BITTER BUSH, CHROMOLAENA ODORATA (L.) HAD BIOACTIVE METABOLITE AGAINST BACTERIAL PATHOGEN IN TILAPIA AND SHRIMP.

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ABSTRACT

Bitter bush or Siam weed had the specific name is *Chromolaena odorata* (L.) R.M. King & H. Rob the classified in order Asterales and family Asteraceae. The plant grows best in sunny or open areas such as roadsides, abandoned fields, pastures, and disturbed forests, but tolerates semi-shade conditions. A single shrub can produce as many as 80,000 seeds in dry season. The species has the tenacity to invade human-induced disturbed and undisturbed lands, posing a significant economic and ecological burden in many countries in its introduced ranges. The unique aroma odour and may poisonous toxic for cattle stomach that the advantage gave the bioactive metabolite against various aquatic infectious microorganisms including *Streptococcus agalactiae* (2809) in bighead carp and tilapia infection and *Vibrio parahaemolyticus* (NX89) and *V. parahaemolyticus* (5HP) in that cause the Acute Hepatopancreatic Necrosis Disease (AHPND), or Early Mortality Syndrome (EMS), in shrimp infection. Isopropanol was the appropriated solvent found antimicrobial screening performance and Its metabolite extraction could beneficial for aquatic treatment on further.

Keywords: bitter bush, Chromolaena odorata (L.), tilapia, shrimp, Streptococcus agalactiae, Vibrio parahaemolyticus

INTRODUCTION

Chromolaena odorata had a common name is Siam weed or Bitter bush. It was an invasive species or pest crop. This plant classified in the phylum Spermatophyta with Subphylum Angiospermae class Dicotyledonae order Asterales family Asteraceae genus *Chromolaena*, and species *odorata* respectively [16]. *C. odorata* is a very widely distributed tropical shrub that is still expanding its range and is considered one of the world's worst weeds. The originate *C. odorata* is often noted as a native of tropical Central and South America, from Mexico and the Caribbean to Brazil; however, the exact northern and southern limits of its native range remain uncertain and are likely to also include some regions outside of the tropics. The estimate distribution of *C. odorata* to Thailand was occurring since World War I during 1914 to 1918 [14] or may start from the Historic City of Ayutthaya, founded in 1350 which time it grew to be one of the world's largest and most cosmopolitan urban areas and a center of global diplomacy and commerce. Because of Ayutthaya was strategically located on an island surrounded by three rivers connecting the city to the sea. This site was chosen because it was located above the tidal bore of the Gulf of Siam as it existed at that time, thus important center of global diplomacy and commerce and distribution of the world's worst weeds of *C. odorata* to Thailand. It continues to spread due to its effective short- and long-distance dispersal. It can form pure stands where established, often in disturbed areas, grasslands, fallow areas and forestry plantations, and is highly competitive. It is viewed as a

major environmental weed but is appreciated by some agriculturalists as it shortens the fallow time in shifting cultivation.

C. odorata has long been referred to as *Eupatorium odoratum*. It was transferred to the genus Chromolaena by King and Robinson (1970), and although this conception of the tribe Eupatorieae has met some disagreement among botanists, the new binomial of *Chromolaena odorata* (L.) R.M. King & H. Rob. is now widely accepted [16]. This plant had several bioactive metabolites such as used for wound healing and inflammation-related diseases. The Siam weed leaves extracted gave the mixture compound of scutellarein tetramethyl ether (scu), stigmasterol, and isosakur-anetin affect anti-inflammatory activity. Those components were suppression cyclooxygenase-2 (COX-2) and inducible nitricoxide synthase (iNOS) are critical pro-inflammatory proteins [13]. Doris and colleague were reported that the dichloromethane and aqueous extracts obtained from the leaves and the ethyl acetate extract from the flowers of *C. moritziana* had anti *Staphylococcus aureus* and *Bacillus cereus* activity [3]. Furthermore, the antioxidant activities and anti-malarial against Plasmodium parasite had been determine the content of *C odorata* metabolite extraction respectively [20].

