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Efficacy of Vegetable Crude Extracts to Inhibit Bacteria, *Exiguobacterium Indicum*

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Abstract

Crude vegetable extracts are very interesting to study and then applied to inhibit bacteria. In this study, we obtained crude extracts from vegetable wastes, including yard-long bean leaf, pumpkin peel, and Chinese kale leaf, and studied their efficacies of those crude extracts to inhibit the bacteria, and investigated their phytochemicals using Gas chromatography-mass spectrometry. The efficacy of the crude vegetable extracts in inhibiting bacteria was evaluated based on the size of the inhibition zone. The results showed that the pathogenic bacteria isolated from the infected Nile tilapia fish was *Exiguobacterium indicum* presenting yellow colony. This species showed the highest inhibition activity at 15.33 mm from Chinese kale leaf crude extract. After testing antibacterial activity, crude extract from Chinese kale leaf had the highest antibacterial efficiency; however, there was no significant difference ($P>0.05$) with yard-long bean leaf extract. Phytochemical studies of all three vegetable wastes showed that crude extracts from Chinese kale leaves and yard-long bean leaves contained high quantities of 3,7,11,15-Tetramethyl-2-hexadecen-1-ol Phytol, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-, Butyl 9,12,15-octadecatrienoate, and Ethyl 9,12,15-octadecatrienoate in high quantity to antimicrobials, which are antimicrobials. In contrast, upon studying the phytochemical compounds, it was found that the crude extracts from yard-long bean leaves contained the highest number of various compounds. This study indicated that the crude extracts from Chinese kale leaf and yard-long bean leaf can be applied in preventing pathogenic bacteria in aquaculture, especially Nile tilapia fish.

Keywords: *antibacterial; Chinese kale; crude extract; Exiguobacterium indicum; pumpkin; yard-long bean*

1. Introduction

Thailand can produce about 1.0 tons of food from aquaculture per year, but the yield is uncertain due to climatic epidemics, market variability, and diseases' increase in aquaculture. Some factors such as harvesting, grading, pond transferring, transporting, and too high or low, and rapid temperature changing can reduce immunity and weakness in aquatic animals and be easy to catch disease accumulations. Vaccination and chemicals are effective methods of disease prevention. But, the vaccine and chemical will increase costs and are typically only effective against a single species of pathogen (Harikrishnan et al., 2011). For this reason, there is a tendency to use plants' extracts and their derivatives as an alternative to prevent and control aquatic animal diseases (Reverter et al., 2014; Harikrishnan et al., 2011). Additionally, major problems caused by an application of chemicals and antibiotics are drug resistance in organisms and their residues in the environmental compartments including air, water and organisms.

Nile tilapia (*Oreochromis niloticus*) is one of the most consumed freshwater fish in Thailand, with 220,000 tons produced for the local market per year, according to the Department of Fisheries. It has been regularly consumed and found on dishes in Thai households and restaurants. This fish is mostly susceptible to bacterial diseases during farming and marketing. Much research reported bacteria in fish such as *Aeromonas hydrophila*, *Streptococcus agalactiae*, *Flavobacterium columnaris* were found and caused diseases in Nile tilapia. The diseases have been controlled by plants' extracts such as *Psidium guajava*, *Andrographis paniculata* and *Allium tuberosum* (Rattanachaikunsopon & Phumkhachorn, 2009).

An alternative way to decrease these problems is to apply extracts from plants and herbs instead of those chemicals and antibiotics. Much research showed the benefits of applying plants, herbs and vegetable wastes to inhibit some pathogens (pathogenic bacteria) and microbials (Mongkolvai et al, 2021; Naiumsawang, 2019; Nithikulworawong, 2012). Generally, the solvents used for their extraction are water, ethanol, hexane, etc. (Mongkolvai et al., 2021). For examples, Naiumsawang (2019) reported that some plants such as cloves, gooseberry and horseradish contain important substances could inhibit some pathogens and have antioxidant capabilities. Mongkolvai et al. (2021) found chemicals, including alkaloids, flavonoids, phenolics, terpenoids and essential oils, in many plants having the abilities to inhibit microbial growth, stimulate an immune system, reduce stress, and prevent diseases. Moreover, Nithikulworawong (2012) suggested that extracts from *Bauhinia siridhorniae* tree have the capability to inhibit *Streptococcus agalactiae* in Nile tilapia.

However, the use of vegetables, including yard-long bean leaf, pumpkin peel, and Chinese kale leaf, as a means to inhibit pathogens or microorganisms, remains unexplored. We therefore investigated the efficacies of vegetable waste extracts comprising yard-long bean leaf, pumpkin peel and kale leaf, to inhibit pathogens in Nile tilapia, and studied their phytochemicals. The results of this research could be applied to use these vegetable extracts for pathogen inhibition in fish farms.

2. Objectives

The objective of the study was to investigate the efficacies of vegetable waste extracts including yard-long bean leaf, pumpkin peel and Chinese kale leaf, in inhibiting pathogens in Nile tilapia, and to study their phytochemicals.

