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Exploring Antimicrobial Potential of Cinnamon, Clove, Peppermint and Black Cumin Essential Oils against Fish Bacterial Pathogens with an Emphasis on the Dietary Supplementation Effects of Cinnamon Oil on Striped Catfish (*Pangasianodon hypophthalmus*)

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Abstract

Emergence of antimicrobial resistance (AMR) among fish bacterial pathogens is one of the major global public threats. Attempts are being made to develop novel alternatives as a promising approach to combat multidrug resistance disease-causing bacteria. Natural antimicrobials such as essential oils (EOs) are a potential unique strategy to treat bacterial infections with a reduced risk of resistance developing. This study aimed to evaluate the antimicrobial activity of some essential oils (EOs) namely, cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), Peppermint (*Mentha piperita*) , and black cumin (*Nigella sativa*) against some fish pathogens implicated with aquaculture disease outbreaks like *Aeromonas* hydrophilia, *Pseudomonas* fluorescence, *Photobacterium damselae* and *Streptococcus agalactiae* using agar well diffusion assay. We found significant differences on the antibacterial activity depending on the type of essential oils and bacterial strain. Among all the tested EOs, cinnamon essential oil (CEO) was shown to be the most effective with minimum bactericidal concentration (MBC) ranged from 0.0156-0.125 ml/ml. As a result, it was selected for our in vivo investigations. We next aimed to investigate the effects of dietary CEO on growth performance, disease resistance and immune response of fish. A total of 150 striped catfish (*Pangasianodon hypophthalmus*) were fed with different levels of CEO (0, 1.50, 2.0, 2.50, and 3.0 mL/kg diets) (assigned as control, Diet 1, Diet 2, Diet 3 and Diet 4) for 60 days. Compared to control, fish fed with graded levels of dietary CEO showed significant ($P < 0.05$) increase in final body weight, weight gain %, and specific growth rate particularly at fish group fed diet 3. Of interest, there were no significant differences ($P > 0.05$) in feed conversion ratio and survival rates among control and CEO-supplemented groups. Moreover, we found significant ($P < 0.05$) increases in plasma lysozyme activity and total IgM levels in a dose dependent manner with dietary CEO supplementation. After feeding trials, we investigated their potential to defend striped catfish against *A. hydrophila* challenge. Fish fed control diet had the highest mortality rates; in contrast, fish fed diets supplemented with CEO had higher levels of resistance to the bacterial infection, with the lowest mortality rates in the fish group fed diet 3. Overall, these findings showed that EOs exhibit a great potential to be used as antimicrobial agents against fish pathogens. Moreover, dietary administration of CEO, particularly at 2.5 ml/kg feed, can be regarded as a promising component for improving growth, immunological responses, and potential alternatives to conventional antimicrobials for control of microbial infections in fish.

KEYWORDS
Essential oils, Antimicrobial resistance, Striped catfish, Performance,Immune response, Resistance

INTRODUCTION

In recent years, global aquaculture expanded rapidly, and become one of the most promising and fastest-growing sectors in the animal-derived food production system (FAO, 2016). However, several bacterial infections are frequently encountered in aquaculture, resulting in enormous financial losses. Among these, *Aeromonads*, *Pseudomonads, Photobacterium* and *Streptococcus* (Aziz and Abdullah, 2021).

Indiscriminate antimicrobial use resulted in the emergence of antimicrobial resistance (AMR), a global health issue that accounts for yearly millions of deaths (WHO, 2019; Pepi and Focardi, 2021) combined by a problematic economic loss estimated at $6 billion losses in 2014 (Subasinghe *et al*., 2019). In aquaculture, AMR has been reported even in open water systems of rivers, lakes and oceans worthily (Zhang *et al*., 2014; Suzuki *et al*., 2017). Production intensification and rising prevalence of infectious diseases attributed to bacterial fish pathogens, which accounts for 90% of all infections recorded in aquaculture, have increased the need of antimicrobials (Mozirandi and Mukanganyama, 2017).

The rise of antimicrobial resistance has recently prompted researchers to find novel natural antimicrobial agents. Among such alternatives, essential oils (EOs), volatile chemicals that develop naturally in various plant parts during secondary metabolism (Swamy *et al*., 2016). Several essential oils derived from diverse plants have been pharmaceutically stated to possess a safe strong antimicrobial activity (Ali *et al*., 2015). Their content of different types of antimicrobial compounds explains their effect against a wide scope of pathogens (Gurumurthy *et al*., 2013).

Several studies demonstrated that EOs have a potent ef-

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fectiveness against several infectious fish bacterial infections as *A. salmonicida*, *Vibrio harveyi*, and *S. agalactiae* (Dawood *et al*., 2021). Where sick fish will frequently stop feeding and feeding dependent therapy (Craig *et al*., 2017), its critical to previously protect fish from infection onset, therefore we aimed to evaluate the antibacterial efficacy of some economic EOs such as cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), Peppermint (*Mentha piperita*) , and black cumin (*Nigella sativa*) against four of the most outbreak causing bacteria in aquaculture; namely (*A. hydrophila*, *P. fluorescence* and *P. damselae* and *S. agalactiae*) Additionally, we aimed to investigate the effects of the most potent antimicrobial essential oil as a feed additive supplementation on growth performance, immunological response, and disease resistance to experimental infection in striped catfish (*Pangasianodon hypophthalmus*).

