Antimicrobial Activities of Some Culinary Spice Extracts against *Streptococcus agalactiae* and Its Prophylactic Uses to Prevent Streptococcal Infection in Red Hybrid Tilapia (*Oreochromis* sp.)*

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**Abstract:** The extracts of ten culinary spices were screened to identify their antimicrobial activities against *Streptococcus agalactiae* by using disk diffusion assay. Only *Cinnamomum verum*, *Allium sativum* Linn, *Eugenia caryophyllus* and *Thymus vulgaris* displayed antimicrobial activity. The bark *C. verum* extract displayed the highest antimicrobial activity with a 18 mm inhibition zone. The minimum inhibitory concentration (MIC) values for spice extracts were determined by utilizing the agar diffusion method. The lowest MIC value with high efficacy against *S. agalactiae* was 0.15 mg/mL, which was obtained from *C. verum* extract. The median lethal dose (LD₅₀) of *S. agalactiae* to tilapia fingerlings was measured to be 1.56×10⁶ CFU/mL. The in vivo antimicrobial effect of *C. verum* was tested by feeding tilapia fingerlings fish feed supplemented with different ratios of *C. verum* extract and bark powder for 17 days after experimentally injecting the fish with *S. agalactiae* intraperitoneally (IP). The mortality was significantly lower (p<0.05) in the fish fed on feed supplemented with bark *C. verum* extract with a ratio of 3:26 (w/w) compared to other groups. These results indicated that the *C. verum* bark extract supplement is promising as a prophylactic against tilapia streptococcosis and for fish health improvement.

**Key words:** *Streptococcus agalactiae* %*Cinnamomum verum* %Antimicrobial activity %Tilapia

**INTRODUCTION**

Global population increase and food demand have been motivating factors leading to additional expansion of intensive animal and fish production. This steady production increase has led to a massive emergence of new, previously unknown diseases. Streptococcosis is one of the diseases that has affected numerous fish species and it has been detected in numerous countries. Streptococcosis can cause yearly economic losses in fish farms, especially tilapia fish farms [1]. Consequently, it is now discerned as an “emerging pathological problem” of considerable significance to aquacultural health.

*Streptococcus agalactiae* is one of bacterial species that can cause Streptococcosis infection in marine and fresh water fish, especially in warm water regions. Generally, it’s characterized as a Gram-positive coccus that is catalase negative, oxidase negative, non-spor forming, non-motile and typically arranged in short chains [2, 3]. *Streptococcus agalactiae* is the most common tilapia bacterial disease and has been isolated from some commercial tilapia farms in different parts of Malaysia [4].

The amount of antibiotics utilized in the treatment of fish diseases has increased because of intensive fish production increases. Many studies have documented that faulty and the indiscriminate use of antibiotics in aquaculture has led to an increase of antibiotic resistance in various pathogens of fish [5, 6] and the presence of residual antibiotics in seafood and fish products has increased as well [7, 8]. Furthermore, the emergence of bacterial antibiotic resistance might be transmitting from aquaculture environments to humans and animals [9, 10].
Herbs have been extensively utilized in human and veterinary medicine. Currently herbs play a considerable role in aquaculture. Several studies have reported that herbal extracts such as garlic and clove, have potential as antimicrobials against various fish pathogens [11, 12]. Traditionally, Cinnamonum (Cinnamomum verum) has been used widely as a human medicine in Asia for the treatment and control of several diseases such as spasmodic, chronic diarrhea and rheumatism [13].

Cinnamon extract possesses antioxidant, antifungal and antibacterial properties that can be utilized to retard and circumvent microbial spoilage in food due to bacterial contamination [14-16] Therefore, in this present study, water extracts of ten culinary spices were examined to determine the potential of their antimicrobial properties as alternative prophylactics against S. agalactiae infection in red hybrid tilapia (Oreochromis sp.). Accordingly, we studied the effects of C. verum bark extract during in vitro and in vivo experiments.

MATERIALS AND METHODS

Fish Preparation: All experiments were performed at an aquatic animal health unit, Faculty of Veterinary Medicine, University Putra Malaysia. Red hybrid tilapia fingerlings with an average weight of 11 ±2 g and 8± 1 cm of length of both sexes were obtained from a commercial tilapia farm. The fish were maintained in 500L fiberglass tank, the water quality and temperature were monitored daily and kept within the acceptable range for tilapia. The fish were fed commercial feed twice daily at a ratio of 3% of their body weight and kept under observation for 2 weeks prior to the experiment. During the observation period, the fish were randomly sampled and their kidneys and livers aseptically streaked on brain heart infusion agar (BHI) to determine whether the fish were free of bacterial infection. All experiments were conducted in 60L aquarium and 12 fish were placed in each aquarium 24 hours prior to the experiments.

