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Evaluation of Anti-Microbial Activity of Extracts of *Moringa oleifera* Lam. Cultivated in Cuba

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ABSTRACT

Background: *Moringa oleifera* (Moringaceae) is a highly praised plant that has spread in many tropical and subtropical countries. It has an impressive variety of medicinal uses with a high medicinal value. **Aim**. To evaluate the *in vitro* anti-microbial activity of aqueous and hydro-alcoholic extracts of *Moringa oleifera* Lam. cultivated in Cuba. **Methods**: The MTT method was used to evaluate *in vitro* cytotoxicity and antiviral activity of different concentrations of the extracts from dry leaves of *Moringa oleifera* against Type 1 Herpes Simplex Virus (HSV-1), and type 2 Herpes Simplex Virus (HSV-2). The determinations were made by microdilution, antimicrobial activity against the reference bacterial strains, and clinical isolates, then the minimum inhibitory concentration (MIC) was calculated. **Results**: The hydroalcoholic extract showed toxicity at the 125 µg/ml concentration, whereas the aqueous extract stopped being toxic at 500 µg/ml in the *Vero* cells within the range of the concentrations evaluated. None of the extracts inhibited the *in vitro* replication of the HSV-1 and HSV-2 viruses, but they showed a powerful activity against the bacterial strains and yeast assayed at different levels. **Conclusions**: The extracts did not show *in vitro* antiviral activity against HSV-1 and HSV-2 in *Vero* cells, whereas the antibacterial activity was strong, suggesting that the leaves of *Moringa oleifera* might be a good antibacterial agent candidate from natural products against the different pathogens.

Keywords: Antimicrobial activity, antiviral, herpes simplex, *Moringa oleifera* Lam. (*Source*: *NAL-USDA)*

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INTRODUCTION

The proliferation of diseases caused by pathogenic microorganisms is a general concern that poses a health risk. Antibiotic and antiviral resistance is one of the most significant public health issues worldwide. The utilization of natural substances to treat different diseases, including infectious diseases, is currently a challenge to medicine, but at the same time, an alternative (Domingo, 2003).

Moringa oleifera is the most relevant species of the family Moringaceae, genus *Moringa* (Alegbeleye, 2018) cultivated in Cuba. The main interest is in the leaves, as they preserve a remarkable quantity of macro and micronutrients. It is internationally known as the tree of life or miraculous plant for its several uses and applications, especially in medicine and nutrition. The plant has numberless nutritional and therapeutic properties, which can be found in the seeds, roots, leaves, flowers, pods, and fruits. Various research studies have demonstrated that the medical properties of Moringa are helpful as an antioxidant, to treat respiratory, cardiovascular, gastrointestinal, and endocrine diseases. They are also useful to the central nervous system, cancer, the immunological system, as an antiviral, and antibacterial (Jung, 2014).

The literature reports several studies aiming to discover new compounds with biological activities based on natural sources, whereas others are intended to check the already attributed properties (Miranda and Cúellar, 2001). Considering the potentialities of *Moringa oleifera*, the purpose of this paper was to evaluate the antimicrobial activity of different extracts of dry *Moringa oleifera Lam.* leaves cultivated in Cuba.

MATERIALS AND METHODS

Plant material

The plant material consisted of *Moringa oleifera* Lam. harvested on the *Futuro Lechero Farm*, in areas used for pharmaceutical purposes in Cuba. The leaves were washed with drinking water, dried in a solar oven at 45--50 °C, and milled. The material was stored in polyethylene bags at room temperature, in a cool dark place until the analysis.

Extract preparation

The dry *Moringa oleifera* leaves were used to make aqueous and alcoholic extracts (70% V/V), according to the methodology described by Miranda and Cúellar (2001). The hydro-alcoholic extraction (alcohol/water) was done by crushing at a 1/10 proportion, for 6 days, and included a change of solvent on the 3rd day, at 25-28 ºC. The aqueous extraction was done by crushing below 50 ºC.

Cell culture

The cell-substrate used was the *Vero* cell line (African green monkey kidney) from the *American Type Culture Collection* (ATCC), grown in 199 (ICN, FLOW), medium, supplemented with 10%

Inactivated Fetal Bovine Serum (SFBI, HYCLONE), and 1 mg/ml neomycin sulfate (SIGMA).

