

Original Article

Therapeutic potentials of *Excoecaria agallocha* against gram-positive and gram-negative fish bacterial pathogens

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Abstract

Background: The present investigation of a mangrove plant, *Excoecaria agallocha*, which is a popular medicinal substitute for the treatment of microbial ailments, were evaluated for potential antimicrobial activity against pathogenic bacteria *Escherichia coli* and *Streptococcus agalactiae* in tilapia, *Oreochromis niloticus*.

Methods: Antibacterial activity was performed using agar diffusion method, disc diffusion method, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and antibiotic susceptibility assays. Experimental fish fed diet containing 0 (control), 5, 25, and 50 mg kg⁻¹ *E. agallocha* leaf methanol extract for 28 days then challenged individually with *E. coli* or *S. agalactiae* and mortalities were recorded over a ten-day post-infection period.

Results: Results indicated that both bacterial species are sensitive to tetracycline, ampicillin, and amoxicillin. *E. coli* was found to be resistant to neomycin. *E. agallocha* extract concentration of 50 mg/ml produced a zone of inhibition of 18 mm against *E. coli*, in contrast to 13 mm against *S. agalactiae*. *E. agallocha* showed bactericidal activity against *E. coli* and bacteriostatic activity against *S. agalactiae*. The highest *E. agallocha* LC50 activity was 83 mg/ml. The highest cumulative mortality was 90.0 ± 10.0% in control as compared to 26.7 ± 11.5% in the group fed with 50 mg kg⁻¹ *E. agallocha* extract, significant differences ($P < 0.05$).

Conclusion: Hence, *E. agallocha* showed antibacterial activity against fish pathogens *Escherichia coli* and *Streptococcus agalactiae* in tilapia, *Oreochromis niloticus*; therefore, *E. agallocha* may be used as an alternative therapeutic agent against fish pathogenic bacteria as an additive to feed at a concentration depend, safe, non-cytotoxic doses.

Keywords: *Excoecaria agallocha*; antimicrobial activities; aquaculture; feed additive; fish pathogens; LC50.

Background

The Bacterial infections in aquaculture are a rising concern across the globe. The effectiveness of conventional antibiotics is decreasing due to the global emergence of multi-drug resistant (MDR) bacterial pathogens. The use of broad-spectrum antibacterial agents has led to one of the largest recent global health problems as a result of the development of bacterial resistance. Several bacterial species have developed multidrug resistance, including *Escherichia coli* [1].

E. coli is a common disease of freshwater fish, especially under cultured conditions [2], and has an important role in economic losses among the fish industry. This pathogen has a variable clinical course ranging from mild to severe infection, possibly leading to death due to septicemia, depending on the

bacterial strain and its virulence as well as host-related factors such as age and immunity [3]. Likewise, *Streptococcus agalactiae* is an important human and animal pathogen associated with a variety of diseases in fish [4]. Septicemia is a common condition in tilapia fish infected with *Streptococcus agalactiae* [5] becoming highly problematic in freshwater fishes, constantly threatening the sustainability of the global tilapia aquaculture industry [6]. Therefore, efforts have been now shifted to focus on identifying novel drugs of natural plant sources to be used as an alternative to antibiotics in disease management in aquaculture [7].

Excoecaria agallocha contains a copious number of polyphenols and terpenoids, which have been reported to have an epidemic and endemic disease control as an antimicrobial, anti-cancer, and anti-diabetic agent [8]. However, there is sparse information regarding the capability of *E. agallocha* for the treatment of infections caused by aquaculture bacterial pathogens [9-11]. Thus, new compounds that target bacterial

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virulence can be developed to control the threat posed by multidrug resistance found in economically valuable fish. The search for a new treatment is urgently needed predominantly in those who are non-therapeutic in nature as well as being environmentally friendly. Therefore, the objective of this study was conducted to evaluate the effect of food additive with *E. agallocha* leaf methanolic extract on artificially infected tilapia, *Oreochromis niloticus*, with zoonotic bacteria *Escherichia coli* and *Streptococcus agalactiae*.

Methods

Preparation of plant and extraction

Excoecaria agallocha leaves were collected from the east coast in Terengganu and was identified at the Plant Taxonomy Laboratory in University Malaysia Terengganu (UMT). The leaves were washed and air-dried in the oven overnight at 40°C. The leaves were then ground to a fine powder using a blender, weighing 1 kg. Then, 25 g of plant sample was Soxhlet extracted with 250 mL of MeOH at 60°C for 12 h [12]. Extracts in methanol solvents were evaporated and concentrated to dryness using a rotary evaporator at 45°C. The weight after extraction was determined, and the percent yield (%) for extraction was calculated [11].

Microbial assessment

Fish pathogens as One Gram-negative bacteria *Escherichia coli* and One Gram-positive bacteria *Streptococcus agalactiae*, deposited in GenBank (Accession No. KT869025), were used in this study. Both were obtained from Fish Disease Laboratory, School of Fisheries and Aquaculture Sciences, University Malaysia Terengganu (UMT), Malaysia.

