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Evaluation of antibacterial activity from different *Aloe vera* (L.) Burm. F. extracts against *Staphylococcus aureus* strains

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Abstract. This study aimed to evaluate the susceptibility of *Staphylococcus aureus* strains isolated from the milk of cows with subclinical mastitis to *Aloe vera in natura* sap, ethanolic extract and *in natura* gel. The substances were tested using agar well diffusion and agar disk-diffusion methods. There was no inhibition of bacterial growth in the agar disk-diffusion method. On the other hand, the agar well diffusion method displayed *in vitro* growth inhibition of *S. aureus* in ethanolic extract and *in natura* sap of 91.66% (22/24) and 35.29% (6/17), respectively. Although the gel showed no effect against the strains tested in both methods, the agar well diffusion method was the most efficient to evaluate the antimicrobial activity of *Aloe vera* extracts.

Keywords: bovine mastitis, bacteria, agar disk-diffusion, agar well diffusion, *Aloe vera*.

Introduction

Aloe Vera (L.) Burm.f. belongs to the Asphodelaceae family, genus *Aloe* and has a viscous gel extracted through its mucilage (Castro & Ramos, 2002; The Linnean Society of London, 2016). This plant originated from the Mediterranean, Canary Island and Madeira Island (Lorenzi & Souza, 2008), and due to its adaptability, today this plant is found worldwide, including many Brazilian regions.

A. vera can be found in households as an ornamental plant, due to the fact that it can be easily cultivated, and is also used for therapeutic and cosmetic purposes (Sartório, 2000; Lorenzi & Souza, 2008; The Linnean Society of London, 2016). This species has a succulent leaf, concave on the top side, grey-green color, grows up to 30-60 centimeters and contains spikes on the leaves' edges (Grandi, 2014; The Linnean Society of London, 2016).

Transverse section of the leaves allows observation of two layers: the external (chlorenchyma), which is dark green, and the internal (parenchyma), which is translucent and mucilaginous from where the aloe gel, also known as mucilage, is obtained. In the zone where parenchyma and chlorenchyma connect, vascular bundles are located. The vascular bundles contain the aloetic cells or aloetic tissue, that produce a yellow viscous latex (sap) known as aloetic liquid or aloetic juice (Brasil, 2010).

Aloe gel is composed of 99,5% water as well as small amounts of hydro- and liposoluble vitamins, minerals, enzymes, polysaccharides, phenolic components, and organics acids. The therapeutic components attributed to aloe gel and its derivatives are related to the diversity of its components (Hamman, 2008). *A. vera* contains barbalodine, aloin, alloylodine, aloethine, aloferon, picric acid, resins, mucilage, vitamins B and C as active components (Sartório, 2000). *The plant* has been used for medicinal purposes due to its bactericidal action, healing properties and anti-inflammatory action (Habeeb et al., 2007; Semenoff Segundo et al., 2007; Semenoff et al., 2008; Ramos & Pimentel, 2011; Stanley et al., 2014). The antibacterial activity of this plant has been attributed to anthraquinones, which are natural components found in the *A. vera*, and present in the sap extracted from its leaves (Hamman, 2008; Sociedade Brasileira de Farmacognosia, 2015; The International Aloe Science Council, 2018).

Several researches have shown that the antimicrobial efficiency of *A. vera* extract in the control of isolated microorganisms can cause diseases in humans such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella sp.*, *Aspergillus niger*, *Escherichia coli* and *Candida sp.* (Semenoff et al, 2008; Shilpakala et al., 2009; Cardoso et al., 2010; Banu et al., 2012; Kedarnath et al., 2013; Stanley et al., 2014).

In veterinary medicine, the use of *A. vera* is not well described as it is primarily a human medicine. Studies performed showed that aqueous extract could be used as post-milking teat dipping in goats as an alternative to glycerin iodine solution. This has been shown to keep the somatic cell count in the reference value and did not affect the milk composition. Furthermore, *A. vera* contains antimicrobial properties, which can be valuable to control causative agents of mastitis such as *S. aureus*, *Streptococcus agalactiae*, and *E. coli* (Amaro et al., 2011; Chikwanda et al., 2013).

Following the rise of antimicrobial resistance, there is a need to identify substances with antimicrobial properties (Silveira et al., 2009; Maddison et al., 2010). This study aims to compare the inhibitory potential from three *A. vera* extracts against *Staphylococcus aureus* strains isolated from milk of cows with subclinical mastitis.

