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EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF THE HYDROALCOHOLIC EXTRACT OF Bixa orellana (Annatto) AND ITS IN VITRO INTERACTION WITH ANTIMICROBIALS FOR CLINICAL USE

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ABSTRACT

Bacterial resistance is a major public health problem, so it is necessary to obtain new treatment alternatives. Research with medicinal plants is an important strategy for the discovery of therapeutic agents. Bixa orellana is a plant species widely used by the population for culinary and medicinal purposes. The objective of this study is to verify the antimicrobial potential of the hydroalcoholic extract of Bixa orellana seeds against strains of Pseudomonas aeruginosa, Escherichia coli and Staphylococcus aureus; to determine the

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MIC and evaluate the interaction of the extract with antimicrobials for clinical use. The determination of the antimicrobial activity as well as the MIC was carried out through the REMA method and the evaluation of the interaction of the extract with antimicrobials for clinical use through the technique of diffusion in solid medium using paper disks. The MIC of the hydroalcoholic extract of Bixa orellana seeds in the different strains ranged from 7.8 to 1000µg/mL. A trend of synergism was observed between the combination of the extract and the antimicrobials tested. It is thus concluded that the results point to Bixa orellana as a promising plant for the development of new products with antimicrobial activity.

Keywords: Bacterial resistance, Bixa orellana, Microorganisms, Antimicrobials, Synergism.

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INTRODUCTION

The bacterial resistance developed by microorganisms is characterized as a biologically normal phenomenon that gives them the ability to adapt, grow and multiply in adverse situations that would normally destroy them (1).

Bacterial resistance, previously considered to occur only in the hospital environment, has become a much broader phenomenon, affecting several environments (2,3,4) and represents a serious public health problem. According to data from the WHO (World Health Organization) and the Pan American Health Organization (PAHO), among the bacteria most evaluated in terms of resistance to antimicrobials are *Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus,* which are important causes of morbidity and mortality (5).

Due to the marked bacterial resistance to antimicrobials, it is necessary to obtain new alternatives for the treatment of infectious diseases (6). The development of research with medicinal plants has been an indication for the discovery of efficient therapeutic agents in obtaining new drugs with satisfactory efficacy and with fewer undesirable effects. The use of extracts for the modulation of bacterial resistance to antimicrobials has been studied, obtaining promising results (7).

Plant biodiversity contributes to the supply of substances useful for the treatment of diseases that affect humans. The secondary metabolites produced by plants have therapeutic properties with variety and complexity (8). This observation arouses interest in the study for the search and development of new drugs from medicinal plants (9). *Bixa orellana* (annatto), a plant native to Brazil and other tropical regions of the planet, belonging to the botanical family *Bixaceae*, is a woody plant known in Brazil as annatto, annatto or turmeric (10). Annatto seed contains components such as cellulose, sucrose, oils, alpha fragrances and beta carotene. This plant has been used in traditional medicine as an expectorant, laxative, stomatic, antihemorrhagic, healing, antipyretic, analgesic and anti-inflammatory (11). Several studies have pointed out different pharmacological activities for this plant, among them: hypocholesterolemic (12), hypoglycemic, antimicrobial and antioxidant (13).

Because it has different types of secondary metabolites, *Bixa orellana* can interact with antimicrobials for clinical use, acting synergistically improving the drug's potential. This strategy is a consequence of a mechanism in which two different compounds are combined to increase their individual activities, and has served as inspiration for new research aimed

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at the discovery of molecules that aim to act on bacterial resistance mechanisms, minimizing them (14).

In view of the above, the present study aimed to evaluate the antimicrobial activity of the hydroalcoholic extract of *Bixa orellana* against strains of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus and to* describe the interaction of this extract with antimicrobials for clinical use.