Antibiotics susceptibility test

Antibiotics resistance patterns were determined using the disk diffusion method, according to CLSI [13].

Antibacterial assay

Disk diffusion assay was carried out on Mueller Hinton Agar (MHA) following the methods described by Barker [14], and the agar well diffusion method, according to Perez [15].

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) was determined using the broth dilution assay technique in 96 wells microtiter plates. This method was carried out according to CLSI [13], while the minimum bactericidal concentration (MBC) extract was determined according to Ilavenil [16].

Toxicity test

Brine shrimp lethality assay was carried out to study the general toxicity of the extract of *E. agallocha* according to the methods of Pisutthanan [17].

Determination of the Median Lethal Dose (LD50)

All assays in experimental fish were divided into two main groups. The first group was challenged with *E. coli*, and the second group challenged with *S. agalactiae*, individually. Both were challenged under the same environmental laboratory conditions.

Fish collection and acclimatization

Healthy juvenile tilapia, *O. niloticus*, (3.5 ± 0.2 g) were obtained from UMT hatchery. Fish were acclimatized to laboratory conditions for 14 days and then stocked into 16 composite tanks (20 L) in a flow-through freshwater supply system, fed twice a day a commercial feed (30% crude proteins). Water temperature ($26 \pm 2^\circ\text{C}$), dissolved oxygen (DO) (5.57 ± 0.01 mg/L) and pH was (7.2 ± 0.2) ranged on acceptable values throughout the experimental period. These were measured with (YSI -Yellow Springs Instruments - model 85/10).

Prepare Bacteria Inoculum

The stock bacteria of *E. coli* or *S. agalactiae* were first passed through healthy fish to potentiate its virulence. Then they were grown on blood agar (Oxoid, U.K.) at 28°C for 24 to 48 hours. Bacterial cells were washed twice with physiological saline and then re-suspended in the same solution to obtain a bacterial suspension. The bacteria suspension was adjusted to McFarland turbidity standard No.5 equivalent to 15×10^8 cfu mL⁻¹. Ten-fold serial dilutions were done to obtain a different concentration of bacteria. Intra-peritoneal injection method (i.p.) inoculated into tilapia, *O. niloticus*.

Experimental design

The LD50 value was determined to obtain the lowest bacterial dose of *E. coli* or *S. agalactiae* (individually) that cause 50 % mortality in tilapia, *O. niloticus*. Six groups of tilapia, *O. niloticus*, with ten fishes per group, were housed in a 20 L aquarium supplied with proper aeration. The environmental conditions were maintained as optimum as possible. The temperature was kept at $26 \pm 2^\circ\text{C}$, dissolved oxygen (DO) was 5.4 ± 0.5 mg L⁻¹, and pH was 7.2 ± 0.2 . Fish were kept in a fasting state for 24 h before beginning the experiment.

The fishes were anesthetized using Tricaine Methane sulfonate (MS-222) (Sigma, Aldrich). The fish in groups 1, 2, 3, 4 and 5 were artificially injected intraperitoneally (i.p.) with 0.1 ml of culture suspension of pathogenic *E. coli* or *S. agalactiae* containing 15×10^8 , 15×10^7 , 15×10^6 , 15×10^5 and 15×10^4 CFU mL⁻¹, respectively. The 6th group, control group, was injected intraperitoneally (i.p.) with 0.1 ml with physiological saline. Fish mortality was recorded every 24 h for ten days. Dead fish were removed from the aquarium daily.

Efficacy of dietary doses of *E. agallocha* on disease resistance

Preparation of *E. agallocha* diets

During the acclimation period of 14 days, fish were fed with commercial diets (Analytical Laboratories of UMT hatchery) at 3% BW d⁻¹ twice a daily between the hours of 8.00-9.00 and 15.00- 16.00. During the experimental period, the fish were fed for 28 days with 5, 25, and 50 mg extract per ⁻¹ kg food, respectively, and the basal diet acted as controls. The extract at a concentration of 5, 25, and 50 mg was dissolved in distilled water and sprayed on the thin layer of basal food. The pellets were dried in an oven at 30°C for 18 h, packed in polythene bags and stored in a freezer at -20°C until used. All fish were deprived of food for 24 h before the challenge test.

Experimental design

A total of 300 healthy juvenile tilapia, *Oreochromis niloticus*, (3.5 ± 0.2 g) were obtained from UMT hatchery. Fish were acclimatized for 14 days before being used for experiments. The experimental fish were divided into two groups; the first group was the control group, included positive and negative fed basic diet (no additive of plant extract). The second group acted as a treatment group, which consisted of three subgroups which received three different levels of *E. agallocha* leaf methanol extract at concentration 5, 25, and 50 mg kg⁻¹, respectively. Each group for three replicates (10 fish per 20L tank). Fish were fed at 3% BW d⁻¹ twice daily (9:00 and 18:00) for 28 days. On the 29th day, all fish in the treatment group were injected intraperitoneally (i.p.) with 100 µl bacteria *S. agalactiae* at dose 15×10⁵ CFU mL⁻¹ (This dose was calculated previously from the determination of Lethal dose LD₅₀). Meanwhile, negative control groups were injected intraperitoneally (i.p.) with 100 µl bacteria *S. agalactiae* at dose 15×10⁵ CFU mL⁻¹. The positive control group was injected intraperitoneally (i.p.) with 100 µl physiological saline. The same design applied but a different type of bacteria, using *E. coli* at dose 15×10⁸ CFU mL⁻¹. The cumulative mortality was monitored for a group of fishes in all experiments for ten days post-infection.