Methods

For this study twenty-four (24) *Staphylococcus aureus* samples were previously isolated from the milk of cows with subclinical mastitis and stored in the Laboratory of Veterinary Microbiology Federal University of Mato Grosso (UFMT), Sinop extension (Koneman, 2008).

The *A. vera* extract was prepared in the Laboratory of Veterinary Microbiology, Federal University of Mato Grosso (UFMT), Sinop extension. From the *A. vera* leaves, three extracts were obtained: *in natura* gel, *in natura* sap and ethanolic extract from dehydrated leaf lamina adapting the methodology described by Bussmann et al., 2010.

In order to obtain *in natura* sap, *A. vera* leaves were washed with distilled water and neutral detergent and then disinfected with 70% alcohol. After drying, the leaves were sectioned transversely and the sap was collected and then stored in a sterile plastic tube.

Three leaves of *A. vera* were collected and sent to the laboratory to obtain ethanolic extract. They were hygienized with neutral detergent and running tap water and then rinsed with distilled water. The chlorenchyma and parenchyma were separated using a sterile scalpel and spoon, as well as a plastic cutting board disinfected with 70% alcohol. Subsequently, the chlorenchyma was kept for 15 minutes in a solution containing 15 mL of 2.5% sodium hypochlorite and 1000 mL of distilled water. Then, the leaves were thoroughly rinsed with distilled water to remove the sanitizing solution. The chlorenchyma was deposited on a paper towel to dry. Afterward, the leaves were placed in a plastic tray and then kept in a drying oven at 45 ° C for 72 hours to dry and concentrate the active compounds.

During the drying period, the drying oven door was opened once a day for five minutes for spontaneous air exchange. After 72 hours, the material was stored in a sterile glass beaker, wrapped in aluminum foil and kept at room temperature. The dehydrated leaf lamina was manually fragmented and then macerated in a

porcelain mortar. Five grams of dehydrated leaf lamina were weighed in a petri dish and placed in an Erlenmeyer flask, and 50 mL of 70% alcohol was added thereafter. This mixture was homogenized and the container sealed with aluminum Kraft paper. It was then wrapped in aluminum foil and kept at room temperature for ten days. After this period, the mixture was filtered in a paper filter n° 1 (Melitta®). For the evaporation of the remaining alcohol, the extract was placed in a water bath at 45 ° C for four days and then stored in plastic microtubes type Eppendorf® and then refrigerated.

The mucilage was extracted from the leaves according to Agarry et al. 2005, with the following adaptations: they were triturated with the aid of a sterile stainless-steel spoon and then sieved to promote the release of the gel which was stored in a glass beaker until use.

For the agar disk-diffusion method, the sap and gel were extracted 24 hours prior the experiment. In this case, microdisks of six millimeters in diameter were made using filter paper Nalgon® (reference 3550) and Melitta®, which were stored in plastic microtubes and sterilized before being immersed in the extracts (*in natura* sap, ethanolic extract, and *in natura* gel). With the aid of pre-flamed forceps, the disks were inserted into the microtubes containing the respective extracts and kept submerged for 24 hours. Petri dishes containing *S. aureus* were prepared to perform susceptibility test using *A. vera* extracts, in accordance to "Clinical and Laboratory Standards Institute" (CLSI, 2012). The observation of the growth inhibition zone was done following the CLSI recommendation (CLSI, 2012). In this procedure, five disks were used: vancomycin 30 micrograms (µg) as positive control (C+); sterile dry filter paper disk as negative control (C-); disk impregnated with *A. vera* ethanolic extract (E); disk impregnated with *A. vera in natura* gel (G), and disk impregnated with *A. vera in natura* sap (S).

For well diffusion method, the sap and gel extraction procedure of both was performed at the moment of use, in order to have minimal changes in its physicochemical properties. In this method, the approach described by Silveira et al. 2009, with adaptations was followed. Five 8 mm wells were made with a 1000 microliter sterile pipet tips on Mueller Hinton Agar plates, which were previously inoculated with a swab containing *S. aureus* samples corresponding to 0.5 degrees on the McFarland Nephelometric Scale. In these wells, 50 microliters of the following were added: 2% chlorhexidine soap (Higimax®) as a positive control (C +), sterile distilled water as negative control (C-), *A. vera in natura* gel (G), *A. vera* ethanolic extract (E), and *A. vera in natura* sap (S).

In both methods, the plates were incubated at 37 ° C for 24 hours and then the zone of growth inhibition was measured with a millimeter ruler.

