RESEARCH ARTICLE

The *in vitro* Antibacterial Activity and Ornamental Fish Toxicity of the Water Extract of Indian Almond Leaves (*Terminalia catappa* Linn.)

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Abstract

Objective — To determine concentration of tannin, an antimicrobial substance, in the water extract of Indian almond leaves (*Terminalia catappa* Linn.), evaluate *in vitro* antibacterial activity against bacteria isolated from aquatic animals, and assess toxicity of the extract in three species of ornamental fish: a guppy, a fancy carp, and the Siam fighting fish.

Materials and Methods — The dried leaves of Indian almond were extracted with water for 1, 3, and 7 days. Then, the amount of tannin in the extract was measured. Based on tannin analysis, only the extract for 3 days was used in this study. For *in vitro* antimicrobial activity test, 15 strains of bacteria isolated from ill aquatic animals were used. Minimal inhibitory concentration (MIC) of the extract was determined by agar dilution technique. For *in vivo* toxicity test, guppies, fancy carps, and Siamese fighting fish, with 30 fish in each species, were used. Fifty percent lethal concentration (LC_{50}) was also determined.

Results — Total tannin levels of the extracts for 1, 3, and 7 days were 4.02, 13.60, and 14.08 mg/ml, respectively. For antimicrobial test, MIC of the extract for 3 days was ranged from 0.8-2.0 mg/ml. For toxicity test, a guppy were more sensitive to the extract than a fancy carp and the Siamese fighting fish, respectively. In a guppy, a fancy carp, and the Siamese fighting fish, LC_{50} at 24 hours were 6.2, 7.6 and 8.6 mg/ml; LC_{50} at 48 hours were 5.4, 7.0 and 8.2 mg/ml; LC_{50} at 72 hours were 5.8, 5.9 and 7.6 mg/ml; and LC_{50} at 96 hours were 5.6, 5.8 and 7.0 mg/ml, respectively.

Conclusion — This study indicated that the extract had a potential to use as an antibacterial alternative for ornamental fish culture.

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Key words: *Aeromonas hydrophila;* Fish; Indian almond leaves; Toxicity

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Introduction

Indian almond tree (*Terminalia catappa* Linn.) is a Combretaceous plant (tropical almond family), presenting throughout any region of Thailand. The plant is a large tree, which can reach up to 30 m height with a thick broad trunk; the leaves cluster toward the end of the branches with glossy, obovate blades mostly 8-30 cm in length and turn red before turning brown and falling [1].

In Southeast Asia, leaves and barks of Indian almond tree are widely used in human as a folk medicine to treat dermatosis, hepatitis, thrush and other oral infections, and intestinal ailments in children. Decoction of the leaves is used to treat indigestion, furred tongue, bronchitis, and tuberculosis. The crushed leaves mixed with coconut oil or coconut cream were used to relieve muscle pain from fractures and sprain [1]. On the other hand, in modern medicine, many pharmacological studies on various extracts of the leaves and barks have been reported to possess anti-cancer [2], antioxidation [3], anti-HIV reverse transcriptase [4], hepatoprotection [5], anti-inflammation [6], aphrodisiac activities [7], antifungal properties againt *Pythium ultimum, Rhizoctonia solani, Sclerotium rolfsii,* and Aspergillus fumigatus [8], and antibacterial properties against; *Staphylococcus epidermidis, S.aureus, Bacillus cereus, B. subtilis,* and *Pseudomonas aeruginosa* [9].

The chemical compositions of this plant consist of tannins (punicalagin, punicalin, terflavin A and B, tergallagin, tercatain, chebulagic acid, geranin, granatin B, corilagin), flavanoids, isovitexin, vitexin, isoorientin, rutin and triterpenoiods (ursolic acid, 2α, 3β, 23-trihydroxyurs-12-en-28 oic acid) [10]. Tannin, a polyphenolic compound commonly found in most herbs, has antibacterial properties. [11]