Disease resistance

The effect of *E. agallocha* leaf extract incorporated feed for the disease resistance (survival percentage) on fish (n = 10/group) was determined. The fish were artificially challenged with a dose of 15×10⁵ CFU mL⁻¹, 15×10⁸ CFU mL⁻¹ of live virulent pathogen *S. agalactiae*, and *E. coli*, respectively.

The mortality was observed for ten days, and the average of the triplicate set was used to express in terms of percentage of survival.

Statistical analysis

Effects of *E. agallocha* diets on survival were expressed as the arithmetic mean ± standard deviation (SD) by using Tukey statistical analysis using one-way analysis of variance (ANOVA) to reveal the significant difference between the treatments at the 5% (P < 0.05) level of significance by using SPSS version 20.0 for windows. Data were expressed as concentration ± mortality (%) of triplicate measurements. Probit analysis was used to determine 120 h (LD₅₀) and (LC₅₀) using SPSS 20.0.

Results

Clinical signs

There were no clinical signs apparent on the experimental fish infected with *E. coli* and *S. agalactiae* except for sudden death and petechial hemorrhages on the site of injection in some fish.

Antibiotic assay

Antibiotic susceptibility test was done on selected samples of gram-positive *S. agalactiae* and gram-negative bacteria *E. coli*. The inhibition zone (mm) was recorded for five types of antibiotics. Both isolates showed 100% sensitivity to amoxicillin, ampicillin, and tetracycline. However, gram-negative bacteria *E. coli* isolates showed resistance to neomycin (Table 1 & Fig. 1, 2).

Table 1: Antibiotic susceptibility test of *Escherichia coli* and *Streptococcus agalactiae* by agar disc diffusion method

Antibiotic	Disc potency (mcg)	Isolates		Diameter Zone of Inhibition (mm)		
		<i>E. coli</i>	<i>S. agalactiae</i>	R	I	S
Amoxicillin	10	22/ S	20/ S	13	14-17	18
Neomycin	30	12/ R	19/ S	12	13-15	17
Penicillin	10	23/ I	24/ I	20	21-28	29
Ampicillin	10	20/ S	18/ S	14	15-16	17
Tetracycline	30	20/ S	20/ S	14	15-18	19

*R=resistance, I= intermediately sensitive, S=sensitive.

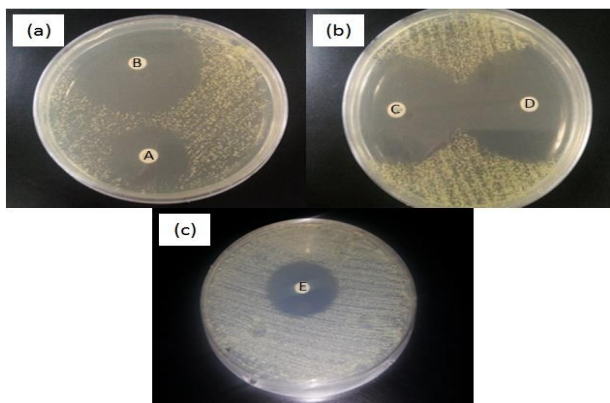


Figure 1: Zone inhibition of antibiotic susceptibilities: A=Neomycin (N 30ug), B=Amoxicillin (AML 10 ug), C=Ampicillin (AMP 25ug), D=Penicillin (P 10ug), E=Tetracycline (TE 30ug) against *Escherichia coli*.

Antibacterial assay

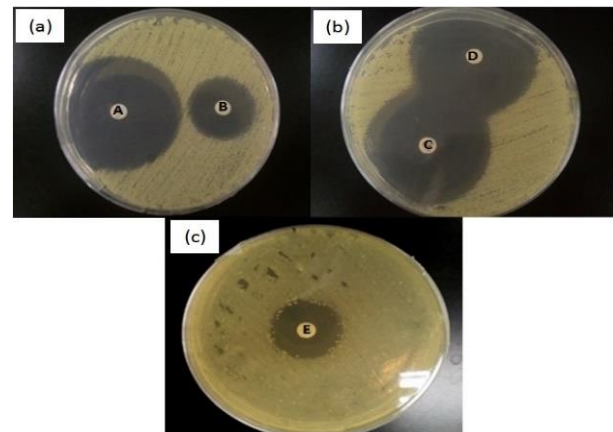


Figure 2: Zone inhibition of antibiotic susceptibilities: A=Neomycin (N 30ug), B=Amoxicillin (AML 10 ug), C=Ampicillin (AMP 25ug), D=Penicillin (P 10ug), E=Tetracycline (TE 30ug) against *Streptococcus agalactiae*.

