# First clues of *Flaveria trinervia* (Spreng.) C. Mohr as an adjuvant against bacteria responsible for Pneumonia

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***Abstract***

*In some regions of Mexico, Flaveria trinervia (Spreng.) C. Mohr, also known as "Scotch broom", is used to treat respiratory diseases, but no scientific studies have been carried out to confirm their possible therapeutic use. For this reason, in this research, the antimicrobial and antioxidant activities were evaluated along with its possible mechanism of action. 16 extracts of flowers, leaves, stems and the whole plant of Flaveria trinervia (Spreng.) C. Mohr were tested using hexane, chloroform, acetone and methanol as extraction solvents. The higher yield and antioxidant activity were obtained when methanol and acetone were used as extraction solvents for the leaves. The antimicrobial activity against microorganisms associated with pneumonia showed that the highest sensitivity was obtained for the two tested strains of S. aureus (50F and ATCC-43300) when acetone was used as extraction solvent (0.57 and 1.53 mg/mL respectively).*

*On the other hand, the sensitivity of the tested Gram-negative microorganisms [A. baumannii (A164) and P. aeruginosa (ATCC-14)] was in the range of 0.36 and 1.9 mg/mL, similar to the positive control. Similar cell membrane damage was observed in all tested microorganisms compared to the control group, especially regarding nucleic acid leakage. These results were confirmed by the increase in penetration of the crystal violet dye and the decrease in microbial density. The results are important considering the greater antimicrobial activity against Gram-positive bacteria.*

**Keywords:** *Acinetobacter baumannii*, antimicrobial activity, *Flaveria trinervia*, pneumonia, *Pseudomona aeruginosa*, *Staphylococcus aureus*.

## Introduction

Respiratory diseases are one of the main causes of annual deaths in the world[19]. However, the problem is greatest in Latin American countries, reaching an incidence of 30.8 deaths per 1,000,000 inhabitants in Mexico[32]. Bacteria-associated respiratory diseases have increased worldwide, mainly due to resistance to antibiotics caused by their inappropriate administration. For this reason, the mortality rate associated with antibiotic-resistant pathogens has risen to 1.27 million[52]. In recent years, a list of bacteria has been published that are considered critical for pneumonia, mainly due to their resistance to antibiotics.

Some Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were highlighted, while a Gram-positive bacterium such as *Staphylococcus aureus* was given a high priority[53]. Due to their resistance to antibiotics, various plant extracts have been used against these bacteria. However, both the part of the plant and the solvent used are important factors in their antimicrobial activity[31]. For this reason, proper identification and analysis of underutilized or new ethnobotanical plants are necessary to identify bioactive compounds as pharmaceutical adjuvants. In Mexico, one of the plants used in alternative medicine for pneumonia is *Flaveria trinervia* (Spreng.) C. Mohr or *Flaveria australasica*[46], which is also used in traditional medicine for the treatment of kidney disease, diarrhea, dysentery and gastritis[4,33,45].

On the other hand, the extracts of *Flaveria trinervia* extracts have shown high efficiency as remedies that reduce lipid peroxidation in the skin[4,47]. Considering that the members of the Asteraceae family, in addition to their medicinal properties, especially those of the genus *Flaveria*, have the potential to be used as phytoremediation agents and that they are considered accumulators of As[20]. An important factor that characterizes *Flaveria trinervia* is its high adaptability to different environments[41], which makes its cultivation possible and for this reason, several studies have been carried out on *Flaveria trinervia* as a possible aid in modern medicine. For example, it has been observed that the extract from the leaves of *Flaveria trinervia* has hepatoprotective effects and high antioxidant activity.

It has been reported that both extracts, the methanolic and the aqueous of *Flaveria trinervia*, are an analgesic that suppresses the central nervous system[27] and also acts as an anthelmintic and bactericidal agent[26]. On the other hand, an *in silico* study has shown that the ethanolic extract of *Flaveria trinervia* is a potential natural antidiabetic[50]. However, the potential effect of the individual parts of *Flaveria trinervia* against bacteria involved in pneumonia has not yet been reported. In addition, we are testing the hypothesis that there are differences in antimicrobial activity between Gram-positive and Gram-negative bacteria responsible for pneumonia. Therefore, this study aimed to

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evaluate the antimicrobial activity of hexane, methanolic, acetone and chloroform extracts from the whole plant and three parts of *Flaveria trinervia*, the stem, leaves and flowers.

## Material and Methods

**Vegetal material:** *Flaveria trinervia* (Spreng.) C. Mohr was collected manually during the flowering period in Celaya, Guanajuato, Mexico. Flowers, leaves, stems and the whole plant were analyzed separately. Each sample was cut into 5 mm pieces, dried in a convection oven (model Binder FD115-UL, USA) at 40 °C for 6 h, then ground to a fine powder and sieved to a particle size of 425 µm. The material was then stored in glass containers at room temperature until further analysis.

**Preparation of extracts:** For each extract, 30 g of the respective sample (flowers, leaves, stems and the whole plant) were used and organic solvents with different polarity (acetone, hexane, chloroform and methanol) were added in a ratio of 1:50 (plant material: solvent). Extraction was performed using a Soxhlet apparatus for approximately 20 cycles at the boiling points of the respective solvents: acetone (56 °C), hexane (69 °C), chloroform (61 °C) and methanol (65 °C). After extraction, the solvents were removed using a rotary evaporator (DragonLab RE100-Pro, China) and the extracts were stored in the dark at 4 °C under a nitrogen atmosphere until analysis.

The extracts obtained were labeled as follows: flower acetone (FA), flower hexane (FH), flower chloroform (FC), flower methanol (FM), leaf hexane (LH), leaf acetone (LA), leaf chloroform (LC), leaf methanol (LM), stem hexane (SH), stem acetone (SA), stem chloroform (SC), stem methanol (SM), whole plant acetone (WPA), whole plant hexane (WPH), whole plant chloroform (WPC) and whole plant methanol (WPM).

