RESEARCH ARTICLE

Antibacterial effect of the hydroalcoholic extract of *Mauritia flexuosa* leaves on gram-negative and gram-positive bacteria

[version 1; peer review: 2 approved with reservations]

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Abstract

Background: Plant-derived compounds are sometimes used as substitutes for pharmaceuticals. *Mauritia flexuosa* is a palm tree that is widely distributed in South America, especially in the Amazon region. The San Martín region of Peru, in which this species of the Arecaceae family is found, has great biological diversity and there is economic potential in the utilization of natural resources in the region.

Methods: In this study, the antibacterial effect of the hydroalcoholic extract of *Mauritia flexuosa* leaves was evaluated for gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633 and gram-negative *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella enterica* subsp. *enterica* ser. Typhi ATCC 11011. *Mauritia flexuosa* leaves were used to prepare concentrations of 10, 20, 40 and 60mg/ml. Phytochemical analysis was performed to identify secondary metabolites in the plants. For the experiment, 10 Mueller-Hinton agar plates were prepared and 1ml of bacterial inoculum, standardized to 0.5 McFarland, was added to each plate. The hydroalcoholic extract was added via the diffusion method, making five holes of 5mm each (four with extract concentrations and one with distilled water as a control group), and the plates were incubated for 24 hours at 36°C. The inhibition halo was measured in mm using a digital vernier caliper.

Results: For gram-negative bacteria, an antibacterial effect was demonstrated for *Pseudomonas aeruginosa* only, at an extract concentration of 60mg/ml, with an inhibition halo of 14.8 mm. For gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, an antibacterial effect was demonstrated at an extract concentration of 60mg/ml, with inhibition halos of 13.2mm and 15.4mm in diameter, respectively.

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Approval Status ? ?

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2. Mario R. Esparza Mantilla , University of Antofagasta, Antofagasta, Chile

Any reports and responses or comments on the article can be found at the end of the article.

Conclusion: It can be concluded that the hydroalcoholic extract of *Mauritia flexuosa* does not inhibit bacterial growth for gram-negative bacteria *Salmonella* Typhi and *Escherichia coli*.

Keywords

Antibacterial, hydro-alcoholic extract, *Mauritia flexuosa*, gram negative bacteria, gram positive bacteria

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Introduction

Flora is important, because of its diversity, for the production of phytochemical compounds, used as a natural alternative to pharmaceuticals for the treatment of diseases caused by fungi, bacteria and other organisms¹. Plant-derived compounds have been used as substitutes for pharmaceuticals; for example, one out of three people use medicinal plants for healing purposes in Europe^{2,3}. A study conducted in the United States found that 25% of drugs are derived from plants^{4,5}.

Mauritia flexuosa is a palm tree that is widely distributed in South America, especially in the Amazon region. The San Martín region of Peru, in which this species of the Arecaceae family is found, has great biological diversity and there is economic potential in the utilization of natural resources in the region. In the food industry, the shell and the endocarp of the *Mauritia flexuosa* fruit are usually discarded or underused for the preparation of sweets, ice creams, juices, jams, infant food and oils⁶. Some studies have emphasized the pharmacological potential of these parts, which contain bioactive compounds, to play a role in antimicrobial defense⁷⁻⁹, lipid metabolism¹⁰, hypoglycaemia¹¹ and curative activities¹². *Mauritia flexuosa* also produces secondary metabolites belonging to chemical groups, such as alkaloids and cyanogenic glycosides, and non-nitrogenous compounds, such as tannins, flavonoids, terpenes and anthocyanin¹³.

Bacteria have become resistant to many medicines; thus, infections have been disseminating quickly between people and animals. Gram-positive bacteria, including *Staphylococcus aureus* (causes a variety of infectious diseases, including skin infections, wound infections and pneumonia) and *Bacillus subtilis* (which is not considered a human pathogen but can cause intoxication and food contamination), as well as gram-negative bacteria, including *Salmonella enterica* subsp. *enterica* ser. Typhi (*Salmonella* Typhi, causes typhoid fever), *Pseudomonas aeruginosa* (resistant to multiple drugs and responsible for healthcare-associated infections) and *Escherichia coli* (causes diarrhea and renal insufficiency, which can lead to death) are involved in human bacterial infection¹⁴.

