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Antimicrobial Activity of the Methanolic Fraction of Bamboo Pyroligneous Liquor

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Abstract: Considering that the pyroligneous liquor obtained from pyrolysis of various types of biomass has antiviral activity, the main objective of the present study was to evaluate the antimicrobial action of the methanolic fraction extracted from bamboo (*Dendrocalamus asper*) pyroligneous liquor in relation to *Escherichia coli* and *Staphylococcus aureus* bacteria. The pyroligneous liquor was recovered by laboratorial bamboo pyrolysis at 350 °C and then fractioned by liquid-liquid separation with methanol and hexane. After solvent evaporation, we collected the methanolic extract, which was later diluted in methanol and used in the diffusion disc assay of *E. coli* and *S. aureus* at concentrations of 0.125, 0.500, 0.750 and 1.000 mg. Through GC/MS analysis, it was possible to qualitatively identify 92 chemical compounds in the methanolic fraction. Treatment with the methanolic fraction inhibited cellular growth and caused a variety of morphological variations in size as well as deformities in the bacterial cell walls, establishing an antimicrobial activity profile. This finding supports the National Policy for Incentives for Sustainable Management and Cultivation of Bamboo (PNMCB) and has benefits to the society and environment as a whole, as it demonstrates the possibility of adding value to a byproduct of pyrolysis.

Key words: Dendrocalamus asper, Escherichia coli, Staphylococcus aureus.

1. Introduction

The overuse of antibiotics increases bacterial resistance, promoting an increase in infections, especially in the hospital context. Bacteria such as *Escherichia coli* and *Staphylococcus aureus* represent pathogenicity, and studies of new chemical compounds with antimicrobial activity are imperative for community health. Considering the action profile of these compounds as potential preservatives makes possible their application in the food, cosmetic, and chemical industries, among others [1-3]. For this reason, the exploration of undiscovered treatment possibilities is needed.

Crude pyroligneous liquor is a source of chemical compounds with a diversity of applications, mostly as liquid smoke. It is characterized as a brown liquid containing two distinct fractions: a water-soluble substance which may contain more than 200 chemical compounds, and a lipid-soluble substance containing tar [4–7].

Dendrocalamus asper (Schult & Schult) Backer ex. K. Heyneke is an important bamboo species in Brazil that is commonly misidentified as *Dendrocalamus giganteus* because both species are a kind of giant bamboo with large culm diameters [8, 9].

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In Brazil, various programs exist to research and develop the use of bamboo fibers for home building, either alone or as reinforcement in different types of matrices, such as soil and cement composites [9-11]. These kinds of incentives were established by Law 12,484/2011, which established the National Policy for Incentives for Sustainable Management and Cultivation of Bamboo [12], aiming to promote the development of bamboo culture in Brazil as well to incentivize research on this non-timber product of lignocellulosic constitution as a source of biomass for charcoal production and other diverse applications.

Of major interest is the use of bamboo as an alternative raw material to produce charcoal, torrefied biomass, and activated carbon, in contrast to the relative disinterest in other products of pyrolysis [13] such as pyroligneous liquor (PL).

Several properties of bamboo leaves and stem are already recognized [14, 15]. A study conducted on methanolic extracts of *D. asper* leaves and pathogenic *E. coli* demonstrated its potential antimicrobial activity [16].

The antimicrobial activity of chemical composites obtained from pyroligneous liquor is poorly studied; however, a comparative study of different lignocellulosic biomasses, including bamboo, its pyroligneous liquor showed antiviral activity [17].

Variables such as lignocellulosic material, temperature used to obtain crude pyroligneous liquor, chemical treatment, and pharmacological activation of the PL are even less well-explored.

The aim of this original study was to determine the antimicrobial activity toward two different pathogenic bacteria of the methanolic fraction obtained at 350 °C of the pyroligneous liquor from *D. asper*, a low-value byproduct obtained in the production of charcoal via pyrolysis.

2. Materials and Methods

2.1 Obtaining the Timber Material

D. asper was collected at the Research and Development Unit of the Agronomic Institute of Campinas, located in the city of Tatuí, State of São Paulo. Culms 3.0 m long were cut from bamboo clumps older than three years and transported to the Department of Forest Sciences facilities. The culms were ripped into small pieces, milled in a Wiley mill (mesh 0.85), and oven-dried to approximately 0% moisture content.

