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PROOF

Evaluation of Antibacterial Activity of Green Tea (*Camellia sinensis* L.) Seeds Against Some Fish Pathogens in Rainbow Trout (*Oncorhynchus mykiss*, Walbaum)

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Abstract

In aquaculture, bacterial fish diseases are still one of the most serious problem and fish mortality of bacterial origin can cause significant economic losses worldwide. The evolution of microorganism resistance to antibiotics has resulted in growing need for new antibacterial compounds. In the present study, the antibacterial activity of green tea (*Camellia sinensis* L.)seed and its secondary metabolite saponin were evaluated on five bacterial fish pathogens that cause diseases in rainbow trout (*Oncorhynchus mykiss*, Walbaum). In addition to this, possible histological effects were investigated on the vital organs of fish. The antibacterial activity of tea seed and saponin were determined using the agar diffusion method. Watered tea seed (WTS) powder and saponin used in this research as the feed additives demonstrated antibacterial activity against the five pathogens but statistically significant inhibition was observed only with *Listonella anguillarum*. Accordingly, significantly higher survival levels were observed in fish infected with *L. anguillarum* in WTS and saponin diet groups. However, organs of fish fed with WTS and saponin showed some nonvital symptoms such as hyperplasia and epithelial lifting in the gills and lipid droplets, intercellular edema and nuclear degeneration in liver. Results from this study confirmed the potential use of WTS as a source of antibacterial compounds or as a feed additive for prevention of disease against *L. anguillarum* infection in trout culture.

Keywords: Tea seed, saponin, antibacterial activity, histopathology, rainbow trout.

Yeşil Çay (*Camellia sinensis* L.) Tohumunun Bazı Balık Patojenlerine Karşı Antibakteriyel Aktivitesinin Gökkuşağı Alabalıklarında (*Oncorhynchus mykiss*, Walbaum) Değerlendirilmesi

Özet

Akuakültürde bakteriyel balık hastalıkları günümüzde hala en önemli problemlerden bir tanesini oluşturmakta ve bakteriyel kaynaklı balık ölümleri dünya çapında önemli ekonomik kayıplara sebep olabilmektedir. Mikroorganizmalarda antibiyotiklere karşı direnç gelişimi, yeni antibakteriyel bileşiklere olan ihtiyacın artmasına neden olmuştur. Bu çalışmada, yeşil çay (*Camellia sinensis* L.) tohumu ve onun ikincil bir metaboliti olan saponinin gökkuşağı alabalıklarında (*Oncorhynchus mykiss*, Walbaum) hastalığa sebep olan beş farklı bakteriyel balık patojeni üzerindeki antibakteriyel aktiviteleri araştırılmıştır. Ayrıca, balıkların hayati organlarına ait dokularda oluşabilecek histopatolojik etkiler incelenmiştir. Çay tohumu ve saponinin antibakteriyel aktivitesi agar difüzyon yöntemi kullanılarak belirlenmiştir. Bu çalışmada yem katkı maddesi olarak kullanılan sulandırılmış çay tohumu (SÇT) tozu ve saponin beş balık patojenine karşı antibakteriyel aktivite göstermiştir, fakat sadece *Listonella anguillarum* bakterisine karşı istatistiksel olarak önemli derecede bir etki tespit edilmiştir. Benzer şekilde, *L. anguillarum* ile enfekte edilen balıklarda, SÇT ve saponin diyet gruplarında istatistiksel olarak önemli derecede bir hayatta kalma oranı gözlenmiştir. Bunun yanında, SÇT ve saponin katkılı yemler ile beslenen balıkların solungaç dokularında, hayati önemi olmayan hiperplazi ve epitelyum doku ayrılması, karaciğer dokularında ise hafif düzeyde yağ damlacıkları, hücreler arası ödem ve çekirdek dejenerasyonu gibi semptomlar tespit edilmiştir. Bu çalışmanın sonuçları, sulandırılmış çay tohumunun özellikle alabalık yetiştiriciliğinde karşılaşılan *L. anguillarum*enfeksiyonuna karşı potansiyel bir antibakteriyel bileşik kaynağı olabileceği veya sağlığı koruyucu bir yem katkı maddesi olarak kullanılabileceğini ortaya koymuştur.

