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ANTIMICROBIAL POTENTIAL OF BYRSONIMA INTERMEDIA IN ISOLATES FROM THE INDUSTRIAL FERMENTATION PROCESS

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Abstract: The traditional industrial fermentation process to obtain bioethanol takes place in a favorable environment not only for the action of yeast, but also for the development of undesirable microorganisms that end up compromising production by consuming the raw material, producing inhibitory substances, altering the ideal conditions. of the process, metabolize the ethanol produced among other actions. The great challenge is to control the invaders, without affecting the action of the selected yeasts. In this sense, the present study sought natural and viable forms of control with the use of plant extracts, minimally affecting the environment and allowing the subsequent trade of by-products generated by leaving no residues. The aqueous and hydroalcoholic extracts of *Byrsonima intermedia*, a plant from the Brazilian cerrado. For the analyses, the plant extracts were solubilized in water. Bacterial contaminants were isolated from material from the yeast treatment vat from three different production units in the Jaboticabal region and also from a fermentation process carried out at Fatec Jaboticabal. It was possible to verify that the extracts showed greater action on the bacterium *S. aureus* regarding *E. coli* and also showed effectiveness on the four groups of isolated bacteria. The action was verified for both the hydroalcoholic extract and the aqueous extract, with similar values. In the test carried out with the isolated yeasts, the formation of inhibition halo was not observed for any of the evaluated extracts. The results were encouraging, revealing that the extracts have potential of antibacterial action to meet the needs of the sugar-energy sector.

Keywords: Vegetable extract. Antimicrobial. contaminants. Fermentation.

INTRODUCTION

The presence of contaminating microorganisms in the industrial fermentation

process has been one of the major concerns of the sugar-energy sector because it causes a drop in the fermentation yield, and microbiological control is of fundamental importance (CARVALHO*et al*., 2021). Traditionally, bacterial control in the process is done by the addition of sulfuric acid, but for high levels of contamination, antibiotics are used. (MUTHAIYAN; LIMAYEM; RICKE, 2011). However, this use has generated a certain limitation due to the high cost and for making the commercialization of dry yeast for animal feed and other purposes unfeasible, as they leave residues in the cells (FREITAS; ROMANO, 2013; BREXÓ; SANT'ANA, 2017).

Faced with this reality, the study of alternative forms of control starts to arouse much interest. (CAETANO; MADALENO, 2011; MADALENO *et al*., 2016; RICH *et al*., 2018).

Natural products derived from plants enter this context, as they have great potential for product development as they are currently the main source of new bioactive chemical molecules (ANAND, et al, 2020) because they are readily available, have low cost of obtaining, have almost no side effects, some of them proving to be effective in eliminating antibiotic-resistant bacteria and others, although not effective on their own, showed promise when combined with antibiotics, thus, attractive tools in the research of bioactive products in the coming years (KHAMENEH, 2019).

In the production of bioethanol, the study of the use of natural extracts has been considered promising with ample possibility of exploitation. (SALAM; QUAVE, 2018), being more economically viable for industrial use the lower the level of purity is needed, that is, the more crude the extract (MUTHAIYAN; LIMAYEM; RICKE, 2011).

Currently, the most used vegetable in the bioethanol industry is hops (Humuluslupulus), from which derive the α-ácidos(humulonas) and β-ácidos (lupulonas), effective mainly against Gram-positive bacteria and found commercially under the names of IsoStab®, LactoStab® and Betabio45® (CECCATOANTONINI, 2018).

Given this reality, the present study sought to evaluate the potential of the antimicrobial use of extracts of *Byrsonima intermedia,* a vegetable from the Brazilian Cerrado in isolates from the industrial fermentation process comparing two forms of extraction (aqueous and hydroalcoholic).

B. intermedia presents itself as a shrub 1–2.5 m high, forming a canopy up to 3 m in diameter, blooming with yellow clusters from October to December known as "murici-do-campo", "murici-anão", "murici-pequeno", "canjica", "baga-de-tucano" or "saratudo" which has its bark and leaves popularly used in the treatment of diarrhea, dysentery, stomach pain, ulcer and inflammation, actions confirmed by pre-clinical pharmacological studies (SANTOS *et al*., 2018) and showed positive results for the antibacterial action of its extracts against *Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* on a screening realized for several plants of popular use (ALVES *et al*., 2000).

