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Plectranthus amboinicus Essential Oil Incorporated into Fish Feed Shows Strong Antimicrobial Activity against *Aeromonas hydrophila*, an Opportunistic Bacterium of Aquaculture

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Plectranthus amboinicus is an aromatic herb often used in traditional medicine due to its antimicrobial properties. Based on it, the present study aimed to assess the toxic, antioxidant and, antibacterial properties of *P. amboinicus* essential oil (EO) either separated or mixed into fish feed. The chemical composition of the EO was also investigated. All samples were prepared with over 50% of carvacrol. The results revealed that the EO is a weak antioxidant and highly toxic against *Artemia salina*, showing greater antibacterial activity than positive control of chloramphenicol and synergistic effects. The EO also presented high antimicrobial activity against *Aeromonas hydrophila* strains when free or feed-incorporated, thus being a promising product for the treatment of opportunistic infections in fish.

Keywords: carvacrol, checkerboard, *Artemia salina*, malvão, qNMR

Introduction

Bacterial resistance is a natural adaptation process toward changes in the environment; however, the overuse of antimicrobials has been accelerating it.[1] One of the key factors is the addition of antimicrobials to the diet of animals intended for human consumption, leading to the emergence of multi-resistant bacteria.[2,3]

Bacteria from the genus *Aeromonas* are found in water, soil, food, human feces and, contaminated animals, responsible for gastroenteric disorders in humans.[4] In fish farming, the species *Aeromonas hydrophila* impairs the production process. This causes major economic losses owing to the importance of these animals as protein and essential micronutrients sources, such as omega-3 and 6.[5-7]

The use of antibiotics in fish farming depends on many factors, which hampers the determination of standardized and rational therapeutic regimens. Hence, the use of new substances for the treatment of opportunistic infections becomes required since antibiotics such as oxytetracycline and florfenicol, widely used to treat fish in *Aeromonas* isolates, have been proven inefficient.[8,9]

Plectranthus amboinicus is an herbaceous plant commonly used by popular medicine to treat chronic diseases for its considerable amounts of monoterpenes carvacrol and thymol, bactericidal compounds against different pathogenic bacteria.[10-14] Its essential oil is

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considered effective against Gram-positive bacteria such as *Staphylococcus aureus*, *S. aureus* MRSA and *S. epidermidis*,[15] Gram-negative bacteria such as *Escherichia coli*,[16] *Klebsiella pneumoniae*[12] and *Pseudomonas aeruginosa*,[17] and *Mycobacterium tuberculosis*.[18]

Given the animal and human health effects triggered by multi-resistant microorganisms, this study assessed the chemical composition of essential oils made from *P. amboinicus* and their biological activity against *A. hydrophila* at *in vitro* experiments as well as combined with fish feed.

Experimental

Plant material

Aerial parts of *P. amboinicus* were collected in the medicinal garden of IF Sertão Pernambucano, sited at "Zona Rural" Campus, Km 22, N4, Petrolina, PE, Brazil. Yields were taken monthly, between March/2019 and January/2020, in the second half of each month during the morning. The access to these materials was registered on SisGen (Registration No. A27F234). Once in the laboratory, aerial parts (stems, leaves, and flowers), 1.5-3.0 kg *per* extraction, were milled using the turbolysis method (drug-solvent weight proportion of 1:1 m/m) for 10 min at room temperature. The essential oils were extracted by hydrodistillation using Clevenger apparatus for 2 h then dried with sodium sulfate anhydrous and stored in the dark at 4 ºC. The yield was expressed as a percentage of fresh plant drug mass.[19]

Chemical characterization

Gas chromatography-mass spectrometry (GC-MS)

The chemical profiles of essential oils (EOs) were obtained by chromatograms and mass spectra on Shimadzu gas chromatography equipment, model QP-2010, provided with DB-5MS column, Agilent Technologies (30 m × 0.25 mm × 0.25 µm). A steady helium (99.999%, White Martins, Rio de Janeiro, Brazil) flow rate of 1.1 mL min^{-1} was applied and the injection volume was 1.0 µL (split ratio of 1:10 and injector temperature of 250 °C). The oven temperature ranged from 60 to 240 °C, at an increase rate of 3 °C min^{-1}. A linear hydrocarbon mixture (C_8H_{18}-$C_{20}H_{42}$) was injected under the same experimental conditions. Mass spectra (MS) were obtained using electron ionization (EI) with electron energy of 70 eV. The injector temperature was 280 °C and the ion-source temperature was 260 °C. The components identification of the samples was performed by experimental mass spectra data and equipment database (Wiley 7 lib and Nist 08 lib) comparison, applying the retention index (IR). The data were acquired and processed in the Shimadzu GC-MS Solution software (Shimadzu Corporation, Kyoto, Japan).[19]

Nuclear magnetic resonance (NMR) experiments

NMR experiments were performed using Eretic2 technique on NMR (Bruker Ascent™, Germany) 400 MHz BBO spectrometer squared z-gradient shape. Initially, 15.0 µL of samples were solubilized into 500 µL chloroform deuterated ($CDCl_3$) (Sigma-Aldrich, Saint Louis, USA) containing 0.05% of tetramethylsilane (TMS), and transferred to 5 mm NMR tubes. Then, one (1D) and two-dimensional (2D) experiments were performed to select the chemical marker signals. To appraise assays, a longitudinal relaxation time constant (T1) was initially defined from the signals referring to carvacrol present in the samples. The value found was used to determine the optimal recovery time (d1) for the EO samples through the equation $d1 = 7 \times T1$.

