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In vitro evaluation of antibacterial activity of phytobiotics against pathogenic bacteria in continental aquaculture

Avaliação *in vitro* da atividade antibacteriana de fitobióticos contra bactérias patogênicas na piscicultura continental

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Abstract: The aim of this study was to evaluate the phytobiotic potential of the oils from *Copaifera langsdorffii* (copaiba), *Carapa guianensis* (andiroba), *Attalea speciosa* (babassu), *Mauritia flexuosa* (buriti), and *Caryocar brasiliense* (pequi), as well as two types of aqueous extracts from *Terminalia catappa* (tropical almond), as alternatives to antibiotics containing enrofloxacin or oxytetracycline as active ingredients in continental aquaculture. Five species-specific pathogens with high prevalence and dissemination in continental fish farming systems were selected for the study. The virulence potential of the strains was assessed using Gram staining, catalase, and hemolytic activity tests, followed by inhibition halo assays to evaluate the phytobiotic potential. All selected strains exhibited *in vitro* virulence activity and were subjected to inhibition evaluations, where the inhibitory zones of the tested products were measured, along with their bactericidal or bacteriostatic effects. Among the evaluated products, only *A. speciosa* did not exhibit an inhibitory halo against the analyzed pathogens. Conversely, the oils from *C. brasiliense*, *M. flexuosa*, *C. guianensis*, *C. langsdorffii*, and the hot extract of *T. catappa* demonstrated bactericidal effects, yielding superior results ($P > 0.05$) compared to the positive control with oxytetracycline. The oils from *M. flexuosa* and *C. langsdorffii* proved to be competitive *in vitro* compared to antibiotics containing enrofloxacin or oxytetracycline as active ingredients, showing antibacterial action against *Aeromonas hydrophila*, *A. caviae*, *A. jandaei*, and *Streptococcus agalactiae*.

Keywords: antibiogram; extract; natural oil; pathogen.

Resumo: O objetivo dessa pesquisa foi avaliar o potencial fitobiótico dos óleos de *Copaifera langsdorffii* (copaíba), *Carapa guianensis* (andiroba), *Attalea speciosa* (babaçu), *Mauritia flexuosa* (buriti) e *Caryocar brasiliense* (pequi), além de dois tipos de extrato aquoso de *Terminalia catappa* (amendoeira), como alternativas ao uso de antibióticos com princípios ativos em enrofloxacino ou oxitetraciclina na piscicultura continental. Para isso, foram selecionados cinco patógenos espécie-específicos de elevada ocorrência e disseminação em sistemas de produção piscícola continental. O potencial de virulência das cepas foi avaliado por meio de testes de Gram, catalase

e atividade hemolítica, seguido de teste de halo de inibição para os potenciais fitobióticos. Todas as cepas selecionadas apresentaram atividade de virulência *in vitro* e prosseguiram para a avaliação de inibição, na qual foram mensuradas as zonas inibitórias dos produtos testados, além de analisar seus efeitos bactericida ou bacteriostáticos. Dos produtos avaliados, apenas *A. speciosa* não apresentou halo inibitório frente aos patógenos analisados, já os óleos de *C. brasiliense*, *M. flexuosa*, *C. guianensis*, *C. langsdorffii* e o extrato a quente de *T. catappa* apresentaram efeito bactericida, com resultados superiores (P > 0,05) em comparação ao controle positivo com oxitetraciclina. Os óleos de *M. flexuosa* e *C. langsdorffii* mostraram-se competitivos *in vitro* em comparação ao uso dos antibióticos com princípio ativo de enrofloxacino ou oxitetraciclina, demonstrando ação antibactericida contra *Aeromonas hydrophila, A. cavie, A. jandaei* e *Streptococcus agalactiae*.

Palavras-chave: antibiograma; extrato; óleo natural; patógeno.

1. Introduction

Aquaculture has emerged as one of the main agricultural activities in recent years, playing a crucial role in animal protein supply. Accordingly, global fish production has increased by 49%, with an additional expected growth of 53% by 2030. This scenario plays a vital role in food and nutritional security for the 21st century[1]. In Brazil, despite a rise to 860,355 tons of fish from continental aquaculture in 2022[2], the intensification of aquaculture practices has made animals more susceptible to diseases due to stressful environmental conditions that compromise the balance of the pathogen-environment-host triad. This imbalance stems from flawed breeding techniques such as inappropriate stocking density, unbalanced diets, changes in water quality, climate changes, super-intensive production systems with water reuse, and indiscriminate use of antibiotics and antimicrobials[3,4].