MATERIAL AND METHODS

The experiments were carried out in Fatec – Jaboticabal, at the Department of Bioprocess Engineering and Biotechnology at UNESP – Araraquara, at the Federal Institute of São Paulo – IFSP Campus Matão and Laboratory of Medicinal Chemistry and Regenerative Medicine – University of Chimera Araraquara – Uniara.

OBTAINING PLANT EXTRACTS

The plant species from the Brazilian Cerrado, *Byrsonima intermedia* was a participant in the project BIOTA by Fapesp,

which seeks to integrate the characterization, conservation, restoration and sustainable use of biodiversity (BIOTA, 2020). The plant was collected in the city of Itirapina - SP and identified by the teacher Jorge Tamashiro, from the Institute of Biology of the University of Campinas. The IBAMA authorization received the number 32066-1 and the exsiccate was deposited in the Herbarium HUEC, Unicamp, under registration 1484. The leaves were dried in a circulating air oven at 45ºC for seven days and after this period they were pulverized in a knife mill. . Storage was carried out in properly labeled plastic packaging at the Phytochemistry Laboratory of the Department of Organic Chemistry (IQ/ Unesp Araraquara) where it was characterized (CARDOSO; BAUAB; VARANDA, 2015) and later forwarded to the Fatec Jaboticabal Bioprocess Laboratory.

The extracts were obtained by maceration (ANVISA, 2010) in the proportion 1:10 (m/v) in 70% ethanol for 5 days or in water for 3 days. After filtration, the resulting filtrates were frozen in an ultrafreezer – 62ºC until the concentration procedures were carried out in an evaporator and lyophilizer route to eliminate the hydroalcoholic solvent extractor. The aqueous extracts were lyophilized directly.

DETERMINATION OF WATER SOLUBILITY OF EXTRACTS

The determination of water solubility was performed as described by NASCIMENTO, (2008). For this determination, a specific amount of extracts were transferred to a test tube or erlenmeyer, followed by addition of aliquots of the solvent (Table 1), using the mechanical vortex mixer (Phoenix AP56) for homogenization and confirmation of the absence of suspended material. Solubility was expressed according to the descriptive terms defined by ANVISA (2010) and presented in Table 2, which refers to the dissolution of a part (1 g) of the sample in the number of parts (volume, in mL) of the solvent.

STANDARD MICROORGANISMS

Standard strains were used (American TypeColection Culture - ATCC) *Staphyloccoccus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922).

OBTAINING YEASTS AND MICROBIAL CONTAMINANTS FROM THE FERMENTATION PROCESS

Yeasts and microbial contaminants were isolated from the simulation of a fermentation process carried out at Fatec Jaboticabale from material from the yeast treatment vat of three different sugar-energy units in the Jaboticabal region defined in this work as Plants A, B and C. The microorganisms were grown in incubator Shaker (CT 712 Cienter) at 32ºC in YPD medium (yeast extract 10 $g.L^{-1}$, dextrose 20 $g.L^{-1}$ e peptona 20 $g.L^{-1}$) for isolation of yeasts, and in nutrient broth (beef extract 3 $g.L^{-1}$ e peptona 5 $g.L^{-1}$) for bacteria isolation.

When an absorbance of 0.3 was reached at a wavelength of 600nm, the samples were centrifuged (Sppencer 80-2B centrifuge) at 1,600xg for 5 minutes. The precipitate was suspended in a sterile solution of 20% glycerol in water. (H_2O) and distributed in low temperature resistant microcentrifuge tubes (cryotubes). The microorganisms were stored in an ultra freezer until use.

EVALUATION OF THE ANTIBACTERIAL POTENTIAL OF PLANT EXTRACTS IN VITRO

The antimicrobial activity of the plant extracts obtained was evaluated by the holeplate method of diffusion in agar, as described by SILVA, 2012. This technique is also known as the well diffusion test. (BONA *et al.*, 2014).

Sample (g)	Cumulative addition of solvent (mL)	Solvent volume (mL)	Solvent parts for 1g of sample
1,0	-	1	1
	9	10	10
	20	30	30
0,01	-	1	100
	9	10	1.000
	90	100	10.000

Table 1. Quantities of sample and solvents used for solubility assessment

Source: NASCIMENTO, (2008).

