

Ethnobotanical Uses and Antimicrobial Properties of Plants in Small-Scale Tropical Fish Farms: The Case of Indonesian Fish Farmers in Java (Indonesia)

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Abstract

Among the most common plants used by fish farmers in Java, 18 were selected for deeper ethnobotanical and laboratory investigation into their antimicrobial activity. These plants are mainly used against fish diseases, but with no precise therapeutic indication, leading to low specificity of use. Leaves, which were the most commonly used part of plants, were generally placed directly in water or less frequently added to the feed. Disk diffusion tests showed that 15 plants of the 18 (83%) displayed varying degrees of antibacterial activity and that *Streptococcus agalactiae* was more sensitive than *Aeromonas hydrophila*. Crude ethanolic extracts (EE) were more active than water macerates (WM), and minimum inhibitory concentrations (MICs) ranged from 12.5 to 25 mg/mL. Only the EE of *Piper betle* had an inhibitory effect against *A. hydrophila*. No MIC was found for any of the WM studied here. Furthermore, both the EE and, to a lesser extent, the WM of active plants were able to reduce bacterial growth by more than 99%, even at doses below their MICs. These results suggest that these plant extracts have a potential for eco-friendly prevention of bacterial fish diseases.

Fish diseases are considered to be a serious threat to the sustainability of aquaculture, and global outbreaks contribute to the loss of several billion US dollars per year (Subasinghe 2005). It has been shown that diseases and health problems particularly affect small-scale aquaculture, as reported in several Asian countries (Mazid

and Banu 2002; Somga et al. 2002; Faruk et al. 2004; Leung and Bates 2013).

Indeed, poor health management and misdiagnosis of diseases by fish farmers can lead to misuse of antibiotics in aquaculture, with an impact on both human health and the environment, which also threatens the sustainability of the fish-farming industry. Sanitary hazards related to residues of antibiotics in fish flesh and the spread of antibiotic resistance to pathogenic

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or commensal bacteria in aquaculture are well documented (Cabello 2006; Sarter et al. 2007). As a result, the use of antibiotics in aquaculture is now the subject of strict regulations in many countries. Hence, there is an urgent need for sustainable alternatives, in particular, for the development of herbal therapy, which is recognized to be a promising, effective, and eco-friendly alternative to the use of antibiotics or drugs in fish and shrimp farming (Reverter et al. 2014; Valladão et al. 2015).

Considering the extent of plant diversity, the selection of plant candidates is a complex task. Compounds derived from ethnobotanical studies have greater pharmaceutical properties than those selected by random screening and therefore have greater potential for the development of innovative therapeutic products (Flaster 1996). Several ethnobotanical parameters, including the fidelity level or the informant consensus factor, are frequently used to select plants (Ali-Shtayeh et al. 2000). However, they require specific knowledge about disease diagnosis and the corresponding plant use, which the fish farmers generally do not have. The use value (UV) (Upadhyay et al. 2011), a quantitative method that evaluates the relative importance of plant species known locally, may be a valuable alternative. An ethnobotanical survey performed in Western Java revealed that 26 of the 79 plants used by fish farmers had a high UV (above 0.025) (Caruso et al. 2013). These 26 plants can be considered the most frequently used ones in herbal therapy by fish farmers in West Java. Two additional ethnobotanical surveys were carried out in 2013–2014 to collect supplementary and more detailed information about their use. The surveys covered 70 fish farmers in different villages in Bogor district (West Java) and 380 in Central Java province. Among the 26 frequently used plants, 18 were randomly selected for the determination of their antimicrobial activity. The antimicrobial activity of plant extracts depends on the solvents used for extraction or maceration (Eloff 1998). Hence, an organic solvent (95% ethanol) and an inorganic solvent (bidistilled water) were used in this study.

The objectives of this study were (1) to understand how small-scale fish farmers in Java use plants and (2) to perform an *in vitro* evaluation of their antimicrobial properties against two pathogenic fish bacteria, a Gram-positive *Streptococcus agalactiae* and a Gram-negative *Aeromonas hydrophila*, both of which have a high incidence in tropical aquaculture (Pridgeon and Klesius 2013).