Anahtar Kelimeler: Çay tohumu, saponin, antibakteriyel aktivite, histopatoloji, gökkuşağı alabalığı.

Introduction

Green tea (*Camellia sinensis* L.) is an important commercial plant that is produced in over 30 countries and has been consumed world wide primarily as a beverage made from the processed leaf (Chung *et al.*, 2004; Choi *et al.*, 2006). It has a variety of secondary metabolites such as catechin, caffeine, theanine and saponin that are important for human welfare (Ogutuga and Northcote, 1970; Chung *et al.*, 2004; Yoon *et al.*, 2005). These compounds in green tea are well known for their broad spectrum of biological activities such as antibacterial, antioxidant, antifungal, and antitumor functions (Mabe *et al.*, 1999; Sakanaka *et al.*, 2000; Yang *et al.*, 2000; Fassina *et al.*, 2002).

Tea seed saponin, natural surfactant exhibits a variety of biological activities (Sparg *et al.*, 2004). It has been recognized that tea seed saponin account for more than 10% per dry weight of tea seed (Xingtu, 1990). It has been commercially utilized as a foam-stabilizing and emulsifying agent (Chen *et al.*, 2010) and is extensively used in aquaculture to eliminate unwanted fish and harmful insects in prawn ponds (Chaicharoenpong and Petsom, 2009). It also has been shown to have other physiological functions such as antiexpectorant and antiinflammatory properties (Tamura, 1956; Sagesaka *et al.*,1996). Control effect of saponin against insects, pests and mites has been reported (Kawai *et al.*, 1999). In addition, It is used as a medicine for the treatment of intestinal disorders (Huang *et al.*, 2007) and burn injuries (Huang *et al.*, 2005). Saponin has also been reported to exert many pharmacological effects, including antihyperlipidemic (Yoshikawa *et al.*, 2005), antiallergic (Matsuda *et al.*, 2010), and cardioprotective effects (Liao *et al.*, 2009). Saponin as other natural products could be utilized as biological agents since it is less polluting to the environment than its chemical counter parts (Yamauchi *et al.*, 2001).

In aquaculture, diseases of microbial origin cause high mortality rates and lesions on fish, withconsequent economic losses worldwide (Toranzo *et al.*, 2005). Bacteria, mainly of *Yersinia ruckeri*, *Pseudomonas putida*, *P. luteola*, *Aeromonas hydrophila* and *Listonellaanguillarum* (formerly *Vibrio anguillarum*) have been identified as the etiological agents responsible for the disease outbreaks in fish and shellfish (Toranzo *et al.*, 2005; Austin and Austin, 2007; Radjasa *et al.*, 2009). Moreover, these microorganisms can accumulate in the reared animal's flesh and become a serious threat to the health of aquatic organisms (Cavallo and Stabili, 2002). Besides, the use of commercial antibiotics for disease treatment produces undesirable side effects, including toxicity to the reared organisms and release of chemical residues into the environment. These chemical residues can then pose risk to animal and human health (Cabello, 2006). In addition, it was reported that many pathogens have shown antibacterial resistance (Immanuel *et al.*, 2004). This has led to agrowing need for new antibacterial substances that can be effective in veterinary medicine and characterized by limited undesirable side effects (Bansemir *et al.*, 2006).

Although many studies on green tea leaf have been performed, the bioactive substances in green tea seed have not been sufficiently studied. Also, the antibacterial activity and/or histopathological effects of green tea seed or saponin have not been reported in fish. Therefore, the main objective of this study is to investigate the antibacterial effects against some fish pathogens *Yersinia ruckeri*, *Pseudomonas putida*, *P. luteola*, *Aeromonas hydrophila* and, *L. anguillarum*. This study also including assessing histopathogical effects of powdered tea seed, powdered and diluted tea seed (liquid) and saponinin rainbow trout (*Oncorhynchus mykiss*, Walbaum).