Despite the recommended use of oxytetracycline and florfenicol in national aquaculture[5], there is a frequent and indiscriminate rise in the use of antibiotics, regardless of the production system employed. This practice has led to an emerging control situation marked by a lack of knowledge about the infectious agents, proper dosage, and duration of treatment. Consequently, there is an elevated risk of developing antimicrobial resistance, the spread of virulence genes, and the accumulation of chemical substances in fish, the environment, and human health[6]. In fish production, bacterial diseases caused by the genus *Aeromonas* (such as *A. hydrophila*, *A. veronii*, *A. jandaei*, *A. caviae*, *A. sobria*, *A. bestiarum*, *A. dhakensis*, *A. schubertii*), as well as strains of *Streptococcus agalactiae* and *Micrococcus luteus*, have been responsible for disease outbreaks with mortality rates that can vary from 50 to 100% of the batch[7,8,9]. This phenomenon is mainly attributed to production intensification, characterized by high stocking density, improper management, and bacterial resistance[3,9].

In this context, as a safe and alternative measure to the use of antibiotics in aquaculture, phytobiotics may serve as a promising strategy in the prophylaxis and treatment of bacterial diseases through the action of their plant bioactive compounds, environmental safety in residual biodegradation, and synergistic contributions to animal production gain indices and immunomodulatory responses of the target species produced[10,11,12].

Examples of phytobiotics that exhibit antiparasitic properties against fungi, bacteria, and viruses due to their functional bioactive principles, including extracts from *T. catappa*, primarily attributed to the biological activities of its leaf extracts containing punicalin, punicalagin, terflavin A, chebulic acid, benzoic acid, and cinnamic acid[13,14] and plant oils with significant chemical components such as flavonoids, alkaloids, glucosinolates, anthocyanins, terpenes, and coumarins, like the oils from *C. langsdorffii*[15], *C. guianensis* [16], *A. speciosa* [17], *M. flexuosa* [18], and *C. brasiliense* [19,20]. Although previous studies have explored the potential of these products in enhancing growth, feed utilization efficiency, antioxidant activities, and immune response in various fish species[21–22], there remains a significant gap in investigations on the treatment of specific bacterial diseases using almond extracts and oils from andiroba, copaiba, babassu, buriti, and pequi in continental aquaculture.

This study evaluated the phytobiotic potential of oils and extracts readily obtainable in Brazil through *in vitro* assays using species-specific pathogenic strains from Brazilian aquaculture. This approach aims to significantly contribute to promoting more sustainable aquaculture and to developing an innovative protocol of prophylactic and therapeutic measures for the domestic aquaculture scenario.

2. Materials and methods

The Animal Experimentation Ethics Committee of the Brazilian Agricultural Research Corporation (Embrapa) approved this study (n° 0033/2020).

2.1 Acquisition and preparation of phytobiotics

Five natural oils were used: *Copaifera langsdorffii* (copaiba), *Carapa guianensis* (andiroba), *Attalea speciosa* (babassu), *Mauritia flexuosa* (buriti), and *Caryocar brasiliense* (pequi), purchased commercially at the Ver-o-Peso open market in Belém City, Pará State, Brazil. The oils were artisanally extracted by drilling into the tree trunks, allowing the exudate collection through a PVC tube connected to a hose that led to a collecting container. After collecting the oil resin, it was diluted in 70% alcohol at a 1:10 (v/v) ratio and then stored in polyethylene terephthalate bottles and refrigerated at 4 °C until sale.

For *T. catappa* aqueous extracts, two methods adapted from that of Meneses et al. [23] were applied. First, yellowed dry leaves recently detached from the plant were collected from the soil in the recreation area of the city of Pinheiro, Maranhão State (02° 31’ 15” S; 045° 04’ 58” W) (Figure 1). Then, they were sent to the Laboratory of Aquacultural Development of Amazon in the Maranhão State (L’AQUAM), Federal University of Maranhão, Campus at Pinheiro.