MATERIALS AND METHODS

Bacterial strains

Aeromonas hydrophila (ATCC 13037), *Pseudomonas* fluorescens (ATCC 13525) and *Streptococcus agalactiae* (ATCC 13813) were obtained from Faculty of Agriculture, Ain Shams University, Egypt. *Photobacterium damselae* was kindly provided by Microbiology Lab., Marine Environment Division, National Institute of Oceanography and Fisheries, Egypt, and was previously isolated and identified from fishes (Abu-Elala *et al*., 2015).

Essential Oils (EOs)

The ready-made herbal oils of cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), peppermint (*Mentha piperita*) and black cumin (*Nigella sativa*) were purchased from National Research Centre, Cairo, Egypt. These oils were kept in amber-colored bottles at 4°C until usage.
Dimethylsulfoxide (DMSO) was used as oil solvent

Preparation of the bacterial inoculum

In brief, each bacterial strain was adjusted at a concentration of 108 cells/ml using 0.5 MacFarland standard (Andrews, 2001), a cell turbidity adjusted from 0.08 to 0.13 optical density at wavelength of 625 nm (Wiegand *et al*., 2008).

The bacterial suspension was prepared from bacterial cells cultured overnight on a non selective solid or liquid medium. The inoculum was added to the liquid media or placed on solid media within 30 min of preparation so that cell density (CFU/ml) is maintained (Andrews, 2001; Kowalska-Krochmal and Dudek-Wicher, 2021).

Qualitative evaluation of antibacterial activity of EOs

Using agar well diffusion assay, the screening of EOs for antibacterial activity was determined according to Kowalska-Krochmal and Dudek-Wicher (2021) with some modifications. Mueller Hinton agar (Oxoid, UK) was melted and cooled to 45ºC. A standardized inoculum of each bacterial strain at a concentration of 1×10^8 CFU/ml, 0.5 McFarland was added aseptically to the cooled media. The medium was carefully poured into agar plates and allowed to solidify. After solidifying, circular wells (8 mm) were punched, and bottoms were sealed with soft agar media. Oil stock solution (4 ml/ml DEMSO, 50 µl / well) was loaded in the wells. Sterile phosphate buffer saline was used as a control negative. The agar allowed to absorb the inoculums. The plates were incubated at 37°C for 24 h to observe the zones of inhibition. Oils antibacterial potency was assessed by the presence or absence of inhibition zone surrounding the wells. The assay was performed in triplicate.

Quantitative evaluation of antibacterial activity of EOs

The determination of minimum bactericidal concentration (MBC) of EOs was performed by agar dilution method according to Bonev *et al*. (2008). Oil stock solution was two-fold serially diluted in sterile DEMSO solvent up to 10 concentrations (4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.031, 0.0156 and 0.0078 ml/ml). Fifty µl of the different concentrations were added to its representative well in the previously inoculated agar of the four tested bacterial strains. After incubation at 37°C for 24 h, plates were observed for inhibition. The plates were reincubated to ensure inhibition zone stability. The lowest concentration showing an inhibition zone after 48 h was recorded as the minimum bactericidal concentration of the tested oils.

In vivo experiment

Ethical Approval

All investigations and handling of fish were carried out in accordance with relevant guidelines of Ethics Committee of the Animal Health and Welfare of Damanhour University, Egypt.

Preparation of experimental diets

The formulation and approximate composition of the experimental diets based on recommended dietary guidelines (Allam *et al*., 2020), and the proximate composition of the feed ingredient is shown in Table 1. Fish meal (72% crude protein; CP) and soybean meal (45% crude protein; CP) were used as the primary protein sources in the basal diet (30% CP). Cinnamon essential oil (CEO) was mixed into experimental diets at various concentrations (0, 1.50, 2.0, 2.50, and 3.0 mL/kg diets) (assigned as control, Diet 1, Diet 2, Diet 3 and Diet 4). All of the ingredients were thoroughly mixed, and an appropriate amount of water was carefully added to form the paste. Then, the experimental diets were prepared as dry pellets with a 2 mm diameter, placed in clean plastic containers, and stored at 4°C until use.