Materials and Methods

Experimental Animal and Rearing Conditions

Juvenile rainbow trout used in the experiments (0.85 ± 0.12 g, mean ± SD) were obtained from Recep Tayyip Erdoğan University, Faculty of Fisheries, İyidere R&D Unit. Fish were held in a flow-through fiberglass tank systems (150 L, 50 cm in diameter) for at least 15 days to acclimate to laboratory conditions prior to experiments. During the acclimation and experiment periods, about 50% of the water in each tank system was replaced daily. Fish were held under a photoperiod of 12 h each of light and of dark. Fishwere fed 2% body weight twice a day with commercial trout pellets. No fish died during the acclimation period. The use of fish and the experimental protocol were approved by the Animal Experimentation Ethics Committee of the Recep Tayyip Erdoğan University (RTEUAEEC). Spring test water had following quality criteria; temperature 14.7 ± 0.6 °C, dissolved oxygen 8.34 ± 0.27 mgL^{-1}, pH 7.54 ± 0.32, alkalinity 15.9 ± 0.8 mgL^{-1} as $CaCO_3$, total hardness 34.5 ± 1.9 mgL^{-1} as $CaCO_3$, nitrite 3.4 ± 1.8 μgL^{-1} and unionized ammonia 9 ± 4 ngL^{-1}. The same water supply was used during the acclimation and subsequent test periods. Specific growth rate (G, % day^{-1}) of fish was calculated according to the formula of Houde and Schekter (1981): $G = 100\ (e^{g} - 1)$, where $g = [(\ln W_F - \ln W_I)\ t^{-1}]$ and W_I and W_F are the initial and final mean wet masses, respectively, and it is the duration of the experimental period (days).

Bacterial Strains

Yersinia ruckeri, *Pseudomonas putida*, *P. luteola*, *Aeromonas hydrophila* and *Listonella anguillarum*, isolated from fish (Table 1) in our fish diseases laboratory were used as the bacterial species in the experiments. These species were maintained as

a stock culture at -80°C with 20% (v/v) glycerol containing Brain Heart Infusion Broth (BHIB, Merck) until experiments. Before experiments, bacteria were subcultured at 22ºC for 2 days on Tryptic Soy Agar (TSA, Merck) and Thiosulfate Citrate Bile Sucrose Agar (TCBSA, Merck) to check purity and then pure colonies were inoculated in BHIB and incubated for 24 h at 22°C with shaking. Pure cultured colonies were biochemically characterized with API 20E and API 20NE test strips (Biomerieux,Marcy l'Etoile, France).Then, bacteria were inoculated into BHIB for immersion experiments and Mueller Hinton Agar (MHA, Merck) for disk diffusion assays. Also, bacteria were inoculated into Plate Count Agar (PCA, Merck) plates for estimating bacterial colony forming units (CFU) and to confirm the challenge dose.

Preparation of Tea Seed

The samples of tea seed that are not commercially available were supplied from a local farmer's garden in the centre of Rize, Turkey. The tea seed was sealed in a plastic bag and stored at -20 °C until use. Pure (99%) saponin was obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). After rinsing with distilled water, tea seeds were ground using a mortar and pestle and dried under reduced pressure at 40°C for 15 days to obtain a powder (Figure 1A). Powdered tea seeds were added to the fish feed in two different ways. Either powdered tea seeds were added to the fish feed directly with liquid sunflower oil to ensure adhesion or powdered tea seeds were diluted with pure water (0.4 g mL^{-1}) and boiled for 3 min. Boiled tea seed powder was then centrifuged at 3000 rpm for 10 min (Figure 1B). Supernatant were taken and added to the fish feed directly.Sunflower oil was added to all diet groups in the same amount.

Experimental Design

Before feeding experiments, the fish were confirmed to be free of pathogenic bacteria and external parasites. After acclimation, 60 fish from the acclimation tanks were randomly transferred to one of 12 fiberglass tanks containing 150 L of flow-through well water. Powdered tea seed (10 and 20% of feed weight), watered tea seed (WTS) (10 and 20% of feed weight) and saponin (5% of feed weight) were added to the fish feed. Fish were fed with these 5 different feeds and control fish were fed with normal pellets. Duplicate tanks were designated for each group and experiment was conducted for 30 days. During the experiment, water in each tank was aerated and quality parameters (temperature, dissolved oxygen, pH, alkalinity, total hardness, nitrite and unionized ammonia) were regularly monitored. At the end of the study, 10 fish from each group were sampled for histology.

