The in vitro antimicrobial and antioxidant activity of leaves of medicinal plants with phytobiotic potential in animal production

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Abstract

Little is known about which secondary metabolites are responsible for inhibiting pathogenic bacteria and reducing the pro-oxidant effect on the leaves of four medicinal plants used as phytobiotic in animal production. The aim of this study was to evaluate the antimicrobial and antioxidant activity of four medicinal plants (Anacardium occidentale, Psidium guajava, Morinda citrifolia and Moringa oleifera) in vitro. A total of six bacterial strains were inoculated, then minimum bactericidal concentration (MBC) was evaluated in fine powder and minimum inhibitory concentration (MIC) and MBC were determined on the aqueous extract. Also, the in vitro antioxidant activity was evaluated through 1,1-diphenyl-2-picryl-hydrazyl, as well as the main secondary metabolites were identified and quantified by chromatographic analysis. The results showed that Anacardium occidentale and Psidium guajava leaves had higher antimicrobial activity against all bacterial strains. In addition, Morinda citrifolia inhibited S. aureus in the aqueous extract, although without in vitro bactericidal effect, while Moringa oleifera leaf did not show antimicrobial effect. All plants showed antioxidant capacity, standing out Anacardium occidentale and Psidium guajava. Mainly the leaves of Anacardium occidentale showed high concentrations of quercetin 3-O-glucoside-7-O-rhamnoside, kaempferol-7-O-glucoside, quercetin, caffeic acid, and cinnamic acid. Apparently, the antimicrobial and antioxidant activity are due to the main polyphenolic compounds identified in medicinal plants (mainly Anacardium occidentale and Psidium guajava); however, further studies are necessary to elucidate the exact mechanism.

Introduction

The European Union totally eliminated the use of growth-promoting antibiotics in animal production on January 1st, 2006, prompting many countries to reduce or eliminate these synthetic products. Subtherapeutic antibiotics are known to cause microbial resistance and cross-resistance with other microorganisms that inhabit animals and humans. Therefore, there is a growing interest in research to find natural alternatives to antibiotics; especially medicinal plants with beneficial phytochemical compounds and with antimicrobial, anti-inflammatory and antioxidant properties (Martínez et al. 2020).

In that context, phytogenic additives have proven to be an important alternative to enhance the genetic expression of farm animals without the use of dietary antibiotics. Thus, phytogenic feed additives are included among supplements in order to positively affect feed quality, animal health and animal products caused by their specifically effective substances (Karasková et al. 2015). Likewise, approximately 80% of the population in developing countries use medicinal plants systematically for humans and animals, in Cuba 1,170 species of these plants are reported, where 56% are known for their curative and preventive properties (Ramírez et al. 2020). Plants for antibacterial purposes are used to heal wounds, relieve digestive and oral discomfort, and additives in the farm animal diet, among others (Martínez et al. 2013; Más et al. 2016; Aroche Ginarte et al. 2017; Salazar Bell et al. 2017; Aroche et al. 2018). Thus, plants such as Anacardium occidentale (A. occidentale), Psidium guajava (P. guajava), Morinda citrifolia (M. citrifolia), and Moringa oleifera (M. oleifera) are some of the most used as adjuvants to therapeutic treatment in different diseases in humans and animals.

Anacardium occidentale, (family Anacardiaceae) is commonly used in the infection treatment, hemorrhages, diarrheal processes, and diabetes in animals, and also have shown that small concentrations could increase the egg production and egg quality and could decrease pig’s diarrheal syndrome (Sunderam et al. 2019; Khatib et al. 2020; Siracusa et al. 2020). Its antimicrobial activity has also been verified in the ethanolic extract of flowers, bark, and leaves, relating it to the alkaloid, saponin, phenolic acid and tannin contents (da Silva et al. 2016).

Several investigations have shown that leaves, fruits, bark and roots of P. guajava have been used to alleviate several illnesses, such as gastrointestinal diseases, diarrheal syndrome, stomach pain, diabetes mellitus, hypertension, wound healing, inflammations and obesity. Also, in animal production, P. guajava has been shown to promote egg production, eggshell thickness and reduce the liquid feces of pigs after weaning (Gupta et al. 2019; Salihu Abdallah et al. 2019; Welí et al. 2019; Ceballos-Francisco et al. 2020).

Morinda citrifolia are also popular for their variety of benefits in human health and animal production, such as antimicrobial, anticancer, antioxidant, anti-inflammatory, analgesic, cardiovascular, among others (Senthilkumar et al. 2016; Sunder et al. 2016; Thot et al. 2017). Those properties made possible to include its leaves and fruits in poultry and pig diets with positive effects on egg production and body weight in order to increase the animal performance (Sunder et al. 2016; Aroche Ginarte et al. 2017; Salazar Bell et al. 2017; Aroche et al. 2018).

Moringa oleifera as a functional food in human health and animal production is very popular, especially for its high nutritional content of protein, minerals and vitamins (Zhang et al. 2018; Wang et al. 2018; Su and Chen, 2020). Likewise, beneficial effects of M. oleifera have been found due to its anticancer, anti-inflammatory, antidiabetic, antioxidant and antimicrobial activity (Siddhuraju and Becker, 2003; Dhakak et al. 2019). Regarding to animal use of M. oleifera, several authors recommend it as a feasible source of nutrients for ruminant and non-ruminant animals (Mahfuz and Piao, 2019; Su and Chen, 2020; Valdivie et al. 2020).

These four plants have a marked global interest for animal production, due to their nutraceutical properties to improving productive indicators, intestinal health, and quality of the final product (e.g., egg, meat and milk) in animals (Martínez et al. 2012, 2013; Más et al. 2015, 2016; Aroche Ginarte et al. 2017; Cañete Sera et al. 2017; Salazar Bell et al. 2017; Aroche et al. 2018; Ramírez et al. 2020). However, little is known about which of these plants has the highest antibacterial and antioxidant potential related to animal production, which would allow to elucidate the medicinal benefits reported animals of zootechnical interest; therefore, the objective of this investigation was to determine the antimicrobial and antioxidant effect of A. occidentale, P. guajava, M. citrifolia y M. oleifera leaves and aqueous extract in vitro.