Aquaculture plays an increasingly important role in food security and the economy of Thailand. Freshwater aquaculture is mainly for domestic consumption. Small-scale freshwater aquaculture is still very crucial in providing the rural poor with high quality protein food for home consumption. Brackish water aquaculture usually produces high-value products for export. The main freshwater species cultured were Nile tilapia (*Oreochromis niloticus*), hybrid catfish (*Clarias macrocephalus* X *C. gariepinus*), silver barb (*Barbodes gonionotus*), giant river prawn (*Macrobrachium rosenbergii*), snakeskin gourami (*Trichogaster pectoralis*). The main brackish water cultured species were Black giant tiger prawn (*Peneaus monodon*) and Pacific white shrimp (*Penaeus vanamei*). A major factor limiting the promotion of aquaculture practices in Thailand is the poor economic return from investments rather than the lack of production technology. Advanced aquaculture techniques, including intensive pond and cage farming, have been developed and are available, particularly for freshwater aquaculture, but the profit margin is very small and is not attractive to expanded investment [5]. Moreover, infectious disease in aquaculture still the problem of aquaculture. The examples of the outbreaks of Tilapia Lake Virus Infection, Thailand, 2015–2016 called tilapia lake virus (TiLV) [20] and the bacterial infection disease. *Streptococcus* is a bacterial occurs in both marine and freshwater environments and has a global distribution. The disease is characterized by septicemia and meningoencephalitis. Bacteriology data show that the disease is caused by different species of Streptococcus, including *Streptococcus agalactiae*. This bacterial still problem in aqua cultural massive mortality loss, infection of tilapia farm. *S. agalactiae* (Group B streptococcus) has emerged as an important pathogen that affects humans and animals, including aquatic species. *S. agalactiae* infections are becoming an increasing problem in aquaculture and have been reported worldwide in a variety of fish species, especially those living in warm water. Although the reported antibiotic susceptibility, which were sensitive to amoxicillin, ciprofloxacin, lomefloxacin, chloramphenicol, rifampin, vancomycin, azithromycin, florfenicol, cefalexin, cefradine and deoxycycline and resistant to gentamicin, sinomin (SMZ/TMP), penicillin, tenemycin, fradiomycin and streptomycin [10]. The treatment of bacterial infection was often increasing the budget and the drug rest from treatment were released to the environment. The bacteria can become resistant to antibacterial drugs. Resistance can be intrinsic, meaning a basic characteristic that naturally occurs in the bacterium, and is essentially always there, or acquired, which means the bacterium is not naturally resistant but has become resistant somehow over time.

Vibrio parahaemolyticus (NX89) and *V. parahaemolyticus* (5HP) in that case the Acute Hepatopancreatic Necrosis Disease (AHPND), or Early Mortality Syndrome (EMS) [6]. Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of cultured shrimp are also known with the current understanding that EMS/AHPNS has a bacterial aetiology, a strain of *V. parahaemolyticus.* It is caused by specific virulent strains of *V. parahaemolyticus* (Vp AHPND) that contain a binary toxin, PirA and PirB [21]. The disease can cause up to 100% cumulative pond mortality within a week. Early mortality syndrome, more precisely known as acute hepatopancreatic necrosis disease (AHPND), has been attributed as the cause of devastating losses in the shrimp aquaculture industry of South-East Asia was first reported in Thailand in late 2011 [7]. Measures to control disease spread have been implemented on several shrimp farms in Thailand, including the improvement of farm and shrimp health management practices, and the application of probiotics, traditional herbal medicine and molasses. However, the effectiveness of these measures has not been evaluated [6].

The problem with this situation is, unless an antibiotic target an essential, conserved, non-negotiable process within the bacterial cell, antibiotic resistance is very likely to develop over time. It isn't feasible to eliminate the development of resistance; it's a natural process of life and one which has allowed bacteria to evolve and survive for millennia. Limiting the use of antibiotics in animal farming is already being recognized as an important step, as the aquatic products were containing the antibiotic residue.

Herbal antibiotics, which have been used for thousands of years, are garnering attention for their strong antibacterial properties and fewer side effects. The pharmaceutical industry is recognizing the power of herbs by combining them synergistically with synthetic antibiotics, creating phytopharmaceuticals that are more powerful than synthetic antibiotics alone. That come to our objective was to evaluate, by searching appropriate plant as herbal *C. odorata* for monitoring the solvent extraction and antibacterial approaches especially aquatic infectious disease. The susceptible for anti *S. agalactiae* and anti *V. parahaemolyticus* may use for herbal treatment.

MATERIALS AND METHODS

Crude extraction of *Chromolaena odorata* for anti-bacterial susceptibility testing

The herbal plant *C. odorata* (L.) R.M. King & H. Rob was selected for anti-bacterial susceptibility testing. Plant samples were obtained from Kanchanaburi province, Ratchaburi Province, and Chachoengsao Province of Thailand. The stem shoot and leaves of fresh herbs were cut into small pieces and grounded in a mortar and pestle. Then finely ground plant 200 g added with 500 ml isopropanol for solvent extraction. The extraction method using a 1 litter screw cap bottle. Plant material mixed with organic solvent was put in the bottle. Then, the bottle was shaken in horizontal orbit shaker to 150 rpm for 48 hrs at room temperature. After finishing shaking the samples were filtrated pass through (Whatman® No.1) and the residue continues for re-extraction with an organic solvent.