In aquaculture, The Indian almond leaves have been claimed as a promoting substance for wound healing, especially for injured Siamese fighting fish after fighting matches. Chansue *et al.* [12] reported increasing thickness of keratin layer in Siamese fighting fish scale. The leaves have a potential to use as an alternative treatment for chemical substances and antibiotics. Various concentrations of the extracts in water to prevent fish pathogen have been examined. Chansue and Tangtrongpiros [13] found that water extracts of the dry leaves can rapidly promote regeneration of fin tail of fancy carp. Chitmanat *et al.* [14] reported effectiveness of 0.8 mg/l concentration of leaf extracts of Indian almond tree against *Trichodina* and other bacterial infections in tilapia, and against fungal infection in tilapia egg. In addition, the leaf extracts can eliminate *Zoothamnium spp.* infection of black tiger postlarva shrimp within 24 hours after exposure [15], and can significantly decrease the number of *Gyrodactylus* and *Dactylogyrus* infection of gold fish [16].

In Thailand, leaves of Indian almond tree have been widely used in Siamese fighting fish culture as bath supplement for treating the injured fish and promoting the fish breeding. However, the breeders still use their experiences to estimate the concentration of the leaves.

Chansue [17] reported effects of the extract on hematology and blood chemistry of Siamese fighting fish but not reported on its toxicity level. The objectives of this study were to evaluate the antibacterial activity of the water extract of dried Indian almond leaves and to evaluate its toxicity in ornamental fishes.

Materials and Methods

Collection of the leaves and preparation of the extract

Leaves of Indian almond tree were collected from some area in Bangkok, Thailand. The leaves were dried in laboratory room at room temperature. To yield 50 mg/ml concentration, 1 kilogram of dried leaves was minced and soaked in 20 liters of distilled water for 1, 3, and 7 days at room temperature. The extracted solution was filtered (Whatman paper No. 4). Then, various dilutions of the extract were prepared for optical density (OD) measurement by using Spectrophotometer at 245 nm. wave length. Antibacterial activity and toxicity test were determined by using the extract for 3 days.

Tannin analysis

Total tannin concentration was determined by Colorimetric method [18].

Antibacterial activity

Minimum Inhibitory Concentration (MIC) was done by the agar dilution technique. The bacterial isolates used in this study were derived from fish and other aquatic animals from the Veterinary Medicine Aquatic Reserch Center (VMARC), Chulalongkorn University, Thailand. The isolates consisted of: *Aeromonas hydrophila , A. sobria, Photobacterium damsela, Pasteurella pneumotropica, Burkholderia cepacia, P. aeruginosa, P. oryzihabitans, Proteus valgaris, Vibrio parahemolyticus, V. fluvialis, V. alginolytica, Shewanella putrefaciens, Stenotrophomonas maltophilia, Klebsiella pneumoniae, and Enterococcus fecalis.* The API-20E test (bioMerieux®, SA France) was used for bacterial identification.

A. hydrophila, A. sobria, P. aeruginosa, P. damsela, P. valgaris, E. fecalis and *P. pneumotropica* were cultured on Mueller-Hinton agar plate. Other bacterial isolates were cultured on Mueller-Hinton +1% NaCl agar plates. The bacterial suspension was diluted into 10^4 cfu/ml (MacFarland nephelometer tube No. 0.5) and was spreaded on to the surface of agar medium plates containing each concentration (5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.8, 0.6, 0.4 and 0.2 mg/ml) of the 3 days extract. In the control plates, we used distilled water and 95% ethanol. All samples were tested in triplicates.

Acute toxicity test of the extract

Fish

We used 3 species of ornamental fish including 210 guppy fish (*Poecilia reticulate*), fancy carp (*Cyprinus carpio*), and Siamese fighting fish (*Betta splendens*) with average length of 2.0+0.21, 4.2±0.4 cm, 2.25±0.25, respectively. All fish, 30 fish of each type, were acclimated for 14 days. Feed was given at 3% body weight twice daily. Static renewal system was also applied daily.

Fifty percent lethal concentration (LC_{50})

The acute toxicity of the extract was observed in guppy, fancy carp, and Siamese fighting fish. Concentrations (1.0, 2.0, 4.0, 6.0, 8.0, and 10 ppm) of the 3 days extract were tested in 30 fish of each species in the 50 liter glass aquarium without any change of water for 96 hrs. The water was continuously aerated. Mortality of fish was observed daily and the dead fish were instantly removed.