Fish rearing and husbandry

A total of 150 fingerling striped catfish (*Pangasianodon hypophthalmus*) with an average body weight of 17.5±0.5 g were obtained from a private fish farm located at Borg El Arab, Alexandria, Egypt, and acclimated in fiberglass containers with a 1000 L capacity for two weeks. Fish were fed the basal diet during acclimation until evident satiety at 7:00, 12:00, and 17:00 h. After acclimation, fish were assigned into five groups and reared in twelve 100-L rectangular glass tanks at a density of 10 fish per tank in triplicates (n.= 30 fish/ group, 10 fish/replicate). These aquariums were used for acclimation for 10 days before the feeding trial began. Then, fish were given the experimental diets for 60 days, three times a day at 7:00, 12:00, and 17:00 h, until they appeared satiated. All aquariums received constant aeration, and 35% of the water in each tank was drained daily along with waste materials and replaced with fresh water. In each tank, the following parameters were monitored twice daily at 8:00 and 16:00 h: temperature, dissolved oxygen (DO-meter, YSI 518, YSI, Ohio, USA), and pH (pH-meter, Hana 8424, Hungary). The readings

were 27.50±1°C, 6.5±0.2 mg/L, and 7.7±0.5 for temperature, DO and pH respectively. Colorimetric reagents (La Chappelle, France) were used to measure the unionized ammonia and nitrite levels, and they were 0.02±0.01 and 0.04±0.01 mg/L, respectively. All these variables fulfilled with the ideal conditions for fish cultivation (Boyd and Tucker, 2012).

Table 1. Ingredients and proximate chemical composition (% on dry matter basis) of the basal diet used in the current study.

Feed ingredients	%
Fish meal (62% CP)	10
Soybean meal (48% CP)	34
Corn gluten meal (60% CP)	3.5
Rice bran	14
Yellow corn meal	15
Wheat bran	9
Wheat flour	13
Sunflower oil	0.7
Vitamin and Mineral premix [a]	0.3
Dicalcium Phosphate	0.5
Total	100
Proximate chemical analysis (%) on DM basis	
Dry matter (DM)	90.19
Crude protein (CP)	30.82
Ether extract (EE)	6.93
Ash	6.59
Crude fiber (CF)	8.81
Nitrogen free extract (NFE) [b]	46.85
Gross energy (GE; KJ /g diet DM) [c]	18.06
Protein to energy ratio (P/E) ratio [d]	17.06

[a]Composition of vitamin and mineral premix mixture (per kg premix):
Vitamins such as vitamin B1 (700 mg), vitamin C (500 mg), vitamin B2 (3500 mg), vitamin B6 (1000 mg), vitamin B12 (7 mg), vitamin A (8,000,000 IU),
vitamin D3 (2,000,000 IU), vitamin E (7000 mg), vitamin K3 (1500 mg), biotin (50 mg), folic acid (700 mg), nicotinic acid (20,000 mg), and pantothenic acid (7000 mg). Minerals such as zinc (40 g), iron (20 g), copper (2.7 g), iodine (0.34 g), manganese (53 g), selenium (70 mg), cobalt (70 mg) and calcium carbonate as carrier up to 1 kg
[b]NFE = 100 - (CP + EE + CF + ash)
[c]GE was calculated based on 23.60, 39.40 and 17.20 kJ /g of CP, EE and NFE, respectively in accordance with the guidelines of NRC (2011).
[d]P/E ratio was calculated as mg crude protein/KJ GE.

Growth performance analysis

Fish per aquarium were counted and weighed individually at the ending of the experiment. The following parameters for feed utilization and growth performance were determined using the following formulas:
Weight gain % (WG %) = 100 [(final fish weight (g) – initial fish weight (g))/ initial fish weight].
Specific growth rate (SGR; %/day) = 100 [(Ln final fish weight – Ln initial fish weight / experimental time (days)].
Feed conversion ratio (FCR) = Feed intake (g) / weight gain (g).
Fish survival (%) = 100 (final fish number / initial fish number).

Blood sampling

At the end of 60 days, fishes in each aquarium were individually weighed., nine fish per each group (three fish per aquarium) were randomly chosen, and clove oil was used to anesthetize them. Pooled blood samples were taken from the caudal blood vessels (3 fish per replicate), and an aliquot of each blood sample was mixed with an anticoagulant for hematological assessments. Another aliquot of blood without an anticoagulant was used for the immunological evaluations, and it was centrifuged at 3000×g for 15 minutes at room temperature and then kept at - 20°C till examination.

Immunological assays

The turbidimetric assay described by Ellis (1990) was used for estimating plasma lysozyme activity using a suspension of Micrococcus lysodeikticus (Sigma, USA). A standard curve formed using chicken egg white lysozyme (Sigma, USA) was used to determine the lysozyme activity for all samples. According to Siwicki and Anderson (1993), total immunoglobulin M (IgM) in plasma was measured in accordance with the manufacturing procedure (Cusabio Biotech Co. Ltd., Wuhan, Hubei, China).

Bacterial challenge test

After 60 days of feeding and blood samplings, the fish were injected IP with 0.1 mL of phosphate-buffered saline (PBS) containing 1×10^6 CFU/mL of a pathogenic strain of *A. hydrophila* (ATCC 13037), In accordance with Abdelhamid *et al.* (2021) Instructions. The control group was injected with sterile PBS solution. During the challenge test, fish were fed on prescribed diets for each treatment. All groups were observed for 10 days to monitor any mortality. At the end of the observation, the cumulative fish mortality curve and their survival rates were recorded. According to Amend (1981), the relative percent of survival (RPS%) was determined using the formula RPS = 100 [1 – (% mortality in treated fish / % mortality in the control fish)].

