

Hydroalcoholic Extracts of Fruit Leaves from the Peruvian Amazon as Antibacterial Potential of Gram-Negative and Gram-Positive Bacteria

Ana N. Sandoval^{*a}, Jhonny W. Valverde^{a,b}, Kriss M. Calla^a, Rafael A. Alba^a, Herry Lloclla^a, Armando G. Ismiño^c, Santos A. Sotero^d, Marco L. Salazar^e

^aUniversidad César Vallejo, Carr Marg Norte F.B.T KM 8.5 Sec. Maronilla, Cacatachi, San Martín, Perú.

^bUniversidad Nacional Agraria La Molina, Av. La Molina s/n, Lima, Perú

^cAsociación de Productores Jardines de Palma, Pongo de Caynarachi, San Martín, Perú.

^dUniversidad Nacional de San Martín, Jr. Amorarca 344, Tarapoto, Perú.

^eUniversidad Nacional de Trujillo, Av. Juan Pablo II s/n, Trujillo, Perú.

asandoval@ucv.edu.pe

The Peruvian Amazon is considered one of the regions with the greatest diversity in flora, so the importance of its study through its hydroalcoholic extracts. The use of natural resources is an alternative for the discovery of new therapeutic agents. The active compounds derived from plants could be used as substitutes of pharmaceutical products for the control of diseases caused by microorganisms. In this study, the antibacterial potential of the hydroalcoholic extract of the fruit leaves was evaluated in gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633) and gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). *Theobroma cacao* leaves were used. *Cocos nucifera*, *Musa paradisiaca* and *Coffea sp.* The hydroalcoholic extract was prepared by the maceration method. A phytochemical analysis was performed on the extracts to identify secondary metabolites. A total of 48 Mueller-Hinton agar plates with 1 mL of bacterial inoculum were prepared in each plate, standardized to 0.5 McFarland; the hydroalcoholic extract was added through the diffusion method, making five holes of 5 mm each (four with concentrations and one with distilled water as a control group), the plates were incubated for 24 h at 36 °C. The halo of inhibition was measured in mm with a Digital Vernier Caliper. The results obtained for gram-negative bacteria, antibacterial potential was observed only in *Pseudomonas aeruginosa* in all its concentrations, but no activity was seen in the hydroalcoholic extract of *Coffea sp.*; for the gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, its antibacterial potential was demonstrated in the extracts of *Cocos nucifera*, *Musa paradisiaca* in all its concentrations, only antibacterial potential was identified in *Staphylococcus aureus* by *Coffea sp.* extract; However, it should be noted that there was no reaction in *E. coli*.

1. Introduction

Plants have become an essential source of traditional medicine, specifically in developing countries where access to antibiotics is limited (Atanasov et al., 2015). Many populations of the world depend on complementary medicine to combat various diseases caused by fungi, bacteria and other microorganisms (Peiris et al., 2015). One in three people use medicinal plants for healing purposes in Europe (Chilquillo et al., 2018). A study conducted in the United States found that 25 % of antibiotics derived from plants (Rodríguez et al., 2015; Corrales and Reyes, 2015) with this, has increased the importance of the plant extracts study due to their content as compounds Chemicals that may be useful for the development of new antibiotics, estimates that 80 % of the world's population uses traditional medicine to meet their primary health needs (Vega et al., 2013)

The Peruvian Amazon presents a great biological diversity in flora housing thousands of species, with a potential economy in the use of natural resources, many of them are used as an alternative medicine (Ávila et al., 2019). Recent research has given interest to the pharmacological potential that plants possess, because of the content of their active ingredients that plays an essential role in bacterial defense (Heisler et al., 2015; Chaves et al., 2015). *Theobroma cacao* L, *Cocos nucifera*, *Musa paradisiaca* and *Coffea sp* species produce secondary metabolites such as alkaloids, phenolic compounds, tannins, flavonoids, terpenes, among others.

The increase in bacterial resistance to antibiotics has aroused interest in seeking new alternatives to fight diseases (CDC, 2013; Teles and Costa, 2014), some bacterial strains become endemic and are a threat to health. Gram-positive bacteria, including *Staphylococcus aureus* (causes a variety of infectious diseases) and *Bacillus subtilis* (may cause food poisoning and contamination), as well as gram-negative bacteria, *Pseudomonas aeruginosa* (multi-drug resistant and responsible for intrahospital infections) and *Escherichia coli* (causes diarrhea and kidney failure leading to death) involved in bacterial infections in humans (CDC, 2016).