A 0.1 mol L^{-1} 99.5% thymol solution (Sigma-Aldrich, Saint Louis, USA), also in $CDCl_3$, was prepared and used as an external standard. The ^1H experiments were performed at 298 K with a pulse sequence zg30 and the following parameters: 56 scans, spectral width (SW) of 8012.820 (ca. 20.0 ppm), 40.0 s of recycle delay (D1) and, 4.09 s of acquisition time. For each sample, tuning, matching, and shimming were automatically run and the pulse length was adjusted using the command pulsecal. A Laurentian multiplication by a line broadening factor of 0.1 Hz was applied to the FIDs (free induction decays) before Fourier transform (TD equal to 65 K). The resulting spectra were manually phased. The baseline was corrected and referenced to the TMS resonance (methyl groups) at 0.0 ppm. All samples were prepared in triplicate and the carvacrol content was monitored throughout the signal area at 6.5 ppm. Quantification was executed from the acquisition spectra by two analysts. The data were then processed on Brucker's TopSpin 4.1.1 software.[20,21] The carvacrol content average was determined on GraphPad Prism 8.0 version.[22]

Antioxidant assays

The antioxidant potential of EOs was evaluated by the following spectrophotometric methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and β-carotene (Sigma-Aldrich, Saint Louis, USA) as described by Brand-Williams *et al.*[23] and Duarte-Almeida *et al.*,[24] respectively. All experiments were performed in triplicate. The obtained data were processed in GraphPad Prism software 8.0 version.[22] The DDPH experimental results were expressed in terms of inhibitory concentration (IC_{50})

and antioxidant activity index (IAA) average,[25] and for β-carotene, as the antioxidant activity average percentage.

Prior toxicity *Artemia salina* assay

Dry *Artemia salina* cysts (20 mg) were dark-incubated in an aerated glass tank filled with 500 mL of artificial salty water (38 g L^{-1}) at 25 ºC. Dispersions of EOs, Tween 80 (1% m/m) and saline solution (38 g L^{-1}) were prepared. Groups of 10 or fewer free-swimming nauplii were transferred to tubes containing saline dispersions (1 to 1000 μg mL^{-1}) 48 h after hatching. Saline solution and paracetamol (Brazil) (800 mg L^{-1}) were employed as negative and positive control, respectively. The death number was measured every 24 h and 48 h post-incubation under white light at 25 ºC. Results were estimated as IC_{50} average on GraphPad Prism software version 8.0.[22,26,27]

Antimicrobial assays

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

An *Aeromonas hydrophila* strain (G38) was provided by the Laboratory of Animal Microbiology and Immunology (UNIVASF), obtained from Pacamã (*Batrachoides surinamensis*) fish kidneys. The EOs antibacterial potential evaluation was performed following the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) methods. A newly sown bacteria aliquot was transferred into a glass tube conveying saline solution then the turbidity adjusted to McFarland 0.5 (spectrophotometer (Model 1600uv, Nova instruments, Piracicaba, Brazil)) (λ = 600 nm). Afterward, 100 μL was added to 9.9 mL of brain heart infusion (BHI) broth for later use. Before the assays, water-based EOs stock solutions, positive control (gentamicin and chloramphenicol), and carvacrol analytical standard (Sigma-Aldrich, Saint Louis, USA) at 1000 μg mL^{-1} were prepared. For assisting solubilization, 3% (weight/volume) of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, USA) was added to the solutions. 100 μL of this dilution was transferred to 96-well plate with 100 μL of sterile BHI (Kasvi, Italy). Consecutive dilutions were performed, varying the concentration from 500 to 6.25 μg mL^{-1}. For the antibiotics, a series of dilutions were carried out until a 0.046 μg mL^{-1}concentration was reached. Then, a bacterial suspension of *A. hydrophila* containing approximately 1.5×10^8 colony forming units (CFU) mL^{-1} was prepared and 10 μL were added to each microplate well. Microplates were incubated under aerobioses conditions at 37 °C for 24 h. In each plate, wells were kept for positive (bacterial growth) and negative (sterility) controls. After incubation time, 10 μL of 2,3,5-triphenyl-tetrazolium chloride 99% (TTC) (99% purity, Dinâmica Quimica Contemporanea Ltda, Indaiatuba, Brazil) at 1% were added to each well to detect the TTC color (colorless) changes into rose, indicating active bacterial metabolism. MIC was defined as the lowest extract concentration, or the isolated compound that visibly inhibits bacterial growth. A DMSO blank solution (3%) was tested and no influence on cell viability triplicate was noted.[28]

To determine the MBC, 10 μL aliquots were withdrawn from the well sand transferred to Petri plates containing sterile Muller-Hinton agar (Kasvi, Italy). The plates were incubated for 24 h at 37 °C. The presence of bacteria colony at a given concentration indicates that it was not able to kill 99.9% or more of the bacterial inoculum used. All experiments were performed in triplicate.[28]