Bacteria Preparation: Streptococcus agalactiae was isolated in 2008 from a commercial aquaculture tilapia farm infected in the Malaysian state of Selangor. The isolated bacteria were identified as S. agalactiae by using BBL Crystal kit and the RapID™ STR System (Remel, USA) as well as a Streptococcal grouping kit. Latex agglutination was performed to identify the streptococcal group. The reference strain (Streptococcus agalactiae (α-haemolytic) ATCC 27956) was obtained from the American Type Culture Collection (ATCC).

The bacterial culture was grown in a brain heart infusion (BHI) medium at 35°C overnight and the stock was kept frozen in 0.85% saline solution with 15% glycerol at-80°C until used. For infection trials, 100 ml of brain heart infusion broth was inoculated with 50 µl of the frozen isolate. The broth was incubated at 35°C for 24 hours in a shaker then centrifuged at (5000 rpm for 20 minutes at 10°C). The pellet was washed three times with phosphate buffered (PBS) (pH 7.2).

Preparation of Culinary Spice Extracts: Ten culinary spices were purchased from the local market (Table 1), oven-dried at 45°C for 72 hours and finely ground. Fifty grams of each spice was dissolved and extracted with 500 ml of distilled water in a ratio of 1:10 (w/v) then homogenized by using a Heidolph Homogenizer (DIAX 900, Germany). The homogenous preparations were kept on a rotary shaker at 100 rpm for 24 hours. The preparations were then centrifuged at 13,000 rpm for 15 minutes at room temperature and the supernatant collected and filtered through Whatman No.4 filter paper. The solvent was removed under vacuum using a rotary evaporator (BÜCHI Rotavapor®) and frozen at-80°C before freeze drying. The residues obtained were stored at-40°C until use. The final concentration of each extract was adjusted to 20mg/ml.

Screening of Antibacterial Activity of Culinary Spices: The antimicrobial assay was performed using the standard procedure as described [17]. The previously prepared S. agalactiae inoculums were adjusted to 0.5 McFarland standards, which are equal to 1x10⁶ CFU/mL and then 0.1 ml was transferred to Mueller Hinton agar (MHA) plates and spread with cotton swabs. Twenty-five microliters of each spice extract were spotted on 6 mm sterile paper disks, dried in a biological hood for 1 hour and then inoculated aseptically onto MHA. Distilled water was
used as a negative control, whereas a disc containing oxytetracycline (25µg) was used as a positive control. Inoculated plates with disks were incubated at 35°C overnight. After a 24-hour incubation period, the inhibition zone diameters (mm) were measured. The experiment was performed in triplicates and the interpretation of antibacterial properties was conducted according to [18]. Inhibition zones > 15 mm were categorized as strong activity, from 10-15 mm as moderate activity and <10 mm as weak activity.

**Minimum Inhibitory Concentration (MIC) Test:** The minimum inhibitory concentrations of the extracts were determined using the agar well diffusion method. A two-fold serial dilution of each extract was prepared and eighth concentrations of each spieces were prepared (20, 10, 5, 2.5, 1.25, 0.6, 0.3 and 0.15 mg/mL). The previously prepared S. agalactiae inoculums were adjusted to 0.5 McFarland standards, which are equal to $1 \times 10^5$ CFU/mL, then 0.1 ml was transferred to MHA plates and spread with cotton swabs. Wells were made in the agar plate by using a cork borer (7 mm in diameter) as described by Valgas et al. [19] then the wells were filled with 50 µL of each prepared concentration and sterile distilled water was used as a negative control. The plates were incubated at 35°C for 24 hours. Tests were performed in triplicate and the MIC values were determined by observing the minimum concentration that caused a zone of inhibition.

**Preparation of Fish Diets:** The fish diets were prepared by mixing commercial tilapia feed with *C. verum* in different ratios. Each supplemented diet was prepared by mixing commercial fish feed with distilled deionized water (1.1 ml/g) until a homogenous mixture was obtained. The mixture was then passed through a meat mincer to produce extruded string shapes. The diet was reformed into pellets roughly 2-mm long, dried at 30°C for 24 hours and stored at 4°C for the experiments. Diets 1, 2 and 3 were prepared by mixing dried matter of bark *C. verum* extract with commercial fish feed in ratios of 1:30, 2:28 and 3:26 (w/w) respectively. The barks of *C. verum* were ground into fine powder and mixed with commercial fish feed in ratios of 1:20, 2:18 and 3:16 (w/w) to make diets 4, 5 and 6, respectively. Diet 7 was prepared by mixing oxytetracycline with fish feed in a ratio of 1:150 (w/w) and finally Diet 8 was a normal fish feed without any supplements as a control diet. All supplemented diets were fed to uninfected fish for two weeks to screen whether the supplemented feeds had any side effects on fish.

