

Full Length Research Paper

***Genipa americana* fruit ethanolic extract in the control of environmental infecting agents**

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The choice of substances that are not harmful to man and the environment is a priority in the production of sanitizing products. The aim of this study was to evaluate the disinfectant action and biofilm biomass reduction of the ethanolic extract of *Genipa americana* fruit. The extract disinfectant efficacy was assessed by modified Kelsey-Sykes, solid surface, and use-dilution methods. Also, the ability of the extract to disrupt biofilm mass was evaluated. Standard disinfectants and Gentamicin were used as control. *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, MRSA and MLSB *Staphylococcus hominis* and *Staphylococcus haemolyticus* and *Staphylococcus* species were used as infective and biofilm-forming microorganisms. *G. americana* fruit extract was not as effective as C12-C14 and BCB, however, it was more effective over *Staphylococcus* bacteria than 70% alcohol on stainless steel surface for a shorter exposure time. The extract did not show the ability to remove adhered *P. aeruginosa* nor to disrupt the mature biofilm. The ethanolic extract of genipap did not show optimal disinfectant action in the simulated critical environment; however, considering the reported antimicrobial action of this extract, more studies should be considered to find the best formula to improve this extract's disinfectant efficacy.

Key words: *Genipa americana* extract, disinfectant, biofilm, microorganisms.

INTRODUCTION

As pointed out by the Centers for Disease Control and Prevention, antimicrobial resistance was one of our greatest public health concerns prior to the COVID-19 pandemic, and it remains so (CDC, 2022). Only in 2019,

an estimated 4 to 95 million associated deaths and 1 to 27 million deaths were attributed to bacterial resistance. The emergency of new infectious agents and the potential of their dissemination through contact with

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infected surfaces raises a need for effective sanitizing products suitable for different environments. In the food industry, where safe products with acceptable shelf life and quality are necessary, wet surfaces provide favorable conditions for the growth of microbes (Nidaullah et al., 2017). In addition, in healthcare, surfaces are important reservoirs for a variety of microorganisms such as pathogenic bacteria with multiple drug resistance (Blake et al., 2021).

In the environment, the elimination of microorganisms depends on several sanitizing measures being one of the most important, the use of sanitizing solutions such as disinfectants (Terpak and Verstraete, 2012). The performance and choice of disinfectants take into consideration the form of use, the concentration to be used, adequacy to the target surface and activity over the microorganisms to be potentially eliminated (Artasensi et al., 2021).

An ideal chemical disinfectant should have a broad spectrum of action, highly effective, water-soluble, odorless, economical, non-toxic to users, easy to use, have good durability and stability in stock or diluted concentration, and not be polluting to the environment. Also, it should not be inactivated by environmental interferences such as soap, other detergents, fluid or organic matter, nor be corrosive or cause damage to objects (Rutala and Weber, 2019).

Several disinfectants approved by regulatory organizations and available in the market present good results, however, disinfectant resistance, including efflux pumps, resistance genes, and the microbe's ability to metabolize disinfectants (Belter et al., 2022) indicate the need for new substances that meet current needs.

Until then, numerous plants have been studied and reported with broad-spectrum antimicrobial properties (Vaou et al., 2021) and some plants such as *Psidium guajava* L. (guava), *Matricaria chamomilla* (chamomile) or mixed plant extracts may be easily available to prepare eco-friendly and effective hand sanitizers as compared to chemical formulations (Alghamdi, 2021).

Taking into consideration the antibacterial (Sousa Júnior et al., 2019) and antiviral (Codignoto et al., 2017) activity of *Genipa americana* extracts reported in the literature, in this work, we considered to evaluate the disinfectant potential and biofilm biomass reduction of the ethanolic extract of *G. americana* fruit at non-cytotoxic concentration. Thus, the inactivation of bacteria on different kinds of surfaces through spray extract contact, in the presence and absence of organic material, or reducing the adhesion of bacteria from the surface were investigated.

MATERIALS AND METHODS

Microorganisms

The microorganisms used for disinfectant tests were *Pseudomonas*

aeruginosa (Schroeter) Migula (ATCC 15442), *Staphylococcus* species (environmental isolate), *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 6538) and *Escherichia coli* (Migula) Castellani and Chalmers (ATCC 11229).

