

# Antimicrobial Effects Caused by *Aloe barbadensis* Miller on Bacteria Associated with Mastitis in Dairy Cattle

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## Abstract

It is known that the primary etiological agents associated with bovine mastitis show high levels of antimicrobial resistance. In this paper, we studied a possible alternative to antimicrobial treatment, *Aloe barbadensis* Miller (*A. vera*). Our goal was to determine the viability of bacteria upon treatment with a methanolic extract of *A. vera* gel, rich in anthraquinones such as aloin A, aloin B, and aloe emodin. To this purpose, we used fluorescence spectrometry to study the following bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus uberis*, and Methicillin-resistant *Staphylococcus aureus* (MRSA). The results show that treatment with *A. vera* gel extract disrupted the cell membrane causing lysis in 75% of *Staphylococcus aureus*, in 88% of *E. coli*, in 97% of *Streptococcus uberis*, and in 88% of MRSA cells. Cell membrane disruption is attributed to the presence of anthraquinones. Further study is needed to determine whether other phenolic compounds present in the extract, influencing antimicrobial activity, could be used to develop pharmaceutical formulations to treat bovine mastitis.

## Keywords

mastitis, *Aloe barbadensis* Miller, antimicrobial, natural products, aloin, aloe emodin, anthraquinones

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Mastitis, a common disease in dairy cattle, is recognized worldwide for the significant economic losses it causes within the dairy industry. The primary etiological agents for bovine mastitis are *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus uberis*. These agents are known for having a high level of antimicrobial resistance,<sup>1,2</sup> which has motivated the development of alternative treatments. To date, several plants with medicinal properties have been studied, among which *Aloe barbadensis* Miller (*A. vera*) stands out as a promising option. *A. vera* Miller's leaf is composed of 99% water, but also contains approximately 200 active compounds within its dry matter. These compounds vary by location, and include bark, gel, or exudate.<sup>3</sup> There is plenty of evidence demonstrating that *A. vera* inhibits the growth of various microorganisms, including *Staphylococcus aureus*, *E. coli*, and *Streptococcus spp.*<sup>4</sup> This effect is mainly attributed to phenolic compounds,<sup>5,6</sup> like emodin, which inhibits DNA and protein synthesis, and causes alterations at the cellular membrane level.<sup>7</sup>

The objective of this research was to determine, using fluorescence spectrometry, whether methanolic extracts of *A. vera* gel, rich in aloin A, aloin B and aloe emodin, have effects on the viability of the bacteria responsible for mastitis in dairy cattle, by causing alterations at the cellular membrane level.

The presence of aloin A, aloin B, and aloe emodin in the methanolic extract of *A. vera* gel was determined using methodology previously published.<sup>8</sup> On the other hand, retention

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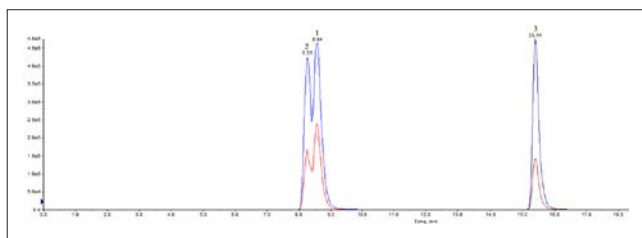
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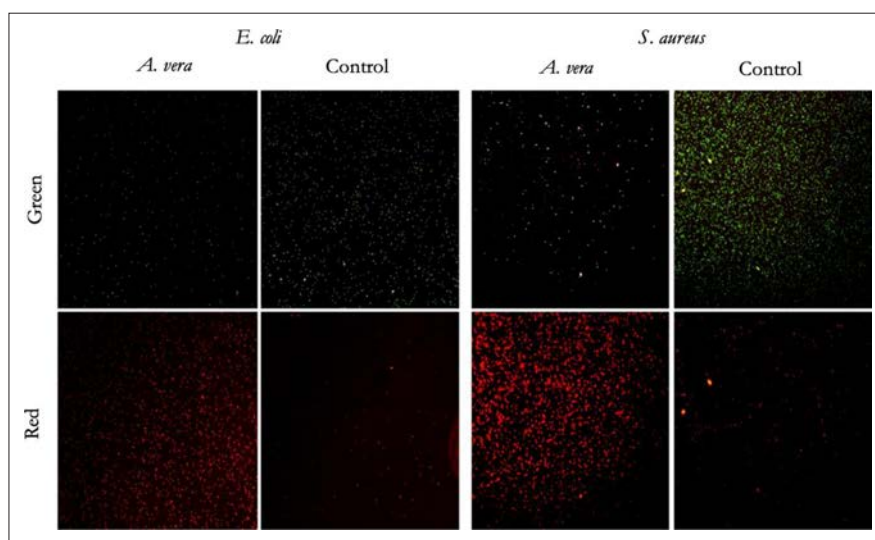
**Figure 1.** High-performance liquid chromatogram of aloin A (1), aloin B (2), and aloe emodin (3) in methanolic extract of *Aloe vera* gel.

