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ISOLATES FROM INDUSTRIAL FERMENTATIVE PROCESSES WOULD BE SENSITIVE TO EXTRACTS OF MYRCIA BELLA?

Luana Beatriz Gonçalves
Faculdade de Tecnologia Nilo de Stéfani – Fatec Jaboticabal
Jaboticabal – SP
http://lattes.cnpq.br/7076292721796382

Paulo Cláudio Claudino Júnior
Faculdade de Tecnologia Nilo de Stéfani – Fatec Jaboticabal
Jaboticabal – SP
http://lattes.cnpq.br/3260031907266771

Julia de Melo Moreira
Centro Universitário Facens – Sorocaba – SP
http://lattes.cnpq.br/5424237754368884

Nádia Figueiredo de Paula
Faculdade de Tecnologia Nilo de Stéfani – Fatec Jaboticabal
Jaboticabal – SP
http://lattes.cnpq.br/6008784007482512

Leonardo Lucas Madaleno
Faculdade de Tecnologia Nilo de Stéfani – Fatec Jaboticabal
Jaboticabal – SP
http://lattes.cnpq.br/3972203417794213

Cássia Regina Primila Cardoso
Universidade Federal de Mato Grosso
Sinop – MT
http://lattes.cnpq.br/6759984980265462

Mariana Carina Frigieri
Faculdade de Tecnologia Nilo de Stéfani – Fatec Jaboticabal
Jaboticabal – SP
http://lattes.cnpq.br/4287525933081582

Abstract: The microbiological control of the industrial production process for obtaining bioethanol has always been a challenge because it is a process involving producer microorganisms (selected yeast) and invaders (contaminants), where invaders must be controlled so that this Control does not interfere with the action. of the producers. The uncontrolled presence of contaminants can make production unfeasible due to competition for raw material in addition to the production of inhibitory substances, changes in the ideal conditions of the process, consumption of the product, among other situations. In this sense, bacterial contaminants isolated from the material of the yeast treatment vat from three different production units in the region of Jaboticabal and also from a fermentation process carried out at Fatec Jaboticabal were tested separately and together for sensitivity to extract of *Myrcia bella* obtained by aqueous and hydroalcoholic extraction. It was possible to verify that the isolates from fermentation processes were sensitive, and it is important to continue the study to evaluate the use during the fermentation process as a potential way of using this species in the microbiological control of the process to optimize the production of bioethanol.
Keywords: Plant extract. Antimicrobial. contaminants. Fermentation.

INTRODUCTION

The industrial production of bioethanol can suffer several interferences, and the microbiological interference is a great "villain" of the process. Microbial contaminants The presence of contaminating microorganisms reduces the fermentation yield, requiring microbiological control actions for the process to be effective (CARVALHO et al., 2021). Control is usually performed with the use of sulfuric acid and, in more extreme cases of contamination, antibiotics are used

(MUTHAIYAN; LIMAYEM; RICKE, 2011), but this use has been questioned due to the high cost of the product and several concerns raised in the field. present as residues in yeast cells, stimulation of bacterial resistance and disposal in the environment (FREITAS; ROMANO, 2013; BREXÓ; SANT'ANA, 2017).

The possibility of finding alternative forms of Control has awakened several researchers to look for less aggressive and more economically attractive sources, such as plant extracts (CARVALHO et al., 2021). the hops (*Humulus lupulus*) has been the most used in the bioethanol industry (CECCATO ANTONINI, 2018).

Natural products derived from plants are attractive due to the ease and cost of obtaining, in addition to having a multitude of bioactive chemical molecules (ANAND, et al. 2020). Khameneh (2019) highlights that several plants are effective against antibiotic-resistant bacteria and also considers the possibility of joint use.

Several studies on microbiological control in the production of bioethanol have been carried out (MADALENO et al., 2016; RICH et al., 2018; CARVALHO et al., 2022) with promising results and with great possibility of exploitation (SALAM; QUAVE, 2018)), mainly in the case of Brazil, as it is a country with great plant diversity. More than 46,000 species have already been recorded and are distributed across the six Brazilian biomes (MMA, 2022). Among these, the Cerrado stands out, considered the savanna with the greatest floristic diversity in the world. Its location, occupying the entire central region of Brazil, makes it have border regions with other biomes, resulting in several ecotones with unique characteristics (COLLI et al. 2020; RIBEIRO et al. 2022).

