TIK-64 Chemicals Profile of Kelakai Leaves Extracts (Stenochlaena palustris) with Antioxidant and Antibacterial Activity against Aeromonas hydrophila

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Chemicals Profile of *Kelakai* Leaves Extracts (*Stenochlaena palustris*) with Antioxidant and Antibacterial Activity against *Aeromonas hydrophila*

(Profil Kimia bagi Ekstrak Daun Kelakai (*Stenochlaena palustris*) dengan Aktiviti Antibakteria dan Antioksidan terhadap *Aeromonas hydrophila*)

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ABSTRACT

Kelakai (*Stenochlaena palustris*) is a typical Kalimantan plant that has been used by Banjar and Dayak communities as traditional medicine and vegetable. This study aimed to examine the potential of *kelakai* leaves extracts as a natural product to inhibit *Aeromonas hydrophila* growth. This research included *kelakai* leaves sampling in Banjarbaru, South Kalimantan, Indonesia, extraction (EtOH 1:4 w/v), phytochemical and chemicals profile screening (LCMS), prediction of biological activity (PASS server), antibacterial activity (broth dilution), antioxidant activity (DPPH), total phenol (gallic acid equivalent), total flavonoid (quercetin equivalent), and total alkaloid (caffeine equivalent). The phytochemical screening showed that the *kelakai* leaves extract contained saponins, tannins, phenolics, flavonoids, alkaloids, anthraquinones, triterpenoids, and steroids. The chemicals profile of the kelakai leaves ethanol extract consisting of alkaloids, alcohols, amines, amine alcohols, amino acids, fatty acids, flavonoids, glycosylglucose, lipid derivatives, monocarboxylic acid, saponins, steroids, and terpenoids. Prediction of biological activity showed *kelakai* leaves extract an inhibitor of the peptidoglycan glycosyltransferase enzyme and free radical scavenger. The antibacterial assay showed that *kelakai* leaves extract could inhibit the growth of *A. hydrophila*. In addition, *kelakai* leaves extract showed very strong antioxidant potential (IC_{50} 42.47 ± 0.98 µg/mL), with a total phenol content of 193.97 ± 0.11 mg GAE/g, total flavonoid 23.45 ± 0.14 mg QE/g, and total alkaloid 11.74 ± 0.10 mg CE/g. These research findings show that the ethanol extract of *kelakai* leaves could be antibacterial against *A. hydrophila*, which is closely related to its antioxidant properties.

Keywords: Alkaloids; flavonoids; Kalimantan; peptidoglycan; phenols

ABSTRAK

Kelakai (*Stenochlaena palustris*) adalah tanaman khas Kalimantan yang telah digunakan oleh masyarakat Banjar dan Dayak sebagai ubatan tradisi dan sayur-sayuran. Penyelidikan ini bertujuan untuk mengkaji potensi ekstrak daun kelakai sebagai produk semula jadi untuk menghalang pertumbuhan *Aeromonas hydrophila*. Penyelidikan ini merangkumi pengambilan sampel daun kelakai di Banjarbaru, Kalimantan Selatan, Indonesia, pengekstrakan (EtOH 1:4 w/v), penyaringan profil fitokimia dan bahan kimia (LCMS), ramalan aktiviti biologi (pelayan PASS), aktiviti antibakteria (pencairan kaldu), aktiviti antioksidan (DPPH) dan total fenol (bersamaan asid galik), total flavonoid (bersamaan kuersetin) dan total alkaloid (bersamaan dengan kafein). Pemeriksaan fitokimia menunjukkan bahawa ekstrak daun kelakai mengandungi saponin, tanin, fenol, flavonoid, alkaloid, antrakuinon, triterpenoid dan steroid. Profil kimia ekstrak etanol daun kelakai terdiri daripada alkaloid, alkohol, amina, alkohol amina, asid amino, asid lemak, flavonoid, glikosilglukosa, terbitan lipid, asid monokarboksilik, saponin, steroid dan terpenoid. Ramalan aktiviti biologi menunjukkan daun kelakai mengekstrak

penghambat enzim peptidoglikan glikosiltransferase dan pemulung radikal bebas. Asai antibakteria menunjukkan bahawa ekstrak daun kelakai dapat merencat pertumbuhan *A. hydrophila*. Selain itu, ekstrak daun kelakai menunjukkan potensi antioksidan yang sangat kuat (IC_{50} 42.47 ± 0.98 µg/mL), dengan jumlah kandungan fenol 193.97 ± 0.11 mg GAE/g, kandungan flavonoid 23.45 ± 0.14 mg QE/g dan kandungan alkaloid 11.74 ± 0.10 mg CE/g. Penyelidikan ini mendapati bahawa ekstrak etanol daun kelakai dapat menjadi antibakteria terhadap *A. hydrophila*, yang sangat berkaitan dengan sifat antioksidannya.

Kata kunci: Alkaloid; fenol; flavonoid; Kalimantan; peptidoglikan

INTRODUCTION

Aeromonas hydrophila is a bacterium that infects freshwater fish and causes red spot disease, better known as *Motile Aeromonad Septicemia* (MAS) disease. *A. hydrophila* is an opportunistic pathogen, always present in water, and will infect fish when the fish's immune condition is weak. According to Rasmussen-Ivey et al. (2016), *A. hydrophila* is a pathogenic-bacteria with high virulence.

Aeromonas hydrophila was reported infection the Siamese catfish cultivated in South Kalimantan (Aisiah et al. 2020). Based on the Freshwater Aquaculture Center (BPBAT) Mandiangin's annual health and environmental monitoring reports from 2009 to 2019, BPBAT reported that *A. hydrophila* infected Siamese catfish in the aquaculture environment. *A. hydrophila* infection could cause catfish mortality to reach 80 - 95% in about one week.

