EFFECT OF pH ON THE ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF WOOD VINEGAR (PYROLIGNEOUS EXTRACT) FROM EUCALYPTUS

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[1] Received on 12.05.2022 accepted for publication on 01.05.2023.
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ABSTRACT – The study aimed to assess the effect of progressive neutralization on the antimicrobial properties against bacteria and yeasts of wood vinegar obtained from the pyrolysis of *Eucalyptus urograndis* (clone I144) wood. Wood samples were carbonized at a heating rate of 0.9 °C min^{-1} until a final temperature of 450 °C, totalizing 8 hours of carbonization. The raw pyrolysis liquids were left to settle, and the aqueous fraction was separated. Then, the aqueous fraction (raw wood vinegar – WV) was purified to yield the WV. WV samples were collected and neutralized from pH 2.5 until 7.5 (2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5, by adding NaOH solution. Through the broth microdilution method, the antimicrobial effect of the neutralized samples at each pH was assessed on *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13076), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (CEPA CLINICA), and *Candida albicans* (ATCC 10231). The minimum inhibitory concentration (MIC) and minimum bactericidal (and fungicidal) concentrations were determined through *in vitro* technics. Results were subjected to logarithmic regression analysis, and statistical models were fitted for each microorganism in the assessed pH range; as pH increased, a progressive decrease in the CIM increased, demanding higher concentrations of WV to inhibit microbial growth. The more resistant strains were *S. aureus* and *S. agalactiae*, which required an increase in WV concentration from a minimum of 6.25% at pH 2.5 to reaching 50% at pH 6.0. When at pH 7.0, both strains were not inhibited even at 50% (the highest concentration evaluated in the study). In contrast, *C. albicans* proved to be the most sensitive strain, starting from 3.12% EP at pH 2.5 and requiring only 25% for inhibition at pH 7.0. The behavior of *P. aeruginosa* and *S. enteritidis* followed the pattern of *C. albicans*, differing only at pH 7.0, where they required 50% of EP. As observed, even at neutral and slightly alkaline pH, the inhibitory activity of EP on microbial growth was maintained to some extent. Nevertheless, even when neutral and slightly alkaline pH values are reached, the inhibitory activity remains at a certain level. Higher pH values of the WV were associated with lower antimicrobial activity. However, its activity remained even at neutral and slightly alkaline pH values.

Keywords: Pyroligneous acid and pH effect; Antibacterial activity; Antifungal activity.

EFFEITO DO pH SOBRE A ATIVIDADE ANTIBACTERIANA E ANTIFÚNGICA DO EXTRATO PIROLENHOSO DE EUCALIPTO

RESUMO – O presente trabalho teve como objetivo avaliar o efeito da neutralização progressiva na atividade antimicrobiana do extrato pirolenhoso (EP) da madeira do híbrido ***Eucalyptus urophylla x Eucalyptus grandis***

(clone I144). Amostras de madeira foram carbonizadas a uma taxa de aquecimento de 0.9 °C min^{-1} até a temperatura final de 450 °C, perfazendo 8 horas de carbonização. Os líquidos brutos da carbonização foram deixados em repouso e a fração aquosa foi separada. A fração aquosa correspondente ao EP bruto foi destilada obtendo o EP purificado. Alíquotas de EP purificado foram progressivamente neutralizadas do pH 2.5 até 7.5, respectivamente, 2,5, 3,0, 3,5, 4,0, 4,5, 5,0, 5,5, 6,0, 6,5, 7,0, e 7,5, por meio da adição de NaOH. Com o método da micro-diluição em caldo, o efeito antimicrobiano das amostras neutralizadas em cada pH foi avaliado contra ***Pseudomonas aeruginosa*** *(ATCC 27853),* ***Salmonella enteritidis*** *(ATCC 13076),* ***Staphylococcus aureus*** *(ATCC 25923),* ***Streptococcus agalactiae*** *(CEPA CLINICA) e* ***Candida albicans*** *(ATCC 10231). As concentrações inibitórias mínimas e as concentrações bactericida e fungicida mínimas foram determinadas por técnicas* ***in vitro****. Os resultados foram analisados por regressão logarítmica e modelos estatísticos foram ajustados para cada microrganismo em cada pH. Na determinação da CIM, foi detectada uma perda progressiva da atividade antimicrobiana do EP com a neutralização, exigindo, conforme o pH caminhava para 7.0, concentrações mais elevadas de EP para resultar em inibição. As cepas que demonstraram maior resistência foram S. aureus e S. agalactiae. Esses microrganismos partiram da exigência de concentrações de 6.25% de EP, em pH inicial (2.5), e chegaram a requerer 50% em pH 6.0. Quando em pH 7.0, ambas as cepas não foram inibidas nem a 50% (maior concentração avaliada no estudo). Em contrapartida,* ***C. albicans*** *se mostrou a cepa mais sensível, partindo de 3.12% de EP, em pH de 2.5, e exigindo apenas 25% para inibição em pH 7.0. O comportamento de* ***P. aeruginosa*** *e* ***S. enteritidis*** *acompanhou o padrão da* ***C. albicans****, diferenciando-se apenas em pH 7.0, onde exigiram 50% de EP. Como observado, mesmo em pH neutro e ligeiramente alcalino, a atividade inibitória do EP ao crescimento microbiano se manteve em certa extensão.*

Palavras-Chave: Carbonização de madeira de eucalipto; Extrato pirolenhoso de eucalipto; Bactericida e fungicida natural.