Checkerboard method

In a sterile 96-well plate, 100 μL of BHI broth was added in 36 wells (columns 1-6 and rows A-F). At well 6 was added 100 μL of chloramphenicol (BlauFarmacêutica S.A., São Paulo, Brazil) solution at a concentration 4× the MIC value, and serial dilutions were performed horizontally from right to left. It was added 100 μL of essential oils with 3% of DMSO and twice the MIC concentration in the row (A) wells. Plus, six serial dilutions were performed vertically. Then, a bacterial suspension of *A. hydrophila* containing approximately 1.5×10^8 CFU mL^{-1} was prepared and 10 μL was added to each well of the microplate. Microplates were incubated under aerobioses conditions at 37 °C for 24 h and the cellular viability was performed using the same steps from MIC. Fractional inhibitory concentration (FIC) was estimated for no color change concentrations. Then, the FICs were summed to classify the effects: synergistic action (FIC ≤ 0.5); additive (0.5 < FIC < 1); indifferent (1 < FIC < 2) and antagonistic (FIC ≥ 2).[29]

$$\text{FIC of natural product (NP)} = \frac{\text{(MIC combinated)}}{\text{(MIC NP)}} \quad (1)$$

$$\text{FIC of antibiotic (ATB)} = \frac{\text{(MIC combinated)}}{\text{(MIC ATB)}} \quad (2)$$

where NP: natural product and ATB: antibiotic.

Fish feeds with essential oils and verification of the final antibacterial potential

Obtaining the feed formulations required a larger volume of EO. Therefore, a new collection and extraction

was necessary so that all the experiments could be performed with the same sample, which was done in January 2021, obtaining the sample EO 1.21. Commercial extruded fish feeds, NUTRIPISCIS TR 32 of 4 mm (Presence, Brazil), were divided into groups, sterilized, and evenly sprayed with alcoholic solutions (EO 1.21) following a 1:2 m/v (g of feed mL of solution) proportion. Then, the groups were dried in sterile paper packaging at 25 °C for 24 h, in a sealed dusk environment. The alcoholic solutions were prepared to obtain feeds with 50 and 1000 mg g^{-1} of EO. Group preparation was performed in triplicate. Feeds sprayed with ethanol 93% purity (Labsynth, Diadema, Brazil) were employed as negative control.[30]

Carvacrol content in fish feeds profile by NMR

After 24 h of drying, the remaining content of carvacrol in each group was determined. Briefly, 700 µL of $CDCl_3$ with 0.05 of TMS were added to 60 mg of fish feed from each group in sterile glass vials. Then, the samples were sonicated for 5 min in ultrasound equipment (LSUC2-120-5.0 model) (LogenScientifc, Brazil) at 25 °C. The carvacrol content was measured in extractive solutions using the mentioned equipment, following the conditions as described for EOs. All experiments were performed in triplicate and the software GraphPad Prism 8.0 was used for statistical analysis.

Antibacterial action of fish feed incorporated with essential oil

To check the antibacterial action of fish feed incorporated with essential oil, MIC and BMC assays were performed, as described previously. For this, the carvacrol content of each sample group was used to determine the feed mass required to stock solutions of 600, 1200, and 2400 µg mL^{-1} of carvacrol.

Results and Discussion

Extraction yields

The research was carried out in the Caatinga, a Brazilian biome acknowledged by high temperatures, thermal stability, short and irregular rainy periods, and endemism.[31] These features contribute to the emergence of endemical species, such as *Plectranthus amboinicus*, used throughout this study.[31] To ease the variability effect, sampling conditions were constant (mornings month-wise). As a result, the extraction yield (0.05%) kept steady over the 11 months appraised.

Essential oils are made up of low-molecular-weight molecules called terpenes or terpenoids. This class of secondary metabolites is characterized by structures ranging from 10-15 carbon atoms that provide them with an oily and volatile appearance. Hence, variables such as temperature, light, environment, farming conditions, harvesting season and procedure influence extractions yields.[32-35] For *P. amboinicus* essential oil obtained from species collected in Petrolina-PE, the high temperature and thermal stability of the city seem to have negatively influenced the yields attained during the EO extractions. This hypothesis gains strength when our results are compared with yields (0.09-0.43%) of essential oils also from *P. amboinicus* collected and extracted in Pelotas-RS, a place with milder climatic conditions than those observed in the Northeastern semi-arid region.[36]

Despite the low yield, the growth constancy observed for *P. amboinicus* in Petrolina is an advantage concerning large-area crops and large-scale production. However, before further conclusions, it is necessary to develop a broad seasonal study to fully assess the climatic variations in the region, and their effect on secondary metabolites outputs.

Chemical composition of essential oils

The substances identified in the EO samples by gas chromatography coupled to mass spectrometry (GC-MS) and their concentrations are presented in Table 1. A total of 32 substances were identified with an average of 20 substances *per* sample. The oxygenated monoterpenes were the highest concentration class in the EOs. Carvacrol was the utmost component throughout the samples, varying from 51.13 to 65.36%. Results were lower than those obtained by Vasconcelos *et al.*[37] and Santos *et al.*[38] 88.1% from the oil of plants collected in the Cariri region (CE), and 90.5% in the city of Fortaleza-CE, respectively. These differences were considered reasonable once the study areas encompassed by the three studies are in different zones of the Brazilian northeast.