Generally, farmers use synthetic drugs and antibiotics as a quick and emergency alternative for MAS disease. However, this action has a negative impact because continuous use for too long can affect not only fish but also bacterial resistance, pollute the environment, and harm consumers (Monteiro et al. 2018). One of the efforts that can be made to avoid antibiotics is by using active compounds from natural products, which are widely distributed in Kalimantan, Indonesia. One of the natural products that could be used to treat MAS disease is *kelakai* (*Stenochlaena palustris*). The Banjar and Dayak communities in Kalimantan know well the *kelakai* as a medicinal plant.

Adenan and Suhartono (2010) reported that the use of *kelakai* leaves in South Kalimantan, Indonesia is still lacking. Literature studies showed that *kelakai* leaves extract has the potential to be antibacterial, namely against *Escherichia coli* (Handayani & Rusmita 2017); *Salmonella typhi* and *Staphylococcus aureus* (Rostinawati et al. 2018); and *Ralstonia solanacearum* and *Streptococcus sobrinus* (Egra et al. 2019). However, *kelakai* leaves extract an antibacterial against *A. hydrophila*, which causes MAS disease, has not been studied. Therefore, this study aimed to examine the potential of *kelakai* leaves extract (*S. palustris*) to inhibit the growth of *A. hydrophila*. In addition, the chemicals profile, potential radical scavenging activity and total phenol content of the *kelakai* extract also evaluated in this study.

MATERIALS AND METHODS

The research was conducted from July to December 2020 at the Fish Disease Laboratory, Faculty of Fisheries and Marine, Universitas Lambung Mangkurat, Indonesia. The *kelakai* leaves were collected from Banjarbaru, South Kalimantan, Indonesia. Isolate of *A. hydrophila* was obtained from the Mandiangin Freshwater Aquaculture Center (BPBAT), South Kalimantan, Indonesia.

KELAKAI LEAVES EXTRACTION

The *kelakai* leaves were cleaned, cut into small pieces, and dried in an oven at 50 °C. The dried *kelakai* leaves were crushed into a fine powder. A total of 100 g of fine powder of *kelakai* leaves was macerated in 400 mL of ethanol (Merck) for 24 h. The extract obtained was then evaporated using a rotary vacuum evaporator (IKA RV 10). Then, it was heated for 5-6 h at 50 °C to dry the *kelakai* leaves extract. *Kelakai* leaves extract was stored in the freezer before use.

PHYTOCHEMICAL SCREENING

The ethanol extract of *kelakai* leaves was subjected to qualitative screening of chemical compounds to determine the presence of phytochemicals compound using conventional standard protocols as described by Harborne (Aisiah et al. 2018).

CHEMICALS PROFILE SCREENING

Chemicals profile screening of *kelakai* leaves extracts using Liquid Chromatography Mass Spectrometry (LCMS

Shimadzu-8040, Japan) with an injection volume of 1 µL. LCMS, equipped with an autosampler, binary pump, column compartment, and a diode array detector for scanning spectroscopy. Chromatographic separation was performed using a C-18 column, Shim Pack FC-ODS (2 mm ø × 150 mm, 3 µm). Two solvents prepared included solvent A (H_2O:MeOH, 8:2) with 0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile). The two solvents were adjusted to 95:5 ratios, respectively, with an elution gradient of 0/0 at 0 min, 15/85 at 5 min, 20/80 at 20 min, 90/10 at 24 min. Mass spectroscopy (MS) analysis was performed by Electrospray Ionization (ESI) with positive ions as the source. MS data were obtained through collision energy traps starting at 5.0 V. The ESI source parameters were regulated, including a capillary voltage of 3.0 kV, source temperature of 100 °C, desolvation temperature of 350 °C, sampling cone 23 V, and desolvation gas flow of 6 L/h. The chromatogram data obtained were compared with the data profile from the mzCloud Library system (Riyadi et al. 2020).

ANTIOXIDANT ACTIVITY ASSAY

Antioxidant activity was determined using the DPPH free radical scavenging method (Tanod et al. 2019). A total of 15 mg of ethanol extract from *kelakai* leaves were added with ethanol as much as 150 mL so that the extract concentration was 100 µg/mL. After that, serial dilutions of 6, 12, 24, 48, and 96 µg/mL were made. A 2 mL aliquot of each concentration's extract solution was added to 2 mL of the 50 µM DPPH (Merck) solution. The mixture was homogenized and left for 30 min in a dark room at room temperature. Then, the mixture measured the free radical scavenging at a wavelength of 517 nm with a spectrophotometer (UV-VIS spectrophotometer T90+PG Instruments Ltd). The absorbance value of the DPPH solution is also measured and determined by IC_{50} (Tanod et al. 2019). Vitamin E was used as a control. The assay was carried out in three repetitions, and the measurement results were expressed with a standard deviation. The DPPH scavenging effects was calculated using:

TOTAL PHENOL ASSAY

The ethanol extract of *kelakai* leaves was evaluated for the total phenol content according to the Folin-Ciocalteu method (Blainski et al. 2013). The first stage was making a standard curve for gallic acid, and the second stage was testing using samples, which is as much as 25 mg of gallic acid and *kelakai* leaves ethanol extracts is weighed, then dissolved in ethanol: water (1: 1) to a volume of 25 mL. The gallic acid solution was made in a series of dilution concentrations of 5, 20, 40, 60, 80, and 100 µg/mL. From each dilution concentration, 1 mL of gallic acid was taken, and 10 mL of distilled water was added. Then, 1 mL of Folin-Ciocalteu reagent (homogeneation) was added. After that, let stand for 8 min, then add 3 mL of 20% Na_2CO_3 solution (homogeneation). Then, let stand for 2 h at room temperature. The absorbance value was measured at a wavelength of 750 nm. The standard curve of gallic acid was prepared with the concentration of gallic acid (µg/mL) against the absorbance value. Then, the absorption was measured with a UV-Vis spectrophotometer at a wavelength of 750 nm which gave a blue colour. Total phenolic was determined using the standard curve regression equation for gallic acid.