**Antioxidant activity:** The DPPH method was applied according to Brand-Williams et al[3] with some modifications. The free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, Germany) was used. Each sample (10 mg/mL) was diluted in absolute methanol, shaken for 2 min and centrifuged at 6000 rpm for 3 min. 50 µL of the supernatant was transferred to a vial and 1950 µL of DPPH in methanol (0.1 mM) was added. The decrease in absorbance (Optima, Model SP-3000nano, Tokyo, Japan) was determined at 515 nm from time 0 and every 10 min until completion of the reaction. Samples were run in triplicate. % DPPH radical was calculated using the equation:

$$DPPH\ inhibition\ (\%) = \frac{A_0 - A_S}{A_0} * 100 \quad (1)$$

where A0 is the absorbance of the control and AS is the absorbance of the test sample.

For the antioxidant activity of 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS), the method of Re[40] with some modifications was used. ABTS was dissolved in distilled water (7 mM) and $K_2S_2O_8$ (2.45 mM) was added. The mixture was then allowed to stand for 16 h at room temperature in the dark. 1980 µL of the ABTS radical was mixed with 20 µL of each sample and reacted for 6 min. The absorbance was measured at 734 nm.

**Total phenolic content**: In brief, 20 µL of each sample was placed in a vial (40 µg of sample) before adding 1580µL of distilled water and 100µL of Folin-Ciocalteu reagent (Folin and Ciocalteu's phenol reagent, Sigma Aldrich, Louis, MO, USA). The mixture was allowed to stand at room temperature for 8 min before adding 300 µL of a 20% sodium carbonate solution. After incubating the resulting reaction mixtures, total phenols were determined by colorimetry at 765 nm (Optima Plus, SP 3000 nano, USA) after resting for 2 h at room temperature in the dark. The results were expressed as gallic acid equivalents (GAE) in mg/g of extract[43].

**Antibacterial activity assays**

**Bacterial strains:** For this study, six bacterial strains were used: Gram-positive [*Staphylococcus aureus* (ATCC 43300) and *Staphylococcus aureus* (50F)] and Gram-negative strains [*Acinetobacter baumannii* (ATCC 17978), *Acinetobacter baumannii* (A164), *Pseudomonas aeruginosa* (ATCC 14) and *Pseudomonas aeruginosa* (42)]. All bacterial strains were obtained from the Ceparium of the Autonomous National University of Mexico.

**Gel diffusion technique:** Bacterial strains (1 pure colony from the SMA plate) were inoculated in 5 mL Luria-Bertani broth (LB) (Merck KGaA Darmstadt, Germany) and incubated at 36 °C ± 1 °C for 18-24 h (Daihan Labtech Co. incubator, model LIB-150M)[22,30]. A volume of 100 µL of each bacterial culture at a concentration of $1 \times 10^6$ CFU/mL was spread on standard SMA agar using the streak plate method. Filter paper disks (Whatmann no. 5) with a diameter of 6 mm were placed on the agar surface. Subsequently, 10 µL aliquots of each extract were added to the paper disks (final dose per disk: 1 mg extract). Disks containing 10% tween 80 (1 mg) were used as negative controls while ciprofloxacin and amoxicillin (1 mg) served as positive controls. Inhibition zones were measured after 24-hour incubation at 36 °C ± 1 °C and the average diameter values were calculated for each extract according to Cruz-Galvez et al[9] and Portillo-Torres et al[38].

**Minimum inhibitory concentration (MIC):** The MIC was determined according to the method of Valgas et al[48] with some modifications. Luria-Bertani broth (LB) was inoculated with extracts at concentrations ranging from 0.1 to 2.0 mg/mL (final microorganism suspension of 1 × 10^7 CFU/mL) and incubated at 36 °C ± 1 °C for 24 h. LB broth alone served as the positive control while the inoculum of each bacterium was the negative control. The MIC was

defined as the lowest extract concentration that completely inhibited bacterial growth after 24 h of incubation at 37 °C.

**Minimum bactericidal concentration (MBC):** The MBC was determined using aliquots of the lowest extract concentration at which no growth was observed (MIC). These were inoculated into standard method agar (SMA) using the pour plate technique and incubated at 36 ± 1 °C for 24 to 48 h. The MBC was defined as the lowest concentration of the extracts at which no colony growth was observed in SMA.

**Bacterial membrane disruption test:** The determination was carried out according to the protocols of Tang et al[44], Yang et al[55] and Zhong et al[57] with some modifications. The cultures with bacteriostatic activity [*S. aureus* (ATCC 43300), *S. aureus* (50F), *P. aeruginosa* (ATCC 14), *A. baumannii* (A164)] were inoculated and incubated in 4 mL of LB broth (20 µL of the 101 dilution) at 36°C ± 1°C for 24 h. Then suspensions were centrifuged for 10 min at 5000 rpm, the pellets were collected and washed with 0.5 M buffered potassium phosphate solution (PBS) at pH 7.4. The analyzed extracts [MIC: 0.57 mg/mL for *S. aureus* (50F); 0.57 to 1.53 mg/mL for *S. aureus* (ATCC 43300); 0.95 to 1.9 mg/mL for *A. baumannii* (A164) and 0.36 mg/mL for *P. aeruginosa* (ATCC 14)] were added to the bacterial suspensions and incubated for 0, 2, 4 and 6 h.

After incubation, the supernatants were separated from the cells by centrifugation for 20 min at 5000 rpm. To determine the release of nucleic acids, the supernatant was analyzed for absorbance at 260 nm using a UV-VIS spectrophotometer (Optima Model SP-3000nano, Tokyo, Japan). For protein content, the absorbance was measured at 595 nm. The positive control was ethylenediaminetetraacetic acid (EDTA) at a concentration of 0.25 mM and the negative control was PBS at a concentration of 0.5 mM.

