EVALUATION OF THE SCIENTIFIC ACTIVITY OF THE LEAF OF THE SPECIES *Campomanesia pubescens* IN WOUND MODEL INFECTED BY *Staphylococcus aureus*

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Abstract

There is an increasing interest in the use of herbal remedies as healing agents, due to their lower cost in relation to other drugs and the vast Brazilian fauna. The objective of this research was to evaluate the cicatrization effect of the guariroba leaf (Campomanesia pubescens) on the healing of infected wounds. We used 45 Wistar rats, distributed in five groups (n = 9) all with surgically induced skin injury, differing in the presence of contamination and treatment, with evaluation periods of 3, 7 and 14 days, being: G1- negative control without contamination, treated with Physiological Solution 0.9%; G2- control with contamination, treated with Physiological Solution 0.9%; G3 negative with contamination, treated with Carbopol in 0.5% gel; G4- positive control with contamination, treated with Colagenase at 0.6 U / g + 0.01 g Chloramphenicol; G5- positive test with contamination treated with Campomanesia pubescens at 3%, whose vehicle was Carbopol at 0.5%. The wound was made with a metal punch 8 mm in diameter, and a cutaneous fragment was removed from the animals' backs and wound infection was applied to S. aureus in groups G2 to G5. Euthanasia was performed for a lethal dose of anesthetic, and the edges of the wounds were removed for histopathological study. The fibrinoleukocytic crust was present in all animals in the groups of 3, 7 and 14 days. The contraction of the wound was also evaluated, and all groups showed low percentage of wound regression in the 3-day treatment and with 14 days presented a high percentage of regression. Of the 5 groups, the only one that presented complete epithelialization was G5. Of the 5 groups, the ones with the best epithelialization were G4 and G5. The group with the highest amount of mature collagen fibers was G4, followed by G5, and the one with the highest proportion of immature fibers was G1. At the end of the experiment, G4 was the group that gained the most weight and G1 the one that had the lowest weight gain. Guariroba leaf extract (*Campomanesia pubescens*) was able to promote healing in infected skin wounds similar to the group treated with antibiotics.

Keywords: Healing. Herbal Medicines. Rats. Wound Infection.

1. Introduction

In Brazil, a suitable strategy to develop natural products is to exploit the therapeutic potential of native species of the different Brazilian biomes in certain situations for which existing medicines are insufficient and where available are costly and have side effects. Owing to this, and to the vast raw material available in Brazil, research opportunities in this area are numerous and pertinent to be used as

nuances for the discovery of new drugs besides ensuring the therapeutic quality and effectiveness of herbal products of popular use (Brasil 2015).

As for the evaluation of the antimicrobial activity of raw extracts of medicinal plants, tests showed that species of Brazilian flora have constituents with antimicrobial activity, such as the species of the genus *Campomanesia* (family Myrtaceae) (Cruz and Kaplan 2004). With special attention to *Campomanesia pubescens* (DC) O. Berg (Myrtaceae), known as "gabiroba pilosa", a native Brazilian species found in the Southeast and Midwest (Chang et al. 2011), whose leaves and stems are used in folk medicine to treat urinary tract infections and diarrhea. As a plant rich in polyphenols, it has been investigated for antioxidant potential, antimicrobial (Cardoso et al. 2010; Chang et al. 2011; Haminiuk et al. 2011).

In the current medical scenario, the treatment of contaminated wounds is a daily challenge, with antibiotic therapy as its major weapon. However, this has been losing its strength due to increasing bacterial resistance and superinfections. Thus, studies on phytotherapy, in an attempt to aid healing and contain infections in cutaneous wounds (Haddad et al. 2000; Muller et al. 2018).

This reality allied to the fact that the already popular use of species for the treatment of cutaneous wounds exposes the necessity of a better exploration of the subject. Therefore, the present study aimed to evaluate the efficacy in the cicatricial process of guavira leaf extract (*Campomanesia pubescens*) in an infected wound model in rats.

2. Material and Methods

The present study was a randomized clinical trial. The collection of the leaves of C. *pubescens* was carried out in areas with native vegetation of Mato Grosso do Sul (MS), in sequence were stabilized and dried in a circulating air oven 40°C, ground in a stainless steel knife mill, and stored in hermetically sealed jars protected from light and heat and labeled. For the preparation of the ethanolic extract, these sheets were used with extraction in an ultrasound bath for 60 minutes followed by maceration at room temperature, repeating until the drug was used up. The solvent was evaporated under vacuum on a rotary evaporator to give the extract. The ethanolic crude extract was incorporated into the vehicle, based on 70% Carbopol Gel.

A total of 45 Wistar male adult rats (250-300 g) were collected from colonies of the Central Biological Station of the University Anhanguera-Uniderp. The procedures were performed at the Laboratory of Toxinology and Medicinal Plants of the University Anhanguera-Uniderp. The animals were kept in the same light/dark period, at temperatures between 20°C and 24°C, in collective cages for twelve days for a period of adaptation and then placed in individual cages, with feed and water *ad libitum*. The research was authorized by the Ethical Committee on the Use of Animals of the University Anhanguera-Uniderp (CEUA 2918). After the adaptation period, the rats were distributed into five groups (n = 9) and the evaluated periods of 3, 7, and 14 days, the groups being: G1 (GWC-FS) - Negative control group without contamination, treated with Physiological Solution 0,9%; G2 (GCC-FS) - Control group with contamination, treated with Physiological Solution 0.9%; G3 (GCC-Car) - Negative group with contamination, treated with Carbopol in 0.5% gel; G4 (GCC + CC) - Positive control group with contamination, treated with Colagenase at 0.6 U / g + 0.01 g Chloramphenicol; G5 (GTC + CP) - Positive test group with contamination treated with *Campomanesia pubescens* at 3%, whose vehicle was Carbopol at 0.5%.

Before skin wound induction, the animals were weighed and anesthetized (ketamine hydrochloride and 2% xylazine hydrochloride, 50mg/kg, intraperitoneally). To induce the wound, an 8 mm diameter metal punch was used, and a cutaneous fragment was removed from the dorsum of the animals circularly in the center of the demarcated areas (1 cm²), already depilated. Hemostasis was performed by digital compression with sterile gauze for a period of two minutes. For infection of the wounds, the culture of the *Staphylococcus aureus* bacterium was previously carried out in Agar with subsequent dilution. A sterile suture line was immersed in this content and the container was placed in a homogenizer during the subsequent sedimentation within 30 min. Subsequently, the suture line contaminated in the groups established as contaminated was superimposed on two opposite ends of the wound (with the animals immobilized on a paraffin plank), and the group established as uncontaminated was exposed to the fixation of the line free of S. aureus. Soon after the procedure, and then with daily frequency, all animals received the treatment by topical application on the wound, according to the inclusion group. According to

the evaluation periods, the euthanasia of the animals (3 of each group on the 3rd, 7th, and 14th days) was performed by administering a lethal anesthetic dose (100mg/kg). After euthanasia, skin samples containing the center and the edges of the lesion (with a distance of 1 cm from the skin beyond the lesion and without reaching the musculature) were removed through a section with a scalpel blade number 15. The skin samples were identified and fixed in 10% buffered formalin and afterward, the material was processed in increasing concentrations of alcohol, diaphanized in xylol, and included in histological paraffin. From the skin samples, 5 μm thick cross-sections were made with the aid of a rotating microtome. The sections obtained were stained by the technique of Hematoxylin-Eosin (HE) and Picrosirius Red. The images of the slides stained with HE were captured in a Carl Zeiss photomicroscope coupled to a Samsung micro-camera connected to a computer with an image capture board.