Materials and Methods

Ethnobotanical Surveys

A knowledge, attitude, and practices (KAP) study (Vigneron et al. 2005) was included in both the surveys. The KAP questionnaire was based on closed (yes/no) and open (what, which, and who) questions (total $n = 81$), including sections related to diseases and health management and to the use of plants. The questionnaire was tested on a subsample of fish farmers, and an inquirer was specially trained to conduct the survey. The resulting ethnobotanical indications (Table 1) correspond to the ratio of specific use of a plant to the total number of respondents who use the plant, expressed as a percentage.

Plants and Preparation of Plant Extracts

Leaves of the plants (Table 1) were purchased from the Indonesian Medicinal and Aromatic Crops Research Institute in Cimanggu, Bogor, and bulbs and rhizomes were purchased at a local market. The leaves, bulbs, and rhizomes were cleaned, wiped, cut, and oven dried at 55–60 C for 15–20 h; dried plants were then ground in an electric mill, sieved with a tea strainer, and stored at 25 C in an airtight container until use. Ethanolic extraction and water maceration were carried out in triplicate for each plant. Ten grams of each plant powder were soaked in either 95% ethanol or bidistilled water at a ratio of 1:10 (w/v), and then gently shaken on a shaking table at room temperature (25 C) for 72 h (in triplicate). Macerates were filtered three times, with two sieves of different sizes (1.2 ± 0.6 mm) and finally filtered with Whatman filter paper (125 mm). For water maceration, the samples were centrifuged at 10,000 g for 10 min after

the third filtration and the supernatants were filtered (0.22 μm sieve) to avoid bacterial contamination. Ethanolic extracts (EE) were evaporated in a rotary evaporator (Ogawa Seiki Ltd., Tokyo, Japan, 40 C; 100–120 rpm, for 4–10 h depending on the plant). For the disk diffusion test, we used ethanol to dissolve the dry extracts and to ensure complete evaporation of the solvent on the disk. For these extracts, the yield was determined as the percentage of dry extract per dry plant. Water macerates (WM) and EE were stored at 4 C until antimicrobial assays, which started about 1 mo later.

Antibacterial Susceptibility Assays

The *S. agalactiae* and *A. hydrophila* used in this study had previously been isolated and identified from diseased *Oreochromis niloticus* and *Clarias gariepinus*, respectively, and stored in 20% glycerol at -20 C . They were grown for 24 h on brain heart (BH) infusion agar (Oxoid) and Muller-Hinton (MH) agar (Difco) plates, respectively, then suspended in sterile phosphate buffered saline (PBS) to reach a concentration of 1.5×10^8 CFU/mL.

The disk diffusion test was carried out according to the CLSI (2005) (at a temperature of $34 \pm 2\text{ C}$ instead of 28 C) for all EE and WM. Sterile Whatman paper disks (diameter 6 mm) were soaked with 20 μL of plant extracts and placed on plates. Plant WM were used directly, whereas EE were first dissolved in 95% ethanol (100 mg/mL) and the corresponding disks were placed on plates after ethanol evaporation for 3 min at room temperature. A disk containing 30 μg of chloramphenicol was used as a positive control and another disk soaked with 95% ethanol (or water as appropriate) was used as a negative control. After 24-h incubation at $34 \pm 2\text{ C}$, the diameter of the inhibition zone was measured with a caliper. Tests were carried out in triplicate. The efficacy comparative index (ECI, in %) was calculated as the ratio of the average inhibition diameters of plant extracts to those induced by the reference antibiotic (chloramphenicol, 30 μg , Oxoid). The plant extracts whose inhibition zone was greater than 6 mm in diameter in the disk diffusion test were

used to determine their minimum inhibitory concentration (MIC) using the macrodilution method. Stock solutions of WM were mixed at a 1:1 ratio with double-strength MH broth for *A. hydrophila* and with BHI broth for *S. agalactiae*. EE (200 mg) were dissolved in 2 mL of sterile distilled water containing 100 mg of Tween 80, then mixed with 2 mL of double-strength MH broth or BHI broth. Both stock solutions had a final concentration of 50 mg/mL. Serial dilutions were carried out in 11 tubes (concentrations ranging from 25 to 0.0475 mg/mL) and an additional one with no extract was used as a negative control. Bacterial inoculum of 1 mL obtained from cultures of *A. hydrophila* and *S. agalactiae* (grown for 24 h at 34 C) at 7.8×10^5 and 6.9×10^5 CFU/mL, respectively, was added to each of the 12 tubes.

