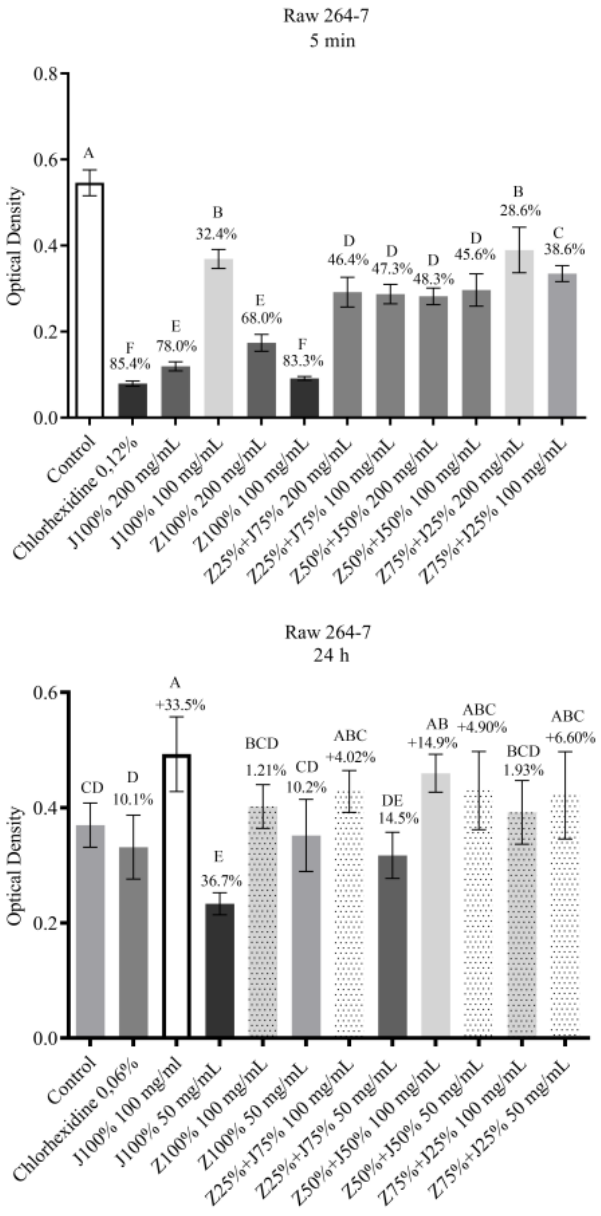

Figure 4. Analysis of cytotoxicity on mouse macrophages (RAW264.7) by MTT test.

a concentration of 100 mg/mL and 50 mg/mL, respectively. Conversely, it was not effective over *P. acnes* showing a growth of 97.7% and 195.5% with a concentration of 100mg/mL and 50mg/mL, respectively.

The study of Akintobi et al. (2013) verified the antimicrobial action of the aqueous extract of *Z. officinale* over *S. aureus* in planktonic culture using the diffusion disc test and founded 9 mm of inhibition zone. The present study founded and effective antimicrobial action of *Z. officinale* over *S. aureus*. It was able to reduce the microbial load to 67.4% inhibition at 50 mg/mL. Even more, it was effective over *S. epidermidis*, since the alone extract showed a reduction of 38.2% with the concentration of 100 mg/mL and 73.6% of reduction with the concentration of 50 mg/mL. However, over *P. acnes*, it showed a reduction of 8.80% and 20.8% with a concentration of 100 mg/mL and 50 mg/mL, respectively.

Lagha et al. (2019) evaluated the effectivity of five medicinal plant essential oils against including *Z. officinale* among others and founded that it was not effective over *E. coli* using disc diffusion and biofilm inhibitory action (by crystal violet test). Still, the same studied indicated plant extracts as good alternatives for antibiotics substitution. In the present study, the glycolic extract of *Z. officinale* showed positive results for the microorganismis tested, however the combination of the extract of *Z. officinale* and *J. regia* showed better antimicrobial action on the same microorganisms. The association of the extracts was also more effective compared to the isolated application of the extracts of *J. regians,* which showed microbial growth when tested separately on planktonic culture and monotypic biofilm of *P. acnes*. Thus it is possible to verify that the association of the extracts is more effective than the application of the isolated extracts.

The acne formation process involves different microorganisms, so a microbial complex needs to be formed for the development of the pathology, despite this, many studies evaluate the application of drugs on monotypic biofilms, with a lack of results on heterotypic biofilms. In the present study, associations of extracts on heterotypic biofilms were applied, increasing the relevance of this paper and obtaining promising results.