Other studies have also identified carvacrol as the greatest component.[16,32] This monoterpene is amply distributed among many plant species, and several biological activities are reported in the literature, such as: antifungal activity,[39] antitumor, antibacterial, antiparasitic,[40] antioxidant, antiviral, hepatoprotective, spasmolytic, acaricidal, anti-inflammatory, vasorelaxant, among others.[41]

As one of the purposes of this study was to use the *P. amboinicus* EO as antimicrobial agent in fish feeds, it was necessary to perform strategies for monitoring essential oil in the formulations. Thus, the carvacrol quantification

Table 1. Percent chemical composition of *Plectranthus amboinicus* essential oils, by GC-MS, collected over 11 months (March/2019 to January/2020)

No.	Compound	Chemical composition / %										
		Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
						Monoterpenes						
1	α-thujene	0.51	0.26	0.60	0.50	0.49	0.43	0.36	0.22	0.49	0.45	0.42
2	α-pinene	0.20	0.11	0.27	0.23	0.27	0.22	0.16	0.09	0.23	0.22	0.19
3	β-phellandrene	0.46	–	–	–	–	–	–	–	0.16	0.14	0.14
4	y-terpinene	7.17	5.74	8.04	2.90	6.16	5.70	4.09	2.45	6.64	4.13	4.30
5	α-terpinene	1.13	0.89	1.29	0.50	0.96	0.94	0.60	–	1.15	0.76	0.78
6	β-mircene	1.11	0.82	1.16	0.99	1.17	0.98	0.80	0.54	0.99	0.87	0.83
7	cymenederivatives	19.4	14.75	17.84	22.93	21.66	18.07	16.35	16.47	15.91	15.15	15.8
8	camphene	–	–	–	–	–	–	–	–	0.05	0.09	0.02
9	β-pinene	–	–	–	–	–	–	–	–	0.05	0.06	0.03
10	α-phellandrene	–	–	–	–	–	–	–	–	0.15	0.08	0.06
11	δ-3-carene	–	–	–	–	–	–	–	–	0.11	0.26	–
12	limonene	–	–	–	–	–	–	–	0.26	0.24	0.24	0.22
13	(*E*)-β-ocimene	–	–	–	–	–	–	–	–	0.04	0.02	0.02
14	terpinolene	–	–	–	–	–	–	–	–	0.06	0.05	0.05
Total		30.22	22.57	29.2	28.05	30.71	26.34	22.36	16.47	26.27	22.52	22.86
						Oxygenated monoterpenes						
15	terpinen-4-ol	1.40	1.24	1.22	1.21	1.25	1.17	0.97	1.16	1.17	1.42	1.47
16	thymol	0.20	0.22	0.19	0.18	0.19	0.21	0.20	0.22	0.33	0.45	0.45
17	carvacrol	52.96	64.12	56.27	58.13	51.13	60.16	63.29	65.36	57.4	58.65	60.66
18	linalool	–	–	–	–	–	–	–	–	0.06	0.07	0.08
19	*trans*-sabinenehydrate	–	–	–	–	–	–	–	–	–	0.03	0.03
20	+(−) borneol	–	–	–	–	–	–	–	–	–	0.21	0.08
21	α-terpineol	–	–	–	–	–	–	–	–	–	0.07	–
Total		54.56	65.58	57.68	59.66	52.57	61.54	64.46	66.74	58.96	60.9	62.77
						Sesquiterpenes						
22	*E*-caryophyllene	5.88	4.49	4.94	4.50	6.41	4.43	4.93	4.62	5.74	4.89	4.48
23	α-bergamotene	3.89	2.76	3.29	3.23	4.83	3.10	3.42	2.97	4.56	3.93	0.06
24	α-humulene	1.51	1.13	1.26	1.14	1.63	1.10	1.26	1.23	1.6	1.49	1.35
25	β-bisabolene	0.11	0.07	0.08	0.07	0.15	N.I.	0.09	0.07	0.23	0.19	0.14
Total		11.39	8.45	9.57	8.94	13.02	8.63	9.7	8.89	12.13	10.5	6.03
						Oxygenated sesquiterpenes						
26	caryophyllene oxide	1.68	1.47	1.65	1.50	1.66	1.48	1.85	2.30	2.65	2.95	2.50
27	humuleneepoxide II	0.22	0.19	0.21	0.20	0.20	0.18	0.26	0.33	0.51	0.58	0.46
28	*E*-sesquisabinene	–	–	–	–	–	–	–	–	0.14	0.14	–
Total		1.9	1.66	1.86	1.7	1.86	1.66	2.11	2.63	3.3	3.67	2.96
						Others						
29	2-hexenal	0.33	0.06	0.14	–	0.12	–	–	0.12	0.27	0.13	0.04
30	1-octen-3-ol	1.60	1.32	1.12	1.37	1.25	1.39	0.90	1.20	0.95	1.47	1.61
31	NI	–	0.34	0.44	–	0.49	0.39	0.35	–	–	–	–
	cis-3-hexenyl formate	–	–	–	–	–	–	–	–	–	0.13	0.08
32	cembrene	–	–	–	–	–	–	–	–	0.06	0.09	0.08
Total		1.93	1.72	1.7	1.37	1.86	1.78	1.25	1.32	1.28	1.82	1.81
Total identified		100	99.98	100.01	99.72	100.02	99.95	99.88	96.05	101.97	99.41	96.43

N.I.: not identified; (−): not found in the determined sample.

through quantitative nuclear magnetic resonance (qNMR) was chosen. The results of qNMR for all samples are presented in Table 2. January and July samples presented the highest and lowest carvacrol content, respectively.