Virus

The viruses used in the study were the 8 WC reference strain from the Type 1 Herpes Simplex Virus (HSV-1), from The Carlos III Institute in Madrid, and the MS strain from Type 2 Herpes Simplex Virus (HSV-2), from the Pasteur Institute. The viruses were inoculated in 25 cm^3 containers, with a *Vero* cell confluent monolayer, at a 0.1 infection multiplicity (imp). Then they were incubated for one hour to favor adsorption. After incubation, the 199 medium and 2% SFBI were added and incubated. The harvest was initiated when 70% of the monolayer showed the characteristic Cytopathic Effect (CPE). Acyclovir was used as a positive control, cellular control, and virus control.

Virus titration

The virus strains were titrated by a 50% final point assay, and the result was expressed as a mean infective dose of the cell culture per milliliter ($DICT50.mL^{-1}$). The estimation of ineffective response was performed following the procedure described by Reed and Muench (1938).

Cytotoxicity assay

Cytotoxicity was estimated by a bromide reduction assay (3-(4,5-dimethyltiazole-2-il)-2,5-diphenyl-2Htetrazolium (MTT). A *Vero* cell confluent monolayer grown 48 hours before was added to shallow 96-well plates. The concentration range evaluated was 62.5, at 1000 µg/ml (seven repetitions per concentration). The plates were incubated at 37 ^{o}C, with 5% CO_2. An inverted microscope was used for daily observations to check the occurrence of morphological changes indicating toxicity. At 72 hours, 10 µl MTT 5 000 µg/ml dissolved in phosphate buffer saline solution (PBS) (0.01 mol/l; pH 7) were added. It was slightly shaken and incubated for 4 hours at the cell line growth temperature, in the dark. Then all the plate's content was carefully removed, and the formazan crystals were dissolved using 100 µl DMSO per well. Absorbance was read at 540 nm in an ELISA reader (Organon Teknika Reader 530).

The cellular viability percentage associated with every concentration of the extract was calculated by dividing the mean absorbance value of the cultures treated with such concentration, by the mean value of absorbance of the cell controls (untreated), with 100% viability. The mean Cytotoxic Concentration (CC50) value was determined by linear regression using a dose-response trend (extract concentration-cellular viability percentage).

The MTT method (Mosmann, 1983; Freshney, 1994)

A volume of 20 µl MTT was added per well, diluted in 0.1 M saline solution, pH 7, in phosphate buffer (SSPB) at a 5 mg/ml concentration, and were incubated for three hours at 37 ºC, in 5% CO_2. Upon incubation, the medium was removed from the wells, and 100 µl dimethylsulfoxide (DMSO) (BDH) was added. The plates were shaken at 300 r.p.m. for 10 minutes, in a KS 500 shaker. It was read at 540 nm in an ELISA reader (Organon Teknika Reader 530).

Antiviral activity study

Determination of the effective mean antiviral concentration (CE_{50})

The CE_{50} assay was performed in shallow 96-well plates containing *Vero* cell confluent monolayers, at $2x10^5$ cells/ml, then 50 µl of different concentrations of each fraction (four repetitions per concentration) were added per well. It was incubated for 1 h at 37 ºC, in 5% CO2. Following incubation, 50 µl of 100 Cell Culture Infectious Dose ($CCID_{50}$) of the virus, were added to the wells. It was incubated at 37 ºC, in 5% CO2, until the appearance of PCE in every control well of the untreated virus. Acyclovir was used as the positive control. The MTT colorimetric method was used to determine cell viability. The experiment was done by triplicate.

Evaluation of antimicrobial activity

Bacterial strains

Reference strains: *Escherichia coli (ATCC10536), Staphylococcus aureus (ATCC 6538), Candida albicans (ATCC 10231) Pseudomonas aeruginosa (ATCC9027), Salmonella thyphi (ATCC9992*), recommended by the National Committee of Clinical Laboratory Standards (NCCLS) to study antimicrobial sensitivity (NCCLS, 2000), and microorganisms isolated from clinical samples: *Staphylococcus aureus* (3cepas), *Acinetobacter iwoffii* (2 strains), *Enterobacter agglomerons* (1 strain), *Pseudomonas aeruginosa* (1 strain), *Escherichia coli* (2 strains) from the Juan Manuel Marquez Pediatric Hospital, Havana.