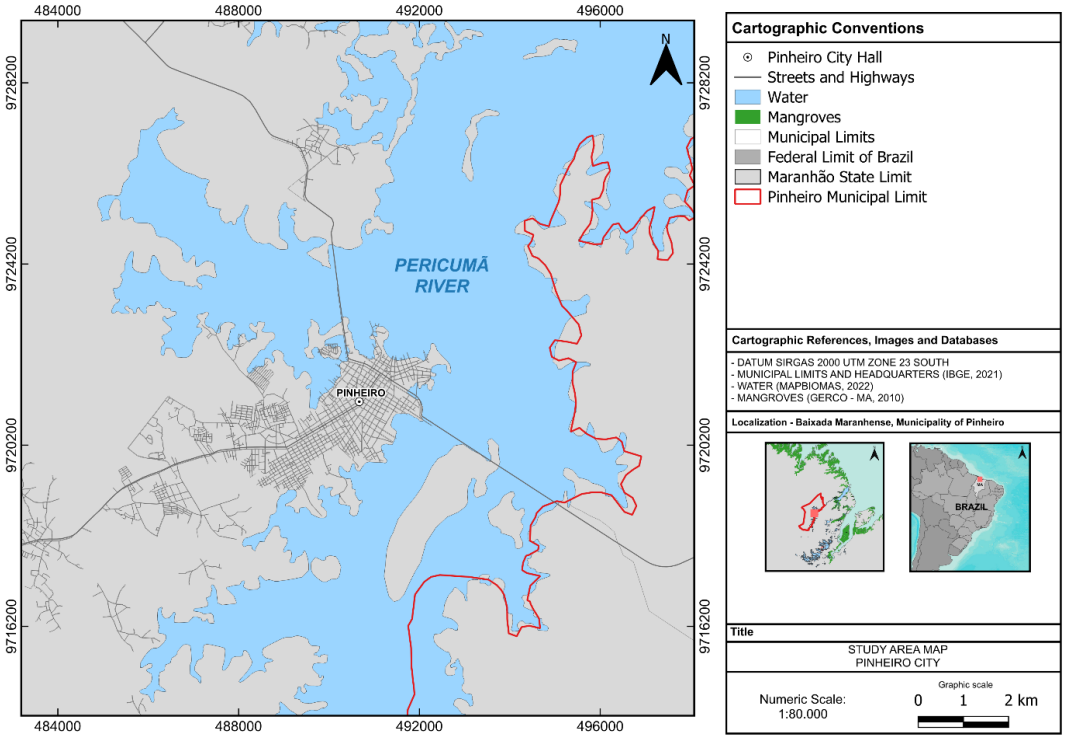

Figure 1. Location map of the municipality of Pinheiro, Maranhão, Brazil.

The leaves were sanitized with running water, sterilized in distilled water, and dried in a forced air circulation oven at 50 °C until moisture loss, following the method of Meneses et al. [23]. After drying, leaves were ground, weighed, and divided into two extraction groups, hot and cold.

For hot extraction, 25g *T. catappa* leaves underwent two extraction fractions. In the first stage, the plant material was wrapped in N°1 filter paper, immersed in 500 mL distilled water, and placed in a water bath (SolidSteel, with circulation) at 80 °C for one hour. The resulting filtrate was stored in a sealed glass container protected from light. The solid residue was then subjected to a second fraction of hot extraction [23,24]. In the end, the two filtrates were combined to form the hot extract and stored at 4 °C refrigeration in a sealed glass container protected from light. Finally, the cold extraction was conducted at room temperature (28 °C), following the same extraction conditions previously described [23].

2.2 Antibiotics

Two antibiotics with active ingredients in oxytetracycline (Terramycin, 5.5 oxytetracycline, Zoetis) and enrofloxacin (Enrofloxacin 150mg, Dechra) were used, both widely employed in continental aquaculture systems. However, to date, only oxytetracycline has been used in aquaculture activities in the United States and Brazil. Although enrofloxacin is recognized by the Ministry of Agriculture and Livestock-MAPA, it is commonly used in continental fish production for bacterial treatment and prophylaxis [25].