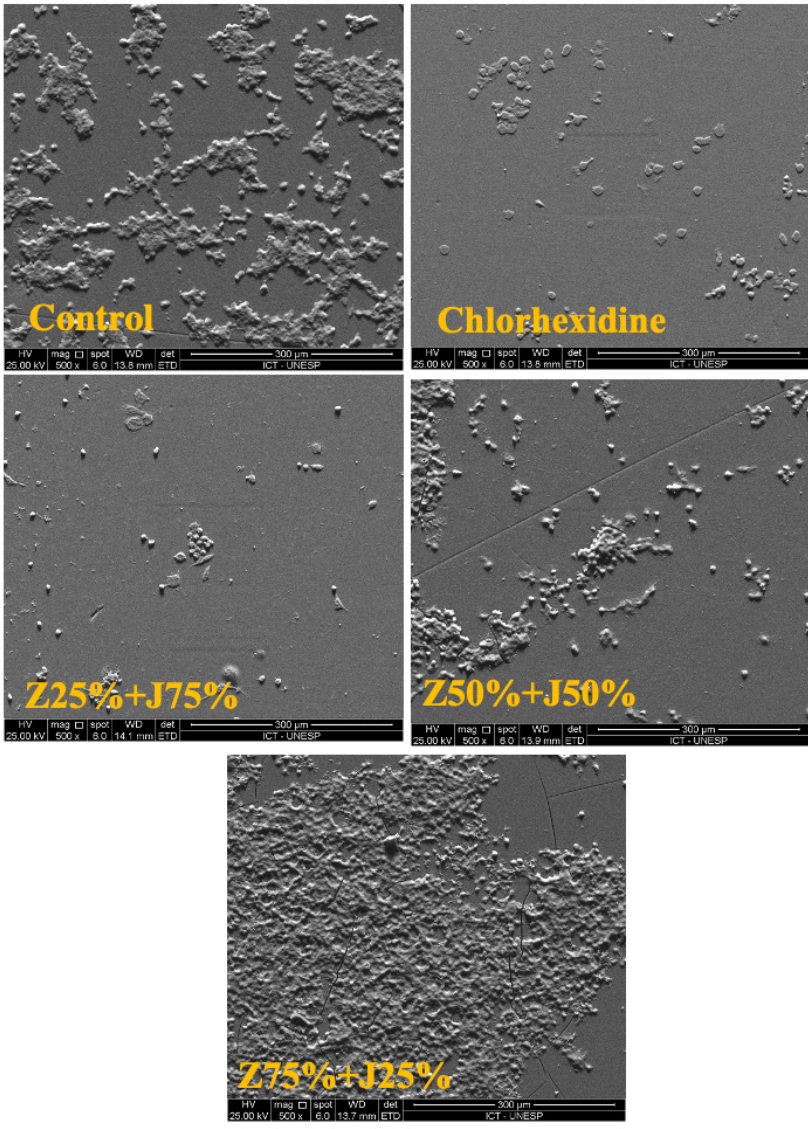

Figure 5. Analysis of cytotoxicity by scanning electron microscopy.

Isotretinoin is considered the gold standard treatment for acne, being used in cases of ineffective antibacterial treatments. Despite presenting good results, it has serious adverse effects that must have great notoriety, such as dryness of the mucosa and skin, depression and even hepatitis, in addition to being considered a highly teratogenic medication. Because of this, new therapeutic methods are used, such as plant extracts. The extracts demonstrated significant clinical efficacy in the treatment of mild and moderate acne, as demonstrated in the study by Yang et al. (2019), significantly reducing non-inflammatory and inflammatory lesions. As it is known that there are patients reluctant to treatment with antimicrobials and isotretinoin, plant extracts are a good candidate for the treatment of acne. In this present study, in addition to promoting the inhibition of pathogenic microorganisms, the association of the extracts also promoted low toxicity on mouse macrophages (RAW 264.7), where it is possible to verify that the Z75% + J25% 100 mg/mL group promoted 98.0 % cell viability, the Z100% 100 mg/mL extract promoted 98.7% viability, whereas

the Z100% 50 mg/mL group obtained 89.8% cell viability similar to the chlorhexidine group.

In view of the results obtained, the Z75% + J25% group showed better percentages of inhibition for all groups of microorganisms tested, in both treatment times (5 min. and 24 h), where for the biofilm isolated from *P. acnes* it inhibited in 5 min. 45.2% and in 24 h it inhibited 59.7%; For the biofilm isolated from *S. aureus*, in 5 min. there was 23.1% inhibition and in 24 h it inhibited 71.8%; For the biofilm isolated from *S. epidermidis*, in 5 min. it inhibited 74.6% and in 24 h, it inhibited 82.05% and finally, for the heterotypic biofilm in 24 h, the inhibition was 85.7%. Reinforcing the results, the Z75% + J25% group showed better cell viability on mouse macrophages (RAW 264.7), resulting in a maximum reduction of 1.93%, without promoting cell toxicity. Being a promising treatment.

In conclusion, the association of extracts demonstrated antimicrobial action on planktonic cultures and monotypic and heterotypic biofilms (*S. epidermidis, S. aureus* and *P. acnes*), in addition to not promoting toxicity on mouse macrophages.

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