In Vitro Antibacterial Effects of Tea Seed to Bacteria

Antimicrobial activity was evaluated using the

Table 1. Bacterial isolates used in the present study

Bacterial species	Host	Isolated organ	Year of isolation
Yersinia ruckeri	Rainbow trout (*Oncorhynchus mykiss*)	Liver	2010
Pseudomonas putida	Goldfish *(Carassius auratus)*	Skin	2010
Pseudomonas luteola	Rainbow trout (*Oncorhynchus mykiss*)	Liver	2009
Aeromonas hydrophila	Rainbow trout (*Oncorhynchus mykiss*)	Kidney	2010
Listonella anguillarum	Rainbow trout (*Oncorhynchus mykiss*)	Spleen	2009

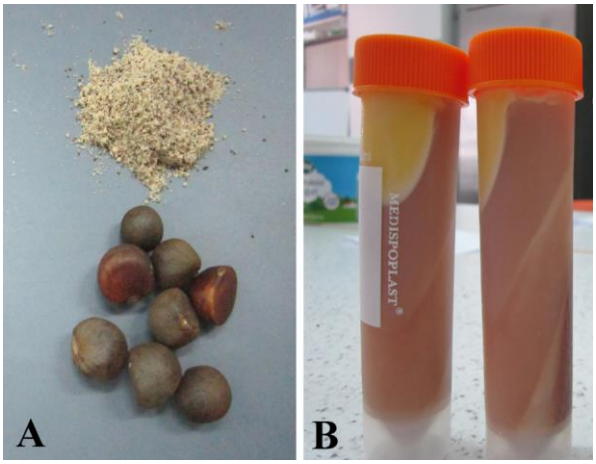

Figure 1. Green tea seed used in the present study. Tea seeds grounded using a mortar and pestle (A), boiled and centrifuged tea seed powder (liquid) (B).

disc diffusion method. Sterile paper discs, 7 mm in diameter (Whatman International Ltd., Maidstone, UK), were impregnated with sterile WTS and left to air-dry at room temperature for 4 h. To verify the possibility that antibiotic activity could be affected by other factors, discs impregnated only with pure water were used as a negative control. For each assay, autoclaved MHA plates were seeded with 100 µL of test bacterial suspension(10^8 CFU mL^{-1}), using a sterile swab to give an uniform covering. Impregnated discs and controls were laid on to the agar surface and the plates were then incubated for 24-48 h at 22°C. The clear zone around the discs was evidence of antibacterial activity and was measured in mm (Figure 2). The diameterof the microbial growth inhibition was taken as the diameterof the clear zone (measured in millimetres). Each test was conducted in triplicate for each bacterial species.

In Vivo Antibacterial Effects of Tea Seed

Trouts fed with powdered tea seed, WTS and saponin combined with fish feed for 30 days were infected with bacteria by immersion in laboratory conditions. After feeding experiment, 10 fish from each experimental group (10 and 20% powdered tea seed, 10 and 20%WTS, 5% saponin, positive and negative control) were transferred to duplicate tanks containing 20 L of flow-through well water. Pure bacteria colonies were inoculated in 240 mL BHI broth and incubated for 24 h at 22°C. At the end of incubation period, fish were infected with bacteria (10^8 CFU mL^{-1},within 20 mL BHIB counted in PCA plates) by immersion for 1 h. Axenic BHI broth were only added to the tanks of negative control group. Fish were not fed during the bacterial challenge period. At the end of challenge period, number of dead fish in different experimental groups were noted down.