Statistical analysis

Using the Kolmogorov-Smirnov and Bartlett's tests, respectively, all data was analyzed for normality of distribution and homogeneity of variances across various treatments. The impact of dietary CEO supplementation on the measured parameters was then assessed using one-way ANOVA. The Duncan test was used as a post-hoc test, and differences between means were considered statistically significant at $P < 0.05$. The SPSS program version 20 (Richmond, VA, USA) was used for all statistical analyses.

RESULTS

Screening of bactericidal activity of essential oils

The antimicrobial activity of tested EOs against the most common bacterial fish pathogens is shown in Table 2. There were significant differences based on the type of essential oils and bacterial strain. Cinnamon EO and clove EO were the most effective against all the tested bacterial isolates. Peppermint EO showed bactericidal activity against both *P. fluorescens* and *S. agalactiae*, however no effect against *A. hydrophila* and *P. damselae*. Black cumin EO displayed bactericidal activity against both *P. damselae* and *S. agalactiae* but had no effect against *A. hydrophila* and *P. fluorescens*.

Minimum bactericidal concentration (MBC) of essential oils

The quantitative bactericidal activity of EOs against the most common bacterial fish pathogens is presented in Table 3. It was found that cinnamon EO was effective against *P. damselae* and *P. fluorescens* in a concertation of 0.0156 and 0.125 ml/ml respectively, and 0.062 ml/ml for *A. hydrophila* and *S. agalactiae*. Clove

EO was effective against *P. fluorescens* and *S. agalactiae* in concentration of 1 and 0.5 ml/ml, respectively and 0.25 ml/ml for both of *A. hydrophila* and *P. damselae*. Peppermint EO was effective against *P. fluorescens* in a concentration of 0.5 ml/ml and *S. agalactiae* in a concentration of 1 ml/ml, where it has no effect against *A. hydrophila* and *P. damselae*. Black cumin EO had no effect against both of *P. fluorescens* and *A. hydrophila*, where its effectiveness was 0.5 and 4 ml/ml for *S. agalactiae* and *P. damselae*, respectively.

Effect of dietary Cinnamon oil on growth performance of striped catfish

Growth performance of striped catfish fingerlings fed with CEO-supplemented diets for 60 days is presented in Table 4 and Fig. 1. Compared to control group, fish fed with graded levels of dietary CEO showed significant ($P < 0.05$) increases in final body weight, weight gain % and specific growth rate particularly at fish group fed diet 3. The highest feed intake values were no-

Table 2. The qualitative bactericidal activity of essential oils against bacterial fish pathogens.

Tested EOs	Bacterial isolates			
	A. hydrophila	*P. fluorescens*	*P. damselae*	*S. agalactiae*
Cinnamon	+VE	+VE	+VE	+VE
Clove	+VE	+VE	+VE	+VE
Peppermint	-VE	+VE	-VE	+VE
Black cumin	-VE	-VE	+VE	+VE

Bacterial susceptibility to essential oils was evaluated by the presence or absence of inhibition zones, where +VE indicates presence of an inhibition zone and -VE indicates absence of an inhibition zone.

Table 3. The quantitative bactericidal activity of essential oils against bacterial fish pathogens.

Tested EOs (mL oil/mL DEMSO)	MBC against the tested bacterial isolates			
	A. hydrophila	*P. fluorescens*	*P. damselae*	*S. agalactiae*
Cinnamon	0.06	0.13	0.02	0.06
Clove	0.25	1	0.25	0.5
Peppermint	-VE	0.5	-VE	1
Black cumin	-VE	-VE	4	0.5

-VE indicates absence of an inhibition zone.