1. INTRODUCTION

Wood vinegar (WV), pyroligneous acid, pyroligneous extract, and water-soluble liquid smoke refer to the same product, namely the aqueous fraction separated through the settling of the raw pyrolysis liquids (Sena et al., 2014). WV has a complex chemical composition, which can reach up to 200 compounds, among them phenols, ketones, furans, aldehydes, pyrans, alcohols, and organic acids (Schnitzer et al., 2015; Araújo et al., 2017; Dias Júnior et al., 2018; Pimenta et al., 2018; Suresh et al., 2019). WV is essentially an acidic product with a pH ranging from 2.5 to 3.6 (Aubin and Roy, 1990; Rahmat et al., 2014; Pimenta et al., 2018), depending on the chemical composition of the pyrolyzed raw material. This acidity is due to organic acids, the most common being acetic and formic acids. The most common organic acid in WV is acetic acid, with concentrations of 3.0 to 7.4% (Sipilä et al., 1998; Theapparat et al., 2018).

Due to its particular chemical composition, preservative, and medicinal properties, WV has been employed for several purposes since ancient times (Tiilikkala et al., 2010). There are millenary reports of its use in the treatment of diseases in China and India (Campos, 2007); salt substitute in 1862 to preserve meats (Kurlansky, 2003); soil disinfection (Doran, 1932), among other applications. This variety of applications has led to increasing interest in this wood-pyrolysis bioproduct by researchers worldwide, with several studies focused on its antimicrobial action and other properties (Araújo et al., 2017; Souza et al., 2018; Chen et al., 2016; Maliang et al., 2020). The role of phenols and other compounds on the antimicrobial activity of WV has been well established in recent works, such as Suresh et al. (2019) and Suresh et al. (2020), for instance. In these works, the authors assessed WV in both acidic and neutral forms and demonstrated that the product had antimicrobial activity even after neutralization, albeit weaker, but not absent. More than that, since the acetic acid is no longer present in WV after neutralization, Suresh et al. (2019) highlighted that the antimicrobial effect cannot be attributed to this compound alone but must be attributed to the combined action with several other compounds.

However, the previous research works involving the antimicrobial activity of neutralized WV were carried out directly with the acidic and neutral versions without verifying what happens to that activity during progressive neutralization. Thus, from the original acidic pH to neutrality, there is a gap in information about the antimicrobial activity of WV in the pH

range from 2.5 to 7.0. In this context, the present work aimed to assess the effect of increasing pH (from the original pH of 2.5 to 7.0) on the antimicrobial activity of wood vinegar (pyroligneous extract) from *Eucalyptus urophylla* x *Eucalyptus grandis* (clone I144) on *Pseudomonas aeruginosa*, *Salmonella enteritides*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Candida albicans*.

2. MATERIAL AND METHODS

2.1. Production and purification of WV

Wood samples from *Eucalyptus urograndis* trees (usual denomination of hybrids of *Eucalyptus urophylla* x *Eucalyptus grandis* in Brazil), clone I144, were collected. Tree selection, harvesting, and wood sample collection followed the procedures described by Carneiro et al. (2013). The wood samples consisted of 3 cm thick disks, and before carbonization, they were cut into wedges and oven-dried at 103 °C (± 2 °C) under forced ventilation until constant weight. The carbonization runs were performed in a laboratory muffle furnace (Labor SP-1200, SP LABOR, São Paulo, Brazil) with dry wood samples weighing about 500 g in each run. Twenty runs were conducted. In each run, the wood samples were placed inside an externally-heated steel container and heated at a rate of 0.9 °C min^{-1} from room temperature to 450 °C, totalizing 8 hours of the process. The liquid fraction from the carbonization was recovered using a water-cooled steel condenser (25 °C). The composite sample of the raw liquids was decanted, and the aqueous supernatant was separated and bi-distilled from 100 to 103 °C to obtain the purified WV. The purified WV was vacuum filtered through a 0.2 μm filter (MF Millipore, Merck, Darmstadt, Germany).

2.2. Neutralization of WV

20 mL aliquots from the purified WV resulting from the previous step were obtained with two replicates of each. The first ones were kept at their original pH of 2.5. Then, two by two, the other aliquots were increasingly neutralized to obtain pH values of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, or 7.5. For neutralization, a 2 mol L^{-1} NaOH solution was employed, and the final pH of the aliquots was monitored with a pHmeter (PG 2000, Gehaka, São Paulo, SP, Brazil). Thus, 11 samples of WV with different pH values were obtained.

2.3. *In vitro* evaluation of the antimicrobial activity

The assays were carried out by the broth microdilution method with 96-well microplates, according to the CLSI (2012) routine procedures. For the assessment tests, five microorganisms were employed: four bacterial strains, *P. aeruginosa* (ATCC 27853), *S. enteritidis* (ATCC 13076), *S. aureus* (ATCC 25923), and *S. agalactiae* (CEPA CLINICA), and a strain of the yeast *C. albicans* (ATCC 10231). All the microorganisms were kept in BHI (brain heart infusion, Kasvi, São José dos Pinhais-PR, Brazil) under refrigeration. Just before each assay, they were cultivated in a bacteriological oven (Fanem model 50, Guarulhos, SP, Brazil) at 37 °C (± 1 °C) for 24 hours with the same culture media (48 hours for *C. albicans*). Then the microbial inocula were prepared. The adjustment of the concentration of bacterial and fungal cells in the culture media was achieved by employing a spectrophotometer (Biospectro SP-22, Labmais, Curitiba, PR, Brazil) at the wavelength of 530 nm with reading values from 0.08 to 0.1, corresponding to 0.5 in the MacFarland scale (density of 1.5 x 10^8 cells mL^{-1}).