The family: Myrtaceae is one of the most expressive botanical families in the Cerrado and the genera: *Myrcia, Eugenia* e *Psidium* are one of the most diverse in number of species (RIBEIRO et al. 2022). Belém et al., (2021) in their study on Cerrado plants with antimicrobial activity highlights the importance of this biome in the development of alternatives to conventional antimicrobials because it has several native plant species with notorious antimicrobial potential with main references to the botanical families Myrtaceae and Arecaceae. The Myrtaceae family is mentioned by Dexheimer; Pozzobon (2017) as possible sources of molecules with pharmacological potential.

Cascaes et al., 2015 highlights that the Myrtaceae Juss. encompasses flowering plants ranging from trees to shrubs found particularly in South America, Australia and Tropical Asia, with 23 genera and 1034 species in Brazil. The genus Myrcia has 260 species in different biomes across all regions of the country, and may be a promising source of biologically active compounds.

Myrcia bella Cambess (Myrtaceae) popularly known as Mercurinho or Murta, it is a common and widely distributed species in the Cerrado. The plant is used in traditional medicine to treat diabetes and gastrointestinal problems (SALDANHA et al. 2020). Several pharmacological properties have been reported in the scientific literature for this species. In a survey carried out by Ribeiro et al. (2022), records of antioxidant, allelopathic, hypoglycemic and antinociceptive activity were found, using mainly the leaves.

Given the evidence that this species has antimicrobial action, as verified by Santos et al., (2018), the present study sought to assess whether this action would also occur in isolates from the industrial fermentation process obtained by two forms of extraction (aqueous and hydroalcoholic).

MATERIAL AND METHODS

The experiments were carried out at the

Bioprocesses Laboratory at Fatec – Jaboticabal.

OBTAINING PLANT EXTRACTS

The plant species: *Myrcia bella* from the Brazilian cerrado, was collected in the city of Itirapina - SP and identified by Prof. Jorge Tamashiro, from the Institute of Biology at the University of Campinas. This activity was part of Fapesp's BIOTA project (BIOTA, 2022) with authorization from the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) for activities with scientific purposes under number 32066-1. The exsiccate was deposited in the Herbarium HUEC, Unicamp, under registration 1482. The vegetable pieces (only leaves) were previously divided, separated and subjected to drying in a circulating air oven at 45ºC, for seven days or the time necessary for complete drying. and homogeneous. After spraying and weighing, they were stored in properly labeled plastic containers and kept away from light, in a dry and air-conditioned environment at the Phytochemistry Laboratory of the Department of Organic Chemistry (IQ/Unesp Araraquara) where they were characterized and later sent to the Bioprocesses Laboratory. by Fatec Jaboticabal.

The plant extracts of *M. bella* were obtained by maceration (ANVISA, 2010) in the proportion 1:10 (m/v) in 70% ethanol for 5 days (hydroalcoholic extraction) or in water for 3 days (aqueous extraction), and after filtration, frozen in an ultra-freezer at –62ºC. The hydroalcoholic extracts were submitted to rotaevaporation to eliminate the extracting solvent. To complete the process, the extracts were lyophilized and stored under refrigeration in an amber bottle. Figure 1 shows the maceration process (A) and the final extract (B).

DETERMINATION OF WATER SOLUBILITY OF EXTRACTS

The determination of the solubility in water was carried out by transferring a certain amount of the extracts to a test tube or erlenmeyer flask where successive volumes of solvent were added, exactly measured, using a mechanical stirrer (vortex) for complete homogenization and solubilization of the samples. Then, an aliquot was centrifuged for 5 minutes at 100 rpm to confirm the absence of suspended material. Solubility was expressed according to descriptive terms in ANVISA (2010) and performed by in NASCIMENTO, (2008) and CARVALHO et al. (2022).

In Table 1 it is possible to verify the descriptive terms defined by and presented used for dissolution of a part (1 g) of the sample in the number of pieces (volume, in mL) of the solvent.