Table 2. Carvacrol concentration in *Plectranthus amboinicus* essential oil samples over the months via qNMR

Sample	Month/Year	Carvacrol / %
EO 1	Mar/2019	50.02 ± 1.43
EO 2	Apr/2019	59.06 ± 2.32
EO 3	May/2019	51.60 ± 1.13
EO 4	Jun/2019	54.92 ± 3.85
EO 5	Jul/2019	46.04 ± 2.21
EO 6	Aug/2019	56.23 ± 2.97
EO 7	Sep/2019	59.51 ± 2.55
EO 8	Oct/2019	56.62 ± 2.45
EO 9	Nov/2019	56.00 ± 1.82
EO 10	Dec/2019	58.29 ± 2.93
EO 11	Jan/2020	61.42 ± 3.64
EO 1.21	Jan/2021	56.29 ± 1.14

Results expressed as mean ± standard deviation, n = 3. EO: essential oil.

Similar results between CG-MS and qNMR were found (Tables 1 and 2), but the differences, which at first glance are questionable, can be easily explained by the particularities of each technique. The GC-MS analysis proposes the relative percentage of the constituents from the ratio between the peak area of each substance by the sum of the total area. On the other hand, in the NMR analysis an internal standard of known concentration is used, which allows to determine the real mass of the analyte in the sample. Despite this difference, the techniques are valuable for the chemical characterization of oils for the GC-MS identifies most of the target substances whereas the NMR grants more reliable quantifications.

No meaningful differences on carvacrol variation by season were observed by both quantification techniques (Figure 1). Despite the steadiness of the content of the major secondary metabolite, the amount and diversity of the trace components impacted the antimicrobial activity results since the inhibitory and bactericidal concentrations varied. Therefore, for the essential oils evaluated, the synergism between these trace compounds was able to modulate the biological response.[42]

Antioxidant activity of essential oils

According to previous studies,[41] the monoterpene carvacrol, present high antioxidant activity *in vitro* due to its chemical structure. Although predominant, the oxygenated

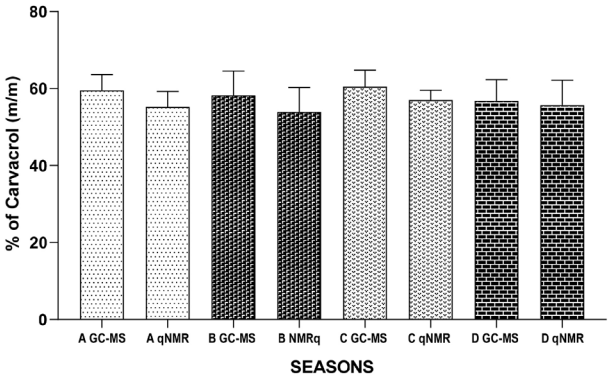

Figure 1. Mean concentration of carvacrol in *Plectranthus amboinicus* essential oils by seasons of the year through GC-MS and qNMR. A: autumn (Apr/May/Jun); B: winter (Jul/Aug/Sept); C: spring (Oct/Nov/Dec); D: summer (Mar and Jan). Results expressed as mean ± standard deviation, n = 3 for each month. Samples presented no statistical difference in one-way analysis of variance (ANOVA) test and Tukey's post-test with $p < 0.05$.

monoterpenes content provided low antioxidant action for the *P. amboinicus* essential oils samples evaluated (Table 3). All samples showed IC_{50} higher than 400 μg mL^{-1}. Also, according to the DPPH method (Scherer and Godoy)[25] all EO samples showed low antioxidant activity presenting an antioxidant activity index (AAI) below 0.5.

Table 3. Antioxidant activities of essential oils produced from *Plectranthus amboinicus* aerial parts collected over 11 months

Sample	Month/year	DPPH (IC_{50}) / (μg mL^{-1})	β-Carotene (AA) / %	AAI
1	Mar/2019	552.20 ± 43.64	20.50	0.020
2	Apr/2019	422.80 ± 3.51	22.50	0.030
3	May/2019	533.40 ± 13.44	22.30	0.020
4	Jun/2019	483.20 ± 7.58	20.00	0.030
5	Jul/2019	638.60 ± 21.41	22.20	0.020
6	Aug/2019	460.70 ±11.81	19.20	0.030
7	Sep/2019	1943 ± 184.50	27.00	0.007
8	Oct/2019	737.10 ± 20.75	18.90	0.019
9	Nov/2019	4352 ± 1267	36.80	0.003
10	Dec/2019	3196 ± 326.40	22.60	0.004
11	Jan/2020	2463 ± 253.20	22.60	0.005
BHA		3.43 ± 0.05	69.70	4.160
Ascorbic acid		2.18 ± 0.03	25.30	6.540

IC_{50} (inhibitory concentration) values were obtained by non-linear regression within a 95% confidence interval. Values are presented as mean ± standard error (n = 3). AA: antioxidant activity; AAI: antioxidant activity index; DPPH: 2,2-diphenyl-1-picrylhydrazyl; BHA: butylated hydroxyanisole.