Then, 100 μL of BHI culture media were added to each well of the microplates. After that, serial dilutions of ½ were performed from 50 to 0.78% for each pH in the BHI medium. For each concentration and each pH, three replicates were conducted. This way, 231 wells for observation were obtained (7 concentrations x 11 pH x 3 replicates, or 77 experimental treatments x 3 replicates). After the dilutions, 0.5 μL of microbial inoculum was added to each well. Next, the microplates were incubated in a bacteriological oven at 37 °C (± 1 °C) for 24 hours (*C. albicans* 48 hours). After that time, the wells were read visually, and the dilutions of each experimental treatment that were completely translucid were considered to establish the minimum inhibitory concentration (MIC). The visual reading was employed since the colorimetric method usually applied using resazurin was inefficient. After the visual assessment, the microplates were read in an ELISA microplate reader (model 660, URIT Medical Electronic, Nanshan, Shenzhen, China) to determine the absorbances, a way to quantify the microbial growth according to the concentration and pH. For each experimental treatment, three replicates were read. The positive control for the readings was achieved with chlorhexidine (Vic Pharma by Shülke,

Taquaritinga, SP, Brazil) at 0.2%. The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined from the MIC value measured for each combination of microorganism and pH, where the solution of the MIC well and those of higher concentrations were inoculated in Petri dishes containing BHI culture medium. The Petri dishes were incubated in a bacteriological oven at 37 °C (± 1 °C) for 24 hours (*C. albicans* 48 hours). After this period, the presence or absence of microbial colonies was assessed.

2.4. Statistical analysis

The experimental data from the absorbance readings were subjected to regression analysis by employing the R software (version 4.1.3) at the moment of the inoculation (zero hours) and after 24 hours for bacterial strains and 48 hours for *C. albicans*. For each type of microorganism, a regression model was fitted at each pH level to describe and predict the behavior of absorbance (Y) as a function of WV concentration (X). Among several models assessed, the logarithmic model [$Y= \beta 1 \ln(X) + \beta 0 + \varepsilon$, recommended by Gujarati and Porter (2009)] was the best to explain the behavior of the absorbances after the incubation period. The data on absorbances were graphically plotted on zero time only to demonstrate their pattern of behavior after inoculation. For the absorbance after the incubation of the microbial cultures, a specific model was adjusted for each combination of pH level and concentration. After the adjustment of the equations, the curves of the factor pH for the same microorganism were compared to each other using the model identity test, according to the routine described by Regazzi (1993, 1996, 1999) and previously applied in a similar experiment (Araújo et al., 2017).

3. RESULTS

3.1. Antimicrobial activity: determination of MIC, MBC, and MFC

Table 1 contains the results obtained for the MIC at each pH for the five microorganisms. The pattern observed was a decrease in the antimicrobial effect as the pH approached neutrality. The results obtained for MBC and MFC, also displayed in Table 1 (numbers in italics), follow the same trend as the MIC. At lower pH levels, the concentrations required to inhibit microbial growth were lower, and as the pH approached neutral, the concentrations needed to achieve the same effect were higher.

3.2. Antimicrobial activity: behavior of the absorbances

Figure 1 contains the graphs based on the models fitted from the regression analysis of the absorbances, each corresponding to a single microorganism. In all components displayed in Figure 1, graphs identified

Table 1 – Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) values against the microorganisms according to increasing pH levels.
Tabela 1 *– Valores de concentraçao inibitória mínima (CIM) e concentraçao bactericida mínima (CBM) contra os microrganismos em funçao do pH.*

Microorganism		WV concentration (%)											
		pH											
		2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	PC
P. aeruginosa	MIC	3.12	3.12	3.12	6.25	6.25	12.5	12.5	25	25	50	25	0.003
	MBC	12.5	6.25	6.25	12.5	12.5	12.5	25	50	50	50	50	0.003
S. enteritidis	MIC	3.12	3.12	3.12	6.25	6.25	6.25	25	25	25	50	25	0.003
	MBC	3.12	6.25	6.25	25	12.5	12.5	25	50	50	>50	50	0.003
S. aureus	MIC	6.25	6.25	6.25	6.25	12.5	12.5	25	50	50	>50	50	0.003
	MBC	6.25	6.25	6.25	12.5	12.5	25	25	50	>50	>50	>50	0.003
S. agalactiae	MIC	6.25	6.25	6.25	6.25	12.5	12.5	25	50	50	>50	>50	0.003
	MBC	6.25	6.25	12.5	12.5	25	25	25	50	50	>50	>50	0.003
C. albicans	MIC	3.12	3.12	3.12	6.25	6.25	12.5	25	25	25	25	25	0.003
	MFC	3.12	3.12	3.12	6.25	6.25	25	25	25	25	50	50	0.003

*PC = positive control (chlorhexidine); **In Table body, for each microorganism, the first line corresponds to the MIC, and the numbers in italics are the MBC; ***For *Candida albicans*, the numbers in italics are the minimum fungicidal concentrations (MFC); ****The sign > is employed to suggest the possibility that a concentration higher than 50% can result in inhibition.
CP = controle positivo (clorexidina); **Na tabela, para cada microrganismo, a primeira linha corresponde à CIM e os números em itálico são a CBM; ***Para* ***Candida albicans**, os números em itálico são a concentração fungicida mínima (CFM); ****O sinal > foi empregado para sugerir a possibilidade de que a concentração acima de 50% pode resultar em inibição.*