Medicinal plants are considered an alternative solution for controlling bacterial diseases; thus, there is a requirement for research with the aim of finding new natural alternatives. The World Health Organization has shown that they can be effective and their percentage of health risk may be minimal¹⁵. Research shows that the hydroalcoholic extracts of leaves have an antibacterial effect on gram-positive and gram-negative bacteria because of their active principles, which play an important role in the development of new therapeutic agents^{16,17}.

The objective of this research was to evaluate the antibacterial effect of the hydroalcoholic extract of *Mauritia flexuosa* leaves on gram-positive and gram-negative bacteria.

Methods

Source of plant material

Leaves of *Mauritia flexuosa* were collected by researchers (ANSV, MFC, MLSC, IMRH and APVR) in the district of

Cacatachi, San Martín at 295 meters above sea level, 12 km to the north of Tarapoto (6°29'40" of South latitude and 76°27'57" of West longitude). The specimen was transferred to the Herbarium Truxillense of Universidad Nacional de Trujillo for identification by specialists, obtaining a registration code. The sample was transported in a wooden press inside a labelled vacuum bag and kept at an ambient temperature of 37°C.

Preparation of the extract

Whole leaves were selected and those with signs of deterioration discarded. Leaves were washed with distilled water and disinfected with cotton dipped in 96% ethanol. Leaves were fragmented to an approximate size of 3mm and the maceration method was carried out as follows. Fresh leaves, with a weight of 200g, were washed with distilled water to remove impurities, wrapped in kraft paper and dried in a universal oven (Memmert GmbH + Co. KG) at 25 °C for approximately 12 hours. The dry sample was cut with scissors to obtain small pieces, placed in an amber glass jar and 500mL of 96% ethanol was added, followed by agitation using a vertical rotavapor (Scilogex RE-100) at 70 rpm for 10 minutes every four hours, except overnight from 10 pm to 7 am, for 15 days. The sample was filtered four times with Whatman N °1 and then N °2 filter paper. Then, the vertical rotavapor (Scilogex RE-100) was used for two hours at 70rpm to obtain a dry extract, which was dissolved in alcohol at 96 °C and used to prepare concentrations of 10, 20, 40 and 60mg/ml.

Phytochemical analysis

The phytochemical analysis of *Mauritia flexuosa* leaves was qualitative and was carried out according to the method described by Miranda and Cuellar¹⁸. Each sample was subjected to solvents of increasing polarity in order to obtain secondary metabolites according to their solubility, using reagents and dyes to determine presence or absence of active components such as terpenes, flavonoids, reducing sugars, among others. The assays used to determine the presence of each type of secondary metabolite are listed in Table 1. The results of the color change were judged by eye according to the method described by Miranda and Cuellar¹⁸ and classified as light, moderate or strong.

Table 1. Phytochemical analysis of hydroalcoholic extracts of *Mauritia flexuosa*.

Assay	Metabolites	Identification
Liebermann-Burchard	Steroids and triterpenes	(++)
Ferric chloride	Phenolic compounds	(+++)
Shinoda	Flavonoids	(+++)
Baljet	Lactones	(+)
Fehling	Reducing sugar	(++)
Gelatin	Tannins	(++)

(+), light: (++) , moderate; (+++), strong.

Source of bacterial strains

Standard bacterial strains (American Type Collection Culture (ATCC)) were provided by the Bacteriology Laboratory of Universidad Nacional de Trujillo. Gram positive bacteria *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633 were used. Gram-negative *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella* Typhi ATCC 11011 were used.

Inhibition experiment protocol

Bacteria were stored at a temperature below 5°C. From there, they were removed and reactivated in brain heart infusion medium at 37°C for 18 days. Mueller-Hinton agar (Merck) was prepared in biosafety level one conditions according to the manufacturer's specifications. 8ml of Mueller-Hinton agar was poured into 100mm Petri dishes and left to dry at 37 °C for 30 minutes. A suspension of 5×10^8 colony forming units (CFU) of each bacterial culture was prepared in a test tube of 10ml isotonic sodium chloride solution, equivalent to 0.5 MacFarland. For the experiment, 1ml of each suspension was spread onto Mueller-Hinton plates and was left to dry for 30 minutes. For the application of the hydroalcoholic extract, the diffusion method in agar was used¹⁹ and five holes of 5mm were made in each Petri dish; four to add 70µl of the prepared extract concentrations (10, 20, 40 and 60mg/ml) and one to add 70µl of the control (distilled water). In total, the experiment involved 50 plaques, with two Petri dishes of five plaques each used for each of the five species of bacteria. The plates were incubated at 36°C for 24 hours. The results were determined using a

digital vernier caliper (CALDI-6MP, Truper), giving the diameter of the halo in mm.