METHODOLOGY

OBTAINING THE STATEMENT

The hydroalcoholic extract of *Bixa orellana seeds* obtained from the Harmonize Laboratory (Ipatinga, Minas Gerais, Brazil) was filtered, subjected to rotationevaporation at 50°C and lyophilized. The generated material was packed in an amber vial at 4°C until the moment of use, and then weighed and used to prepare a stock solution at 1mg/mL in 5% dimethylsulfoxide (DMSO - Synth, São Paulo, Brazil), and was immediately used in the biological tests.

PHYTOCHEMICAL SCREENING

The qualitative phytochemical tests were based on colorimetric methods and were conducted as follows: steroids and triterpenoids were investigated using the Lieberman-Burchard test, flavonoids were screened by Shinoda's test, tannins were identified by observing the formation of blue precipitates after the addition of FeCl3, and saponins and alkaloids were investigated using the Drangendorff test (15).

OBTAINING AND CULTIVATING THE MICROORGANISMS USED

The microorganisms were obtained from the Microbiology Laboratory of the Vale do Rio Doce University (UNIVALE), identified as S1-S5 (*Staphylococcus aureus* strains, catheter tip isolates), E1-E5 (uropathogenic *Escherichia coli* strains) and P1-P5 (*Pseudomonas aeruginosa* strains, tracheal secretion isolates), were submitted to identification with the Vitek 2.0 automated system (version R04.02, bioMérieux), following the manufacturer's instructions. All strains were kept frozen at -20 °C in BHI broth (Difco, Becton Dikinson, USA) with 10% glycerin. To activate the crops, aliquots of the frozen culture were transferred to BHI broth supplemented with 5% sucrose, and incubated at 35 ± 2 °C. After 24 hours, the cultures containing the bacteria were seeded in Petri dishes

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containing Nutrient Agar (Difco, Becton Dikinson, USA) and placed in an incubator at 37°C for 48 hours. After visualization of the growth, colonies of the bacteria were selected and transferred to the BHI broth.

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC of the hydroalcoholic extract of *Bixa orellana* seeds was determined on polystyrene microplates (Nunc, Roskilde, Denmark) from 96 sterile wells as previously described by our group (16). Briefly, the bacterial cultures were prepared in BHI broth on the 0.5 McFarland scale, with 100µL dispensed in the wells. Subsequently, 100µL of the hydroalcoholic extract of the seeds of *Bixa orellana were added* , which was diluted in series. The concentrations tested ranged from 1mg/mL to 7.8µg/mL. The plates were then incubated in a bacteriological incubator at 35 ± 2 °C for the entire night. After this time, 10µL of resazurin solution (Sigma-Aldrich Missouri, USA) previously diluted in sterile distilled water at a final concentration of 0.001mg/mL were added to each well, and the plates were recubated under the previous conditions for 30 minutes. MIC was established as the lowest concentration at which the resazurin color remained unchanged (without changing from blue to pink). All experiments were carried out in independent triplicates.

EVALUATION OF THE INTERACTION OF *BIXA ORELLANA* EXTRACT WITH ANTIMICROBIAL DRUGS

The possible interference of Bixa orellana *extract* with antimicrobial drugs was evaluated using the agar diffusion disk method (17) with modifications (18). Petri dishes were prepared with Mueller-Hinton agar (Difco, Becton Dikinson, USA) on which bacterial suspensions were seeded on the 0.5 McFarland scale. The following antimicrobial discs were used: amoxicillin 500mg, ampicillin 500mg, norfloxacin 500mg, ciprofloxacin 500mg, nitrofurantoin 100mg, (all from CEFAR, São Paulo, Brazil). Briefly, 10µL of Bixa orellana *extract* at a concentration of 1000 µg/mL were dispensed into each disc. The plates were incubated in a bacteriological incubator at 35 ± 2 °C for 24 hours, and the mean diameter of the inhibition zone was measured and compared with the control plates (extract-free discs). Synergism or antagonism were considered if the inhibition of the mean diameter of the inhibition zone was at least 2 mm larger or 2 mm shorter than the control, respectively. All experiments were carried out on independent duplicates.

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RESULTS

The qualitative phytochemical tests showed the presence of flavonoids, tannins, saponins and alkaloids as shown in Table 01.