As visual determination of MIC was not possible owing to the color of the extracts, 50 μL of each dilution was mixed with 1 mL of sterile PBS and then serially diluted down to 10^{-6} . For each dilution, 50 μL was spread on BH-infusion agar or Trypticase Soya Agar (Oxoid) plates and bacterial colonies were counted after 24 h of incubation at 34 C . The MIC (expressed as mg/mL) was defined as the lowest concentration of the plant extract at which the microbial concentration did not increase compared with the initial inoculum.

The inhibition rate (IR) of bacterial growth caused by plant extracts was calculated as follows and expressed as a percentage:

$$\text{IR (\%)} = \left(\frac{\text{TPC}_{\text{cont } 24 \text{ h}} - \text{TPC}_{\text{plant } 24 \text{ h}}}{\text{TPC}_{\text{cont } 24 \text{ h}}} \right) \times 100,$$

where TPC is the total plate count of bacteria after 24-h incubation at $34 \pm 2\text{ C}$.

Statistics

Normality and equal variance were checked with Kolmogorov–Smirnov and Levene median tests, respectively. One-way ANOVA was used for comparisons of means, followed by Holm–Sidak test for pairwise differences. When normality and equal variance were not detected, a nonparametric Kruskal–Wallis one-way ANOVA on rank was used. Yield data were Ln

transformed and percentages and frequencies were compared with χ^2 or z tests. For all tests, α was set at 0.05.

Results

Table 1 summarizes all the ethnobotanical information collected on the uses of the 18 plants used by the fish farmers in Java. Leaves were the most commonly used part of the plants (60%), but in the case of the plants belonging to the Zingiberaceae or Liliaceae families, only the rhizomes were used. Balneation was the most prevalent way to apply the plants. Generally leaves were used whole but in some instances they were chopped or crushed before used. ($z=7.896$, $P<0.001$, $n=203$). The selected plants were mainly used against fish diseases rather than to improve water quality, 70 versus 30% \pm 4.7% (I.C interval of confidence for both percentages), (z test = 6.946, $P<0.001$, $n=367$). Fish farmers used the plants indifferently to prevent or treat diseases and there was no significant difference between the two uses ($z=0.191$, $P=0.849$, $n=251$). Information provided by the fish farmers concerning therapeutic indications was vague and more than 50% of fish farmers chose the response "no symptoms"; the highest ranked symptom, "cutaneous symptoms," was cited by 25% of the fish farmers. It also appeared that fish farmers may use the same plants for many purposes, including improving water quality and increasing fish resistance to diseases.

After ethanol evaporation, the yields of EE ranged from 1.43 to 16.2% (Fig. 1), but yields from the leaves of the trees were significantly higher ($P<0.001$) than from the leaves of herbaceous plants, or rhizomes and bulbs.

Figure 2 shows the results of disk diffusion against *S. agalactiae* and *A. hydrophila* obtained from the EE and WM of plants. With the exception of *Colocasia esculenta*, *Gliricidia sepium*, and *Parkia speciosa*, most plants (15 out of 18 plants, 83%) exhibited antibacterial activity. The number of EE effective against *S. agalactiae* was significantly higher ($n=15$; 83%) than WM ($n=5$; 28%, $\chi^2=9.113$, $df=1$, $P=0.003$) (Fig. 2A, B). The EE of only four plants (22%)

displayed minimal antibacterial activity against *A. hydrophila*, and no inhibition was detected with WM (Fig. 2C). When a plant showed antimicrobial activity after both types of maceration, the inhibition diameters of EE were significantly larger than those of WM ($P<0.001$). The median value of the ECI was significantly higher with EE ($P=0.016$).