3. Materials and methods

3.1 Preparation of crude extracts from vegetable wastes

Preparation of crude extracts from vegetable wastes, comprising yard-long bean leaf (*Vigna unguiculata* ssp. sesquipedalis), pumpkin peel (*Cucurbita moschata* Duchesne) and Chinese kale leaf (*Brassica oleracea*), was modified from Mongkolvai et al. (2021). Briefly, the vegetables taken from local markets at Kalasin province, Thailand were washed carefully with deionized (dI) water and then ground by a blender. Eight grams of samples were weighed using 2 digit-balance and then extracted with 400 mL of ethanol, methanol and DI water. The procedure was performed in room temperature for 5 days. Next, the solution was filtered using glass cones and filter paper. The obtained solution was evaporated in a rotary evaporator to get crude extracts. After that, they were weighed and kept for preliminary screening of phytochemicals, and antibacterial activities.

3.2 Phytochemical of vegetable waste crude extracts by Gas chromatography- mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) analysis of the extracts using ethanol extraction was performed with Perkin Elmer Glarus 680 GC equipped with a capillary column made of Elite-5MS. Helium was the carrier gas at a flow rate of 1 mL/min. The temperature was programmed as follows: 80ºC at 5ºC/min to 100ºC and 3ºC/min to 250ºC. The components of the extracts were identified with the database of the National Institute Standard and Technology MS library (NIST-MS library).

3.3 Isolation of pathogenic bacteria from fresh fish organs

A total of 5 fresh fishes, Nile tilapia (*Oreochromis niloticus*), were collected from a pond of Fisheries Science Technology Department, Kalasin University, Kalasin Province. The fishes, presenting a symptom of disease, were placed in thermal flask and transported to the laboratory for analysis, which each fish was placed in aseptic small plastic bag, labeled and sealed separately to avoid contamination, washed with tap water and kept at 4°C. In this study, all specimens' swabs of gills and body surface were taken for analysis. Each fish was swabbed from gills and body surface, and then cross-streaked on Nutrient Agar media in plates. After the plates were incubated at 37°C for 24 h, single colony was picked up from the plates and streaked on the Agar for pure culture colony and kept at 4°C for next experiment. Different pathogenic bacteria were isolated by single colony colors of yellow or white. Both of colony colors were tested for the efficacy of the crude extracts for antibacterial activity.

3.4 Efficacy of vegetable crude extracts for antibacterial activity

Efficacies of three vegetable crude extracts namely: yard-long bean leaf (*Vigna unguiculata* ssp. *sesquipedalis*), pumpkin peel (*Cucurbita moschata Duchesne*) and Chinese kale leaf (*Brassica oleracea*) to inhibit the growth of yellow and white colonies of bacteria were investigated. We used 50 µL of each bacterium at the concentration of $5x10^8$ cell/mL in the well. The crude of three vegetables at 50 µL of each crude drop to paper disc and 50 µL of chloralmphenicol was used for positive control and normal saline used for negative control and tested 3 replicates in the plate. All plates were incubated at 37°C for 24 h. The diameter of clear zone was measured to analyze and report to the result of efficacy of plant crude extract to antibacterial.

3.5 Methods of bacteria identification; 16s rDNA sequencing

Identification of the yellow isolates was carried out by molecular single strand 16S rDNA sequencing characterization. This method was done by Thailand Bioresource Research Center (TBRC). The processes were as the followings.

PCR amplification of 16S rDNA

DNA templates for PCR amplification were prepared using "Genomic DNA mini kit (Blood/culture cell)" (Geneaid Biotech Ltd., Taiwan). DNA coding for 16S rRNA regions was amplified by means of PCR with *Taq* polymerase, as described by Kawasaki et al. (1993), Yamada et al. (2000), and Katsura et al. (2001). A PCR product for sequencing 16S rDNA regions was prepared using the following two primers: 20F (5'-GAG TTT GAT CCT GGC TCA G-3', position 9-27 on 16S rDNA by the *E coli* numbering system (Brosius et al., 1981), and 1500R (5'-GTT ACC TTG TTA CGA CTT-3', position 1509-1492 on 16S rDNA by the *E. coli* numbering system (Brosius et al., 1981). The PCR amplification was carried out with DNA Engine Dyad® Thermal Cycler (Bio-Rad Laboratories). 100 mL of a reaction mixture contained 15-20 ng of template DNA, 2.5 µmoles in each of the two primers, 2.5 units of *Taq* polymerase, 2.0 mM of $MgCl_2$, 0.2 mM of dNTP and 10 µL of 10x*Taq* buffer, pH 8.8, containing $(NH_4)_2SO_4$, which comprised 750 mM of Tris-HCl, 200 mM of $(NH_4)_2SO_4$ and 0.1% Tween 20. The PCR amplification was programmed to carry out an initial denaturation step at 94ºC for 3 min, 25 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and elongation at 72ºC for 2 min, followed by a final amplification step at 72ºC for 3 min. The PCR product was analyzed by 0.8% (w/v) agarose gel electrophoresis and purified with a GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). The purified PCR product was stored at -20ºC for further step.

Direct sequencing of 16S rDNA

Direct sequencing of the single-banded and purified PCR products (ca. 1500 bases, on 16S

rDNA by the *E. coli* numbering system (Brosius et al., 1981) was carried out. Then, sequencing of the purified PCR products was performed on ABI Prism® 3730XL DNA Sequence (Applied Biosystems, Foster City, California, USA) by sequencing service provider. The two primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') or 800R (5'-TAC CAG GGT ATC TAA TCC-3'), and 518F (5'-CCA GCA GCC GCG CTA ATA CG-3') or 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') for single strand 16S rDNA sequencing, and 4 primers of 27F, 518jF, 800R and 1492R for double strands 16S rDNA sequencing were used.