The mean zone of inhibition for *E. agallocha* methanol extracts of concentration 5, 25, and 50 mg/ml using the disk diffusion method were 10, 12, and 14mm against *E. coli* and 7, 9 and 13 mm, against *S. agalactiae*, respectively (Table 2 & Fig. 3). In using a suitable diffusion method, results showed 13, 15,

and 18mm against *E. coli* and 7, 9, and 13mm against *S. agalactiae*, respectively (Table 3 & Fig. 4). The lowest MIC and MBC values for the *E. agallocha* against *E. coli* were 3.12 and 6.25 mg/ml, and 6.25 and 25 mg/ml against *S. agalactiae*, respectively (Table 4).

Table 2. Comparison of antimicrobial activities of *Excoecaria agallocha* leaf methanol extract on test organisms by agar disc diffusion method

Microorganisms	Diameter Zone of Inhibition (mm)			Tetracycline (30 ug)	Control
	<i>Excoecaria agallocha</i> extract				
	5 mg/ml	25 mg/ml	50 mg/ml		
<i>Escherichia coli</i>	10	12	14	21	0.0
<i>Streptococcus agalactiae</i>	7	9	13	15	0.0

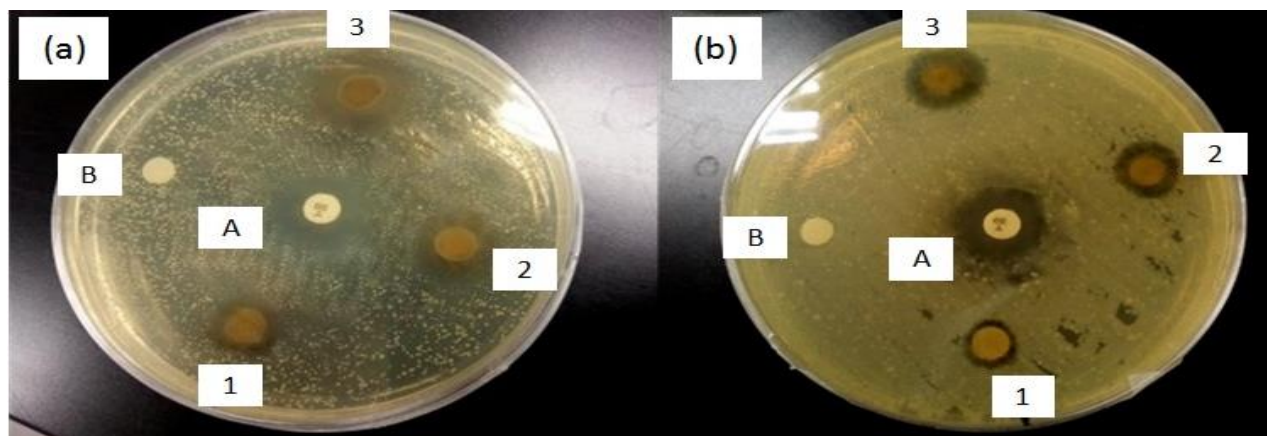


Figure 3: Antibacterial activity of *Excoecaria agallocha* against test organisms by disc diffusion; (a) *Escherichia coli*, (b) *Streptococcus agalactiae*; 1=5mg/ml, 2=25mg/ml, 3=50mg/ml (*Excoecaria agallocha* extract); A= Tetracycline (TE 30ug), B=DMSO.

Table 3. Comparison of antimicrobial activities of *Excoecaria agallocha* leaf methanol extract on test organisms by agar well diffusion method

Microorganisms	Diameter Zone of Inhibition (mm)			Tetracycline (30 ug)	Control
	<i>Excoecaria agallocha</i> extract				
	5 mg/ml	25 mg/ml	50 mg/ml		
<i>Escherichia coli</i>	13	15	18	14	0.0
<i>Streptococcus agalactiae</i>	7	9	13	12	0.0