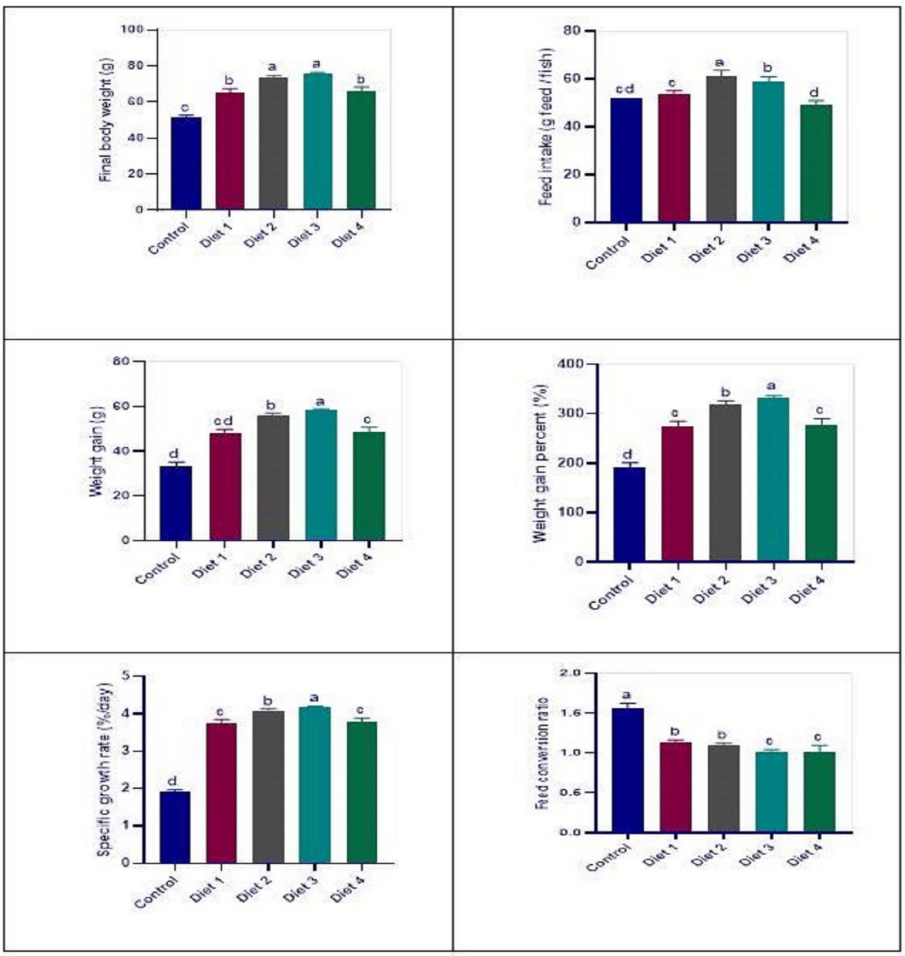

Fig. 1. Growth performance and feed utilization of striped catfish fingerlings fed on diets supplemented with various levels of cinnamon essential oil (CEO) for 60 days.

ticed in fish group fed on diet 2. Meanwhile, the lowest values were recorded in fish group fed on diet 4. Of interest, there were no significant differences (P > 0.05) in feed conversion ratio values among the different treatments. No significant differences were detected in survival rates of fish between the control and CEO-supplemented groups.

Effect of dietary Cinnamon on immune response of striped catfish

Striped catfish fed CEO-supplemented diets exhibited significant (P<0.05) increases in plasma lysozyme activity and total IgM levels in a dose dependent manner. The highest values were recorded in fish group fed diet 4 (Fig. 2 and Table 5).

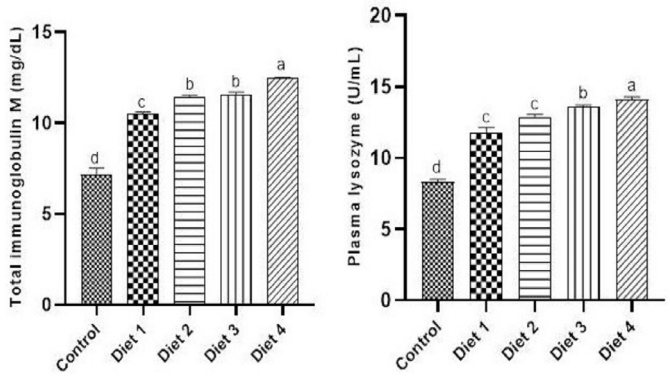

Fig. 2. Changes in plasma immune assays (total immunoglobulin M (IgM) and lysozyme activity) of striped catfish fingerlings fed on diets supplemented with varied concentrations of cinnamon essential oil (CEO) for 60 days. Different superscript letters indicate significant difference (P<0.05). Values represent means ± SEM.

Post-challenge cumulative mortality

The cumulative mortality of the various CEO supplementation groups following a challenge with *A. hydrophila* is shown in Fig. 3 and Table 6. Fish fed the control diet had the highest mortality rates; in contrast, fish given diets supplemented with CEO had higher levels of resistance to the bacterial infection, with the lowest mortality rates in the fish group fed diet 3.

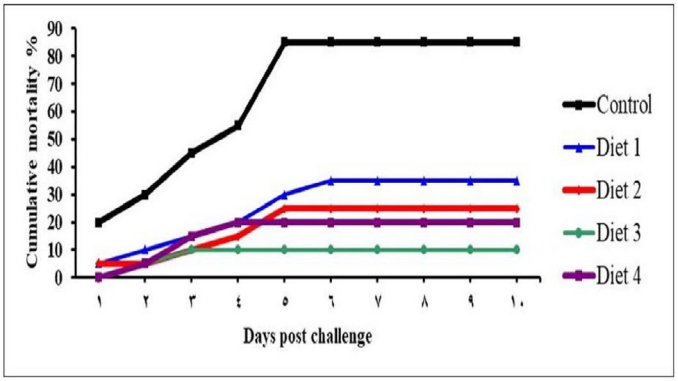

Fig. 3. Cumulative mortality (%) of striped catfish fingerlings fed on diets supplemented with varying concentrations of cinnamon essential oil (CEO) for 60 days, and post-challenged with *A. hydrophila* and observed for 10 days.

DISCUSSION

Exploring the antibacterial properties of natural resources, such as essential oils (EOs) of plants, has become crucial due to the rising resistance of fish pathogens to conventional treat-

Table 4. Growth performance and feed utilization of striped catfish fingerlings fed on diets supplemented with various levels of cinnamon essential oil (CEO) for 60 days.