For *in vitro* analyses, the antibiotics were used according to sensitivity and resistance standards by the disk diffusion method, adapted for pathogenic bacteria isolated from

symptomatic fish [26,27]. The antibiotics were prepared by chemical dilution in sterilized distilled water until they reached therapeutic concentrations of 10 $mg.L^{-1}$ and 15 $mg.L^{-1}$ for oxytetracycline [28] and enrofloxacin [29], respectively.

2.3 Pathogen acquisition

The pathogens (*Aeromonas hydrophila, A. caviae, A. jandaei, Streptococcus agalactiae*, and *Micrococcus luteus*) were isolated from symptomatic animals of the species *Colossoma macropomum* and *Oreochromis niloticus*, which are the main continental species produced in Brazil. The specimens were obtained from a fish farm, in Propriá, Sergipe State, Brazil. For this purpose, the pathogens were isolated from swabs collected at infection sites on the animal backs. The swabs were cultured in Brain Heart Infusion broth (BHI, Kasvi) and incubated at 37 ºC for 24 hours. After, the samples were inoculated into BHI culture media using the streak-plate method for bacterial growth and colony isolation, incubating at 37 ºC for 48 hours, following Jatobá and Mouriño [30].

The isolated strains were assessed for cellular morphotype by the Gram staining method and then identified to the species level using the MALDI-TOF (Matrix Assisted Laser Desorption/ Ionization and Time-of-Flight, Bruker Biotyper) method, with scores above 2.0. Each isolated strain was incubated on a new BHI culture medium at 37 ºC for 48 hours. After microbial growth, each colony from each strain was overlaid by direct smearing onto the target on a MALDI-TOF stainless steel plate. Then, 1 µL of 100% formic acid was directly added to each target with the strain, which was air-dried, added with 1 µL of a matrix (α-cyano-4-hydroxycinnamic, Bruker Daltonics), and left to dry completely on the plate at room temperature. Finally, readings were conducted using the MALDI Biotyper flexControl operating system, Bruker Biotyper 3.0 software, and the taxonomy library [31,32].

MALDI-TOF MS (Bruker Biotyper) analysis was performed automatically, with 240 laser shots for each isolated strain. The bacterial test standard (BTS) (part number 255343, Bruker Daltonics) was used in each run as a calibrator and for quality control [31,32]. After identification, the strains were preserved in glycerin and semi-solid BHI culture medium and added to the strain bank of the Laboratory of Aquaculture of Embrapa Coastal Tablelands in Aracaju, Sergipe State, Brazil [26,27]. They were then ceded to the Laboratory of Aquacultural Development of Amazon in the Maranhão State (L'AQUAM), Federal University of Maranhão, Campus at Pinheiro.

2.4 Microbiological preparations

The pathogenic strains were activated by microbial growth in BHI broth. The culture medium was prepared following the manufacturer's recommendations and sterilized in an autoclave at 120°C, 1 atm, for 20 minutes. The pathogenic cultures were activated in a solution using a sterile 10 µL platinum loop for microbial growth in 10 mL of BHI under conditions of 37°C for 48 hours [26,30].

After microbial growth, the strains were purified on a plate containing BHI Agar culture medium (Kasvi). Then, 10 µL of the microbial culture in a liquid medium was collected

and applied to the culture medium on a plate by the streak plate method for subsequent growth at 37°C for 48 hours. The colonies were collected and subjected to Gram staining to confirm the cellular morphotype and microbiological purity level, and subsequently, they proceeded to tests for catalase activity and hemolytic activity to assess the virulence of the microorganisms. For this, BHI Agar culture medium (Kasvi) enriched with fish blood (1:10) was used [26,30] (Figure 2).