times were established at 8.88, 8.33, and 15.44 minutes for aloin A, aloin B, and aloe emodin (Figure 1) and concentrations of the analytes in the extract were set as 146.72, 122.08, and 21.29 ng/mL, respectively.

The effect of exposure to *A. vera* gel extract on cell viability was explored using the LIVE/DEAD BacLight bacterial viability kit. When grown in cation-adjusted Mueller–Hinton broth (MHBCA) medium, *Staphylococcus aureus*, *E. coli*, *Streptococcus uberis*, and MRSA strains were dominated by viable cells. However, after treatment with *A. vera* gel extract for 24 hours, green fluorescence was dramatically reduced in all strains, indicating a lack of intact cell membranes, as shown in Figure 2 for *E. coli* and *Staphylococcus aureus*. The intense red emission from the same samples indicates that most cell membranes were disrupted, thus supporting previous observations.<sup>9,10</sup> In an effort to quantify viability using the BacLight kit, the fluorescence emissions corresponding to various ratios of live and dead bacteria were measured. When plotted as the percentage of viable bacteria versus the ratio of green-to-red fluorescence, a linear relationship was observed for *Staphylococcus aureus* ( $R^2 = 0.93$ ), *E. coli* ( $R^2 = 0.96$ ), *Streptococcus uberis* ( $R^2 =$

0.94), and MRSA ( $R^2 = 0.92$ ). Thus, that exposure to *A. vera* gel extract decreased viability in 75%, 88%, 97%, and 88% of cells from these strains has been estimated, as shown in Table 1.

The use of fluorescent stains to distinguish and quantify live and dead bacteria cells is a reliable method for assessing bacterial membrane integrity.<sup>11,12</sup> In particular, based on the decrease in the green:red fluorescence ratio observed in each strand after treatment with *A. vera*, this study shows that *A. vera* gel extract causes the disruption of the cell membrane in both Gram-positive and Gram-negative bacteria associated with bovine mastitis. Alterations in the cellular structure might induce expansion and destabilization in the fluidity of membranes, increasing passive permeability and causing a loss of intracellular components.<sup>13</sup> Present results are consistent with those previously described against MRSA using fluorescence spectrometry, where it was observed that treatment with emodin indeed causes a decrease in fluidity and disruption of the cell membrane.<sup>14</sup> The latter study also demonstrated that this effect is not related to the expression of genes associated with cell wall synthesis and lysis. On the other hand, loss of integrity in the cell membrane of *Haemophilus parasuis* by staining damaged cells with propidium iodide has been described after treatment with emodin isolated from *Polygonum cuspidatum*, demonstrating interaction with membrane proteins.<sup>15</sup> Membrane disruption in *E. coli* instead has been associated with the affinity of emodin and aloin for phosphatidylethanolamine and phosphatidylglycerol, 2 of the major phospholipids in this structure.<sup>16</sup> In this research, we have shown that treatment with *A. vera* gel extract produces disruption of the cell membrane in bacteria responsible for bovine mastitis in dairy cattle, probably due to the presence of anthraquinones such as aloin and aloe emodin. This treatment has been shown to be effective, even though the contents of aloin A, aloin B, and



**Figure 2.** Viability of the *Escherichia coli* (left) and *Staphylococcus aureus* (right) following exposure to 9 and 10 mg/mL methanolic extract of *Aloe vera* gel, respectively, and without treatment (control) as measured using the BacLight bacterial viability kit. Bacteria cells were viewed at a magnification of  $\times 10$  using red (620 to 650 nm) and green (510–540 nm) filters.

**Table 1.** Percentage of Bacteria with Cell Membrane Disruption after Incubation for 24 hours with 10, 9, 16, and 10 mg/mL Methanolic Extract of *Aloe vera* Gel, for *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus uberis*, and MRSA, Respectively, as a Function of the Green:Red Fluorescence Quotient.