Sequence analysis

The nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/_BioEdit/BioEdit.htm) . For the identification of phylogenetic neighbors was initially carried out by the BLASTN (Altschul et al., 1997) program against 16S rDNA sequence database of validly published prokaryote. The sequences with the highest scores were calculated pairwise sequence similarity using global alignment algorithm (Myers & Miller, 1988).

3.6 Statistical analysis

All data were expressed as mean ± standard deviation of inhibition zone. The values of each column were tested for a different significant $p<0.05$ according to LSD test by using SPSS 19.0 for Windows program.

4. Results and Discussion

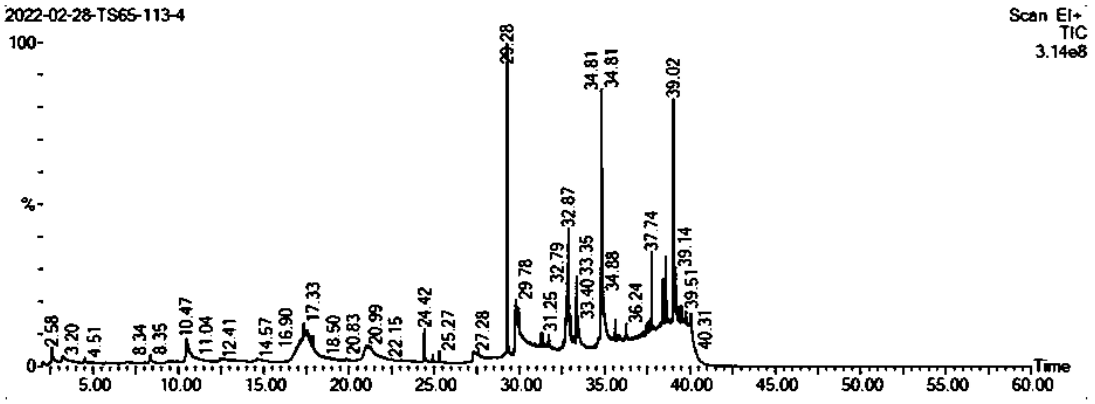
4.1 Phytochemical of vegetable waste crude extracts by Gas chromatography-mass spectrometry

The optimal conditions for crude extraction from this study were found to be 80 g of vegetable material combined with 400 mL of solvent. Ethanol was most suitable for the crude extraction from vegetables. The crude extracts from yard-long bean leaf, pumpkin peel and Chinese kale leaf were 9, 3.5 and 5 grams, respectively (Table 1).

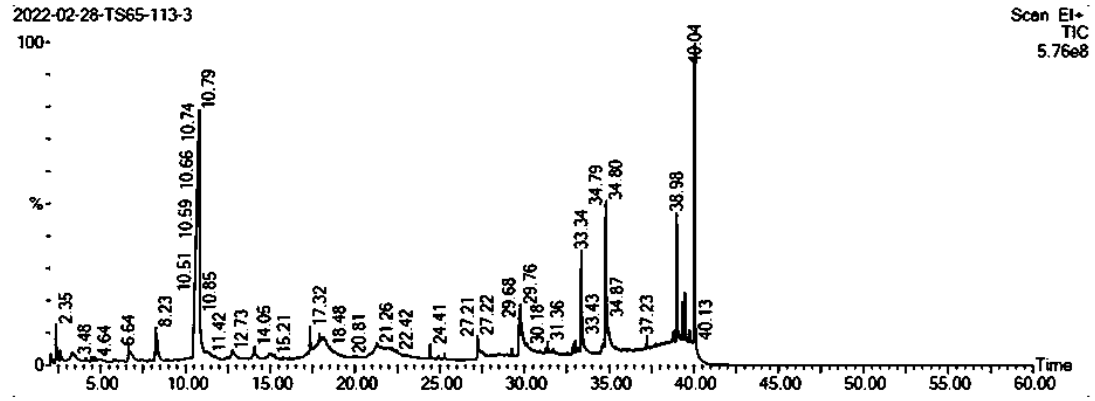
The ethanolic extracts of yard-long bean leaf, pumpkin peel, and Chinese kale leaf were analyzed by GC-MS, and each sample gave a chromatogram illustrating several peaks (see in Figure 1). The GC-MS analysis of the extracts revealed the existence of 67 compounds in ethanolic extracts with their molecular formula and various activities present in table 2. The phytochemical compounds in yard-long bean leaf were Butyl 9,12,15-octadecatrienoate (10.157%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (6.355%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (4.446%), γ-Sitosterol (4.938%), Ethyl 9,12,15-octadecatrienoate (4.804%), 5-Hydroxymethylfurfural (3.167%), Hexadecanoic acid, 2-hydroxy 1-(hydroxymethy) ethyl ester (2.292%), Furfural (1.030%) and Neophytadiene (diterpene) (0.850). The major phytochemicals of pumpkin peel are Stigmasterol (3.139%), n-Hexadecanoic acid (palmitic acid ester) (1.354%), Hexadecanoic acid, 2-hydroxy 1-(hydroxymethyl) ethyl ester (3.449%), Furfural (1.030%) and Neophytadiene (diterpene) (0.359%). Additionally, Chinese kale leaf shows γ-Sitosterol (11.456%), Hexadecanoic acid, 2-hydroxy 1-(hydroxymethy) ethyl ester (1.731%) and Neophytadiene (diterpene) (1.429%).