Material And Methods

Plant Material
Leaves of *A. occidentale*, *P. guajava*, *M. oleifera* and *M. citrifolia* were collected in Granma province, Cuba, in February/2019, during the low rainy season; this zone is characterized by a flat topography and charcoal brown soil, authenticated by specialists from the Faculty of Agricultural Sciences of the University of Granma. The plants were more than one year old and without any sign of pathology. The leaves were dried in the shade, with free air circulation to constant weight and then dried in a stove (WSU 400, German) with air recirculation for 1 hour at 60°C. Subsequently, the leaves were crushed in a hammer mill with parallel blades, at 1 mm of size. The samples were stored at room temperature (26°C) in fully airtight plastic bags until further use.

*In vitro* experiments were performed at the Feed Research Institute of the Chinese Academy of Agricultural Sciences to determine the antibacterial and antioxidant activity of the leaves and their aqueous extracts.

**Preparation of the Fine Powder and Aqueous Extract**

To obtain the fine powder, the leaves were ground in a commercial grain crushing machine (Zhejiang Horus Industry and Trade Co., Ltd., Zhejiang, China) through a 40 mesh (0.45 mm) sieve (Yoston, China) and stored in completely airtight bags until use for microbiological tests.

Also, 16.67 g of the leaves of each plant were weighed and mixed with 500 mL of water (30:1, v/w) for aqueous extraction. The aqueous extract was obtained by the sonication method, using an ultrasonic extractor (model SY-1000E, China) for 50 minutes at 50°C, allowed to stand for 1 hour, and filtered through Whatman filter paper. No. 1. It was subsequently condensed through a rotary evaporator (model RE-2000, China), under reduced pressure at 45°C at 60 rpm. The extract was frozen at -80°C for at least 4 hours, and finally dried in a lyophilizing machine (model LGJ-18, China).

**Minimum Bactericidal Concentration of Fine Powder**

The MBC of the fine powder from leaves of the four plants was determined for triplicated. Bacterial culture was inoculated and incubated for 12 hours, later, 90 mm diameter petri dishes were prepared with Mueller-Hinton Agar (MHA) at different concentrations of the fine powder. Each bacterial culture of 100 µL of was inoculated, which consisted in strains of enterotoxigenic *Escherichia coli* (ETEC) K88*, *Escherichia coli* ATCC 1515, *Staphylococcus aureus* (*S. aureus*): ATCC 43300 and ATCC 25923, *Salmonella enteritidis* (*S. enteritidis*): ATCC 3377, and *Salmonella typhimurium* (*S. typhimurium*): ATCC 14028. In the first period, concentrations of 5, 15 and 30 mg/mL of fine powder in the culture medium were tested to identify the minimum concentration of each plant in that range. Then, concentrations less than 5 mg/mL were used for leaves of *A. occidentale* and *P. guajava*; 5 to 15 mg/mL of fine powder in culture medium in the case of *M. citrifolia* and 15 to 30 mg/mL of fine powder of culture medium with *M. oleifera*.

**Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Aqueous Extract from the Plants**

For this study, aqueous extract of the four plants was used, thus a stock solution of 13 mg/mL was prepared, which was used to prepare in serial dilutions of 13, 6.5, 3.25, 1.63, 0.81, 0.41, 0.2, 0.1, 0.05, 0.03, and 0.01 mg/mL. The inoculum of *E. coli* (ETEC K88*), *S. aureus* (ATCC 43300), and *S. typhimurium* (ATCC 14028) were prepared in culture medium at a concentration of approximately 15 x 10⁷ CFU/mL compared to theoretical optical density (550 nm absorbance) that defines the level of 0.50 in the McFarland turbidimetric scale. Then, 200 µL/well of each dilution was placed in 96-well microplates and 2 µL of each bacterial culture was inoculated for triplicates, incubated for 12 hours at 37°C to determine its absorbance in a plate reader (ELISA, BIO-TEK, Synergy HT).

Determination of the MBC was carried out for triplicated, 100 µL of supernatant from those wells where bacterial growth was inhibited and seeded with a sterile glass triangular spatula in 90 mm diameter petri dishes with Mueller-Hinton Agar (MHA), and incubated for 12 hours at 37°C.

**Antioxidant Activity**

Antioxidant activity of aqueous extract of leaves from the four plants was evaluated with DPPH− (Shen et al. 2010) where a solution of 0.1 mM of DPPH− in methanol was prepared. Later, 1 mL of this solution was taken and vigorously mixed in a vortex with 3 mL of the different concentrations (10, 5, 2.5, 1.25, 0.625, 0.313, 1.156, 0.078, 0.039, 0.020 and 0.010 mg/mL) of the extract, and 200 µL of each concentration were placed in a 96-well microplate. The solutions were left to stand at room temperature in the dark for 30 min and then, the absorbance at 517 nm was measured with the use of a plate reader (ELISA brand, BIO-TEK, Synergy HT). BHT was used as reference. Low absorbance values indicate high free radical scavenging capacity, or high antioxidant capacity, which was calculated using the following formula:

Antioxidant effect of DPPH− (％ inhibition) = [(A0 - A1) / A0 * 100], where A0 is the absorbance of the control reaction, and A1 is the absorbance in the presence of the extracts and the reference. All samples were evaluated in triplicate and the results were averaged and shown as IC₅₀ values (mg/mL).

**Identification and Quantification of Major Compounds from Leaves of the Four Plants**

**Pretreatment Method**

The sample leaves (40 mg) from *A. occidentale*, *P. guajava*, *M. citrifolia* and *M. oleifera* were added to 4 mL of extractant and were shaken under ultrasonic for 30 min. Then, centrifugation for 5 min to take the supernatant and the membrane was done.

1. *A. occidentale* leaves 41.93 mg + extractant (0.8 mL EDTA buffer solution + 3.2 mL methanol)
2. *P. guajava* leaves 41.94 mg + extractant (0.8 mL EDTA buffer solution + 3.2 mL methanol)
3. *M. citrifolia* leaves 44.50 mg + extractant (0.8 mL EDTA buffer solution + 3.2 mL methanol)
4. *M. oleifera* leaves 44.20 mg + extractant (0.8 mL EDTA buffer solution + 3.2 mL methanol)
EDTA buffer solution: weigh 7.10 g of anhydrous sodium hydrogen phosphate, 1.95 g of disodium edetate, 8.40 g of citric acid, and dissolve in 650 mL of water.