MICROORGANISMS USED

Standard strains were used (American Type Colection Culture - ATCC) *Staphyloccoccus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) and also yeasts and microbial contaminants from the fermentation process.

The microbial samples were obtained from the simulation of a fermentation process carried out at Fatec Jaboticabal and from the material from the yeast treatment vat of three different sugar-energy units in the Jaboticabal region (Plants A, B and C). YPD was used to isolate the yeasts (yeast extract 10 g.L-1, dextrose 20 g.L-1 and peptone 20 g.L-1), and for the bacteria, the nutrient broth (meat extract 3 g.L-1 and peptone 5 g.L-1). -1).

With the culture showing an absorbance of 0.3 at a wavelength of 600 nm, centrifugation was performed at 1,600xg for 5 minutes. The precipitate was suspended in a sterile solution of 20% glycerol in water (H_2O) and stored in an ultrafreezer until use.

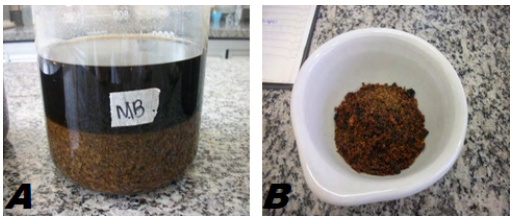

Figure 1: **A.** Vegetable maceration. **B**. final extract obtained
Source: Own file

Descriptive term	Approximate volumes of solvent in milliliters per gram of substance
Very soluble	Less than 1 piece
Easily soluble	From 1 to 10 pieces
Soluble	From 10 to 30 pieces
Slightly soluble	From 30 to 100 pieces
Poorly soluble	From 100 to 1.000 pieces
Very little soluble	From 1.000 to 10.000 pieces
Practically insoluble or insoluble	Over 10.000 pieces

Table 1. Descriptive terms to express solubility
Source: ANVISA, 2010.

Plant species	Plant extract	Solubility
Myrcia bella	Hydroalcoholic	Slightly soluble
	Aqueous	Soluble

Table 2. Solubility of plant extracts in descriptive terms
Source: Research data

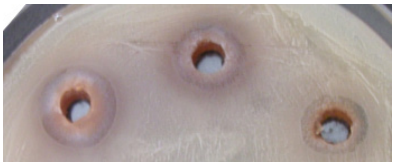

Figure 2: Inhibition halos obtained.
Source: Own file

Plant species	Plant extract	Diameter of halos (mm)	
		S. aureus	***E. coli***
Myrcia bella	Hydroalcoholic	12,17 ± 0,29	10,52 ± 0,48
	Aqueous	11,48 ± 0,93	9,85 ± 0,61
Control	-	*	*

* Halo measurement < 7mm. Source: Research data

Table 3. Measurement of inhibition halos obtained for standard microorganisms.

Plant extract	Diameter of halos (mm)				
	Fatec	**Power plant: A**	**Power plant: B**	**Power plant: C**	**Bacterial mix**
Hydroalcoholic	11,25 ± 0,90	11,07 ± 0,67	10,67 ± 0,29	14,50 ± 0,87	11,60 ± 0,96
Aqueous	10,58 ± 0,88	11,02 ± 0,50	10,75 ± 0,30	12,67 ± 0,76	9,45 ± 0,49
Control	*	*	*	*	*

* Halo measurement < 7mm. Source: Research data

Table 4. Measurement of inhibition halos for microorganisms isolated from the fermentation process.

EVALUATION OF THE ANTIBACTERIAL POTENTIAL OF PLANT EXTRACTS IN VITR

The antimicrobial activity of the plant extracts obtained was evaluated using the hole plate or well diffusion method (SILVA, 2012; BONA et al., 2014), essentially as described in Carvalho et al. al., 2022). The microorganisms were grown in Mueller-Hinton broth (2g/L bovine extract, 17.5g/L casein acid hydrolyzate and 1.5g/L starch) until the exponential phase and the microbial culture was subsequently adjusted to an optical density corresponding to the standard 0.5 of the Mac-Farland scale (OD620=0.10) and 100 µL of the same suspension were spread with a Drigalsky loop on Mueller Hinton agar plates (broth described above with 2% agar added). Holes of approximately 5 mm in diameter and 3 mm in height were made in the agar and 30 µL of hydroalcoholic and Aqueous extracts (150 mg/mL) were added.