Histopathology

Histological analysis were performed to determine the possible damage of tea seed and saponin to the vital organs of fish. After completing 30 d of feeding with tea seed and saponin (mixed with fish feed), fish from each experimental and control tanks were anesthetized with overdose of benzocaine (as approved by the Ethics Committee of the Recep Tayyip Erdoğan University). Then, the primary lamellae of gill, trunk kidney, spleen, liver, intestine, and skin of fish were carefully removed and preserved in 10% neutral buffered formalin(NBF) for 48 h. Tissues were rinsed in 2 changes of 50% ethanol (EtOH), and stored in 50% EtOH until further processing. Then, the tissues werede hydrated in isopropanol, cleared in xylene, infiltrated in paraffin and sectioned at a thickness of 5-7 µm. Sections were stained with hematoxylin and eosin, and examined using light microscope.

Statistical Analysis

Data were subjected to statistics analysis by using the software package SPSS Statistics 17.0 for Windows (SPSS Inc., Chicago, IL, USA, 2008). Results were expressed as means ± SEM. ANOVA was carried out to assess significant differences in the inhibition of bacterial growth produced by tea seed and saponin against each bacteria species ($P<0.05$).

Results

No mortality occurred during acclimation or 30 days experimental period before bacterial challenge, and no control fish died during the tests. WTS and saponin used in this studyas feed additives demonstrated antibacterial activity in the bacterial disc diffusion assay against five bacterial fish pathogens. However, statistically significant inhibition was observed only in *L. anguillarum*(Figure 2, 3) ($P<0.05$).In addition, WTS(20%) exhibited higher level of antibacterial activity than saponin (5%)for antibacterial activity (Figure 3).

There was no statistical difference in the survival rates of rainbow trout infected with *Y.*

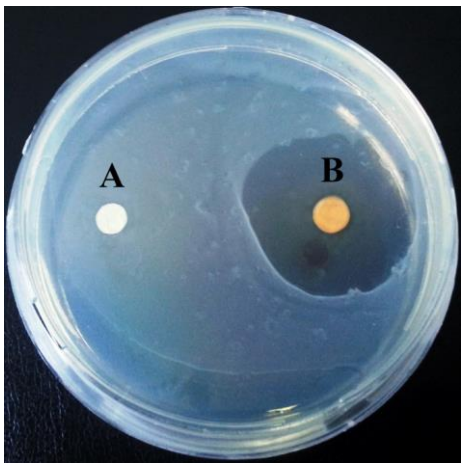

Figure 2. Bacterial disc diffusion assay. Liquid green tea seed against *Listonella anguillarum*, Negative control (A), Disc impregnated with WTS (B).

ruckeri, *P. putida*, *P. luteola* and *A. hydrophila*compared to the control groups. Significant differences in survival rates were observed between fish groups fed WTS (100% survival of first day) and saponin (77% survival of first day) when infected with *L. anguillarum*in WTS(20%) and saponin diet groups on the first and subsequent days (Figure 4) ($P<0.05$). Furthermore, fish fed with WTS were recorded higher survival rates than other groups (Figure 4).

After 30 day study period, no significant differences were observed in the specific or total weight of fish fed tea seed or saponin mixed pellets compared to control groups (Figure 5).Moreover, no histopathological changes were observed in the gills (Figure 6A), trunk kidney, liver (Figure 7A), spleen, intestine and skin of control fish. Organs of fish fed with powdered tea seed showed no tissue damage. WTS (20%) and saponin fed fish showed mild symptoms such as hyperplasia and epithelial lifting in the secondary lamellae of gills (Figure 6B, 6C) and presence of lipid droplets, intercellular edema and nuclear degeneration in liver tissue (Figure 7B, 7C). No damage was observed in the other organs (trunk kidney, spleen, intestine and skin) of fish fed WTS and saponin.