Statistical analysis

SPSS (version 22) software was used for the summation, averages, tables and graphs. For the calculation of the inhibition halo, the mean average of the two repetitions for each extract concentration for each bacterium was calculated.

Results

It was observed that *Mauritia flexuosa* has a large number of phenolic compounds and flavonoids (Table 1)²⁰.

As shown in Figure 1, no inhibition halos were formed in the control group (distilled water) around any of the gram-negative bacteria. For *Pseudomonas aeruginosa*, there was an inhibition halo of 14.8mm of diameter at a *Mauritia flexuosa* extract concentration of 60mg/ml, a halo of 12.4mm at 40mg/ml, a halo of 10.2mm at 20mg/ml and a halo of 8.0mm at 10mg/ml. However, for *Salmonella* Typhi and *Escherichia coli*, no inhibition halos were observed^{21,22}.

As shown in Figure 2, the control (distilled water) did not produce inhibition halos around any of the gram-positive bacteria. For *Bacillus subtilis*, there was an inhibition halo with a diameter of 13.2mm at a *Mauritia flexuosa* extract concentration of 60mg/ml, a halo of 11.1mm at 40mg/ml, a halo of 9.4mm at 20mg/ml and a halo of 6.6mm at 10mg/ml. For *Staphylococcus aureus*, diameter of the inhibition halo was 15.4mm at an

Figure 1. Halo diameter in mm of gram-negative bacteria.

Figure 2. Halo diameter in mm of gram-positive bacteria.

extract concentration of 60mg/ml, a halo of 13.2mm at 40mg/ml, a halo of 11.3mm at 20mg/ml and a halo of 8.1mm at 10mg/ml^{21,22}.

Discussion

Medicinal plants have become common in today's research, analyzing different parts of the plant, in order to ensure the diversity, and evaluating and their potential as therapeutic agents^{23–25}. Therefore, the increase in bacterial genetic mutations conferring bacterial resistance to antibiotics and the increase in difficulty in treating these infections has led to the investigation of new antibacterial agents, especially those of natural origin²⁶.

As shown in Table 1, secondary metabolites were qualitatively identified in *Mauritia flexuosa* leaves, such as steroids, triterpenes, phenolic compounds, flavonoids, lactones, reducing sugars and tannins. The qualitative analysis of leaves of *Mauritia flexuosa* is important, since the evidence that there is a large presence of secondary metabolites, such as steroids, triterpenes, phenolic compounds and flavonoids, allows comparison of its potential against growth of bacteria with other research.

As shown in Figure 1, three gram-negative bacteria were studied, of which only *Pseudomonas aeruginosa* was inhibited by the hydroalcoholic extract, at a concentration of 60mg/ml and producing an inhibition halo of 14.8 mm in diameter. There was no inhibitory effect on *Salmonella Typhi* and *Escherichia coli*. In a study carried out in Brazil, in which *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used, no sensitivity to the extract of *Mauritia flexuosa*

was evidenced²⁷. Therefore, it should be pointed out that the chemical composition of the soil and the climatic conditions may result in variation of the active constituents; therefore, differences in the results may be observed despite the use of the same bacterial species. Furthermore, it has been found previously that the hydroalcoholic extract inhibited the bacterial growth of the same species used for this study^{28,29}. Therefore, the chemical composition of the leaves plays an essential role in the antibacterial effect, since the effect is composed of a set of active principles that work synergistically.