The active ingredients play an important role in antimicrobial activity, inhibition of neurotransmitters, antioxidants because it could be used as a synergistic resource for the treatment of diseases. (Rezende et al., 2019). Other plant species also have antimicrobial action and would be an alternative use of synthetic substances to control pathogenic microorganisms. (Marchi et al., 2019)

Medicinal plants are an alternative for the control of bacterial diseases, the World Health Organization shows that such therapeutic agents based on plants can be effective and that their percentage of health risk is minimal (WHO, 2013). Research shows that the hydroalcoholic extracts of the leaves have an antibacterial effect on gram-positive and gram-negative bacteria due to their secondary metabolites, which play an important role in the development of new therapeutic agents (Sika et al., 2014). The objective of this research was to evaluate the hydroalcoholic extracts of the fruit leaves from the Peruvian Amazon as an antibacterial potential.

2. Methods

2.1 Vegetal material.

Theobroma cacao L, *Cocos nucifera*, *Musa paradisiaca* and *Coffea sp* leaves were selected and collected, in the district of Cacatachi, San Martín at 295 meters above sea level, 12 kilometres Northern Tarapoto (6 ° 29'40 " south latitude and 76 ° 27'57 "west longitude). The samples were placed in a wooden press inside vacuum bags to maintain an ambient temperature of 37 °C; the copies were transferred to the Truxillense Herbarium of the National University of Trujillo for identification, depositary and registration code for each species: *Theobroma cacao* L (COD. 59599), *Cocos nucifera* (COD. 59603), *Musa paradisiaca* (COD. 59608) and *Coffea sp* (COD. 59609).

2.2 Preparation of hydroalcoholic extract.

Whole leaves were selected discarding those with signs of deterioration, washed with distilled water and disinfected with cotton dipped in 96% ethanol wrapped in kraft paper to be dried in a universal oven (Memmert GmbH + Co. KG) at 25 ° C for 12 h; subsequently, the samples were cut with scissors to obtain small pieces at an approximate size of 3mm and prepare the hydroalcoholic and by the maceration method being carried out as follows. It was placed in a 500 mL amber glass jar of ethanol 96% with 200 g of leaves allowed to macerate for 15 d, the solution obtained was carried on a vertical rotary evaporator (Scilogex RE-100) at 70 rpm for 10 minutes every four hours, except at night of 10 pm at 7 a.m. The sample was filtered four times with Whatman No. 1 paper to obtain a dry extract, which was dissolved in alcohol at 96 ° C to prepare concentrations of 10, 20, 40 and 60 mg/mL.

2.3 Phytochemical analysis

The phytochemical analysis of the fruit leaves from the Peruvian Amazon was qualitative and was carried out by the method referred to by Lock (2016), each sample was subjected to solvents of increasing polarity in order to show secondary metabolites, were used reagents and dyes to identify the presence or absence of active compounds such as tannins, triterpenes, flavonoids, phenolic compounds, reducing sugars, and others. The color change of each plant sample was classified as light, moderate or strong; Finally, the tests are listed in Table 1.

Table 1: Phytochemical analysis of the hydroalcoholic extract of the fruit leaves from the Peruvian Amazon

Essay	Metabolites	Coffea sp	Cocos nucifera	Musa paradisiaca	Theobroma cacao L
Lieberman-	Steroids	+	++	++	++
Bouchard	Triterpenes	+	++	++	++
Borntrager	Quinones	-	-	-	-
Ferric Chloride	Phenolic compounds	+	+	++	+
Shinoda	Flavonoids	+	+	+++	-
Baljet	Lactones	-	+	++	-
Dragendorff	Alkaloids	+	-	-	-
Mayer	Alkaloids	+	-	-	-
Fehling	Reducing sugars	+	++	++	++
Gelatin	Tannins	+	++	-	-
Foam	Saponins	-	-	-	-

(+): Light (++) : Moderate and (+++) : Strong

2.4 Source of bacterial strains

Standard bacterial strains American Type Collection Culture (ATCC) were provided by the Bacteriology Laboratory of the National University of Trujillo. Gram positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633) and gram negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used.