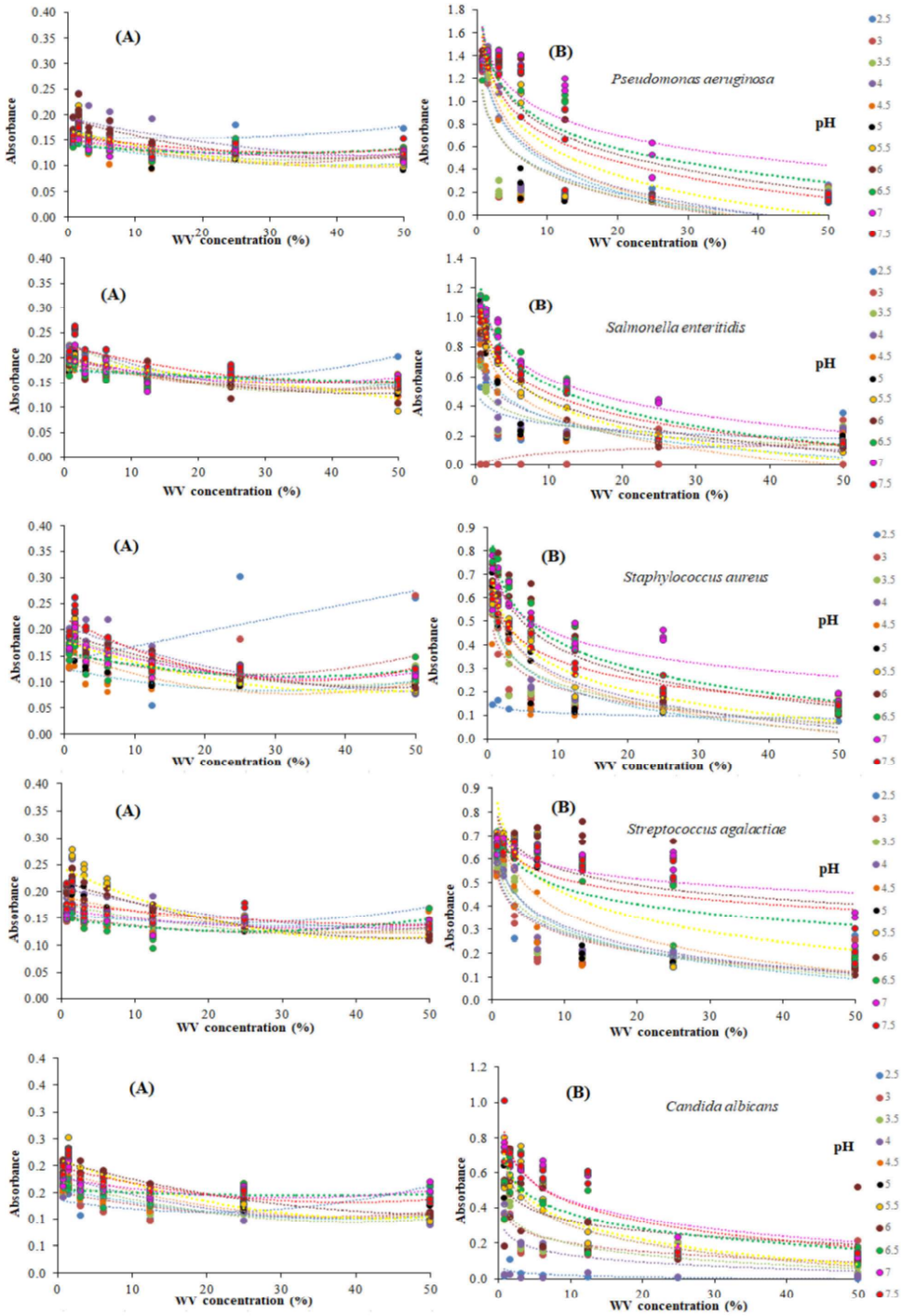

Figure 1 – Graphs representing the effect of the pH of WV on the growth of *Pseudomonas aeruginosa*, *Salmonella enteritides*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Candida albicans* as a function of the concentration at zero time (A) and 24 hours (B) after incubation (48 hours for C. albicans).

***Figura 1** – Gráficos representando o efeito do pH do extrato pirolenhoso (EP) sobre o crescimento de **Pseudomonas aeruginosa**, **Salmonella enteritides**, **Staphylococcus aureus**, **Streptococcus agalactiae** e **Candida albicans** em função da concentração no tempo zero (A) e após 24 h (B) de incubação (48 horas para C. albicans).*

with the letter A are representations of the behavior of the absorbances of the culture media at 0 h (just after the inoculation) and after 24 hours of incubation for bacterial strains and 48 for *C. albicans* (B). The status of the culture at 0 h was performed to know if any immediate effect of WV on microorganisms could occur. Although the MIC, MBC, and MFC values could not be determined from the graphs in Figure 1, the knowledge of the absorbances was still a valuable tool to quantify the interaction between pH, WV concentration, and microbial growth precisely, as will be discussed in the next section. In the graphs marked with the B letter, at the same pH, higher absorbances are associated with lower efficacy of the WV.