Significantly more plants were effective against *S. agalactiae* than against *A. hydrophila* ($\chi^2=11,146$, $df=1$, $P<0.001$), and their diameter of inhibition was larger ($P<0.001$ for all comparisons). For the EE of the four plants showing effective activity against both bacteria, the median value of inhibition was higher against *S. agalactiae* than against *A. hydrophila* ($P<0.001$ for all comparisons) (Fig. 2A, B).

The MIC against *S. agalactiae* was 12.5 mg/mL for *Curcuma domestica*, *Curcuma zanthorrhiza*, *Curcuma zedoaria*, *Phyllanthus niruri*, *Piper betle*, and *Plectranthus scutellarioides*; and 25 mg/mL for *Ageratum conyzoides*, *Austroeupeatorium inulaefolium*, *Terminalia catappa*, and *Tithonia diversifolia*. On the other hand, among the EE of the four plants effective against *A. hydrophila*, *P. betle* was the only one with an MIC of 25 mg/mL. The MICs of the other plants were higher than 25 mg/mL. The IRs of the EE and WM of plants against both bacteria after 24 h of growth at $34 \pm 2^\circ\text{C}$ are listed in Table 2. IRs of over 90% were observed for all effective plants. Three plants, *Plectranthus scutellaroides*, *A. inulaefolium*, and *P. niruri*, had an IR >90% at the lowest concentration tested (0.781 mg/mL).

Discussion

The limited knowledge of fish farmers about fish diseases proved to be an obstacle to a clear understanding of the relationships between plants and diseases. The use of plants with no clearly identified therapeutic indications suggests that the fish farmers use the plants pragmatically and with low specificity. However, our ethnobotanical surveys revealed that use of natural compounds plays an important role in health management in small-scale fish farming in Java. Fish farmers recognize that