**Chromatographic Method**

The column used was an Agilent’s Zorbax Eclipse Plus-C18 (3.0 x 150 mm, 1.8 µm). Mobile phase A: water (0.1% formic acid and 0.2 mmol/L ammonium acetate); mobile phase B: methanol (0.1% formic acid and 0.2 mmol/L ammonium acetate). Separation gradient (0-1 min: 10% B; 1-9 min: 10% B-90% B; 9-11 min: 90% B-100% B; 11-11.1 min: 100% B-10% B; 11.1-13 min: 10% B). The column temperature was 29.5 °C, the injection volume was 2 µL and the flow rate 0.25 mL/min (Fang et al. 2007).

**Mass spectrometry (MS)**

Electrospray ionization (ESI) in positive/negative mode with a MS2 Scan was used in MS. The drying gas temperature was 250°C and dry gas flow rate of 7 L/min, with an atomizing gas pressure of 35 psi. Sheath gas temperature of 325°C and sheath gas flow of 11 L/min was used, and a fragmenter of 80 V, 100 V, 120 V with a cell accelerator voltage of 5 V was used.

**Description**

The pretreatment was separately extracted with methanol and acetonitrile. The results showed that the extraction with methanol was better. In the full scan of the parent ion, the positive and negative ion modes are used for simultaneous scanning, and the results can be mutually verified. The addition of formic acid to the mobile phase increased the sensitivity of the compound both in positive ions and in negative ion mode. The addition of ammonium acetate improved the peak shape of the chromatogram. There may be ions in positive ion mode: [M+H] +, [M+Na] +, [M+NH4] +, [2M+H] +; and there may be ions in the negative ion mode: [M+CH3COO]-, [M+COO]-.

**Qualitative Method**

Forty mg sample of leaves from *A. occidentale* and *P. guajava* were added to 4 mL of extractant and were shaken under ultrasonic for 30 min. Then, centrifugation for 5 min to take the supernatant and the membrane was done.

EDTA buffer solution: weight 7.1 g of anhydrous sodium hydrogen phosphate, 1.95 g of disodium edetate, 8.4 g of citric acid, and dissolve in 650 mL of water.

**UHPLC-MS/MS Conditions**

Chromatographic analysis was performed on a Waters acquity ultrahigh-performance liquid chromatography system, using an Agilent Zorbax Eclipse Plus C18 column (3.0 x 150 mm, 1.8 µm). Mobile phase A: water (0.1% formic acid and 0.2 mmol/L ammonium acetate); mobile phase B: methanol (0.1% formic acid and 0.2 mmol/L ammonium acetate). Separation gradient (0-1 min: 10% B; 1-2 min: 10% B-60% B; 2-7.5 min: 60% B-90% B; 7.5-8.0 min: 90% B-100% B; 8.0-8.1 min: 10% B). The injection volume was 2 µL and the flow rate 0.30 mL/min (Fang et al. 2007).

MS was performed on a Sciex Triple Quad 4500 MS/MS, and electrospray ionization coupled with multiple reaction monitoring (MRM) model. The resulting optimized values were as follows: source temperature 450°C; ion spray voltage 4500 V; collision gas: 9 psi; curtain gas 10 psi; ion source gas (GS 1) 18 psi; and ion source gas (GS 2) 0 psi.

**Statistical Analysis**

Data were processed by simple classification ANOVA in a completely randomized design. Before this, the normality of the data was verified using the Kolmogorov-Smirnov test and for uniformity of variance, the Bartlett test. When the effects were significant, the means were separated using Duncan’s test at the significance level of *P* ≤ 0.05. All analyzes were carried out in accordance with the SPSS statistical software, version 21.0 (SPSS Inc., Chicago, IL, USA).

**Results**

The MBC of the leaves of *A. occidentale*, *P. guajava*, *M. citrifolia* and *M. oleifera* against six strains of pathogenic bacteria is showed in Table 1. Leaves of *A. occidentale* showed the greatest bactericidal effect in the study, mainly against *Escherichia coli* K88 and *Staphylococcus aureus* (ATCC 25923) with concentrations of 4 and 1 mg/mL, respectively. Likewise, the leaves of *P. guajava* showed a bactericidal effect by reducing the growth of Gram negative and Gram-positive bacteria with a concentration of 11 mg/mL for *E. coli* K88. Also, the leaves of *M. citrifolia* and *M. oleifera* only showed bactericidal activity against the strains of *S. aureus* (ATCC 43300; ATCC 25923) although with higher doses (8-16 mg/ml) than the inhibitory effects of the leaves of *A. occidentale* and *P. guajava*. 
Table 1
MBC of the leaf powder of four plants against six bacterial strains (mg/mL)

| Bacteria                        | AO | PG  | MC  | MO  |
|---------------------------------|----|-----|-----|-----|
| *E. coli* K88*                  | 4.0| 11.0| NI  | NI  |
| *E. coli* (ATCC 1515)          | 4.0| 5.0 | NI  | NI  |
| *S. aureus* (ATCC 43300)       | 1.0| 1.0 | 8.0 | 16.0|
| *S. aureus* (ATCC 25923)       | 0.5| 5.0 | 15.0| NI  |
| *S. enteritidis* (ATCC 3377)   | 4.0| 4.0 | NI  | NI  |
| *S. typhimurium* (ATCC 14028)  | 2.0| 2.0 | NI  | NI  |

AO: Anacardium occidentale. PG: Psidium guajava. MC: Morinda citrifolia. MO: Moringa oleifera. NI: No inhibition.

MIC and MBC of the aqueous extract of the leaves of the four plants in study are shown in Table 2. Similar to the fine powder of the leaves, the aqueous extracts of *A. occidentale* and *P. guajava* had the highest bactericidal activity. It should be noted that MIC and MBC to inhibit the growth of *E. coli* K88*+* is the same in both medicinal plants (6.5 mg/ml).