Parameters	Control	Diet 1	Diet 2	Diet 3	Diet 4	*P value*
Final body weight (g)	51.08±0.87c	65.33±1.17b	73.33±0.73a	75.83±0.33a	66.17±1.20b	<0.0001
Feed intake (g)	52.00±0.00 cd	53.83±0.73c	61.00±1.53a	59.00±1.16b	49.00±1.16d	0.00
Weight gain (g)	33.58 ± 0.87d	47.83 ± 1.17 cd	55.83 ± 0.73b	58.33±0.33a	48.67±1.20c	<0.0001
Weight gain %	191.9 ± 4.97d	273.3 ± 6.67c	319.0 ± 4.15b	333.3±1.90a	278.1±6.87c	<0.0001
Feed conversion ratio	1.01±0.51	1.127±0.02	1.093±0.01	1.01±0.02	1.01±0.05	0.10
Specific growth rate	1.913±0.03d	3.763 ± 0.05c	4.093±0.03b	4.19±0.01a	3.8±0.05c	<0.0001
Survival rate (%)	100	100	100	100	100	0

In each row, different letters indicate significant differences between groups (p<0.05). Values represent means ± SEM

Table 5. Plasma immune assays of striped catfish fingerlings fed on diets supplemented with various levels of cinnamon essential oil (CEO) for 60 days

Parameters	Control	Diet 1	Diet 2	Diet 3	Diet 4	*P value*
Total IgM (mg/dL)	7.17±0.33d	10.52±0.07c	11.41±0.11b	11.55± 0.14b	12.46± 0.05a	<0.0001
Plasma lysozyme (U/mL)	8.32±0.19d	11.75±0.38c	12.88±0.17c	13.63± 0.07b	14.14± 0.15a	<0.0001

In each row, different letters indicate significant differences between groups (p<0.05). Values represent means ± SEM

Table 6. Mortality rate (%), survival rate (%), and relative percentage survival (RPS; %) of striped catfish given diets that included varying amounts of cinnamon essential oil (CEO) for 60 days.

Groups	Mortality rate (%) *	Survival rate (%)	RPS (%)
Control	85.0a	15.0e	
Diet 1	35.0b	65.0d	58.8
Diet 2	25.0c	75.0c	70.6
Diet 3	10.0e	90.0a	88.23
Diet 4	20.0d	80.0b	76.47

According to Tukey's post-hoc tests (Means ± SE), different letters imply significant variations.
* Mortality (%) equal (number of dead fish / total number of fish) × 100. RPS (%) = [1 – (cumulative mortality of the treated group/cumulative mortality of the control group)] × 100.

ments. Development of products that effectively lower or prevent bacterial infections in fish is essential (Da Cunha *et al*., 2012). Thus, the aim of this study was to assess in vitro antimicrobial activity of some essential oils such as cinnamon, clove, peppermint, and black cumin against some fish pathogens implicated with aquaculture disease outbreaks like *A. hydrophila*, *P. fluorescence* and *P. damselae* and *S. agalactiae*.

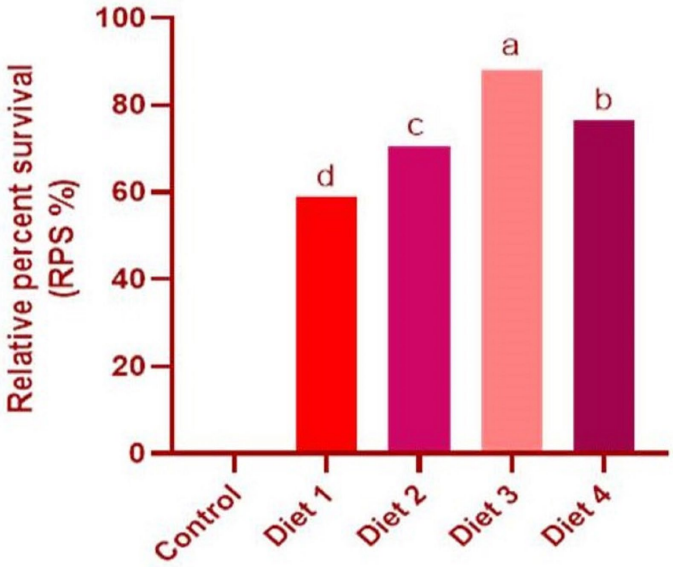

Fig. 4. Variations in the relative percentage survival (RPS) of striped catfish that were given diets supplemented with varying concentrations of cinnamon essential oil (CEO) for 60 days, then post-challenged with *A. hydrophila* and monitored for 10 days afterward. Values are shown as the mean ± SE; bars with various letter designations are significantly different ($P < 0.05$).