2.2 Preparation of the Methanolic Fraction of the Pyroligneous Liquor of D. asper

The milled and dried material was submitted to a pyrolysis process at a controlled temperature of 350 °C, and pyroligneous liquor was obtained by condensation of the volatile gases within 4 hours of reaching the desired pyrolytic temperature.

The pyroligneous liquor was fractioned by chemical liquid-liquid separation, utilizing methanol and hexane [20:100 mL (3 times)] as organic solvents (Merck, Sigma-Aldrich). Solvents were evaporated at 40 °C in a rotary evaporator, and the extracted fractions were stored at 4 °C.

The obtained pyroligneous methanolic fraction (PMF) was a dark brown liquid, with high viscosity and the characteristic smelt of liquid smoke; and highly soluble in methanol.

2.3 GC/MS Analysis

This methanolic fraction was analyzed by GC/MS quadrupole mass spectrometer with linear type model 5975C (Agilent) fitted with an injector at 280 °C, column 30 m long × 0.25 mm i.d., and 0.25 μm film thickness (Stabilwax). The GC oven temperature program was held at 62 °C for 6 min, and then increased to 110 °C at a rate of 10 °C/min and finally to 215 °C at a rate of 3 °C/min for 15 min. The other GC/MS parameters employed were an injection and ion source temperature of 280 °C, helium carrier gas at 1.0 mL/min, an injection volume of 1.0 μL, a split ratio of 50:1, ion source energy of 70 eV, and a mass range of m/z 33–550 Da.

For identification of the compounds detected in the chromatograms, the NIST11 mass spectral database and the Automated Mass Spectral Deconvolution and Identification System (AMDIS) programs were used.

2.4 Diffusion Disc Assay

Diffusion disc assay is accepted by the ANVISA (National Agency of Sanitary Vigilance), FDA (Food and Drug Administration), and NCCLS (National Committee for Clinical Laboratory Standards) [18–20]. All materials used in the tests were autoclaved at 110 °C for 20 min. Two strains *Escherichia coli* (ATCC 23282) and *Staphylococcus aureus* (ATCC 35696) were used for research in Applied Microbiology Laboratory.

First, the bacteria were defrosted from -80 °C, and a volume of 50 µL at room temperature was inoculated in liquid Luria-Bertani (LB) culture medium at 37 °C for 24 hours in a shaking incubator. Simultaneously, the same LB medium, now containing agar, was poured into Petri dishes (15 cm diameter) inside the laminar flow chamber. Next, the plates were incubated in a chemical oxygen demand incubator (ODI) at 37 °C for 24 hours. After the incubation period, 100 µL of each bacterium was inoculated by scattering in the Petri dishes, remaining in residence for 15 min in the ODI for adhesion and growth of the bacteria.

2.4.1 Application of Samples

For the diffusion disc assay, a standardized paper disc (6.0 mm diameter) impregnated with 0.01 mg of ampicillin (Cefar Diagnóstica Ltda), produced according to the parameters established by the Clinical & Laboratorial Standards Institute (CLSI), was used as a positive control; and methanol was used as a negative control.

Samples of methanolic fraction were diluted and homogenized in methanol at four different concentrations (0.25, 1.0, 1.5 and 2.0 mg/µL). From each one solution, 0.5 µL was added with a volumetric pipette onto paper discs (6.0 mm diameter) inside the laminar flow chamber. Discs samples of PMF and negative control with methanol were submitted to an evaporation process to eliminate the solvent.

After the solvent evaporation, the disc samples impregnated with concentrations of 0.25, 1.0, 1.5 and 2.0 mg/µL give a PMF content of 0.125, 0.500, 0.750 and 1.000 mg per disc, respectively.

Then, the discs were inoculated onto the Petri dishes, previously prepared as described above, and then incubated in an ODI for 24 hours.

Absence of bacteria radial growth around the paper discs was measured using two perpendicular measurements of the inhibition halo diameter with an electronic pachymeter (Mitutoyo).

2.5 Scanning Electron Microscopy Imaging

A sample of 0.25 mg/µL (0.125 mg) collected at the end of the disk diffusion assay was infiltrated in Karnovisky fixative and stored at 4 °C. The cell pellet of each bacteria to be analyzed was homogenized in the Karnovisky fixative and 5 µL was added to a glass grid covered with ε-poly-l-lysine for 15 min, followed by an ethanol dehydration sequence in series concentrations [30, 50, 70, 90, and 100% (3 times)] for 10 min at each stage. Soon after, they were dried to the critical point using liquid CO_2, glued to stubs, and metalized with gold. The samples were then analyzed by scanning electron microscopy (SEM, model LEO 435VP, Zeiss, Germany) to produce images saved in TIFF format [20].