The extractions were then triple time repeating the step all above of solvent extraction by adding the isopropanol 500 ml into the plant residue. To collect filtrated of each solvent was pulled and evaporated by a rotary evaporator (Rotavapor® R-300, BUCHI) to remove the solvent and dried in a desiccator. The crude continued to weigh and dissolve in DMSO (dimethyl sulfoxide) for the next experiment [1, 11].

Bacterial strains and culture conditions

The bacteria culture was grown in Mueller–Hinton agar (MHA) medium (pH 7.3). The bacteria used were *Vibrio parahaemolyticus* (5HP, Centex Shrimp), *V. parahaemolyticus* (NX89, Centex Shrimp) and *Streptococcus agalactiae* (2809). *S. agalactiae* isolated from bighead carp (*Hypophthalmichthys nobilis*) is a species of freshwater fish and tilapia (*Oreochromis niloticus*) infection. *Vibrio* isolate were shrimp pathogen Acute Hepatopancreatic Necrosis Disease (AHPND) which has also been referred to as Early Mortality Syndrome (EMS), initially emerged as a destructive disease of cultured shrimp species in Asia in 2009. All the bacterial culture samples were supported by Center for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Mahidol University Thailand.

Antimicrobial by agar disc diffusion assay

Bacterial culture and infectious yeast culture were then performed testing with the crude extract of *Chromolaena odorata* (L.) R.M. King & H. Rob by agar disc diffusion assay. The method for antibacterial disk diffusion susceptibility following a manual of antimicrobial susceptibility testing in a manual of antimicrobial susceptibility testing guideline [2]. All crude extract was dissolved in dimethyl sulfoxide (DMSO). For initial screening, 40-75 µl of the extract was loaded onto each Whatman No. 1 filter paper disks (Ø, 6 mm) and air dried for 20 min. The disc was weighing and calculated the amount of residue on the paper disc. Those of bacteria were grown on Mueller–Hinton agar (MHA) medium (pH 7.3) except *Vibrio* spp. were supplement with 1.5% w/v NaCl. The microbial suspensions were prepared by spectrophotometer using culture broth adding sufficient sterile medium to adjust the transmittance to that produced by a 0.5 McFarland standard match to an optical density (OD) 0.1 at 625 nm wavelength. This procedure will obtain bacterial yields stock suspension $1x10^8$ cfu/ml. Then the microbial suspension was swab over the surface of media using a sterile cotton swab to ensure the confluent growth of the organism. The disks used were Whatman® No. 1 papers, 6 mm in diameter were then aseptically applied to the surface of the agar plates at well-spaced intervals. The plates were incubated at 37 °C for 24 h and observed growth inhibition zones, including the diameter of the disks, were measured. Control disks impregnated with 10 µl of the dissolved solvent and DMSO.

Determination of the minimum inhibitory concentration (MIC)

The bacterial cultures were grown in broth MHB using the horizontal orbital incubator shaker (Forma®435) at 150 rpm and 37 °C for several hours till logarithmic phase (O.D.625 ~0.1) was reached. After growth, the cells suspensions were prepared by spectrophotometer using culture broth adding a sufficient sterile medium to adjust the transmittance that related turbidity standard equivalent to a 0.5 McFarland standard match to an optical density (OD) 0.1 at 625 nm wavelength [13]. This results in a suspension containing approximately 1 to 2×10^8 colony-forming units (CFU)/mL [1,11,17]. Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in MHB so, after inoculation, each well contains approximately 5×10^5 CFU/mL (range 2 to 8×10^5 CFU/mL). For example, if the volume of broth in the well is 0.1 mL and the inoculum volume is 0.01 mL, then the 0.5 McFarland suspension (1×10^8 CFU/mL) should be diluted 1:20 to yield Clinical and Laboratory Standards Institute [1,11,17]. The crude extract was two-fold dilution with appropriate medium 100 µl in 96-well plate then the inoculums were applied in each well with a final concentration $1x10^5$ cfu/ml for bacteria. The microplate was incubated at 37°C for 24 hr. After incubation colorimetric reading a result using 30 µl Resazurin sodium salt (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) was purchased from Sigma-Aldrich (cat. R0717) 50 µM. After incubation for 30 min-1 hr, the MIC was read as the lowest concentration of antimicrobial agent at which no color change occurred [1, 11, 17, 18].