TOTAL FLAVONOID ASSAY

The total flavonoid was evaluated by the colourimetric method with quercetin (Quercetin equivalent - QE) as the standard referring to the procedure (Ahmad et al. 2015; Aminah et al. 2016). The first stage of making a standard quercetin solution, and the second stage was using samples, which is as much as 10 mg of quercetin and *kelakai* extracts, is added with ethanol (1000 mg/L). Then made a series of solutions of 5, 20, 40, 60, 80, and 100 mg/L. Next, 1 mL of each solution was taken, then 1.5 mL of 95% ethanol was added; 0.1 mL aluminum chloride ($AlCl_3$) 10%; 0.1 mL of 1M potassium acetate; and 2.8 mL of distilled water. After that, it was incubated for 30 min and measured the absorbance value at 433.5 nm using a UV Vis T90+ PG Instruments Ltd spectrophotometer. Total flavonoid was determined using the standard curve regression equation for quercetin.

TOTAL ALKALOID ASSAY

The total alkaloid content was evaluated using a UV-Vis spectrophotometer with caffeine curve calibration (caffeine equivalent - CE) (John et al. 2015). The first stage is making a caffeine standard curve, and the second stage is using the extract, which is 40 mg of caffeine and *kelakai* extracts, and the extract is dissolved in ethanol (100 mg/L). Then, made a series of dilutions of 5, 20, 40, 60, 80, 100 mg/L. Then, from each dilution series, added 2 mL of phosphate buffer pH 4.7 and 2 mL of bromocresol green. After that, 3 mL of chloroform was extracted three times, and chloroform was added up to 10 mL. The absorbance value was measured at a wavelength of 430 nm using a UV Vis T90+ PG Instruments Ltd spectrophotometer. Total alkaloids were determined using the standard curve regression equation for caffeine.

PREDICTION OF BIOLOGICAL ACTIVITY

The chemicals profile detected from the ethanol extract of *kelakai* leaves with LCMS were predicted for biological activity using the PASS server http://www.pharmaexpert.ru/passonline/index.ph. PASS server is software that is useful for predicting the biological activity of a compound (Aisiah et al. 2020). The PASS server can predict the biological activity of compounds based on the formula with an accuracy of 95% (Filimonov et al. 2014). The predicted biological activity requires a structural formula in the form of canonical SMILE obtained from the National Center for Biotechnology Information https://pubchem.ncbi.nlm.nih.gov/. The prediction of biological activity with PASS Server was expressed by the probability of being active (Pa). Pa describes the correlation between the structure of a compound and its biological activity. If Pa>0.7 indicates that the compound was predicted to have a high potential for biological activity, both in computational and laboratory assays. Suppose the value is 0.3<Pa<0.7, it indicates that the compound was predicted to have computational biological activity but has not been proven in laboratory assays. Meanwhile, if the value of Pa <0.3, it indicates the compound has no potential.

ANTIBACTERIAL ACTIVITY ASSAY

Antibacterial activity was evaluated using the broth dilution method based on the guidelines (Wiegand et al. 2008) with modifications. A total of 10 mL of tryptic soy broth (TSB, Merck) was inoculated with 100 µL of *A. hydrophila* (density 1×10^7 colony/mL), then incubated at 37 °C for 24 h. After that, 100 µL of the *kelakai* leaves ethanol extract (200 mg/mL) was added. *Aeromonas hydrophila* culture with aquadest was used as the negative control, and cefadroxil and tetracycline 1 mg/mL as positive controls. The total colony count was carried out on glutamate starch phenol-agar (GSP agar), based on total plate count methods following the Indonesian National Standard No. 01-2332.3 of 2006 with modifications (Indonesian National Standardization Agency–BSN 2006). Modifications made using TSB on broth media and solid media using GSP selective media. If the GSP is red, it indicates *A. hydrophila* was not growing, whereas if the GSP was yellow, it indicates *A. hydrophila* growth. All experimental measurement data were carried out in three replications and expressed as Mean ± Standard Deviation (n=3).

RESULTS AND DISCUSSION

Stenochlaena palustris (local name *kelakai*) is a shrub that grows around the Dayak and Banjar communities', Kalimantan, Indonesia. *Kelakai* is widely distributed throughout almost all Indonesia regions, such as Sumatera, Kalimantan, Java, Sulawesi, and Papua. Kalimantan is one of the largest islands in Indonesia, which has high biodiversity. One of the plants commonly consumed as food by the Dayak and Banjar people in South Kalimantan was *kelakai*. *Kelakai* was used by the Dayak and Banjar people as a vegetable and has been used from generation to generation as traditional medicine, where the Dayak community is believed to treat anemia and is used to increase postpartum energy (Negara et al. 2017). The distribution of *kelakai* (*S. palustris*) in Indonesia is presented in Figure 1.

FIGURE 1. Distribution of *Stenochlaena palustris* in Indonesia
(Source: https://www.gbif.org/species/4079023)

Phytochemical was carried out to determine the type of bioactive content in the ethanol extract of *kelakai* leaves. The phytochemical screening of the ethanol extract of *kelakai* leaves (*S. palustris*) was presented in Table 1.