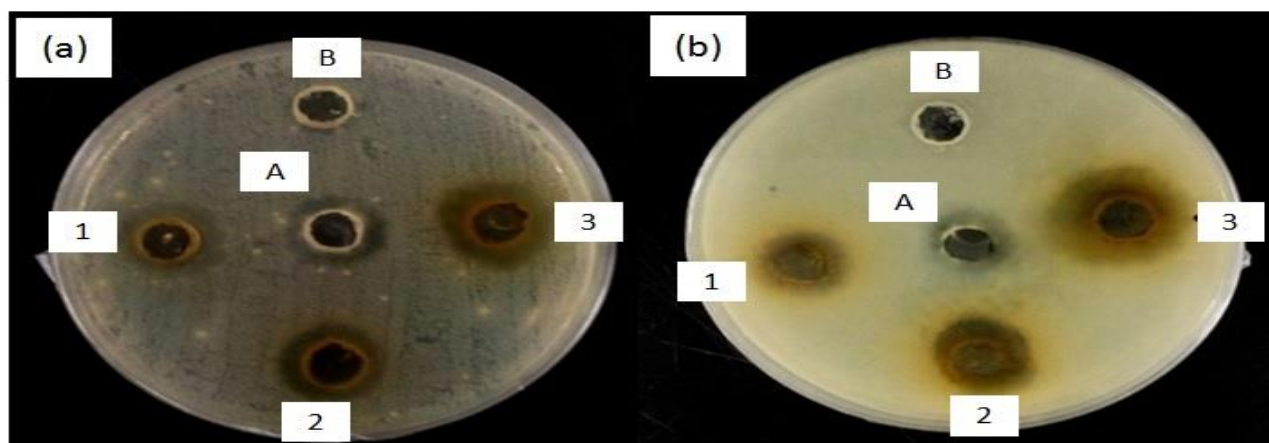


Figure 4: Antibacterial activity of *Excoecaria agallocha* against test organisms by agar well diffusion; (a) *Escherichia coli*, (b) *Streptococcus agalactiae*; 1=5mg/ml, 2=25mg/ml, 3=50mg/ml (*Excoecaria agallocha* extract); A= Tetracycline (TE 30ug), B=DMSO.

Table 4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) (mg/ml) of *Excoecaria agallocha* leaf methanol extract on test organisms

Bacteria	MIC	MBC	MBC/MIC
<i>Escherichia coli</i>	3.12	6.25	2
<i>Streptococcus agalactiae</i>	6.25	25	4

Toxicity test

The toxicity of the brine shrimp test was analyzed by Excel to obtain the 50% lethality concentration (LC_{50}), showing the value of LC_{50} to be 83 mg/ml (Fig. 5).

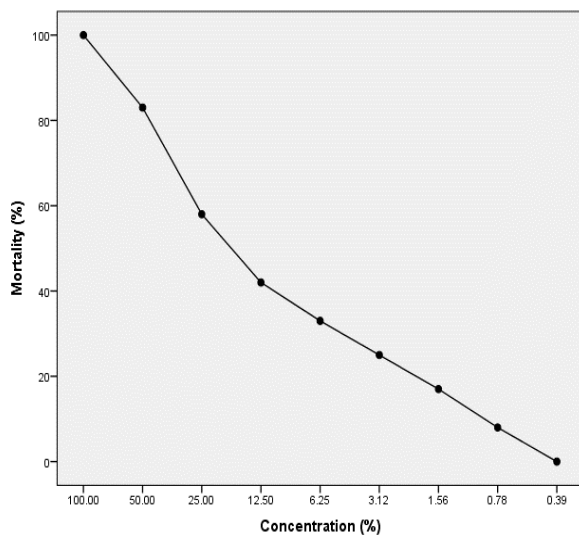


Figure 5: Brine shrimp toxicity test at 50% lethality concentration (LC_{50})

Disease Resistance

Total mortalities recorded during the experiment were attributed to the challenge infection with pathogenic bacteria *E. coli* and *S. agalactiae*. These mortalities were observed from the first day until the fifth day in fish injected with *S. agalactiae* and sixth day in fish injected with *E. coli* post-challenge. There were no mortalities up to 10 days post-challenge. Among the treatment groups that were challenged with *S. agalactiae*, the highest cumulative mortality was $90.0 \pm 10.0\%$ in the control group as compared to $26.7 \pm 11.5\%$, with the group fed with 50 mg kg^{-1} of *E. agallocha*, a significant difference ($p < 0.05$) (Table 5 & Fig 6). On the other hand, the cumulative mortality was $70.0 \pm 17.3\%$, and $40.0 \pm 0.0\%$ in the group fed with 5 mg kg^{-1} and 25 mg kg^{-1} of *E. agallocha* leave crude extract, respectively, with significant difference ($p < 0.05$) (Table 6 & Fig. 7). In contrast, the control group challenged with *E. coli* showed the highest cumulative mortality was $100.0 \pm 0.0\%$ as compared to $37.0 \pm 5.7\%$ in the group fed at 50 mg kg^{-1} of *E. agallocha* extract, a significant difference ($p < 0.05$). Moreover, the cumulative mortality was $73.3 \pm 5.7\%$ and $47.0 \pm 5.7\%$ in the group fed with 5 mg kg^{-1} and 25 mg kg^{-1} of *E. agallocha*, respectively, with significant difference ($p < 0.05$) (Table 6 & 5). To best our knowledge, this study was the first, which discussed the gender differences in the domains of emotional exhaustion among a sample of medical doctors in Iraq and Arab region using the validated MBI questionnaire.