Results and discussion

The susceptibility profile of 24 samples of *S. aureus* from cows with subclinical mastitis was analyzed through the agar well diffusion method in addition to analysis of nine samples applying the agar disk-diffusion method. For these analyses, two types of filter paper were used for both the methods.

In the agar disk-diffusion method, zone of growth inhibition of *S. aureus* strains was not observed in both filter papers when using *in natura* gel, ethanolic extract and *in natura* sap (Table 1).

Although the agar disk-diffusion method is widely used in laboratories to verify the sensitivity profile of bacteria against antimicrobials, in this study this technique could not demonstrate the real effectiveness from the extracts of *A. vera* tested. This fact may be related to the lack of solubility of substances in the growth medium, which may have

compromised diffusibility of the tested product (Semenoff et al., 2008). In addition, the concentration of antimicrobial active component impregnated in the disk may be less than the minimum inhibitory concentration capable of inhibiting the growth of the tested strains. Thus, information obtained from other studies and the present results emphasize that the agar disk-diffusion method is not suitable to evaluate the inhibition of microbial growth in *A. vera* substrate.

In the agar well diffusion method, there was growth inhibition of 22 *S. aureus* strains (91.66%) in ethanolic extract. In the case of *in natura* gel, no inhibition of bacterial growth was observed. However, *in natura* sap was effective against 35.29% (6/17) of the *S. aureus* strains tested (table 2).

Table 1. Zone of growth inhibition from different extracts of *Aloe vera* against strains of *S. aureus* using the agar disk-diffusion method.

Agar disk-diffusion method – filter paper Melitta®						Agar disk-diffusion method –filter paper Nalgon®					
Sample	C+	C-	Gel	EA	Sap	Sample	C+	C-	Gel	EA	Sap
1	20	0	0	0	-	1	18	0	0	0	-
2	19	0	0	0	-	2	18	0	0	0	-
3	11	0	0	0	-	3	19	0	0	0	-
4	16	0	0	0	-	4	20	0	0	0	-
5	18	0	0	0	-	5	20	0	0	0	-
6	11	0	0	0	-	6	20	0	0	0	-
7	19	0	0	0	-	7	19	0	0	0	-
8	18	0	0	0	-	8	18	0	0	0	-
9	22	0	0	0	-	9	21	0	0	0	-
Mean	17,11*	0	0	0	-	Mean	19,22	0	0	0	-

Abbreviations: C+ (positive control); C- (negative control); EA (ethanolic extract);* diameters of inhibition halo in millimeters.

Table 2. Zone of growth inhibition (mm) of the substances tested against *S. aureus* using agar well diffusion method.

Agar well diffusion method						
Sample	C+	C-	Gel	EA	Sap	
1	29	0	0	15	-	
2	28	0	0	16	-	
3	35	0	0	18	0	
4	23	0	0	11	-	
5	26	0	0	15	-	
6	29	0	0	12	-	
7	30	0	0	12	-	
8	25	0	0	13	-	
9	39	0	0	25	0	
10	33	0	0	20	0	
11	32	0	0	19	0	
12	33	0	0	20	0	
13	31	0	0	11	0	
14	29	0	0	13	13	
15	37	0	0	22	0	
16	28	0	0	12	0	
17	25	0	0	13	0	
18	30	0	0	0	30	
19	32	0	0	12	12	
20	29	0	0	12	0	
21	25	0	0	16	0	
22	38	0	0	13	13	
23	38	0	0	0	16	
24	31	0	0	13	16	
Mean	30,63*	0	0	15,14	16,67	

Abbreviations: C+ (2% chlorhexidine soap); C- (negative control); EA (ethanolic extract); * diameters of inhibition halo in millimeters.

Researchers observed a 4mm zone of growth inhibition in *S. aureus* colony using 100µl *A. vera* ethanolic extract in wells of 10 mm (Agarry et al., 2005). In another study, *A. vera* ethanolic extract was tested in concentrations of 100, 25 and 6.25 mg/mL against *S. aureus* strains, using 60µl of the extract deposited in 8 mm wells, and zone of growth inhibition of 20 ± 0.3 mm was noted in the extract concentration of 100 mg/mL (Moody et al., 2004). In the present study, 8 mm wells were used and 50µl ethanolic extract was added, with inhibition zone ranging from 11 mm to 25 mm in 22 *S. aureus* samples. The discrepancy evidenced in this study, when comparing the results obtained by other researchers, is due to the difference in the amount of extracts used or could also be associated to a lower concentration of active components in the plant, since several environmental factors may alter the production of secondary metabolites, altering its therapeutic properties (Globbo-Neto & Lopes, 2007). The results obtained in the present study reinforce the efficacy of the ethanolic extract against *S. aureus* strains and can be used *in vivo* to establish alternative therapies in the treatment of bovine mastitis caused by this bacterial species.