Bezerra *et al.*[43] also evaluated the antioxidant action of carvacrol using the DPPH radical scavenging method. In general, the essential oils evaluated by the authors showed greater antioxidant potential at high carvacrol

concentrations than those observed in the present study. Samples containing 73.4 and 68.1% of carvacrol presented the highest antioxidant capacity, with IC_{50} results equal to 132.6 and 125.0 μg mL^{-1}, respectively. Similarly, Mendez *et al*.[44] appraised EOs from Colombia and 64.55 and 69.97% of carvacrol reached IC_{50} values of 327.5 ± 2.35 and 240.3 ± 4.60 μg mL^{-1}, respectively. According to the literature available, Suntres *et al*.[41] theory explain the current experimental results. Also, essential oils from Thailand containing 51.57 and 17.78% of carvacrol showed IC_{50} of 9518 and 18760 μg mL^{-1}, respectively.[45]

Regarding mechanisms of β-carotene auto-oxidation inhibition, the EOs showed similar results to the natural antioxidant ascorbic acid. Thanaseelungkoon *et al*.[45] evaluated the a EO by the β-carotene method, and observed IC_{50} values of 1345 and 3850 μg mL^{-1}, indicating low antioxidant action. EOs antioxidant potential has many reasons, especially the amount and synergism between the substances and their chemical complexity. Therefore, it is complex to compare the activity of oils with different compositions, which is why the major metabolite is often used as a comparison parameter. In this context, the association of methods with different mechanisms of action is recommended.[46]

Preliminary toxicity against *Artemia salina*

For essential oils, the lethality rate was 100% from the second concentration tested after 24 h (50 μg mL^{-1}). This result was superior to paracetamol, which showed lethality rates of 100% after 48 h. The EOs were classified as highly toxic with lethal concentration (LC_{50}) of 4.58 ± 0.27 μg mL^{-1}. The isolated tween 80 did not influence viability with LC_{50} corresponding to 1106 ± 52.54 μg mL^{-1}.[47]

When in an essential oil mixtures, carvacrol doses up to 200 mg kg^{-1} in Nile Tilapia (*Oreochromis niloticus*) was not toxic to this species. In contrary, it showed toxicity against *Artemia salina*.[48] In another study, Nile Tilapia diet enhanced with fennel EO (*Foeniculum vulgare*) (1 mL kg^{-1}) was able to protect the animals from the toxic action of aflatoxin B1 produced by fungi.[49] These results support the use of *P. amboinicus* essential oil as an antimicrobial agent.

Antimicrobial activity of essential oils against *Aeromonas hydrophila*

Every EOs sample showed high antimicrobial activity (Table 4). Oils 5, 7, 8, 10 and 11 presented the lowest MIC (62.5 μg mL^{-1}). This value was smaller than the chloramphenicol standard MIC of 250 μg mL^{-1}.[50] The chemical characterization of the *P. amboinicus* EO showed an abundance of monoterpenes. Such compounds act by disorganizing and breaking the bacterial cell membrane, given its lipid nature.[51,52]

The NMR allowed the determination of the carvacrol concentration in the EOs' MICs. Each mg of essential oil used to prepare the main solutions contained, on average, 0.48 mg of carvacrol. Thus, the bacteria were subjected to concentrations of carvacrol ranging from 240 to 3.75 μg mL^{-1}.

Table 4. Antimicrobial activities of essential oils against *Aeromonas hydrophila*

Sample	Month/year	MIC / (μg mL^{-1})	MBC / (μg mL^{-1})
1	Mar/2019	125	125
2	Apr/2019	125	250
3	May/2019	125	125
4	Jun/2019	125	125
5	Jul/2019	62.5	125
6	Aug/2019	125	125
7	Sep/2019	62.5	125
8	Oct/2019	62.5	125
9	Nov/2019	125	125
10	Dec/2019	62.5	250
11	Jan/2020	62.5	125
1.21	Jan/2021	500	500
Carvacrol		500	500
Gentamicin		1.7	1.7
Chloramphenicol		250	250

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

Based on the results, no straight correlation between carvacrol concentration and antimicrobial activities could be pointed out. For example, the EOs that showed high percentages of this compound (September, October, December, and January) as well as the lowest one (July) had MIC values equal to 62.5 μg mL^{-1}. Thus, the major compound cannot be considered responsible for biological activity. This reiterates the need to use tracers even at low concentrations since they directly influence the modulation of biological activity.[40,51]

Given the experimental results obtained and the goal of this work, despite not showing biological activity, carvacrol, present in the essential oil of *P. amboinicus*, can be applied as a chemical marker monitored through the analytical techniques already described. The correlation between the chemical constitution of the compounds and antimicrobial

activities can be carried out by multivariate analyses, which are beyond the scope of our study.