2.6 Statistical Analyses

The statistical delineation adopted was a factorial 2 × 5 (bacteria × concentration) design with 4 replications. ANOVA was applied to the total results and the Tukey test was used to compare means. The relationship between inhibition halo diameter and PMF concentration was analyzed by linear regression.

3. Results

The GC/MS results were expressed according to the

following parameters: retention time (t_R) of each compound in the chromatogram, expressed in minutes; percentage of normalized area (A%), which indicates the relative distribution of the compounds; and qualitative analysis (QA, with score ranging from 0 to 100), which is the mass spectra database that reflects the similarity of the mass spectrum obtained with those registered in the used libraries, and considering as a limit for compound identification a score equal or greater than 70.

Through GC/MS analysis it was possible to qualitatively identify 92 chemical compounds in the methanolic fraction of the pyroligneous liquor obtained at 350 °C, as can be observed in the obtained chromatogram (Fig. 1). The phenols and derivative compounds present in the methanolic fraction are related in Table 1.

A concentration of 0.25 mg/μL (0.125 mg) was initially used in a comparative screening of the PL methanol and hexane fractions at 350 °C, in the disk diffusion test, to assess possible antimicrobial activity. The results allowed us to select the most suitable fractionation and to determine the concentrations to be subsequently evaluated.