Water and Amoxicillin (1µg/µL) were used as negative and positive controls, respectively. Assays were performed in triplicate. After 24 hours of incubation at 32ºC, the halos obtained were measured, and halos larger than 7mm were considered positive.

RESULTS AND DISCUSSION

OBTAINING AND EVALUATING THE SOLUBILITY OF PLANT EXTRACTS

To carry out the analysis, the crude extracts of *M. bella* were obtained by hydroalcoholic and aqueous extraction. MUTHAIYAN; LIMAYEM; RICKE, (2011) highlight as an economic advantage the use of extracts that require a lower level of purity. The maceration process is important to obtain thermolabile components, with the volume of solvent used being a disadvantage (ZHANG; LIN; YE, 2018), but if the solvent is low-cost as in the case of this analysis, the disadvantage is practically eliminated. ALTEMIMI et al. (2017) points out that the use of water in extractive processes contributes to obtaining compounds with polar characteristics. In addition to being a safe and biocompatible handling solvent (KUBILIENE et al., 2018), essential characteristics for processes that use live microorganisms.

The water solubility of the plant extracts obtained was determined because it is desirable to have a product for industrial use with practicality of use because it is easily soluble, a characteristic that was confirmed by the solubility evaluation as shown in Table 2. It was possible to verify the greater solubility in water of the Aqueous extract, in relation to hydroalcoholic. The same fact was observed by NASCIMENTO, (2008) and CARVALHO et al. (2022).

EVALUATION OF THE ANTIMICROBIAL POTENTIAL OF PLANT EXTRACTS IN VITRO

Each extract was tested for the standard microorganisms (Table 4) and for the isolates from the fermentation process carried out at Fatec and at the three sugar-energy plants in the Jaboticabal region, separately and for the microorganisms isolated from the fermentation process carried out at Fatec, in the sugar-energy units in the region. of Jaboticabal (Plants A, B and C) separately and also together (Table 5). The samples that showed inhibition had their halos measured through the diameter of the circumference obtained (Figure 2).

It was possible to observe that the extracts of *Myrcia bella* showed greater action on the bacterium S. aureus in relation to *E. coli* (Table 3), the hydroalcoholic extract being the most effective in both situations. The effectiveness of the extracts was also verified on the four bacterial groups isolated (Table 4), with more expressive values for the isolates from Plant C in the presence of the hydroalcoholic

extract. The action was verified for both the hydroalcoholic extract and the Aqueous extract, with similar values for the isolates from Fatec and from Plants A and B. Sensitivity was maintained when the isolates were tested together (bacterial mix) also with emphasis on the extract hydroalcoholic. In tests carried out in*Saccharomyces cerevisiae,* no inhibition halo formation was observed, as shown in CARVALHO et al. (2022).

Diffusion methods are used in the preliminary evaluation of the antimicrobial action of plant extracts and other natural products (OSTROSKY et al., 2008), especially the Hole plate method or well diffusion, which allows the evaluation of a larger volume of sample versus the disk diffusion method. BONA et al., (2014) evaluating plant extracts of the family: Myrtaceae verified that the inhibition promoted by the extracts of *Psidium guajava*, *Myrciaria cauliflora* and *Syzygium cumini* about the microbial samples in the well method was higher than the values obtained by disk, using the same volume of the tested plant extract.

SANTOS *et al.*, (2018) also verified antimicrobial activity for hydroalcoholic extracts of *M. bella* and highlights that the activities found are related to the phenolic composition of this plant.

The fact that the extracts act on the isolates from the industrial fermentation process raises the awareness of the potential use of this species in the microbiological control of the process to optimize the production of bioethanol.

CONCLUSION

With the accomplishment of the study, it was possible to conclude that the isolates from industrial fermentation processes were sensitive to Myrcia bella extracts, both those obtained by aqueous extraction and those obtained by hydroalcoholic extraction, being important to continue the study to evaluate the use during the fermentation process.