Table 01: Result of the qualitative analysis of the phytochemical assay of annatto seed extract (*Bixa orellana*)

Metabolites analyzed	Results
Steroids/triterpenoids	-
Flavonoids	+
Tannins	+
Saponins	+
Alkaloids	+

(+) presence of the chemical compound, (-) absence of the chemical compound. Source (The authors)

The data obtained in the evaluation of the antimicrobial activity of *B. orellana* are shown in Table 2.

Table 02: Minimum Inhibitory Concentration **(**MIC) of the hydroalcoholic extract of *Bixa orellana seeds*, expressed in µg/mL, compared to clinical isolates.

			STRAINS		
	P1	**P2**	**P3**	**P4**	**P5**
	< 7.8	< 7.8	>1000	< 7.8	< 7.8
	S1	**S2**	**S3**	**S4**	**S5**
CIM (µg/mL)	125	125	125	125	125
	E1	**E2**	**E3**	**E4**	**E5**
	>1000	>1000	>1000	>1000	>1000

Concentrations tested ranged from 7.8 to 1000 µg/mL. Source (The authors)

The results regarding the sensitivity of bacterial strains to the action of antimicrobials are shown in Table 3.

Table 03: Evaluation of the interaction of *Bixa orellana* extract with antimicrobial drugs

Strains	Nor	Nor+E	Amp	Amp+E	Cip	Cip+E	Fishhook	I love +E	Nit	Nit+E
S1	15 ± 4,2	12 ± 0	27 ± 4,2	36 ± 4,2	18 ± 0	16 ± 5,0	27 ± 4,2	35 ± 1,4	18 ± 0	20 ± 6,4
S2	18 ± 0	21 ± 4,2	11 ± 7,8	12 ± 0,7	18 ± 0	21 ± 4,2	17 ± 12,0	12 ± 0,70	18 ± 0	18 ± 0
S3	15 ± 4,2	18 ± 0	18 ± 17,0	20 ± 14,1	21 ± 4,2	19 ± 1,4	16 ± 11,3	22 ± 17,0	15 ± 4,2	16 ± 5,0
S4	12 ± 0	12 ± 0	24 ± 0	21 ± 4,2	12 ± 0	12 ± 0	24 ± 0	21 ± 4,2	18 ± 0	16 ± 3,3
S5	18 ± 12,7	16 ± 2,8	30 ± 21,2	33 ± 3,5	15 ± 4,2	18 ± 7,8	24 ± 8,5	29 ± 3,5	15 ± 4,2	16 ± 5,0
S ATCC	09 ± 4,2	12 ± 1,4	18 ± 8,5	32 ± 5,7	12 ± 8,5	18 ± 0	24 ± 0	27 ± 4,2	12 ± 8,5	19 ± 7,0
Strains	**Nor**	**Nor+E**	**Amp**	**Amp+E**	**Cip**	**Cip+E**	**Fishhook**	**I love +E**	**Nit**	**Nit+E**
P1	15 ± 4,2	12 ± 4,2	36 ± 8,5	36 ± 8,5	18 ± 0	21 ± 4,2	36 ± 8,5	39 ± 4,2	09 ± 4,2	09 ± 4,2
P2	12 ± 8,5	09 ± 4,2	30 ± 0	26 ± 5,7	11 ± 1,4	12 ± 8,5	27 ± 4,2	27 ± 4,2	12 ± 0	13 ± 7,0

