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Original Article

In Vitro Antimicrobial Action of Bixa Orellana L.

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Abstract

INTRODUCTION: *Bixa orellana L.* is a plant that grows in Cuba, which is held to have medicinal properties.

MATERIALS AND METHODS: An experimental study was carried out to evaluate the in vitro antimicrobial action of 50% fluid extract and 10% and 20% tinctures made of the leaves of the species *Bixa orellana L.* The method of macrodilution in broth was used and minimal concentration with antimicrobial activity, expressed in dilutions, was determined. Strains of international reference were used.

RESULTS: The tinctures at 10% and 20% of the leaf showed bactericidal action against *Staphylococcus aureus* at a concentration of 1:8 and 1:32 respectively, whereas only 10% tincture showed antimicrobial activity against *Escherichia coli* at a concentration of 1:8, however, they were not active against *Pseudomona aeruginosa* and *Candida albicans*. As to 50% fluid extract, it only showed bactericidal activity against *Staphylococcus aureus* at a concentration of 1:4.

CONCLUSION: Both, fluid extract at 50% and tinctures at 10% and 20% of the leaf were active against *Staphylococcus aureus* at lower concentrations compared to the rest of microorganisms, but they had no antimicrobial activity against *Candida albicans* or *Pseudomona aeruginosa*. 50% fluid extract showed antimicrobial activity only against *Staphylococcus aureus*. Tincture at 10% confirmed activity against *Staphylococcus aureus* and *Escherichia coli*. Tincture at 20%was of use only against *Staphylococcus aureus*. *Staphylococcus aureus* turned out to be the most sensitive germ to the pharmaceutical forms studied at lower concentrations. Tincture at 10% showed a wider spectrum of antimicrobial activity.

Keywords: *Bixa orellana L.,* Antimicrobial Action, Fluid Extract, Tincture.

Introduction

Natural and traditional medicine is a system arisen from people's beliefs and well accepted by their cultures. Given this understanding, herbal medicine or phythotherapy, as it is usually named, is as old as mankind.[1-3]

Leaves, stalks, roots and other organs provide food, spices and active principles against several disorders. A number of research has been focused on finding new compounds with biological activities from natural sources. Among them a considerable number of studies have been aimed at evaluating antimicrobial activity in extract and essential oils from medicinal and aromatic plants. [4-6]

With that purpose, in vitro techniques have been used due to their simplicity and reproducibility. Cuba has been including this international trend and along the country there are scientific institutions dedicated to study different pharmacological activities within the vegetable kingdom, and the richness of the Cuban flora offers infinite possibilities. [7-9]

Bixa Orellana L is a vegetable specie that grows in Cuba. A number of actions such as antitumoral species (mainly in buccal cancer), aphrodisiac, anti-inflammatory, astringent, emollient, antiseptic, antibacterial, fungicide, leishmanicide, antioxidant, expectorant, cicatrizant, diuretic, hypoglycemic, laxative, vermifuge, febrifuge and a source of vitamines are attributed to its leaves and seeds. [10, 11]

Antimicrobial activity in this plant varies according to the species (due to the genetic differences that may exist among them), the characteristics of the ground and the weather conditions of the place where it grows as well as the period in which the vegetable material is picked up, the organ employed, the concentration of active principles used and the pharmaceutical form made. All what is stated above encouraged the researchers to carry out this

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research with the species that grows in Camagüey (there are no specific studies about it) using 50% fluid extract and 10% and 20% tinctures made of the leaves.[12]

Taking into account what it is stated above, *Bixa orellana* should be considered a potential therapy for the search of new powerful and efficacious pharmaceutical forms that might be effective in the treatment of infections caused by pathogenic microorganisms, and also in the prevention of the appearance of microbial resistance to synthetic antibiotics traditionally used in modern medicine.[12]

It is important to make known that bacterial resistance has become a serious health problem worldwide in which some factors get involved, for example: the use and abuse of antimicrobials, debilitation of infections control programs, highest complexity and terminally ill patients care, and the use of antimicrobials for non-medical usage among others. [13,14]