Histological analysis of the stained slides with Hematoxylin-eosin (HE) showed tissue repair according to the treatment period. For the treatment of 3 days, the following parameters were considered: inflammatory process, neovascularization, presence of crust, hemorrhage, and epithelization. Already for the treatment of 14 days were considered: epidermal crests, lesion size, attachments, and epithelium.

The histological results were grouped qualitatively, using a scale of symbols, being: 0 absent; + mild; ++ moderate and +++ intense; for crust was determined whether absent (0) or present (+); for lesion size: + small, medium ++ and large +++; for the epithelium: + fine, ++ normal and +++ thick (Shimizu et al. 2009). This histological classification will allow the analysis of the inflammatory/healing process, as well as the comparison with similar publications that used the same evaluation.

A spreadsheet was created to record data on the characteristics of the animals used in each group.

Measurements of the variables of the wound regression and histological analysis were expressed as mean ± standard deviation. The results were tabulated to observe the disposition of the values regarding the evaluation of the wound areas between the groups and periods analyzed. The normality analysis was done using the Shapiro-Wilk test.

Intragroup comparisons were performed using the Student t-test for related samples in the normal distributions and the Wilcoxon test in the non-normal distribution samples.

The magnitude of inter-group variation was assessed through the Analysis of Variance (ANOVA) with Tukey's post hoc test in the normal distribution samples and the Kruskal-Wallis test with Dunn's post hoc test in the non-normal distribution samples.

Factorial type ANOVA was used to test in vitro antibacterial activity and ANOVA followed by the Tukey test for analysis of total phenolic and flavonoid data.

Comparisons with $p < 0.05$ were considered statistically significant. Data were tabulated using Microsoft Office Excel 2010 software and statistical analysis was performed with the GraphPad Prism 4 program.

Measurements were expressed as mean and standard deviation. For comparison between groups, Variance Analysis (ANOVA) with post hoc Bonferroni test was used. The ignificance level was considered $p < 0.05$. The data were organized in the software Microsoft Excel 2013, and the analyzes performed using the program GraphPadPrism 5.

3. Results

The fibrinoleukocytic crust was present in all animals in the groups of 3, 7, and 14 days. In the group of 14 days, the presence of crust was not observed from the twelfth day.

The weight of the animals was also evaluated and figure 1 and table 1 show the variation of the weight in grams that the animals had during the experiment.

It can be seen in figure 1 that in the 7-day experiment, only the G4 group gained weight after treatment and the G1 group lost the most weight. In the 14-day experiment, the G2 and G4 groups were the ones that gained the most weight, and the G1 group had the lowest weight gain, differing from the G2 and G4 groups, which obtained the highest weight gain (Figure 1).
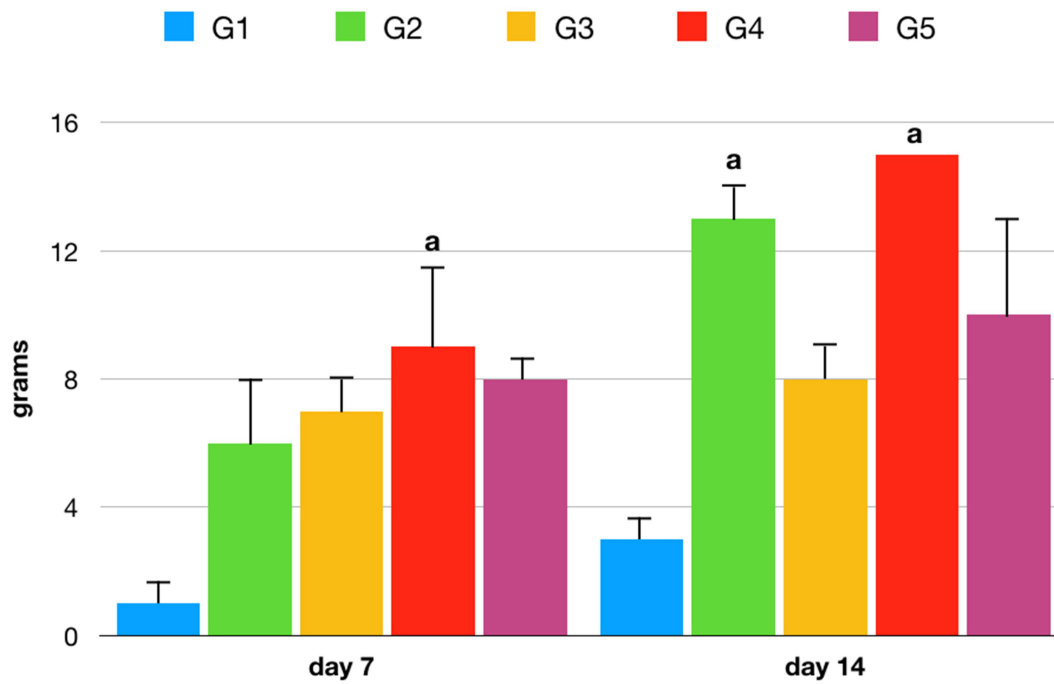
Figure 1. Comparison between the weight changes of the animals at 7 and 14 days
("a" p <0.05 in relation to group 1).

Figure 1 shows that in the 7-day experiment, group G4 gained the most weight after treatment and G1 the least gained. These results persisted in the 14-day experiment, in which groups G2 and G4 gained the most weight and group G1 remained with the lowest gain.

Table 1. Distribution of mean values and standard deviation of weight variations (in grams) and wound regression for the different groups and times observed.