**Alteration in membrane permeability:** The crystal violet assay according to Devi et al[13] and Portillo-Torres et al[38] with modifications was used to assess changes in membrane permeability. The bacterial strains with bacteriostatic activity [*S. aureus* (ATCC 43300), *S. aureus* (50F), *P. aeruginosa* (ATCC 14) and *A. baumannii* (A164)] were inoculated in LB broth and incubated at 36 °C ± 1 °C for 6 h. After incubation, the bacterial suspension was centrifuged at 5000 rpm for 10 min. The pellets were washed with 0.5 mM PBS and incubated with different concentrations of extracts [as bacterial strain with MIC (0.1 to 2.0 mg/mL) and MBC (0.5 to 3.0 mg/mL)]. EDTA (0.25M) was used as a positive control while PBS (0.5 mM) served as a negative control. Both controls were prepared in the same way as the samples.

Cells were centrifuged at 5000 rpm for 5 min and then suspended in PBS containing 10 µg/mL crystal violet. The cell suspension was incubated at 36 °C for 10 min and then centrifuged at 5000 rpm for further 5 min. The optical density (OD) at 590 nm of the supernatant was measured using a UV-VIS spectrophotometer. The OD value of the crystal violet solution was considered 100% excluded. The OD value of the wash suspension without extract was taken as blank. The percentage of uptake of crystal violet for all samples was calculated using equation 2:

$$Crystal\ violet\ uptake(\%) = \frac{OD\ value\ of\ sample}{OD\ of\ crystal\ violet\ solution} \times 100 \quad (2)$$

**Bacterial density:** Bacterial density was measured according to Peña-Martín et al[35], using ImageJ© software. Images were captured with the epifluorescence microscope (DFC450C Camera, DM500B Microscope, Leica Microsystems) at 100x magnification with immersion oil, 1/10s exposure time and a resolution of 1280 x 960 pixels in JPG format with 24-bit depth per channel. The results of the bacterial count were returned as a binary image with a coding between 0 and 1.

**Changes in the mobility of bacteria:** Mobility was carried out according to Gill and Holley[21] with modifications; *P. aeruginosa* (ATCC 14) was used for the determination, as it is the only flagellated bacteria with bacteriostatic activity in this work. Bacteria were inoculated in LB broth (enriched with yeast extract), incubated at 36°C ± 1 °C for 24 h and centrifuged for 10 min at 5000 rpm. Cells were washed and re-suspended in PBS 0.5 M, the extracts were added according to the results of MIC obtained. The sample was placed on slides and observed with an epifluorescence microscope (Leica DM 5000B, Germany) at a magnification of 100 X for 5 min. The results were expressed as positive mobility, reduced mobility or immobility. The positive control was EDTA and the negative control was the wash suspension without extract.

**Statistical analysis:** All data are expressed as mean ± standard deviation. ANOVA was performed with Tukey´s test. The Pearson correlation test was determined at $p < 0.05$, using the Origin Pro, version 2021 (OriginLab Corporation, Northampton, MA, USA).

## Results and Discussion

**Yield of the extraction:** As seen in table 1, a significant difference in the extracts yield can be observed, which can be attributed to the two variables evaluated, the solvent and the plant part studied. The methanolic extract from the leaves of *Flaveria trinervia* showed the highest yield; it was 50 % higher than the methanolic extracts from the flower, stem and whole plant. The higher yield observed in the leaf extracts is probably due to the biochemical machinery in the leaves which is responsible for the absorption and conversion of energy and for this reason, a high concentration of compounds is present in this part of the plant[5].

On the other hand, the differences in the yield of *Flaveria trinervia* extracts depending on the solvents are mainly due to their polarity[51]. The results obtained are in agreement with

those of Dieu-Hien et al[14], who indicated that extraction with methanol is more efficient than with acetone or chloroform as solvents.

**Antioxidant activity**: The values of *Flaveria trinervia* extracts using the DPPH method for free radicals are shown in table 2. Most of the extracts exhibited antioxidant activity, with the acetone and methanol extracts showing higher activity values. In contrast, the hexane and chloroform extracts showed lower antioxidant activity. For most of the plant parts tested, the acetone extract showed the highest antioxidant activity, but for the leaves, it was the methanolic extract. It has been reported that the DPPH radical scavenging activity of alcoholic leaf extracts is higher than that of acetonic leaf extracts[24], apparently, the specificity of methanol for the extraction of phenolic compounds contributes to its better antioxidant activity.

As shown in table 3, similar to the DPPH radical scavenging assay, the ABTS assay showed that the best antioxidant capacity was obtained with the acetonic and methanolic extracts. However, lower concentrations were required to achieve the $IC_{50}$ for the ABTS assay with the acetone extract than for the DPPH assay and a reverse result was observed with the methanolic extract. For the hexane and chloroform extracts, the concentrations required to achieve the $IC_{50}$ were higher than 10 mg and reached concentrations of up to 58 mg. The DPPH and ABTS assays showed comparable results for the antioxidant activity measured in the extracts.

Other studies have shown similar trends in both assays[17,37], however, the plant matrix is an important factor to consider as is the extraction solvent. Here, we found that the acetonic and methanolic extracts of *Flaveria trinervia* have strong antioxidant activities, so they could be considered an important potential source of natural antioxidants.

**Total phenolic content:** The results show that the methanolic and acetonic extract of the leaf and flower have a higher content of phenolic compounds than that of the stem and whole plant (Table 4) which could be due to the fact that in these structures, there is a greater accumulation of these metabolites, which can serve as a defense against predators[7,18]. On the other hand, the content of phenolic compounds was lower in the stem extracts. This result could be because these compounds are not transported through the phloem of the stem, so that only a small amount of phenolic substances is present which can play a regulatory role in the plant[7,18].

The differences between the content of phenolic compounds in the structures of the plant could be due to the fact that it has been reported that the distribution of phenolic compounds can vary significantly, which is due to their role in the life cycle and growth stages of the plant[18,25]. On the other hand, the absence of phenolic compounds in the hexane and chloroform extracts may be due to the fact that these compounds are not soluble in solvents with low polarity, but are soluble in polar solvents such as methanol, ethanol, acetone, ethyl acetate and their mixtures with different proportions of water[11,15].