TABLE 1. Pytochemical screening of *kelakai* leaves ethanol extract (*S. palustris*)

Phytochemicals	Methods		Standards
Flavonoids	Alkaline Reagent	+	The greenish yellow colour (sample + NaOH) faded after dilute acid was added
	Lead Acetate	+	Yellowish brown precipitate formed
Alkaloids	Dragendorff's		Red colour produced
Steroids	Libermann Burchard's	+	A brown ring formed
Terpenoids	Salkowski's	+	Formed golden yellow colour

(+) : present; (-) : Absent

Table 1 showed the *kelakai* leaves extract contained saponins, tannins, phenolics, flavonoids, alkaloids, anthraquinones, triterpenoids, and steroids. Previous research has reported *kelakai* also contains vitamin C, protein, beta carotene, folic acid (Chotimah et al. 2013). *Kelakai* extracts can be anti-inflammatory, antimalarial, anti-glucoside, and antioxidant. Active products in *kelakai* such as phenolic, tannins, and β-carotene compounds can reduce free radicals (Chai et al. 2015). *Kelakai* extracts are also reported to have the potential to inhibit the production of TNF-α (Margono et al. 2016). Suhartono and Bahriansyah (2016) reported that the aquadest extract of *kelakai* leaves could inhibit cadmium glycation reactions and *in vitro* fructation reactions in the body. *Kelakai* bioactivity research also showed moderate antibacterial properties against *S. aureus* and *E. coli* (Erwin et al. 2016).

Each class of compounds has a different ability to inhibit bacterial growth. The difference in an activity that occurs is due to the active ingredients having different synergistic effects depending on the bacteria's type and morphology. Phenolic compounds are classified into four main groups: flavonoids, phenolic acids, lignans, and polymer tannins (Nohynek et al. 2006). Górniak (2019) reported that flavonoids inhibit bacterial growth by damaging cell walls, deactivating enzymes, binding to cell adhesion, and damaging cell membranes. The instability of the bacterial cell wall and cytoplasmic membrane causes selective permeability, active transport function, and control of the bacterial cell's protein structure to be disturbed. This disturbance results in the escape of macromolecules and ions from the cell so that the bacterial cell loses its shape, and lysis occurs. Flavonoids have reported to antioxidant effects, so it can inhibit lipid peroxidation (Gündoğan & Özdemir 2021). Phenols have a mechanism of action in inhibiting bacterial growth by inactivating proteins (enzymes) in the cell membrane. Phenol binds to proteins through hydrogen bonds resulting in the protein structure being damaged where most of the cell wall structure and cytoplasmic membrane of bacteria contain protein and fat (Susanti et al. 2008). Tannin can work like a siderophore to chelate iron from the medium and make iron unavailable to microorganisms. Tannins that target cell wall polypeptides cause damage to cell walls because tannins are phenolic compounds. Phospholipid

disorders are unable to maintain the cell membrane's shape so that the membrane will leak, and bacteria will experience growth retardation and even death. According to Ogbuagu (2008), tannins have antimicrobial activity and are responsible for preventing and treating urinary tract infections and other bacterial infections.

Terpenoids have antibacterial activity by damaging the bacterial cell membrane, and these compounds will react with the active side of the membrane, dissolve lipid constituents and increase their permeability (Górniak 2019). An important factor that plays a role in the antibacterial activity of terpenoids is their chemical composition, including functional groups and hydroxyl groups of phenolic terpenoids and the number of single components. Previous *in vitro* assays showed that terpenoids used as single compounds were less effective as antibacterial agents. In addition, the antibacterial activity of terpenoids also depends on the number of compounds produced. At low concentrations, terpenoids only affect enzymes involved in energy production, while at high concentrations, they can lyse membranes (Daisy et al. 2008).

Saponins are complex glycoside compounds, namely compounds resulting from the condensation of sugar with an organic hydroxyl compound that will produce sugar (glycons) and non-sugars (aglycones) and foam. These saponins fall into two groups: triterpenoids and saponins. Saponins are fat-soluble and water-soluble. These compounds will be concentrated in the cell membrane, which is a delicate and important part. Saponins are strong surface tension-reducing compounds. Saponins act as antimicrobials by disrupting the bacterial cell membrane's stability, causing the bacterial cell to undergo lysis. Saponins are also known to be naturally antibacterial and energy booster, but they are also reported to be useful in reducing inflammation in the upper respiratory system (Ogbuagu 2008).

The chemicals profile screening of *kelakai* leaves extract using LCMS detected 81 peaks (66 compounds), with a retention time range of 0.873 - 27.498 min. However, only 53 peaks (44 compounds) were above 85% quality. The screening of the ethanol extracts profile of the *kelakai* leaves with LCMS can be seen in Table 2.

TABLE 2. Chemicals profile of the *kelakai* leaves ethanol extract (*S. palustris*) with LCMS

RT (min)	Chemicals profile	Formula	Molecular weight (g/mol)	Area (%)	Quality	Group	Bioactivity potential	References
0.873	Trifolin	$C_{21}H_{20}O_{11}$	448.10	0.29	96.2	Flavonoids	Antifungal, Radical Scavengers, Induces Apoptosis	Kim et al. (2016), Li et al. (2005), Sientzoff et al. (2015)
8.073					97.7			
0.921	4-(4-hydroxy-2,6,6-trimethyl-3-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] oxy}cyclohex-1-en-1-yl) butan-2-one	$C_{19}H_{32}O_8$	410.19	0.55	98.1	Terpenoids	–	–
6.462					98.7			
0.928	5-amino-1-(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-1H-pyrazole-4-carbonitrile	$C_{15}H_{10}N_8$	302.10	0.15	90.2	Alkaloids	Antimicrobial	Sureja et al. (2016)
0.936	Isoamylamine	$C_5H_{13}N$	87.11	3.50	94.9	Amines	Anti-inflammatory	Yen et al. (2017)
0.940	Decaethylene glycol	$C_{20}H_{42}O_{11}$	475.30	0.12	90.4	Alcohols	Antibacterial	Moghayedi et al. (2017)
0.944	Maltobiose	$C_{12}H_{22}O_{11}$	342.30	1.89	88.5	Glyco-sylglucoses	Antigenic	Otvos et al. (1994)