In the present investigation, when *in natura* sap was used through the agar well diffusion method, growth inhibition of 35.29% (6/17) was observed in the tested samples. The absence of efficacy of the *in natura* sap against other strains, however, can be explained by the fact that not all bacterial strains were tested with the same sap sample. This result occurred due to the necessity of extracting the sap at the moment of its use, with minimum change in the physical-chemical properties. Although the samples came from the same plant, concentrations of antimicrobial substances in the sap may have changed since environmental changes could alter the chemical components. In addition, dilution and lower production of the sap's active components can occur if there are a greater water availability and lack of solar radiation, respectively (Globbo-Neto & Lopes, 2007).

In a research conducted with *S. aureus* strains, growth inhibitions of 18 mm were observed using 100 µl of *A. vera in natura* gel in 10 mm wells (Agarry et al., 2005). However, other researchers tested the *A. vera in natura* gel using agar well diffusion method at concentrations of 100%, 25%, and 6.5% and did not found a zone of growth inhibition in any bacteria tested including *S. aureus* - the same result was observed in the present study (Moody et al., 2004). The divergence regarding the results obtained in the sensibility test to the *A. vera* gel can be related to the number of active compounds present in each plant that can be influenced by environmental factors, which alter the chemical composition of the therapeutic components (Reynolds & Dweck, 1999; Globbo-Neto & Lopes, 2007). In addition, when comparing the action of sap and gel *in natura A. vera* against *S. aureus*

strains, the absence of antibacterial activity can be explained by the low concentration of anthraquinone in the gel (Hamman, 2008).

Although the results obtained in this research did not demonstrate the inhibition of the growth of *S. aureus* strains, *A. vera* gel was used *in vivo* to treat chronic ulcers in legs of humans infected with multiresistant microorganisms (Banu et al., 2012). In the study mentioned above, from the 30 patients in the gel treated group, only two presented positive cultures after 11 days of experiment, demonstrating *in vivo* antibacterial effect of *A. vera*. The response observed in the use of gel *in vivo* is due to immunomodulatory properties present in this substance, in which polysaccharides, mainly acenamamin, activate macrophages and increase the number of lymphocytes, boosting the immune system (Reynolds & Dweck, 1999; Ramos & Pimentel 2011). Furthermore, an experiment with aqueous extract of the *A. vera* gel had shown an antibacterial action similar to iodine glycerine, postulating that it can be used as post-dipping solution in goats (Amaro et al., 2011). Thus, due to the difference between the *in vivo* and *in vitro* actions of the *A. vera* gel, *in vivo* studies are required to verify the potential of this substance in the treatment of bovine mastitis caused by *S. aureus*.

Among the methods used, the agar well diffusion method was the most appropriate to test the antimicrobial properties of *A. vera* extracts. The results obtained are consistent with the results obtained by other researchers, who showed a greater sensitivity of the agar well diffusion method in comparison to the agar disk-diffusion method when determining the antibacterial activity of natural products from plants, fungi, and lichens (Valgas et al., 2007). The difference in the results obtained among both methods, as such in this study, is correlated to the precipitation of insoluble substances present in the extract on the filter paper used (Whatman® n ° 3) in agar disk-diffusion method, thus did not show diffusion of these substances in the agar as was the case in the diffusion well technique. Besides that, the difference between the results obtained in the techniques used may be related to amount of inhibitory substances in which they were used 50 µl of extract in the agar well diffusion method and approximately 10 µl in the agar disk-diffusion method. Thus, considering *in vitro* antimicrobial activity of *A. vera* on *S. aureus* strains isolated from milk of cows with subclinical mastitis, it is possible that this plant can be used an alternative treatment in this disease to reduce the presence of *S. aureus* in the dairy herd.

Conclusion

Aloe vera ethanolic extract and the sap presented *in vitro* antimicrobial activity against *S. aureus* strains isolated from milk of cows with subclinical mastitis. However, *A. vera* gel did not show growth inhibition of the strains tested. In

addition, the agar well diffusion method was the most adequate to evaluate the antimicrobial potential of *A. vera* extracts.

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