TABLE 1. *Ethnobotanical indications of selected plants.*

Species of plants (family)	Part of plants	Mode of administration	Plant used for	Type of treatment	Therapeutic indications
<i>A. sativum</i> ^a (Amaryllidaceae)	R/B 100% (21)	B 60%, TF 40% (5)	DT 88%, Wm 12% (8) DT 57%, Wm 43% (7)	P 50%, T 50% (10)	ASD 5%, LA 2%, C 11%, M 11% (9) ASD 100% (7)
<i>Anacardium occidentale</i> ^c (Anacardiaceae)					
<i>C. esculenta</i> ^b (Araaceae)	L 100% (11)	B 100% (11)	DT 73%, Wm 27% (15)	P 79%, T 21% (14)	ASD 64%, C 14%, LA 7%, LG 7% (14)
<i>T. catappa</i> ^c (Combretaceae)	L 100% (56)	B 97%, TF 3% (74)	DT 51%, Wm 49% (7)	T 47%, P 53% (53)	ASD 63%, C 15%, LA 12%, GBC 10% (41)
<i>A. conyzoides</i> ^b (Compositae)	L 55%, Ov 45% (22)	B 92%, TF 8% (12)	DT 79%, Wm 21% (28)	T 60%, P 40% (28)	ASD 41%, C 21%, M 17%, LA 12%, GBC 3%, RD 3% (29)
<i>A. inulaefolium</i> ^b (Compositae)	L 60%, Ov 40% (5)	B 100% (5)	DT 59%, Wm 41% (22)	P 71%, T 29% (7)	ASD 50%, C 33%, GBC 17% (6)
<i>T. diversifolia</i> ^d (Compositae)	L 70%, Ov 30% (46)	B 91%, TF 9% (44)	DT 72%, Wm 28% (74)	T 52%, P 48% (62)	C 35%, ASD 5%, M 3%, LA 9%, RD 9%, GBC 2%, LG 2% (56)
<i>P. scutellaroides</i> ^b (Lamiaceae)	L 100% (3)	B 100% (3)	DT 91%, Wm 9% (11)	T 100% (3)	C 33%, GBC 33%, ASD 33% (3)
<i>G. sepium</i> ^c (Leguminosae)	L 100% (19)	B 100% (19)	DT 3%, Wm 47% (36)	P 52%, T 48% (19)	C 58%, ASD 42% (19)
<i>Leucaena leucocephala</i> ^d (Leguminosae)	L 100% (2)	B 100% (2)	Wm 71%, DT 29% (7)	P 100% (2)	C 50%, ASD 50% (2)
<i>P. spectiosa</i> ^c (Leguminosae)			DT 100% (11)		ASD 100% (7)
<i>Senna alata</i> ^b (Leguminosae)	L 100% (2)	TF 50%, B 50% (2)	Wm 60%, DT 40% (5)	P 50%, T 50% (4)	LA 50%, ASD 50% (2)
<i>P. niruri</i> ^{b,d} (Phyllanthaceae)	Ov 66%, L 44% (9)	B 78%, TF 22% (9)	DT 100% (9)	T 60%, P 40% (10)	C 33%, GBC 25%, ASD 25%, RD 17% (12)
<i>P. betle</i> ^b (Piperaceae)	L 93%, Ov 7% (14)	B 85%, TF 15% (13)	DT 88%, Wm 12% (16)	T 52%, P 48% (21)	ASD 61%, C 17%, LA 11%, M 11% (18)
<i>C. longa</i> ^a (Zingiberaceae)	R/B 98%, L 2% (44)	TF 32%, B 68% (40)	DT 89%, Wm 11% (37)	P 51%, T 49% (59)	ASD 52%, C 22%, RD 10%, LA 6%, GBC 6%, M 2%, LG 2% (50)
<i>C. zanthorrhiza</i> ^a (Zingiberaceae)	R/B 100% (7)	TF 57%, B 43% (7)	DT 86%, Wm 14% (7)	P 67%, T 33% (9)	ASD 100% (7)
<i>C. zedoaria</i> ^a (Zingiberaceae)	R/B 75%, L 25% (3)	TF 50%, B 50% (2)	DT 100% (2)	P 100% (2)	GBC 50%, ASD 50% (2)
<i>Etilingera elatior</i> ^b (Zingiberaceae)	R/B 75%, Ov 25% (3)	B 100% (3)	DT 100% (3)	T 75%, P 25% (3)	ASD 75%, C 25% (3)

Parts of plant: R/B = rhizome or bulb; L = leaves; Ov = overall. Mode of administration: B = by bath; TF = by feeding. Plant used for: Wm = water management; DT = disease treatment. Type of treatment: T = therapy; P = prevention. Therapeutic indications (symptom of diseases for which plants are used): ASD = any symptoms described; C = cutaneous; RD = respiratory distress; LA = low appetite; GBC = general bad condition; LG = low growth; M = mortality.

Type of plant: ^arhizome or bulb; ^bherbaceous; ^ctree; ^d*P. urinaria* and/or *Phyllanthus fraternus*. Data are from two surveys in Java (2013–2014). Numbers in bracket indicate the total number of informants using the plant. The results expressed in % are related to the specific use of plant.

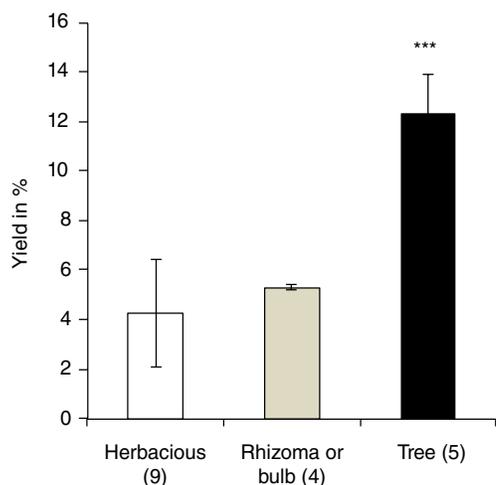


FIGURE 1. Yields of dry extract obtained after ethanolic extraction and evaporation according to the type of plants. The number in bracket indicates the number of species of plants that have been processed. Herbaceous plants were *A. conyzoides*, *A. inulaefolium*, *C. esculenta*, *E. elatior*, *P. scutellaroides*, *P. niruri*, *P. betle*, *S. alata*, and *T. diversifolia*. Rhizome plants were *A. sativum*, *C. longa*, *C. zanthorrhiza*, and *C. zedoaria*. Tree plants were *A. occidentale*, *G. sepium*, *L. leucocephala*, *P. speciosa*, and *T. catappa*. Data are expressed as average \pm SD, *** $P < 0.001$ (one-way ANOVA and Holm-Sidak method).