In Table 2, the regression models that were adjusted to each microorganism are presented. As can be observed in Figure 1, as the concentration decreased and the pH increased, the absorbances rose. A higher absorbance value indicates that the culture medium became more turbid due to the higher concentration of developed microbial cells. For the models that were adjusted to explain the effect of pH in the growth of *P. aeruginosa* (Table 2), a low value of the coefficient of correlation was determined at pH 2.5, implicating a relatively high dispersion of the experimental data having the regression as reference. However, a trend of behavior is reflected by the regression line, and for the other pH values, this pattern is confirmed since the values of R^2 for the same type of model are higher, with a maximum of 0.83 for the pH of 5.5.

Regarding the models that were adjusted to predict the growth of *S. enteritides* (Table 2) under the effect of variable concentrations of WV at different pHs, for the values of pH of 2.5 and 3.0, the correlation coefficients were low, 0.30, and 0.23, respectively. From pH 3.0 and higher, the R^2 values were higher, reaching 0.96 at pH 7.0. For *S. enteritides*, as a general trend, the values of R^2 from pH of 5.5 up to 7.5 were the highest among the microorganisms assessed, representing good power of predictability of growth pattern by the logarithmic model.

For the growth of *S. aureus*, the value of R^2 at pH 2.5 was the lowest among all the assessed microorganisms, with a value of 0.11 (Table 2). However, from this point forward, the correlation coefficient values were higher than 0.70, except at

Table 2 – Logarithmic regression models models that were adjusted to explain the behavior of absorbances from cultures of *Pseudomonas aeruginosa*, *Salmonella enteritides*, *Staphylococcus aureus*, *Streptococcus agalactiae* after 24 hours of incubation, and *Candida albicans* after 48 hours at each pH level according to WV concentration.

Tabela 2 *– Modelos logarítmicos de regressão ajustados para explicar o comportamento das absorbâncias das culturas de* ***Salmonella enteritides****,* ***Staphylococcus aureus****,* ***Streptococcus agalactiae*** *após 24 h de incubação e* ***Candida albicans*** *após 48 h para cada pH em função da concentração de EP.*

pH	Regression Model	R^2
	Pseudomonas aeruginosa	
2.5	Y = – 0.266 ln X + 0.9922	0.55
3.0	Y = – 0.287 ln X + 1.0194	0.60
3.5	Y = – 0.284 ln X + 1.0201	0.64
4.0	Y = – 0.344 ln X + 1.2765	0.81
4.5	Y = – 0.358 ln X + 1.2777	0.75
5.0	Y = – 0.384 ln X + 1.3839	0.79
5.5	Y = – 0.379 ln X + 1.4791	0.83
6.0	Y = – 0.346 ln X + 1.5663	0.82
6.5	Y = – 0.323 ln X + 1.5487	0.72
7.0	Y = – 0.292 ln X + 1.5721	0.73
7.5	Y = – 0,337 ln X + 1,4743	0.80
	Salmonella enteritides	
2.5	Y = – 0.063 ln X + 0.4273	0.31
3.0	Y = 0.0369 ln X – 0.0014	0.23
3.5	Y = – 0.099 ln X + 0.5175	0.62
4.0	Y = – 0.136 ln X + 0.6351	0.73
4.5	Y = – 0.164 ln X + 0.6957	0.78
5.0	Y = – 0.224 ln X + 0.8705	0.83
5.5	Y = – 0.243 ln X + 0.9836	0.94
6.0	Y = – 0.213 ln X + 0.9241	0.89
6.5	Y = – 0.253 ln X + 1.1261	0.95
7.0	Y = – 0.223 ln X + 1.1013	0.96
7.5	Y = – 0.217 ln X + 0.9850	0.96
	Staphylococcus aureus	
2.5	Y = – 0.014 ln X + 0.1428	0.11
3.0	Y = – 0.094 ln X + 0.4339	0.67
3.5	Y = – 0.109 ln X + 0.4865	0.78
4.0	Y = – 0.140 ln X + 0.5964	0.87
4.5	Y = – 0.121 ln X + 0.5055	0.73
5.0	Y = – 0.146 ln X + 0.5980	0.87
5.5	Y = – 0.144 ln X + 0.6379	0.94
6.0	Y = – 0.154 ln X + 0.7425	0.84
6.5	Y = – 0.158 ln X + 0.7783	0.91
7.0	Y = – 0.110 ln X + 0.6978	0.80
7.5	Y = – 0.114 ln X + 0.5974	0.94
	Streptococcus agalactiae	
2.5	Y = – 0.100 ln X + 0.5120	0.54
3.0	Y = – 0.098 ln X + 0.4993	0.67
3.5	Y = – 0.114 ln X + 0.5509	0.79
4.0	Y = – 0.122 ln X + 0.5932	0.81

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Table 2 ...
Tabela 2 ...

4.5	Y = – 0.130 ln X + 0,5996	0.81
5.0	Y = – 0.155 ln X + 0.7264	0.80
5.5	Y = – 0.150 ln X + 0.7993	0.72
6.0	Y = – 0.089 ln X + 0.7560	0.39
6.5	Y = – 0,100 ln X + 0.7058	0.64
7.0	Y = – 0.064 ln X + 0.7066	0.54
7.5	Y = – 0,080 ln X + 0.6972	0.59
	Candida albicans	
2.5	Y = – 0.014 ln X + 0.0513	0.29
3.0	Y = – 0.066 ln X + 0.3515	0.54
3.5	Y = – 0.084 ln X + 0.3891	0.70
4.0	Y = – 0.056 ln X + 0.2616	0.26
4.5	Y = – 0.106 ln X + 0.4449	0,58
5.0	Y = – 0.142 ln X + 0.6274	0.77
5.5	Y = – 0.149 ln X + 0.6668	0.78
6.0	Y = – 0.083 ln X + 0.5174	0,30
6.5	Y = – 0.123 ln X + 0.6511	0.58
7.0	Y = – 0.146 ln X + 0.7763	0.78
7.5	Y = – 0.158 ln X + 0.7951	0.78
3.0 and 4.0**	Y = – 0.061 ln X + 0.3065	0.35
3.5 and 4.0**	Y = – 0.070 ln X + 0.3254	0.41