Table 1 Conditions for separating and amount of vegetable crude extracts

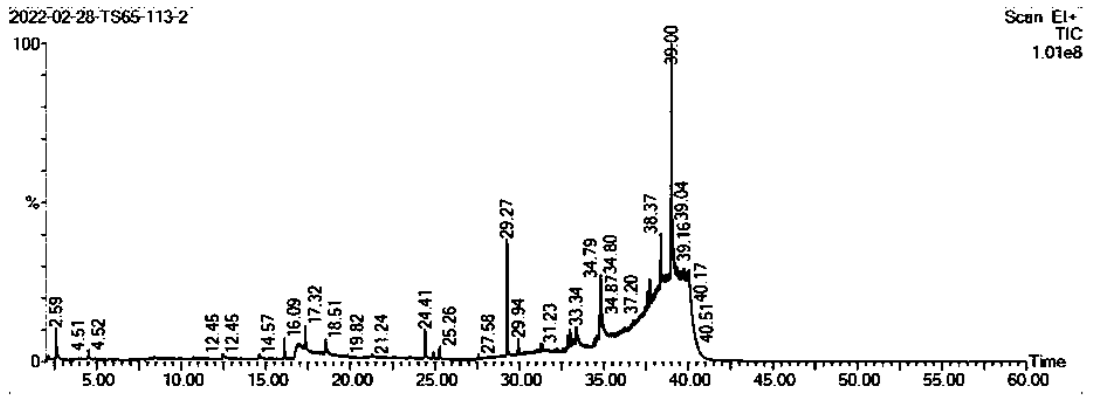
Type of vegetable	Type of solvent	Amount of vegetable waste (g)	Solvent (mL)	Amount of crude extract (g)
Yard-long bean leaf	methanol	80	400	9.0
	ethanol	80	400	12.0
	distilled water	80	400	4.0
Pumpkin peel	methanol	80	400	3.5
	ethanol	80	400	3.9
	distilled water	80	400	2.6
Chinese kale leaf	methanol	80	400	5.0
	ethanol	80	400	6.2
	distilled water	80	400	4.0



Yardlong bean leaf crude extract (*Vigna unguiculata* ssp. *sesquipedalis*)



Pumpkin peel crude extract (*Cucurbita moschata Duchesne*)



Chinese kale leaf crude extract (*Brassica oleracea*)

Figure 1 GC-MS chromatogram of ethanolic extracts of yard-long bean leaf, pumpkin peel, and Chinese kale leaf

Table 2 Phytochemical components in ethanolic extracts of yard-long bean leaf, pumpkin peel, and Chinese kale leaf

No.	Name of the compound	Molecular formula	Activity	Relative abundance (%)		
				Yard long bean leaf	Pumpkin peel	Chinese kale leaf
1	1,2.3,4,5-Cyclopentanepentol	$C_5H_{10}O_5$	unknown	-	1.353	
2	1-(2-Acetoxyethyl)-3,6-diazahomoadamantan-9-one oxime	$C_{13}H_{21}N_3O_3$	unknown	-	-	0.544
3	1,13-Tridecanediol, diacetate	$C_{17}H_{32}O_4$	unknown	0.482	-	-
4	1,3-Propanediol, 2-(hydroxymethyl) -2-nitro-	$C_4H_9NO_5$	unknown	2.901	-	-
5	1-Heptatriacotanol	$C_{37}H_{76}O$	unknown	0.446		
6	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	unknown	3.158	1.145	2.239
7	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	$C_{13}H_{22}OSi_2$	unknown	-	-	0.425
8	2,5-Dimethoxy-4-ethylamphetamine	$C_{13}H_{21}NO_2$	unknown	-	-	1.233
9	2,5-Octadecadiynoic acid, methyl ester	$C_{19}H_{34}O_2$	unknown	-	-	0.463
10	2-Butene-1,4-diol, TMS derivative	$C_7H_{16}O_2Si$	unknown	-	-	0.510
11	3,7,11,15-Tetramethyl-2-hexadecen-1-ol Phytol	$C_{20}H_{40}O$	Antimicrobial, anti-inflammatory, anticancer, diuretic, antifungal agent *S. typhi*, *resistant gonorrhea*, *joint* dislocation, headache, hernia, stimulant and antimalarial (Tyagi & Agarwal, 2017)	6.355	-	4.975
12	3-Cyclopentylpropionic acid, 2-methylpropył ester	$C_{13}H_{24}O_2$	unknown	-	0.371	-
13	3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$	Antibacterial activity (Ghosh et al., 2015).	-	2.020	-
14	3-Heptanol, 2-methyl-5-nitro-	$C_8H_{17}NO_3$	unknown	5.316	-	-
15	3-Hexen-1-ol	$C_6H_{12}O$	unknown	0.575	-	1.760
16	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	Strong free radical scavenging activity (Ahmed et al., 2019)	-	1.320	-
17	4-Hydroxybenzyl ethyl ether	$C_9H_{12}O_2$	unknown	-	0.656	
18	4-Octanol, propanoate	$C_{11}H_{22}O_2$	unknown	-	-	0.474
19	5-Hydroxymethylfurfural	$C_6H_6O_3$	Cancer preventive, antioxidant, and hepatic and renal protective effects (Ahmed et al., 2019)	3.167	27.813	-
20	6-Methoxy-2,7,8-trimethyl-2-(4,8,12-trimethyltridecylchroman	$C_{29}H_{50}O_2$	unknown	-	-	1.057
21	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	unknown	0.468	-	-
22	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{19}H_{32}O_2$	Antimicrobial (Shaheed et al., 2019)	4.446	3.692	6.523
23	9.12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	$C_{21}H_{36}O_4$	Antioxidant, antimicrobial activity, anti-inflammatory, nematicide, antihistaminic, antieczemic, insectifuge (Shaheed et al., 2019)	-	7.893	-

Table 2 Cont.