Resazurin was prepared by dissolves in sterile distilled water with 50 µM concentration then added to 30 µl in each 96-well plates from the total volume of 200 µl. At the end of 40 min incubation, color development was observed. Resazurin was a dark blue reduction to pink color

RESULTS AND DISCUSSIONS

Herbal plant *Chromolaena odorata* morphology

Bitter bush or Siam weed had the scientific name of *Chromolaena odorata* (L.) R.M. King & H. Robinson and a synonym as *Eupatorium conyzoides* Vahl or *Eupatorium odoratum* L. This plat was classified in family Asteraceae and the common names were bitter bush, butterfly weed, Siam weed, turpentine weed, and triffid weed. This original species is native to the warmer parts of southeastern USA (i.e. southern Florida and Texas), Mexico, the Caribbean and tropical South America (i.e. French Guiana, Guyana, Surinam, Venezuela, Bolivia, Colombia, Ecuador, Peru, Paraguay, Brazil and northern Argentina) [4]. The habitat found in a potential weed in tropical and sub-tropical regions that inhabits waterways (i.e. riparian areas), bushland, forest margins, roadsides, disturbed sites, waste areas, neglected pastures, crops and plantations.

The stems grow up to 7 m or more in length and several are usually produced from the plants long-lived rootstock (i.e. crown). They are many-branched, with the side (i.e. lateral) branches usually being produced in pairs in the leaf forks (i.e. access). The oppositely arranged leaves (5-12 cm long and 3-7 cm wide) are triangular or egg-shaped in outline (i.e. ovate) and have a pointed tip (i.e. acute apex) (Fig. 1A). They are hairy (i.e. pubescent) on both surfaces and have coarsely toothed (i.e. serrated) margins. These leaves are borne on stalks (i.e. petioles) up to 6 cm long (usually 10-15 mm) and give off a strong odour when crushed. The small flower-heads (i.e. capitula) do not have any petals (i.e. ray florets) and are borne in dense clusters at the ends of the branches (i.e. in terminal panicles). These flower-heads (about 10 mm long and 3 mm wide) are pale pink or pale mauve in colour (sometimes appearing whitish when older) and consist of numerous (15-30) tiny flowers (i.e. tubular florets) (Fig. 1B).

These tiny flowers (10-12 mm long) are surrounded by several layers of overlapping slender bracts (i.e. an involucre) 8-9 mm long. Each flower-head (i.e. capitulum) is borne on a stalk (i.e. peduncle) 10-30 mm long. Flowering occurs from late summer through to early spring, but is most abundant during winter (Fig. 1B, 1C). The black or dark brown 'seeds' (i.e. achenes) are 4-5 mm long and topped with a ring (i.e. pappus) of white to brownish coloured hairs (5-6 mm long) (Fig. 1D) [4].

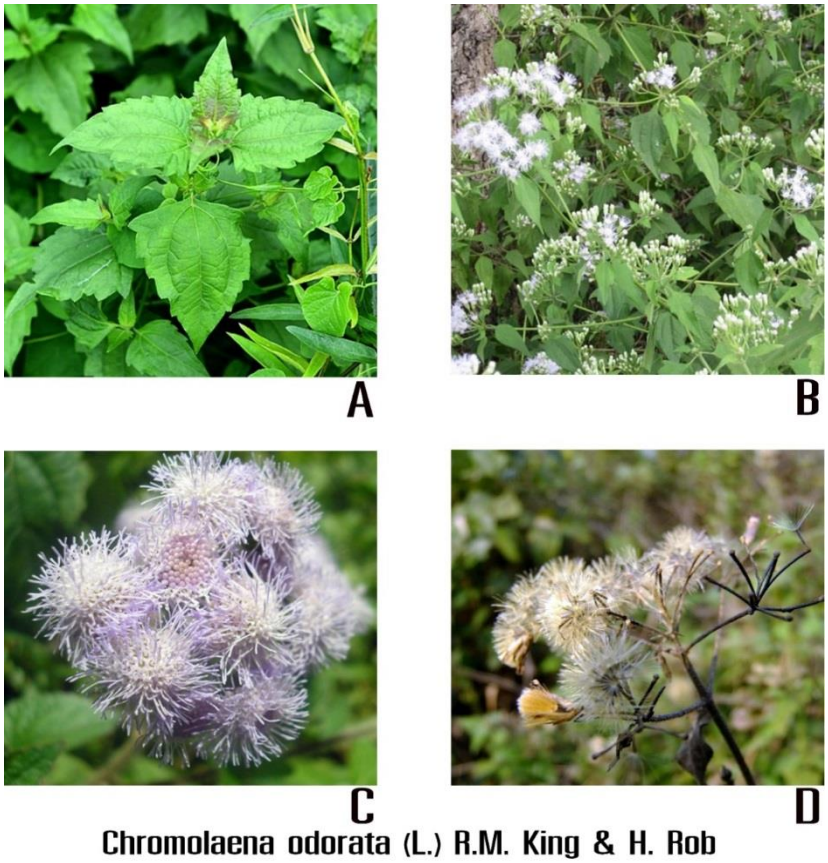

Figure 1. Herbal plant *Chromolaena odorata* (L.) R.M. King & H. Rob., bitter bush or Siam weed. These leaves are borne on stalks up to 6 cm long and give off a strong odour when crushed (A). Flowering occurs from late summer through to early spring, but is most abundant during winter (B, C). The black or dark brown 'seeds' (i.e. achenes) are 4-5 mm long and topped with a ring (i.e. pappus) of white to brownish coloured hairs (5-6 mm long) (D).