	Group 1	Group 2	Group 3	Group 4	Group 5	p-value
WEIGHT						
7 days	0.7 ± 0.6	6.0 ± 2.6	6.7 ± 2.3	9.0 ± 5.0	7.7 ± 1.5	0.0392
14 days	2.3 ± 1.5	13.7 ± 3.5	8.7 ± 3.1	15.0 ± 0.0	10.0 ± 6.2	0.0108
WOUND REGRESSION						
3 days	0.16 ± 0.06	0.02 ± 0.00	0.79 ± 0.64	0.87 ± 0.64	1.360.80	0.3821
7 days	1.13 ± 1.05	1.50 ± 0.43	0.92 ± 0.19	5.16 ± 2.99	2.97 ± 0.69	0.0224
14 days	3.42 ± 0.38	2.62 ± 0.03	2.65 ± 0.48	3.22 ± 0.52	2.89 ± 0.07	0.0228

The contraction of the wound was also evaluated because it indicates how the healing process is. For the contraction of the wound, the regression percentages were calculated in the treatments of 7 and 14 days (Figure 2).

All groups presented a low percentage of wound regression in the treatment of 3 days and with 14 days presented a high percentage of regression, which characterizes the two phases of healing, the initial inflammatory phase in the treatment of 3 days and from 14 days to stage of maturation of the wound called remodeling.
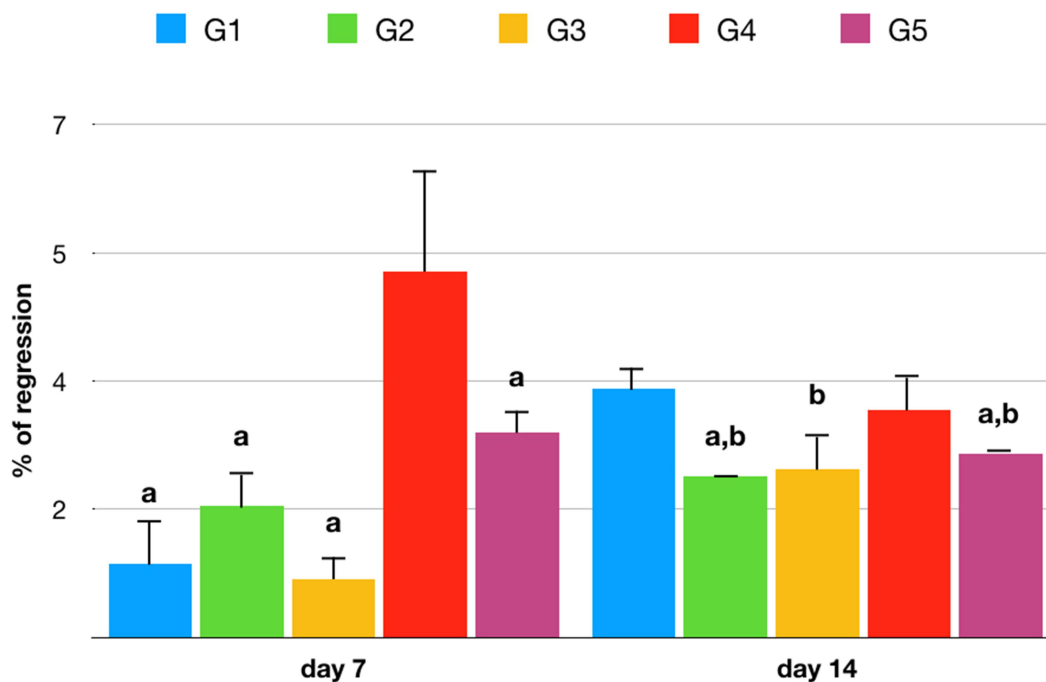
Figure 2. Comparison between the percentages of wound regression at 7 and 14 days ("a" $p < 0.05$ in relation to group 4; "b" $p < 0.05$ in relation to group 1).

The evaluation of type I and III collagen concentration in the evaluation periods of 3, 7, and 14 days is shown in table 2. Figures 3 to 5 show comparisons of types of collagens in different periods.

Table 2. Comparison between the percentages of type I and III collagens between the groups in the analyzed periods.

	Type I collagen (%)					
	G1	G2	G3	G4	G5	p-value
3 days	3.58 ± 0.83	5.50 ± 0.28	3.41 ± 0.9	3.68 ± 3.53	11.20 ± 5.82	0.0137
7 days	1.49 ± 0.67	2.54 ± 0.46	2.57 ± 0.18	4.20 ± 5.85	2.39 ± 0.3	0.3565
14 days	3.13 ± 1.0	3.93 ± 2.37	2.33 ± 0.63	6.52 ± 0.87	4.16 ± 0.77	0.0313
	Type III collagen (%)					
	G1	G2	G3	G4	G5	p-value
3 days	0.66 ± 1.38	3.02 ± 0.26	0.43 ± 0.12	0.72 ± 1.26	0.37 ± 0.19	0.0550
7 days	0.33 ± 0.16	1.71 ± 0.16	0.40 ± 0.28	0.21 ± 0.55	0.54 ± 0.10	0.0791
14 days	6.14 ± 4.7	0.32 ± 0.15	0.22 ± 0.23	1.08 ± 0.63	0.39 ± 0.32	0.0154

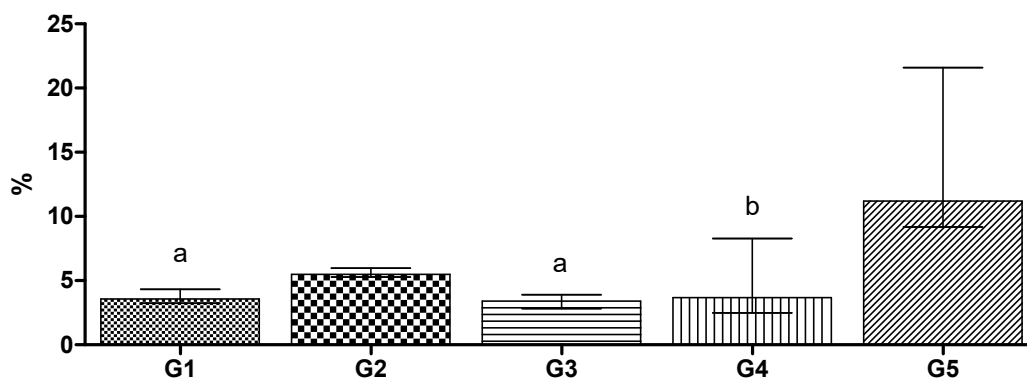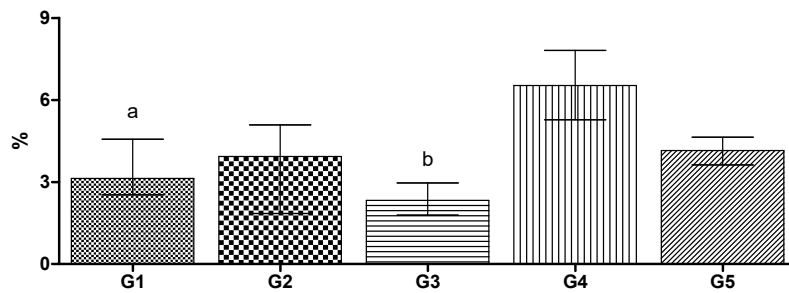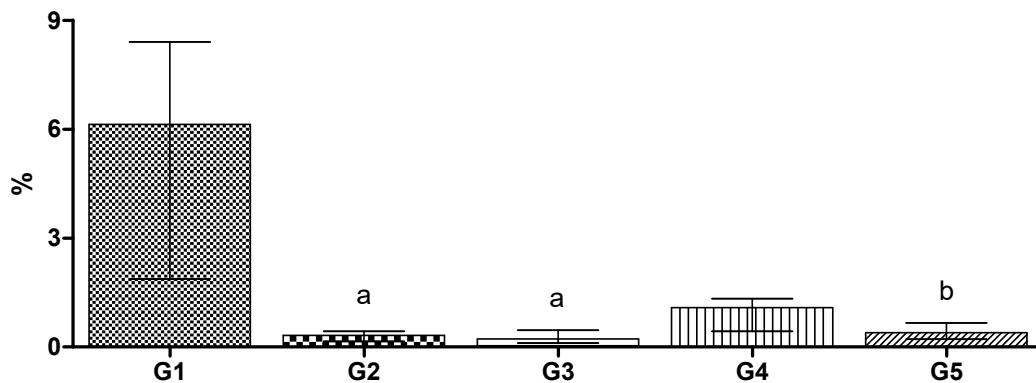*Kruskal-Wallis*, median ± interquartile range.