No.	Name of the compound	Molecular formula	Activity	Relative abundance (%)		
				Yard long bean leaf	Pumpkin peel	Chinese kale leaf
24	Alpha-l-rhamnopyranose	$C_6H_{12}O_5$	unknown	3.331	-	-
25	Androst-4-en-9-thiocyanomethyl-11-ol-3,17-dione	$C_{21}H_{27}NO_3S$	unknown	-	-	0.727
26	Butoxyacetic acid	$C_6H_{12}O_3$	unknown	-	5.267	-
27	Butyl 9,12,15-octadecatrienoate	$C_{22}H_{38}O_2$	Antiinflammatory, hypocholesterolemic, cancer preventive, hepatoprotective (Devakumar et al., 2017)	10.157	-	-
28	Campesterol	$C_{28}H_{48}O$	Anti-inflammatory (Al-Marzoqi et al., 2016)	-	-	3.302
29	Carbonic acid, heptyl prop-1-en-2-yl ester	$C_{11}H_{20}O_3$	unknown	-	-	1.233
30	cis-5,8,11,14,17-Eicosapentaenoic acid	$C_{20}H_{30}O_2$	unknown	-	-	0.448
31	Cyclopropane, 1,1-dimethyl-2-(2-propeny)-	$C_8H_{14}O$	unknown	-	-	0.432
32	d-Gala-l-ido-octonic lactone	$C_8H_{17}NO_8$	Anti-diabetic activity (Kadhim et al., 2017)	-	2.190	-
33	dl- α -Tocopherol	$C_{31}H_{52}O_3$	unknown	1.390	-	-
34	dl-Glyceraldehyde dimer	$C_6H_{12}O_6$	unknown	0.446	-	-
35	Dihydroxyacetone	$C_3H_6O_3$	unknown	-	0.364	-
36	Dodecane, 5,8-diethyl-	$C_{16}H_{34}$	unknown	-	0.416	-
37	E-10,13,13-Trimethyl-11-tetradecen-1-ol acetate	$C_{19}H_{36}O_2$	unknown	-	-	0.800
38	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	$C_{19}H_{34}O_2$	unknown	0.639	-	-
39	Ergost-5-en-3-ol, (3 α)	$C_{28}H_{48}O$	unknown	1.141	-	-
40	Ethanamine, 2-propoxy	$C_5H_{13}NO$	unknown	-	0.948	-
41	Ethanol, 2-(9-octadecenylaxy), (Z)	$C_{20}H_{40}O_2$	unknown	0.527	-	-
42	Ethyl 9,12,15-octadecatrienoate	$C_{20}H_{34}O_2$	Anti-inflammatory and antimicrobial activity (Siswadi & Saragih, 2021)	4.804	-	-
43	Furfural	$C_5H_4O_2$	Anti-inflammatory activity (Ahmed et al., 2019)	1.030	1.030	-
44	Furyl hydroxymethyl ketone	$C_6H_6O_3$	unknown	-	0.497	-
45	Geldaramycin	$C_{29}H_{40}N_2O_9$	unknown	-	0.448	-
46	Hexadecanoic acid, 2-hydroxy 1-(hydroxymethy) ethyl ester	$C_{19}H_{38}O_4$	Hemolytic, pesticide, flavor, antioxidant (Tyagi & Agarwal, 2017)	2.292	3.449	1.731
47	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	Antioxidant, hemolytic, hypocholesterolemic, flavor, nematicide, anti-androgenic (Tyagi & Agarwal, 2017)	0.464	-	-
48	Linoelaidic acid	$C_{18}H_{32}O_2$	unknown	-	1.112	-
49	Melezitose	$C_{18}H_{32}O_{16}$	unknown	-	-	1.209

Table 2 Cont.