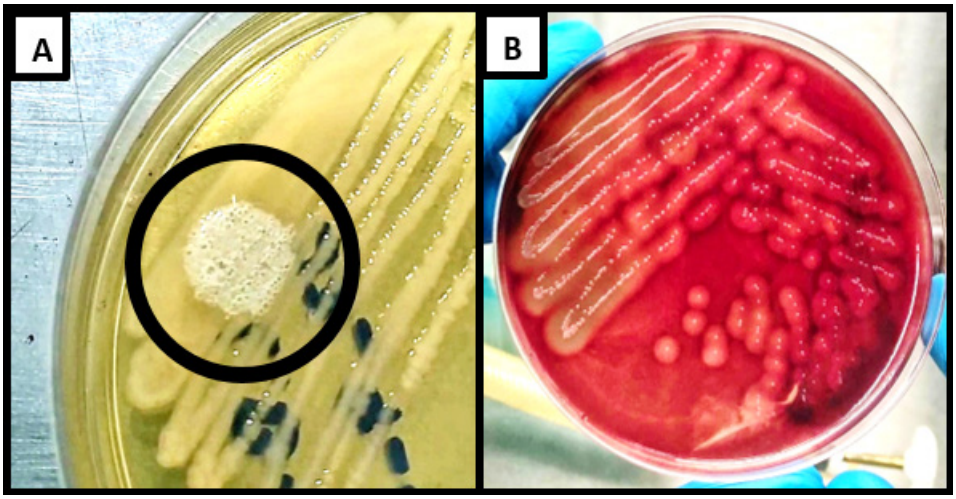

Figure 2. A - Catalase activity of the isolated pathogens; B - Positive hemolysis of the isolated pathogens.

Following the pathogenic microbiological premises, the strains were inoculated into a new BHI broth (Kasvi) for logarithmic microbial growth until reaching infective concentrations of 10^9 CFU.mL^{-1} [26], according to McFarland scale No. 4, with the bacterial density confirmed on a plate by the serial dilution method for subsequent use in bacteriological tests.

2.5 Microbiological challenge

The test was conducted on a non-selective Mueller Hinton Agar culture medium (Himedia) [26], where 100 µL of each pathogen was seeded on plates and homogenized with a sterile Drigalski loop. Subsequently, sterile filter paper discs (25 µm porosity), 6mm in diameter, soaked with 10 µL of each product (oil, extract, or antibiotics), were overlaid on the agar plates, with four repetitions each. The plates were incubated at 30°C for 48 hours, and the occurrence of inhibitory halos was measured by the inhibition zone using a digital caliper and evaluated for the bactericidal or bacteriostatic effect of the tested products [23,28] (Figure 3).