2.5 Inhibition experiment protocol

The bacteria were stored at a temperature below 5 °C. Then, they were removed and reactivated in BHI Brain Heart infusion (BHI) culture medium at 37 °C for 8 h. Subsequently, another culture medium was prepared using Mueller-Hinton Agar (Merck) which was prepared under level one biosecurity conditions according to the manufacturer's specifications, 8 mL of Mueller-Hinton agar was poured into 100 mm Petri dishes and it was allowed 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 10 mL test tube with isotonic sodium chloride solution, equivalent to 0.5 MacFarland, for the experiment 1 mL of each bacterial suspension was used on the Petri dishes containing Mueller-Hinton agar allowing to dry for 30 minutes, for the application of the hydroalcoholic extract the agar diffusion method was used (Sánchez and Muhammad, 2016), making five 5 mm holes in each Petri dish; four to add 70 µL of the prepared extract concentrations (10, 20, 40 and 60 mg/mL) and one to add 70 µL of distilled water (control group). The plates were incubated at 37 °C for 24 h. The analyzes were performed in triplicate, involving a total of 48 petri dishes, the results were determined using a Digital Vernier Caliper (CALDI-6MP, Truper), giving the diameter of the halo in mm.

3. Results and discussion

In Figure 1, the effect generated by the hydroalcoholic extract of *Musa paradisiaca* (HEMP) is observed in the gram negative bacterium *Pseudomonas aeruginosa*, appearing a halo of inhibition of 7.3 mm in 10 mg/ml HEMP, 8.2 mm in 20 mg/ml HEMP, 10.7 mm in 40 mg/ml HEMP and 12.9 mm in 60 mg/ml HEMP. The gram positive bacteria, *Staphylococcus aureus* has an inhibition halo of 8.3 mm in 10 mg/ml HEMP, 10.5 mm in 20mg/ml HEMP, 13.6 mm in 40 mg/ml HEMP and 16.4 mm in 60 mg/ml HEMP, and *Bacillus subtilis* has a halo 7.2 mm in 10 mg/ml HEMP, 7.8 mm in 20 mg/ml HEMP, 10.3 mm in 40 mg/ml HEMP and 11.3 mm in 60 mg/ml HEMP. It is observed that the higher concentration of the hydroalcoholic extract generate greater growth of the inhibition halo; Therefore, the chemical composition of the leaves plays an essential role as an antibacterial potential.

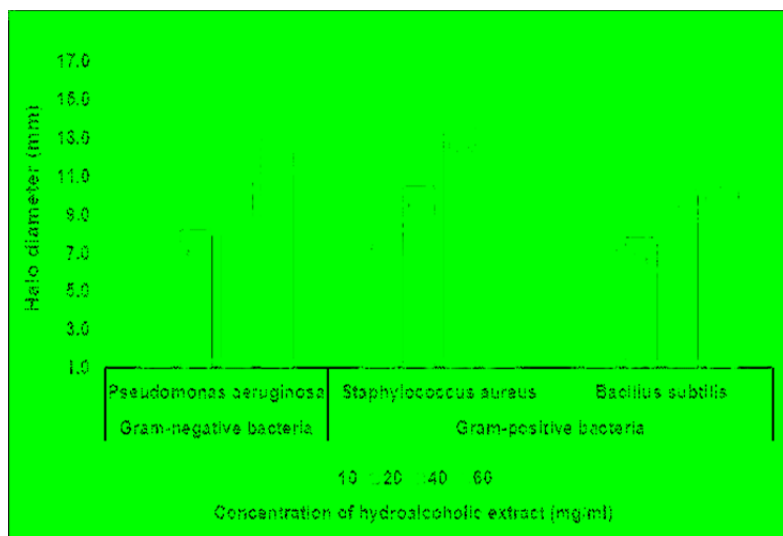


Figure 1: Halo diameter of the bacteria in hydroalcoholic extract of *Musa paradisiaca*

In Figure 2, the effect generated by the hydroalcoholic extract *Cocos nucifera* (HECN) is observed in the gram negative bacterium *Pseudomonas aeruginosa*, showing a 5.8 mm inhibition halo in 10 mg/ml (HECN), 7.1 mm in 20 mg/ml (HECN), 8.8 mm in 40 mg/ml (HECN) and 11.1 mm in 60 mg/ml (HECN). In gram positive bacteria, *Staphylococcus aureus* has an inhibition halo of 6.8 mm in 10 mg/ml (HECN), 8.6 mm in 20 mg/ml (HECN), 11.1 mm in 40 mg/ml (HECN) and 14.4 mm in 60 mg/ml (HECN). *Bacillus subtilis* shows a halo of 5.3 mm in 10 mg/ml (HECN), 6.4 mm in 20 mg/ml (HECN), 8.4 mm in 40 mg/ml (HECN) and 10 mm in 60 mg/ml (HECN). It is important to emphasize that the chemical composition of the leaves plays an essential role, since the hydroalcoholic extracts of plants release phenolic compounds, flavonoids and other compounds, which can explain their antibacterial action.