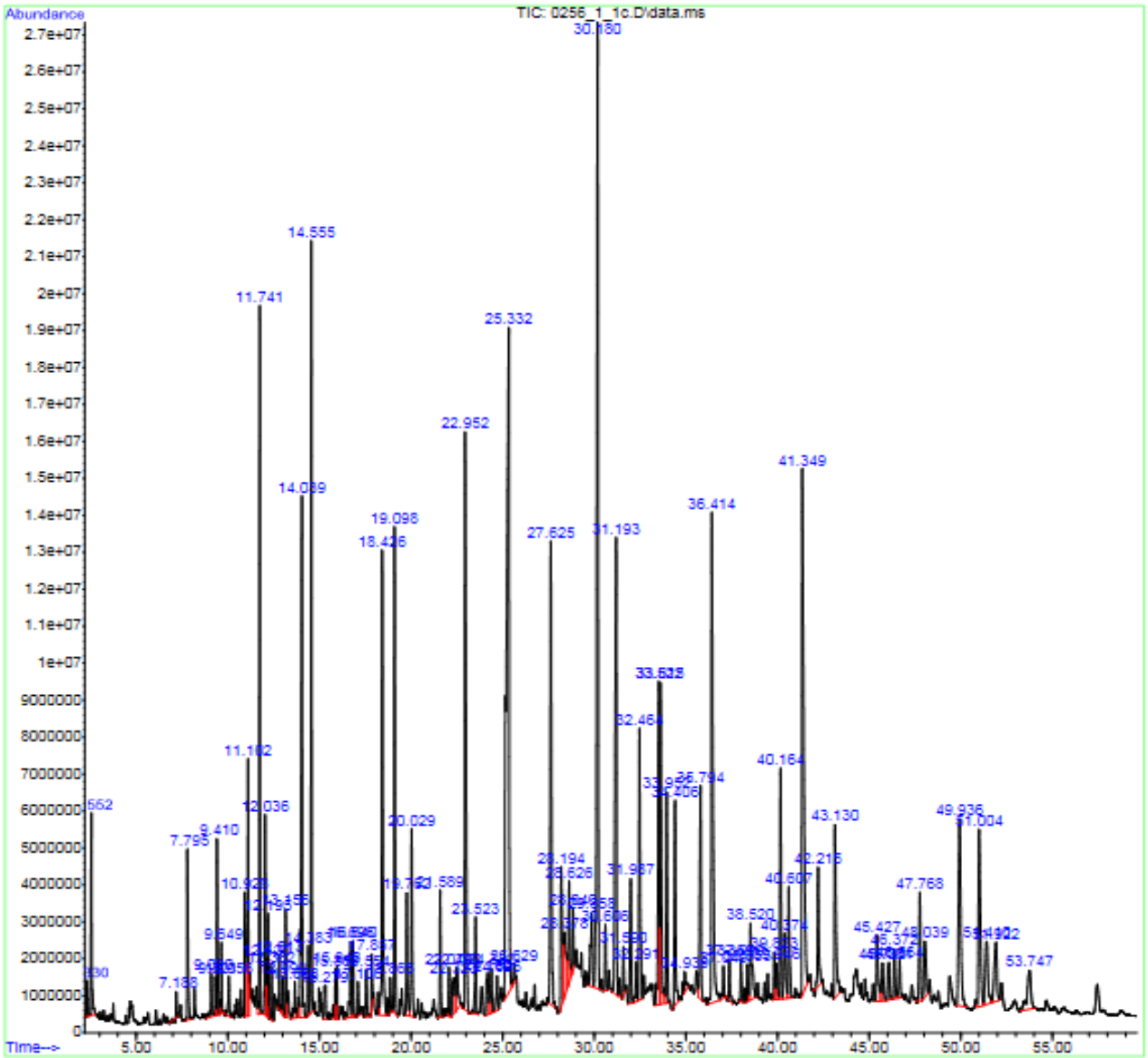

Fig. 1 Chromatogram of chemical compounds identified through GC/MS analysis.

Table 1 The phenols and derivatives of chemical composts found in the GC/MS of the methanolic fraction of pyroligneous liquor of *Dendrocalamus asper* obtained at 350 °C.

Phenolic compost and derivatives	T_r (min)	A%	QA
Methoxyphenol (guaiacol)	19.1	2.4	97
Creosol	21.588	0.66	96
Maltol	22.323	0.45	94
Phenol	22.952	3.85	95
Dimethoxyphenol	30.179	6.81	97
Dimethoxy-4-propenylphenol	37.367	0.26	97
Vanillin	38.001	0.28	97
Catechol	41.348	5.16	95

t_R: retention time; A%: percentage of normalized area; QA: Score of qualitative analysis.

The results of the diffusion disk assay, which are presented in Table 2, corroborate the antimicrobial effect of the PMF on the growth of *E. coli* and *S. aureus*. These results clearly demonstrate that the effects of the methanolic fraction at different concentrations, as well as the ampicillin effect, are similar for both bacteria (*E. coli* and *S. aureus*).

In relation to *E. coli*, statistical analysis showed that PMF at 0.125, 0.500 and 0.750 mg has similar antimicrobial action, and that the same interpretation can be applied to concentrations of 0.500, 0.750 and 1.000 mg. Only the higher PMF dosage, 1.000 mg, is statistically equivalent to ampicillin.

The results obtained with *S. aureus* are very similar. Methanolic extract at 0.125 and 0.500 mg showed the same effect, according to the Tukey test; and dosages of 0.500 and 0.750 mg, as well dosages of 0.750 and 1.000 mg, were equivalent.

The statistical equivalence between PMF dosage of 1.000 mg and ampicillin dosage of 0.010 mg in relation to *E. coli* was not observed on *S. aureus*.

The values from Table 2 also make evident a direct relationship between methanolic extract concentration and inhibition halo diameter, which is analyzed in Fig. 2. The linear regression analysis showed that for both bacteria, the inhibition halo diameter increased mainly as a response to the increase in concentration, and the linear equation that expresses this relationship has a coefficient of determination (R^2) greater than 0.75 ($p < 0.01$). This result can be interpreted as evidence of the microbial action of the methanolic extract.

Samples collected at the edges of the inhibition halo initially used in the screening, 0.25 mg/μL (0.125 mg), were analyzed by SEM, verifying the morphological differences between the control (untreated) bacteria and the bacteria submitted to treatment with the methanolic fraction, as demonstrated in Figs. 3 and 4. To make these comparisons, it is necessary to know the normal morphological patterns of the bacteria, in which they manifest their pathogenicity.

In Fig. 3, it is possible to analyze SEM images of *S. aureus*. The bacterial control shows cells that were not subjected to any treatment, which appear in the shape of a coccus with regular morphology, standard diameter, and regular grouping (Fig. 3A). After treatment with the methanolic fraction, the cells are irregular in size and cell walls present alterations as rugosity, mostly without the spherical shape; some with a visible section in the axial axis. The presence of an extracellular substance can be noted (Fig. 3B). Those differences were not observed in the control bacteria. Differences between control and treated bacterial cells may be indicative of cell growth inhibition, thus configuring an antimicrobial activity profile.

In Fig. 4, it is possible to analyze SEM images of *E. coli*. In the bacterial control, cells that were not subjected to any treatment have regular morphology and rod shape, with standard diameter and regular grouping (Fig. 4A).