REFERENCES

ALTEMIMI, A.; LAKHSSASSI, N.; BAHARLOUEI, A.; WATSON, D. G.; LIGHTFOOT, D. A. **Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts.** Plants, v. 6, n. 4, p. 42, 2017.

ANAND, U.; NANDY, S.; MUNDHRA, A.; DAS, N.; PANDEY, D. K.; DEY, A. **A review on antimicrobial botanicals, phytochemicals and natural resistance modifying agents from Apocynaceae family: Possible therapeutic approaches against multidrug resistance in pathogenic microorganisms.** Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy, v. 51, p. 100695-100695, 2020.

ANVISA - Agência Nacional de Vigilância Sanitária. **Farmacopeia Brasileira, 5a edição**, v. 2, p. 1-523, 2010.

BIOTA. **Integrando a caracterização, conservação, restauração e o uso sustentável da biodiversidade**. Disponível em: http://www.biota.org.br/. Acesso em: 21 Jul. 2022.

BONA, E. A. M. D.; PINTO, F. G. D. S.; FRUET, T. K.; JORGE, T. C. M.; MOURA, A. C. D. **Comparação de métodos para avaliação da atividade antimicrobiana e determinação da concentração inibitória mínima (cim) de extratos vegetais Aqueouss e etanólicos**. Arquivos do Instituto Biológico, v. 81, n. 3, p. 218-225, 2014.

BREXÓ, R. P.; SANT'ANA, A. S. **Impact and significance of microbial contamination during fermentation for bioethanol production**. Renewable and Sustainable Energy Reviews, v. 73, p. 423–434, 2017.

CARVALHO, A. J. L.; FRIGIERI, M. C.; MADALENO, L. L.; LUSTRI, W. R.; FRAJÁCOMO, S. C. L.; FLUMIGNAN, D. L.; PAULA, A. V.; CARDOSO, C. R. P. **Produção de bioetanol e Control microbiológico do processo**, In: Microbiologia: Clínica, Ambiental e Alimentos. Atena Editora, 2021. p. 1-388–416.

CARVALHO, A. J. L.; FRIGIERI, M. C.; MADALENO, L. L.; SENA; I. A. LUSTRI, W. R.; FRAJÁCOMO, S. C. L.; FLUMIGNAN, D. L.; PAULA, A. V.; CARDOSO, C. R. P. **Potential of byrsonima intermedia in isolates from the industrial fermentation process**, International Journal of Biological and Natural Sciences, v. 2, n. 1, p. 1-10, 2022.

CASCAES, M. M.; GUILHON, G. M. S. P.; ANDRADE, E. H. D. A.; ZOGHBI, M. D. G. B.; SANTOS, L. D. S. **Constituents and pharmacological activities of Myrcia (Myrtaceae): A review of an aromatic and medicinal group of plants.** International Journal of Molecular Sciences, v. 16, n. 10, p. 23881-23904, 2015.

CECCATO-ANTONINI, S. R. **Conventional and nonconventional strategies for controlling bacterial contamination in fuel ethanol fermentations.** World Journal of Microbiology and Biotechnology, v. 34, n. 6, p. 80, 2018.

COLLI, R. G.; VIEIRA, R, C.; DIANESE, C. J. **Biodiversity and conservation of the Cerrado: recent advances and old challenges.** Biodiversity and Conservation (2020) 29:1465–1475 https://doi.org/10.1007/s10531-020-01967-x

DEXHEIMER, G. M.; POZZOBON, A. Atividade biológica de plantas da família Myrtaceae: revisão sistemática de artigos entre 1989 e 2015. Revista Cubana de Plantas Medicinales, v. 22, n. 2, 2017.

FREITAS, M. D.; ROMANO, F. P. **Tipos de contaminações bacterianas presentes no processo de fermentação alcoólica.** Bioenergia em Revista, v. 3, n. 2, p. 29–37, 2013.

KHAMENEH, B.; IRANSHAHY, M.; SOHEILI, V.; BAZZAZ, B. S. F. **Review on plant antimicrobials: A mechanistic viewpoint.** Antimicrobial Resistance & Infection Control, v. 8, n. 1, p. 118, 2019.