**Antimicrobial activity:** The antimicrobial activity showed that both the ATCC 43300 strain and the 50F clinical isolate of *S. aureus* were most sensitive to the extracts. The acetone extracts of the stem, flower and whole plant were more effective against *S. aureus* than the acetone extract of the leaves. Apparently, the terpenoids in the acetone extracts of the flowers and leaves contribute to the antimicrobial activity against *S. aureus* in addition to their high content of phenolic compounds.

**Minimum inhibitory concentration (MIC):** The minimum inhibitory concentration (MIC) against *S. aureus* for the acetone extracts was between 0.57 and 1.53 mg/mL for flowers and stems, while for the leaves only, the chloroform extract showed inhibition at a concentration of 1.23 mg/mL as shown in table 5. Differences in MICs obtained by extracts with different solvents have been reported[1,6,42]. For the groups treated with acetone extracts, our results are in agreement with those of Aqil et al[2], who obtained MICs ranging from 0.59 to 1.75 mg/mL against *S. aureus* with acetone extracts of *Hemidesmus indicus* and *Holarrhena antidysenterica*, respectively. On the other hand, the MIC for *A. baumannii* (A164) was only achieved with the hexane and acetone extracts from the stems at a concentration of 0.95 mg/mL and 1.90 mg/mL respectively. Finally, for *P. aeruginosa* (ATCC14), only the acetone extract from the whole plant showed a minimum inhibitory concentration of 0.36 mg/mL (Table 6).

**Table 1**
**Yield (%) of the extracts of *Flaveria trinervia* using different solvents**

| Solvent | Vegetable Material | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf | | | Flower | | | Stem | | | Whole plant | | |
| | Mean | CI of mean 95% | | Mean | CI of mean 95% | | Mean | CI of mean 95% | | Mean | CI of mean 95% | |
| | | Lower | Upper | | Lower | Upper | | Lower | Upper | | Lower | Upper |
| Hexane | 10.24 | 9.64 | 10.77 | 9.55 | 9.28 | 9.82 | 14.7 | 14.27 | 15.12 | 12.21 | 11.82 | 12.60 |
| Chloroform | 12.77 | 12.41 | 13.25 | 5.25 | 3.51 | 6.98 | 10.7 | 10.54 | 15.12 | 13.56 | 13.40 | 13.73 |
| Acetone | 15.23 | 13.62 | 15.19 | 11.19 | 10.46 | 11.91 | 8.34 | 6.92 | 9.75 | 13.37 | 12.64 | 14.11 |
| Methanol | 32.62 | 28.19 | 35.92 | 21.41 | 18.00 | 24.82 | 22.6 | 17.05 | 28.14 | 22.55 | 22.41 | 22.68 |

Reported as mean and confidence intervals (CI) of mean at 95%.

**Table 2**
**Antioxidant properties of extracts and hydrolates of *Flaveria trinervia* expressed as scavenge 50% of free radical DPPH ($IC_{50}$).**

| Solvent | Vegetable Material | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf | | | Flower | | | Stem | | | Whole plant | | |
| | Mean | CI of mean 95% | | Mean | CI of mean 95% | | Mean | CI of mean 95% | | Mean | CI of mean 95% | |
| | | Lower | Upper | | Lower | Upper | | Lower | Upper | | Lower | Upper |
| Hexane | 62.47 | 58.50 | 66.50 | 101.23 | 69.92 | 132.55 | 47.61 | 41.83 | 53.39 | 77.05 | 73.24 | 82.70 |
| Chloroform | - | - | - | - | - | - | 23.89 | 23.81 | 23.96 | 20.28 | 19.81 | 20.75 |
| Acetone | 0.405 | 0.39 | 0.42 | 0.6 | 0.35 | 0.85 | 1.26 | 1.05 | 1.47 | 1.32 | 1.16 | 1.49 |
| Methanol | 0.672 | 0.64 | 0.71 | 3.17 | 3.023 | 3.31 | 1.63 | 1.56 | 1.71 | 3.95 | 3.54 | 4.37 |

Reported as mean and confidence intervals (CI) of mean at 95% at mg/mL. (-): Non-activity.

**Table 3**
**Antioxidant properties of extracts of *Flaveria trinervia* expressed as scavenge 50% of free radical ABTS (IC50).**

| Solvent | Vegetable Material | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf | | | Flower | | | Stem | | | Whole plant | | |
| | Mean | CI of mean 95% | | Mean | CI of mean 95% | | Mean | CI of mean 95% | | Mean | CI of mean 95% | |
| | | Lower | Upper | | Lower | Upper | | Lower | Upper | | Lower | Upper |
| Hexane | 18.39 | 17.44 | 19.35 | 58.09 | 51.35 | 64.82 | 45.07 | 41.51 | 48.62 | 58.96 | 42.04 | 75.87 |
| Chloroform | 12.42 | 11.23 | 13.61 | 73.15 | 61.59 | 84.71 | 52.68 | 38.81 | 66.55 | 22.11 | 20.78 | 23.44 |
| Acetone | 0.15 | 0.12 | 0.19 | 2.93 | 2.72 | 3.14 | 1.86 | 1.68 | 2.05 | 2.63 | 2.19 | 3.08 |
| Methanol | 1.34 | 1.30 | 1.40 | 4.28 | 3.83 | 4.73 | 2.18 | 1.76 | 2.61 | 3.02 | 2.54 | 3.51 |

Reported as mean and confidence intervals (CI) of mean at 95%. (-): Non-activity.

**Table 4**
**Total Phenolic Compounds (mg GA/g extract) of *Flaveria trinervia* extracts**

| Solvent | Vegetable Material | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf | | | Flower | | | Stem | | | Whole plant | | |
| | Mean | CI of mean 95% | | Mean | CI of mean 95% | | Mean | CI of mean 95% | | Mean | CI of mean 95% | |
| | | Lower | Upper | | Lower | Upper | | Lower | Upper | | Lower | Upper |
| Hexane | - | - | - | - | - | - | - | - | - | - | - | - |
| Chloroform | - | - | - | - | - | - | - | - | - | - | - | - |
| Acetone | 419 | 304 | 535 | 460 | 417 | 504 | 77 | 66 | 87 | 100 | 68 | 132 |
| Methanol | 106 | 104 | 109 | 126 | 96 | 155 | 64 | 47 | 80 | 62 | 33 | 91 |

Reported as mean and confidence intervals (CI) of mean at 95%. (-): No detected.