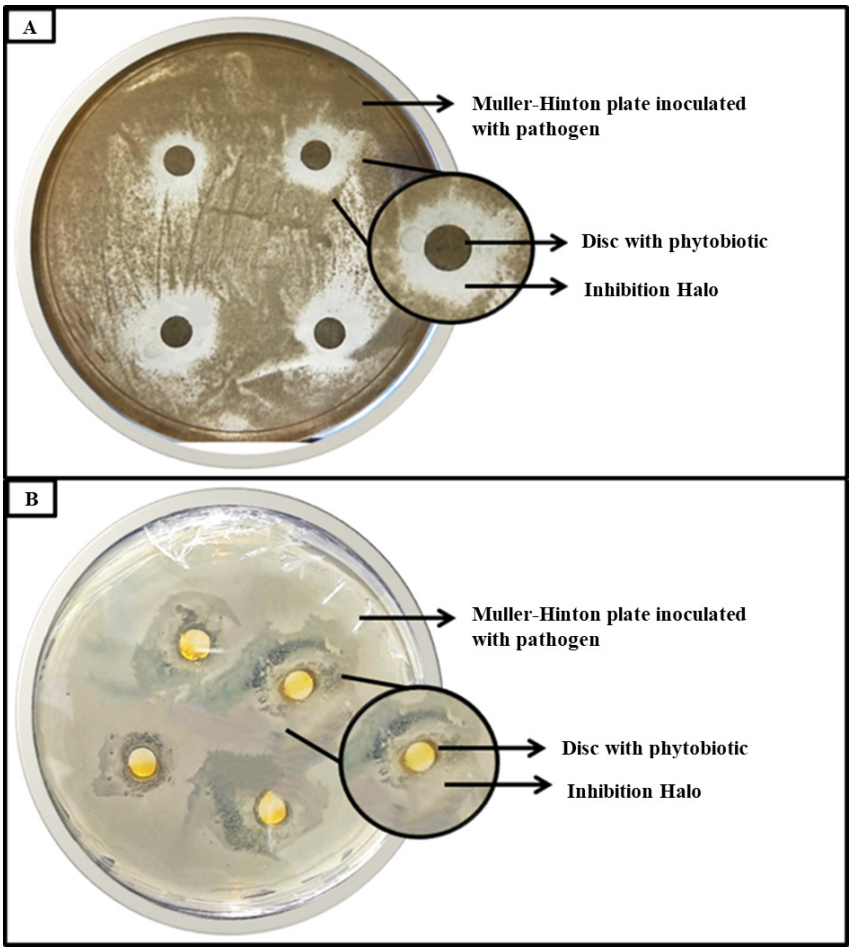

Figure 3. A - Inhibition halo indicating bactericidal effect; B - Inhibitory halo indicating bacteriostatic effect.

2.6 Statistical analysis

The data were analyzed for normality and homoscedasticity using the Shapiro-Wilk and Bartlett tests, respectively. In the case of variance heterogeneity, the data were transformed to log10 (x + 1) and then Analysis of Variance (ANOVA). In the case of significant F-values, the means were compared using the Tukey test at a 5% probability level.

3. Results

The pathogens displayed morphology compatible with the species identified by MALDI-TOF, and their virulence activity was evidenced by catalase and hemolytic activity tests (Table 1).

Table 1. Bacterial morphology and catalase test.

Strains	Gram		Catalase		Hemolytic activity		Morphology	
	+	-	+	-	+	-	Coccus	Bacillus
Aeromonas hydrophila		*	*		*			*
Aeromonas caviae		*	*		*			*
Aeromonas jandaei		*	*		*			*
Streptococcus agalactiae	*			*	*		*	
Micrococcus luteus	*		*		*		*	

(+) Positive; (-) Negative; (*) Microbiological occurrence.

In the antagonism test, the oily phytobiotics from buriti and pequi demonstrated a satisfactory inhibitory zone against the pathogen *A. hydrophila*, with responses up to 49.77% greater than the positive control with oxytetracycline. Additionally, a similar effect (P>0.05) was observed in the inhibitory zone of the pathogens when compared to the efficacy of the positive control with enrofloxacin (Table 2).

Table 2. Inhibitory zone (mm) of phytobiotics from *C. brasiliense*, *M. flexuosa*, *C. langsdorffii*, *A. speciosa* and *C. guianensis* oil, hot extract of *T. catappa* (HE), cold extract of T. catappa (CE), enrofloxacin and oxytetracycline, against the pathogens *A. hydrophila*, *A. cavie*, *A. jandaei*, *S. agalactiae* and *M. luteus*.

Treatment	*A. hydrophila*	*A. cavie*	*A. jandaei*	*S. agalactiae*	*M. luteus*
Pequi	24.22±4.77a	6.42±0.42b	0.00±0.00d	7.43±0.50c	10.03±2.24c
Buriti	23.39±4.30a	12.60±2.40b	9.90±2.00b	6.90±4.73c	16.54±2.26b
Babassu	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0,00d	0.00±0.00d
Copaíba	11.57±1.19c	9.39±0.41b	10.56±4.43b	10.58±0.88bc	9.96±2.76c
Andiroba	0.00±0.00d	0.00±0.00d	10.61±7.19b	14.74±1,45b	0.00±0.00d
HE	17.29±2.03b	7.03±1.12b	5.07±3.57bc	7.09±0.69c	6.80±1.36c
CE	0.00±0.00d	5.46±3.67b	0.00±0.00c	7.70±0.80c	0.00±0.00d
Enrofloxacin	23.92±0.00a	19.04±1.52a	22.40±1.28a	22.30±3.59a	21.44±1.21a
Oxytetracycline	11.85±0.00c	7.73±1.16c	5.79±0.88bc	7.24±1.80c	7.70±0.85c

Mean values ± standard deviation, followed by different letters in the same column indicate significant differences by Tukey's test (p <0.05).

For both Gram-positive and Gram-negative pathogen inhibition, the oily phytobiotics from *M. flexuosa*, *C. brasiliense*, *C. langsdorffii*, *C. guianensis*, and hot extract from *T. catappa* showed inhibitory zones equal to or greater than the positive control with oxytetracycline (Table 2). These responses are complemented by the inhibitory effect against bacteria (Table 3), which indicated the phytobiotics from *M. flexuosa* and *C. langsdorffii* bactericidal efficacy for most of the tested microorganisms, with promising responses compared to the bacteriostatic effect of the drug with the active principle of oxytetracycline (Table 3).