*Y = absorbance; X = WV concentration; R^2 = coefficient of determination
**treatments with statistical similarity.
**Y = absorbância; X = concentração de EP; R2 = coeficiente de determinação; **Tratamentos com similaridade estatística.*

the pH of 3.0, where it was 0.67. Once again, some variation in the microorganism's growth at these pH values (and included in the error of the adjustment) resulted in lower values of R^2. As displayed in Table 2, the development of *S. agalactiae* was best explained by the logarithmic model in the pH range of 3.5 to 5.5, reflected by the higher correlation coefficient values in this range. Despite presenting good correlation coefficients in general, the logarithmic model was ineffective in explaining the growth of the yeast *C. albicans* at the pH values of 2.5, 4.0, and 6.0. At these points, the R^2 had low values, which shows a variable response of the microorganism to the action of WV as pH was varying. The results of the model identity test determined, as a general trend, that as the pH increased, the quality of WV as an antimicrobial changed, probably due to the differential neutralization of its phenolic components. This difference in WV quality at each pH was positively determined by the statistical dissimilarity between the compared models through the identity test, which showed a statistical difference for all models, except for *C. albicans* in the comparison between the models at pHs 3.0 and 3.5, when compared with the model at pH 4.0.

4. DISCUSSION

4.1. Antimicrobial activity: determination of MIC, MBC, and MFC

The data displayed in Table 1 corroborate the results of the positive antimicrobial effects achieved by several researchers listed in the review published by Tiilikkala et al. (2010) and, more recently, by Souza et al. (2018). Other studies have demonstrated the antibacterial and antifungal properties of WV from varied lignocellulosic sources (Ibrahim et al., 2013; Abas et al., 2018, Souza et al., 2018). The experimental results presented here corroborate the results reported by Velmurugan et al. (2009) and Suresh et al. (2019), who assessed the antimicrobial effect of different types of WV both in their original acid form and after neutralization. Both those studies described the antimicrobial impact of WV before and after neutralization. They found a decrease in the activity but not the disappearance of the biological effect when pH became neutral.

As shown in Table 1, the results demonstrated that as the neutralization increased and the pH approached 7.0, the concentration of WV required for microbial inhibition increased. Even at neutral pH, the WV still had antimicrobial activity, although needing higher concentrations for this purpose. For *P. aeruginosa*, the MIC of WV at pH 2.5 was 3.12% and increased to 50% at neutral pH. When the pH reached 7.5, the MIC returned to the same value of 25% observed for pH 6.5. The same relationship between MIC and pH was determined for *S. enteritides*. For *C. albicans*, the MIC at pH 2.5 was 3.12% and increased to 25% at pH 5.5, which remained constant until the pH reached 7.5. The pattern of inhibition as a function of the concentration and pH of the WV is different for each microorganism. As Suresh et al. (2019) commented, the activity of neutralized WV indicates that the antibacterial property of the product is due to its complex chemical composition and not the presence of acetic acid. Citing other authors, Suresh et al. (2019) highlighted that the inhibition of the WV against microorganisms, especially fungi, is caused by the antioxidative property of the phenolic compounds. In this respect, previous studies have reported that the inhibition of lipid oxidation caused by phenolics is enhanced at acidic pH.

However, a different inhibition pattern was observed for *S. aureus* and *S. agalactiae*. For the first microorganism, when the pH was equal to 7.0 (neutral), no inhibition in the growth of the culture was observed. When the pH became slightly alkaline (7.5), the MIC was 50%, the same concentration required to inhibit the culture growth at pH 6.5 completely. In the case of *S. agalactiae*, for both pH levels, 7.0 and 7.5, a WV concentration of 50% was not enough to inhibit microbial growth. Both microorganisms required higher WV concentration to inhibit growth, starting with the original pH of 2.5. For the other three microorganisms, the initial MIC value was 3.12%. Several compounds (phenolics and ketones) in WV's chemical composition have antimicrobial activity, so they most likely interact differently with one microorganism due to differences in cell wall structure and composition. These differences among microorganisms combined with the degree of response to the action of one or another compound in the chemical composition of WV probably explain why the product does not inhibit microbial growth with the same inhibition results at the same concentration. In the present study, *S. aureus* and *S. agalactiae* were the most resistant species to the inhibitory effect of WV, while *C. albicans* was the most sensitive one (Table 1).

The work from Suresh et al. (2019) reported a decrease in the effect of the product after neutralization but not complete disappearance, the same trend reported in the present work. Still, based on their experimental data, those authors stated categorically that the antimicrobial effect of the WV, even after neutralization indicated that the antibacterial property of the product was due to its complex chemical composition rather than the significant presence of acetic acid. In their experiments assessing the antimicrobial action of *Escherichia coli*, *Enterobacter aerogenes*, *P. aeruginosa*, *Listeria monocytogenes*, and *Enterococcus faecalis*, the authors found a loss of activity ranging from 10 to 24%, with the degree of loss varying according to the species.