Microbial resistance is considered the silent epidemic of the XXI. This process acquires greater dimensions in hospital environments where very aggressive germs have arisen and can easily spread from patient to patient. US statistics show that hospital infections contribute to the death of 60,000 people every year, with a cost for health institutions of 4.5 billion dollars per year and it is estimated that 90% of such infections are caused by multi-resistant germs.[15-17]

Studies carried out in Cuba have proved that *Streptococcus pneumonia* progressively increased resistance to penicillin. Other researches show that *Salmonella typhi* in the country have shown resistance fhave shown Salmonella typhi resistance to chloranphenicol from 21 to 22%, to trymethropin from 41 to 46%, of 12% to nalidixic acid and it is systematically increasing its resistance to Ampicillin. *Neisseria meningitidis* type B nowadays presents a resistance of 88.9%. [19]

Materials and Methods

An experimental study was carried out to evaluate the In vitro antimicrobial action of the fluid extract and the elaborated tinctures from *Bixa orellana L* leaves, in the Center of Immunology and Biological products (CENIPBI) of the University of Medical Sciences of Camagüey, during the period comprised between January 2013 and January 2014.

Leaves were collected early in the morning and packed in polyethylene bags to be taken to the lab. A sample of the collected stuff was taken to the herbarium of the Academy of Sciences (HACC), in Camagüey city for its taxonomic identification, and the corresponding certificate was issued.

Once the plant was identified, the leaves were washed with tap water and later with chlorine water to eliminate impurity. Later they were dried in a stove with air re-circulation, at a temperature of 45º C, for 72 hours and subsequently crushed. Three preparations or pharmaceutical forms were prepared.

- Hydro-alcoholic extract at 50%: elaborated through the technological process of repercollation, at 1g of dry vegetable material per mL of menstruum or solvent (alcohol at 50%)
- Tinctures at 10% and 20%: made through maceration technique, at 19g and 20g of dry vegetable material respectively, per each 10ml of menstruum (alcohol at 50%).

After the products were made, they were passed through filter paper to eliminate impurities and sterilized by filtration through milliporum membranes. Later on, they underwent a quality control process in which the constituted parameters were established (pH, refraction range, relative density and total solids). A negative control was used; it was done by taking 1 ml of the substance to be studied and a mL of the culture medium was added in order to check if there was a growth of germs.

Once the process was concluded, the product was bottled in amber bottles; they were labeled on and stored at room temperature.

Different culture medua were used depending on the germs used for the research:

- Agar and Triptone Soy broth.
- Agar and Mueller Hinton broth (doubled concentrated).
- Agar and Sabouraud broth.

Control of Sterility

A sample of 1mL of each culture mean was analyzed, and incubated at temperature and time established, without microorganism growing confirmed by sterility.

Mean of Culture Quality Control

The positive control was carried out taking a sample of 1 mL of the culture media and 25µL of the inoculum were added. The growth of microorganisms was observed, which proved the quality of the culture mean.

International reference stocks from the American type of Culture Collection (ATCC) that were used:

- Staphylococcus aereus. ATCC 25293
- Escherichia coli. ATCC 25922
- Pseudomona aeruginosa. ATCC 25853
- Candida albicans. ATCC 10231

Before determining the antimicrobial activity of the preparation, work dilutions of the different pharmaceutical forms and alcohol were prepared, the last one was taken as a reference, to discard that antimicrobial activity was due to alcohol.

Alcohol Dilutions

Alcohol dilutions at 50% with sterile bidistilled water were done using macrodilution technique.

Tube #1: Alcohol at 50% not diluted.

Tube #2: 10ml of sterile bidistilled water to pass 10mLof alcohol at 50% (dilution 1:2).

Tube #3 to tube #9, 10mL of sterile distilled water plus doing passes of 10mL of alcohol at 50% of the previous dilution, until dilution 1:256 and discard there 10mL.

It is considered that the product studied had great antimicrobial activity, as this one exceeded in two dilutions or more the one shown by the alcohol.

Dilutions of Preparations in Study

The preparation of dilutions of different pharmaceutical forms was also performed by using macrodilution technique.

Tube #1: Substance in study not diluted.