**Table 5**
**Minimum Inhibitory Concentration (mg/mL) of *Flaveria trinervia* extracts**

| Extracts | Bacterial strains | | | | | |
|---|---|---|---|---|---|---|
| | ***P. aeruginosa* ATCC 14** | ***P. aeruginosa* 42** | ***A. baumannii* ATCC 17978** | ***A. baumannii* A164** | ***S. aureus* ATCC 43300** | ***S. aureus* 50F** |
| FA | - | - | - | - | 0.57 | 0.57 |
| LC | - | - | - | - | 1.23 | 0.57 |
| SH | - | - | - | 0.95 | - | - |
| SA | - | - | - | 1.90 | 1.53 | 0.57 |
| WPA | 0.36 | - | - | - | 0.57 | 0.57 |
| **Antibiotics tested at 1 mg/mL** | | | | | | |
| Ciprofloxacin | + | nt | nt | + | + | + |
| Amoxicillin | + | nt | nt | - | + | + |

Abbreviations: (-) = non-activity; (+) = indicates complete absence of bacterial growth; nt = not tested; FA = flower acetone extract; LC = leaf chloroform extract; SH = stem hexane extract; SA = stem acetone extract; WPA = whole plant acetone extract.

**Table 6**
**Minimum Bactericidal Concentration (mg/mL) of *Flaveria trinervia* extracts.**

| Extracts | Bacterial strains | | | | | |
|---|---|---|---|---|---|---|
| | ***P.aeruginosa* ATCC 14** | ***P.aeruginosa* 42** | ***A.baumannii* ATCC 17978** | ***A.baumannii* A164** | ***S.aureus* ATCC 43300** | ***S.aureus* 50F** |
| FA | - | - | - | - | 0.57 | 0.57 |
| LC | - | - | - | - | - | - |
| SH | - | - | - | - | - | - |
| SA | - | - | - | - | - | - |
| WPA | - | - | - | - | 0.57 | 0.57 |
| **Antibiotics tested at 1 mg/mL** | | | | | | |
| Ciprofloxacin | + | nt | nt | + | + | - |
| Amoxicillin | + | nt | nt | - | - | - |

Abbreviations: (-) = non-activity; (+) = indicates total absence of bacterial growth; nt = not tested; FA= flower acetone extract; LC = leaf chloroform extract; SH = stem hexane extract; SA = stem acetone extract; WPA = whole plant acetone extract.

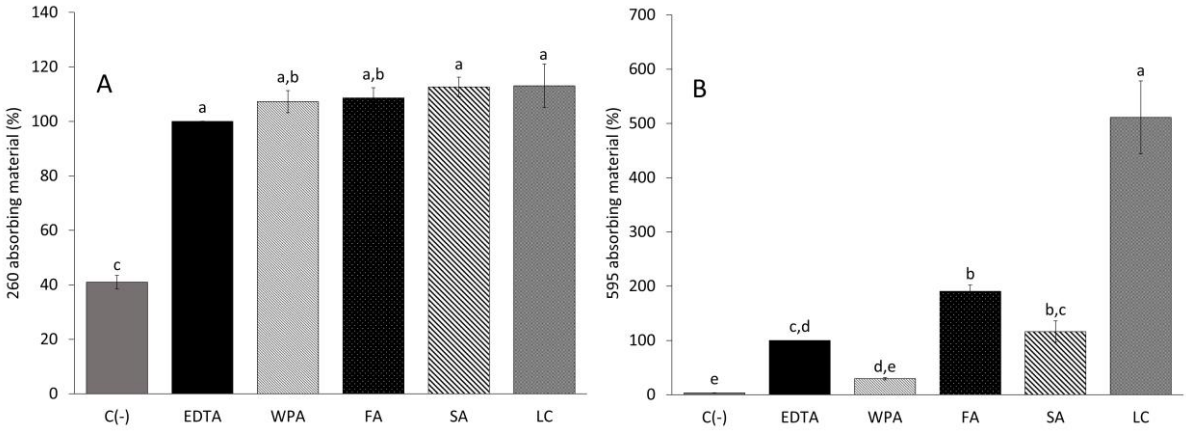


**Figure 1: Rate of leakage material from *S. aureus* ATCC (43300) in the absence and presence of experimental extracts. The lysing agent (EDTA 0.25M) induced 100% of the release of intracellular material; absence is the negative control. A) Nucleic acids (260 nm); B) proteins (595 nm). Abbreviations: WPA = acetone extract of whole plant; FA = acetone extract of flower; SA = acetone extract of stems; LC = chloroform extract of leaves**

Apparently, the presence of phenolic compounds contributes to the inhibition of the tested microorganisms, considering that the highest TPC values were obtained from the acetone extracts which showed antimicrobial activity for some microorganisms depending on the plant part used. In this study, the antimicrobial activity of whole plant, flower and stem acetone extracts was higher for both *S. aureus* (ATCC43300) and *S. aureus* (50F) than that reported by Hoskeri and Krishna[23] for *S. aureus*. For *P. aureginosa*, the whole plant acetone extract showed higher antimicrobial activity than that reported by the same authors using methanol and water extracts of *Flaveria trinervia* against *P. aureginosa*. In addition, the results obtained are in agreement with those of Kenny et al[28] for five plant species from the Asteraceae family with MICs between 187 and 365 µg/mL of water and ethanol extracts, probably due to the presence of various phenolic compounds such as phenolic acids, flavonoids and procyanidins.

**Intracellular material leakage:** In *S. aureus*, both the nucleic acid and protein concentrations were increased compared to the control (without extract) after 6 h of interaction ($p < 0.05$) (Figure 1A). Significant leakage of intracellular material was used as an indicator of severe damage to the microbial cell membrane[12]. For nucleic acids, the absorbance of samples treated with extracts was higher than that of samples treated with EDTA (Figure 1B), while in the quantification of proteins, only samples treated with acetone extract of flowers and stems showed higher absorbance than those treated with EDTA. Similar results were observed when chloroform was used as extraction solvent for leaves. It has been reported that RNA, protein and lipid synthesis of *S. aureus* are inhibited by the presence of flavonoids[49], which were identified in all extracts of *Flaveria trinervia*.