Microorganism	Treatment with <i>A. vera</i>		Without treatment	
	Bacteria with cell membrane disruption (%)	Live/dead fluorescence ratio (nm)	Bacteria with cell membrane disruption (%)	Live/dead fluorescence ratio (nm)
<i>S. aureus</i>	75.80	4.23	25.37	9.11
<i>E. coli</i>	88.70	5.06	31.50	12.03
<i>S. uberis</i>	97.51	1.72	10.71	25.09
MRSA	88.24	2.18	7.41	6.63

MRSA, Methicillin-resistant *Staphylococcus aureus*.

A positive control treated with 70% isopropyl alcohol was included for each bacteria, considering 100% of bacteria with cell membrane disruption as 0 nm live/dead fluorescence ratio.

aloe emodin were lower than the contents of these compounds in other works.<sup>17,18</sup> In particular, the minimum inhibitory concentration (MIC) value for emodin against MRSA was determined as 2–8 µg/mL,<sup>14</sup> whereas the MIC value against MRSA in this study was 10 mg/mL, corresponding to 1.825 ng/mL. In spite of these results, it is noted that the antibacterial effect should not be related to this compound by itself, due the reported synergic effect between several compounds of natural products.<sup>19</sup> The methanolic extract of *A. vera* gel used in this study contains aloin A and B, in greater proportions than aloe emodin, which could increase the antibacterial effect of the extract against MRSA.

According to these results, this extract of *A. vera* gel could be used to develop alternative pharmaceuticals to drugs commonly used to treat bovine mastitis caused by Gram-positive and Gram-negative bacteria. An intramammary formulation based on *A. vera* gel combined with other plants (Mastiliber) has been developed and registered in Mexico. Unfortunately, according to a previous study, this formulation did not show a complete inhibition of bacterial growth in isolates obtained from cows with clinical mastitis.<sup>20</sup> However, the use of a methanolic extract of *A. vera* gel with a specific composition of the active compounds, particularly anthraquinones, to develop an intramammary formulation to treat bovine mastitis could be effective against Gram-positive and Gram-negative bacteria.

It is important to consider that some adverse effects are commonly associated with anthraquinones, including hypersensitivity reactions to *A. vera* administration.<sup>21</sup> Actually, in case of products for nonoral administration, derivatives of *A. vera* should not contain more than 50 ppm of aloin.<sup>22</sup> Therefore, the aloin content (aloin A + aloin B) of the extract used in this study (0.268 ppm) is far below that limit.

In conclusion, more studies are needed to determine whether other types of compounds present in the methanolic extract of *A. vera*, separately or in combination, cause disruption of the cellular structure. These studies would be considered to develop a new pharmaceutical product based on the extract, selecting the most appropriate compounds to optimize the efficacy and safety of the product.

## Experimental

### General

This study was conducted at the Laboratory of Veterinary Pharmacology (FARMAVET) and the Biotechnological Veterinary Center, Faculty of Veterinary and Animal Sciences, Universidad de Chile, where the following laboratory instruments were used: Agilent 1200 Infinity high-performance liquid chromatography (HPLC; Santa Clara, CA, USA); SunFire C18 column (Milford, MA, USA); AB Sciex API 4000 Applied Biosystems Sciex mass spectrometer (Concord, Canada); Spectrophotometer (Glomax Multi JR, Glomax, Madison, WI, USA); Olympus MVX10 fluorescence microscope (Olympus, Center Valley, PA, USA) equipped with filter cube U-MCFPHQ/XL and Olympus DC71 camera.

### Fractionation of *A. vera*

A methanolic extraction<sup>20</sup> was made from lyophilized *A. vera* gel powder obtained from Aloe Vera of America, Inc. (AZ, USA). Methanol (40 mL) and acetic acid (0.5 mL) were added to 5 g of the lyophilized gel. The sample was shaken, sonicated, and centrifuged at 5000 rpm for 10 minutes. The supernatant was moved to a round bottom flask and the extraction process was repeated with the sediment. The supernatant obtained from the 2 extractions was rotavaporised at 25°C using the maximum vacuum setting. The resulting extract was reconstituted with Mueller–Hinton Cation Adjusted Broth (MHCAB) BD (Franklin Lakes, NJ, USA) and stored at –80°C until use.

