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Antibacterial Activity of *Kaempferia parviflora* and *Curcuma longa* at Different Harvest Periods on Pathogenic Bacterial Isolates of Fish and Shrimp

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Abstract: Due to food safety and public health concerns, much interest has been placed on antibacterials derived from natural products for use in aquaculture. *Kaempferia parviflora* and *Curcuma longa*, herbs that can be found in Thailand, have been shown to possess antibacterial properties. The biological activities of these herbs, however, was found to be dependent on age. Here, ethanol extracts of *K. parviflora* and *C. longa* harvested at different periods were evaluated for their antibacterial activity against 5 strains of bacteria, pathogenic to aquatic animals, using disc diffusion method. Our results revealed that *K. parviflora* ethanol extracts at 9 and 10 months after planting showed antibacterial activity only against *Vibrio harveyi* and *V. parahaemolyticus*, while *C. longa* ethanol extracts at 7, 8, 9 and 10 months after planting exhibited antibacterial activity against *V. harveyi*, *V. parahaemolyticus*, *Edwardsiella tarda* and *Streptococcus agalactiae*. Both *K. parviflora* and *C. longa* ethanol extracts show no inhibitory effect on *Escherichia coli*. Comparison of the zone of inhibitions suggest that the suitable time to harvest *K. parviflora* and *C. longa* for ethanol extraction was 9 and 10 months after planting, respectively. The minimum inhibitory concentrations of *K. parviflora* and *C. longa* ethanol extracts during the above mentioned periods ranged from 12.50 to 50.00 and 3.12 to 50.00 mg/ml, respectively. In conclusion, both herbs have exhibited antibacterial activity against *V. harveyi*, *V. parahaemolyticus*, *E. tarda* and *S. agalactiae*. *C. longa* ethanol extract, specifically, showed better inhibitory properties and can thus be potentially useful for aquaculture in the treatment of bacterial infections.

Keywords: *Kaempferia parviflora*, *Curcuma longa*, ethanol extracts, antibacterial activity, harvest period

Introduction

Various problems posed by microorganisms developing resistance to commercial antibiotics have placed particular emphasis on researches targeting pharmacologically active ingredients in plants, particularly those with antibacterial potential (Yasunaka *et al*., 2005). In aquaculture, for instance, a number of plant extracts have shown significant potential as antibacterials and therefore can be used as an alternative to commonly used chemotherapeutants (Reverter *et al*., 2014). Harikrishnan *et al*. (2003) reported that aqueous extract of *Azadirachta indica* leaf could effectively control *Aeromonas hydrophila* infection in common carp, *Cyprinus carpio*. In another study, kelp grouper (*Epinephelus bruneus*) fed with *Inonotus obliquus* ethanolic extract supplemented diets had a lower cumulative mortality after *Vibrio harveyi* infection compared to the control group (Harikrishnan *et al*., 2012).

Kaempferia parviflora and *Curcuma longa* are herbs commonly known in Thailand as Krachai-Dum and Khamin Chan, respectively. These plants are traditional herbal medicine used in Asia particularly in Malaysia, India, China and Thailand (Elshamy *et al*., 2019; Remadevi *et al.*, 2007). Pharmacological studies of these herbs include, among others, their antiviral, antifungal, antibacterial properties (Anand *et al*., 2007; Elshamy *et al*.,

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2019; Kim *et al*., 2009; Moghadamtousi *et al*., 2014; Rudrappa & Bais, 2008; Sookkongwaree *et al*., 2006; Yenjai *et al*., 2004; Yenjai *et al*., 2009). Previous research on both herbs have demonstrated their antimicrobial activity but failed to mention the age of the plant material that was used (Chaichanawongsaroj *et al*., 2010; Gupta & Ravishankar, 2005; Jeong *et al*., 2016; Kummee *et al*., 2008; Lawhavinit *et al*., 2010; Naz *et al*., 2010; Niamsa & Sittiwet, 2009; Raji *et al*., 2018; Yenjai *et al*., 2004). The chemical profile of plants has been reported to be dependent upon geographical location, age, time of collection, mode of extraction, etc. (Cimanga *et al*., 2002; Rimkiene *et al*., 2017; Yao *et al*., 2012). Given the few published data dealing with this, it would be interesting to study how the difference in harvest period affect antibacterial activity of these herbs. The aim of this research therefore, is to find the suitable harvest period for *K. parviflora* and *C. longa* that could effectively inhibit pathogenic bacteria of fish and shrimp. In addition, we aim to establish the respective minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these herbs.