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P3	18 ± 8,5	22 ± 11,3	27 ± 4,2	25 ± 9,9	15 ± 4,2	16 ± 2,8	27 ± 4,2	25 ± 9,9	18 ± 12,7	12 ± 8,5
P4	15 ± 4,2	16 ± 2,8	30 ± 0	30 ± 0	15 ± 4,2	16 ± 2,8	27 ± 4,2	31 ± 1,4	15 ± 4,2	14 ± 2,8
P5	12 ± 0	12 ± 0	27 ± 4,2	28 ± 2,8	12 ± 0	14 ± 5,7	30 ± 0	25 ± 1,4	12 ± 0	14 ± 2,8
P ATCC	21 ± 4,2	19 ± 1,4	15 ± 4,2	18 ± 0	24 ± 0	18 ± 0,4	15 ± 4,2	18 ± 1,0	09 ± 4,2	16 ± 2,8
Strains	**Nor**	**Nor+E**	**Amp**	**Amp+E**	**Cip**	**Cip+E**	**Fishhook**	**I love +E**	**Nit**	**Nit+E**
E1	22 ± 11,3	27 ± 4,2	24 ± 0	26 ± 2,8	24 ± 0	28 ± 11,3	24 ± 0	25 ± 1,4	07 ± 0,7	10 ± 5,7
E2	30 ± 0	31 ± 1,0	24 ± 0	24 ± 0	27 ± 4,2	30 ± 0	24 ± 0	20 ± 3,2	09 ± 3,5	09 ± 3,5
E3	21 ± 4,2	22 ± 2,8	06 ± 4,2	09 ± 4,2	21 ± 4,2	23 ± 2,1	06 ± 4,2	10 ± 5,7	06 ± 0	07 ± 1,4
E4	27 ± 4,2	27 ± 4,2	21 ± 4,2	25 ± 1,4	21 ± 12,7	20 ± 2,8	21 ± 4,2	21 ± 4,2	08 ± 2,8	09 ± 4,2
E5	21 ± 4,2	25 ± 7,0	24 ± 0	23 ± 1,0	15 ± 4,2	20 ± 5,7	24 ± 0	24 ± 0	06 ± 0	10 ± 5,1
THE ATCC	24 ± 0	25 ± 7,0	24 ± 0	24 ± 0	21 ± 4,2	22 ± 3,8	24 ± 0	24 ± 0	06 ± 0	10 ± 1,0

Nor: Norfloxacin; Amp: Ampicillin; Cip: Ciprofloxacin; Love: Amoxicillin; Nit: Nitrofurantoin; E: Extract; Synergism; Antagonism; Indifferent. Values presented as mean ± standard deviation. Source (The authors)

DISCUSSION

Through phytochemical analyses, relevant information is obtained about the presence of secondary metabolites in the plant, this is important, as they provide protection against attacks by insects and herbivores, ultraviolet radiation and diseases. In addition, they offer benefits to human health, as they have biological activities (19).

The presence of secondary metabolites can be confirmed through conventional techniques such as thin layer chromatography and spectroscopy, but the use of hyphenated techniques that refers to the coupling between two or more analytical techniques such as high performance liquid chromatography and gas chromatography, brings additional information about the chemical structure of the sample components, functioning as detectors, in order to obtain a more efficient and faster analysis tool than conventional techniques (20).

According to Table 01, although the presence of steroids was not detected, a previous study identified this metabolite in annatto seeds (21). This difference in the concentration of secondary metabolites is justified by the fact that, despite being genetically controlled, there may be variations in plant tissues as a result of the age and size of the plant, the part collected, place of collection, seasonality (22), environmental factors as well as interactions with insects and predators (23).

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As previously mentioned, bacterial resistance generates an overload for health systems, putting patient safety at risk, as it prolongs the length of hospital stay and, consequently, treatment costs, and can be a cause of morbidity and mortality(24). Thus, our study aims to evaluate the presence of secondary metabolites that have antimicrobial activity in *B. orellana* that may somehow present synergistic action and/or potentiate the action of antimicrobials used in the clinic, a plant used for culinary and ethnobotanical purposes.