Table 2
MIC and MBC of the aqueous extract of the leaves of the plants (mg / mL)

| Extracts | *E. coli* (K88*) | *S. aureus* (43300) | *S. typhimurium* (14028) |
|-----------|------------------|---------------------|--------------------------|
|           | MIC   | MBC   | MIC  | MBC   | MIC  | MBC  |
| AO        | 6.5   | 6.5   | 0.81 | 0.81  | 3.25 | 3.25 |
| PG        | 6.5   | 6.5   | 0.81 | 1.63  | 6.5  | 6.5  |
| MC        | NI    | NI    | 6.5  | NI    | NI   | NI   |
| MO        | NI    | NI    | NI   | NI    | NI   | NI   |

MIC: Minimum Inhibitory Concentration. MBC: Minimum Bactericidal Concentration. AO: Anacardium occidentale. PG: Psidium guajava. MC: Morinda citrifolia. MO: Moringa oleifera. NI: No inhibition.

Likewise, a higher concentration of the aqueous extract of *P. guajava* (compared to the aqueous extract of *A. occidentale* leaves) is necessary to inhibit and eliminate *S. typhimurium* (ATCC 14028), similar occurred for the bactericidal effect of this product against *S. aureus* (ATCC 43300). The aqueous extract of *M. citrifolia* only inhibited the growth of *S. aureus* (ATCC 43300) at doses of 6.5 mg/mL, however, it did not show bactericidal activity at the concentrations studied (maximum concentration of 13 mg/mL). Also, the *M. oleifera* extract did not show inhibitory or bactericidal activity.

Table 3 shows the IC<sub>50</sub> of the aqueous extract of the leaves of the four plants. *A. occidentale* plant with the highest free radical trapping activity compared to the other three plants, as it reflects the lower IC<sub>50</sub> being even lower (P<0.001) than the positive control butylated hydroxytoluene (BHT). Furthermore, *P. guajava* did not show (P>0.05) statistical differences with *A. occidentale* and BHT. However, *M. oleifera* and *M. citrifolia* had the lowest results in antioxidant activity, as they require the highest concentration to inhibit the 1,1-diphenyl-2-picryl-hydrazyl (DPPH−) reaction.

Table 3
IC<sub>50</sub> of the aqueous extract of the leaves of the four plants

| Extracts            | IC<sub>50</sub> (mg/mL) |
|---------------------|-------------------------|
| *Anacardium occidentale* | 0.028±0.0006<sup>a</sup> |
| *Psidium guajava*    | 0.069±0.0061<sup>ab</sup> |
| *Morinda citrifolia* | 6.269±0.0665<sup>d</sup> |
| *Moringa oleifera*   | 0.603±0.0102<sup>c</sup> |
| BHT                  | 0.093±0.0101<sup>b</sup> |

IC<sub>50</sub>: Extract concentration required to inhibit the DPPH− reaction by 50%. Data are mean ± SD (n = 3). Values followed by different letters within a column are significantly different (P<0.05) according to the Duncan. BHT used as a positive control.
The phytochemical compounds identified in the four plants and compared with those reported in the literature are shown in Table 4, where the information on antibacterial and antioxidant activity was compared based on the scientific literature.
| Phytochemical subclass | Name                                         | AO | PG | MC | MO | Properties | References                      |
|------------------------|----------------------------------------------|----|----|----|----|------------|----------------------------------|
| Flavonoids             | Flavonoids                                   |    |    |    |    |            |                                  |
|                        | Anthocyanins                                 |    |    |    |    |            |                                  |
|                        | Cyanidin 3-O-xylosyl-rutinoside               | X  | X  | X  | X  | X          | (Diaconeasa et al. 2020)         |
|                        | Cyanidin 3-O-(6''-acetyl-galactoside)         | X  | X  |    |    | X          | (Diaconeasa et al. 2020)         |
|                        | Cyanidin 3-O-(6''-acetyl-glucoside)           | X  | X  |    |    | X          | (Einbond et al. 2004)            |
|                        | Petunidin 3-O-rhamnoside                     | X  | X  |    | X  |            | (Diaconeasa et al. 2020)         |
|                        | Delphinidin 3-O-galactoside                  | X  | X  | X  |    | X          | (Einbond et al. 2004)            |
|                        | Delphinidin 3-O-glucoside                    | X  | X  | X  |    |            | (Einbond et al. 2004)            |
|                        | Pelargonidin 3-O-galactoside                 | X  | X  |    | X  |            | (Martínez et al. 2020)           |
|                        | Delphinidin 3-O-sambubioside                 |    |    |    | X  |            | (Einbond et al. 2004)            |
|                        | Malvidin 3-O-glucoside                       | X  | X  |    |    |            | (Diaconeasa et al. 2020)         |
|                        | Peonidin 3-O-(6''-p-coumaroyl-glucoside)     |    |    |    | X  |            | (Salehi et al. 2019)             |
|                        | Peonidin 3-O-rutinoside                      |    |    |    | X  |            | (Salehi et al. 2019)             |
|                        | Dihydrochalcones                             | X  |    |    |    | X          | (Zamroziewicz and Barbery, 2016)  |
|                        | Dihydroflavonols                             | X  | X  |    |    |            | (Hajimahmoodi et al. 2014)        |
|                        | Flavanones                                  | X  | X  | X  |    |            | (Chotphruethipong et al. 2019)    |
|                        | Flavanones                                  | X  |    |    |    |            | (Hajimahmoodi et al. 2014)        |
|                        | Flavanones                                  | X  |    |    |    |            | (Sangweni et al. 2020)            |
|                        | Flavanones                                  |    | X  | X  |    |            | (Samarakoon et al. 2012)          |
|                        | Flavanones                                  |    |    |    | X  |            | (Bannour et al. 2017)             |
|                        | Flavones                                    |    |    |    |    |            | (Hajimahmoodi et al. 2014)        |
|                        | Flavones                                    |    |    |    |    |            | (Martínez et al. 2020)            |
|                        | Flavones                                    |    |    |    |    |            | (Martínez et al. 2020)            |
|                        | Flavones                                    |    |    |    |    |            | (Martínez et al. 2020)            |
|                        | Flavones                                    |    |    |    |    |            | (Martínez et al. 2020)            |
|                        | Flavones                                    |    |    |    |    |            | (Martínez et al. 2020)            |
|                        | Flavones                                    |    |    |    |    |            | (Dsouza and Nanjiaiah, 2018)      |
|                        | Flavones                                    |    |    |    |    |            | (Liu et al. 2016)                |
|                        | Flavones                                    |    |    |    |    |            | (Osman et al. 2014)              |