Discussion

Discovery of potent plant and plant seed extracts; feed additives that can effectively inhibit bacterial growth and having antioxidant properties is a continuing challenge and opportunity. Therefore, the main objective of this work was to evaluate the capability of green tea seed and its secondary metabolite saponin to inhibit the growth of

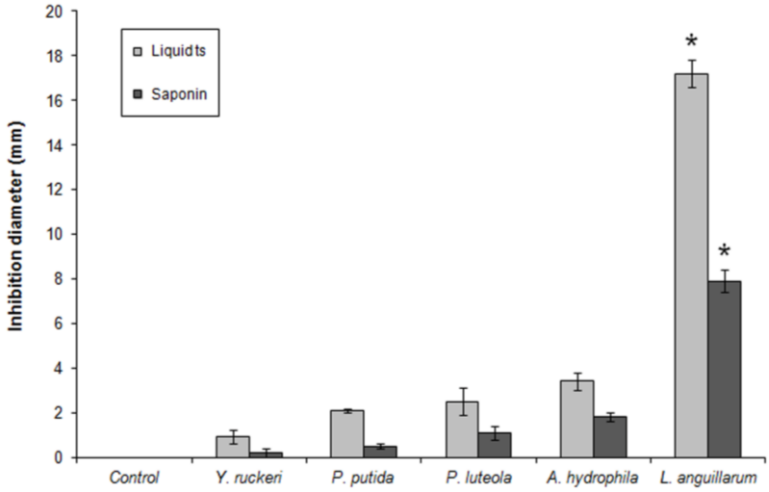

Figure 3. Antibacterial activity of WTS (20% of feed weight) and saponin (5% of feed weight) against the important fish pathogens. Zone of inhibition in mm (mean ± SD). Asterisks above individual bars indicate the significantly ($P<0.05$) higher diamaters of inhibition.

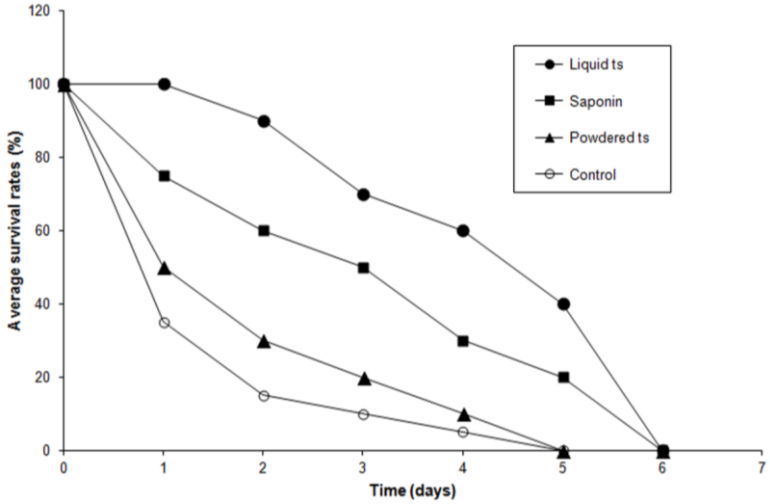

Figure 4. Average survival of rainbow trout infected with *L. anguillarum*. Before challenge experiments, fish were fed with WTS (20% of feed weight), powdered tea seed (20% of feed weight), and saponin (5% of feed weight) mixed pellets for 30 days.

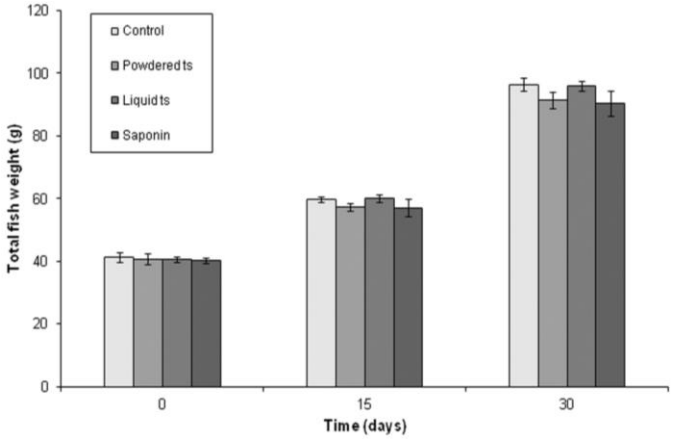

Figure 5. Effects of powdered tea seed, WTS and saponin diets (mixed with feed) on weight gain in rainbow trout over time. During the 30 day feeding period, mean weights were not significantly different between diet groups (P>0.05).