plants have different actions, but in the case of the plants studied here, they mainly use them for the prevention or treatment of fish diseases. Moreover, 15 of the 18 plants (i.e. 83%) with a high UV were shown to have antibacterial activity in this analysis. This high validation rate suggests that this generic ethnobotanical indicator may be a reliable way to select plant candidates for sustainable pharmacopeia in aquaculture. Considering the widespread use of these plants against disease, ethnobotanical surveys can help to advance research on herbal therapy for aquaculture. In this study, yields of ethanolic dry extracts depended more on the species of plants (herbaceous versus ligneous plants) than on the part of the plant (leaf or rhizome) used for extraction. These differences may be linked to the higher amount of polysaccharides (both cellulose and lignin) in the leaves of trees than in leaves of herbaceous plants (Schädel et al. 2010).

Our results confirm that EE have higher antimicrobial activity than WM, as already reported in the literature (Parekh et al. 2005; Parekh and Chanda 2008; Das et al. 2010). The entire extraction process is of major importance for the antimicrobial activity of plants, and obviously, a high rate of extraction or yield of dry extract will play a decisive role in the selection of a plant. Yield and other important pharmaceutical properties – including antimicrobial activity – are closely linked with the process of extraction (Eloff 1998; Cowan 1999; Nostro et al. 2000), and new techniques may improve the efficacy and safety of herbal compounds.

However, because all, even the simplest, methods are costly and time consuming, they cannot be afforded by Javanese fish farmers. We therefore suggest the use of WM of active plants only for small volumes of water such as aquaria or small tanks, where an effective concentration can be reached. In larger facilities, which would require larger quantities of plants, it may be more appropriate to use dry plants in fish feed.

The antimicrobial activities of EE and WM revealed by disk diffusion tests are in agreement with the results obtained with the liquid method used here to determine MIC. The disk diffusion test allows rapid detection of antimicrobial activity and is suitable for screening a wide range of plants and extracts. However, it is only a qualitative indication of bioactive plants (Nostro et al. 2000; Langfield et al. 2004), which should be followed up with more accurate quantitative methods.

Our results support other findings showing that Gram-positive bacteria are generally more sensitive to plant extracts than Gram-negative ones (Burt 2004; Hussain et al. 2010). Yet several natural compounds can be effective against Gram-negative bacteria (Genovese et al. 2012; Fankam et al. 2014). Natural compounds may cause structural damage, particularly to the bacterial membrane (Cowan 1999). In contrast to Gram-positive bacteria, Gram-negative bacteria possess an outer bilayered membrane with porins (Pagès 2004). These structural differences may explain the variability in antibacterial activity against the bacteria evaluated here.