Materials and Methods

Plant materials and extraction

Rhizomes of *K. parviflora* and *C. longa* were planted in an experimental plot at King Mongkut's Institute of Technology Ladkrabang, Prince of Chumphon Campus. *K. parviflora* was collected at 9 and 10 months after planting while *C. longa* was collected at 7, 8, 9 and 10 months after planting. The collected samples were shade dried and ground using a blender. The powder was extracted with 95% ethanol by soaking for 3 days (0.3 g sample per 20 ml). The mixture was filtered using double filter paper (Whatman™). The filtered solutions were dried using a rotary evaporator.

Microbial strains

Vibrio harveyi, *V. parahaemolyticus* and *Streptococcus agalactiae* strains were kindly provided by the Coastal Aquatic Animal Health Research Center, Department of Fisheries, Songkhla Province, Thailand. Strains of *Escherichia coli* and *Edwardsiella tarda* were generous provided by the Aquatic Animal Health Research and Development Division, Department of Fisheries, Bangkok Province, Thailand.

Antibacterial susceptibility testing

The antibacterial activity of *K. parviflora* and *C. longa* ethanol extracts were tested on *V. harveyi*, *V. parahaemolyticus*, *E.coli*, *E. tarda* and *S. agalactiae* using agar disc diffusion method following a method previously described (Habtom & Gebrehiwot, 2019). Briefly, sterile paper discs (6 mm in diameter) were impregnated with 20 µl of the ethanol extracts. Discs, which were placed on agar plates as prescribed, were spread with suspension of test bacterial strains that were adjusted to give a turbidity of a 0.5 McFarland standard (1.5×10^8 CFU/ml). Plates were incubated at 37 ^{o}C for 24 h. The diameter of the inhibition zones was measured in mm.

Minimum inhibitory concentrations/Minimum bactericidal concentrations (MIC/MBC)

After screening the antibacterial activity of *K. parviflora* and *C. longa* ethanol extracts, the planting period of each herb that produced good inhibition zones was chosen for MIC testing. Briefly, the bacterial tests were cultured in Brain Heart Infusion (BHI) broth for *E.coli*, *E. tarda* and *S. agalactiae* and BHI broth added 3% NaCl for *V. harveyi* and *V. parahaemolyticus*, and then incubated at 37 ^{o}C for 24 h. The bacterial cultures were adjusted for turbidity to a 0.5 McFarland standard before use. To prepare for *K. parviflora* and *C. longa* ethanol extracts, the extracts were first dissolved in a little amount of dimethyl sulfoxide (DMSO) then added with appropriate BHI broth to a final concentration of 100 mg/ml (stock solution). Then, two fold serial dilutions of the stock solution (1.56, 3.12, 6.25, 12.50, 25.00 and 50.00 mg/ml) were also prepared in broth. Next, 50 µl of

each diluted ethanol extracts and 50 µl of bacterial suspension were added to a 96-well plate and incubated at 37 ºC for 24 h (from Raeisi *et al*., 2012 with modifications). Subsequently, 10 µl of 1 mg/ml resazurin (Sigma, UK) solution was added into the 96-well plate and incubated again at 37 ºC for 2 h. The MIC value was defined as the lowest concentration of *K. parviflora* and *C. longa* ethanol extracts that prevented the color of resazurin from changing from blue to pink. The MBC was determined by subculturing 10 µl of the bacterial suspension from the well with no color change on agar plates. After 24 h of incubation at 37 ºC, the lowest concentration that shows no colony growth was determined as the MBC (Boonyanugomol *et al*., 2017).