0.952	Flurandrenolide	$C_{24}H_{33}FO_6$	436.23	0.58	93.5	Steroids	Anti-inflammatory	de Oliveira Bezerra et al. (2019)
0.992 1.217	Betaine	$C_5H_{11}NO_2$	117.08	7.28	98.1 98.0	Amino acids	Antimicrobial, Cytotoxic and Antioxidant	Radošević et al. (2018), Angelini et al. (2019)
26.395 1.001 1.207	Choline	$C_5H_{13}NO$	104.17	26.97	87.6 97.5 97.8	Amino acids	Antioxidant, Antibacterial	Wu et al. (2014), Zhao et al. (2016), Wu et al. (2017)
1.001	Pandaroside B	$C_{35}H_{54}O_{10}$	672.33	0.13	96.8	Saponnins	Antileishmanial	França et al. (2017)
1.004 1.314	2-Mercaptoethanol	C_2H_6OS	78.01	23.44	99.7 99.7	Alcohols	Reduce plasma lipid peroxidation, reduce inflammation, Antioxidant	Siu Wonga et al. (2014), Nikseresht et al. (2017)
1.005	Roseoside	$C_{19}H_{30}O_8$	408.18	0.65	98.7	Terpenoids	Antioxidant, Antitumor	Mahmoud et al. (2009)
1.018	N-({(2R,4S,5R)-5-[3-(2-Methoxyphenyl)-1-methyl-1H-pyrazol-5-yl]-1-azabicyclo[2.2.2]oct-2-yl} methyl)methanesulfonamide	$C_{20}H_{28}N_4O_3S$	444.18	0.21	98.0	Alkaloids	–	–
5.179	Chlorogenic acid	$C_{16}H_{18}O_9$	354.09	0.18	99.4	Flavonoids	Antioxidant, Antibacterial	Naveed et al. (2018)
5.770	(-)-Epicatechin	$C_{15}H_{14}O_6$	290.08	0.04	99.0	Flavonoids	Antioxidant, Antimicrobial, Anti-inflammatory	Prakash et al. (2019)
7.812	Nictoflorin	$C_{27}H_{30}O_{15}$	594.16	0.11	98.6	Flavonoids	Hepatoprotective, Anti-inflammatory	Zhao et al. (2017), Yu (2021)
8.069	Kaempferol	$C_{15}H_{10}O_6$	286.05	0.11	99.3	Flavonoids	Antitumor, Antioxidant and Anti-inflammatory	Wang et al. (2018)
10.166	Eucalyptol	$C_{10}H_{18}O$	136.12	0.16	96.1	Terpenoids	Antibacterial, Antioxidant	Zengin & Baysal (2014)
14.309	2-(Methylthio)benzothiazole	$C_8H_7NS_2$	181.00	0.07	95.1	Alkaloids	Antimicrobial	Prasad et al. (2012)
14.885	Palmitic Acid	$C_{16}H_{32}O_2$	256.42	0.18	85.3	Fatty Acids	Antibacterial, Anti-Diarrhoeal, Analgesic, Cytotoxic	Hossain et al. (2017)
15.992	12-oxo Phytodienoic Acid	$C_{18}H_{28}O_3$	292.20	0.15	96.6	Fatty Acids	Antimicrobial	Boeira et al. (2020)

16.394	Cafestol	$C_{20}H_{28}O_3$	316.40	0.09	88.1	Terpenoids	Anti-inflammatory	Muhammad et al. (2008)
17.313	α-Eleostearic acid	$C_{18}H_{30}O_2$	278.22	0.20	94.5	Fatty Acids	Antioxidant	Anjum et al. (2013)
17.525	acridine-9(10H)-thione	$C_{13}H_9NS$	211.05	0.19	97.1	Alkaloids	Antimicrobial	Parikh et al. (2011)
17.783	(±)13-HpODE	$C_{18}H_{32}O_4$	312.40	0.19	94.3	Fatty Acids	Antioxidant	Faizo et al. (2021)
17.984	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	294.22	0.68	94.8	Fatty Acids	Antioxidant, Antimicrobial	Abdel-Wareth et al. (2019), Kim et al. (2020)
18.172	Palmitoleic acid	$C_{16}H_{30}O_2$	254.22	0.26	96.5	Fatty Acids	Antioxidant, Antibacterial	Huang et al. (2010), Karimi et al. (2015)
18.334	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	0.42	88.6	Fatty Acids	Antifungal, Antibacterial, Antioxidant	Shobi et al. (2018), Khan & Javaid (2019)
19.323	Methylpentanediol dineopentanoate	$C_{16}H_{30}O_4$	286.41	0.29	97.4	Fatty Acids	–	–
19.894 20.245	octadec-9-ynoic acid	$C_{18}H_{32}O_2$	262.23	0.42	96.6 96.9	Fatty Acids	Antibacterial, Antioxidant	(Thippeswamy et al. 2015)
19.912 20.279	1-Linoleoyl glycerol	$C_{21}H_{38}O_4$	354.28	0.74	96.3 97.1	Lipid Derivatives	Antioxidant	Shuai et al. (2021)
19.924	2-Arachidonoyl glycerol	$C_{23}H_{38}O_4$	378.50	0.41	88.0	Fatty Acids	Antimicrobial	Adnan et al. (2021)
20.431	α-Linolenic acid	$C_{18}H_{30}O_2$	278.22	0.43	97.9	Fatty Acids	Antioxidant, Antimicrobial	Gıdık (2021)
22.180	Oleamide	$C_{18}H_{35}NO$	281.27	5.38	98.9	Fatty Acids	Antimicrobial, Anticancer	Adnan et al. (2021)
22.860	Hexadecanamide	$C_{16}H_{33}NO$	255.26	1.29	98.2	Fatty Acids	–	–
23.342	Bis(2-ethylhexyl) adipate	$C_{22}H_{42}O_4$	370.31	0.06	91.5	Lipid Derivatives	–	–
23.355 27.492	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.28	0.36	99.4 99.4	Lipid Derivatives	Antimicrobial, Cytotoxic	Lotfy et al. (2018)
24.876	Stearamide	$C_{18}H_{37}NO$	283.29	0.58	97.9	Fatty Acids	Cytotoxic	Ifandari et al. (2020)
26.409	Triethanolamine	$C_6H_{15}NO_3$	149.10	1.32	96.3	Amine Alcohols	Antibacterial, Antioxidant	Gyawali et al. (2016)
26.420	Urocanic Acid	$C_6H_6N_2O_2$	138.12	0.40	87.6	Mono-carboxylic acid	Antioxidant, Anticancer	Adnan et al. (2021)
27.491	2-[(2-chlorobenzyl)sulfanyl]-4,6-dimethylnicotinonitrile	$C_{15}H_{13}ClN_2S$	326.00	0.96	97.6	Alkaloids	–	–
27.495	Bis(7-methyloctyl) adipate	$C_{24}H_{46}O_4$	398.34	0.29	96.4	Lipid Derivatives	–	–
27.497	Bis(3,5,5-trimethylhexyl) phthalate	$C_{26}H_{42}O_4$	418.31	1.49	98.4	Lipid Derivatives	Antiviral	Tumilaar et al. (2021)
27.498	N-Butylbenzenesulfonamide	$C_{10}H_{15}NO_2S$	213.30	0.21	88.1	Alkaloids	Antimicrobial, Antioxidant	Angelini et al. (2019)