AO: Anacardium occidentale. PG: Psidium guajava. MC: Morinda citrifolia. MO: Moringa oleifera.
| Phytochemical subclass | Name                                                                 | AO | PG | MC | MO | Properties          | References                              |
|------------------------|----------------------------------------------------------------------|----|----|----|----|----------------------|-----------------------------------------|
| Flavones               | Cirsimaritin                                                         | X  | X  | X  |    | Antibacterial        | (Ren et al. 2019)                       |
| Flavones               | Apigenin 6,8-di-C-glucoside                                          | X  | X  | X  |    |                      | (Călinoiu and Vodnar, 2020)             |
| Flavones               | Chrysoeriol 7-O-apiosyl-glucoside                                    | X  |    | X  |    |                      | (Bannour et al. 2017)                   |
| Flavones               | Luteolin 7-O-rutinoside                                               | X  | X  | X  |    |                      | (Pereira et al. 2016)                   |
| Flavones               | Luteolin 7-O-malonyl-glucoside                                        | X  | X  | X  |    |                      | (Pereira et al. 2016)                   |
| Flavonols              | Quercetin 3-O-(6''-acetyl-galactoside) 7-O-rhamnoside                 | X  | X  | X  | X  |                      | (Hajimahmoodi et al. 2014)              |
| Flavonols              | Methylgalangin                                                       | X  | X  | X  |    |                      | (Echeverría et al. 2017)                |
| Flavonols              | 3-Methoxynobiletin                                                   | X  | X  | X  |    |                      | (Bannour et al. 2017)                   |
| Flavonols              | Kaempferol 7-O-glucoside                                              | X  |    | X  |    |                      | (Salehi et al. 2019)                    |
| Flavonols              | Quercetin 3-O-rhamnoside                                              | X  | X  |    |    |                      | (Hajimahmoodi et al. 2014)              |
| Flavonols              | Kaempferol 3-O-galactoside                                            | X  | X  | X  |    |                      | (Almeida et al. 2019)                   |
| Flavonols              | Kaempferol 3-O-glucoside                                              | X  | X  | X  |    |                      | (Salehi et al. 2019)                    |
| Flavonols              | Quercetin 3-O-arabinoside                                             | X  | X  | X  |    |                      | (Hajimahmoodi et al. 2014)              |
| Flavonols              | Quercetin 3-O-xylloside                                               | X  | X  | X  |    |                      | (Hajimahmoodi et al. 2014)              |
| Flavonols              | Myricetin 3-O-galactoside                                             | X  | X  | X  |    |                      | (Marín et al. 2018)                    |
| Flavonols              | Myricetin 3-O-glucoside                                               | X  | X  | X  |    |                      | (Marín et al. 2018)                    |
| Flavonols              | Myricetin 3-O-arabinoside                                             | X  | X  | X  |    |                      | (Marín et al. 2018)                    |
| Flavonols              | Quercetin 3-O-glucosyl-xylloside                                      | X  | X  | X  |    |                      | (Hajimahmoodi et al. 2014)              |
| Flavonols              | 3-Methoxysinensetin                                                  | X  |    |    |    |                      | (Biswas et al. 2019)                   |
| Flavonols              | Isorhamnetin                                                         | X  | X  | X  |    |                      | (Gong et al. 2020)                     |
| Isoflavonoids          | Biochanin A                                                          | X  | X  | X  |    |                      | (Rufatto et al. 2018)                   |
| Isoflavonoids          | Glycitein                                                            | X  |    |    |    |                      | (Doughari, 2012)                       |
| Isoflavonoids          | 6''-O-Acetylgenistin                                                 | X  | X  |    |    |                      | (Bannour et al. 2017)                   |
| Isoflavonoids          | Genistin                                                             | X  | X  | X  |    |                      | (Devi et al. 2009)                     |
| Isoflavonoids          | 6''-O-Acetylglycitin                                                 | X  |    |    |    |                      | (Iqbal et al. 2018)                    |
| Isoflavonoids          | Glycitin                                                             | X  |    |    |    |                      | (Iqbal et al. 2018)                    |
| Lignans                | Sesamol                                                              | X  | X  | X  |    |                      | (Alshahrani et al. 2020)                |
| Lignans                | Dimethylmatairesinol                                                 | X  | X  | X  |    |                      | (Chiodelli et al. 2017)                |
| Phenolic acids         | Schottenol ferulate                                                  | X  | X  |    |    |                      | (Biswas et al. 2019)                   |
| Phenolic acids         | Sitosterol ferulate                                                  | X  | X  |    |    |                      | (Moussa et al. 2020)                   |