Determination of the minimum inhibitory concentration (MIC)

The antimicrobial activity was determined by the microdilution method, according to the NCCLS (NCCLS, 2000), to determine the Minimum Inhibitory Concentration (MIC). The inoculate of the microbial strains adjusted to the 0.5 Mc Farland scale was prepared. From a bacterial growth in plates containing Tryptone Soy Agar and yeasts in Sabouraud medium, at 37 ºC, for 18-24 hours. Double dilutions of the extracts were made, between 100 mg/ml and 0.78 mg/ml. The MIC was determined as the lowest concentration of the extract, capable of inhibiting bacterial and yeast growth. The negative control was the incubation using 70% ethanol solution (v/v).

RESULTS AND DISCUSSION

Cytotoxicity assay and antiviral activity

The exposure of the *Vero* cells to the extracts demonstrated that the aqueous extract at the 500 µg/ml concentration did not cause toxic effects on the cells, whereas the alcoholic extract did not show toxicity at the 125 µg/ml concentration and further, with cell viability of 80% in the two extracts. From these values on, cell viability decreased while the extract concentration rose. The microscopic observation of cultures evidenced characteristic damage caused by the extracts, such as the production of granules in the cytoplasm, and the alteration of cell morphology, which increased along with the concentration. Hence, the extracts did not produce toxicity at low

concentrations.

Based on the previous knowledge about cell fractions, the antiviral activity against HSV-1 and HSV-2 was determined. The extract concentrations used did not show any cytotoxic activity, and corresponded to cell viability above 50%.

The results of the primary assays of *in vitro* antiviral activity justified that none of the extracts inhibited the multiplication of the Herpes Simplex viruses assayed.

Antimicrobial activity

The antimicrobial activity of the extracts against proven pathogens is shown in table 1. The 70% hydroalcoholic extract showed higher activity compared to the aqueous extract, both against bacterial strains and yeast at different concentrations, inhibiting 100% of the strains evaluated. The best CML values (3.13 mg/g) were achieved against *Vibrio cholerae* (ATTC7258), *Salmonella typhi* (ATCC9992), *Acinetobacter iwoffii* (1 clinically isolated strain), *Staphylococcus aureus* reference strain (ATCC6538), and the three clinically isolated strains, whereas the aqueous extract inhibited 46.66% of the strains studied: *Salmonella typhi* (ATCC9992), *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (ATCC6538), and the 3 clinically isolated strains. The activity dropped along with the reduction of the concentrations.

Table 1. Anti-microbial activity of *Moringa oleifera* Lam. extracts

Microorganisms	**Extracts (MIC)**	
	Aqueous	Hydro-alcoholic
Escherichia coli (ATCC 10536)	N	12.55
Pseudomonas aeruginosa (ATCC9027)	N	12.55
Vibrio cholerae (ATCC 7258)	N	3.13
Candida albicans (ATCC 10231)	11.1	3.13
Salmonella tiphi (ATCC9992)	11.1	3.13
Staphylococcus aureus (ATCC6538)	11.1	3.13
Staphylococcus aureus (ac)	11.1	3.13
Staphylococcus aureus (ac)	11.1	3.13
Staphylococcus aureus (ac)	11.1	3.13
Acinetobacter iwoffii (ac)	N	3.13
Acinetobacter iwoffii (ac)	N	12.55
Enterobacter agglomerons (ac)	N	12.55
Pseudomonas aeruginosa (ac)	N	12.55
Escherichia coli (ac)	N	12.55
Escherichia coli (ac)	N	12.55

Legend: N negative.

Cytotoxic and antiviral activity

The MTT colorimetric method was used in the assay, which is widely recognized as a helpful tool to evaluate cytotoxicity and cell proliferation. In the two extracts, high concentrations were demonstrated to produce toxicity in the cell cultures, which might be caused by the presence of cytotoxic plat constituents in them. These results are similar to other cytotoxicity studies on

Moringa oleifera leaf extracts, with variabilities, coinciding with the fact that toxicity is not detected in the cells at low concentrations. In Thailand, a study using alcoholic and aqueous extracts from *Moringa oleifera* showed cytotoxicity at concentrations above 100 µg/ml in cancerous cells. (Saetung *et al*., 2005). The cytotoxicity of aqueous extracts of *Moringa oleifera* leaves in *Hela* cells caused high cell mortality from de100 µg/ml (Nair and Varalakshmi, 2011).

Assays performed on methanol extracts of *A. indica* and *M. oleifera* demonstrated to inhibit cellular viability (CC_{50}) at a concentration of 70 µg /ml, and showed no toxicity against the MDCK cell line at low concentrations (Arévalo-Híjar *et al.*, 2018; Jumba *et al*., 2015), evaluated the cytotoxicity of the methanol extract of *M. oleifera* against the *Vero-E-6* cell line and determined CC_{50} at 149 µg/ml.