Table 2 showed the chemicals profile of the *kelakai* leaves ethanol extract consisting of alkaloids (2.19%), alcohols (23.56%), amines (3.50%), amine alcohols (1.32%), amino acids (34.25%), fatty acids (10.88%), flavonoids (0.74%), glycosylglucose (1.89%), lipid derivatives (2.94%), monocarboxylic acid (0.40%), saponins (0.13%), steroids (0.58%), and terpenoids (1.44%). Based on the literature, Table 2 also showed 29 compounds that have potential as antibacterial and antioxidant (71.4%). All the compounds contained in the extracts contribute to the biological activity of the extracts. The compounds in Table 2 can act alone or interact to provide antioxidant and antibacterial properties. According to Merzenich et al. (2010), compounds from natural ingredients work synergistically between compounds. Compounds from natural ingredients can work through multi-targets and multi-compounds synergistic modes (Long et al. 2015). This result indicates that the *kelakai* leaves ethanolic extract has strong potential as an antibacterial and antioxidant.

This study also evaluated the antioxidant activity of the *kelakai* leaves extract using the DPPH radical scavenging method. The percentage of DPPH radical scavenging for *kelakai* leaves extract and vitamin E as a control can be seen in Figure 2. In addition, this study evaluated IC_{50} value and the total phenol, flavonoid and alkaloid content of the *kelakai* leaves ethanol extract (*S. palustris*) (Table 3).

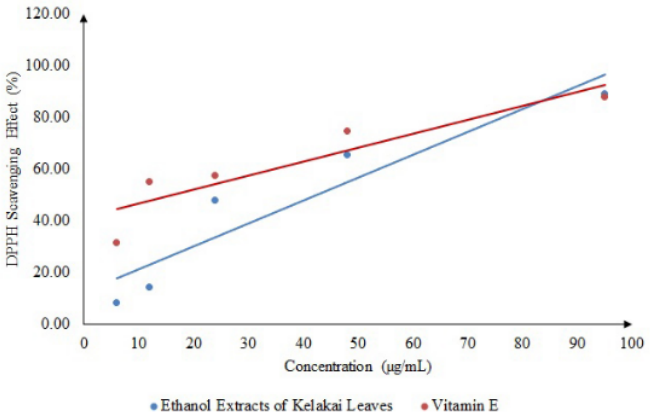


FIGURE 2. DPPH scavenging effect of *kelakai* leaves extract compared with vitamin E

Figure 2 showed the increase in *kelakai* leaves extract concentration could increase the inhibition of DPPH radical. The antioxidant activity shows a substance's ability to inhibit oxidation reactions which are expressed as a percentage of inhibition (Dewanto et al. 2019). This is presumably because the ethanol extract of kelakai leaves can donate H atoms to scavenge DPPH radicals. *Kelakai* leaves ethanol extract was also evaluated for IC_{50} determination. The ethanol extract of *kelakai* leaves showed very strong potential as an antioxidant because it can donate hydrogen atoms/electrons to react with DPPH radicals. There are four categories of antioxidant activity: very strong (IC_{50}<50 µg/mL), strong (IC_{50} between 50-100 µg/mL), moderate (IC_{50} between 100-150 µg/mL) and weak (IC_{50} between 150-200 µg/mL) (Blois 1958).

TABLE 3. IC_{50} total phenol, flavonoid, and alkaloid of the *kelakai* leaves ethanol extract

Evaluation	Value	Regression
IC_{50} of *kelakai* leaves	42.47 ± 0.98 µg/mL	$y = 0.5404x + 41.273$, $R^2 = 0.8308$
IC_{50} of Vitamin E	16.15 ± 0.80 µg/mL	$y = 0.8868x + 12.176$, $R^2 = 0.8873$
Total phenol	193.97 ± 0.11 mg GAE/g	$y = 0.01x - 0.032$, $R^2 = 0.9987$
Total flavonoid	23.45 ± 0.14 mg QE/g	$y = 0.0079x - 0.0099$, $R^2 = 0.9964$
Total alkaloid	11.74 ± 0.10 mg CE/g	$y = 0.0095x - 0.0365$, $R^2 = 0.9998$

This study is supported by previous studies which reported the antioxidant properties of the *kelakai* leaves (*S. palustris*). Chai et al. (2012) reported that the EC_{50} value of *kelakai* leaves extracts ranged from 72.51 ± 0.69 to 180.29 ± 6.92 µg dry matter/mL (depends on young and mature leaves). Kusmardiyani et al. (2016) reported a very strong antioxidant for leaves extracts (old and young) and *kelakai* roots with IC_{50} values varying between 0.8 and 14.13 µg/mL. Rahmawati et al. (2017) also reported the antioxidant activity of the extract of *kelakai* (*S. palustris*) with an IC_{50} value of 4.20 ± 0.01 µg/mL. The ethanol extracts of *S. palustris* leaves had an IC_{50} DPPH value of 24.24 ± 0.174 µg/mL (Ndanusa et al. 2020).