Figure 3. comparisons between the quantification of type I collagen between the groups evaluated at 3 days of experiment (Kruskal-Wallis, $p = 0.0137$, "a" $p < 0.01$ in relation to G5; "b" $p < 0.05$ in relation to G5).

Figure 4. Comparisons between the quantification of type I collagen between the groups evaluated at 14 days of experiment (Kruskal-Wallis, p=0.0313, "a" p<0.01 in relation to G1, "b" p<0.05 in relation to G1).

Figure 5. Comparisons between the quantification of type III collagen between the groups evaluated at 14 days of experiment (Kruskal-Wallis, p=0.0154, "a" p<0.01 in relation to G1, "b" p<0.05 in relation to G1).

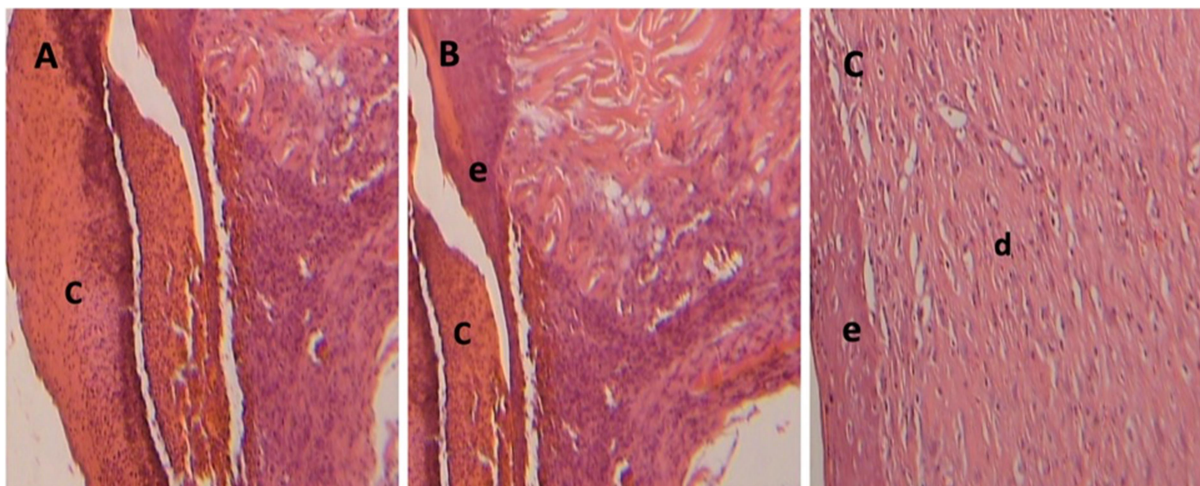Figures 6 to 10 demonstrate the main histological findings of the groups studied.

Figure 6. Photomicrography of Negative control group without contamination treated with Physiological Solution. A – 3 days, with fibrinoleukocytic crust (C); B – 7 days, with fibrinoleukocytic crust (C) and initiation of epithelization (e); C – 14 days, with fine epithelium already formed.
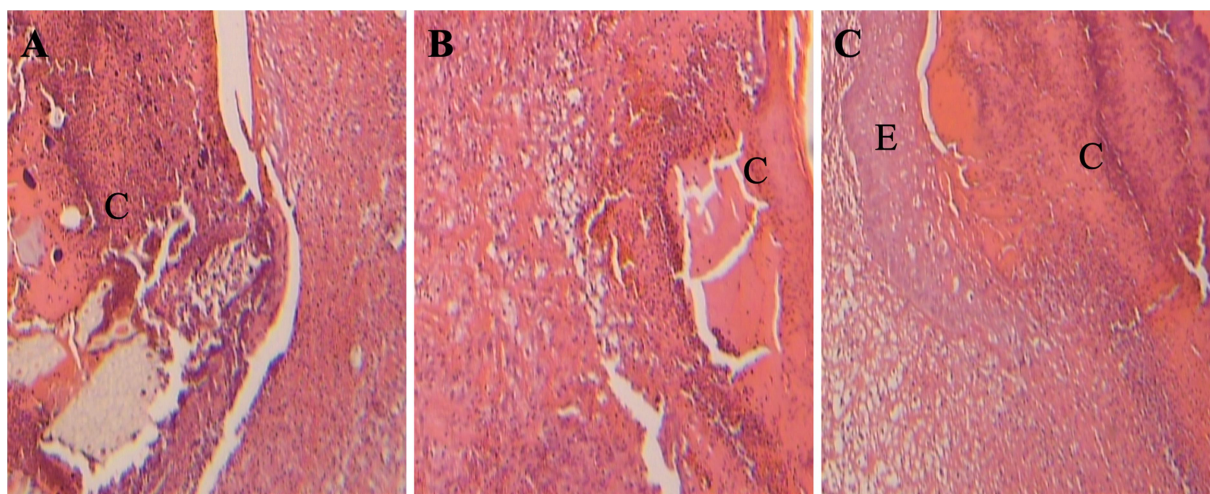Crosta (C); epiderme (e); derme (d) (HE 10x).