Other essential oils and plant extracts are antimicrobial against *A. hydrophila* in fish. They reduced mortality and improved the life quality of these animals owning to their antioxidant, immunomodulatory and haematological effects.[53-55] The present study is the first to evaluate the activity of *P. amboinicus* EO against *A. hydrophila*. Species of Lamiaceae family, such as *Salvia pisidica*, *Ocimum gratissimum* and *Hesperozy gisringens* showed low or no antimicrobial activity with MIC and MBC values ranging from 400 to 1600 μg mL^{-1}.[56,57] In this study, *P. amboinicus*is excels against *A. hydrophila* when compared to other species of the family, thus, it is an alternative to antibiotics in the treatment of animals.

Synergism of EOs by the checkerboard method

Oils 3, 5, 7 and 10 were selected for the checkerboard assay as they had the highest MIC values and were representative of the four seasons. The synergism is observed when there is a fourfold reduction in the MIC of the agents combined. Then, all our samples showed synergistic effect.[29] The synergism obtained by the oil association was able to reduce the MIC quantity (250 μg mL^{-1}) of chloramphenicol by 4 and 8×, as shown in Table 5.

Previous studies showed that associations of *P. amboinicus* EO and other antimicrobial agents can reduce the MIC of these substances. When associated with the aminoglycosides amikacin and gentamicin, the EO provided synergistic effect against *S. aureus*, *E. coli* and *P. aeruginosa.*[17] The terpenes isolated: carvacrol, citral, eugenol, linalool and thymol also showed activity against *Aeromonas* spp., and other penicillin-resistant fish bacteria.[58]

In fish, *A. hydrophila* can cause loss of balance, apathy, exophthalmos, necrosis, hemorrhage and inflammatory infiltrate, affecting the epidermis, dermis and muscles.[59] Results imply that the *P. amboinicus* EO can be used either isolated or combined, reducing the need of antibiotics in the treatment of these animals.

Commercial extruded feed containing the essential oil of *P. amboinicus*

In the first moment, NMR spectra of bare fish feed were obtained and used as a comparison and monitoring parameter of the carvacrol content. After the EO incorporation, signals in the region of aromatic hydrogens between 6 and 8 ppm referring to the presence of carvacrol, the majority compound in the essential oil (Figure 2) were observed (Figure S1, Supplementary Information (SI) section).

The fish feed samples were sprayed with an EO volume sufficient to obtain groups containing 5 and 10% of essential oil. Also, as previously mentioned, the qNMR technique was useful to measure the EO content in samples from its chemical marker after drying. The EO used for incorporation had, in average, 562.9 mg g^{-1} (56.29 ± 1.14%) of carvacrol *per* g of sample. Figure 3 presents the carvacrol average before and after drying.

Results show a substantial loss of carvacrol (Figure 3) and hence, OE in the feed after drying. These considerable drops are related to its evaporation alongside the solvent. The OE loss data described here are preliminary and do not accurately predict the long-term behavior of the OE in the formulation. Therefore, further research and analysis of formulations is required to determine the evaporation rate of the oil under different preparation and storage conditions. With the information about carvacrol content, after drying, 30, 60 and 125 mg of dried samples, initially sprayed with 10% of EO, were weighed (Table S1, SI section). For each concentration, were prepared 3 groups ($T1_{10\%}$-$T3_{10\%}$) and for each group, the 3 measures were carried out.

Table 5. Modulatory effect of *Plectranthus amboinicus* essential oils in association with chloramphenicol against *Aeromonas hydrophila*

Sample	Month/year	MIC / (μg mL^{-1})		Σ FIC	Result
		Isolated	Combined		
3	Mar/2019	125	31.25	0.50	synergic
	chloramphenicol	250	62.5		
5	Jul/2019	62.5	15.6	0.38	synergic
	chloramphenicol	250	31.25		
7	Sep/2019	62.5	15.6	0.38	synergic
	chloramphenicol	250	31.25		
10	Dec/2019	62.5	15.6	0.38	synergic
	chloramphenicol	250	31.25		

FIC: fractional inhibitory concentration; MIC: minimum inhibitory concentration.