The measurement of total phenol, flavonoid and alkaloid of *kelakai* leaves extract was supported by the screening of chemicals profile. The 44 compounds (quality above 85%) detected by LCMS, there were 14 compounds with phenolic structures and have hydroxyl bond (–OH), 5 compounds are flavonoid derivatives, and 6 compounds are alkaloid derivatives. The literature studies also showed the total phenol content of *kelakai* leaves extract. Chai et al. (2012) reported that the total phenol content of *kelakai* leaves extract ranged from 18.78 ± 0.51 to 51.69 ± 1.28 mg/GAE g. Kusmardiyani et al. (2016) reported that the total phenol content of *kelakai* extracts (young leaves, old leaves, and *kelakai* roots) varied between 1.87 and 24.22 g GAE/100 g. Fahruni et al. (2018) also reported the total phenol content of *kelakai* extracts collected from Central Kalimantan, Indonesia 4.85 ± 0.07 mg GAE/g, whereas Ndanusa et al. (2020) reported the total phenol content of *kelakai* extracts collected from Kuala Belait district of Brunei Darussalam 3.80 ± 0.22 mg GAE/g.

The literature studies also showed the total flavonoid of *kelakai* leaves extract. The *kelakai* leaves extract collected from Gambut subdistrict, South Kalimantan, was reported to have a total flavonoid content of 14.5 ± 0.7 µg/mL (Suhartono et al. 2012). Margono et al. (2015) reported 14.5 µg/mL of total flavonoids in the aqueous extract of the kelakai leaves collected from Sungai Tabuk District, South Kalimantan. The flavonoid content of the *kelakai* leaves ethanolic extract was also reported to be 2.2159 ± 0.083% (Syamsul et al. 2019). Until now, no literature has been found that reports the total alkaloid content of the *kelakai* leaves extract, even though from screening using LCMS, it is known that the ethanolic extract of *kelakai* leaves also contains alkaloids derivatives.

Furthermore, the compounds in Table 2 predicted the biological activity using PASS Server. The predicted value was expressed as a probability to be active (Pa). Prediction of activity was carried out related to antibacterial and antioxidant, namely as an inhibitor of cell wall biosynthesis, peptidoglycan membrane inhibitor, protein synthesis inhibitor, nucleic acid synthesis inhibitor, and as a free radical scavenger. The prediction of the biological activity of the ethanolic extract of the leaves of *kelakai* (*S. palustris*) is presented in Figure 3.

The prediction of the biological activity of the *kelakai* leaves extract is adjusted to the mechanism of the antibacterial agent, namely disrupting microbial cell metabolism, inhibiting microbial cell wall synthesis, damage the integrity of microbial cell membranes, inhibiting microbial cell protein synthesis, and inhibiting the synthesis or damage of microbial cell nucleic acids (Madigan et al. 2019). In addition, *kelakai* extracts were also predicted as scavenge free radicals.

Figure 3 showed the potential of *kelakai* leaves ethanol extract as an antibacterial against the growth of *A. hydrophila*, closely related to its mechanism, which is thought to be an inhibitor of the peptidoglycan glycosyltransferase enzyme and free radical scavenger. Peptidoglycan glycosyltransferase is an enzyme that plays a role in peptidoglycan biosynthesis in the formation of bacterial cell walls (Derouaux et al. 2013). By inhibiting the peptidoglycan glycosyltransferase enzyme action, bacteria cannot synthesize peptidoglycan, so bacteria cannot maintain their shape and protect themselves from osmotic pressure. Prediction of the biological activity of the *kelakai* leaves extract as an inhibitor of the peptidoglycan glycosyltransferase enzyme and free radical scavenger was supported by the results of phytochemical screening and chemicals profile with LCMS. The flavonoid and phenol compounds in the extract are thought to deactivate and inhibit the peptidoglycan glycosyltransferase enzyme's performance. In addition, the flavonoids, phenols, and alkaloids derivatives in Table 2, which have a hydroxyl group (–OH), are thought to act as a free radical scavenger. This study observed the antibacterial power of the ethanol extract of *kelakai* leaves in inhibiting the growth of *A. hydrophila* using the broth dilution method. The results show that *kelakai* leaves ethanol extract (200 mg/mL) could suppress the amount of *A. hydrophila* on GSP-agar (Table 4).