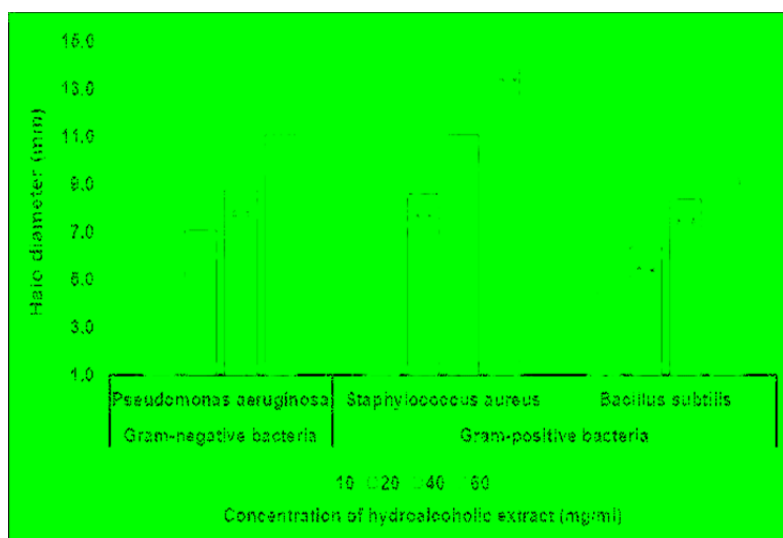


Figure 2: Halo diameter of bacteria in hydroalcoholic extract *Cocos nucifera*

In Figure 3, the effect generated by the hydroalcoholic extract of *Coffea sp* (HECS) on the gram positive bacterium *Staphylococcus aureus* is observed, with an inhibition halo of 3.9 mm in 10 mg/ml (HECS), 4.9 mm in 20 mg/ml (HECS), 6.3 mm in 40 mg/ml (HECS) and 7.9 mm in 60 mg/ml (HECS). Only antibacterial potential is evidenced in a single species of bacteria, probably due to a slight presence of its active ingredients

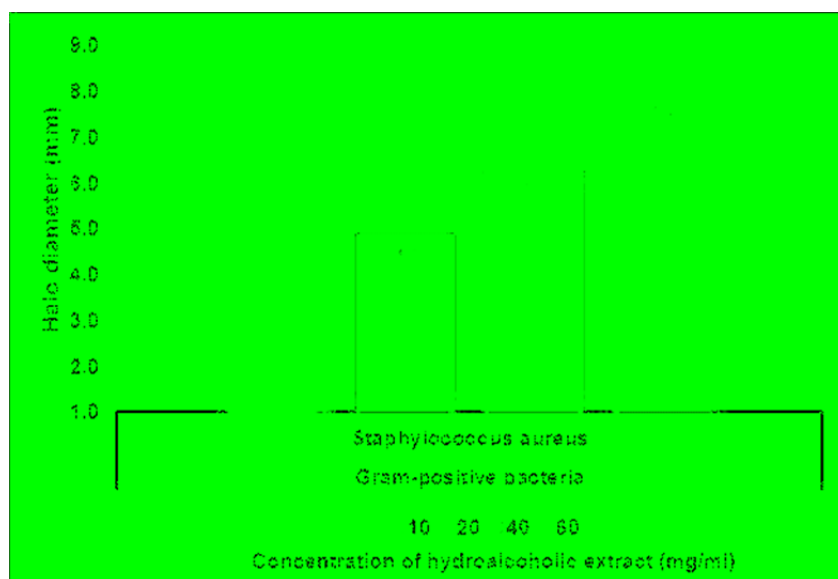


Figure 3: Halo diameter of bacteria in hydroalcoholic extract of *Coffea sp*

4. Conclusions

It is evidenced that a higher concentration (60 mg/mL) of hydroalcoholic extracts, the halo of inhibition is higher with respect to the growth of bacteria. But, there is not antibacterial potential of hydroalcoholic extracts in *E. coli*.

The existence of the antibacterial potential in the hydroalcoholic extract of *Musa paradisiaca* and *Cocos nucifera* for gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* and gram negative is confirmed only in *Pseudomonas aeruginosa*, in the *Coffea sp* extract only bacterial action is observed in *Staphylococcus aureus*; That is why it is important to study regional plants as an alternative for the development of new phytosanitary products.