Water quality analysis

Water quality parameters were measured in both the experimental and control groups. The pH was determined by pH meter (Salinger & Mack®). Alkalinity, hardness, ammonium, and nitrite were determined by spectrophotometric kit (AQUA-VBC®). Dissolved oxygen was measured by Oxygen meter (YSI MODEL 57®). Each parameter was determined in triplicates for each group.

Statistical analysis

For warter parameters, the experimental data was calculated in the mean percentage of each water parameter, compared with control groups. Differences between two means were evaluated by the Student's t-test. For acute toxicity test, statistical analysis to determine median lethal concentrations at 24, 48, 72, and 96 hrs used the binomial/nonlinear progression method, nominal concentrations were used for the calculations [19].

Results

The results showed that total tannin level increased when duration of extraction increased. The total tannin levels of 1, 3, and 7 days extracts were 4.02, 13.60, and 14.08 mg/ml, respectively (**Table 1.**).

MICs of the extracts ranged between 0.8-2.0 mg/ml (**Table 2.**). No growth inhibition was observed in the control group. The liquid extractions showed higher effect against *P. pneumotropica* (0.8 mg/ml), *Photobacterium damsela* and *Enterococcus faecalis* (1.0 mg/ml), and lower effect against other bacterial organisms (1.5-2.0 mg/ml).

Table 1. Correlation of optical density (OD) and tannin level of the extract of dried Indian almond leaves with various durations of extraction.

Duration of extraction	OD	Tannin (mg/ml)
Control	0	0
1 day	0.045	4.02
3 days	0.211	13.60
7 days	0.243	14.08

Table 2. MICs (mg/ml) of the extract of dried Indian almond leaves.

Bacteria species	VMARC Code	MIC (mg/ml)
Gram negative		
Aeromonas hydrophila	Ah001	1.5
	Ah002	1.5
	Ah003	2.0
	Ah004	1.5
Aeromonas sobria	As001	2.0
	As002	2.0
Burkholderia cepacia	Buc001	2.0
Pseudomonas aeruginosa	Psa001	2.0
Pseudomonas oryzihabitans	Pso001	2.0
Pasteurella pneumotropica	Pap001	0.8
Photobacterium damsela	Phd001	1.0
	Phd002	1.5
Proteus valgaris	Prv001	1.5
Shewanella putrefaciens	Shp001	2.0
Stenotrophomonas maltophilia	Stm001	2.0

Table 2. MICs (mg/ml) of the extract of dried Indian almond leaves. (Cont.)

Bacteria species	VMARC Code	MIC (mg/ml)
Vibrio Parahemolyticus	Vp001	2.0
Vibrio fluvialis	Vf001	2.0
Vibrio alginolytica	Va001	1.5
Gram positive		
Enterococcus faecalis	Enf001	1.0
	Enf002	2.0
Klebsiella pneumoniae	Klp001	2.0

When dried Indian almond leaves were extracted in water, the water gradually turned brown, like tea color and generated acidic condition of the water as shown in **Table 3**. At the concentrations of 0-4.0 mg/ml, the water pH of the extract was slightly lower (7.6-6.7) than that of the control group, but pH of the extract at the concentrations of 6.0-10.0 mg/ml were more acidic (pH 6.5-6.0) and significantly lower than that of control ($p<0.05$). The extract of dried Indian almond leaves affects water quality by increasing ammonium ion level in the water. Other parameter changes were not significantly different.

Table 3. Water parameters of various concentrations dried Indian almond leaves.

Conc. (mg/ml)	pH	Alkalinity (ppm)	Hardness (ppm)	Ammonium (ppm)	Nitrite (ppm)	D.O. (ppm)
Control	7.60±0.14	60±2.5	180±10	0.00	0.000	4.28±0.36
1.0	7.52±0.13	60±0	140±10	0.10±0.05 *	0.000	4.80±0.09
2.0	7.43±0.13	60±2.5	120±15	0.15±0.05 *	0.000	3.98±0.34
4.0	6.78±0.22	60±0	160±10	0.15±0 *	0.000	4.78±0.03
6.0	6.52±0.14*	50±5.0	180±15	0.20±0 *	0.000	5.00±0.07
8.0	6.29±0.12*	50±5.0	140±15	0.15±0.05 *	0.000	4.80±0.07
10.0	6.09±0.16*	60±0	160±10	0.10±0 *	0.000	4.42±0.37

* Value differs significantly ($p<0.05$) from value of control groups.