Estimation of Medial Lethal Dose (LD$_{50}$): Nine groups of 10 healthy red tilapia fingerlings weighing approximately 11 ± 2 g were used to estimate the pathogenicity of *S. agalactiae*. Graded doses ranging from $10^0$ to $10^8$ CFU/ml were utilized. Each group of fish was IP injected with 0.1 ml of the designed dilution of *S. agalactiae* and the ninth group was injected with 0.1 ml of 0.9% normal saline solution and served as a control group. Mortalities were recorded for 10 days after infection. Dead fish were removed from the aquarium during the experiments and the freshly dead fish were submitted for bacterial isolation to confirm specificity of mortality. The LD$_{50}$ values were calculated according to the Reed-Muensch method [20].

**Experiment Trials:** To study the effect of *C. verum* on the *S. agalactiae* infection in vivo, after 7 days of feeding diets (1-8), 12 fish from each group were anaesthetized using MS-222 to reduce stress and were injected intraperitoneally with 0.1 ml of bacterial suspension at a dose causing 50% mortality (LD$_{50}$). Mortality was recorded until 17 days after post challenge. Behavioral alterations, feeding response and mortality were observed daily. Dead fish were removed and samples were taken from livers, kidneys, brains and eyes for bacteria isolation. At the end of the experiment, the surviving fish were sacrificed and internal organs were streaked on sheep blood agar (SBA) to confirm that they were free of infection. The experiment was conducted in triplicate.

**Statistical Analysis:** Mortality rates were performed using the Kaplan-Meier method and a log rank test and by analysis of variance (ANOVA). All the statistical analyses were done using MATLAB program version 7.8 (R2009a). Statistical significance was designated as a P value <0.05.

**RESULTS**

Only four spice extracts (*Cinnamomum verum, Allium sativum Linn, Eugenia caryophyllus and Thymus vulgaris*) displayed antibacterial activity against *S. agalactiae* and the rest did not exhibit any antibacterial properties. The extract of bark *C. verum* showed the strongest antimicrobial activity against *S.agalactiae* whereas the other three extracts displayed moderate to weak inhibitory activity (Table 2).

The Minimum inhibitory concentrations (MIC) of spice extracts against *S.agalactiae* results extract of bark *C.verum* showed the lowest MIC 0.15 mg/ml followed by *E.caryophyllus* 0.3 mg/ml and *T. vulgaris* 0.6 mg/ml,
Fig. 1: Cumulative mortality (%) of red hybrid tilapia fed diets supplemented with C. verum barks (CB), dried water extract of C. verum (EC), Oxytetracycline (OX) and unsupplemented diet (UN) after challenged with S. agalactiae.

Table 2: The results of screening for antibacterial activities of spice extracts against S. agalactiae

<table>
<thead>
<tr>
<th>Common name</th>
<th>Inhibition zones (mm)</th>
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</thead>
<tbody>
<tr>
<td>Garlic</td>
<td>10</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>18</td>
</tr>
<tr>
<td>Ginger</td>
<td>-</td>
</tr>
<tr>
<td>Lemongrass</td>
<td>-</td>
</tr>
<tr>
<td>Thyme</td>
<td>14</td>
</tr>
<tr>
<td>Curry</td>
<td>-</td>
</tr>
<tr>
<td>Mustard</td>
<td>-</td>
</tr>
<tr>
<td>Turmeric</td>
<td>-</td>
</tr>
<tr>
<td>Cubeb</td>
<td>16</td>
</tr>
</tbody>
</table>

(-) No inhibition was observed.

while the A. sativum extract showed the highest value of 2.50 mg/ml. Therefore, the MIC determination is important to offer an indication concerning which extracts are very active, possess antibacterial properties and to determine the effective concentration of a therapeutic substance. In the present study, cinnamon extract was chosen for experimental trials based on the antibacterial activity results obtained.