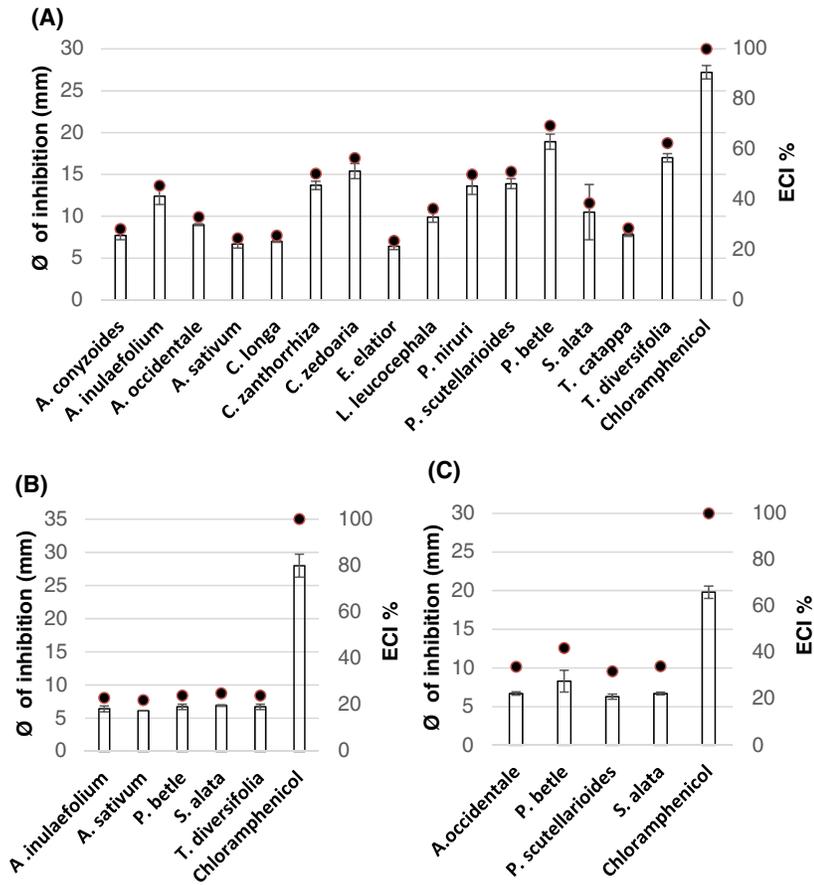


FIGURE 2. Disk diffusion test of ethanolic extracts of plants against *S. agalactiae*. (A) Disk diffusion test of water macerates of plants against *S. agalactiae*. (B) Disk diffusion test of ethanolic extracts of plants against *A. hydrophila*. (C) Bars show the diameters of bacterial inhibition as mean \pm SD (n = 9). The black dots show the efficiency comparative index (ECI).

According to Pankey and Sabath (2004), a compound has a bacteriostatic effect when it reduces bacterial growth by 90 to 99% and a bactericidal effect when it kills >99.9% of bacteria in the initial bacterial inoculum. According to this definition, only four plants (*C. zanthorrhiza*, *P. betle*, *P. niruri*, and *P. scutellarioides*) among those tested here can be considered as bacteriostatic against *S. agalactiae*. Two plants of the Zingiberaceae family (*Curcuma longa* and *C. zedoaria*) have already been evaluated in fish health management (Sahu et al. 2008; Chowdhury and Rahman 2012). In contrast, there has been no study of *C. zanthorrhiza*. This rhizome is capable of stimulating the immune function of macrophage cells in rats (Kim et al. 2007) and is a promising candidate for aquaculture.

Piper betle is also a promising candidate for aquaculture; in this study, it displayed activity against both bacteria; its EE was the only one able to reduce bacterial growth of *A. hydrophila*, even at a low concentration (6.25 mg/mL). The effect of *P. betle* against fish pathogens has already been highlighted (Muniruzzaman and Chowdhury 2004; Albert and Ransangan 2013), but as far as we know, the antibacterial properties of *P. niruri*, *Austroeupatorium inulifolium*, and *P. scutellaroides* against fish pathogens in aquaculture have never been reported. EEs of *P. niruri* have already been successfully tested against human enteropathogenic bacteria (Ekwenye and Njoku 2006), and activity against fish bacteria has been reported for *Phyllanthus urinaria*, a species belonging to the same genus and very

TABLE 2. Inhibition rate (IR %) of ethanolic extracts (EE) and water macerates (WM) of plants both against *S. agalactiae* and *A. hydrophila* growth after 24 h at 34 C.