After treatment with the PMF, the bacteria did not present the standard morphological conformation.

Table 2 Mean diameter (mm) of the inhibition halo (absence of bacteria radial growth) due to the presence of the methanolic fraction and ampicillin.

Bacteria	Dosage (mg)				
	Pyroligneous Methanolic Extract				Ampicillin
	0.125	0.500	0.750	1.000	0.010
E. coli	$11.66^{A,a}$	$13.42^{A,a,b}$	$14.28^{A,a,b}$	$16.73^{A,b,c}$	$19.91^{A,c}$
S. aureus	$10.77^{A,a}$	$12.74^{A,a,b}$	$14.91^{A,b,c}$	$16.95^{A,c}$	$21.69^{A,d}$

Means with the same capital letter in the vertical direction do not differ among itself, Tukey test, $p < 0.05$.
Means with same small letter in horizontal direction do not differ among itself, Tukey test, $p < 0.05$.
ANOVA (5, 10) = 58.98, $p < 0.01$.

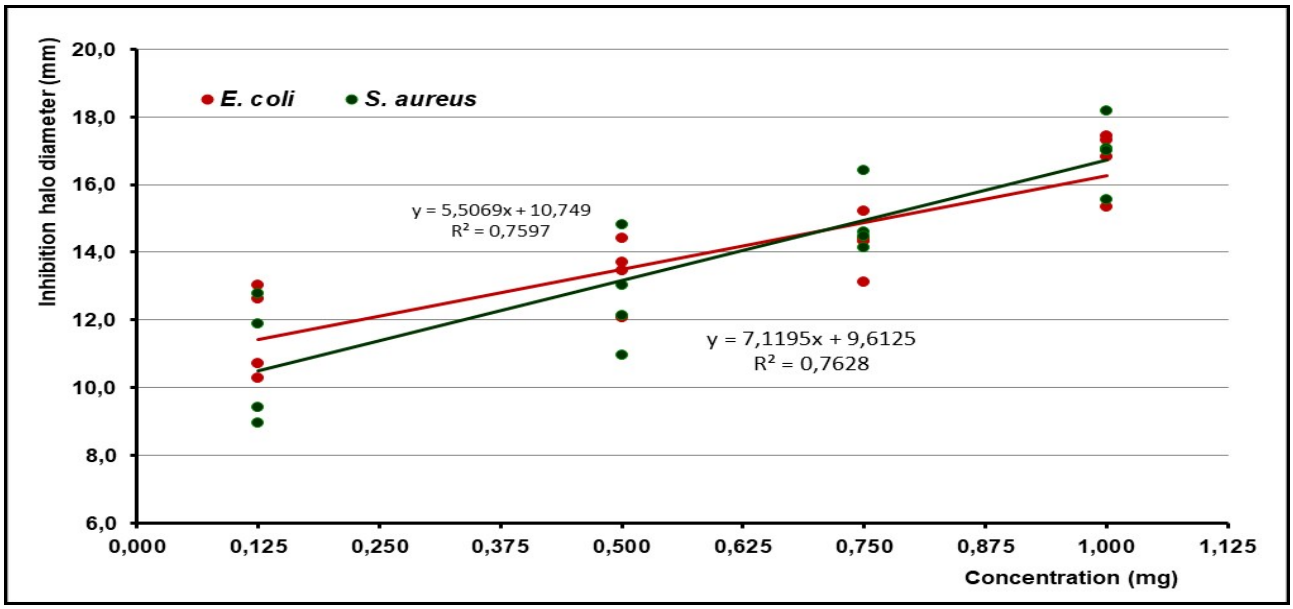

Fig. 2 Linear regression analysis applied to the relationship between methanolic extract concentration and inhibition halo diameter.