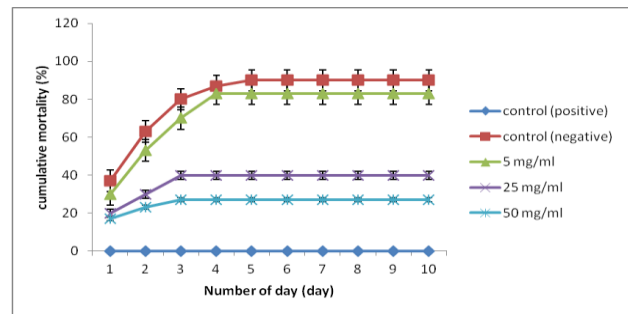


Figure 6: Cumulative mortality (%) of tilapia, *Oreochromis niloticus* fed with *Excoecaria agallocha* supplementation diets and challenged with *Streptococcus agalactiae* for 10 days. Values are mean \pm SD of triplicate.

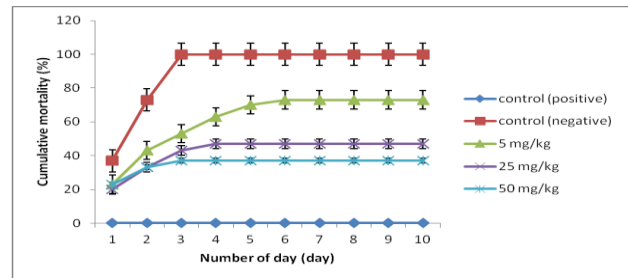


Figure 7: Cumulative mortality (%) of tilapia, *Oreochromis niloticus* fed with *Excoecaria agallocha* supplementation diets and challenged with *Escherichia coli* for ten days. Values are mean \pm SD of triplicate.

Discussion

Mangroves are biochemically unique, producing a wide range of novel natural products. In this study, the antimicrobial activity of *Excoecaria agallocha* was established on pathogenic bacteria *Escherichia coli* and *Streptococcus agalactiae* in fish. Antimicrobial agents can be defined as substances that can kill and/or inhibit the growth of microorganisms. The antimicrobial action of such compounds is related to the inactivation of cellular enzymes or by membrane permeability changes causing a loss of cellular integrity and eventual cell death [18].

Mangrove plants are a rich source of bioactive composites and are utilized in many parts of the world as a renewable resource and as a treatment option to medical illness in various cultural communities globally. Nowadays, mangrove plants play a significant role in the treatment of aquatic and fish disease because of the minimal side effects and therapeutic potential [11]. The present study focuses on the use of *E. agallocha* leaf extract to control bacterial infection in fish. *E. agallocha* extract exhibited significant in vitro antibacterial activity against bacteria. Similarly, Laith [11] observed in previous studies that *E. agallocha* extract exhibited antibacterial activity against fish pathogens bacteria, including *Flavobacterium Indicum*, *Chryseobacterium indologenes*, *Chryseobacterium gleum*, and *Elizabethkingia meningoseptica*. Moreover, our findings receive support from the works of Bakshi [19] who recorded the antimicrobial activity of leaf extracts of mangrove plants: *Avicennia alba*, *Avicennia marina*, *Avicennia officinalis*, *Excoecaria agallocha*, *Sonneratia caseolaris*, *Sonneratia apetela*, *Aegiceris corniculatum*, *Acanthus ilicifolius*, *Nypa fruticans*, and *Ceriops decandra* to have inhibitory effect against *Escherichia coli* with the most active extracts being from *Excoecaria agallocha* plant with a zone of inhibition of 10.3 mm.

Table 5. Cumulative mortality (%) of tilapia, *Oreochromis niloticus* treated with *Exocaria agallocha* and challenged with *Streptococcus agalactiae* for ten days. Values are mean \pm SD of triplicate. Means with the same letters were not significantly different ($p > 0.05$).

Treatment/Number of days	Control (+Ve)	Control (-Ve)	5 mg/kg	25 mg/kg	50 mg/kg
1	0.0 \pm 0.0 ^a	36.7 \pm 5.8 ^b	30 \pm 10.0 ^{bc}	20.0 \pm 0.0 ^b	16.7 \pm 5.8 ^b
2	0.0 \pm 0.0 ^a	63.3 \pm 5.8 ^c	53.3 \pm 11.5 ^c	30.0 \pm 0.0 ^b	23.3 \pm 5.8 ^b
3	0.0 \pm 0.0 ^a	80.0 \pm 0.0 ^c	70 \pm 17.3 ^c	40.0 \pm 0.0 ^b	26.7 \pm 11.5 ^b
4	0.0 \pm 0.0 ^a	86.7 \pm 5.8 ^c	70 \pm 17.3 ^c	40.0 \pm 0.0 ^b	26.7 \pm 11.5 ^b
5	0.0 \pm 0.0 ^a	90.0 \pm 10.0 ^c	70 \pm 17.3 ^c	40.0 \pm 0.0 ^b	26.7 \pm 11.5 ^b
6	0.0 \pm 0.0 ^a	90.0 \pm 10.0 ^c	70 \pm 17.3 ^c	40.0 \pm 0.0 ^b	26.7 \pm 11.5 ^b
7	0.0 \pm 0.0 ^a	90.0 \pm 10.0 ^c	70 \pm 17.3 ^c	40.0 \pm 0.0 ^b	26.7 \pm 11.5 ^b
8	0.0 \pm 0.0 ^a	90.0 \pm 10.0 ^c	70 \pm 17.3 ^c	40.0 \pm 0.0 ^b	26.7 \pm 11.5 ^b
9	0.0 \pm 0.0 ^a	90.0 \pm 10.0 ^c	70 \pm 17.3 ^c	40.0 \pm 0.0 ^b	26.7 \pm 11.5 ^b
10	0.0 \pm 0.0 ^a	90.0 \pm 10.0 ^c	70 \pm 17.3 ^c	40.0 \pm 0.0 ^c	26.7 \pm 11.5 ^b