**AO**: Anacardium occidentale. **PG**: Psidium guajava. **MC**: Morinda citrifolia. **MO**: Moringa oleifera.
| Phytochemical subclass | Name                                | AO | PG | MC | MO | Properties | References                           |
|------------------------|-------------------------------------|----|----|----|----|------------|---------------------------------------|
| Hydroxycinnamic acids  | Chicoric acid                       | X  | X  | X  | X  | Antibacterial | (Zhu et al. 2018)                     |
| Hydroxybenzoic acids   | Ellagic acid acetyl-arabinoside      | X  | X  | X  | X  | Antioxidant  | (Arifuzzaman et al. 2018)             |
| Hydroxybenzoic acids   | Ellagic acid acetyl-xiloside         | X  | X  | X  | X  |             | (Arifuzzaman et al. 2018)             |
| Hydroxycinnamic acids  | Sinapic acid                        | X  | X  | X  | X  |             | (Kim et al. 2017)                     |
| Hydroxycinnamic acids  | Caffeic acid                        | X  | X  | X  | X  |             | (Liu et al. 2016)                     |
| Hydroxycinnamic acids  | Hydroxycaffeic acid                 | X  | X  | X  | X  |             | (Amato et al. 2018)                   |
| Hydroxyphenylacetic acids | Homoveratric acid                 | X  | X  | X  | X  |             | (Rocchetti et al. 2019)               |
| Hydroxybenzoic acids   | 2-Hydroxybenzoic acid               | X  | X  | X  | X  |             | (Martínez et al. 2020)                |
| Hydroxybenzoic acids   | 3-Hydroxybenzoic acid               | X  | X  | X  | X  |             | (Martínez et al. 2020)                |
| Hydroxybenzoic acids   | 4-Hydroxybenzoic acid               | X  | X  | X  | X  |             | (Martínez et al. 2020)                |
| Hydroxybenzoic acids   | Ellagic acid                        | X  | X  | X  | X  |             | (Wong et al. 2012)                    |
| Hydroxybenzoic acids   | Gallic acid                         | X  | X  | X  | X  |             | (Martínez et al. 2020)                |
| Hydroxycinnamic acids  | p-Coumaroyl malic acid              | X  | X  | X  | X  |             | (Mouterde et al. 2020)                |
| Hydroxybenzoic acids   | Stigmasterol ferulate               | X  | X  | X  | X  |             | (Odhiambo et al. 2018)                |
| Hydroxyphenylacetic acids | Methoxyphenylacetic acid           | X  | X  | X  | X  |             | (Bannour et al. 2017)                 |
| Hydroxyphenylpropanoic acids | Dihydro-p-coumaric acid            | X  | X  | X  | X  |             | (Casadey et al. 2021)                 |
| Hydroxycinnamic acids  | 5′-Dehydrodiferulic acid            | X  | X  | X  | X  |             | (Bannour et al. 2017)                 |
| Hydroxycinnamic acids  | 5′-8′-Dehydrodiferulic acid         | X  | X  | X  | X  |             | (Bannour et al. 2017)                 |
| Hydroxycinnamic acids  | 5′-8′-Benzo furan dehydrodiferulic acid | X  | X  | X  | X  |             | (Bannour et al. 2017)                 |
| Hydroxycinnamic acids  | 5′-8′-Dehydrodiferulic acid         | X  | X  | X  | X  |             | (Bannour et al. 2017)                 |
| Hydroxycinnamic acids  | 8-O-4′-Dehydrodiferulic acid        | X  | X  | X  | X  |             | (Bannour et al. 2017)                 |
| Hydroxycinnamic acids  | Avenanthramide 2c                   | X  | X  | X  | X  |             | (Jágr et al. 2020)                    |
| Hydroxycinnamic acids  | Avenanthramide K                    | X  | X  | X  | X  |             | (Jágr et al. 2020)                    |
| Hydroxybenzoic acids   | Protocatechuic acid 4-O-glucoside   | X  | X  | X  | X  |             | (Sánchez-Maldonado et al. 2011)       |
| Hydroxybenzoic acids   | Gallic acid 4-O-glucoside           | X  | X  | X  | X  |             | (Martínez et al. 2020)                |
| Hydroxybenzoic acids   | Galloyl glucose                     | X  | X  | X  | X  |             | (Bouarab-Chibane et al. 2019)         |
| Hydroxycinnamic acids  | Cinnamoyl glucose                  | X  | X  | X  | X  |             | (Arifuzzaman et al. 2018)             |
| Hydroxycinnamic acids  | Sinapine                           | X  | X  | X  | X  |             | (Mouterde et al. 2020)                |

**Triterpenoids**

AO: *Anacardium occidentale*. PG: *Psidium guajava*. MC: *Morinda citrifolia*. MO: *Moringa oleifera.*
| Phytochemical subclass | Name                        | AO  | PG  | MC  | MO  | Properties                              | References                |
|------------------------|-----------------------------|-----|-----|-----|-----|-----------------------------------------|---------------------------|
|                        |                             | X   | X   | X   |     | Antibacterial                           |                           |
| jacoumaric acid        |                             |     |     |     |     |                                         | (Egharevba et al. 2010)   |
| isoneriucoumaric acid  |                             |     |     |     |     |                                         | (Ngbolua, 2018)           |
| 2α-hydroxyursolic acid |                             |     |     |     |     |                                         | (Ngbolua, 2018)           |
| Stilbenes              |                             |     |     |     |     |                                         |                           |
|                        | Pinosylvin                  |     |     |     |     |                                         | (Plumed-Ferrer et al. 2013)|
|                        | Resveratrol                 |     |     |     |     |                                         | (Florence et al. 2018)   |
|                        | Pterostilbene               |     |     |     |     |                                         | (Lee et al. 2017)         |
| Other polyphenols      |                             |     |     |     |     |                                         |                           |
|                        | Acetyl eugenol              | X   | X   | X   | X   |                                         | (Shankar et al. 2018)    |
|                        | 5- Tricosylresorcinol       | X   |     | X   |     |                                         | (Kamal-Eldin et al. 2001) |
|                        | Oleuropein                  |     | X   | X   | X   |                                         | (Amini et al. 2017)      |
|                        | Anethole                    |     | X   | X   | X   |                                         | (Ponte et al. 2012)      |
|                        | Estragole                   |     | X   | X   | X   |                                         | (Song et al. 2016)       |
|                        | p-HPEA-AC                   | X   | X   | X   |     |                                         | (Ma et al. 2019)         |
|                        | Psoralen                    |     |     | X   | X   |                                         | (Li et al. 2018)         |
|                        | 4-Vinylguaiacol             | X   | X   | X   |     |                                         | (Ahmed et al. 2019)      |
|                        | 3-Methoxyacetophenone       | X   | X   | X   | X   |                                         | (Türkkan et al. 2017)    |
|                        | Carvacrol                   | X   | X   | X   | X   |                                         | (Du et al. 2015)         |
|                        | Thymol                      | X   | X   | X   | X   |                                         | (Shankar et al. 2018)    |
|                        | Pyrogallol                  |     | X   | X   | X   |                                         | (Florence et al. 2018)   |
|                        | 3,4-DHPEA-AC                |     |     |     |     |                                         | (Ma et al. 2019)         |
|                        | 4-Ethylcatechol             | X   |     | X   |     |                                         | (Sova and Saso, 2020)    |
|                        | Protocatechuic aldehyde     |     |     |     |     |                                         | (Takos and Rook, 2013)   |
|                        | Tyrosol                     |     |     |     |     |                                         | (Casadey et al. 2021)    |
|                        | 2,3-Dihydroxy-1-guaiacylpropanone |     |     |     |     |                                         | (Zemek et al. 1987)      |
|                        | Hydroxytyrosol 4-O-glucoside | X   |     | X   |     |                                         | (Amini et al. 2017)      |
|                        | Carnosic acid               |     |     |     |     |                                         | (Park et al. 2019)       |
|                        | 3-Methylcatechol            | X   |     | X   | X   |                                         | (Capasso et al. 1995)    |
|                        | Phlorin                     |     | X   | X   | X   |                                         | (Miyake and Hiramitsu, 2011) |