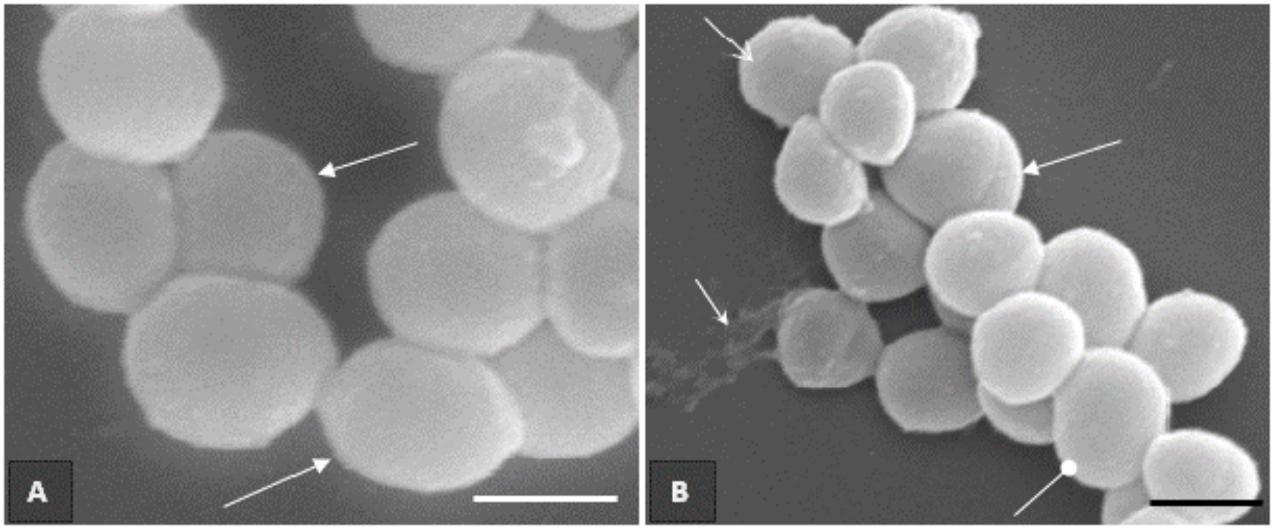

Fig. 3 Scanning electron microscopy: *Staphylococcus aureus*.

3A: Bacteria without treatment; cocci show spherical form and normal sizes (arrows). 3B Bacteria submitted to treatment with the methanolic fraction, with oval coccus morphology (arrow in ellipse); a secessional line in a cell of abnormal size and form (arrow); rugosity in the coccus cellular wall (open arrow); and an extracellular substance (furtive arrow). (Bars = 0.5 μm).