The fact that the absorbance of the material at 260 nm was greater than that of the control, suggests that this was the result of a direct effect on the cell membrane and cellular cytoplasmic components. The unexpectedly greater leakage of material by the chloroform extract of the leaves compared to the leakage of proteins in the presence of acetone extracts

from the whole plant, flower and stem could be due to the solubilizing effect of the chloroform-extracted components on the cell membranes, resulting in the release of membrane proteins.

The effect of the acetone whole plant extract on the integrity of the membrane of *P. aeruginosa* (ATCC14) was similar for the nucleic acids as for the EDTA-treated groups (Figure 2A). However, for the components that absorb at 595 nm, such as the proteins, the EDTA-treated groups are significantly higher than the acetone whole plant extract-treated groups (Figure 2B). These results confirm damage to the microbial cell membrane caused by *Flaveria trinervia* extracts. In *A. baumannii* (A164), both nucleic acids and proteins are released in similar concentrations (Figure 2C and 2D). These results indicate that the cell membranes of *A. baumannii* (A164) were initially damaged, leading to losses of intracellular components, resulting in an increase in material absorbing at 260 nm (nucleic acids).

**Cell rupture:** For the two *S. aureus* strains ATCC43300 and 50F treated with the *Flaveria trinervia* extracts, a lower permeability to crystal violet was achieved than for the samples treated with EDTA (Figure 3). In the Gram-negative bacteria, no significant difference in the permeability of the bacterial cell membrane was observed for *P. aeruginosa* (ATCC14) with the whole plant acetone extract (Figure 4), while for *A. baumannii* (A164) a significantly lower permeability of the bacterial cell membrane was observed with the acetone extract of the stem than with the control (EDTA).

However, when the hexane extract of the stem was used, a significantly higher permeability of the bacterial cell membrane was observed than with the control (Figure 4). The different resistance of microorganisms to *Flaveria trinervia* extracts, could be due to that their composition includes polysaccharides, lipids, proteins and nucleic acids. It has been reported that Gram-negative bacteria contain a greater number of amino acids, resulting in different biochemical properties between Gram-positive and Gram-negative bacteria[39].

According to some authors, the differences observed in the permeability of the bacterial cell membrane between Gram-positive and Gram-negative bacteria in the present study are due to the fact that the destabilization of the cell membrane is more complex in Gram-negative bacteria than in Gram-positive bacteria. Some authors associate this phenomenon with the differences in the outer membrane while Gram-positive bacteria only have the outer membrane, an additional restrictive barrier is observed in Gram-negative bacteria[29].

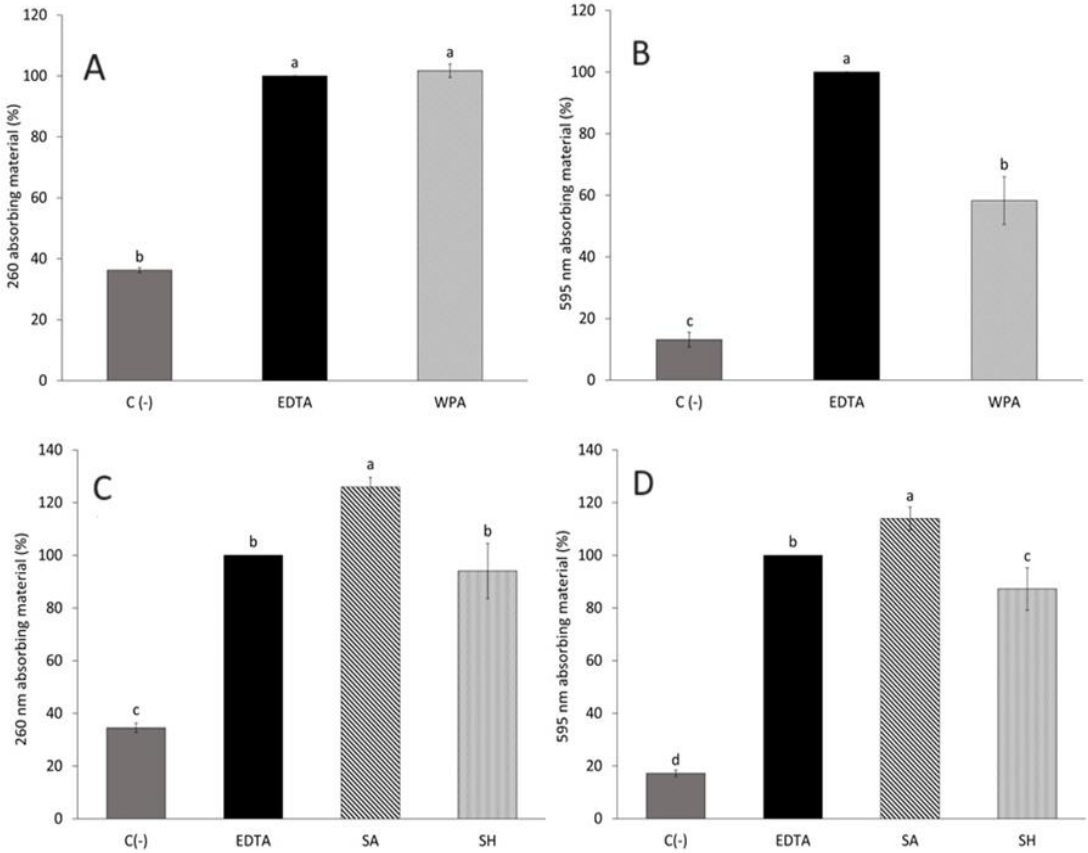


**Figure 2: Rate of leakage material. A) and B) *P. aeruginosa* (ATCC14), C) and D) *A. baumannii* (A164), in the absence and presence of experimental extracts. Lysing agent (EDTA 0.25M) induced 100 % of the release of intracellular material; absence is the negative control. A) and C) Nucleic acids (260 nm); B) and D) Proteins (595 nm).**