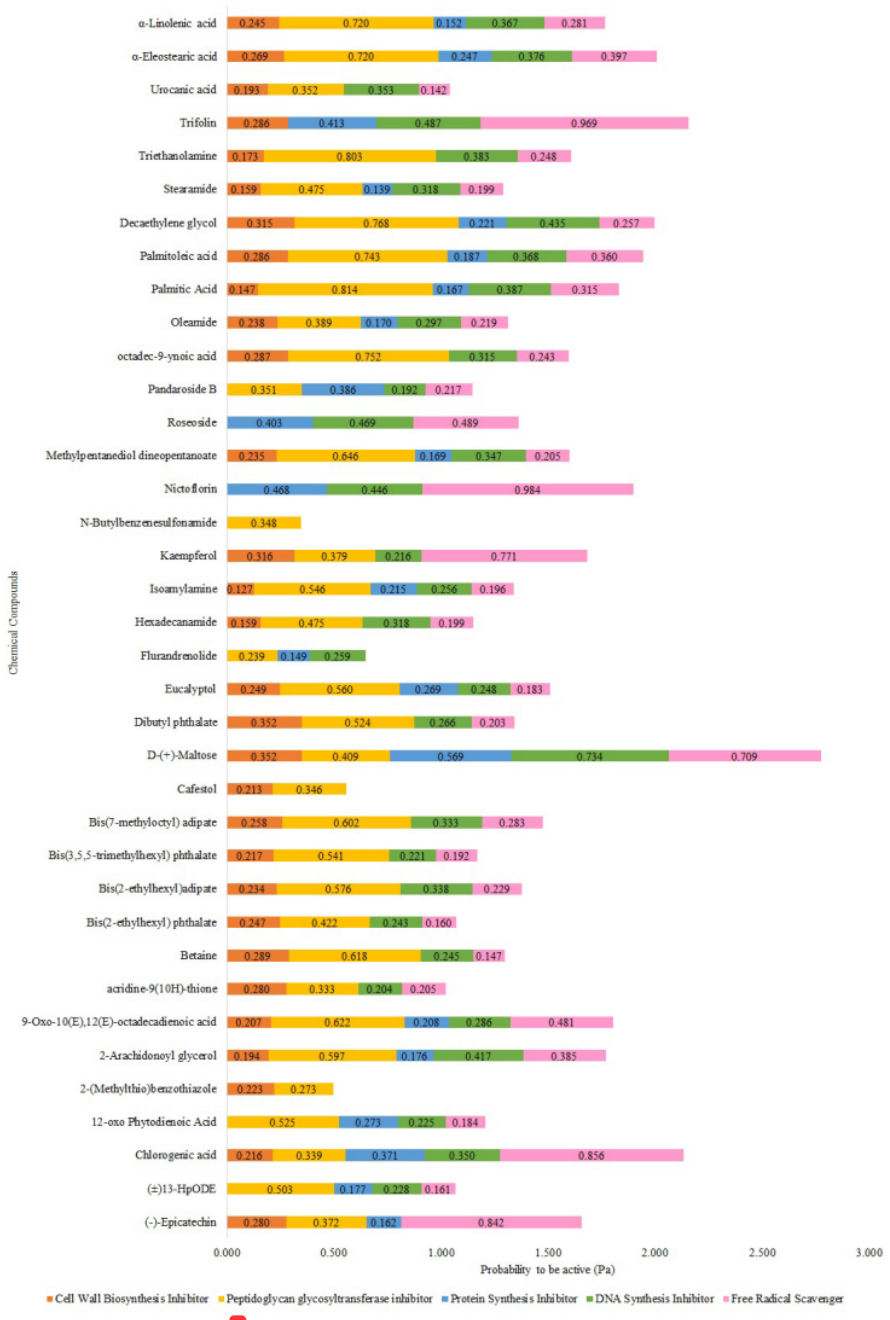
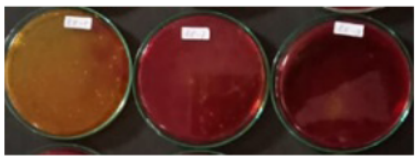
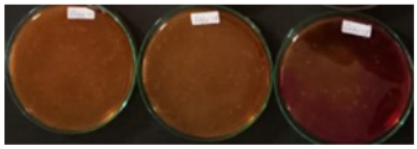
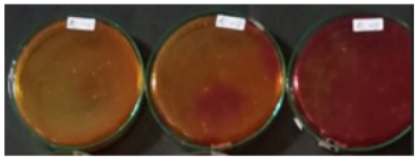


FIGURE 3. Prediction of the biological activity of the *kelakai* leaves extract

TABLE 4. Antibacterial activity of *kelakai* leaves against *A. hydrophila* after 24 h

Sample	Antibacterial activity	
	Broth dilution method (Colony/mL)	Selective media (GSP agar)
Kelakai leaves extract 200 mg/mL	$1.61\times10^{9} \pm 3.53\times10^{7}$	
Cefadroxil 1 mg/mL	$2.46\times10^{10} \pm 9.46\times10^{7}$	
Tetracycline 1 mg/mL	NG	
Aquadest	NC	

GSP is red = *A. hydrophila*, not growth; GSP is yellow = *A. hydrophila* growth; NG = Not growth; NC = too many to count

Antibacterial assay with broth dilution method using GSP media. Table 4 showed *kelakai* leaves extract can change the red colour of the GSP-agar medium to yellow. The colour change is because *A. hydrophila* grows and degrades the starch in GSP-agar by producing acid, causing the phenol red to turn yellow (Naviner et al. 2006).

Kelakai leaves extract contains phenolic compounds, flavonoids, tannins, and saponins, which are hydrophilic. *Aeromonas hydrophila* has a hydrophilic side, namely carboxyl, amino acid, and hydroxyl, sensitive to polar antibacterial compounds (Madigan et al. 2019). The difference in sensitivity relates to the cell wall structure, such as peptidoglycan thickness (presence of receptors, pores, and lipids), nature of cross-linking, and autolytic enzyme activity. The hydrophilic side is a factor that determines the penetration, binding, and activity of antibacterial compounds (Tanod et al. 2018). Overall, the results shows that the ethanolic extract of the *kelakai* leaves from Banjarbaru, South Kalimantan has the potential as a natural alternative to treat MAS in South Kalimantan.

CONCLUSION

These research findings show that the ethanol extract of *kelakai* leaves could be antibacterial against *A. hydrophila*, which is closely related to its antioxidant properties. *Kelakai* leaves extracts to contain flavonoids and phenols thought to act as inhibitors of the peptidoglycan glycosyltransferase enzyme and free radical scavenger. However, *kelakai* leaves extract could be a candidate for natural products to control Motile Aeromonad Septicemia (MAS) disease in freshwater fish aquaculture.

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