Descriptive term	Approximate volumes of solvent in milliliters per gram of substance
Very soluble	less than 1 part
Easily soluble	From 1 to 10 parts
Soluble	From 10 to 30 parts
Slightly soluble	From 30 to 100 parts
Poorly soluble	From 100 to 1,000 parts
Very little soluble	From 1,000 to 10,000 parts
Practically insoluble or insoluble	More than 10,000 parts

Table 2. Descriptive terms to express solubility

Source: ANVISA, 2010.

For the assays, bacterial samples were grown in Mueller-Hinton broth (bovine extract 2g.L^{-1}, casein acid hydrolyzate 17,5g.L^{-1} and starch 1,5g.L^{-1}) or ágar Mueller-Hinton (added from ágar the 2%). After growth in incubator Shaker (CT 712 Cienter) at 32ºC, the culture was diluted to optical density corresponding to the standard 0.5 of the Mac-Farland (OD620 = 0,10). After this, 100 µL of the microbial suspension were spread with a Drigalsky loop on ágar Mueller-Hinton (Broth described above with 2% agar added). Then, holes of approximately 5 mm in diameter and 3 mm in height were made in the agar and 30 µL of extracts solubilized in water (H_2O) in the concentration of 150mg.mL^{-1} were added.

All tests were performed in triplicate. The resulting plates were incubated in a bacteriological oven at 32 °C for 48 hours. After this period, the development of inhibition halos was observed, whose diameters were measured in millimeters. Halo diameter values greater than 7mm were considered positive.

Susceptibility analyzes were also performed using the yeast samples *Saccharomyces cerevisiae*, as described above, but instead of placing the extracts in holes, they were applied on absorbent paper discs (disk-diffusion method), after the yeasts were grown in YPD medium (Yeast Extract 10g.L^{-1}, peptona 20g.L^{-1} and glicose 20g.L^{-1}) and applied on plates containing the same medium in solid form (added 2% agar).

RESULTS AND DISCUSSION

OBTAINING AND EVALUATING THE SOLUBILITY OF PLANT EXTRACTS

Initially, the preparation of crude extracts of *Byrsonima intermedia*. Aqueous and hydroalcoholic extracts were obtained by

the maceration process, which has great importance in obtaining thermolabile components, especially phenolic compounds, although the waiting time and the solvent volume are characteristics of disadvantages of this method (ZHANG; LIN; YE, 2018). A vantagem econômica do uso industrial de extratos que necessitem de menor nível de pureza, ou seja, quanto mais bruto for o extrato mais atrativo, como é o nosso caso, foram ressaltadas por MUTHAIYAN; LIMAYEM; RICKE,(2011).

The interest in the use of water in the preparation of the extract was to obtain a product of low cost and practicality of use because it is easily soluble, a characteristic that was confirmed by the solubility evaluation as shown in Table 3. The use of water becomes also an attractive in obtaining compounds with polar characteristics (ALTEMIMI *et al.*, 2017).

The presence of antimicrobial activity in the aqueous extract in the present study has been desired, since, TIWARI *et al.*, (2011), studying the aqueous and ethanolic extracts of *Tinosporacordifolia*, showed the presence of bioactivity in both cases.

After obtaining the extract, the determination of water solubility (Table 3) was performed to verify the possibility of using this solvent in the dilution of the extract (or possible commercial product) at the time of use, which becomes attractive for the sector. industrial because it is a universal solvent, abundant and relatively low cost. It was possible to verify the ease of solubilization of extracts in aqueous medium, while extracts without hydroalcoholic medium presented greater difficulty. This observation was also reported by NASCIMENTO, (2008). In a study carried out by KUBILIENE *et al.*, (2018), using aqueous, ethanolic and aqueous extracts with the addition of polyethylene glycol of propolis was highlighted the importance of water solubility as a safer and biocompatible form of use, which is very significant for the present study, since the obtained extract will be manipulated by the operators and will come into direct contact with the yeasts of the process, being very important the innocuousness of the solvent.

EVALUATION OF THE ANTIMICROBIAL POTENTIAL OF PLANT EXTRACTS IN VITRO

In the assays to evaluate the antimicrobial potential carried out in Saccharomyces cerevisiae, the formation of an inhibition halo was not observed for any of the evaluated extracts (Figure 1). This result is very interesting because the objective is to use the extract during the fermentation process and for that it is necessary that only the contaminants present sensitivity and that the action of the yeast is not influenced.