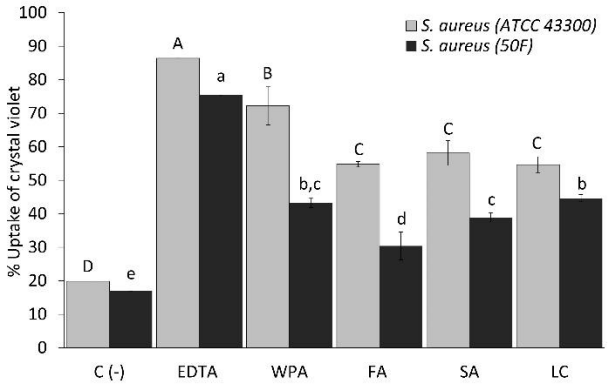


**Figure 3: Alteration of bacterial cell membrane permeability of Gram-positive bacteria with various extracts.**

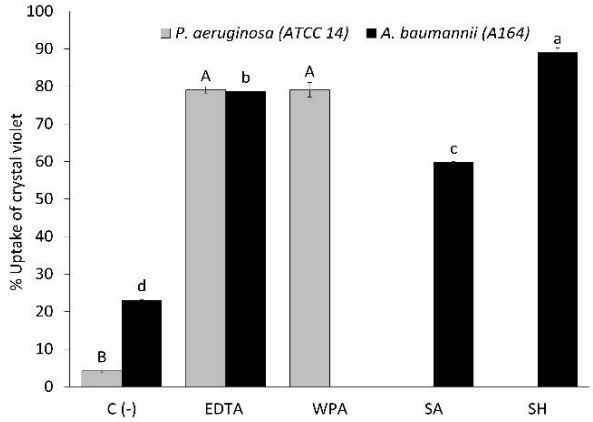


**Figure 4: Alteration of bacterial cell membrane permeability of Gram-negative bacteria with various extracts.**

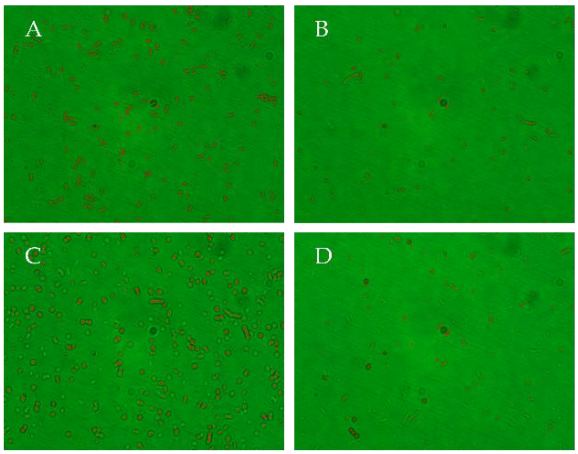


**Figure 5: Bacterial density of *A. baumannii* (A164) after treatments. A) negative control B) positive control (EDTA) C) stem acetone extract and D) stem hexane extract**

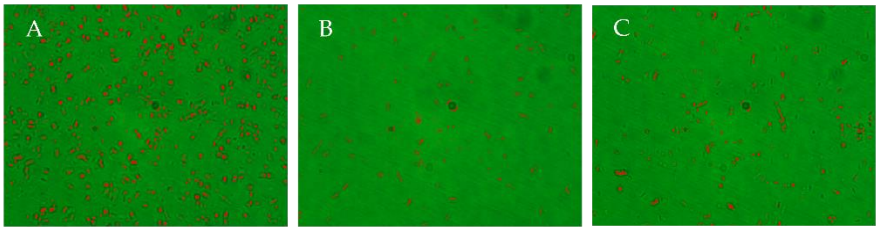


**Figure 6: Bacterial density of *P. aeruginosa* (ATCC14) after treatments. A) negative control B) positive control (EDTA) and C) whole plant acetone extract**

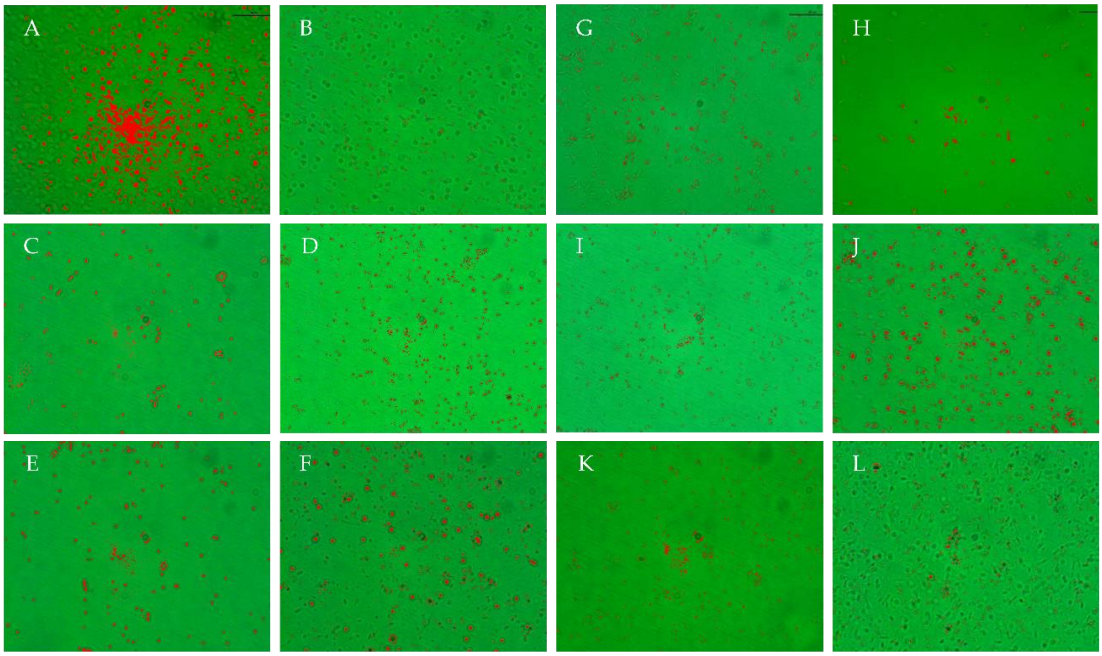


**Figure 7: Bacterial density of *S. aureus* (ATCC43300) after treatments. A) negative control, B) positive control (EDTA), C) whole plant acetone extract, D) flower acetone extract, E) stem acetone extract, F) leaf chloroform extract. *S. aureus* (50F) after treatments. G) Negative control, H) positive control (EDTA), I) whole plant acetone extract, J) flower acetone extract, K) stem acetone extract, L) leaf chloroform extract**