Table 6. Cumulative mortality (%) of tilapia, *Oreochromis niloticus* treated with *Exocaria agallocha* and challenged with *Escherichia coli* for ten days. Values are mean \pm SD of triplicate. Means with the same letters were not significantly different ($p > 0.05$).

Treatment/Number of days	Control (+Ve)	Control (-Ve)	5 mg/kg	25 mg/kg	50 mg/kg
1	0.0 \pm 0.0 ^a	37.0 \pm 5.7 ^c	23 \pm 5.7 ^{bc}	20 \pm 10.0 ^b	23 \pm 0.0 ^{ab}
2	0.0 \pm 0.0 ^a	73 \pm 5.7 ^d	43 \pm 5.7 ^c	33 \pm 5.7 ^{bc}	33 \pm 5.7 ^b
3	0.0 \pm 0.0 ^a	100 \pm 0.0 ^d	53 \pm 5.7 ^c	43 \pm 5.7 ^{bc}	37 \pm 5.7 ^b
4	0.0 \pm 0.0 ^a	100 \pm 0.0 ^d	63 \pm 5.7 ^c	47 \pm 5.7 ^b	37 \pm 5.7 ^b
5	0.0 \pm 0.0 ^a	100 \pm 0.0 ^e	70 \pm 0.0 ^d	47 \pm 5.7 ^c	37 \pm 5.7 ^b
6	0.0 \pm 0.0 ^a	100 \pm 0.0 ^c	73 \pm 5.7 ^d	47 \pm 5.7 ^b	37 \pm 5.7 ^b
7	0.0 \pm 0.0 ^a	100 \pm 0.0 ^c	73 \pm 5.7 ^d	47 \pm 5.7 ^b	37 \pm 5.7 ^b
8	0.0 \pm 0.0 ^a	100 \pm 0.0 ^c	73 \pm 5.7 ^d	47 \pm 5.7 ^b	37 \pm 5.7 ^b
9	0.0 \pm 0.0 ^a	100 \pm 0.0 ^c	73 \pm 5.7 ^d	47 \pm 5.7 ^b	37 \pm 5.7 ^b
10	0.0 \pm 0.0 ^a	100 \pm 0.0 ^c	73 \pm 5.7 ^d	47 \pm 5.7 ^b	37 \pm 5.7 ^b

In vitro assays indicated that the growth of the tested fish pathogens was suppressed by *E. agallocha* extract. Gram-negative bacteria *E. coli* showed a high inhibition zone of 18 mm as compared to 13 mm from Gram-positive bacteria *S. agalactiae*. These results were confirmed by in vivo assays which showed cumulative mortality to be $26.7 \pm 11.5\%$ in the group fed with 50mgkg⁻¹ of *E. agallocha* leave crude extract injected with gram-positive *S. agalactiae*, while this percentage was $37.3 \pm 5.7\%$ of the same concentration dose of plant extract but injected with gram-negative *E. coli*. Jayanta [20] reported that both methanol and aqueous extract of *E. agallocha* were effective against *E. coli*. These findings are in agreement with Agoramorthy [21], who determined that antimicrobial compounds extracted from plant sources are considered to be more active on Gram-negative than Gram-positive bacteria due to cell wall structure differences among the two. Similar results were also obtained by Laith [22] in that active constituents of plants interfere with the growth and metabolism of microorganisms in a negative manner. Moreover, Packialakshmi [23] evaluated the antibacterial activity of aqueous *E. agallocha* leaf extracts using the disc diffusion method against gram-negative and positive bacteria.

The result revealed that the gram-negative bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* had a higher inhibition zone of 17, 15, and 16 mm, respectively, as compared to gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* with zones of 15, 14 mm, respectively. This antibacterial activity that is possessed by *E. agallocha* against infectious pathogens, as shown in this study, may be attributed to the presence of diterpenes and benzoxazole derivatives and other bioactive compounds found in this mangrove plant [24]. In contrast, our results were not in