Table 1. Yield of crude extract from herbal plant, *Chromolaena odorata* (L.) R.M. King & H. Rob

Plant species	Organic solvent	Weight of plant (g)	Crude Extract (g)	Percent yield (%)
Chromolaena odorata	Isopropanol	200	2.5738 ± 0.0687	1.2931 ± 0.0344

Plant extraction and yields

The results of crude extraction from herbal plants *Chromolaena odorata* (L.) R.M. King & H. Rob produced a crude yield of 1.2931 ± 0.0344 g per 100 g of the fresh herb plant as the data in Table 1. The metabolite from isopropanol extraction was a dark brown color and acrid smell. The isopropanol (2-Propanol or Propan-2-ol) had 0.546 relative polarities organic solvent that was commonly used in chemistry and molecular biology laboratories [15]. It will dissolve in a wide range of chemicals and evaporates quickly. After evaporating to remove solvent, the residue was difficult to dissolve with water while crude extracted from isopropanol was amphoteric. Anyway, most of them were dissolved in DMSO before an attempt to perform testing.

Zone of inhibition by agar disc diffusion assay

Development of a disk diffusion assay for determining the microbial susceptibility was the technique modified from NCCLS guideline in 2015 [8]. A filter-paper disk impregnated with the *C. odorata* isopropanol extracted metabolite to be tested. The disk placed on the surface of the agar medium that already spread on top with microbial suspension as described in above. The bioactive compound diffuses from the filter paper into the agar. The concentration of the compound will be higher next to the disk and will decrease as a distance from the disk circle. If the compound was effective against microbes at a certain concentration, no microbial colonies will grow around the filter-paper disk where the clear zone of inhibition were measured (Fig. 2).

An example of zone of inhibition by agar disc diffusion assay was present in Figure 2. The *C. odorata* were given clear zone or inhibition zone around the paper disc of both compare to positive control of Amp (ampicillin) had large area and negative control isopropanol were not shown the inhibition zone. The measurement of the inhibition zone was shown in Table 2.

The solvents isopropanol is used for *C. odorata* extraction and dissolved the metabolite was examined agar disk diffusion assay in 30 microbial isolates is the control. There were performed clear zone resulting of crude extracted metabolite represented compared to antibiotic drug, ampicillin 300 µg as positive control. All of the paper disc was dried under 60^{O}C for 30 min. The result shows an effective metabolite of crude extracted from isopropanol at 16.9 ± 0.9 mg gave the inhibition clear zone against *S. agalactiae* (2809)with the clear zone 11.5 mm compared to ampicillin 13.0 mm, *E. faecalis* with the clear zone 13.0 mm compared to ampicillin 23.0 mm, *S. aureus* with the clear zone 10.0 mm compare to ampicillin 40.0 mm, *V. parahaemolyticus* (5HP) with the clear zone 11.0 mm compared to ampicillin 35.0 mm, *V. parahaemolyticus* (5HP) with the clear zone 10.0 mm compared to ampicillin 28.0 mm, and *V. parahaemolyticus* (NX89) with the clear zone 7.0 mm compared to ampicillin 25.0 mm whereas the solvent isopropanol were not inhibit all of testing (Fig. 2 and Table 2).

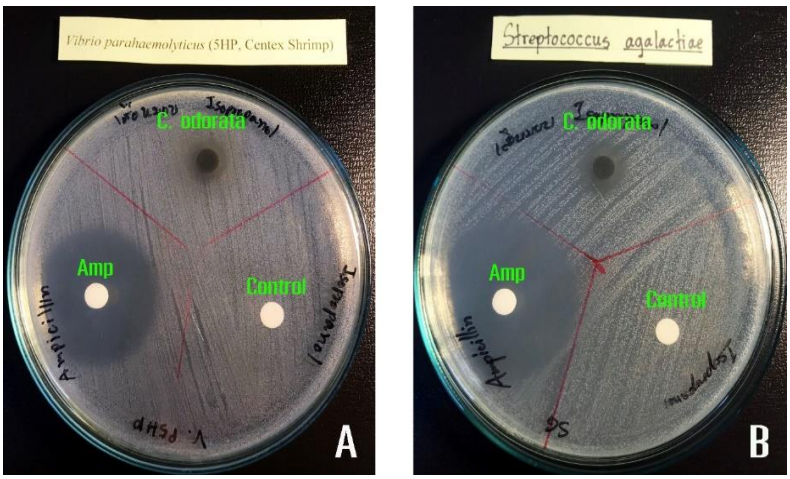

Figure 2. Inhibition zone metabolite extract *C. odorata* (L.) R.M. King & H. Rob against *V. parahaemolyticus* (5HP, Centex Shrimp) (Fig. A) and *S. agalactiae* (2809, Centex Shrimp) (Fig. B). *C. odorata*, metabolite; Control, Isopropanol; Am, ampicillin.