**Bacterial density estimation by image analysis:** As observed in figure 5, the estimation of bacterial density after treatment with the acetone and hexane extracts of the stems, showed a decrease in the total number of colonies of *A. baumannii* (A164). The greatest effect on the bacterial density of *A. baumannii* (A164) was observed with the hexane extract of the stems (Figure 5D) which decreased by 88 % compared to the negative control (Figure 5A), with the number of colonies being 22 % higher than the positive control (EDTA, 66 %). For *P. aeruginosa* (ATCC14), only the extract that showed an antimicrobial effect was evaluated (Figure 6). However, the efficacy of the whole plant acetone extract (56 %) was lower than that observed with the positive control (EDTA) which showed a 66 % reduction in the number of colonies.

Regarding the bacterial density of *S. aureus*, a reduction of 26% was observed with the addition of FA extract (Figure 7D). For both strains (ATCC43300 and 50F), the WPA extract showed a reduction in bacterial density of about 50% compared to the negative control. A similar reduction was observed with the extracts SA (Figure 7E) and LC (Figure 7F), but the reduction was about 50% lower than the bacterial density obtained with the positive control (EDTA).

The destabilization of the cell membrane caused by the extracts, is apparently a consequence of the presence of some volatile compounds such as terpenoids and alkaloids which are mainly present in the LC extract, since these compounds not only disrupt the cell membrane but also affect the bacterial enzyme system[34], that is why an increase in the permeability of the cell membrane produces a lower number of viable colonies. Similar results were obtained by Pinto et al[36] against Gram-positive bacteria such as *S. aureus* and *E. faecalis*, in which the total number of colonies decreased by 28 % and 40 % respectively. Further studies are needed considering that extracts from different vegetables cause cell membrane damage related to the presence of phenolic compounds[16].

The terpenoids contained in the extracts damage cell membranes by interacting with the phospholipids of the cell membrane and increase their permeability. The results are consistent with the data of Xu et al[54], who used a methanol-water extract of *Taraxacum officinale* from the Asteraceae family to evaluate its antimicrobial activity against *S. aureus* and observed that the cell wall and membrane increase the permeability of the bacteria, which appears to be due to the change in alkaline phosphatase acid (AKPA) concentration, electrical conductivity, intracellular protein content and DNA after exposure to the extract, which appears to be due to the presence of chlorogenic acid, luteolin, ferulic acid, caffeic acid and rutin.

**Bacterial motility:** The test was only carried out with *P. aeruginosa*, considering that is the only flagellated microorganism. The results obtained showed a drastic decrease in the motility of *P. aeruginosa* (ATCC 14) when the acetone extract was added to the whole plant, especially in terms of twitching and swarming motility. According to the qualitative results, flavonoids were identified in the whole plant acetone extract and are in agreement with the results reported by Vargas-Sánchez et al[49]. Bacterial motility

decreases when the flavonoids quercetin or naringenin are present.

**Minimal bactericidal concentration (MBC):** The minimal bactericidal concentration of the *Flaveria trinervia* extracts for the two strains of *S. aureus*, (ATCC 43300 and 50F), was 0.57 mg/mL and showed no bactericidal activity against *P. aeruginosa* (ATCC 14) and *A. baumannii* (A164) (Table 6). It has been reported that most natural extracts show higher antimicrobial activity against Gram-positive bacteria than against Gram-negative bacteria[39,56]. This is one reason why they apparently did not show bactericidal activity against the tested Gram-negative bacteria. Other authors point out that some compounds such as the flavonoids could be responsible for the bactericidal or bacteriostatic effect. However, due to the formation of aggregates in *S. aureus* strains in the presence of flavonoids, it is thought to be a single colony forming unit (CFU), giving the false impression that the number is reduced[10].

For this reason, extensive studies are required to evaluate the potential of the isolated *Flaveria trinervia* compounds. Nevertheless, the results obtained against *S. aureus* are first indication of its potential as a nutraceutical agent, considering that Chiavari-Frederico et al[8] reported MIC values > 10 mg/mL and MBC values > 500 mg/mL for the extracts of *Bidens pilosa*, *Tanacetum vulgare* and *Bidens sulphurea*, which may indicate a lower antimicrobial activity against *Staphylococcus aureus* than the extracts of *Flaveria trinervia*.

## Conclusion

The highest yield of *Flaveria trinervia* extracts was obtained when methanol or hexane were used as extraction solvents. However, the highest antioxidant activity was obtained with the methanol and acetone extracts obtained from the leaves. On the other hand, the two *S. aureus* strains ATCC 43300 and clinical isolate 50F were most sensitive to the extracts obtained mainly from the stem, flowers and whole plant with acetone extract. The MIC for *P. aeruginosa* (ATCC 14) was lower than that for *A. baumannii* (A164) and *S. aureus* using the acetone extracts. The highest intracellular material leakage was observed for *S. aureus*, while the lowest cell membrane permeability was observed for the Gram-negative bacteria.

The results to date indicate a different mechanism of action of the extracts of *Flaveria trinervia* against the microorganisms tested, that is why further studies need to be carried out. This study should be complemented by the investigation of other bacterial strains that are resistant to antibiotics, as the study was limited to four bacterial strains. For this reason, it is necessary to carry out further studies to complement the results obtained.

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