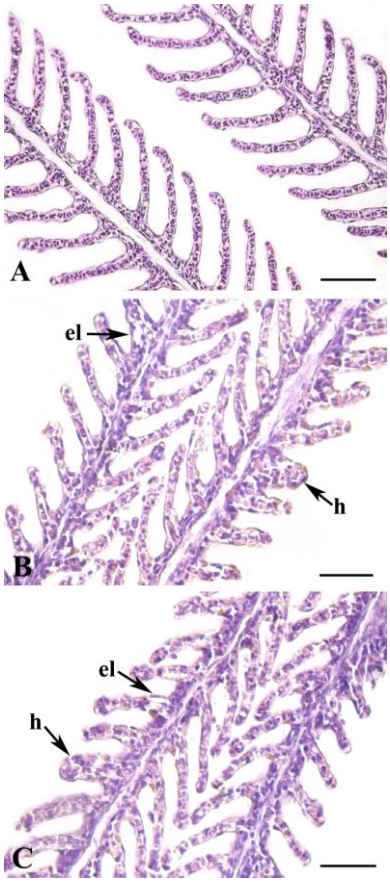

Figure 6. Longitudinal sections of rainbow trout gills. (A) Control gill filament and lamellae. Fish were only fed with normal trout pellets for 30 days. (B) Fish were fed with WTS (20% of feed weight) mixed pellets. (C) Fish were fed with saponin (5% of feed weight) mixed pellets. h: gill exhibiting hyperplasia, el: epithelial lifting. Scale bars = 50μm.

somespecies of fish pathogenic bacteria (*Y. ruckeri, P. putida, P. luteola, A. hydrophila* and *L. anguillarum*) with the aim of assessing them as possible disease preventive additives in aquaculture.

This study revealed different levels of bioactivity in the powdered tea seed, WTS and saponin extracted from the green tea seeds.The products also exhibited various levels of antibacterial activity with bacterial strains investigated. WTS had the broadest antibacterial spectrum; indeed it was active against the five bacteria species, although with different inhibition strength. Similarly, a previous study demonstrated that extracts and fractions of green tea had bacteriostatic activity against some vibrio species (Toda *et al.*, 1989). Also, multiple lethality hurdles to the growth of pathogens including *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium* can be provided by using

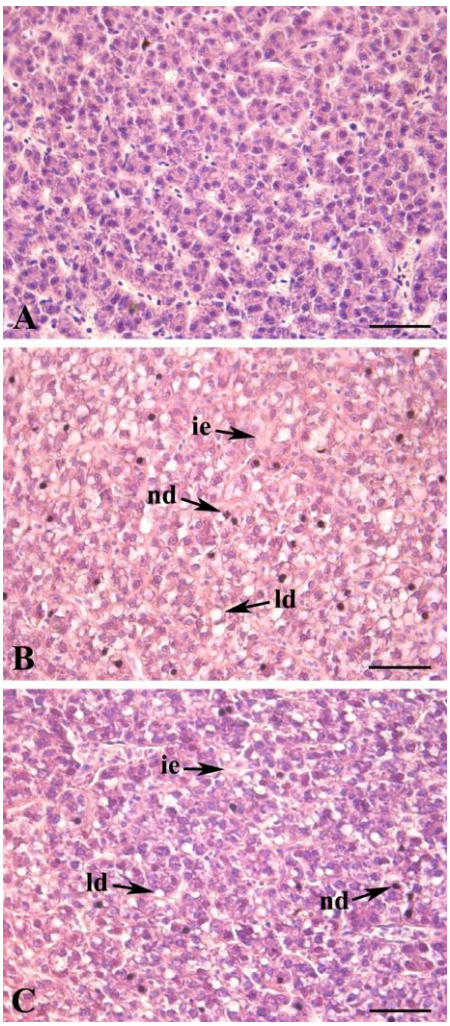

Figure 7. Histological section of rainbow trout liver 30 d after feeding experiment. (A) Control liver. Fish were only fed with normal trout pellets. (B) Fish were fed with WTS (20% of feed weight) mixed pellets. (C) ld: lipid droplets, nd: nuclear degeneration, ie: intercellular edema. Scale bars = 50μm.

lesser amount of chemical antimicrobials in combination with natural antimicrobials from green tea and grape seed extracts (Gadang *et al.*, 2008; Chan*et al.*, 2011).