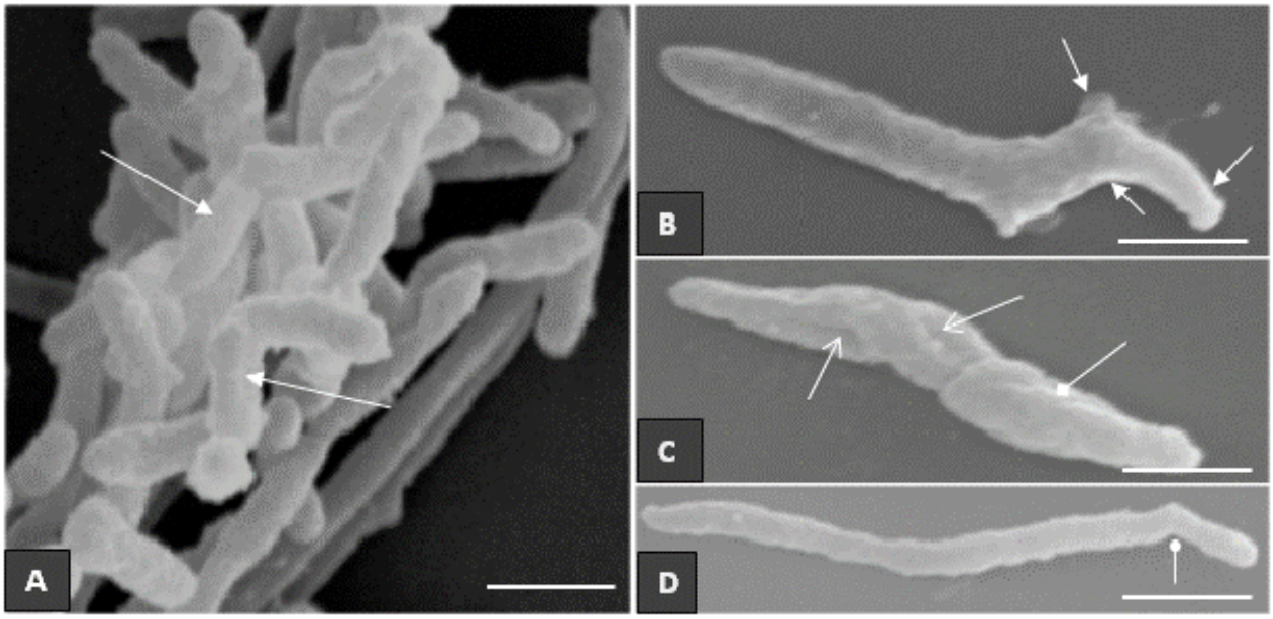

Fig. 4 Scanning electron microscopy: *Escherichia coli.*

4A: Bacteria without treatment showing rod form and normal size (arrow). 4B, 4C, and 4D: Bacteria submitted to treatment with the methanolic fraction. 3B: Deformations in the bacterium, whose normal rod morphology is modified, showing curves (arrow). 4C: Presence of longitudinal sections in the bacterium as well as rugosity in the total extension of the cellular wall (open arrow). 4D: Bending in the final prolongation of the bacterium (arrow in ellipse). (Bars = 1.0 μm).

Bacterial cells, after treatment, exhibited a variety of morphological variations in size and deformities in the cell wall (Figs. 4B-4D), confirming the antimicrobial activity profile.

4. Discussion

Bamboo species are classified as grasses, having many lignocellulosic similarities with wood, except for the alkaline extracts, ashes, and silica, which are present in greater quantity in the bamboos. Its chemical composition has as its main components cellulose (55%) and lignin (25%), and it also contains hemicellulose, resins, tannic acid, waxes, and organic acids [21, 22].

Differences in chemical composition of the lignocellulosic material and pyrolysis temperature have qualitative and quantitative effects on the chemical composition of the pyroligneous liquor.

Biological activity studies are performed with crude PL, indifferent to lignocellulosic material [16, 17, 27, 28], or temperature. The temperature at 350 °C shows an exothermic process, the composition of the condensable gases containing hundreds of organic (some recoverable) chemical components [29].

The liquid-liquid fractionation was performed with the organic solvents methanol and hexane. Methanol is a polar solvent, which extracts by affinity compounds with more polar or medium polarity characteristics from crude PL. Hexane, due to its nonpolar characteristics, extracts compounds of similar polarity. Using this fractionation, the most toxic compounds, such as tar and polycyclic aromatic hydrocarbons (PAH), are excluded from the methanolic fraction. For this reason, the methanolic fraction was selected for the proposed tests.

The chromatogram in Fig. 1 shows all 92 chemical compounds are identified through absorbance of the methanolic fraction; including phenolic compounds, ketones, aldehydes and lactones, among others. However, the compounds with greater focus in the literature are the phenolic.

Previous studies on crude bamboo PL have focused on phenolic compounds. Most phenolic compounds present an aromatic ring and a short carbon chain,

including guaiacol, vanillin, and apocin, among others, whose antimicrobial activities and employment by food companies are widely known [14, 22–26]. Table 1 highlights the phenolic compounds whose qualitative identification by MS are comparable to those ones described by other authors.

Many of the phenolic compounds present in the PMF show pharmacological activity in diverse organic systems; however, other chemical compounds may be related to antimicrobial activity, as shown in Table 2. In a fraction of such chemical complexity, it is impossible to assign this activity to a single chemical compound.

The images in Figs. 3 and 4 from SEM evidence the alterations found in *E. coli* and *S. aureus*, untreated and with treatment. Therefore, to know the normal morphological characteristics of each bacterium, which configure them as pathogenic, allowed comparing and describing the differences discovered in this analysis.

S. aureus is a Gram and catalase-positive bacterium whose size varies from 0.5 to 1.5 μm in diameter. It is unmoving, not encapsulated, and presents itself with diverse forms: isolated, in pairs, in short chains, and in irregular arrangements. In the healthy human organism, it is found in the skin and nasal cavities and can cause infections such as spines, furuncles, cellulites, pneumonia, meningitis, endocarditis, septicemia, and syndrome of septic shock, among others [30].

E. coli it is a Gram-negative bacterium of the rod type, not sporulated, with a facultative anaerobic metabolism, whose mobility is allowed by the presence of flagella. Its dimensions are 1.1 to 1.5 μm by 2.0 to 6.0 μm is a bacterium present in the intestinal flora of humans and animals and does not present risks to healthy individuals. However, when pathogenic as a result of ingestion or immune system disequilibrium, it may cause diseases of the gastrointestinal and urinary systems [31].