Tube #2: 10mL of sterile distilled water plus passes of 10 mL of the substance in study not diluted. (dilution 1:2)

Tube #3 until tube #9, 10mL of sterile distilled plus passes of 10mL of the previous dilution, until dilution 1:256 and discard there 10mL.

Once dilutions were ready, they worked in the preparation of bacterial stocks to obtain inoculum to be planted in the tubes. To do it, bacterial stocks were replanted in triptone soy broth and incubated in the stove at 37º, for 24 hours, for its reproduction. In the case of the yeast, they were cultured in sabouraud broth and incubated at room temperature.

Later on, bacterial stocks were replanted in plaques of Petri with Triptone agar soy and the yeasts in the saboureaud agar. Gram coloration was done to the culture, to corroborate its purity and finally a suspension was prepared for each stock with turbidity at 0.5 Mc Farland in soup Mueller Hinton, which constituted the inoculum.

Inoculums' Plantation

A total of 12 tubes were prepared, numbered from 1 to 12, and a ml of the mean of culture Mueller Hilton (double concentrated) was put in them. Tubes from #1 to 9 were inoculated 25µL of a bacterial suspension at 0.5 Mc Farland of the microorganism chosen for the study and 1 mL of different dilutions of *Bixa orellana* preparations. In tube #10 a culture mean and the stocks were added as a positive control.

Tubes #11 and 12 were not inoculated with germs because the controls were negative and of the experiment sterility, respectively. Thus, it was done with each microorganism. All tubes were incubated in a stove at 37ºC for 24–48 hours as negative and sterility controls respectively.

Determination of Antimicrobial Activity

At the end of the incubation period, bacteria (100µL) were replanted in plaques of Triptone soy agar and *Candida albicans* in Saboraud agar.

Plaques with bacterial inoculums were incubated from 24 to 48 hours, at 37º C and *Candida albicans* at room temperature for 24 to 72 hours, after that time a definite reading was carried out to check whether there was a growth of microorganisms. In environments where growth occurred the coincidence with the planted germ was confirmed, the gram coloration was done and was observed in microscopes to check morphology and purity of stocks.

Variables Studied

- Minimal concentration with antimicrobial activity.
- Sensitivity of microorganisms to pharmaceutical forms
- Spectrum of antimicrobial activity.

Table 1. In *vitro* antimicrobial action of *Bixa orellana L.* Camagüey. 2014.

Distribution of the antimicrobial activity of the fluid extract of *Bixa orellana L.* at 50% before microorganisms, taking ethanol as reference.

Pharmaceutical forms	Minimal concentration with antimicrobial activity on microorganisms taking as a reference			
	Staphylococcus aureus	*Escherichia coli*	*Pseudomona aeruginosa*	*Candida albicans*
Fluid Extract at 50%.	1:4	0	0	0
Ethanol at 50%	1:2	1:2	1:4	1:4

Source: Data Collection Index

Results and Discussion

Table 1 illustrates the distribution of antimicrobial activity of the different dilutions of fluid extract at 50% taking Ethanol as reference. It is shown that this pharmaceutical form presented activity only on *Staphylococcus aureus*, with the dilution 1:4, very near to alcohol activity.

These results coincide with those obtained in the research of authors like Lourido Perez H and collaborators[20] who agree state that Hydro alcoholic extracts of the plant have found antimicrobial actions against *Staphylococcus aureus*, while activity of Bixa on *Lactobacillus plantarum, Bifidobacterium bifidum*, yeasts and some gramnegative bacteria wasn't detected generally presenting a higher activity against grampositive germs.

In other study carried out by Rojas J and collaborators[12], where the antimicrobial gram positive activity pattern of ten species of plants was analyzed, among them *Bixa orellana L,* a similar pattern of antimicrobial activity of an ethanolic extract on *Staphylococcus aureus* was shown.

The Table 2 shows the minimal concentration of the tincture at 10% with activity on microorganisms of reference. It can be seen on *Staphylococcus aureus* and *Escherichia coli,* the dilution with antimicrobial activity was 1:8, which makes this product useful against these microorganisms, because its activity overcame ethanol in more than two dilutions. Meanwhile, no any activity was observed against *Pseudo*

Table 2.