To perform mature biofilm inhibition, *E. coli* (ATCC 25922), *S. aureus* subsp. *aureus* Rosenbach (ATCC 25904) and *S. aureus* subsp. *aureus* Rosenbach (ATCC 29213), MRSA and MLSB *Staphylococcus hominis* and *Staphylococcus haemolyticus* isolated from hospital environment were used.

All microorganisms were recovered from -20°C and 15-20% glycerol (LGCBIO, Brazil) storage through brain heart infusion broth (KASVI, Brazil) culture and maintained in agar nutrient until use. All bacteria were kindly provided by Acassia Lippi from Belo Horizonte Fleury Laboratory in Minas Gerais State, Brazil.

G. americana extract

The *G. Americana* L. (Rubiaceae) fruit was collected through botanical field work in January 2006, in the Rio de Engenho district, Ilhéus, Bahia, Brazil. The voucher specimens were identified and deposited in the herbarium of the Universidade Estadual de Santa Cruz under identification number 13.923. After collection, plant material was separated and measured. The fruits were dried in an incubator under forced ventilation for 8 h at 28°C.

To obtain the *G. americana* fruits ethanolic extract (GAFEE), 10 g of dried and powdered plant material was macerated in 100 ml ethanol for 24 to 48 h through direct contact with solvent and with mechanical agitation. The marc was filtered through Whatmann number 1 filter paper and evaporated to dryness under reduced pressure. The plant material was soaked in recovered solvent once more to make a new extraction. The plant access is supported by the Brazilian Biodiversity Regulation (Law nº 13.123, of 2015) and duly registered in Sisgen under number A2153BD.

Fresh solutions at 1 mg/mL in sterile distilled water solution were prepared for modified Kelsey-Sykes or at 500 µg/mL for other tests. The concentration chosen was based on the previous test being the minimal antimicrobial concentration with no cytotoxicity to mammal cells. The solution showed slight turbidity, but no odor or precipitation were observed.

Disinfectants used as reference of effectiveness

The 0.45% N-alkyl ethyl benzyl dimethyl ammonium chloride (C12-C14) (Lysoform®), 1% Benzalkonium Chloride and Biguanide mixture (BCB) solution (Surfic®) and 70% ethanol (Araucária, Brazil) were used for comparison of effectiveness.

Disinfectants tests

Solid surface

The solid surface test was performed using the methodology described by Jang et al. (2017) with adaptation. For this, three kinds of material were used as a solid surface: glass, stainless steel, and granite. Three areas (triplicate) of 10 cm² were bound in each surface area.

To inoculate surfaces, *S. aureus* (ATCC 25904), *Staphylococcus* spp. and *P. aeruginosa* (ATCC 27853) were standardized at 0.5 in McFarland scale in NaCl 0.9% solution and diluted at 1:20. From this dilution, 100 µL were seeded all over each square and spread with a sterile swab. At the same time, another 100 µL was seeded and spread in a nutrient agar plate to count the initial (time zero) number of colonies forming units (CFU).

Following the inoculation, GAFEE at 500 µg/mL, 70% ethanol, C12-C14 and BCB solutions were sprayed over the delimited surfaces at a distance of 30 cm high. After 1, 5 and 10 min, sterile swabs were passed over the surfaces and thereafter on the nutrient agar plates. Plates were incubated at 37°C for 24 h. Then, the plate images were obtained using a digital camera and the CFU were obtained using the Open CFU 3.9.0 program (by Quentin Geissmann; <http://opencfu.sourceforge.net/>).

All experiments were made in triplicate and repeated three times. The percentage of bacterial inhibition was obtained using Excel 2010 (Microsoft Corporation) and graphs and statistical analysis were done using GraphPad Prism 5.0 version for Windows (GraphPad Software, San Diego California USA).