Studies with antimicrobial agents, of natural or synthetic origin [32-34], do not present the same alterations discovery with the treatment of the PMF at 350 °C, as show in Figs. 3 and 4. The inhibition of cell growth was observed in both bacteria submitted to treatment. Although it was not possible to determine whether this activity was bactericidal or bacteriostatic, the dose/response relationship presented in Fig. 2 proved a linear relationship between dose and the antimicrobial effect, for both bacteria tested.

This antimicrobial activity is important because of hospital infections related to these two bacteria, especially in intensive therapy units. The etiology of sepsis in children and adolescents is related to bacterial pathogenicity and can cause death [35].

Along with hospital infections, other items such as personal hygiene products, cosmetics, perfumes, and foods can also cause bacterial pathogenicity in humans. In Brazil, according to ANVISA Resolution no. 79, those items should incorporate a preservative, which is defined as "a chemical compound, of natural or synthetic origin, that acts as a preservative system, whose intention is to guarantee the integrity of the individuals as well as of products" [36].

In the Clinical & Laboratory Standards Institute (CLSI) MD100 ED28: 2018 guidelines, there are categories of definitions to interpret the breakpoint of inhibition halo diameter values (mm) in order to categorize an organism as sensitive (inhibition of halo ≥ 20 mm), intermediate (inhibition of halo 15–19 mm), or resistant (inhibition of halo ≤ 14 mm) [36].

If an antimicrobial compound is categorized as sensitive, the infection caused by the bacteria can be properly treated with the antimicrobial agent at the dose used in clinical therapy; the intermediary category corresponds to infections caused by the bacterium that can be treated appropriately in places of the body that physiologically allow the accumulation of the antimicrobial used in treatment, or for which a greater dose can be used in clinical therapy; the resistant category corresponds to infections in which the action of the antimicrobial agent, at its habitual dose and

Table 3 Categories definitions to interpret the breakpoint of inhibition halo diameter values (mm) of PMF and ampicillin in bacteria *Escherichia coli* and *Staphylococcus aureus.*

Treatment	Dosage (mg)	Mean values of the inhibition halo diameter (mm)					
		Escherichia coli			*Staphylococcus aureus*		
		S*	I**	R***	S*	I**	R***
PMF (Pyroligneous methanolic fraction)	0.125	-	-	11.66	-	-	10.77
	0.500	-	-	13.42	-	-	12.74
	0.750	-	-	14.28	-	-	14.91
	1.000	-	16.73	-	-	16.95	-
Ampicillin	0.010	-	19.91	-	21.69	-	-

* Sensitive; ** Intermediate; *** Resistant.

frequency, does not inhibit the manifestation of the bacterial infection, thus allowing it to be associated clinically with mechanisms of microbial resistance [37].

Analysis of Table 3 shows that, for both bacteria, dosage of 0.125, 5.000 and 7.500 mg could be categorized as resistant. For *E. coli*, the dosage of 1.000 mg and ampicillin are categorized as intermediate. For *S. aureus*, the dosage of 1.000 mg is intermediate and ampicillin is considered sensitive.

Since the breakpoint category of inhibition halos is based on pharmacological and clinical data sets obtained from *in vitro* and *in vivo* evaluations, they are considered robust predictors of probable clinical outcomes [33].

It should be pointed that the dosage of PMF with effect equivalent to ampicillin is 100 times greater, respectively 1.00 mg and 0.01 mg. At first sight this may seem an unfavorable result, but it is necessary to consider that the PMF is an extract and ampicillin is a well known synthetized antibiotic.

Even in higher dosages when compared to ampicillin, the antimicrobial action of PMF allows its use as a preservative in the food, cosmetic, and chemical industries; adding value to a byproduct of bamboo pyrolysis.

The correlation of chemical composition with SEM analyses and data from the inhibition halo test confirm that the methanolic fraction of pyroligneous liquor recovered from bamboo pyrolysis at 350°C demonstrates potential as an antimicrobial agent for *S. aureus* and *E. coli*.

6. Conclusions

The unpublished results obtained from the methanolic fraction of *D. asper* pyroligneous liquor obtained at 350 °C demonstrate complexity in its chemical composition, with emphasis on the phenolic compounds. A dose-response relationship was found in the four different concentrations evaluated, confirming antimicrobial activity. Through SEM analysis, it was possible to verify morphological differences between the controls and treated *S. aureus* and *E. coli*. This finding supports the National Policy for Incentives for Sustainable Management and Cultivation of Bamboo (PNMCB), and has benefits to society and to the environment as a whole because it demonstrates the possibility of adding value to a byproduct of pyrolysis.

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