Figure 7. Photomicrography of Negative control group with contamination treated with Physiological Solution. A – 3 days, with fibrinoleukocytic crust (C) without signs of epithelization; B – 7 days, with fibrinoleukocytic crust (C) without signs of epithelization; C – 14 days, with primordial epithelium (E) (HE 10x).
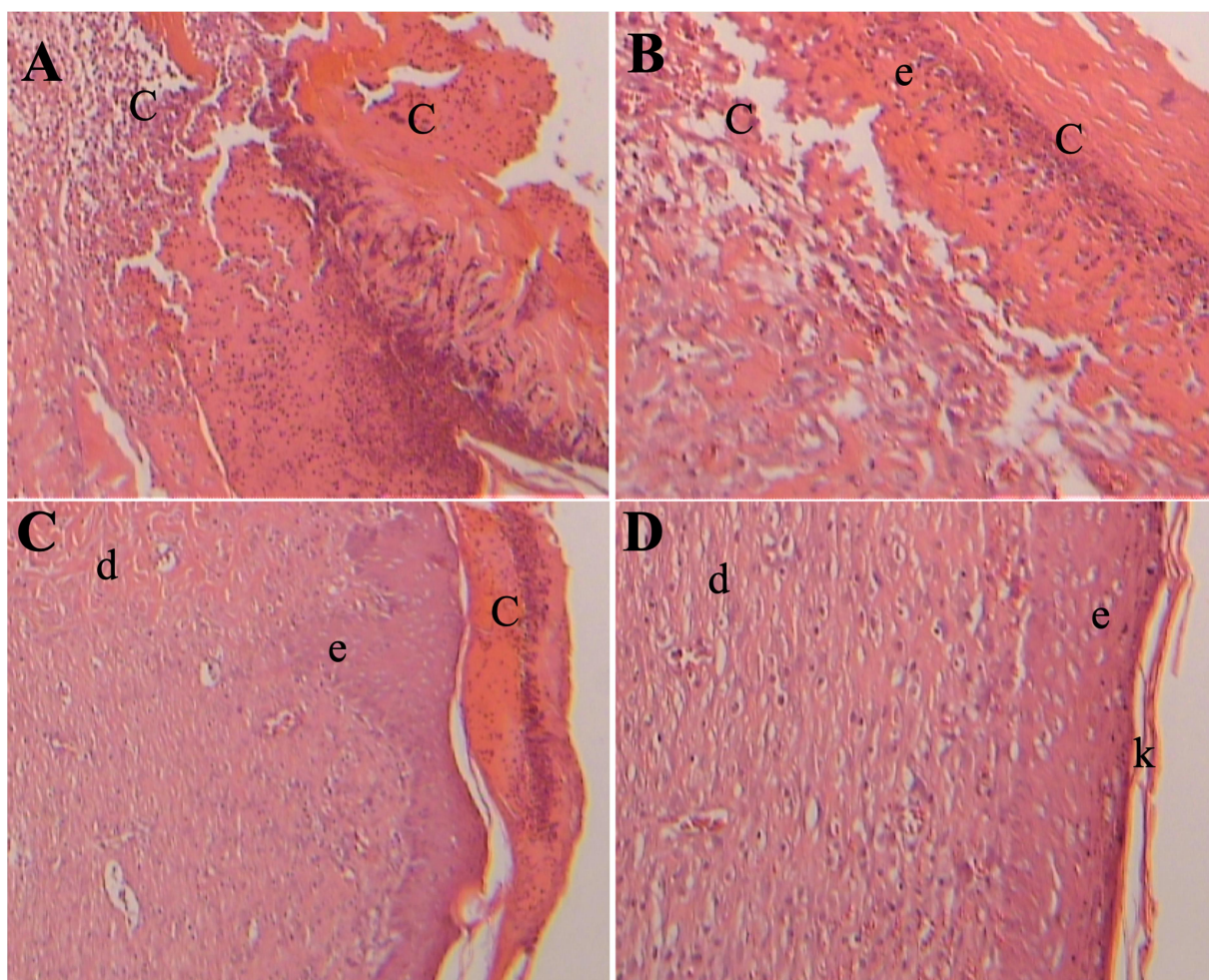