AO: Anacardium occidentale. PG: Psidium guajava. MC: Morinda citrifolia. MO: Moringa oleifera.

Is remarkable the presence of powerful antibacterial compounds (Table 4) such as quercetin 3-O-(6'-acetyl-galactoside) 7-O-rhamnoside, methylgalangin, 3-methoxyboline, kaempferol 7-O-glucoside, quercetin 3-O-rhamnoside, kaempferol 3-o-galactoside, kaempferol 3-O-glucoside, quercetin 3-O-arabinoside, quercetin 3-O-xylloside, quercetin 3-O-glucoxyloside, myricetin 3-O-galactoside, myricetin 3-O-glucoside, and myricetin 3-O-arabinoside, where mainly present in A. occidentale and P. guajava leaves. This is consistent with the results obtained in the antimicrobial experiment shown in Table 1 and 2.
Also, the content of principal compounds from \textit{A. occidentale} and \textit{P. guajava} leaves are shown on Table 5, where it is observed the higher concentration of quercetin 3-O-glucoside-7-O-rhamnosome, kaempferol-7-O-glucoside, quercetin, caffeic acid and cinnamic acid from \textit{A. occidentale} leaves compared to \textit{P. guajava}.

| Compounds                              | \textit{A. occidentale} (µg/g) | \textit{P. guajava} (µg/g) |
|----------------------------------------|-------------------------------|---------------------------|
| Quercetin 3-O-glucoside-7-O-rhamnosome | 0.54                          | 0.12                      |
| Chicorid acid                          | 0.62                          | 1.3                       |
| Kaempferol-7-O-glucoside               | 1.95                          | <0                        |
| Quercetin                              | 10.25                         | <0                        |
| Caffeic acid                           | 0.22                          | <0                        |
| Cinnamic acid                          | 0.25                          | 0.07                      |

## Discussion

\textit{A. occidentale} is known for its antibacterial properties, mainly in its flowers, bark and leaves (da Silva et al. 2016). In addition, it has been used in the prevention and treatment of oral diseases (being the first contact of the digestive system with the food) by inhibiting the bacteria in this cavity and therefore the formation of biofilm (Anand et al. 2015). Also, Melo Menezes et al. (2014) found that both crude extract and isolated tannins of \textit{A. occidentale} have inhibitory activity against microorganisms that are part of the composition of oral biofilm. Therefore, they hypothesized that the mechanisms of the antimicrobial action of tannins, the enzymatic inhibition, the modification of cellular metabolism by its action on the membranes and binding with metal ions, decrease the access to metabolism to the microorganisms that are outside the biofilm. The present study results showed a potent antimicrobial and antioxidant activity, which is related to the high content of polyphenols and flavonoids contained in its leaves, in addition to other medicinal compounds.

Souza et al. (2017) observed the antioxidant and anti-inflammatory activity \textit{in vitro} in \textit{A. occidentale} leaves extract when used in RAW 264.7 macrophage cells due to the lower oxidative damage of these cells and the decrease in inflammatory parameters induced by LPS stimulation. Additionally, Brito et al. (2020), pointed out that pentagalolli hexoside, a precursor to the formation of hydrolyzed tannins such as ellagitannins and gallotannins, was found in all the organs of \textit{A. occidentale}, these chemical compounds are responsible for several functional properties, with higher emphasis on the antimicrobial activity. Thus, the present study showed that \textit{A. occidentale} was the plant with the highest antimicrobial and antioxidant capacity compared to the other three plants studied.

Regarding the effect of \textit{A. occidentale} in animal production, specifically in poultry and pig production, Aroche-Ginarte et al. (2017) found that dietary supplementation with 1.0% of a mixed powder made from 40% of \textit{A. occidentale} leaves powder increased growth performance and decreased the diarrhea incidence in weaned piglets. Furthermore, Mâs et al. (2016) showed that the dietary inclusion in low concentrations of \textit{A. occidentale} and \textit{P. guajava} leaves powder promoted growth and reduced dehydration in pigs before and after weaning. In this sense, Aroche et al. (2018) showed positive results in feed efficiency and IgG production when they added 0.5% of a mixture of plants representing 60% of \textit{A. occidentale} in broiler diets.

\textit{P. guajava} has also shown strong bactericidal activity on its leaves and aqueous extract, as it requires a small amount to eliminate bacteria such as \textit{E. coli}, \textit{S. aureus}, and \textit{Salmonella}. Similarly, Salihu Abdallah et al. (2019) verified that the aqueous and methanol extracts of \textit{P. guajava} leaves have antimicrobial activity against \textit{S. aureus} and \textit{S. typhi}. The aqueous extract was effective with MIC of 12.5 mg/mL for both bacteria and MBC between 25 and 50 mg/mL for \textit{S. aureus} and \textit{S. typhi} respectively. In this study, the concentrations of aqueous extract were necessary to obtain the MIC and MBC against these bacteria, and were lower than those aforementioned, which may be due to the variety of the plant used, the origin, the extraction methods, among other factors. Also, Chero Nepo and Ruiz Barrueto (2016) determined that the alcoholic extract of \textit{P. guajava} inhibits the growth of \textit{Streptococcus mutans} due to its bactericidal power.