Table 2. Inhibition zone of *C. odorata* (L.) R.M. King & H. Rob against microbes.

Microbial isolates	Inhibition zone of testing disc		
	C. odorata 16.9 ± 0.9 mg	Ampicillin 300 µg	Isopropanol
Streptococcus agalactiae (2809)	11.0	35.0	NI
Vibrio parahaemolyticus (5HP)	10.0	28.0	NI
Vibrio parahaemolyticus (NX89)	7.0	25.0	NI

*NI: non inhibition

The minimum inhibitory concentration (MIC)

In this experiment, we used the whole part of plant extraction, especially leaves in order to primarily screen the antibacterial activity. The result of cured extract screening for antibacterial of the minimum inhibitory concentration (MIC) (Table 3) indicated that crude extracted of *C. odorata* (L.) R.M. King & H. Rob had the strongest bacteria inhibition at MIC 1,562.5 µg/ml for *Streptococcus agalactiae (2809)*, at MIC 3,125.0µg/ml for *V. parahaemolyticus* (5HP), and *V. parahaemolyticus* (NX89) (Table 3).

Table 3. Comparative MIC values of crude extraction against aquatic infection bacteria.

Microorganisms	Minimum Inhibitory Concentration µg/ml	Ampicillin µg/ml
Streptococcus agalactiae (2809)	1,562.5	≤ 50
Vibrio parahaemolyticus (5HP)	3,125.0	≤ 50
Vibrio parahaemolyticus (NX89)	3,125.0	≤ 50

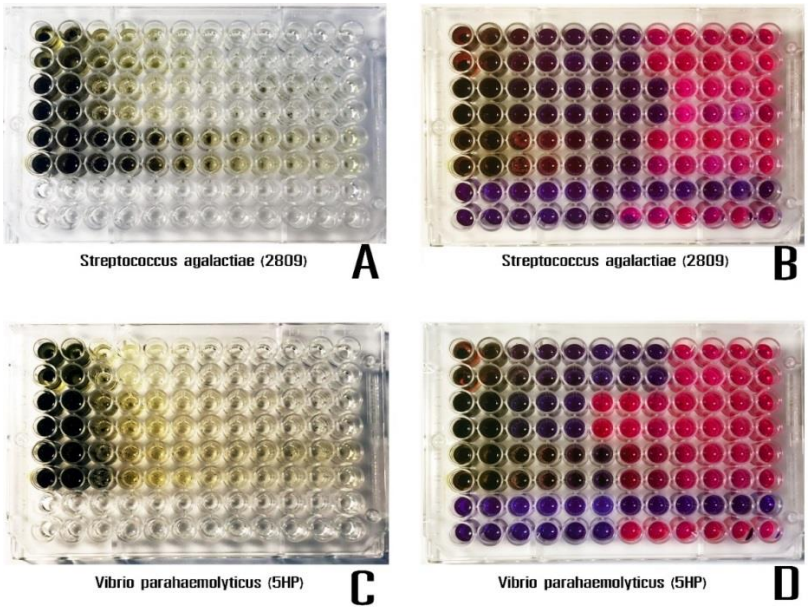

Figure 3. The colorless inhibition of bacterial *Streptococcus agalactiae* (A) *Vibrio parahaemolyticus* (C) and was oxidized form developed to the red color indicated bacterial growth. Colorimetric viability assay for minimum inhibitory concentration (MIC) in 96-well plate comparison using Resazurin (B&D). Resazurin has a dark blue color for bacterial inhibition and then reduced to pink color that means the non-inhibitory concentration of testing well.

The colorimetric viability assay for minimum inhibitory concentration (MIC)

The MICs obtained by the rapid colorimetric assay using tetrazolium salt, resazurin to enhance the visual. of detection. The experiment had performed crude metabolite from plant extracts to interpretive categories of anti-microbial susceptible inhibition, intermediate, and resistant strains in the diluted 96-well plate (Fig. 3).

After incubation 16-24 hr the resazurin has developed the colorimetric viability assay for minimum inhibitory concentration (MIC) by changing from dark blue color to reduced form pink color. Inhibition bacterial was dark blue effected by native resazurin whereas the intermediate changing was dark pink meanwhile the bacteria were moderately resistance and no resistance bacteria were reduced to resorufin has developed in pink color [13, 14, 16].