Table 4. Mortality of guppy, fancy carp, and Siamese fighting fish exposed to the extract of dried Indian almond leaves at LC_{50} values.

Fish	LC_{50}			
	24 h.	48 h.	72 h.	96 h.
Guppy (n=30)	6.2	5.4	5.8	5.6
Fancy carp (n=30)	7.6	7.0	5.9	5.8
Siamese fighting (n=30)	8.6	8.2	7.6	7.0

The extracts cause acute toxic to guppy, fancy carp, and Siamese fighting fish. The toxicity of the extracts was shown in **Table 4**. There was no mortality in the control groups (0 mg/ml). All fish showed similar clinical signs when initially exposed to all concentrations. From observation, their rate of respiration increased according to faster opercula movement. The survived fish returned to normal within 24 hrs. after treatment. Heavy solid suspension adhered to the gills was observed during necropsy.

Discussion and Conclusion

Tannins possess antibacterial properties [11]. Tannic acid can inhibit the growth of intestinal bacteria by binding with metal ions especially strong binding with iron and then forming a chelate. The chelate, like a siderophore, is toxic to the membranes of microorganisms. When tannins form chelating complex with iron in the medium, this action makes no iron available for microorganisms to grow under aerobic condition. Bacterial growth was inhibited due in part to the malfunction of the reduction of ribonucleotide precursor of DNA, formation of heme, and other essential mechanisms [20]. According to this study, the total tannin level was rapidly increased within the first 3 day of extraction then gradually increased. We can get higher concentration of tannin when allowing longer time of extraction. However, the longer the time, the higher the number of microorganisms contaminated [21]. Therefore, the extract of Indian almond leaves for 3 days was the most appropriate for use.

In this study, MICs of the extracts were ranged from 0.8-2.0 mg/ml. The extracts were more effective against *P. pneumotropica* (0.8 mg/ml), *Photobacterium damsela* and *Enterococcus faecalis* (1.0 mg/ml), but less effective against other bacterial organisms (1.5-2.0 mg/ml). Chitmanat *et al.* [22] also reported that water solution of dried Indian almond leaves (0.8 ppm) was able to inhibit *A. hydrophila* infection in tilapia.

The extracts are gradually acidic when their concentrations increase. Acceptable pH for most fish cultures ranges from 6.2 to 7.8, but the rapidly change of pH over 0.2 may affect the fish growth and can cause mortality [23]. The extracts also affect quality of water by increasing level of ammonium ion in the water. Although ammonium ion (ionized ammonia) is less toxic to fish than ammonia, increasing in ammonium ion may indicate increasing in ammonia. Thus, balancing between ammonium ion and toxic ammonia should be determined by measuring pH and temperature of the water [23].

Results from this study indicated that the extracts were toxic to any individuals depending on the species of fish. Guppies were more sensitive than fancy carp and Siamese fighting fish, respectively. Gills of the fish adhered with heavy solid suspension could be observed when the fish underwent necropsy. This occurring might be another cause of death because the adhered gills were blocked for oxygen and were irritated by high concentration of tannins [24]. Tannic acid exhibits chelating properties when soaked in water, and can bind cation in water to form colloid that possibly causes adhesion in fish gills [11]. Borisutpeth et al. [25] reported that tannic acid caused hyperplasia of epithelial cells of gill filaments, fusion, disarray, and aneurysm of gill lamellae, but no histopathological changes of other organs in tilapia when the acid was used with concentration of 97.5 mg/ml for 96 hrs. However, in this study, the treatment concentrations against aquatic bacteria (1.5-2.0 mg/ml) were much lower than the lethal concentration. Therefore, Indian almond leaves can be developed for safer treatment of bacterial infection in fish.

In conclusion, the water extracts of Indian almond leaves have potential to use as an alternative of antibacterial agents and chemical substances. As natural products, the extracts may overcome the problems of chemical residues and antibiotic resistances in fish cultures.

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