In the present study, the secondary metabolite saponin of green tea seed was also showed antibacterial activity, although it was not as active as WTS against the five pathogenic bacteria. In a previous study, saponin demonstrated antibacterial activity againstsome Gram-negative bacteria, (Rasheed and Haider, 1998).To our knowledge no other reprint is available related to potential use of bioactive compounds from green tea seed. Thepositive results obtained in the present study could,therefore, represent a valid basis for the development of research on green tea seed or its secondary metabolites, considering that green tea is one of the most widely consumed beverages in the world (Kubo *et al.*, 1992).

With WTS, our results showed substantial bioactivity against *L. anguillarum*, with a higher inhibition powercompared to the other four bacteria species. In a similar study, the liquid extracts of green tea seedhave shown inhibitory effects on some Gram-positive as well as Gram-negative bacteria (Gadang *et al.*, 2008). No *Vibrio*species were tested in this study.On the other hand, the green tea extract had a weak/medium activity against *Vibrio cholera*(Hamilton-Miller, 1995). Therefore, the antibacterial activity of green tea seed extracts against *L. anguillarum*tested, could confirm a good perspectivefor the use of WTSas a food additive used for prevention of disease. These results provideencouragement for continuation of research on green tea seed extracts in order to verify their antibacterial activityagainst *Vibrio* spp. with different extraction methods.

In the present study, the least bioactivity against pathogens was noticed with powdered tea seed in fish at the end of the experiments. Antimicrobial effect of plant extracts depends on pH and solubilityof the extract in the model systems (Hao*et al.*, 1998). Plant extracts that are having low pH are more effective in inhibiting the microbial growth (Conner and Beuchat, 1984). Green tea extracts demonstrated inhibitory properties against major pathogens including

L.monocytogenes, *E. coli*,*Salmonella typhimurium*, *Campylobacter jejuni*, and others including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella Enteriditis*, *Shigellaflexneri*, *Shigella dysenteriae*, and *V. cholera* (Toda *et al.*, 1991; An *et al.*, 2004; Gadang*et al.*, 2008). Irradiation of some green tea extracts could enhance the antimicrobial properties against *S. aureus*, *S. epidermidis*, *E. coli* and *S. mutans* (An *et al.*, 2004).Chinese green tea extracts showed stronginhibitory effect on major pathogens (Si*et al.*, 2006). Over *et al*. (2009) demonstrated that green tea extracts alone or in combination with tartaric acid reduced *Salmonella*, *Listeria* and *E. coli* by at least 3.5 log $CFUmL^{-1}$ in broth culture studies. The antimicrobial activities of green tea extracts when combined with bacteriocins have demonstrated more effectiveness than when used alone against major pathogen like *L. monocytogenes*.This may be due to the synergistic mechanism of action of bioactive compounds present in green tea extract (Sivaroobana*et al.*, 2008).

Despite the potential benefits of green tea extracts in the prevention of bacterial diseases and reducing the effects of bacterial pathogens, there are concerns regarding the side effects of these extracts when taken as dietary supplement (Perumalla and Hettiarachchy, 2011). In the present study, WTS and saponin as dietary supplement mildly affected some of the vital organs of fish. Therefore, further research is required to address this concern and validate the beneficial effects and safety of using green tea seed extracts. However, we should consider the fairlylow concentration level of green tea seed extracts in feed applications when compared to pharmacological doses for beneficial effects in animals. Potential use of these natural extracts could offer the aquaculture industry an alternative solution to synthetic chemical antimicrobials.

The concentrations of WTS and saponin used in this study were higher than those used in other applications in aquaculture. Therefore, theseantibacterial agents may be effective at higher dosages. However, high concentrations not onlyinhibit the tested bacteria, but could also affect rainbow trout gill and liver slightly. The observations suggest that primarily WTS and secondarily saponin are effective agents against *L. anguillarum*in vivo and in vitro.

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