Figure 8. Photomicrography of negative group with contamination treated with Carbopol gel in 0.5%. A – 3 days, with thick fibrinoleukocytic crust (C) without beginning epithelization (e); B – 7 days, with thick fibrinoleukocytic crust (C) without beginning epithelization (e); C – 14 days, with fine epithelium formed (e) and at some sites of fibrinoleukocytic crust (C) on the surface of the epithelium remains; D – 14 days, with epithelium formed with all layers and above fine keratin (k) (HE 10x).
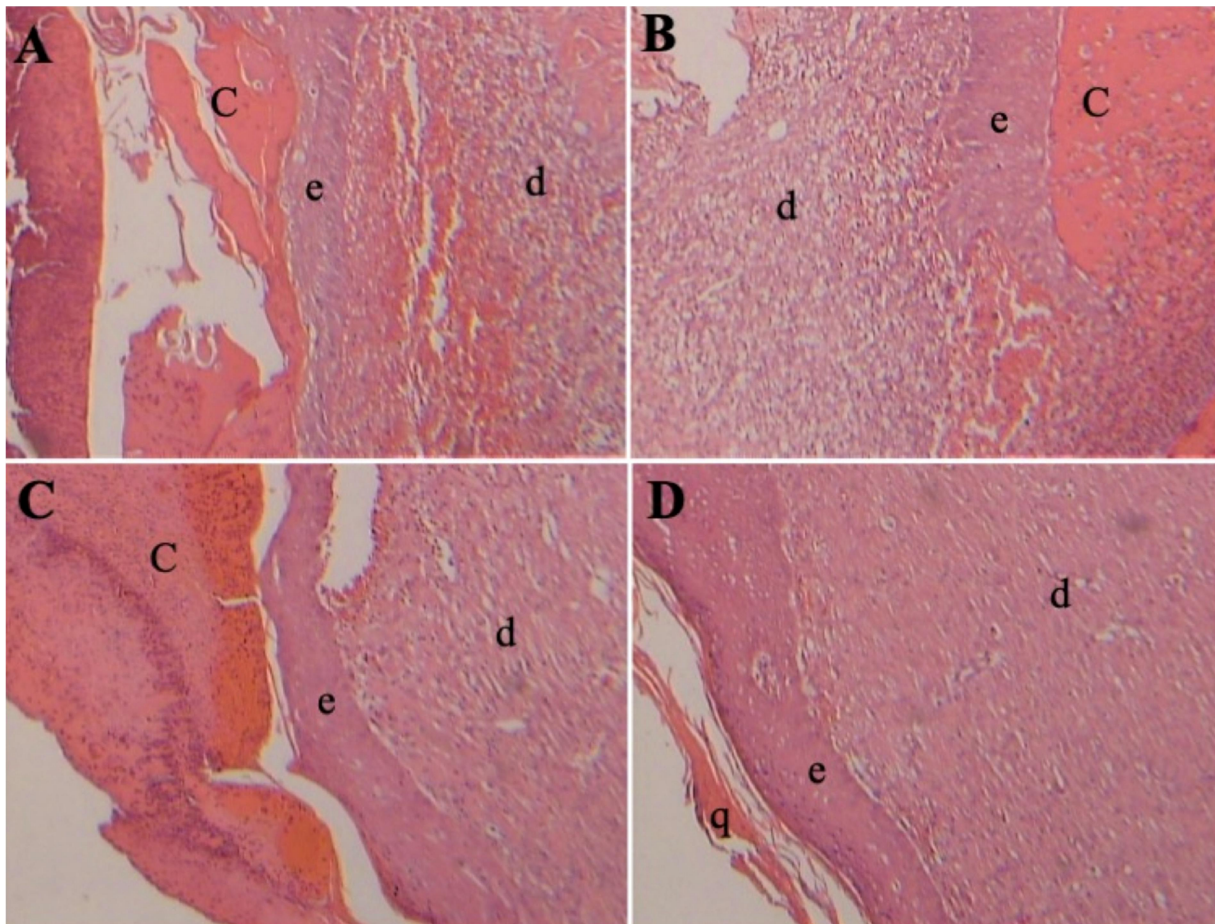
Figure 9. Photomicrographs of the positive control group treated with Colagenase at 0.6 U / g + Chloramphenicol at 0.01g. A – 3 days, with thick fibrinoleukocytic crust (C) and initiation of epithelization (e); B – 7 days, with thick fibrinoleukocytic crust (C) and initiation of epithelization (e); C – 14 days, with thin epithelium proliferating on the surface of the epithelium (e) with fibrinoleukocytic crust (C); D – 14 days, with epithelium formed with all layers and above fine keratin (k) (HE 10x).
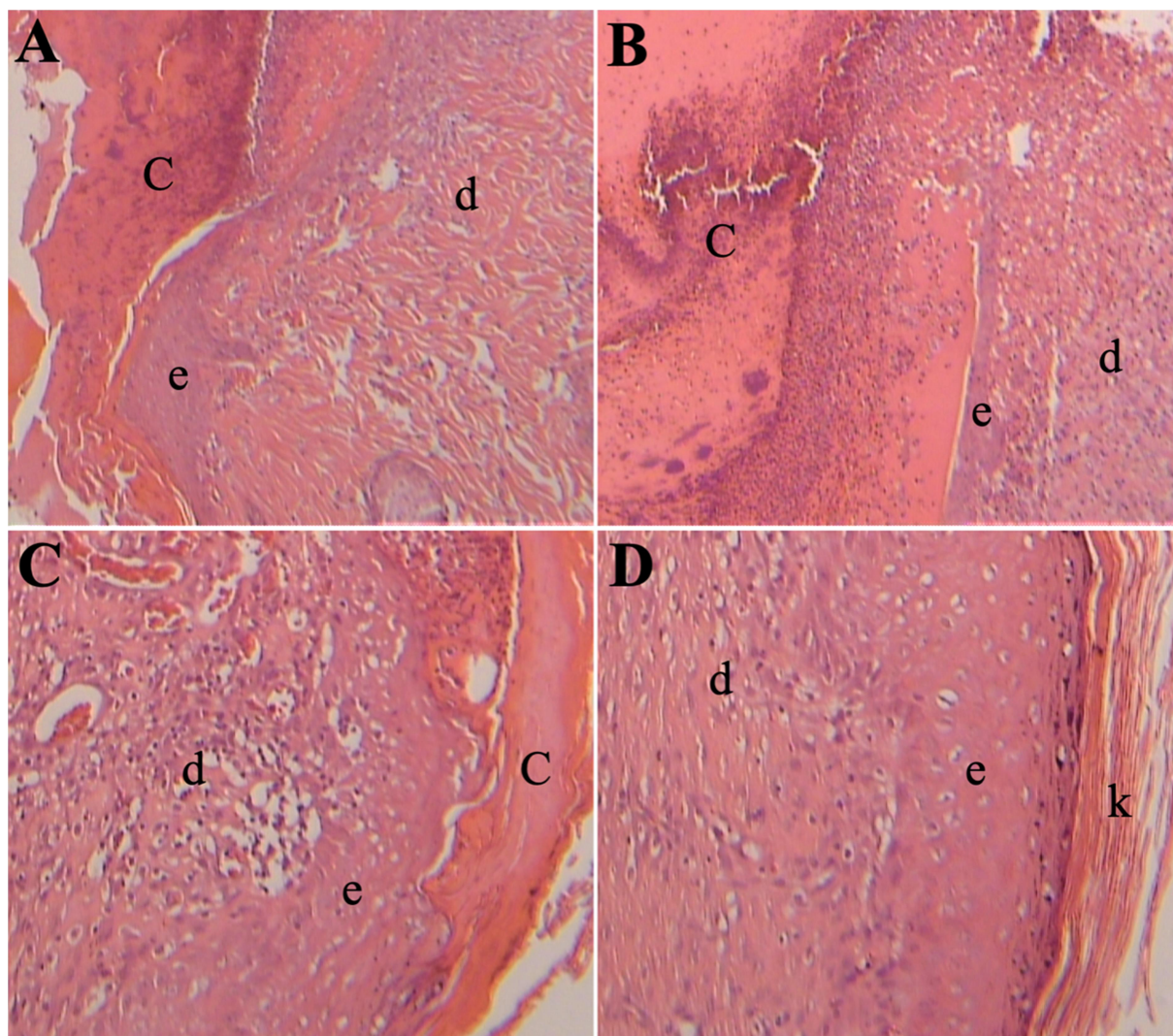
Figure 10. Photomicrographs of positive test group with contamination treated with Campomanesia ssp at 3%. A – 3 days, with thick fibrinoleukocytic crust (C) and beginning of epithelization (e); B – 7 days, with thick fibrinoleukocytic crust (C) and beginning of epithelization (e); C – 14 days, with thick epithelium proliferating (e) and at some sites on the epithelial surface (e) with fibrinoleukocytic crust (C); D – 14 days, with epithelium formed with all layers and above thick keratin (HE 10x).