Statistical analysis

The inhibition of *K. parviflora* and *C. longa* ethanol extracts against *V. harveyi*, *V. parahaemolyticus*, *E.coli*, *E. tarda* and *S. agalactiae* were evaluated by Analysis of Variance and Duncan's Multiple Range Test. Statistical significance was accepted at the $P < 0.05$ level.

Results and Discussion

K. parviflora and *C. longa* ethanol extracts were tested for antimicrobial activity against Gram-positive and Gram-negative bacteria that are pathogenic to aquatic animals. Our results revealed that *K. parviflora* ethanol extract can inhibit the growth of *V. harveyi* and *V. parahaemolyticus* but ineffective against *E. coli*, *E. tarda* and *S. agalactiae* (Table 1). This result was similar to a previous report showing that ethanol extract of *K. parviflora* was inactive against *E. coli* and some Gram-positive bacteria, such as *Staphylococcus aureus*, *Staph. epidermidis* and *Enterococcus faecalis* (Kummee *et al*., 2008). Moreover, Wungsintaweekul *et al*. (2010) also reported that the volatile oils and the methanol extracts of *K. parviflora* did not show any inhibitory activity against *E. coli*. These are in contrast to an earlier report showing that the volatile oil from *K. parviflora* could inhibit the growth of *Staph. aureus* (Tanasiriwattana *et al*., 1997). The difference in antimicrobial activity of natural extracts has been described to be dependent on the solvent extraction methods applied (Chaichanawongsaroj *et al*., 2010, Mbata *et al*., 2008). The efficacy of *K. parviflora* extracts against bacteria comes from the flavonoids component in the rhizome as suggested by earlier reports on the biochemistry and pharmacology of flavonoids displaying antibacterial, antiviral and antifungal activities (Ahmad *et al*., 2015; Cushnie & Lamb, 2005; De Conti Lourenço *et al*., 2013). Wattanapitayakul *et al*. (2007) reported that the main phytochemicals of *K. parviflora* are methoxyflavone derivatives; 3,5,7,4´-tetramethoxyflavone. The 5,7,4´-trimethoxyflavone isolated from *K. parviflora* possessed antimycobacterial activity (Yenjai *et al*., 2004).

Curcumin is the major phytoconstituent of *C. longa* (Ammon & Wahl, 1991). This component also showed properties similar to flavonoids such as antibacterial, antiviral and antifungal activities (Moghadamtousi *et al*., 2014). Ethanolic extract of *C. longa* in this study exhibited antibacterial activity against *V. harveyi*, *V. parahaemolyticus*, *E. tarda* and *S. agalactiae* but failed to inhibit *E. coli* growth (Table 2). These results conforms with that of Lawhavinit *et al*. (2010), who reported ethanol turmeric extract showed inhibitory effects against 13 pathogenic bacteria of shrimp including *V. harveyi*, *V. cholerae*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *Aeromonas hydrophila*, *S. agalactiae*, *Staph. aureus*, *Staph. epidermidis*, *Staph. intermidis*, *Bacillus subtilis*, *B. cereus* and *E. tarda*, but not against Salmonella serv. Typhi, Salmonella serv. Typhimurium, Salmonella serv. Enteritidis, *E. coli*, *Proteus mirabilis*, *P. vulgaris*, *Shigella sonnei*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Erwinia carotovora* and *Citrobacter frundii*.

Table 1 Antimicrobial activity of Kaempferia parviflora ethanolic extract harvested at 9 and 10 months after planting by the disc diffusion method.

Microbes	Gram -/+	Inhibition zone (mm)	
		9	10
Vibrio harveyi[ns]	-	16.00±2.65	16.67±1.53
V. parahaemolyticus[ns]	-	12.67±1.15	14.00±2.00
Escherichia coli	-	na	na
Edwardsiella tarda	-	na	na
Streptococcus agalactiae	+	na	na

ns = no significant, na = no activity

Table 2 Antimicrobial activity of Curcuma longa ethanolic extract harvested at 7, 8, 9 and 10 months after planting by the disc diffusion method.