The comparison of colorimetric assay was easier to observe within 30 min after colorimetric development. The Figure 3A and 3C shows the colorless inhibition of bacterial *S. agalactiae* (A) *V. parahaemolyticus* that were difficult to observe by naked eye viability and or may detection be turbidity of microbial growth. Moreover, the colour of testing metabolite may interfere the observation. For the colorimetric assay of minimum inhibitory concentration (MIC) in 96-well plate shown in the Figure 3B was *S. agalactiae* and 3D was *V. parahaemolyticus* using resazurin to perform the color inhibition of bacteria. There was differential displayed blue or red color. Resazurin has a dark blue color for bacterial inhibition and then reduced to pink color that means the non-inhibitory concentration of testing well.

CONCLUSION

Bitter bush or Siam weed had the specific name is *Chromolaena odorata* (L.) R.M. King & H. Rob was classified with the invasive species or pest crop, but the bioactive metabolite properties had been studying and report the chemical component [1, 2, 3, 4, 9, 14]. This experiment confirmed the beneficial of *C. odorata* shown the bioactive metabolite against aquatic bacterial infection in tilapia and penaeid shrimp. Thrush Herbal plants *C. odorata* has been used in assessing natural biotherapeutic potential of medicinal plants and to select plants for future phytochemical research. This plant is annual, monoecious, and grows well during humid, rainy season in several regions of Thailand (Fig. 1). The dominance of this plant was unique characteristic that observed the fruits and seeds hanging under the leaf (Fig. 1).

The extraction technique using with appropriate solvent, isopropanol had 0.546 relative polarities organic solvent [15]. This solvent had miscible in water that advantage for extraction the bioactive metabolite related to the wide range of hydrophobic to hydrophilic properties and easily to evaporate the solvent residue. The triple solvent extraction technique and grounded in a blender was given the percentage yields 1.2931 ± 0.0344 from the fresh plant (Table 1). Agar disc diffusion assay and colorimetric microdilution assay were confirming the crude extract of *C. odorata* had inhibit shrimp pathogen *V. parahaemolyticus* (5HP), *V. parahaemolyticus* (NX89), and fish pathogen *S. agalactiae* (2809) (Fig. 2, Fog. 3, Table 2, Table 3).

The properties of resazurin were a phenoxazine dye that was weakly fluorescent, nontoxic, cell-permeable, and redox-sensitive [1, 12]. Resazurin has a blue to purple color (at pH > 6.5) and is used in microbiological, cellular, and enzymatic assays because it can be irreversibly reduced to the pink-colored and highly fluorescent resorufin (7-Hydroxy-3H-phenoxazin-3-one) [1, 12]. That could the advantage for using the detection dye for antimicrobial screening. Finally, bitter bush or Siam weed had the specific name is *C. odorata* (L.) R.M. King & H. Rob could be a potential herb using as the therapeutic drug for aquatic farm culture on the future.

According to this plant was easy growing in the tropics of Thailand this was might reduce cost of drug treatment and no aware for drug residue in aquaculture producers, more than that the problem of microbial drug resistance were not a problem. Anywhere this research could continue to define the biochemical properties and chemical properties of this plant on both *in vitro* and *in vivo* for safety and effectiveness.

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REFERENCES

[1] Bueno, C., Villegas, M. L., Bertolotti, S. G., Previtali, C. M., Neumann, M. G., Encinas, M. V. (2002). The Excited-State Interaction of Resazurin and Resorufin with Aminesin Aqueous Solutions: Photophysics and Photochemical Reaction. *Photochemistry and Photobiology*, 76(4), pp. 385–90. doi:10.1562/0031-8655(2002)0760385TESIOR2.0.CO2.PMID12405144

[2] Chen, J. L., Steele, T. W., Stuckey, D. C. (2015). Modeling and application of a rapid fluorescence-based assay for biotoxicity in anaerobic digestion. *Environmental science & technology*, 49(22), pp. 13463-13471. http://pubs.acs.org/doi/10.1021/acs.est.5b03050

[3] Doris, H. B., Carmen, de los R., Oscar, C., Adela, C. (1998). Antibacterial and chemical evaluation of *Chromolaena moritziana. Journal of Ethnopharmacology,* 59, pp. 203–206.

[4] Fact Sheet Index. (2016). Queensland Government, Weeds of Australia - Biosecurity Queensland Edition. Retrieved January 2, 2020 from https://keyserver.lucidcentral.org/weeds/data/media/Html/chromolaena_odorata.htm

[5] FAO. (2004). Fisheries and Aquaculture Department, National Aquaculture Sector Overview Thailand. Retrieved January 2, 2020 from http://www.fao.org/fishery/countrysector/naso_thailand/en

[6] FAO. (2013). Report of the FAO /MARD Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp (under TCP/VIE/3304). Hanoi, Viet Nam, on 25–27 June 2013. *FAO Fisheries and Aquaculture Report No. 1053*. Rome, p.54.