Regarding the antioxidant activity, Flores et al. (2015) identified the chemical composition of seven cultivars of \textit{P. guajava} and found a high content of flavonoids, in addition of anthocyanins, proanthocyanins, triterpenes and other compounds. Likewise, Feng et al. (2015) and Flores et al. (2015) showed that there is high correlation between flavonoid content and the antioxidant capacity of the plant, which agree with our findings, where \textit{P. guajava} was the second plant to show a high antioxidant power.

On the other hand, \textit{M. oleifera} is a multipurpose plant with multiple nutritional benefits, but also has been studied for its antimicrobial and antioxidant effects, since its use in human and animal nutrition is increasingly popular (Wang et al. 2018). Likewise, \textit{M. citrifolia} has innumerable health benefits, however, when these two plants are compared with \textit{A. occidentale} and \textit{P. guajava}, they may be at a disadvantage due to the lower content of secondary metabolites responsible for the aforementioned activity. This research demonstrated the marked difference for antimicrobial and antioxidant effect of both the leaves and the aqueous extract of \textit{A. occidentale} and \textit{P. guajava} compared to \textit{M. citrifolia} and \textit{M. oleifera}.

However, in the case of \textit{M. oleifera}, researchers such as Siddhiraju and Becker (2003), determined that this plant presents high antioxidant power in its ethanolic and methanol extracts, which was related to abundant flavonoid content, especially quercetin and kaempferol. Shih et al. (2011) found high antioxidant activity in the ethanolic extract of various parts of this plant, where the leaves showed the highest activity, with an IC50 of 0.287 mg/mL, which is less than that found in this study (0.603 mg/mL). This difference could be due to the difference on the extraction (aqueous) method used this study. In
relation to animal production, authors such as Zhang et al. (2018) found positive effects of *M. oleifera* on performance of fattening pigs, with a marked effect due to increased activity of the enzyme superoxide dismutase and decreased serum malondialdehyde concentration.

*M. citrifolia* only inhibited the growth of staphylococcal strains, in both forms, as fine powder and as aqueous extract, however, did not show any antimicrobial effect with the other bacterial strains. These results agree with Almeida et al. (2019), whom reported several studies that probe the antimicrobial and antioxidant properties of *M. citrifolia* based on its chemical compounds in the plant parts. Also, antibacterial activity was found by Pandiselvi et al. (2019) and Sunder et al. (2012) specifically with *Staphylococcus aureus*. The difference in terms of the least antimicrobial effect in this study could be due to the use of methanolic extract. The antioxidant activity of the leaves of *M. citrifolia* was the lowest of among the four plants. Very little literature has been published about the antioxidant capacity of the leaves of this plant. Besides, there are several investigations that show this quality in its fruits (Senthilkumar et al. 2016; Sunder et al. 2016; Thorat et al. 2017). Sunder et al. (2016) demonstrated the multiple uses of *M. citrifolia* in livestock and poultry as a natural growth promoter due to its immunomodulatory, antioxidant, and hypocholesterolemic properties.

Polyphenols are the major secondary metabolites distributed in all plants, with higher emphasis on isoflavonoids, anthocyanins, flavonols, and flavones in *A. occidentale* and *P. guajava*. The quantification of the main secondary metabolites in these two plants (*A. occidentale* and *P. guajava*) such as quercetin 3-O-glucoside-7-O-rhamnoside, chicoric acid, kaempferol-7-O-glucoside, caffeic acid, and cinnamic acid could support the antibacterial and antioxidant effects found in this study.

Theoretically, authors such as Sharaf et al. (2000) and Roepke and Bozzo (2013) have mentioned that 3-O-glucoside-7-O-rhamnoside is a rare secondary metabolite in plants with proven antioxidant and antimicrobial properties against *E. coli*. Furthermore, caffeic and chicoric acids have potential as antidiabetic agents (Tousch et al. 2008), already demonstrated by Kamtchouing et al. (1998) and Mukhtar et al. (2004) who found a reduction in glucose concentration in laboratory mice when they used extracts of *A. occidentale* and *P. guajava*, respectively. In addition, the flavonoid kaempferol-7-O-glucoside was identified and quantified in the leaves of *A. occidentale*, which is a phytochemical widely studied for its antimicrobial properties (Singh et al. 2011). Moreover, cinnamic acid is an organic acid that occurs naturally in many medicinal plants and quantified in both medicinal plants, it has low toxicity and a wide spectrum of functional activities, this secondary metabolite has antibacterial, antiviral and antifungal properties (Sova, 2012), which supports the effect antimicrobial found in the leaves of the plant in study (Tables 1 and 2). Although positive results have been found for the secondary metabolites quantified in the leaves of the plant under study (mainly *A. occidentale*), the results in farm animals are not conclusive. Thus, these results could contribute to understand how medicinal plants (mainly leaves of *A. occidentale* and *P. guajava* and their extracts), due to their antimicrobial and antioxidant function, can completely replace growth-promoting antibiotics in farm animals, as demonstrated by Martínez et al. (2013), Más et al. (2016), Aroche et al. (2017), Salazar et al. (2017) and Aroche et al. (2018) in poultry and pigs.

**Conclusions**

It is concluded that *A. occidentale* and *P. guajava* are the plants with the highest antimicrobial and antioxidant activity in their leaves and aqueous extract. *M. oleifera* has good antioxidant *in vitro* activity, although it does not have high antimicrobial power; and *M. citrifolia* is the plant that has the least antioxidant activity in its aqueous extract.

**Declarations**

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**Data Availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Author Contributions** Conceptualization, Roisbel Aroche, Xilong Li and Yordan Martínez; methodology, Roisbel Aroche, Xianren Jiang; data analysis, Roisbel Aroche, Xianren Jiang; investigation, Roisbel Aroche; writing—original draft preparation, Roisbel Aroche; writing—review and editing, Román Rodríguez, Xilong Li, Ana Carolina Arévalo, Mavir Carolina Avellaneda and Yordan Martínez; supervision, Xilong Li; funding acquisition, Xilong Li. All authors have read and agreed to the published version of the manuscript.

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