Modified Kelsey-Sykes

The modified Kelsey-Sykes test (Mattila, 1987) was used to determine the ideal concentration to achieve disinfection of a product in the presence or absence of organic material. To perform the technique, 500 µL of GAFEE at 500 µg/mL, sanitizers C12, C14 or BCB at 1% and BHI only were prepared. To simulate organic material interference, a 0.9% NaCl solution of *Candida albicans* at 0.5 Mac Farland scale was produced and submitted to 121°C for 20 min and added at 50% (v/v) to each treatment. To each test tube a bacterial suspension was added at time 0, 10 and 20 min. After 8 min of each time, samples were incubated at 36±1°C for 24 h. Then, 100 µL of each tube were transferred to microwell plates to evaluate the bacterial viability and growth. The bacterial viability was measured using 0.01% Resazurin and the bacterial density was established by optical density at 492 nm wavelength in plate reader EZRead (Nova Analítica, BR). To perform this test, *E. coli* ATCC 25922, *Staphylococcus* spp., *S. aureus* ATCC 25904 and *P. aeruginosa* ATCC 27853 were used. All experiments were done in triplicates and repeated three times. One-way ANOVA followed by Tukey test was used to compare treatments considering $p < 0.05$.

Use-dilution test

The use-dilution method followed Sridhar Rao (2020) description with modifications. For that, 8 cm nylon threads were immersed in the bacterial suspension standardized at 0.5 in Mac Farland scale. After 10 min incubation, threads were completely dried and transferred to 12x75 mm sterile tubes containing 3 mL of GAFEE at 500 µg/mL or control solutions. Each thread was immersed in one tube and five tubes were used for each solution. Controls consisted of sterile water and C12-C14. After 10 min at room temperature, threads were individually transferred to 12x75 mm sterile tubes containing brain heart infusion (BHI) broth and tubes were incubated for 24 h at 37°C. To consider the solution, a good disinfectant in the absence of turbidity in all five replicates was expected.

Biofilm biomass inhibition

The mature biofilm test was performed following Santos et al. (2018) method. For that, 200 µL of 24 h bacteria cultured in BHI were seeded in 96 microwell plates. After a 48-h incubation period at 36±1°C, the supernatant was removed and wells were washed with sterile distilled water to remove planktonic bacteria. Then, mature bacterial biofilm was treated with *G. americana* extract at 500 µg/mL and controls. Controls consisted of gentamicin sulfate at 20 or 30 µg/mL in accordance with minimal bacterial inhibition through microdilution technique previously performed for each bacteria or phosphate buffer saline (Thermo Fisher, EUA) for 24 h

at 36±1°C. The plate wells were washed with sterile distilled water and fixed with 200 µL of methanol for 15 min. After drying at room temperature for around 15 min, 200 µL of crystal violet (1%; v/v) were added to each well. After 5 min, the biofilm was gently washed with sterile distilled water. Subsequently, 100 µL of 96° ethanol was added to promote the solubility of the biofilm biomass. The microplate was then read in a microplate reader (EZReader) at 570 nm wavelength.

RESULTS AND DISCUSSION

G. americana disinfectant activity on solid surfaces

Initially, the efficacy of the GAFEE and commercial disinfectants to kill bacteria in different solid surfaces was evaluated. Results showed variation of efficacy between sanitizer, surface, time of exposition and microorganism tested (Table 1). The C12-C14 and the BCB solutions proved 100% efficacy for all surfaces and microorganisms in any time of substance contact with the surface. However, 70% ethanol was more variable. The 70% ethanol best results were seen for standard microorganisms, in a short period (1 min) of contact with surface and in less porous surfaces such as glass and stainless steel. Similar to ethanol, GAFEE at 500 µg/ml, the percentage of inhibition is varied. For this product it is worth to note that the percentage of inhibition was superior to ethanol on granite surface and its best action was 70% inhibition after 5 min of contact on stainless steel surface. It is known that microorganisms vary greatly in their resistance to chemical germicides and that there is a variation in the intrinsic resistance mechanisms in microorganisms to disinfectants (Rozman et al., 2021). Structures such as envelope, porins, periplasm space, etc., responsible for Gram-negative bacterial impermeability (Denyer and Maillard, 2002), for example. In this study, although with weak efficacy (less than 99%) through surface test and confirmed by modified Kelsey-Sykes test, it seemed that genipap ethanolic extract is more prone to attack Gram positive than negative bacteria. Another point of disinfectant property that must be considered is the time of exposure to a disinfectant to be effective. According to European regulations, exposure times lie at 5 min for bacteria and 15 min for yeasts and molds (ECA, 2022). In this criterion, the genipap ethanolic extract best performance was restricted to stainless steel (Table 1).