No.	Name of the compound	Molecular formula	Activity	Relative abundance (%)		
				Yard long bean leaf	Pumpkin peel	Chinese kale leaf
50	Neophytadiene (diterpene)	$C_{20}H_{38}$	Fragrance, antimicrobial, analgesic, antipyretic, anti-inflammatory, antimicrobial, and the antioxidant (Muthulakshmi et al., 2012; Hazarika et al., 2002; Raman et al., 2012)	0.850	0.359	1.429
51	N-Nitroso-2,4,4-trimethyloxazolidine	$C_6H_{12}N_2O_2$	unknown	-	0.889	-
52	n-Hexadecanoic acid (palmitic acid ester)	$C_{16}H_{32}O_2$	Antioxidant, nematicide, hypocholesterolemic, anti-androgenic, hemolytic, pesticide, lubricant, 5-Alphareductase inhibitor, antipsychotic (Enerijiofi et al., 2021)	0.852	1.354	-
53	Octadecanal, 2-bromo-	$C_{18}H_{35}BrO$	unknown	-	-	0.606
54	Octadecanoic acid	$C_{18}H_{36}O_2$	unknown	-	0.925	
55	Octanoic acid, 2-dimethylaminoethyl ester	$C_{12}H_{25}NO_2$	unknown	-	-	0.431
56	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	unknown	1.348	1.725	-
57	Octahydropyrano[3,2-b]pyridin-6-one	$C_8H_{13}NO_2$	unknown	-	3.296	-
58	Olean-13(18)-ene	$C_{30}H_{50}$	unknown	-	0.796	-
59	Phosphoric acid, diethyl octyl ester	$C_{12}H_{27}O_4P$	unknown	1.959	-	-
60	Propene, 3-tert-butoxy-2-(methoxymethyl)-	$C_9H_{18}O_2$	unknown	-	-	1.065
61	Stigmast-7-en-3-ol, (3α,5α)-	$C_{29}H_{50}O$	unknown	-	1.246	-
62	Stigmasta-5,24(28)-dien-3-ol, (3α,24Z)	$C_{29}H_{48}O$	unknown	1.065	-	-
63	Stigmasterol	$C_{29}H_{48}O$	Antioxidant, hypoglycemic and thyroid inhibiting properties, precursor of progesterone, antimicrobial, anticancer, anti-arthritic, anti-asthama, anti-inflammatory, diuretic. It has a role as a vitamin and a plant metabolite, bronchodilator (Tyagi & Agarwal, 2017).	1.417	3.139	-
64	trans-2-undecenoic acid	$C_{11}H_{20}O_2$	unknown	-	0.400	-
65	Uracil, 1-methyl-	$C_5H_6N_2O_2$	unknown	-	1.177	-
66	Urs-12-en-24-oic acid, 3-oxo-, methyl ester,	$C_{31}H_{48}O_3$	unknown	3.041	-	-

Table 2 Cont.

No.	Name of the compound	Molecular formula	Activity	Relative abundance (%)		
				Yard long bean leaf	Pumpkin peel	Chinese kale leaf
67	γ-Sitosterol	$C_{29}H_{50}O$	Antihyperglycemic activity by increasing insulin secretion in response to glucose (Sirikhansaeng et al., 2017)	4.938	-	11.456

Table 3 Inhibition zone of antibacterial activity of vegetable crude extracts

Isolate	Negative control (normal saline) (mm)	Positive control (chloramphenicol 30 µg) (mm)	Yard-long bean leaf crude extract (mm)	Pumpkin peel crude extract (mm)	Chinese kale leaf crude extract (mm)
Yellow colony	0	66	18	10	18
	0	58	14	8	15
	0	70	12	6	13
Average	**0** ^{d}	**64.66** ^{a}	**14.66** ^{b}	**8.0** ^{c}	**15.33** ^{b}
White colony	0	66	1	18	16
	0	58	2	16	14
	0	70	2	16	14
Average	**0** ^{c}	**64.66** ^{a}	**1.66** ^{c}	**16.66** ^{b}	**14.66** ^{b}

After analyzing phytochemical components of vegetable wastes using GC- MS, we found 3,7,11,15- Tetramethyl- 2- hexadecen- 1- ol Phytol, 9,12,15- Octadecatrienoic acid, (Z,Z,Z) - , Butyl 9,12,15- octadecatrienoate and Ethyl 9,12 ,15- octadecatrienoate in high quantities. These types of substances possess antimicrobial properties (Devakumar et al., 2017; Siswadi & Saragih, 2021; Shaheed et al., 2019). Especially, the compound of 3,7,11,15- Tetramethyl- 2- hexadecen- 1- ol Phytol found in yard- long bean leaf and Chinese kale leaf had high ability to inhibit *E. indicum* in laboratory because this compound has abilities of antimicrobial, anti-inflammatory, antifugal agent of *S. typhi* and inhibitory to *Gonorrhea* sp. (Tyagi & Agarwal, 2017) . This finding corresponds with previous studies which indicated that crude extracts from plants could inhibit pathogenic bacteria in fish.

Among the phytochemical compounds in the vegetable waste crude extracts in this study, the yard-long bean leaf crude extract contained the highest number of important substances. However, Potiwong et al. (2020) reported that plants have a variety of properties, such as a dietary supplement for stimulating appetite, increasing growth and applying as an anesthetic in fish transportation, boosting the immune system, which is resistant to pathogens, reducing stress in fish and improving flesh quality (Chakraborty & Hancz, 2011; Chitmanat et al., 2005). These effects might be caused by chemicals or plant derivatives containing alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides and flavonoids. Many medicinal plants are available and inexpensive; thus, they have been widely used in both fresh or dried forms or in extract form. Additionally, the phytochemicals and antibacterial activity may vary depending on the solvents used for extraction.

4.2 Antibacterial activity of vegetable crude extracts

The efficacy of vegetable crude extracts to inhibit pathogenic bacteria showed that the yellow-colony bacteria treated with Chinese kale leaf crude extract had the highest inhibition activity at 15.33 mm, followed by yard-long bean leaf crude extract at 14.66 mm, and pumpkin peel crude extract at 8.00 mm, respectively. For the bacteria in white colony found that pumpkin peel crude extract had the highest inhibitory activity at 16.55 mm, followed by Chinese kale leaf crude extract at 14.66 mm, and yard-long bean leaf crude extract at 1.66 mm, respectively. All the averages of inhibitory

activity had different significance ($p<0.05$). All inhibition zones showed better results than the negative control but were less effective than the positive control. The results are shown in Table 3 and Figure 2.