In the LD<sub>50</sub> trial, the fish started to die on the first day post challenge and mortality stopped on the 6<sup>th</sup> day post challenge. Clinical signs such as lethargy, erratic swimming, eye opacity and exophthalmia were observed during this experiment. The LD<sub>50</sub> of S. agalactiae were determined to be 1.65×10<sup>6</sup>CFU/mL. No mortality or visible changes were observed in the control group. Streptococcus agalactiae was re-isolated from the kidneys and brains of all dead fish. The in vivo antibacterial activity of cinnamon extract and powder were experimentally examined. The tilapia were fed supplemented feed in different ratios and then injected with S. agalactiae.

At the end of the experiment, the mortality rates were noticed in fish that received a diet containing cinnamon extract (3:26) and powder cinnamon bark (3:16) were significantly lower than other groups. No significant differences in fish mortality were observed between the cinnamon extract diet (3:26) and the Oxytetracycline treatment diet (1:50) Fig. 1, p<0.05.

DISCUSSION

The use of herbal extracts is widely expected to become an alternative therapy in aquaculture as a prophylactic and to control fish diseases. Studies concerning antimicrobial properties of herbal extracts against bacteria with fish culture importance in vitro and in vivo are still limited.

We tested ten popular culinary spices to determine which has the strongest antimicrobial activity against S. agalactiae. The spices used in this study are inexpensive and easy to obtain from markets in Southeast
Asia. Most of these spices are reported to have antimicrobial properties [21, 22]. Our study determined that in vitro experiments utilizing the water extracts of some spices were not able to inhibit the growth of *S. agalactiae*. It may be that the active compounds of the spices are not able to inhibit the growth of *S. agalactiae* activity and provide antimicrobial activity. This study found that the *C. verum* bark extract had the highest inhibitory activity when compared with the other extracts.

The bark of *C. verum* is known to contain biologically active compounds, i.e., Cinnamaldehyde and eugenol which were reported to have antibacterial properties [23, 24]. Cinnamon extract has been observed to display antimicrobial activity and to be effective against many types of bacteria, such as *Mycobacterium, Staphylococcus, Enterococcus, Pseudomonas* and *Micrococcus* [25]. Furthermore, it has been widely used in veterinary and human medicine [26].

Harris *et al.* [27] stated that the antimicrobial property of garlic is due to the Allicin compound. Antibacterial and antifungal activity of thyme extract has been previously reported [28, 29]. Bagamboula *et al.* [30] referred that the antibacterial properties in thyme may be related to the presence of phenolic components such as thymol. However, lemongrass and ginger extracts did not show inhibitory activity against *S. agalactiae*. Similar findings have been reported by Ushimaru *et al.* [31] who made the observation that lemongrass and ginger aqueous extracts did not inhibit the growth of *Escherichia coli, Salmonella, Staphylococcus aureus* and *Enterococcus sp.* In this study, the MIC of bark *C. verum* displayed the strongest antimicrobial activity 0.15 mg/ml while garlic showed the least activity 2.5 mg/ml. [32] found in 2008 that 62.5 mg/ml of Cinnamon extract can inhibit *Bacillus sp.* and *Staphylococcus aureus* on Mueller Hinton agar plates. Hili *et al.* [33] reported that cinnamon had the most effective antimicrobial activity in the range of 10-150 μg/mL. These differences could be due to the character and level of the antimicrobial agents present in the extracts and their mode of action on different test microorganisms.

Some medicinal herbs have been demonstrated to control fish pathogenic bacteria such as *Euphorbia humifusa, Leoncana glauca, Eclipta alba* and garlic [34, 35]. Nile tilapia that were fed lonicera extract were observed to have higher survival rates than the control fish, following a challenge with *Aeromonas hydrophila* [36].

The present results showed that feeding the fish with feed supplemented with dry bark powder of cinnamon (18.7%) or extract of *C. verum* (11.5%) significantly helped to reduce cumulative mortality after challenging the fish with *S. agalactiae* and had no toxic influence on the fish. Similar results showed that feeding catfish (*Clarias gariepinus*) fingerlings with garlic (*Allium sativum* L.) peel reduced mortality after challenging them with *A. hydrophila* [37]. Also, Harikrishnan *et al.* [38] demonstrated in 2009 that there was a reduction in mortality when feeding Goldfish (*Carassius auratus*) with tri-herbal extract supplementation feeds when the fish were challenged with *A. hydrophila*.

In conclusion the results of this study show that some culinary spices could be used as an alternative therapy as an additive to fish food in order to prevent diseases. Administering feed supplemented with bark *C. verum* extract as a prophylactic may possibly avoid and/or decrease fish mortalities. The mechanism of action of the components of bark *C. verum* extract could be difficult to speculate and further studies are required to understand the mechanism and toxicology of these spice extracts.

REFERENCES