Figures 11 and 12 represent the images captured from the histological sections stained by the picrossirius-red technique. With 3 days of the experiment, the group with the highest amount of mature collagen fibers was G5, but with 14 days of treatment, G4 was the group in which mature collagen predominated, followed by G5. The group with the most immature fibers at the end of the experiment was G1.
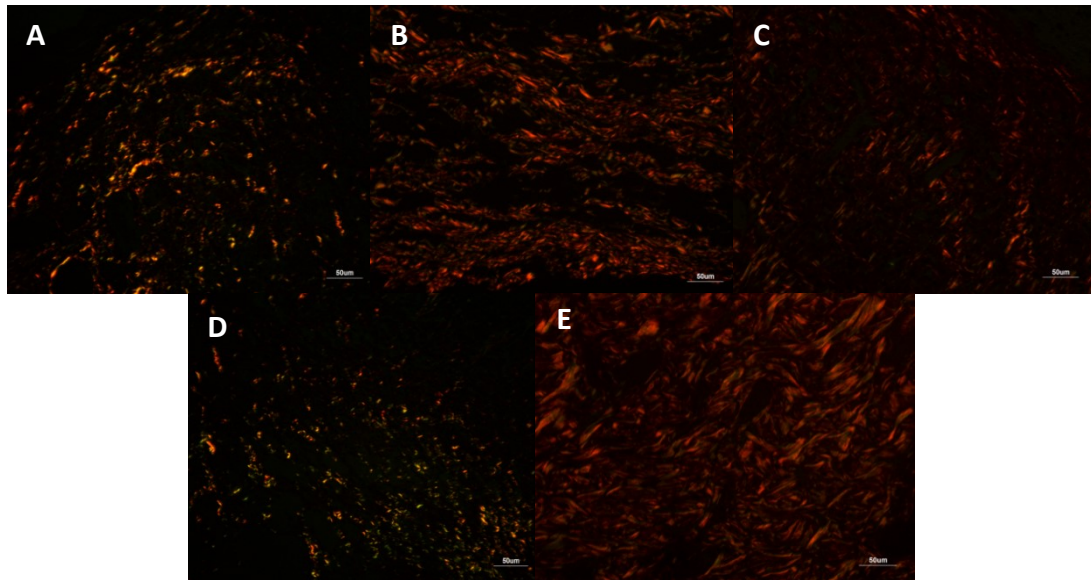
Figure 11. Photomicrograph of the groups with 7 days of experiment. A – (G1) negative control without contamination, treated with 0.9% Saline Solution; B – (G2) contamination control, treated with 0.9% Saline Solution; C – (G3) negative with contamination, treated with Carbopol in 0.5% gel; D – (G4) positive control with contamination, treated with 0.6 U / g Collagenase + 0.01 g Chloramphenicol; E – (G5) positive test with contamination treated with 3% Campomanesia pubescens, whose vehicle was 0.5% Carbopol.

Figure 12. Photomicrograph of the groups with 14 days of experiment. A – (G1) negative control without contamination, treated with 0.9% Saline Solution; B – (G2) contamination control, treated with 0.9% Saline Solution; C – (G3) negative with contamination, treated with Carbopol in 0.5% gel; D – (G4) positive control with contamination, treated with 0.6 U / g Collagenase + 0.01 g Chloramphenicol; E – (G5) positive test with contamination treated with 3% Campomanesia pubescens, whose vehicle was 0.5% Carbopol.
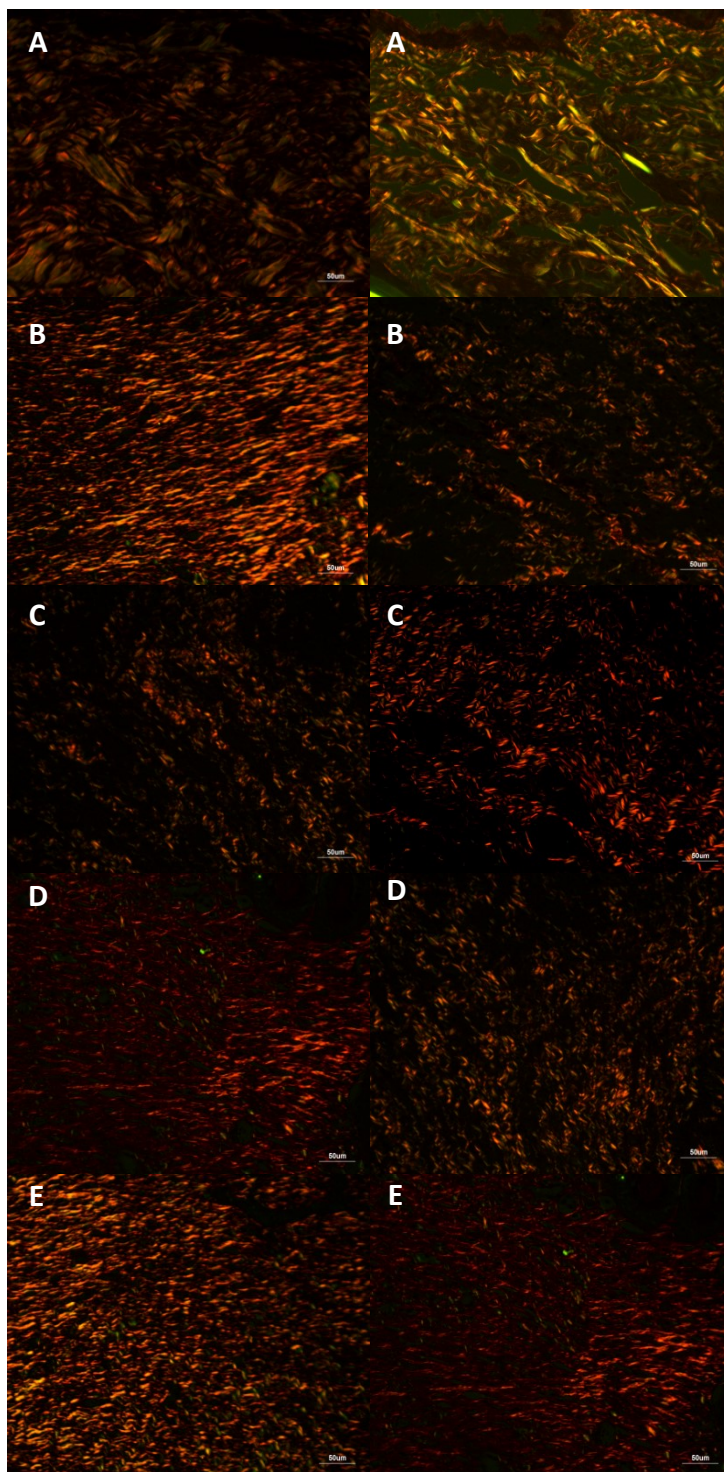
4. Discussion

The genus *Campomanesia* of the family *Myrtaceae* of popular name guavira originates from Brazil, and of great abundance in the cerrado region. Its leaves and fruits have some medicinal properties like anti-inflammatory, antidiarrheal, and antiseptic of the urinary tracts, and the leaves are also used in the treatment of influenza. These actions are due to the presence of flavonoids (chemical studies with leaves of the species reported the presence of myricetin, myricitrin, rutin, and quercitrin in *C. pubescens*), components that can be related to several of the medicinal activities described for the plant, since have

shown proven anti-inflammatory and anti-inflammatory actions, factors related to their use as antidiarrheal, antiseptic, and cicatrizant (Ramos et al. 2007; Cardoso et al. 2013).