To determine the concentration of these analytes in the extract, we performed a chromatographic analysis using liquid chromatography (HPLC)<sup>8</sup> and a mass spectrometer. Standards for aloin A, aloin B, and aloe emodin with 87.0%, 92.9%, and 96.3% purity were supplied by ChromaDex, Inc. (Irvine, CA, USA). The mobile phase consisted of 2 solutions of water/acetic acid (90:10; v/v) and water/acetonitrile/acetic acid (10:80:10; v/v) with a flow of 0.2 mL/min. The injection volume was 20 µL, the column temperature was 35°C and the corresponding flow rate was 24 mL/min.

Analyte ionization was performed using the following settings: curtain gas (N<sub>2</sub>) 15 psi, ion source gas 1 (GS1) and 2 (GS2) at 50 and 40 psi, respectively, source temperature at 400°C and voltage ionization at -4200 and 4200 V. Data on precursor ions and products were taken using the Multiple Reaction Monitoring option from Analyst Software Sciex (Concord, Canada).

### Microorganisms

The strains of *Staphylococcus aureus* ATCC29213, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Streptococcus uberis* ATCC 9927, and Methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA) (KWIK-STIK) were obtained from Microbiologics (St. Cloud, MN, USA).

### Treatment with *A. vera* Gel

A suspension of 10 mL of each strain, adjusted to 0.5 McFarland (A0,83-143), was prepared in MHCAB, using a spectrophotometer at 625 nm, as recommended by the Clinical and Laboratory Standards Institute (CLSI).<sup>23</sup> Each inoculum was treated with *A. vera* gel extract at concentrations of 10, 9, 16, and 10 mg/mL for *Staphylococcus aureus*, *E. coli*, *Streptococcus uberis*, and MRSA, respectively, considering the MIC for each strain, at a concentration of 0.5 McFarland, then incubated at 36 ± 1°C for 24 hours. A control of bacterial growth without *A. vera* gel extract was readjusted to 0.5 McFarland post incubation. All bacterial suspensions were centrifuged for 15 minutes at 10,000 × *g* and washed twice with a 0.85% NaCl solution.

### Fluorescence Spectrophotometry

The LIVE/DEAD BacLight bacterial viability kit (Molecular Probes Inc., Eugene, OR, USA) was used to determine the viability of the bacteria, as indicated by disruption of the cell membrane after treatment with *A. vera* gel extract, following the manufacturer's instructions.

Briefly, 100 µL of each bacterial suspension was incubated separately with 100 µL of a solution of 6 µL of propidium iodide and 6 µL of Syto 9 (viability kit) (diluted in 2 mL of filtered-sterilized water) at room temperature for 15 minutes in the dark. The intensity of the fluorescence was quantified by a spectrophotometer as the ratio of green fluorescence intensity emitted by the sample at 530 nm (excitation centered at 485 nm) in the case of living bacteria or when the cell membrane was undamaged. In cases when the cells were dead or the membranes damaged, quantification was performed according to the red fluorescence intensity emitted from the sample at 630 nm (excitation centered at 485 nm).

To determine the relationship between bacteria with cell membrane damage and fluorescence intensity, a calibration curve using bacterial suspensions of each strain in 10 mL of MHBCA, adjusted to 1-2 × 10<sup>8</sup> CFU/mL under the

mentioned conditions, was made. Samples were put in 1.5 mL Eppendorf tubes, and centrifuged for 15 minutes at 10,000 × *g*. In half of the tubes, the supernatant was replaced with 70% isopropyl alcohol to cause disruption in the cell membrane. In the rest of the tubes, the supernatant was replaced with 0.85% NaCl solution to maintain membrane integrity. The tubes were incubated for 1 hour at room temperature, in light and shaken every 15 minutes. From the latter, new bacterial suspensions were prepared using different proportions of bacteria with and without cell membrane damage (0:100, 10:90, 50:50, 90:10, 100:0). The suspensions were treated using the same staining procedure as the samples treated with the *A. vera* gel extract.

### Fluorescence Microscopy

Qualitative analysis was performed through a fluorescence microscope. For this purpose, 20 µL of the sample treated with *A. vera* gel extract and its respective growth control, stained and incubated under the aforementioned conditions, was placed on a glass microscope slide and covered with an 18 mm square coverslip. The samples were observed at a magnification of 10× using a fluorescence microscope. Fluorescence associated with viable (green) and nonviable (red) cells was measured at 510-540 and 620-650 nm, respectively. Images of 10 different fields were captured for each treatment using a camera operated by DP Manager software. The experiment was repeated 3 times for each strain. The samples were covered and observed under the microscope, and digital images were captured.

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### Declaration of Conflicting Interests

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