References

- Atanasov A.G., Waltenberger B., Pferschy-Wenzig E.M., Linder T., Wawrosch C., Uhrin P., Temml V., Wang L., Schwaiger S., Heiss E.H., Rollinger J.M., Schuster D., Breuss J.M., Bochkov V., Mihovilovic M.D., Kopp B., Bauer R., Dirsch V.M., Stuppner H., 2015, Discovery and resupply of pharmacologically active plant-derived natural products, *A review Biotechnol*, 33, 1582-1614.
- Ávila R., Montero A.F., Aguilar P., Vera O., Lazcano M., Morales J.C., Navarro A.R., 2019, Antioxidant Properties of Amazonian Fruits, *A Mini Review of In Vivo and In Vitro Studies*, *Oxid Med Cell Longev*.
- CDC (Center for Disease Control and Prevention), 2013, National Center for Health Statistics. Health, United States with special feature on prescription drugs. <www.cdc.gov> accessed 20.10.2019
- CDC (Center for Disease Control and Prevention), 2016, Antibiotic/Antimicrobial Resistance. <www.cdc.gov> accessed 28.10.2019
- Chaves T.L, Ricardo L., De Paula J., Brandão M.G.L., 2015, Useful Brazilian plants under the view of the writer-naturalist João Guimarães Rosa, *Revista Brasileira de Farmacognosia*, 25, 437– 444.
- Chilquillo E.A, Albán J., Muñoz A., 2018, Estudio etnobotánico de plantas medicinales utilizadas en comunidades adyacentes al Área de Conservación Privada San Antonio, Chachapoyas, Amazonas, Perú, *Revista de Investigación Científica UNTRM Ciencias Naturales e Ingeniería*, 1, 65-73.
- Corrales I.E y Reyes J.J., 2015, Actividad etnofarmacológica y antimicrobiana de los componentes químicos de las plantas medicinales utilizadas en Estomatología, *April 16 student magazine*, 54, 71-83.
- Heisler E.V., Budó M.D., Schimith M.D., Badke M.R., Ceolin S., Heck R.M., 2015, Uso de plantas medicinais no cuidado á saúde, produção científica das teses e dissertações da enfermagem brasileira, *Enfermería Global*, 14, 390-417.
- Lock O., 2016, *Investigación Fitoquímica: Métodos en el estudio de productos naturales*. 3era edición, Editorial del Departamento de Ciencias. Universidad Pontificia Católica del Perú, Lima, Perú, 6-8.

- Marchi L., Dornellas F., Polonio J., Pamphile J., Monteiro A., Goncalves O., Perdoncini M., 2019, Antifungal Activity of Curcuma Longa L. (zingiberaceae) Against Degrading Filamentous Fungi, Chemical Engineering Transactions, 75, 319-324
- Peiris L.D., Dhanushka M.A., Jayatilleka T.A., 2015, Evaluation of Aqueous Leaf Extract of *Cardiospermum halicacabum* (L.) on Fertility of Male Rats, Hindawi Bio Med Research International, 2015: 175726.
- Rezende F., Sande D., Coelho A.C., Oliveira G., Boaventura M.A., Takahashi J.A., 2019, Edible Flowers as Innovative Ingredients for Future Food Development: Anti-alzheimer, Antimicrobial and Antioxidant Potential, Chemical Engineering Transactions, 75, 337-342
- Rodríguez F.A., Pérez A.F., Iglesias C.A., Gallego R.M., Veiga B.L., Coteló N.V., 2015, Actualidad de las plantas medicinales en terapéutica, Acta Farmacéutica Portuguesa, 4, 42-52.
- Sánchez E; Castillo S.L y García P., 2016, Actividad antimicrobiana. En Rivas, C; Oranday, M y Verde, M (Eds.), Investigación en plantas de importancia médica. Barcelona, España, 77-100.
- Sika C., Sina H., Adoukonou H., Ahoton G.O., Roko G., Saidou A., Adéoti K., Ahanchede A., Baba L., 2014, Antimicrobial activity of Anacardium occidentale L. leaves and barks extracts on pathogenic bacteria, African journal of microbiology research, 8, 2458–2467.
- Teles D.G y Costa M.M., 2014, Estudo da ação antimicrobiana conjunta de extratos aquosos de Tansagem (*Plantago major* L., Plantaginaceae) y Romã (*Punica granatum* L., Punicaceae) e interferencia de dos meses en una dosis de amoxicilina *in vitro*, Revista Brasileira de Plantas Mediciniais, 16, 323–328.
- Vega M.C., Verde J., Oranday A., Morales M.E., Nuñez M.A., Rivera M.A., Serrano L.B., Rivas C., 2013, Actividad antibacteriana y citotóxica de *Leucophyllum frutense* (Berl) I. M. Johnst del Norte de México contra *Staphylococcus aureus* de aislados clínicos, Revista Mexicana de Ciencias farmacéuticas, 44, 24-30.
- WHO. 2013, Estrategia de la OMS sobre medicina tradicional 2014-2023.