Remark: Results are as mean of inhibition zone. Letters a, b, c are in the same row, and there was a statistically difference at the 95%

The efficacy of the crude extracts in inhibiting bacteria showed that the yellow colonies had a higher average inhibition zone compared to the white colonies. This colony was selected to identify the species of pathogenic bacteria by molecular characteristic and this strain namely SurinY1 isolate (Figure 3).

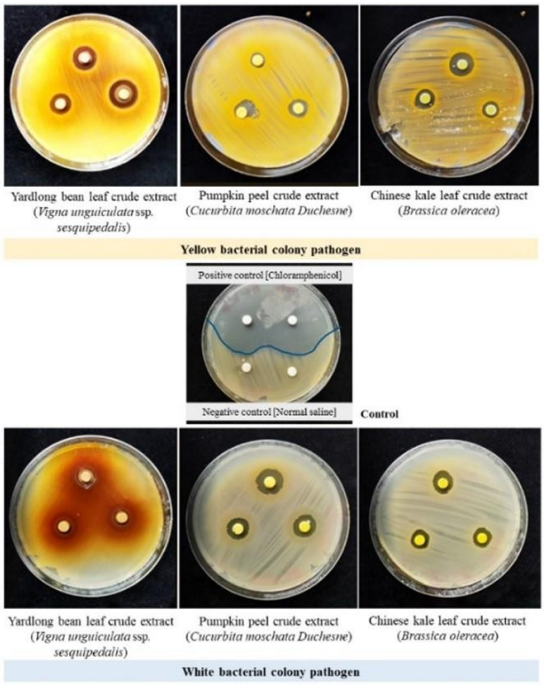


Figure 2 Efficacies of vegetable crude extracts to inhibit bacteria of yellow and white colonies from infected fish

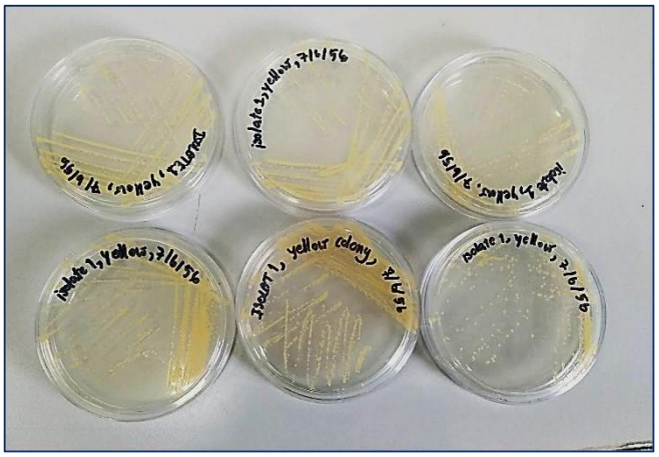


Figure 3 Yellow colony of SurinY1 isolate

4.3 Identification of bacteria from fresh fish organs

The bacteria isolate SurinY1 that appeared in yellow colony had the highest antibacterial activity, identified by single strand 16S rDNA sequencing. The sequence was compared using the basis local alignment search tool (BLAST), with the difference in nucleotide sequence database of NCBI. The partial sequence of 16S rDNA from isolated SurinY1 showed high levels of sequence with similarity to the strains of *Exiguobacterium indicum* (99.86%). These percentages of similarity and sequencing of *E. indicum* are shown in Table 4.

Table 4 Comparison of nucleotide sequences with reference strain(s) by 16s rDNA on National Center of Biotechnology Information (NCBI) database

Rank	Name	Strain	Authors	Accession	Pairwise Similarity (%)	Mismatch/ Total (nt)
1	*Exiguobacterium indicum*	HHS31	Chaturvedi & Shivaji (2006)	AJ846291	99.86	2/1456
2	*Exiguobacterium acetylicum*	DSM 20416	Levine & Soppeland (1926) Farrow et al. (1994)	JNIR01000001	99.66	5/1456
3	*Exiguobacterium enclense*	NIO-1109	Dastager et al. (2015)	JF893462	99.50	7/1414
4	*Exiguobacterium sibiricum*	255-15	Rodrigues et al. (2006)	CP001022	98.42	23/1456
5	*Exiguobacterium oxidotolerans*	JCM 12280	Yumoto et al. (2004)	JNIS01000001	98.35	24/1456
6	*Exiguobacterium artemiae*	9AN	Lõpez-Cortés et al. (2006)	AM072763	98.27	24/1385
7	*Exiguobacterium antarcticum*	DSM 14480	Frühling et al. (2002)	JMKS01000003	98.03	28/1456
8	*Exiguobacterium undae*	DSM 14481	Frühling et al. (2002)	JHZV01000003	98.01	29/1456
9	*Exiguobacterium soli*	DVS3Y	Chaturvedi et al. (2008)	AY864633	97.87	31/1454
10	*Exiguobacterium flavidum*	HF60	Meng et al. (2020)	MH375463	96.83	45/1440
11	*Exiguobacterium profundum*	10C	Crapart et al. (2007)	AY818050	94.80	75/1441
12	*Exiguobacterium marinum*	DSM 16307	Kim et al. (2005)	JHAT01000001	94.78	76/1456
13	*Exiguobacterium aestuarii*	TF-16	Kim et al. (2005)	AY594264	94.57	79/1456
14	*Exiguobacterium mexicanum*	8N	Lõpez-Cortés et al. (2006)	AM072764	94.48	77/1394
15	*Exiguobacterium aquaticum*	IMTB-3094	Raichand et al. (2012)	JF775503	94.30	83/1456
16	*Exiguobacterium aurantiacum*	DSM 6208	Collins et al. (1984)	JNIQ01000001	93.96	88/1456
17	*Exiguobacterium alkaliphilum*	12/1	Kulshreshtha et al. (2013)	EU379016	93.67	91/1438
18	*Ornithinibacillus contaminans*	CCUG 53201	Kämpfer et al. (2010)	FN597064	92.83	88/1228
19	*Kurthia senegalensis*	JC8E	Roux et al. (2016)	CAEW01000048	92.21	100/1284
20	*Domibacillus indicus*	SD111	Sharma et al. (2014)	KF732820	92.17	100/1284
21	*Metabacillus mangrovi*	AK61	Gupta et al. (2017) Gupta et al. (2020)	HG974242	92.02	114/1429
22	*Metabacillus indicus*	LMG 22858	Suresh et al. (2004) Patel & Gupta (2020)	JGVU01000003	91.88	116/1429