Table 3. Bactericidal and/or bacteriostatic evaluation of the inhibitory halo for treatments with oil from *Caryocar brasiliense* (pequi), *Mauritia flexuosa* (buriti), *Copaifera langsdorffii* (copaíba), *Attalea speciosa* (babassu) and *Carapa guianensis* (andiroba), hot extract of Terminalia catappa (HE), cold extract of Terminalia catappa (CE), in comparison to antibiotics with enrofloxacin and oxytetracycline.

Treatment	*Aeromonas hydrophila*	*Aeromonas caviae*	*Aeromonas jandaei*	*Streptococcus agalactiae*	*Micrococcus luteus*
Pequi	BC	BT	-	BT	BT
Buriti	BC	BC	BT	BT	BC
Babassu	-	-	-	-	-
Copaíba	BT	BC	BC	BC	BT
Andiroba	-	-	BC	BC	-
HE	BC	BT	BT	BT	BT
CE	-	BT	-	BT	-
Enrofloxacin	BC	BC	BC	BC	BC
Oxytetracycline	BT	BT	BT	BT	BT

(BC) Bactericidal; (BT) Bacteriostatic; (-) No inhibition.

4. Discussion

Natural disease prevention and control measures in aquaculture are part of a global sustainable management plan to achieve the European Union's model of chemical-free aquaculture trade [23, 33]. *M. flexuosa* oil, rich in antimicrobial compounds like fatty acids, tocopherols, and carotenoids, has shown promising bactericidal activity against *Aeromonas hydrophila*, *A. caviae*, and *Micrococcus luteus*, offering a safe and natural approach to combatting bacterial diseases in Brazilian aquaculture [34]. Compared to antibiotics, especially oxytetracycline, this oil can be considered a promising alternative to combating bacterial diseases in continental aquaculture.

As observed in Table 3, the overuse of oxytetracycline likely reduced its effectiveness by promoting the spread of antibiotic-resistance genes. While the second antibiotic, enrofloxacin, demonstrated satisfactory inhibition of the tested pathogens, its use should be carefully monitored to ensure appropriate concentrations and durations. The indiscriminate use of this drug should be avoided to prevent the development of antibiotic resistance, which can lead to the emergence of superbugs. Additionally, it is important to consider reducing chemical bioaccumulation in the muscle tissue and the production environment of the aquatic organisms being raised [7].

In this study, the use of *M. flexuosa* oil as a phytobiotic against Aeromonas and M. luteus strains corroborates those found by Santos-Morais et al. [18] against varieties of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Bacillus subtilis*. They attributed the antimicrobial activity against Gram-positive pathogens primarily to tocopherols in the oil, while carotenoids from the extraction process exhibited higher inhibitory effects against Gram-negative pathogens.

On the other hand, Silveira et al. [34] linked the bactericidal effect of *M. flexuosa* oil to both Gram-positive and Gram-negative microbiota to the compounds of its fatty acids, as in the inhibitory effect against *Staphylococcus* and *Pseudomonas aeruginosa*. In this sense, we can infer that the inhibitory effect of *M. flexuosa* may be associated with the synergism of its antimicrobial compounds, which still need to be elucidated, as well as the application concentration, as observed by Carvalho et al. [35] and Chaves et al. [36]. When using minimal inhibitory concentrations (MIC) of *M. flexuosa* oil, these studies achieved a bactericidal effect against strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Candida albicans*, and multidrug-resistant *S. aureus*.

The phytobiotic from *C. brasiliense* showed bactericidal effects only for *Aeromonas hydrophila*, demonstrating a competitive response compared to the studied antibiotics. This antimicrobial activity can vary depending on the origin of the extracted plant material. According to Sousa et al. [37], in the bactericidal evaluation of *C. brasiliense* against the pathogens *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*, promising results were obtained with average inhibition halos of 33mm. These extracts, derived from the flowers of *C. brasiliense*, exhibited high concentrations of flavonoids and terpenoids, which may have contributed to their efficacy.