Microbes	Gram -/+	Inhibition zone (mm)			
		7	8	9	10
Vibrio harveyi	-	11.33±0.58^{b}	12.33±0.58^{b}	12.67±0.58^{b}	14.00±1.00^{a}
V. parahaemolyticus	-	11.33±0.58^{b}	11.67±0.58^{b}	12.33±0.58^{b}	15.00±1.00^{a}
Escherichia coli	-	na	na	na	na
Edwardsiella tarda	-	9.67±0.58^{b}	10.00±0.00^{b}	10.33±0.58^{b}	12.00±0.00^{a}
Streptococcus agalactiae	+	10.00±0.00^{c}	10.67±1.15^{bc}	12.00±1.00^{ab}	13.33±1.15^{a}

Inhibition zones on the same row followed by the same letters are not significantly different (P>0.05), na = no activity

The variation in the chemical profile of natural extracts, aside from solvent extraction methods, may have also resulted from the geographical location, seasonal changes, climate, time of collection and age of plant (Cimanga *et al*., 2002). Here, we compared the antimicrobial activity of extracts from *K. parviflora* and *C. longa* harvested at different periods. We found that *K. parviflora* ethanol extract collected at 9 and 10 months after planting produced no significant difference in the zone of inhibition against *V. harveyi* and *V. parahaemolyticus*. In contrast, ethanol extract from *C. longa* collected at 10 months after planting produced the largest zone of inhibition against *V. harveyi*, *V. parahaemolyticus*, *E. tarda* and *S. agalactiae* which is significantly different from extracts obtained from 7, 8 and 9 months after planting. Subsequently, we chose *K. parviflora* ethanol extract collected at 9 months and *C. longa* ethanol extract collected at 10 months to conduct MIC and MBC experiments. The MIC values of *K. parviflora* ethanol extract against *V. harveyi* and *V. parahaemolyticus* were 50.00 and 12.50 mg/ml (Figure 1), whereas MBC values were 100.00 and 25.00 mg/ml, respectively (Figure 2). The MIC values of *C. longa* ethanol extract against *V. harveyi*, *V. parahaemolyticus*, *E. tarda* and *S. agalactiae* were 12.50, 3.12, 50.00, 6.25 mg/ml (Figure 1), whereas MBC values were 50.00, 25.00, 50.00 and 25.00 mg/ml, respectively (Figure 2).

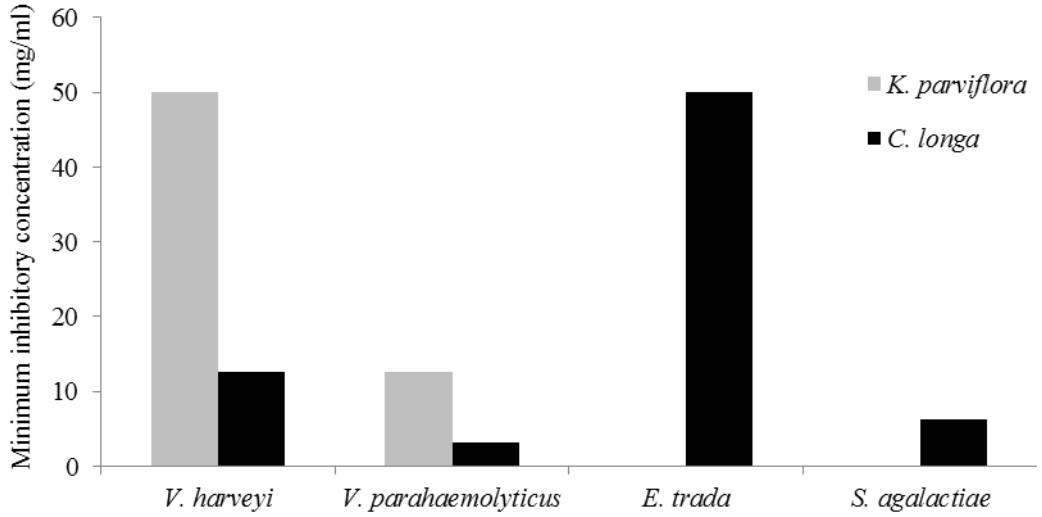

Figure 1 Minimum inhibitory concentration values of Kaempferia parviflora and Curcuma longa ethanolic extracts harvested at 9 and 10 months after planting, respectively, against aquatic bacterial pathogens.