Although these beneficial effects, of the action of flavonoids, have already been demonstrated in numerous publications (Corrêa et al. 2013; Carmignan et al. 2020), the present research demonstrates a different approach in which it demonstrated the efficacy of the plant in both wound healing and infection, since the infected wound group treated by *C. pubescens* presented better results than the control groups.

One of the variables analyzed was the animals' weight during the experiment (Table 1). The literature demonstrates that several situations may contribute to the weight loss or decrease in the weight gain of the animals, among them the infection. Considering that all the animals had access to water and food without restriction, they were exposed to the same treatment conditions, having as differences only the experiment performed, it is demonstrated that the weight variation can be used as a parameter of better or worse response to treatment (Tazima et al. 2008).

Thus, the finding that the G4 group obtained the highest weight gain demonstrates that local control of topical antibiotic infection established in the literature and commonly used in clinical practice may have been determinant for this fact. However, at the end of the experiment (14 days) the group G2, contaminated and treated with saline, presented weight gain similar to G4, which may reflect the interference of other variables not subject to control and measurement, such as the acceptance of diet by each animal, the degree of wound colonization by the bacteria of the induced contamination, the animal's microbiota and the environment, as well as the level of systemic involvement by the local infection (Goldim 2012).

These considerations, although relevant, do not guarantee a direct evaluation of the action of the tested treatments. In contrast, wound healing, assessed by contraction and histopathological features of the lesions, is a direct and objective means of evaluating the response to *Campomanesia pubescens* in the treatment of infected skin wounds.

All groups presented a low wound regression percentage in the 3-day treatment and at 14 days showed a high regression, a finding that reflects the two healing phases, the initial inflammatory phase in the 3-day treatment, and from 14 days the maturation of the wound called remodeling. These processes are highly influenced and dependent on intrinsic factors of the affected organism (immunological competence, nutritional status, comorbidities) and external variables (environmental hygiene condition, treatment instituted, presence of infection (Tazima et al. 2008). Of the variables mentioned, the emphasis of the evaluation were the treatments that, in this analysis did not show any difference between the groups.

In the captured images it can be observed that of the five groups, the only one that presented complete epithelialization, despite the contamination, was the one that received treatment with *Campomanesia pubescens*. In this group (G5), the epithelium presented all the epithelial layers formed, besides the thick keratin layer, the main difference to the others. Such guavira properties, combined with the efficacy of the vehicle used (greater tissue penetration), probably favored a more effective healing process, making healing a more harmonic event in all its complexity.

Cardoso et al. (2010) had already suggested an antimicrobial potential of *Campomanesia pubescens* when analyzing its components and identifying 34 volatile components and flavonoids, which would be responsible for action against pathogens. The safety of this herbal remedy seems to be comparable to the drugs already used in the treatment of infected wounds. In the present research, there were no deaths in any group and, although the weight gain was higher in the G4 group, the one treated by *Campomanesia pubescens* was not inferior to the others. In recent research on the toxicity of this herbal remedy, Villas Boas et al. (2018), are shown to be safe enough to be tested in humans under conditions similar to those in which the animals were exposed.

Another method used in the experiment to expand the analysis of the results was the technique of histological staining with picrossirius-red. It is a selective staining of connective tissue that allows a qualitative analysis of collagen fibers, which allows differentiation mainly of type I and type III fibers when observed under polarized light (Bedoya et al. 2016). Thus, different shades are observed according to the type of molecular arrangement present. Collagen I (mature) fibers are thick and orange in color and those of collagen III are thin and yellow-green in color (Costa et al. 2017).

This coloring has been used in the evaluation of skin healing, as it allows correlating the quality and quantity of collagen with the success of the healing process (Lemos 2011). The healthy dermis contains approximately 80% type I collagen and 20% type III collagen, however, the granulation tissue of the healing process expresses 30% to 40% type III collagen, being considered immature collagen. What happens is that the collagen produced initially (type III), in the inflammatory phase (up to 3-7 days), is thinner and overtime is reabsorbed and a thicker collagen (type I) is produced, starting this process at proliferative phase (7-14 days) and extending to the remodeling phase (14 days - 3 months) (Campos et al. 2007).

What was observed in the present study is that with 7 days of experiment the group treated with the extract of *C. pubescens* (G5) was the one that presented the greatest amount of type I collagen. However, with 14 days the group with the predominance of mature collagen fibers was the "gold standard" treatment group, with collagenase and chloramphenicol (G4), followed by the G5 group. In addition, it was shown that at 14 days, the negative control group (G1) had a greater amount of type III collagen, that is, it was still at an early stage of the remodeling process.

In his thesis on the evaluation of skin healing for forensic purposes (determining the time of evolution of the wounds), Goldim (2012) presented results demonstrating the predominance of immature collagen until the 14th day of the evolution of the lesions, and only after the 20th day there was an inversion of the proportion. In the results of the present study, this proportion was observed only in the negative control group (G1), a fact that can be justified by the contamination and different treatments in the other groups since the groups treated with antibiotics (G4) and with ethanolic extract of *C. pubescens* were the ones with the highest amount of mature collagen.

In a study by Ribeiro et al. (2015), who analyzed the concentration of collagen types I and III in the repair of uncontaminated surgical wounds treated with Mitomycin-C in rats, the results obtained represent later stages of healing, and the analyzes are made of samples with 30 and 60 days of the experiment, that is, a clear remodeling phase, in which the expected according to the literature for this period of evolution was demonstrated, the predominance of mature collagen in both study groups.

The finding of the best healing of G5 in relation to G4 is a finding that should be valued and certainly deepened in future research since it has demonstrated the superiority of *Campomanesia pubescens*, a low cost phytotherapeutic and widely available in our flora, in comparison to topical antimicrobials widely used in daily clinical practice and which are of high cost, in addition to what has been widely reported in research in the field of infectology, is increasing the rate of bacterial resistance to the drugs in use. Thus, in confirming in other research the results obtained here, *Campomanesia pubescens* could be used with the advantage of lower cost and as an option in cases of bacterial resistance.

The finding of a similar cure of G5 in relation to G4 is a result that should be valued and certainly deepened in future research, as it demonstrated a good performance of *Campomanesia pubescens*, a low-cost herbal medicine widely available in our flora, in comparison with antimicrobials topics widely used in daily clinical practice and high cost, in addition to what has been widely reported in research in the field of infectious diseases, are increasing the rate of bacterial resistance to drugs in use. Thus, when confirming in other research the results obtained here, *Campomanesia pubescens* could be used with the advantage of lower cost and as an option in cases of bacterial resistance.

5. Conclusions

The ethanolic extract of *Campomanesia pubescens* was able to accelerate the healing process of cutaneous wounds in rats infected with *S. aureus* in comparison to topical antimicrobials.

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