G. americana disinfectant activity in the presence of organic material

As the organic matter can interfere in the disinfectant performance through substances complex forming which prevent its activity or a physical barrier, protecting microorganisms from attack (Querido et al., 2019), the antibacterial efficacy of the extract as a disinfectant in the

Table 1. Efficacy of *G. americana* ethanolic fruit extract and commercial disinfectants on different solid surfaces at different times.

Surface material time (min)	Glass (%)			Stainless steel (%)			Granite (%)		
	1	5	10	1	5	10	1	5	10
<i>S. aureus</i> (ATCC 25904)									
1% BCB	100	100	100	100	100	100	100	100	100
2.5% C12-C14	100	100	100	100	100	100	100	100	100
70% Ethanol	0	12.9	0	1.49	0	0	0	0	20.7
GAFEE 500 µg/ml	0	0	0	40.2	40.4	24.1	48.8	37.7	40.5
<i>Staphylococcus</i> spp.									
1% BCB	100	100	100	100	100	100	100	100	100
2.5% C12-C14	100	100	100	100	100	100	100	100	100
70% ethanol	41.3	65.8	48.7	31.2	33.6	21.3	4.2	21.6	17.8
GAFEE 500 µg/ml	20.2	0	27.7	37.8	70.1	71.4	17.3	0	0
<i>P. aeruginosa</i> (ATCC 27853)									
1% BCB	100	100	100	100	100	100	100	100	100
2.5% C12-C14	100	100	100	100	100	100	100	100	100
70% ethanol	18.7	26.8	34.0	0	0	0	19.7	2.7	21.4
GAFEE 500 µg/ml	11.8	3.7	0	0	0	0	33.5	34.4	42.2

Data are expressed in percentage of UCF decreasing in relation to inoculum (time zero). BCB - Benzalkonium Chloride and Biguanide mixture; C12-C14 - 0.45% N-alkyl ethyl benzyl dimethyl ammonium chloride; GAFEE – *Genipa americana* ethanolic fruits extract.

Source: Authors

presence or absence of organic matter was also investigated. Through the modified Kelsey-Sykes method, the results indicated the maintenance of efficacy percentage even in the presence of organic matter (Figure 1) with best results on environmental species of *Staphylococcus*. It has 66.2% of efficacy in the absence of organic matter and 77.5% in the presence of organic matter at 10 min contact with the disinfectant. In brief, taking together surface and modified Kelsey-Sykes methods, although the poor efficacy compared to commercial disinfectants, isolated sterol or higher concentration of the extract should be tested before dismissal of its disinfectant quality.

***G. americana* bactericidal activity on adhered bacteria and mature biofilm**

Considering that bacteria can be tightly attached to surfaces and cannot be easily removed, forming biofilms, an ideal disinfectant (products such as hydrogen peroxide and sodium hypochlorite) must disrupt biofilms or reduce numbers of viable bacteria within a biofilm (Lineback et al., 2018). In this study, we use the nylon material as a carrier to verify the reduction of adhesion or the elimination from the surface of *P. aeruginosa*. This bacterium easily forms biofilm on various types of surfaces leading to human infections (Tuon et al., 2022). No inhibition was observed using the GAFEE. The hypotheses of the absence of action of the extract may

be: (1) the low concentration of the extract used for this test; (2) the inability to inhibit the bacteria adhesion to abiotic surfaces; or (3) the absence of direct antimicrobial activity on *P. aeruginosa*.

As observed in the literature, genipap derived extract presents weak activity on *P. aeruginosa* (Santos et al., 2017), although a promising modulatory activity on antimicrobial peptides over this bacterium was demonstrated (de Sousa Júnior et al., 2019). Furthermore, the *P. aeruginosa* multiple interconnected signal transduction pathways (Thi et al., 2020) could be activated during the management of the technique increasing the possibility to form biofilm and reducing the chance of eliminating the bacteria.