KUBILIENE, L.; JEKABSONE, A.; ZILIUS, M.; TRUMBECKAITE, S.; SIMANAVICIUTE, D.; GERBUTAVICIENE, R.; MAJIENE, D. **Comparison of aqueous, polyethylene glycol-aqueous and ethanolic propolis extracts: antioxidant and mitochondria modulating properties**. BMC complementary and alternative medicine, v. 18, n. 1, p. 165, 2018.

MADALENO, L. L.; MINARI, G. D.; DE ANNUNZIO, F. R.; DE CARVALHO, M. R.; JÚNIOR, G. R. B.; SALES, D. C.; FRIGIERI, M. C. **Use of antimicrobials for contamination control during ethanolic fermentation**. Científica, v. 44, n. 2, p. 226–234, 2016.

MMA. Ministério do meio Ambiente. https://www.gov.br/mma/pt-br/assuntos/biodiversidade. Acesso em 06/08/2022.

MUTHAIYAN, A.; LIMAYEM, A.; RICKE, S. C. **Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations**. Progress in Energy and Combustion Science, v. 37, n. 3, p. 351–370, 2011.

NASCIMENTO, A.M. **Avaliação da qualidade de extratos de Stryphnodendron adstringens (Martius) Coville**. 2008. 159p. Dissertação (Mestrado em Ciências Farmacêuticas), Universidade Federal de Minas Gerais, Belo Horizonte, 2008.

OSTROSKY, E. A.; MIZUMOTO, M. K.; LIMA, M. E.; KANEKO, T. M.; NISHIKAWA, S. O.; FREITAS, B. R. **Métodos para avaliação da atividade antimicrobiana e determinação da concentração mínima inibitória (CMI) de plantas medicinais.** Revista Brasileira de Farmacognosia, v. 18, n. 2, p. 301-307, 2008.

RIBEIRO, C. L.; PAULA, J. A. M.; PEIXOTO, J. C. **Propriedades farmacológicas de espécies dos gêneros*: Myrcia*, *Eugenia* e *Psidium* – Myrtaceae, típicas do Cerrado: Uma revisão de escopo**. Research, Society and Development, v. 11, n. 8, e44711830356, 2022

RICH, J. O.; BISCHOFF, K. M.; LEATHERS, T. D.; ANDERSON, A. M.; LIU, S.; SKORY, C. D. **Resolving bacterial contamination of fuel ethanol fermentations with beneficial bacteria – An alternative to antibiotic treatment.** Bioresource technology, v. 247, p. 357–362, 2018.

SALAM, A. M.; QUAVE, C. L. **Opportunities for plant natural products in infection control**. Current opinion in microbiology, v. 45, p. 189-194, 2018

SALDANHA, L.L.; ALLARD, P.M.; AFZAM, A.; MELO, F.P.S.P.; MARCOURT, L.; QUEIROZ, E.F.; VILEGAS, W.; FURLAN, C.M.; DOKKEDAL. A.C.; WOLFENDER, J.C. **Metabolomics of *Myrcia bella* Populations in Brazilian Savanna Reveals Strong Influence of Environmental Factors on Its Specialized Metabolism.** Molecules 2020, 25, 2954; doi:10.3390/molecules 25122954

SANTOS, C.; GALAVERNA, R. S.; ANGOLINI, C. F.; NUNES, V. V.; DE ALMEIDA, L. F.; RUIZ, A. L.; CARVALHO, J. E.; DUARTE, R. M. T.; DUARTE, M. C. T.; EBERLIN, M. N. **Antioxidative, antiproliferative and antimicrobial activities of phenolic compounds from three Myrcia species**. Molecules, v. 23, n. 5, p. 986, 2018.

SILVA, D. M. **Efeito de extratos vegetais e antibióticos sobre *Staphylococcus aureus* de origem bovina**. 2012. 45p. Dissertação (Mestrado em Bioquímica Agrícola), Universidade Federal de Viçosa, Minas Gerais, 2012.

ZHANG, Q. W.; LIN, L. G.; YE, W. C. **Techniques for extraction and isolation of natural products: a comprehensive review**. Chinese medicine, v. 13, n. 1, p. 20, 2018.