Studies on *Moringa oleifera* using a primary culture of leukocytes of *S. aurata* HK showed that both the aqueous and alcoholic extracts remained viable for 24 h of incubation, except when a high concentration of the aqueous extract was used (Othmen *et al.*, 2020). Rahaman *et al.* (2015) conducted an assay of the effect of aqueous extracts of *Moringa oleifera* leaves and seeds on MCF-7 cells and confirmed that cell viability decreased along with the reduction in the level of the drug used for that purpose.

Antiviral activity

The extract concentrations used did not show any significant cytotoxic activity, and corresponded to cell viability above 50%.

The results of the primary assays of *in vitro* antiviral activity justified that none of the extracts inhibited the multiplication of the Herpes Simplex viruses in the *Vero* cells. In that sense, perhaps the absence of activity is linked to the characteristics of the cellular receptors that mediate the infections process of *Vero* cells, having a differential susceptibility in the different cell line types. Although the activity against these viruses has been particularly reported *in vivo* assays, delaying the development of cutaneous herpetic lesions, prolonging the mean survival times, and reducing the mortality of mice infected with the Herpes Simplex.

Several papers refer to the activity of different parts of the plant *Moringa* with activity against the Herpes in *vivo* assays, which demonstrated to delay the progression of lesions caused by these viruses. Lipipuna *et al*. (2003), upon evaluating extracts from different plants, including *Moringa*, found that when *moringa* leaf extracts were administered to mice according to their weight, their lesions were retarded, prolonging the survival time, and reducing mortality after being infected with HSV-1. The protective effect of *Moringa oleifera* in the mice was not associated with a direct antiviral effect but could have resulted from an immunomodulating activity, or greater bioavailability of *Moringa oleifera*, and therefore, this extract might contain the active compounds necessary to treat the cutaneous infection caused by HSV-1 effectively.

Kurokawa *et al*. (2016), using HSV-1 in mice, administered 300 mg/kg doses of aqueous extracts of Moringa oleifera Lam leaves, and was able to limit the development of herpetic lesions, and reduce the virus titers in the brain, inducing the production of interferon, with an inducer effect

on cellular immunity. The *in vitro* assay of *Vero* cells, using alcoholic and aqueous extracts found activity against HSV-1, at a CE_{50} of 100 μg/ml, whereas the aqueous extract did not show *in vitro* activity, which possibly meant that the components with an activity in the extracts were removed during the extraction.

In this study, the *Moringa oleifera* extracts did not neutralize the Herpes virus strains studied. However, further research is needed in this area to study its potential against the Herpes Simplex viruses, assaying several cell lines and other viruses where the *M. oleifera* extracts have been reported to have an inhibiting effect, as on Herpes zoster, Human Immunodeficiency Virus (HIV), Hepatitis B, etc. (Abd rani, 2018; Mattar, 2019).

Antibacterial activity

Several studies refer to the properties of *Moringa oleifera* in experimental research, using several types of extracts (Franco-Ospina *et al.*, 2013). In this paper, the activity of the hydroalcoholic and aqueous extracts from Moringa oleifera Lam. against bacteremia-causing pathogens was evaluated.

Both extracts were active against the genus *Staphylococcus*, showing a better response with the hydroalcoholic extract than the aqueous one. The former showed activity against all the strains assayed whereas the latter only inhibited six strains. This antimicrobial activity confirms that the phytochemicals present in the leaves of *Moringa oleifera* confer these properties. These results corroborate the reports of Vivian *et al*. (2021), who found the presence of high concentrations of polyphenols and flavonoids in the leaves of different *Moringa oleifera* ecotypes cultivated in Cuba. It demonstrated that these compounds contribute to the inhibition of microbial growth by several mechanisms. Akinpelu (2001) noted that the secondary compounds comprise the active principles, with antibacterial, fungicide, antiviral, antitumor, analgesic, anti-inflammatory, hypotensive, and immunostimulating properties.