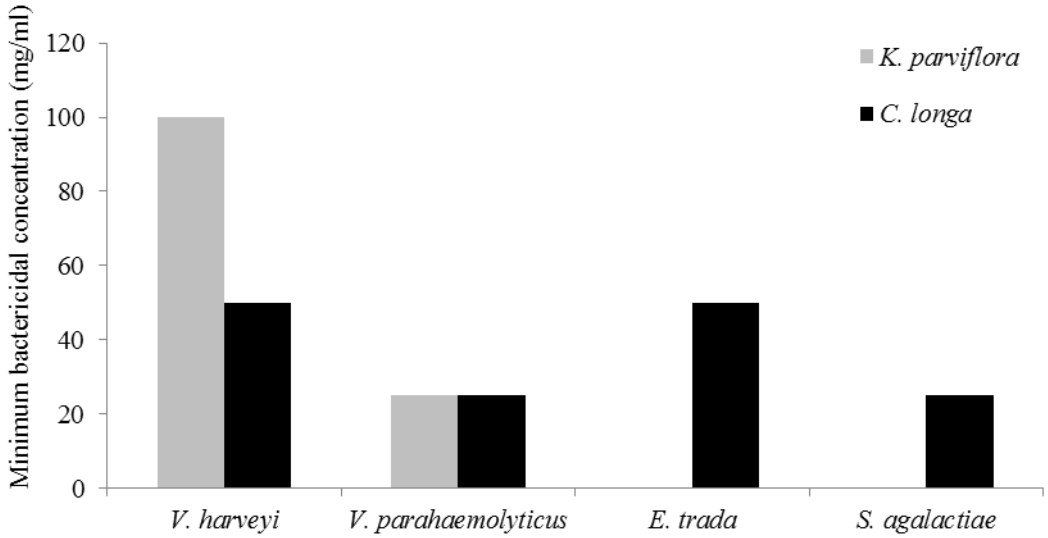

Figure 2 Minimum bactericidal concentration values of Kaempferia parviflora and Curcuma longa ethanolic extracts harvested at 9 and 10 months after planting, respectively, against aquatic bacterial pathogens.

Kitwetcharoen *et al*. (2020) reported that the best time to harvest rhizomes of *K. parviflora* to produce the highest mass or volume is approximately 9-10 months after planting. Furthermore, Rahman *et al*. (2018) reported that the flavonoid component in the rhizomes of *K. parviflora* was highest at 8 months after planting but starts to decrease after 10 months. Our results show that ethanol crude extract from *K. parviflora* at 9 months after planting can inhibit bacterial growth. Thus, the utilization of *K. parviflora* at 9 months after planting is suitable because this period gives the highest production volume of rhizomes and the extract obtained during this time could inhibit the growth of bacteria. For *C. longa*, our results suggested that the best period to harvest for ethanol crude extract is 10 months. This is in contrast to that of Cooray *et al*. (1988), who recommended that the ideal time to harvest Sri Lankan cultivar of *C. longa* for curcumin extraction is 9 months after planting. The

disparity in the harvest period of *C. longa* might be because of the difference in habitats which may ultimately affect curcumin yield in the rhizomes (Remadevi *et al*., 2007).

Taken together, our results suggest that both Thai herbs exhibit antimicrobial activity against bacteria pathogenic to cultured aquatic organisms. *C. longa* ethanol extract, in particular, remarkably exhibited a broader spectrum of activity against these pathogenic bacteria as compared to *K. parviflora* ethanol extract. Therefore, *C. longa* ethanol extract has a potential for use as a natural antibiotics to treat diseases in cultured aquatic animals which may reduce the use of synthetic antibiotics in aquaculture. Other potential applications and further studies on the other biological activities, *in vivo*, of both Thai herbs are currently being explored.

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