As shown in Figure 2, gram-positive strains of *Bacillus subtilis* and *Staphylococcus aureus* had inhibition halos of 13.2mm and 15.4mm in diameter, respectively, at a hydroalcoholic extract concentration of 60mg/ml, identifying that at a higher concentration there is lower bacterial growth. It is known that hydroalcoholic extracts of plants release a large quantity of phenols and flavonoids and other compounds, including great diversity of secondary metabolites, which may explain this antibacterial action^{30–32}. Similar results were identified in other studies that tested hydroalcoholic extracts on gram-positive bacteria, finding that the extract had an antibacterial effect^{28,33,34}. Therefore, it is important to emphasize the importance of each active principle since act synergistically to obtain better results, being more efficient and effective³⁵. Finally, gram-positive and gram-negative bacteria can be pathogenic; hence, the importance of identifying new alternatives to mitigate the pathologies caused by these bacteria³⁶. Therefore, it is necessary to study regional plants as a treatment alternative and develop new plant products with protective effects.

In conclusion, the hydroalcoholic extract of *Mauritia flexuosa* does not inhibit bacterial growth of gram-negative bacteria *Salmonella* Typhi and *Escherichia coli*. However, in *Pseudomonas aeruginosa* the extract inhibits growth at a concentration of 60mg/ml, forming an inhibition halo of 14.8 mm. For gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, bacterial inhibition is observed at a concentration of 60mg/ml, with halos of 13.2mm and 15.4mm of diameter, respectively, demonstrating an antibacterial effect in these species.

Data availability

Underlying data

Figshare: Supporting data for Figure 1 and Figure 2. <https://doi.org/10.6084/m9.figshare.8051870.v3>²¹

Figshare: Photographs of each agar plate. <https://doi.org/10.6084/m9.figshare.8312777.v3>²²

Photographs of the phytochemical analyses. <https://doi.org/10.6084/m9.figshare.8862431.v1>²⁰

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Version 1

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Mario R. Esparza Mantilla

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The research proposes to demonstrate the antimicrobial effect of a natural extraction of plants, this is well known and the test models also, in that direction to work it requires more evidence or generate new contributions to change the models or use more robust ones for the tests, perhaps the systems of broths of cultures in 96-plate plates and evaluate the effect in vivo allow a result of greater impact, on the other hand the controls applied to the experiment such as water are not so blunt should the solvent with which the plant extract.

On the application of more robust methods, I suggest applying the MIC-MBC (minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), the methodology recommended by the Clinical and Laboratory Standards Institute), as well as the technique of viable microdrops.

It is also suggested to construct microbial kinetic curves to have a more precise data of the effect of the dose vs the bacterial cell density in a certain time and can be contributed in the knowledge of microbial physiology since under these conditions the extract could also have another form of action closer to the real one (within the host) that provides an approach to results that allow a future biomedical application of the plant extract.

Researchers should improve the conclusion since their writing only reports one result, they should be able to generate new knowledge based on the analysis and discussion of the results that will allow a substantive contribution to the execution of the proposed research.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology, Biotechnology, Environmental Microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 27 September 2019

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Flor de María Evangelista Montoya

Wilkinson Scientific Consulting, Provincia de Trujillo, Peru

Sandoval and collaborators investigated the antibacterial effect of the hydroalcoholic extract of *Mauritia flexuosa* leaves on gram-negative and gram-positive bacteria. They tested this effect on standard strains of 3 gram-negative and 2 gram-positive bacteria using the agar diffusion method. They found that the extract had antibacterial effects against the pathogens *S. aureus*, *B. subtilis* and *P. aeruginosa* but not against *E. coli* and *S. typhi*. Inhibition was observed at 60 mg/mL (4.2 mg/well).

Overall the article is clearly written, however it could benefit from some English grammar and style editing.

Although the Introduction expresses the present art well, I suggest including a short explanation of why the authors decided to use the leaves of *M. flexuosa* for their study. Additionally, references 16 and 17 do not seem appropriate to support the use of leaves because they deal with fruit and total aerial parts.

In the Methods section authors say: "The sample was transported in a wooden press inside a labelled vacuum bag and kept at an ambient temperature of 37°C ". Usually ambient temperature is approximately 25°C. I request the authors clarify the temperature used.

Why was it necessary to reactivate the bacterial strains for 18 days? That seems like a very long time, please clarify.

The reference used (18) to describe the method used for the phytochemical analysis of the extract is not easily accessible to the reader. I recommend referencing a standard review or book in which these common procedures are explained.