agreement with the findings of Rajia [25] who stated that methanol extract of *E. agallocha* did not show any antibacterial activity against the clinical isolates; gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, or gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Pseudomonas Vulgaris* at different concentrations of 1, 2 and 3 mg/ml due to bacterial resistance which could cause this ineffectiveness of the mangrove extract. Nonetheless, Laith [11] indicated that *E. agallocha* leaf extracts displayed antimicrobial activity due to secondary metabolites, and the potential mechanisms of these components on bacteria may be related to the alteration in bacteria cell wall structures and inhibition of bacterial proteins. MIC and MBC values are used to assess the antimicrobial efficacy of metabolites or synthetic compounds of *E. agallocha* [26]. Therefore, MIC and MBC values are useful as guidelines to choose the appropriate and effective concentrations for therapeutic substances [27]. In this study, it was determined that the lowest MIC and MBC values for the *E. agallocha* against *E. coli* were 3.12 and 6.25 mg/ml, and 6.25 and 25 mg/ml against *S. agalactiae*, respectively. Similar results observed by Jayanta [20] who determined the MIC values of *E. agallocha* against *Streptococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *E. coli* ranged from 5 to 7 mg/ml. Similar observations were recorded by Selvam [28] in that the MIC of mangrove plants tested against bacterial pathogens ranged between 20 mg/ml to 640 mg/ml. According to Manilal [29], a substance is considered bactericidal when the ratio of MBC/MIC ≤ 2 , is bacteriostatic if the ratio of MBC/MIC ≥ 2 and is ineffective if the ratio of MBC/MIC ≥ 16 . Therefore, it was concluded that *E. agallocha* leaf extract was bactericidal against *E. coli* and bacteriostatic against *S. agalactiae*. Additionally, the toxicity of

plant extract plays an important role in medicine; therefore, brine shrimp larvae bioassay, which is a simple and useful tool for the isolation of potentially cytotoxic compounds from plant extracts [10], was used to determine the toxic level of extract substance. In this study, the LC₅₀ value of *E. agalocha* was 83 mg/ml, similar to the results observed by Laith [10], who recorded the LC₅₀ value of *E. agalocha* to be 94.19 mg/ml. The difference in values of LC₅₀ may be attributed to the different locations of the mangrove plant. Antimicrobial resistance is a progressively global problem, and the emerging antimicrobial resistance has become a major public health issue [30]. Our result revealed *E. coli* was resistant to neomycin and susceptible to tetracycline, which is similar to observations recorded by Noryawati [31] in that isolated *E. coli* was the most sensitive to tetracycline. Also, our finding is supported by the works of Sabarinath [32], who stated that *E. coli* was susceptible to neomycin and even to Kibret [33], who said that *E. coli* was resistant to tetracycline. Bacteria resistance towards antibiotics might be due to their close genetic relatedness [34]. Moreover, sudden death was the most common sign of tilapia fish infected with *E. coli* and *S. agalactiae*. This can be attributed to septicemia caused by these pathogenic bacteria [3, 5]. Regarding the overall survival rate, the present study revealed that *E. agalocha* leaf extracts increased the survival rate of fish after a challenge trial with *E. coli* and *S. agalactiae*. The highest survival rate was in the group fed at 50 mg kg⁻¹, and the lowest survival was observed in control. This finding is supported by the works of Pavraj [35] in common Carp (*Cyprinus carpio*) administered with plant extract of *Ocimum sanctum* and challenged with *Aeromonas hydrophila* showed no mortality as compared to control group which showed 40% mortality. Decreased cumulative mortality of fish fed with *E. agalocha* diets and infected with *E. coli* and *S. agalactiae* are in agreement with the previous study by Laith [10, 22] in that *E. agalocha* has the potential as a dietary additive to mitigate mortality of fish infected with pathogenic bacteria. Therefore, the efficacy of in vivo assay may establish the antimicrobial action properties and provide a tailor-made alternative source to combat infectious agents. Hence, our findings revealed a potent in vitro and in vivo activity of the *E. agalocha* leaves extracts on pathogenic fish bacteria *E. coli* and *S. agalactiae*.

Conclusion

The results of the present study conclude crude extract of mangrove plants is a suitable agent in providing a non-medicinal antimicrobial activity to combat bacterial infections in fish. Further work on the purification of bioactive compounds is currently underway to discover additional effective solutions for treating fish diseases. Therefore, we recommend the use of *E. agalocha* as an alternative therapeutic agent against both gram-negative and gram-positive bacterial infections in the aquaculture industry.

Abbreviations

UMT: University Malaysia Terengganu. MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, LD₅₀: Median Lethal Dose

Declarations

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Availability of data and materials

Data will be available by emailing laith.abdul@umt.edu.my

Authors' contributions

LA is the principal investigator of the study who designed the study and coordinated all aspects of the research, including all steps of the manuscript preparation. He is responsible for the study concept, design, writing, reviewing, editing, and approving the manuscript in its final form. NM, AJ, ANM, NM contributed to the study design, data collecting, analysis, and interpretation of data, drafting the work, writing the manuscript and reviewed and approved the manuscript. All authors have read, reviewed, and approved the final manuscript. Ethics approval and consent to participate

Ethics approval and consent to participate

The study design was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) (Approval Number: UTM/FKK/2013/Fadzilah/15-May/506-June-2013-June-2016).

Consent for publication

Not applicable

Competing interest

The authors declare that they have no competing interests.

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