Distribution of antimicrobial activity of tincture at 10% of *Bixa orellana L* against microorganisms, with ethanol as reference.

Pharmaceutical forms	Minimal concentration with antimicrobial activity against microorganism of reference.			
	Staphylococcus aureus	*Escherichia coli*	*Pseudomona aeruginosa*	*Candida albicans*
Tincture at 10%	1:8	1:8	0	0
Ethanol at 50%	1:2	1:2	1:4	1:4

Source: Data Collection Index

Table 3.

Distribution of antimicrobial activity of Tincture at 20% of *Bixa orellana L* against microorganisms with Ethanol as reference.

Pharmaceutical forms	Minimal concentration with antimicrobial activity against microorganisms of reference.			
	Staphylococcus aureus	*Escherichia coli*	*Pseudomona aeruginosa*	*Candida albicans*
Tincture at 20%	1:32	0	0	0
Ethanol at 50%	1:2	1:2	1:4	1:4

Source: Data Collection Index

Table 4.

Spectrum of antimicrobial activity of the different pharmaceutical forms, against the microorganisms reference.

Pharmaceutical forms	SENSITIVE MICROORGANISMS		
	No. of microorganisms	*Species*	*%*
Fluid Extract at 50%.	1	S. aureus	25,0
Tincture of the leaf at 10%	2	S. aureus, E.coli	50,0*
Tincture of the leaf at 20%	1	S. aureus	25,0

Source: Data Collection Index

mona aeruginosa and *Candida albicans* because dilutions in which it was observed didn't overcome the results obtained with ethanol at 50%.

Similar results are shown in Rojas[12] research where antimicrobial activity of the plant with ethanolic preparations against *Staphylococcus aureus* and *Escherichia coli* were seen. A very similar result was obtained in a research carried out by Silva RB and collaborators.[21] In this study minimal inhibitory concentrations (MIC) of different ethanolic preparations from leaves, seeds and stalks of *Bixa orellana* against five bacteria *Salmonella typhimurium*, *Proteus mirabilis, Bacilus cereus*, *Staphylococcus aureus, Pseudomona aeruginosa* and *Mycobaterium tuberculosis*, were determined and antimicrobial activity of the preparation made of leaves before *Staphylococcus aureus* con una MIC of 3,95 mg/ml could be detected. However, the lowest MIC of 0,66 mg/ml against *Pseudomona aeruginosa* is shown.

These variations, as it is reported in literature, can take place due to differences in concentrations of substances in vegetal stuff and in pharmaceutical preparations, to environmental factors where the plant is located, to the period in which the stuff was picked up and due to genetic differences that may exist in plant species.

Table 3 shows antimicrobial activity in dilutions of the tincture at 20% against microorganisms of reference. It can be seen that such pharmaceutical form only presented antimicrobial activity against *Staphylococcus aureus*, in the dilution at 1:32. There was no activity against the rest of microorganisms where dilutions couldn't overcome the activity shown by alcohol.

Pseudomona aeruginosa was just shown in one dilution of 1:4 (as in ethanol) and against *Candida albicans* activity was only shown with undiluted product. Therefore it could be attributed to the presence of ethanol at 50% in its formulation. In a study carried out by Raga et al,[22] preparations produced from *Bixa orellana* leaves were used, in which a terrain with antimicrobial properties, mainly antifungal, before *Candida albicans*, but with very low activity against *Staphylococcus aureus*, *Pseudomona aeruginosa* and *Escherichia coli*, was isolated, which does not coincide with the results of the present research, where the pharmaceutical form presented higher activity against *Staphylococcus aureus*, and *Candida albicans* fungus was the least affected.

It is necessary to make known that in most of the researches checked, the work has been done experimentally with ethanolic extracts of aerial organs of the plants (leaves, seeds, and stalks) and not with tinctures as in the present study. Therefore, there will hopefully be variations concerning efficacy, of the pharmaceutical forms and in reference to their microorganisms sensitivity, as it had been previously stated. In this respect, antimicrobial activity of ethanolic preparations of *Bixa orellana L* is proved by Viuda-Martos et al [23] who demonstrate their activity against P*seudomona aeruginosa* and the *Bacillus cereus* at variance with the present results.