In the Results section, the authors show that the extract has antibacterial effect against some bacteria. This is useful data as it may point to new leads for antibiotics. However, it seems that there are some deficiencies in proper experimental controls. First, distilled water was used as a negative control, but the extract was dissolved in ethanol. Therefore, it is possible that some residual ethanol may be causing a bacteriostatic effect. Second, no positive control with a standard antibiotic was performed. This makes it difficult to make clear comparisons of the antibiotic activity of the extract to known antibiotics and to establish what halo size determines sensitivity.

The discussion expresses the importance of this type of study and makes some comparisons of the present results with other studies. However, the way it is written can mislead the reader to the idea that any hydroalcoholic extract from any plant has an antibacterial effect against the bacteria tested and does not highlight the importance of using the extract from *M. flexuosa* leaves for antimicrobial use. I recommend including a statement and explanation about this.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology, antibiotic resistance.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 Oct 2019

ANA SANDOVAL VERGARA, Universidad César Vallejo, Carretera Marginal Norte Fernando Belaúnde Terry Km. 8.5, Peru

Dear.

Flor de María Evangelista Montoya.

I appreciate your comments, below I detail each suggestion:

1. *M. flexuosa* it is one of the most consumed fruit species in the Peruvian Amazon, the inhabitants use by-products such as leaves, fruits, seeds to cure diseases; Therefore, the importance of its study as an alternative to traditional medicine.
The references indicated were considered by the authors since it is a plant extract, they refer to the importance of the active ingredients and the antibacterial effect they possess; Starting from that premise is a valuable criterion to demonstrate the presence of toxic components in plants that could be an option for the treatment of diseases caused by bacteria.
2. The temperature at which the plant samples were transported was 25 ° C.
3. The bacteria mentioned only reactivate in 18 hours.
4. Another author who clearly describes the phytochemical march in plants has been considered.
Lock, O. *Phytochemical Research: Methods in the study of natural products*. 3rd edition, Editorial of the Department of Sciences. Pontifical Catholic University of Perú, 2016: p 6-8
5. **First.** - When the ethanol extract is prepared, the ethanol is completely removed by evaporation, therefore it is not possible to find residual alcohol consequently does not cause bacteriostatic effect
Second. - The antibiotic is a drug synthesized and industrially purified, while the plant extract has a set of naturally produced drugs and the authors did not consider it as a positive control, since comparisons cannot be made only serve as a reference.
6. This study contributes to the knowledge of the species and the expansion of its application in biotechnology, as well as the results obtained contribute to a better understanding of the relationship between the chemical composition of the hydroalcoholic leaf extract and its antibacterial potential, this plant is widely used in the food industry, taking into account the diversity of its active ingredients, future research should be encouraged and the main objective should be to isolate specific compounds to produce safe phytotherapies

Competing Interests: No competing interests were disclosed.

Comments on this article

Version 1

Reader Comment 29 Aug 2019

Mitchel Okumu, University Of Nairobi, Nairobi, Kenya

The authors have made an attempt to report on the effect of the hydroalcoholic extract of *Mauritia flexuosa* on gram positive (*S. aureus*, and *B. subtilis*) and gram negative bacteria (*E. Coli*, and *P. aeruginosa*).

1. Overall, the manuscript has too little information in its present form to qualify as scientific communication leave alone passing as a paper in such a reputable outlet as F1000 research.
2. Why are there no controls (standard antimicrobial drugs for both gram positive and gram negative bacteria) by which the effects of the extracts can be compared against?. This is a major oversight on the part of the authors!
3. Authors should consider using present/absent when describing the phytochemical composition of the extracts. Using light, moderate or strong as descriptors is misleading as this kind of description is subjective.
4. Under the results section, the authors report that the extracts had a large number of phenolic compounds and flavonoids. This is not accurate at all since what has been carried out is QUALITATIVE phytochemical screening therefore it is not possible to QUANTIFY how much phenolic compounds and flavonoids are available in the said extracts. Ideally, this statement would have been relevant if the authors had carried out a determination of the total phenolic content and total flavonoid content.
5. The range of doses tested (10-60mg/ml) is too narrow. More doses/concentrations should have been tested.
6. The discussion is really poorly done. Authors have made no attempt to discuss their results but have rather repeated their results
7. I wonder what the conclusion of this study is? As far as I can see, the authors have simply repeated their results in the conclusion section as well.
8. In conclusion, this manuscript requires extensive changes if it is to reach acceptable standards of scientific quality.

Competing Interests: No competing interests.

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