For the hot extract of *Terminalia catappa*, the effect was similar to that observed with the oil from *C. brasiliense* for *A. hydrophila*, but it exhibited a different mode of action compared to the cold extract. This result could be due to the high temperatures during the extraction process that may result in chemical alterations of the extracted biomolecules[13]. Meneses et al. [23] confirmed this through high-precision chromatographic analysis of *Terminalia catappa* extracts, showing that the temperature of 80°C produced higher peaks of gallic acid, ellagic acid, and phenols responsible for the antimicrobial effect [13,23].

The phytobiotics from *C. guianensis* and *C. langsdorffii* exhibited a bactericidal inhibition spectrum for two or more tested pathogens. These results should be considered and investigated for other pathogens affecting continental aquaculture. According to the literature, inhibition halos derived from *C. guianensis* oil are due to the active principle of limonoids [38], a compound with bactericidal action. However, the origin, extraction time, and collection region of oils can influence the concentration of limonoids, which may impact the bactericidal activity. This fact was noted by Lacerda et al. [39] against strains of *Staphylococcus aureus* and *Escherichia coli*.

The oil of *C. langsdorffii* contains active ingredients such as β-bisabolene, which has anti-inflammatory and analgesic properties, and β-caryophyllene, which has bactericidal and insecticidal effects [40]. Masson et al. [41] confirmed the antimicrobial action of *C. langsdorffii* oil sold in open markets, corroborating our results. These authors observed a bactericidal effect against pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Candida albicans*.

5. Conclusion

Phytobiotics derived from oils of pequi (*Caryocar brasiliense*), buriti (*Mauritia flexuosa*), copaiba (*Copaifera langsdorffii*), and andiroba (*Carapa guianensis*), as well as hot extract of *Terminalia catappa*, have promising bactericidal activity against pathogens such as *Aeromonas hydrophila, A. cavie, A. jandaei, Streptococcus agalactiae*, and *Micrococcus luteus*. Notably, oils from *M. flexuosa* and *C. langsdorffii* exhibited competitive *in vitro* activity against pathogens like *Aeromonas hidrophila, A. cavie, A. jandaei*, and *Streptococcus agalactiae*, comparable to the antibiotics enrofloxacin and oxytetracycline. However, further research is needed to determine the specific activity of each active compound in these phytobiotics against fish pathogens and to develop long-term biosecurity protocols to ensure food safety. *In vivo* studies are also crucial to assess the toxicology, efficacy, and potential synergistic effects of these phytobiotics on aquatic organisms in aquaculture.

Conflict of Interest statement

The authors declare no conflict of interest.

Data availability statement

The data will be provided upon request.

Author contributions

Conceptualization: J. A R. Dias, N. C. S. Sarges, A. Silva and Y. F. Marinho. Data curation: J. A R. Dias, N. C. S. Sarges, A. Silva, A. M. B. Machado and Y. F. Marinho. Formal analysis: J. A R. Dias, N. C. S. Sarges, Y. V. A. Lopes, A. M. B. Machado and Y. F. Marinho. Funding acquisition: J. A R. Dias and Y. F. Project administration: J. A R. Dias, N. C. S. Sarges, I. R. A. Dos Santos, W. B. Barros and Y. F. Marinho. Methodology: J. A R. Dias, N. C. S. Sarges, I. R. A. Dos Santos, W. B. Barros and Y. F. Marinho. Supervision: J. A R. Dias and Y. F. Marinho. Investigation: J. A R. Dias, N. C. S. Sarges, I. R. A. Dos Santos, W. B. Barros and Y. F. Marinho. Visualization: J. A R. Dias, N. C. S. Sarges, A. Silva, I. R. A. Dos Santos, W. B. Barros, Y. V. A. Lopes, A. M. B. Machado and Y. F. Marinho. Writing (original draft): J. A R. Dias, N. C. S. Sarges, A. Silva, I. R. A. Dos Santos, W. B. Barros, Y. V. A. Lopes, A. M. B. Machado and Y. F. Marinho. Writing (review and editing): J. A R. Dias, N. C. S. Sarges, A. Silva, I. R. A. Dos Santos, W. B. Barros, Y. V. A. Lopes, A. M. B. Machado and Y. F. Marinho.

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