Tables 4 and 5 present the results of bacterial sensitivity by the Hole plate method. Each extract was tested for the standard microorganisms (Table 4) and for the microorganisms isolated from the fermentation process carried out at Fatec and at the sugar-energy units in the Jaboticabal region (Plants A, B and C), separately (Table 5).

Plant species	Plant extract	Solubility
Byrsonima intermedia	Hydroalcoholic	Slightly soluble
	Aqueous	Soluble

Table 3. Solubility of plant extracts in descriptive terms

Source: Research data

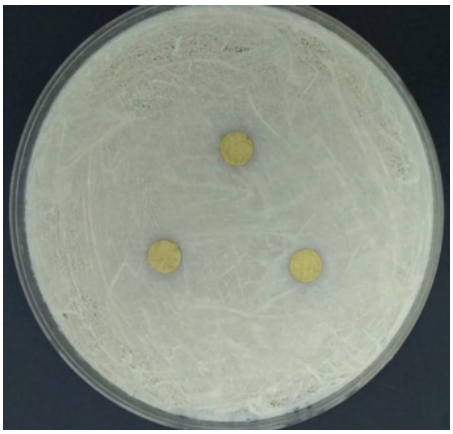

Figure 1. No observation of halo for yeast in aqueous extract.

Plant species	Plant extract	Diameter of halos (mm)	
		S. aureus	E. coli
Byrsonima intermedia	Hydroalcoholic	14,12 ± 0,97	9,64 ± 0,71
	Aqueous	13,95 ± 1,07	10,63 ± 0,21
Control	-	*	*

Table 4. Measurement of inhibition halos obtained for standard microorganisms.

* Halo measurement < 7mm. Source: Research data

Plant species	Plant extract	Diameter of halos (mm)			
		Fatec	Power plant A	Power plant B	Power plant C
Byrsonima intermedia	Hydroalcoholic	9,26 ± 0,39	9,85 ± 0,59	15,30 ± 0,49	15,70 ± 0,61
	Aqueous	9,65 ± 0,31	10,17 ± 0,26	15,13 ± 0,60	15,05 ± 0,79
Control	-	*	*	*	*

Table 5. Measurement of inhibition halos for microorganisms isolated from the fermentation process.

* Halo measurement < 7mm. Source: Research data

It was possible to observe that the extracts of *Byrsonima intermedia*, showed greater action on the bacteria *S. aureus* regarding *E. coli*(Table 4). The extracts also showed effectiveness on the four groups of isolated bacteria (Table 5), with the highest halo values for bacterial samples from plants B and C. The action was verified for both the hydroalcoholic extract and the aqueous extract, with similar values.

Diffusion methods are the initial choice when selecting new antimicrobial agents from plant extracts and other natural products, mainly due to the ease of execution and low cost (OSTROSKY *et al.*, 2008).BONA *et al.*, (2014) emphasize the importance of the Hole plate method or well diffusion test in the preliminary evaluation of plant extracts with antimicrobial potential.

MICHELIN *et al.*, (2008), verified positive results of antibacterial action by diffusion of methanolic extracts of *Byrsonimaintermedia* against *Bacillus subtilis*, *Enterococcusfaecalis*, *Proteusmirabilis*, *Salmonella* sp., *Shigella* sp., *Staphylococcusepidermidis*. In agreement with the results obtained for the aqueous

extract, ALVES *et al*., (2000), studying several plants, observed antibacterial activity in the diffusion assay of the aqueous extract of *Byrsonimaintermedia*contra *Bacillus cereus*, *Pseudomonas aeruginosa* e *Staphylococcus aureus*. MOREIRA, (2010), also using the aqueous extract verified by the Hole plate method the presence of antibacterial activity for *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Micrococusluteus*e *Proteusmirabilis.*

CONCLUSION

The study carried out revealed effectiveness of antibacterial action for both the hydroalcoholic extract and the aqueous extract in the four groups of isolated bacteria and no inhibitory action was observed on the yeasts of the process, revealing that the extracts have the potential to meet the needs of the sugar-energy sector.

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