The antimicrobial efficacy was evaluated qualitatively by presence or absence of inhibition zones using agar well diffusion method. Moreover, quantitatively, the bactericidal activity was evaluated by determining its MBC. Significant differences in the antibacterial effect of essential oils, depending on type of essential oils and bacterial strain were found (Table 2). The highest antibacterial activity against all tested bacteria was detected in cinnamon and clove EOs with MBC ranged from 0.0156-0.125 ml/ml for cinnamon EO and 0.25^{-1} ml/ml for clove EO. Peppermint EO showed bactericidal activity against both *P. fluorescens* and *S. agalactiae* with MBC of 0.5 and 1 ml/ml, respectively, however no effect against *A. hydrophila* and *P. damselae*. Black cumin EO displayed bactericidal efficacy against both *S. agalactiae* and *P. damselae* with MBC of 0.5 and 4 ml/ml, respectively, whereas *A. hydrophila* and *P. fluorescens* were unaffected. The antimicrobial property of EOs depend on the type and the quantity of their functional bioactive constituents (Nazzaro *et al*., 2013), that can explain the different antimicrobial activities among oils against the examined bacterial strains.

These findings are relatively in agreement with the previous investigations described the antibacterial activity of cinnamon, clove, mint, and black cumin against Gram positive and Gram negative bacteria and revealed a wide range in the tested strains susceptibility to the tested essential oils. According to Rios and Recio (2005), clove oil exhibited substantial inhibitory effects against pathogenic bacteria in fish, being able to suppress bacterial growth with a MIC as low as 0·015 μg ml^{-1}. The antibacterial effects of four essential oils against *Streptococcus iniae* were investigated by Rattanachaikunsopon and Phumkhachorn (2010), cinnamon oil was the most effective antibacterial agent. According to Kačániová *et al.* (2017), all of the evaluated essential oils had antibacterial action, however *Cinnamomum zeylanicum* was the most potent against seven *Pseudomonas* species isolated from fish. *Mentha piperita* was found to have antibacterial action against *S. agalactiae*, which was isolated from a diseased tilapia (*Oreochromis niloticus*) (Majolo *et al*., 2018). According to Chagas *et al.* (2020), the essential oils of Mentha species demonstrated antibacterial action against 12 isolates of *Aeromonas* spp. Cinnamon oils exhibited promising antimicrobial effect against bacteria isolated from diseased silver catfish, according to Bandeira Junior *et al*. (2022).

It is crucial to note that some EO components may be lost during estimation due to their volatility (Burt, 2004; Scorzoni *et al*., 2007), which may account for the negative results obtained by black cumin EO against *A. hydrophila* and *P. fluorescens* and by peppermint EO against *A. hydrophila* and *P. damselae* and may predict the more applicable stability of cinnamon and clove EO.

The hydrophobicity of EOs has a substantial correlation with their antimicrobial action (Dorman and Deans, 2000). It has been shown that Gram negative bacteria typically exhibit greater resistance to EOs than Gram positive bacteria due to the differences in the structure of Gram positive and Gram negative bacteria's cell walls. The main component of the cell walls of Gram-positive bacteria is peptidoglycan, which is connected to other molecules like proteins and teichoic acid that enables hydrophobic compounds to act on both the cell wall and the cytoplasm by easily entering the cells. In addition, phenolic compounds in EOs typically exhibit antibacterial action against Gram positive bacteria. Whereas Gram negative bacteria are assumed to be less sensitive to the effects of EOs than Gram positive bacteria due to the hydrophilic lipopolysaccharides (LPS) on their outer membranes, which act as a barrier to the hydrophobic components like those in Eos. However, due to the porin proteins in Gram negative cell wall, the hydrophobic components of EOs can slowly pass through the outer membrane and enter the periplasm of the bacteria (Nazzaro *et al*., 2013). These confirm our findings that the Gram positive bacterium *S. agalactiae* was susceptible to all of the tested EOs, whereas other Gram negative strains, including *A. hydrophila*, *P. fluorescens*, and *P. damselae*, showed varied degrees of susceptibility. Additionally, as reported in a number of previous studies (Burt, 2004; Dhifi *et al*., 2016; Fokou *et al*., 2020), it is noticeable that EO compositions vary from one another substantially to influence the susceptibility of both Gram-negative and Gram-positive bacteria.

Using different estimating techniques which are not universally standardized make the comparison between results is a challenge. A detailed study on the component's nature of EOs and the development of a standardized technique to evaluate and compare the essential oils antibacterial activity will be helpful to improve our understanding of their application as a novel antibiotic drug (Trong *et al*., 2020).

Dietary supplementation with herbal essential oils is suggested for aquatic animals due to their potential for enhancing growth, improving the immune system and reducing free radicals (Magouz *et al*., 2021). Reviews on the use of medicinal plants and EOs in aquaculture for the prevention and/or treatment of disease, and their antibacterial capabilities have already been published (Bulfon *et al*., 2015; Da Cunha *et al*., 2012). Our in vitro findings revealed that cinnamon EO exhibited the most potent antibacterial activity, however, little is known about cinnamon's efficacy in aquatic organisms. Thus, we next aimed to determine how CEO dietary supplements affected striped catfish (*Pangasianodon hypophthalmus*) performance, immunological function, and disease resistance.