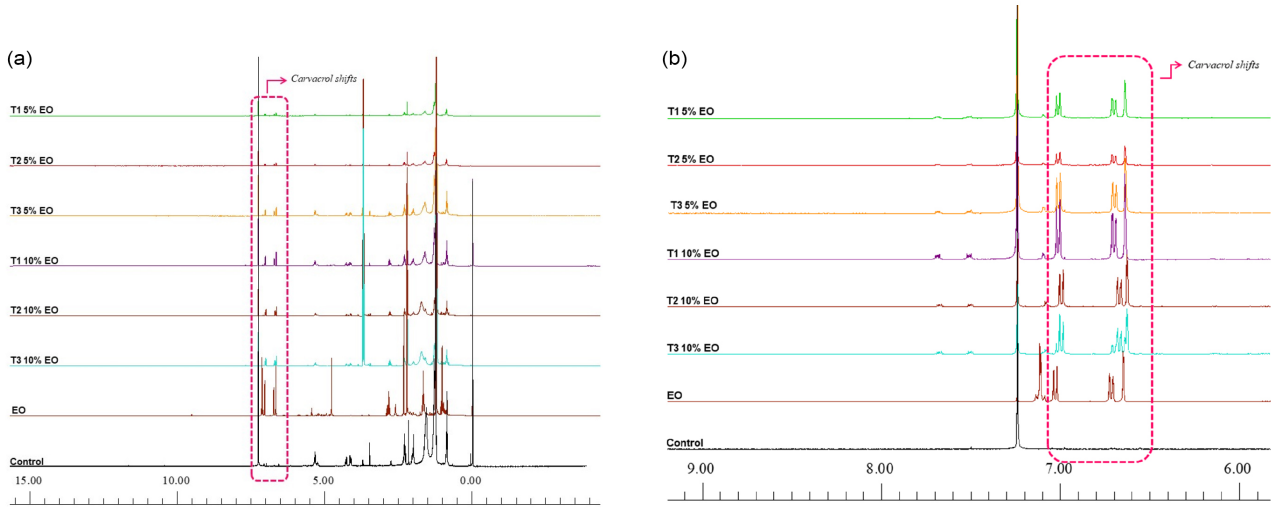

Figure 2. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of extruded feed samples with and without *Plectranthus amboinicus* essential oil. (a) Total ^{1}H NMR from fish feed samples; (b) aromatic region of ^{1}H NMR; T: triplicate; control: feed sample with essential oil replaced by ethanol 93%.

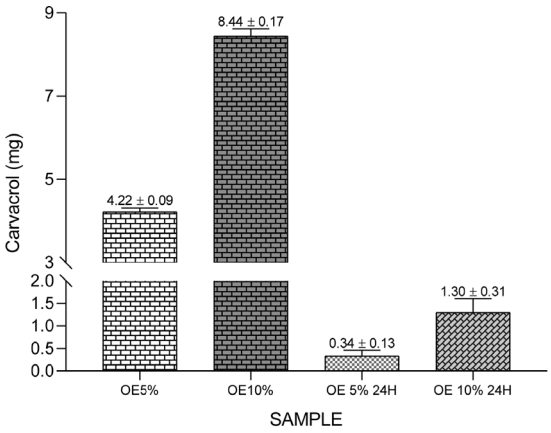

Figure 3. Carvacrol content in fish feed before and after drying. EO5%: groups with 5% (m/m) of essential oil from *Plechtranthus amboinicus*; OE10%: groups with 10% (m/m) of essential oil from *Plechtranthus amboinicus*. Results expressed as mean ± standard deviation, n = 3.

Despite the significant evaporation, the feeds were able to inhibit bacterial growth and promote the death of *Aeromonas hydrophila*, both on MIC and MBC assays. In these tests, the bacteria were exposed to carvacrol concentrations ranging from 5.23 to 1280 μg mL^{-1}, approximately (Table S1). Values varied between 310 and 1280 μg mL^{-1} of carvacrol, and between 640 and 1280 μg mL^{-1} for MIC and MBC, respectively.

Feed should contain sufficient nutrients for the development of the animals, with protein (25 to 70%) being the main components. In addition to high protein content, they should be formulated with sources of starch (5 to 60%), lipids (up to 22%), fibers and vitamins (15 to 20%).[60] It is known that different food components can reduce the activity of EOs components, mainly fats, proteins and complex sugars such as starch, besides the influence of pH and temperature.[61,62] In the NMR spectra obtained from the feed it is possible to observe signals among 0-2.5 ppm, a region commonly attributed to the presence of fatty acids.

Thus, these components promoted interaction with the EO, hampering its extraction and directly interfering with the antimicrobial activity since the results were slightly lower than expected. Strategies to overcome interaction between the components and evaporation loss are nanoencapsulation of the EO or the use of cyclodextrins. However, these alternatives can increase the production cost.[63,64]

Like this, the data discussed indicate: (*i*) the potential of the *P. amboinicus* EO incorporated into feeds for infections caused by *A. hydrophila* in fish; (*ii*) benefits on growth, immunomodulation and protection of the intestinal microbiota of these animals.[65]

Conclusions

Seasonal analysis of the essential oils of *P. amboinicus* was made. No noteworthy difference in the amount of the major metabolite was noticed. Low antioxidant activity results suggest an influence of trace compounds, and despite the high toxicity, *in vivo* assays are necessary for understanding the impacts of the toxicity on fish.

The EO displayed excellent synergistic results when combined with chloramphenicol. Likewise, the *P. amboinicus* OE maintained its antibacterial activity when added to fish feed. Hence, *P. amboinicus* can be considered a promising species for controlling infectious diseases in fish, dipping the over use of antibiotics related to rapid bacterial resistance. The present study brings pioneering results concerning the application of the EO in feed for fish farming.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Juliane M. S. Silva was responsible for research, formal analysis and writing of the original manuscript; Flávia C. R. Vilar and Gabriel A. B. Lima for cultivation, maintenance of the medicinal herb garden, monthly collections; Elizangela Maria de Souza for planning, research, and formal data analysis; Livia M. Dutra, Jackson Roberto G. da Silva Almeida, Larissa A. Rolim and Norberto P. Lopes for research, formal data analysis and review of the manuscript; Ana Paula de Oliveira for research, formal data analysis, supervision, and review of the manuscript.

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