It is reported in the literature the antibiofilm activity against *Escherichia coli* isolates of β -sitosterol glucoside plant compounds (Vikram et al., 2013). Thus, it was expected that this plant extract would have some influence on mature biofilm. However, no degradation of established biofilms by the contact of GAFEE was evident for both *Staphylococcus* spp. and *E. coli* strains in this study. On the contrary, it seemed that the extract improved the biofilm's stability for MRSA and MSLB *Staphylococcus* isolates (Figure 2).

Conclusion

The results presented here show for the first time the disinfectant potential of *G. americana* fruit ethanolic

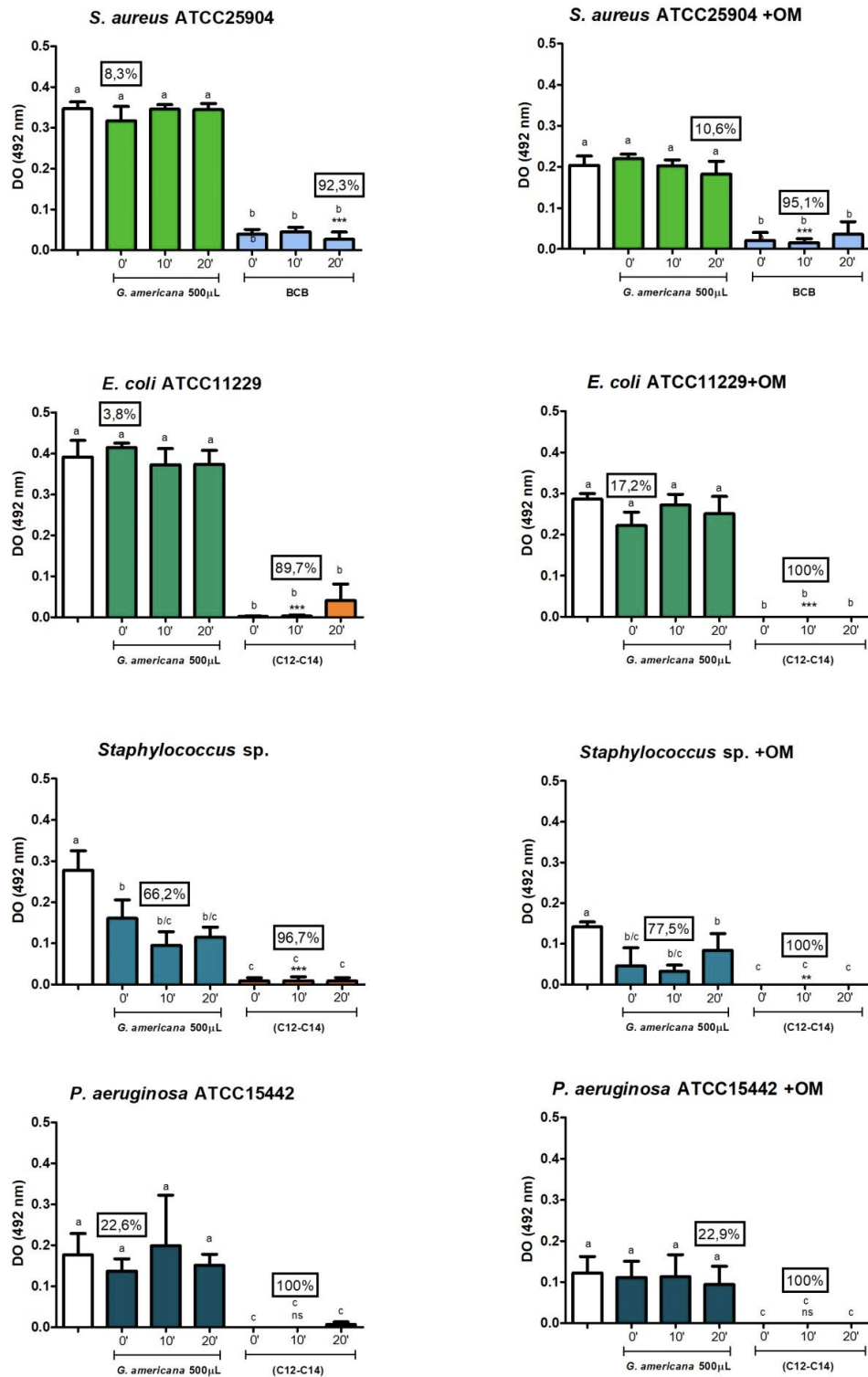


Figure 1. *Genipa americana* ethanolic fruit extract (500 µg/ml) efficacy test as disinfectant by modified Kelsey-Sykes method. Squares show the best percentage of bacterial growth inhibition at time point. Controls consisted on inoculum without treatment ±organic matter (OM); 0,45% N-alkyl ethylbenzyl dimethyl ammonium chloride (C12-C14) ± OM, 1% benzalkonium chloride and biguanide mixture (BCB) solution±OM.
Source: Authors