[7] Flegel 2012; Flegel, T.W. (2012) Historic emergence, impact and current status of shrimp pathogens in Asia. *Journal of Invertebrate Pathology*, 110, pp.166–173.

[8] Franklin, R. C., Matthew, A. W., Jeff, A., Michael, N. D., George, M. E., Mary, J. F., Dwight, J. H., David, W. H., et al. (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. *NCCLS M07-A9*, 32(1).

[9] Funmilayo, I. D., Afolayan, O. M., Adegbolagun, B., Irungu, L., Kangethe, J. O., Chiaka, I. A. (N.D.). Antimalarial actions of *Lawsonia lnermis, Tithonia diversifolia* and *Chromolaena odorata* in combination. *Journal of Ethnopharmacology.* Retrieved from http://dx.doi.org/10.1016/j.jep.2016.06.045

[10] Geng, Y., Wang, K. Y., Huang, X. L., Chen, D. F., Li, C. W., Ren, S. Y., Liao, Y. T., Zhou, Z. Y., Liu, Q. F., Du, Z. J., Lai, W. M. (2011). *Streptococcus agalactiae*, an Emerging Pathogen for Cultured Ya-Fish, *Schizothorax prenanti*, in China. *Transboundary and Emerging Diseases*, 1-7. Retrieved from doi:10.1111/j.1865-1682.2011.01280.x

[11] Grare, M., Fontanay, S., Cornil, C., Finance, C., Duval, R.E. (2008). Trazolium salts for MIC determination in microplates: Why? Which salt to select? How?. *Journal of Microbiological Methods*, 75, pp.156–159.

[12] Haggerty, R., Martí, E., Argerich, A., Schiller, D. von., Grimm, N. B. (2009). Resazurin as a smart tracer for quantifying metabolically active transient storage in stream ecosystems. *Journal of Geophysical Research: Biogeosciences*, 114(G3). Retrieved from doi*:*10.1029/2008JG000942. *hdl*:10261/38263. ISSN 2156-2202.

[13] Hataichanok, P., Xiaobo, Z., Suchitra, T., Yuvadee, W., Wandee, G., Seung, J. (2013). Effect of Siam weed extract and its bioactive component scutellarein tetramethyle theron anti-inflammatory activity through NF-κB pathway. *Journal of Ethnopharmacology*, 147, pp. 434–441. Retrieved from doi.org/10.1016/j.jep.2013.03.033

[14] Teampanpong, J. (2015). *BLOG. Posted on July 15, 2015. © 2019 Baimai.org – Thailand's 1st Environmental Enterprise – Social Enterprise*. Retrieved January 2, 2020, from http://baimai.org/stories

[15] Miller's Home. (1998). Solvent Polarity Table. *Chemistry Webercises Directory*. Retrieved January 2, 2020, from https://sites.google.com/site/miller00828/in/solvent-polarity-table

[16] Pasiecznik, N. (2019). Invasive Species Compendium *Chromolaena odorata* (Siam weed). © Copyright *2019 CAB International*. Retrieved January 2, 2020, from https://www.cabi.org/isc/datasheet/23248#tosummaryOfInvasiveness

[17] Panphut, W. (2016). Potential of extracted *Piper retrofractum* Vahl against plant pathogenic fungi. *8th World Conference on Educational Sciences*. Madrid-Spain, pp.741-6.

[18] Panphut, W. (2017). Anti Plant Pathogen of Bioactive Metabolite from *Piper retrofractum* Vahl. J. *Engineering and Applied Sciences*, 12, pp. 7321-7324.

[19] Srinivasa, R. K., Pradeep, K. C. and Anshuman, P. (2010). Evaluation of anti-oxidant activities and total phenolic content of *Chromolaena odorata*. *Food and Chemical Toxicology*, 48, pp. 729–732. Retrieved from doi: 10.1016/j.fct.2009.12.005

[20] Win, S., Taveesak, J., Nutthawan, N., P., Tattiyapong, Kwanrawee, S. and Alongkorn, A. (2017). Outbreaks of Tilapia Lake Virus Infection, Thailand, 2015–2016. *Emerging Infectious Diseases*, 23(6), pp. 1031-33. Retrieved from doi: https://dx.doi.org/10.3201/eid2306.161278

[21] Yang et al., 2014 Yang Y.T., Chen I.T., Lee C.T., Chen C.Y., Lin S.S., Hor L.I., Tseng T.C., Huang Y.T., Sritunyalucksana K., Thitamadee S., Wang H.C. & Lo C.F. (2014). Draft genome sequences of four strains of *Vibrio parahaemolyticus*, three of which cause early mortality syndrome/Acute hepatopancreatic necrosis disease in shrimp in China and Thailand. *Genome Announcements*, 2, pp. e00816–14.