In-vivo application of cinnamon oil as a feed additive for pangasius fish showed that final body weight, weight gain percentage and specific growth rate of fish fed with graded amounts of dietary CEO increased significantly ($P < 0.05$) compared to control group and appeared to peak in the fish group fed diet 3. Of interest, there were no significant differences ($P > 0.05$) among treatments and control in terms of FCR and survival rate. We suggest that dietary supplementation with CEO significantly enhances the palatability, feed intake, growth rate and the final weight gain of pangisus fish in a dose-dependent manner. Nevertheless, we noticed that a dose of 3 ml/kg in group 4 has a less feed intake and a less weight gain than group 3. We propose that increase of cinnamon oil in a dose over 2.5 mg/kg may decrease the feed palatability and subsequently decrease the feed intake and the

final body gain of the fish. These findings are consistent with several investigations that explored into the impact of cinnamon in boosting growth in various finfish species. Rattanachaikunsopon and Phunkhachorn (2010) found that feeding Nile tilapia, *Oreochromis niloticus*, 0.4% cinnamon oil during 28-day trial improved growth parameters. Moreover, dietary cinnamon oil, at a dosage of 4 mL/kg, has been shown by Kesbiç (2019) to improve rainbow trout growth performance.

Recently, there has been a rising interest in using herbal immunostimulants in aquaculture to strengthen fish immune systems and defend them from infection (Wang *et al*., 2016; Tadese *et al*., 2022). Our findings showed that dietary cinnamon supplementation significantly improved immunological response, as evidenced by significantly higher levels of total IgM and lysozyme activity in striped catfish fed 3 ml/kg feed cinnamon than fish fed 0, 1.5, 2 and 2.5 mL/kg ($P < 0.05$) compared to control group. Where, lysozyme is an important tool to estimate the innate immune system (Amphan *et al*., 2019). Furthermore, IgM also serves as the first line of the host natural non-specific (Liu *et al*., 2019) and specific humoral immune responses (Ouchida *et al*., 2012). IgM binds to antigen and activate complement system to activate the classical specific immunity pathway (Sathe and Cusick, 2021). Accordingly, we assume that CEO supplementation promotes the striped catfish's innate and specific immune response. These findings were consistent with previous studies by Dawood *et al*. (2022) suggested that essential could enhance fish immunity.

Following the feeding trial, *A. hydrophila* was IP injected into the fish, and the 10-day cumulative mortality was determined. Fish fed the control diet displayed the highest mortality 85%, whereas fish given diets supplemented with CEO were more resistant to the bacterial infection. The fish group fed diet 3 had the lowest mortality. Thus, we propose that CEO supplementation (2.5 ml/kg) boost diseased fish survival by 75%. Similarly, Rattanachaikunsopon and Phumkhachorn (2010) demonstrated that experimental S. iniae infection in Nile tilapia was protected by cinnamon oil, supporting the assertion that EOs can protect fish against infections.

Nevertheless, we also noticed that a dose of 3 ml/kg in group 4 recorded a higher immune parameter with a less survival rate than group 3. We can suggest that a dose of 3 ml/kg may possess a damaging effect stimulate the immune defense response but lower survival rate. Horky *et al*. (2019) reported that some oils can be toxic causing damage to the liver, kidneys, or gastrointestinal tissues although its strong antimicrobial effect.

Our in-vivo results are nearly similar to those previously published studies, where Rattanachaikunsopon and Phumkhachorn (2010) found that dietary application of Cinnamomum verum oil significantly increased the resistance of Nile tilapia against S. iniae infection. Ahmad *et al*. (2011) documented that 1% *Cinnamomum zeylanicum* improved the growth performance, feed utilization, whole-body composition, and resistance of Nile tilapia to *A. hydrophila* infection. Dos Santos *et al*. (2016) illustrated that Cinnamomum sp. inclusion in diets significantly increased serum lysozyme and bactericidal activities in Nile tilapia. Stoyanova *et al*. (2018) showed that dietary Cinnamon verum extract considerably enhanced the growth rates, feed conversion ratio, and the production efficiency of common carp. Kesbiç (2019) demonstrated that Cinnamomum verum essential oil enhanced the growth performance and improved the blood picture of rainbow trout (*Oncorhynchus mykiss*). Also, Mohammad (2021) found that dietary application of 1.5% Cinnamomum sp. noticeably increased total weight, daily growth rate, protein intake, feed conversion ratio and protein productive value of common carp.

CONCLUSION

The obtained findings revealed significant differences in the antibacterial activity of the examined essential oils based on the type of essential oils and bacterial strain. Cinnamon EO revealed to be the most potent against all the tested bacterial isolates. Dietary cinnamon oil supplementation, specifically at 2.5 ml/kg feed, has a substantial potential to promote pangasius catfish health, growth performance and boosting the immune system against *A. hydrophila* infection. Additionally, the post-challenge reduced mortality rate in cinnamon oil fed groups compared to the control, revealed the positive impact of cinnamon oil on the health and innate immune responses of fish. Thus, CEO could be applied as an alternative to traditional antimicrobials for the prevention and treatment of microbial infections in fish.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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