An important point to consider in our results is the optimal pH range for the growth of the evaluated microorganisms, which could, to a certain extent, influence the results. For *P. aeruginosa*, the ideal growth pH is 7.5 to 8.0 (Charyulu and Gnanamani, 2010). For *Salmonella* sp., this range is between 6.5 and 7.5. However, these microorganisms can grow effectively at more acidic pHs (Chung and Goepfert, 1970). Several factors can interfere with this, even at ideal or adverse pHs, such as temperature, the type of acid present in the medium, and the ability of these strains to achieve pH homeostasis (Keerthirathne et al., 2016). *S. aureus* strains follow the same pattern as the previous strain, with an optimal range between 6.0 and 7.0 but an excellent development capacity between 4.0 and 9.0 (Andrade Júnior et al., 2019; Lyer et al. 2021). *S. agalactiae* also grows over a wide pH range from 3.0 to 11.0, with the optimum pH being 7.0 (Laith et al., 2017). On the other hand, *C. albicans*, according to a review by Rane et al. (2019), can survive in both highly acidic and extremely alkaline environments (pH 2-10), but the optimal conditions for the development of this culture are between pHs of 7.0 and 7.4 (Karam El-Din et al., 2012; Nadeem et al. . 2013). As seen, according to the literature, all microorganisms tested are capable of developing in a wide range of pH, and, even with the influence that this may have played, the results found here only reinforce the antimicrobial potential of WV, because even in the optimum pH range for the growth of microorganisms, WV demonstrated an efficient antimicrobial action for most strains, emphasizing its potential even when neutral. The optimal pH range can also help explain the fact that *S. aureus* and *S. agalactiae* were the most resistant strains at pH 6.0, 7.0, and 7.5 (their optimal growth pH), reinforcing the need to evaluate greater WV concentrations for these strains at this pH.

Another explanation for the more significant antimicrobial activity of EP at more acidic pHs, in addition to the optimal pH range, is precisely the presence of organic acids in its chemical composition. Its existence, as demonstrated, enhances antimicrobial action because these acids interact in different ways in microorganisms. In general, undissociated forms of organic acids, such as phenolic acids and others, can cross the cell membrane and acidify the cytoplasm, disrupting the membrane and causing leakage of essential cell constituents (Ecevit et al., 2022). This acidification happens by dissociating H+ ions from acids inside microbial cells. To reverse the situation, microorganisms activate proton pumps to rebalance the pH, generating a significant energy expenditure and further influencing cell death (Holyoak et al., 1996). Furthermore, according to Lund et al., (2020), organic acids can cause the collapse of proton gradients, generating cellular leakage.

4.2. Antimicrobial activity: behavior of the absorbances

On the right side of Figure 1, the different pH levels are identified by the same colors as the plotted curves. In Table 2, the regression models fitted to explain the effect of the pH of WV on each microorganism according to the concentration are displayed. The models can predict the absorbance (Y) behavior as a function of WV concentration (X). From the curves shown in Figure 1, it is possible to verify that at lower pH levels, the concentrations of WV required to achieve the antimicrobial effect were lower, corroborating the experimental data on MIC described previously. As the pH of WV increased, the requirement for higher concentrations of the product to inhibit microbial growth also increased, reflected by higher absorbance values when WV low concentrations were not effective.

Another trend that corroborates the behavior of the data displayed in Table 1 is that the absorbances at a pH of 7.0 were consistently higher than those at pH 7.5. That fact indicates that the statement that the antimicrobial properties are not solely related to the effect of acetic acid is correct (Suresh et al., 2019). The acetic acid in a given aqueous solution is wholly converted to sodium acetate at pH 7.0 and remains at alkaline pH levels. Consequently, there is no possibility of its being responsible for the antimicrobial effect of WV found in this work at both pHs of 7.0 and 7.5. Other authors found the same pattern cited previously.

Therefore, the pH of the WV undoubtedly influences its antimicrobial activity but does not cause it alone. The chemical composition of the WV reported in the literature shows a product composed of around 200 compounds (Schnitzer et al., 2015; Araújo et al. 2017; Pimenta et al. 2018). Among these components, alcohols, phenolic compounds, furans, ketones, organic acids, phenols, and pyrans are the most representative and abundant (Theapparat et al., 2018).

The phenolic compounds are the leading group with which antimicrobial properties are closely associated, a fact that is long proven by scientific reports (Abas et al., 2018). The mechanisms of action of these compounds are related to possible interactions with proteins and some sulfhydryl groups present in microbial enzymes, which may result in their inhibition (Mason and Wasserman, 1987; Aldulaimi, 2017). According to a review by Miklasińska-Majdanik et al. (2018), the presence of hydroxyl groups in phenolic compounds can result in binding affinity to proteins present in microbial cells, which can suppress the enzymes originated from them and increase affinity for cell membranes, enhancing the antimicrobial effect of the product used. In addition, the literature demonstrates several mechanisms of action that these compounds may present, such as the inhibition of efflux pumps of microorganisms (these pumps throw the antimicrobial agent out of the microbial cells, preventing their action), the interaction with some enzymes and with the cell membrane, in addition to the inhibition of cell wall synthesis (Khameneh et al., 2019). Therefore, this may explain the permanence of the antimicrobial action of WV even at neutral pH.