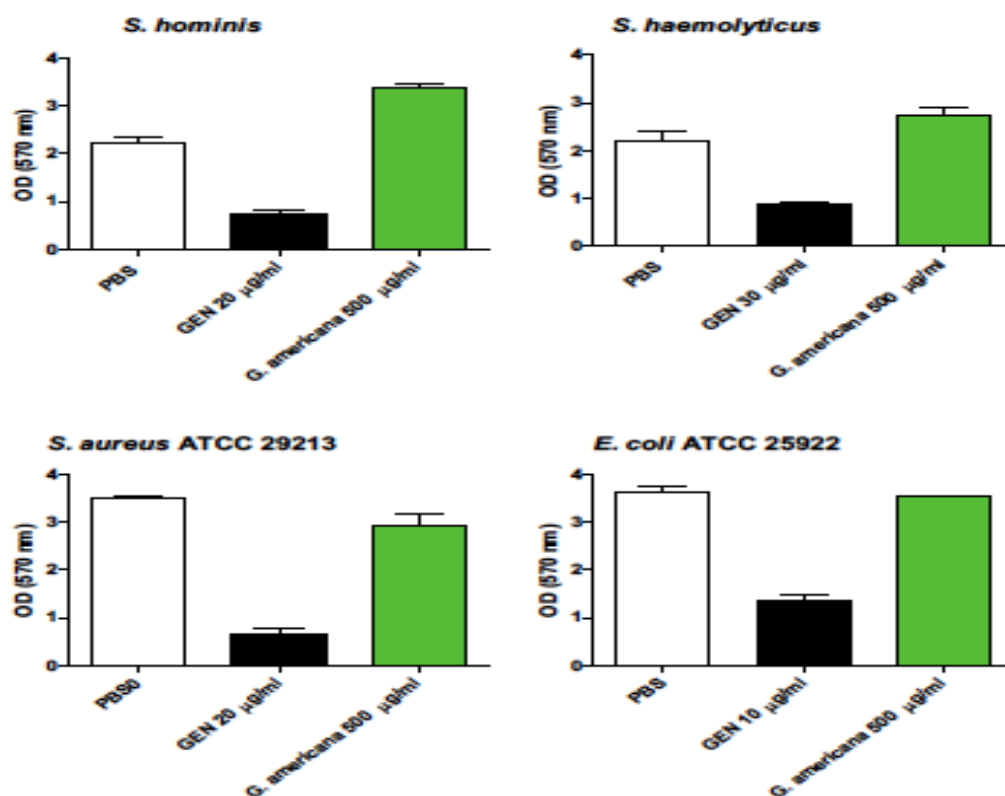


Figure 2. Biofilm biomass inhibition test. Three *Staphylococcus* species were used to test *G. americana* mature biofilm inhibition: (a) *S. hominis*; (b) *S. haemolyticus*; (c) *S. aureus*. Control consisted of Gentamicin (20 µg/ml) and phosphate buffer saline. Experiment was made in triplicate and repeated twice.

Source: Authors

extract. Although this extract may not be indicated for a contaminated critical environment, its potential use can be considered regarding the fact that it is a natural product and it has reported antimicrobial action *in vitro*. Some characteristics of an ideal disinfectant such as broad spectrum of action, water-soluble, odorless, economical, non-toxic to users, and not pollutant to the environment can be seen in this extract. Despite this, aspects such as durability and stability in stock or diluted concentration are still to be analyzed. Also, improvement in steroid extraction and the association with other substances, modulating their activity, should also be analyzed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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