Table 4 Cont.

Rank	Name	Strain	Authors	Accession	Pairwise Similarity (%)	Mismatch/Total (nt)
23	*Domibacillus tundrae*	PAMC 8007	Gyeong et al. (2015)	LAJA010000023	91.80	113/1378
24	*Bacillus benzoevorans*	DSM 5391	Pichinoty et al. (1987)	D78311	91.71	116/1400
25	*Siminovitchia fortis*	DSM 16012	Scheldeman et al. (2004) Gupta et al. (2020)	KZ987423	91.57	121/1436
26	*Oceanobacillus oncorhynchi* subsp. *incaldanensis*	20AG	Romano et al. (2006)	AJ640134	91.54	123/1454
27	*Ectobacillus antri*	SYSU K30001	Rao et al. (2019) Gupta et al. (2020)	MK129420	91.46	122/1428
28	*Metabacillus lacus*	AK74	Singh et al. (2018) Gupta et al. (2020)	LT844664	91.41	121/1409
29	*Ornithinibacillus scapharcae*	TW25	Shin et al. (2012)	AEWH0100000 25	91.40	125/1454
30	*Salinibacillus xinjiangensis*	J4	Yang et al. (2014)	JX402080	91.33	126/1454

Note: All authors from National Center of Biotechnology Information, Retrieved 25 September, 2022, from https://blast.ncbi.nlm.nih.gov/Blast.cgi

The results of antimicrobial inhibition to bacteria, *Exiguobacterium indicum*, from Nile tilapia that presented in symptom of disease showed that *E. indicum* has not been found and reported in Thailand. This study first reported an application of vegetable crude extracts to inhibit this bacterium. The members of the genus *Exiguobacterium* is found in diverse aquatic environments from marine to freshwater. They can grow in a wide range temperature, pH, salinity and heavy metal conditions and this species identified to psychrophilic bacterium that reported from Hamta glacier of the Himlayan mountain range of India. (White et al., 2019; Chaturvedi and Shivaji, 2006). This genus confirmed the highest antibiotic producing and isolating as *E. indicum*, that including strain isolated from lake, river and marine water. The species *E. indicum* is closely related to *Bacillus* species (Lisa et al., 2021). From all reasons this bacterial showed great adaptation to grow and survive in wide environment and maybe it can grow rapidly in fish in fresh water. Addition, this *E. indicum* find a lot from grill of Nile tilapia that showed disease is dark, red, and swollen and lose of grill. This research discovered *E. indicum* from disease of Nile tilapia. Addition reason, this bacteria *E. indicum* can grow fast in the psychrophilic condition and found in fish pond because in Thailand had fish culture in open ponds and rivers which has a high temperature, so this species of bacteria can be found in Nile tilapia fish.

The crude extracts from vegetable, consisting of yard-long bean leaf, pumpkin peel and Chinese kale leaf, could be used in preventing disease in Nile tilapia by evaluating the extracts' efficiencies. For inhibition test, Kamolrat & Chopjit (2018) suggested that it be appropriate because it can be used in inhibiting *Streptococcus* spp., which are pathogenic to two types of Nile tilapias: *S. agalactiae* and *S. iniae*. The main key to inhibit pathogenic of bacteria is their chemical substances. However, an effect of phytochemical compound that from different plant and solvent may be presented the different inhibition to same bacterial. The results of antibacterial activity showed that yard-long bean leaf crude extract and Chinese kale leaf crude extract had better ability to inhibit *E. indicum* when compared to Pumpkin peel crude extract.

5. Conclusion

Fish farming in Thailand is widespread and expanding rapidly due to an increasing demand for aquatic animals to boost the economy, while yields from natural water sources are declining. Many commercial fish farms face the problem of disease outbreaks in fish. Bacteria are responsible for causing disease in about 60-70% of aquatic animals. Much research has been conducted on the

application of extracts to inhibit pathogenic bacteria, including both gram- positive and gram- negative strains. In this study, we found that crude extracts from the yard- long bean leaf and Chinese kale leaf could inhibit these pathogenic bacteria. These findings could be applied in fish farms, particularly for the treatment of *E. indicum* infections in Nile tilapia fish.

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