Table 4 shows the spectrum of antimicrobial activity of the different pharmaceutical forms before microorganisms of reference. Tincture at 10% was the preparation that presented wider spectrum of activity, being effective before *Staphylococcus aureus* and against *Escherichia coli*. The tincture at 20% and the fluid extract at 50%, were just active against *Staphylococcus aureus.* The pharmaceutical forms studied did not present activity against *Pseudomona aeruginosa* or against the levaduriform fungus *Candida albicans.*

It should be stated, based on consulted research and literature, that the ethanolic and aequous exracts of the aerial organs are the most frequently used pharmaceutical forms of this species. Studies that would guarantee the efficacy of antimicrobial activity or tinctures efficacy were not found.

Table 5 shows the sensitivity of microorganisms against minimal concentration to what it was presented in this activity. It can be observed that *Staphylococcus aureus*, was affected by all preparations, with a higher sensitivity to tincture at 20%. Antimicrobial activity show with the use of this tincture to a dilution higher (1:32), compared to the rest of the microorganisms, for that it can be stated that this could be considered the most efficacious pharmaceutical form, considering that it can keep its antimicrobial action at a lower concentration.

For the fluid extract of the leaf at 50%, *Staphylococcus aureus* was the microorganism showed a higher sensitivity to the preparation, which showed its antimicrobial activity with a dilution at 1:4, while the rest of the microorganismos showed a higher resistance and were only sensitive to the undiluted product, in which the antimicrobial activity of ethanol and that of the plant in question couldn't be dis-

Table 5.

Distribution microorganisms' sensitivity against minimal concentrations of antimicrobial activity according to pharmaceutical forms.

Pharmaceutical forms	Sensitivity of microorganisms of reference to pharmaceutical form to the minimal concentration with antimicrobial activity.			
	Staphylococcus aureus	*Escherichia coli*	*Pseudomona aeruginosa*	*Candida albicans*
Fluid Extract at 50%.	1:4	0	0	0
Tincture at 10%	1.8	1:8	0	0
Tincture at 20%	1:32	0	0	0

Source: Data Collection Index

tinguished because the same effect was obtained.

Concerning the tincture of the leaf at 10% with menstruum at 50%, it could be observed that *Staphylococcus aureus* and *Escherichia coli* as well were both sensitive to the preparation to minimal concentrations at 1:8, showing similar patterns according to sensitivity. On the other hand *Pseudomona aeruginosa* and *Candida albicans* showed a lower sensitivity to this pharmaceutical form.

Staphylococcus aureus was the most sensitive microorganism to the three pharmaceutical forms studied showing a higher sensitivity against exposure to tincture at 20% of the leaf with the lowest concentration, followed by *Escherichia coli* which was only sensitive to tincture at 10%. The other microorganisms (*Pseudomona aeruginosa* and *Candida albicans*) were not sensitive to the pharmaceutical forms studied.

In a research carried out by Castello M et al [24] *Staphylococcus aureus* was one of the microorganisms with highest sensitivity of the extracts of the plant. Also Fleischer et al [25] obtained similar results concerning sensitivity of gram positive germs as *Staphylococcus aureus* to preparations made of the leaves and seeds of *Bixa orellana*. Whereas, a study carried out by Irobi et al [26] revealed a great sensitivity of *Staphylococcus aureus* to these Pharmaceutical forms.

Conclusion

A 50% fluid extract as well as 10 and 20% tinctures of *Bixa orellana* leaves were active against *Staphylococcus aureus* at a lower concentration than with respect to the other microorganisms, but they did not present antimicrobial activity against *Candida albicans* and *Pseudomona aeruginosa. Staphylococcus aureus* results show the most sensitive germ to the pharmaceutical forms studied to lower minimal concentration with antimicrobial activity expressed in dilutions. The 10% tincture showed the highest spectrum of antimicrobial activity. The most active pharmaceutical form was the 20% tincture.

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