The susceptibility profile of clinical isolates to hydroalcoholic extract of *B. orellana* varied among isolates of different species and even in the same species. This data can be justified by the genetic variability of each strain, as described by some authors (25) when they report that genetic variability among isolates of the same species has been pointed out as one of the determining factors for variations in the susceptibility profile. In this study, we chose to use clinical isolates to make this evaluation, as they correspond to the strains circulating in the community, instead of using ATCC strains that are commercially acquired. A study analyzing biofilm production by clinical P*. a* isolates from patients with ventilator-associated pneumonia showed that the highest detection of biofilm production was by clinical isolates compared to ATCC strains used (26). Previous studies published by our group have successfully evaluated the effects of medicinal plants on these clinical isolates.

The P. aeruginosa strains showed greater sensitivity to B. orellana seed extract when compared to S. aureus and E. coli. Although E. coli being Gram negative did not present sensitivity, this may possibly be in relation to the characteristics of this group of bacteria, as it has a double membrane forming a complex envelope, being responsible for the lower sensitivity of this microorganism to the plant extract, which hinders the entry of antimicrobial agents or for the concentrations used of the extract (27).

Another study on the antimicrobial and anti-inflammatory activity of natural products on respiratory pathogens demonstrated through the microdilution method that the hydroalcoholic extract of B. orellana shows activity against all pathogens tested, including P. aeruginosa and S. aureus, with reduction rates of 95% under the biofilm formed by P. aeruginosa and 93% under the biofilm formed by S. aureus, both at a concentration of 64 mg/ml (28).

The evaluation of the interaction of the hydroalcoholic extract of the *Bixa orellana seed* was carried out through the measurement of the halos by the diffusion technique in solid medium using filter paper discs.

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The study to evaluate the interaction of *B. orellana* extract with antimicrobial drugs for clinical use is necessary since medicinal plants are used by the general population, but without any proof of their pharmacological activities. The use of the combination of plant extract with antimicrobials can promote a reduction in the minimum dose necessary for the effectiveness of antimicrobials, this is interesting, as it can reduce the chance of side effects, as well as reduce the cost of treatments. Therefore, this study represents a therapeutic alternative in the fight against emerging multidrug-resistant bacteria (29).

A study similar to ours evaluated the interaction of the hydroalcoholic extract of *B. orellana* with antimicrobials and observed that the presence of activity of the extract against Gram-positive bacteria (*S. aureus*) and absence of activity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) where they report greater sensitivity of the former against plant metabolites, corroborating our study (30).

The tests showed that *S. aureus* was more sensitive when the antimicrobials ampicillin and amoxicillin were used in conjunction with the extract, with inhibition halos ranging from 12 to 36 mm, and less sensitive against *E. coli*.

These antimicrobials were chosen because they are frequently used in clinical medicine, and their repeated and inappropriate use is the main cause of the increase in resistant bacteria (31). Norfloxacin and Ciprofloxacin belong to the class of 2nd generation quinolones, they are used in urinary infection (cystitis and pyelonephritis) and prostatitis; Ampicillin and amoxicillin belong to the class of β- Lactams being ampicillin, used in the treatment of bronchitis, endocarditis, gonorrhea, meningitis, pneumonia, amoxicillin used in the treatment of a series of bacterial infections of the respiratory tract and also as part of the treatment scheme against infections caused by the famous bacterium H.pylor. Nitrofurantoin, a class of nitrofurans, is used in the treatment of urinary tract infection.

The hydroalcoholic extract of *Bixa orellana* has a synergistic effect, being justified by the various secondary metabolites produced by the plant and already described in this work, which when isolated or together presented a growth inhibition profile (32).

Bixa orellana *seeds* are widely used in cooking and for dermatological purposes, so it is necessary to observe because when used in association with antimicrobials for clinical use it can enhance their effect.

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CONCLUSION

Considering the results obtained in this study, the hydroalcoholic extract of *Bixa orellana seeds* has antimicrobial activity on clinical isolates, in addition to interacting with antimicrobials for clinical use, causing a synergistic effect, thus improving the activity of these antimicrobials. These data together point to *Bixa orellana* as a promising plant for the development of new products with antimicrobial activity, but the concomitant use of plant products and conventional medicines deserves a very careful look, given the possibility of interfering in the treatment of diseases of bacterial etiology.

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