Plant EE	Inhibition rate (%) against <i>S. agalactiae</i> ^a					
	25 mg/mL	12.5 mg/mL	6.25 mg/mL	3.125 mg/mL	1.562 mg/mL	0.781 mg/mL
<i>A. conyzoides</i>	++	++	+	-	-	-
<i>A. sativum</i>	++	++	-	-	-	-
<i>A. occidentale</i>	++	++	++	+	-	-
<i>A. inulaefolium</i>	++	++	++	++	+	+
<i>C. domestica</i>	++	++	++	-	-	-
<i>C. zanthorrhiza</i>	+++	++	++	++	+	-
<i>C. zedoaria</i>	+++	+++	++	++	+	-
<i>E. elatior</i>	++	++	+	-	-	-
<i>L. leucocephala</i>	++	++	+	+	-	-
<i>P. niruri</i>	+++	++	++	++	+	+
<i>P. betle</i>	+++	++	++	++	+	-
<i>P. scutellarioides</i>	+++	+++	++	++	++	++
<i>S. alata</i>	++	+	+	+	-	-
<i>T. catappa</i>	+++	++	++	+	-	-
<i>T. diversifolia</i>	++	++	++	++	+	-
Plant WM	IR (%) against <i>S. agalactiae</i> ^b					
	Dilution of plant macerates					
	1:2	1:4	1:8	1:16	1:32	1:64
<i>A. sativum</i>	++	-	-	-	-	-
<i>A. inulaefolium</i>	++	+	-	-	-	-
<i>P. betle</i>	++	-	-	-	-	-
<i>T. diversifolia</i>	++	-	-	-	-	-
<i>S. alata</i>	++	-	-	-	-	-
Plant EE	Inhibition rate (%) against <i>A. hydrophila</i> ^c					
	25 mg/mL	12.5 mg/mL	6.25 mg/mL	3.125 mg/mL	1.562 mg/mL	0.781 mg/mL
<i>A. sativum</i>	+	-	-	-	-	-
<i>A. occidentale</i>	+	-	-	-	-	-
<i>P. betle</i>	++	++	++	-	-	-
<i>Cassia alata</i>	+	-	-	-	-	-
<i>P. scutellarioides</i>	-	-	-	-	-	-

IR < 90% = -; IR ≥ 90 = +; IR ≥ 99% = ++; IR ≥ 99.99% = +++.

^aControl concentration = $9.12 \pm 0.16 \log_{10}$ CFU/mL (average ± SD, n = 15).

^bControl concentration = $9.20 \pm 0.12 \log_{10}$ CFU/mL (average ± SD, n = 5).

^cControl concentration = $9.16 \pm 0.20 \log_{10}$ CFU/mL (average ± SD, n = 6).

similar to *P. niruri* (Muniruzzaman and Chowdhury 2004). Likewise, EEs of *A. inulifolium* have been found to act against *Staphylococcus aureus* (Álvarez et al. 2005). No reports of the antimicrobial activity of *P. scutellarioides* have appeared in the literature, but this plant is used to heal skin infections, wounds, and sores in Papua New Guinea (Nick et al. 1995).

Consistent reductions (≥90%) in bacterial concentration may contribute to the effectiveness of plants in controlling populations of opportunistic or pathogenic bacteria in fish farming. Indeed,

T. catappa or *Allium sativum*, which showed low antibacterial activity in this study, have been found to be effective against fish diseases, even at concentrations lower than those we tested *in vitro* (Chitmanat et al. 2005; Nya and Austin 2009). Plant antibacterial activity may also be related to anti-quorum sensing properties (Koh et al. 2013) and the immuno-stimulation induced by plants may improve fish resistance against bacteria (Vaseeharan and Thaya 2014; Van Hai 2015). Hence, a multidisciplinary approach should be considered for a better understanding

of the biological activities of plants in fish. Antibacterial plants may greatly contribute to the reduction of the excessive use of antibiotics. They should be recommended as an alternative because they are inexpensive, renewable, and locally available, and their use is generally recognized to be safe. According to the IR recorded in this study, the use of plants with high antimicrobial activities should be recommended to fish farmers as both a WM and in the feed. These plants may reduce the bacterial load in the fish gut or in the water, thereby reducing the pressure exerted by opportunistic fish pathogens.

At the same time, the current pragmatic use of plants should be scientifically assessed and refined to increase the efficacy and ensure the sustainability of herbal therapy in fish farming.

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