Also, Suresh et al. (2019) highlighted that fact and mentioned that each WV component has a different mode of action. Because of this, according to the authors, WV is even more interesting for use as an antimicrobial agent since it is unlikely that the microorganisms will develop any resistance mechanism against all the product components simultaneously. In the present work, the WV's pH and the product's concentration vary regarding a microorganism. Thus, regression models were fitted for each microorganism, one for each pH level, where the dependent variable is the absorbance, and the independent one is the WV concentration. This way, for each microorganism subjected to the action of WV, the models fitted for each pH could be compared to detect differences in the antimicrobial activity depending on pH. The importance of this comparison is not only to determine the effectiveness of the WV itself at each pH level but also to provide information about the interaction between the microorganism and the WV at a given pH when the concentration is varied.

According to the results of the model identity test, the quality of WV changed as pH increased since the comparison among the models was different from one pH to another. In other words, for each microorganism, the pH variation could result in a different effect of WV and, concomitantly, a specific type of interaction. Something in the WV was decreasing and weakening, so as the pH of the WV increased, higher concentrations of the product were required to continue inhibiting the growth of the five microorganisms in the culture medium. This pattern

corroborates the results of Setiawati et al. (2019), cited previously, who observed that changes in the composition occur at different pH levels. A slightly different pattern was observed for *C. albicans*, where the identity test, when applied to the models fitted for this microorganism, determined that the effect of WV at pH of 3.0 and 3.5 was equal to that at pH 4.0, probably meaning the presence of an interaction of the inhibitory product with this microorganism. Nevertheless, for all other pH levels, the regression models were different from each other.

As commented by Pimenta et al. (2018), the partial loss of the antimicrobial activity can probably be attributed to the reaction of the sodium hydroxide with the phenolic compounds, turning them into salts, a type of chemical change that deactivates their hydroxyl groups, which are responsible for the antiseptic properties presented by most of them in the pristine form. Phenols are acids with pKa around 10.0, so they are weak acids. There are at least 20 types of phenols in WV's chemical composition (Araújo et al., 2017; Pimenta et al., 2018), and since each compound is different, as the pH increases, their hydroxyl groups are not equally neutralized because of the substituent groups that are present in the aromatic ring. This way, as pH increases, different chemical species are most likely generated in aqueous media, ones more available than others to exert an antimicrobial effect.

The results of Setiawati et al. (2019) and the results of the present work corroborate the points raised in the previous paragraph. When evaluating neutralized WV, the cited authors found some change in the percentage of phenolic compounds in the chemical composition of the product obtained from durian wood. According to them, in the acidic version, the main compound was guaiacol, while in the neutralized product, pyrocatechol was the prevailing substance. The explanation presented by the authors was that in the neutralized form, alkyl groups in the para position (carbon 4) of phenolics accept electrons, and that behavior decreases the ionization of the compounds due to the addition of NaOH in WV. The changes in the proportion of phenolic compounds in the neutral version of WV explain why the product becomes less effective than the acidic versions in terms of the power to inhibit the growth of microorganisms. These results were expected to a certain extent because, according to Brown et al. (1997), acid and basic solutions can differ significantly in their chemical properties, so products obtained from the reaction after neutralization do not have the same characteristics as the original solution. However, since WV is a solution containing many kinds of compounds, even with its acid fraction completely neutralized, other compounds still preserve the product's bioactive characteristic.

Further research should be performed to understand the specific chemical species of WV that prevail as inhibitors at each pH level when increasing neutralization. This could predict the product's behavior when employed as a natural antibiotic in varied applications. For example, suppose the product is used as an additive for animal feed. In that case, the WV, after being swallowed, will find strong acidic conditions in the digestive tract of poultry and swine. In this condition, the inhibitory power of WV on microorganisms is maximized. However, suppose the intention is to use the product to compose drug formulations for external uses such as ointments and creams or to deter parasites like ticks. In that case, the importance of the pH in the final benefit may be essential to maximize the action of the product.

5. CONCLUSIONS

The eucalyptus WV maintained its antimicrobial effect even at neutral and slightly alkaline pH, which runs counter to the claims in other studies that the inhibitory action of the product versus microorganisms is related just to the presence of acetic acid in its chemical composition. This is clear with the inhibition of *P. aeruginosa*, *S. enteritidis*, and *C. albicans*, even at pH 7.0. The data obtained here revealed that, with the increase in pH, there was a decrease in WV efficiency, requiring higher concentrations of it to result in inhibition. However, there was no complete cancellation of its antimicrobial activity, reaffirming the union of a set of substances acting to generate this action, refuting data that attribute its antimicrobial potential only to its acidic portion.

Our experimental data indicate the potential of the possible antibacterial and antifungal use of WV. However, it is essential to highlight that all the experiments reported here were conducted *in vitro*, so further research should investigate the behavior of WV under in vivo conditions. Another important aspect is the degree of purification of the WV for such

applications. The model identity test was a valuable tool to detect different responses of microorganisms at each pH level, validating the attribution of the bioactive action of WV to the set of components as a whole and not only to a single compound.

AUTHOR CONTRIBUTIONS

GSP Gama conducted the main experiments and elaborated the manuscript draft in Portuguese; AS Pimenta raised funds, supervised all the research steps, interpreted the statistical analyses, revised and translated the manuscript to English; FMC Feijó provided the reagents and all the infrastructure to conduct the microbiological assays, revised the manuscript draft; CS dos Santos assisted to the microbiological assays; RVO Castro performed the statistical analyses; TKB Azevedo assisted to the fundraising and revised the manuscript draft; LCD de Medeiros established the routine to adjust the pH of wood vinegar samples. An American native revised the final version of the manuscript.

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