The variation of antibacterial activity of the extracts against different strains might be caused by the level of polarity and solubility associated with solvents in capturing active metabolites with a direct action on the cell wall of the pathogens (Seleshe and Kang, 2019). Ethanol tends to drag more polar compounds such as phenols, tannins, flavonoids, and other metabolites. It has been reported that these compounds have antimicrobial activity, since they inhibit the formation of the cell wall and DNA or RNA synthesis, and hinder bacterial growth, inhibiting enzymes through oxidized compounds, perhaps by reactions of the sulfhydryl group, or by non-specific interactions with proteins (Domingo, 2003). Moreover, the hydroalcoholic mixtures favor higher solubility of the active components responsible for the bactericidal activity and increase the permeability of the cell wall (Sahin and Samli, 2013). Thus, the hydroalcoholic extract had greater antimicrobial activity, with better MIC values (3.13 mg/g) than the aqueous extract, which might be caused by the high metabolite concentrations in the hydroalcoholic extracts.

The lower activity of the aqueous extract may be attributed to the fact that water removes other metabolites that might interact antagonistically in this activity, and probably needs high

concentrations to have a better antibacterial effect. Martin (1998) reported that the aqueous extracts have little antimicrobial activity and that the active principles of the plants are not easily removed with water.

Rahman (2009) studied the antibacterial activity of aqueous and alcoholic extracts from the leaves of *Moringa oleifera in vitro*, in which both extracts showed antimicrobial activity against Gram [-] and Gram [+] bacteria; the alcoholic extract's activity was higher than the aqueous extract.

S. aureus is a pathogenic species capable of causing the appearance of numerous skin infections, which might even become hard to eliminate. The biological activity of alcohol and aqueous extracts of *Moringa* inhibited the growth of different *S. aureus* strains. The MIC values against the *S. aureus* strains coincide with the study of Benavides (2019), who demonstrated the antibacterial activity of *Moringa* leaf extracts against *S. aureus.*

The antibacterial activity of the alcoholic extract was stronger against *S. aureus* than *E. coli*., with more sensitive Gram [+] than Gram [-] organisms. Similar studies conducted by Bukar *et al*. (2010) concluded that the alcoholic extracts of *Moringa oleifera* leaves were sensitive to *S. aureus and E. coli at* 200 mg/ml concentrations. The fact of finding higher effectiveness against Gram [+] bacteria than against Gram [-] bacteria may be linked to the structure of the cell wall. The Gram [+] bacteria have an internal cell wall, and a peptidoglycan wall, whereas the Gram [-] bacteria have a more complex cell wall (internal wall), the peptidoglycan wall, and outer lipid double layer or outer membrane, making a rigid sac around the bacterium, an impermeable macromolecule barrier that offers protection in adverse conditions. They act as a barrier against a large number of substances, including antibiotics (Ali e*t al*., 2001). This may also explain why medicinal plants tend to be more effective against Gram [+] bacterial cultures than against Gram [-] bacteria.

The study also revealed the antibacterial activity of the extracts against bacterial strains of *Salmonella tiphi* and *Candida albicans***.** Doughari and Pukuma (2007) reported similar results using the plate-diffusion technique, with an 8 mm inhibition halo. In a study of *Moringa* extracts in several organic solvents at different concentrations, against Gram [+] and Gram [-] bacteria, Manikandan (2016) noted that most extracts inhibited both organisms.

The efficiency of *Moringa oleifera* extracts as antibacterial agents may be caused by certain active components, which might work synergistically as antagonists of cell growth and viability. These compounds, responsible for such activity, may easily pass through the cell membrane due to the presence of alcoholic groups that inhibit the essential enzymes of the cell membrane and the synthesis of peptidoglycan and generate negative-charged free radicals that could disorganize or break the cell membrane. Likewise, the soluble compounds present in *Moringa oleifera* may harm the cell membrane, affecting cell permeability and bacterial growth and survival (Wang *et al.*, 2016).

CONCLUSIONS

The constant search for new antimicrobial substances that offer phytotherapeutic alternatives against multiresistant strains of microorganisms based on biological activity research, offers a system that can be used in healthcare services. The results of this study suggested that the aqueous and alcoholic extracts from dry leaves of *M. oleifera* have a potential number of compounds with promising antimicrobial activities, and inhibiting effects against pathogenic species, including Gram [+] and Gram [-] species. It might contribute to the treatment of infectious diseases caused by these microorganisms. These results validate the need for further research studies on new compounds present in the dry leaves of *M. oleifera* with relevant antimicrobial activity and natural antimicrobial agents that provide an inexpensive and sustainable method to control diseases and improve life quality.

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AUTHOR CONTRIBUTION

Conception and design of research: RMB, OFP, OEA; data analysis and interpretation: RMB, OFP, EAH, VLA, OEA, GBQ: redaction of the manuscript: RMB, EAH, VLA.

CONFLICT OF INTERESTS

The authors declare the existence of no conflicts of interest.