PINEAPPLE LEAF JUICE CHARACTERIZATION AND KINETIC STUDY ON MICROBIAL GROWTH INHIBITION

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Abstract

Microbial growth inhibitor (MGI) is crucial in preventing the spreaders of infection. Pineapple (Ananas comosus) leaf juice (PLJ) is chosen as an alternative MGI agent due to its phenolic compounds content. Phenolic compounds in PLJ were quantified by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) analysis. Phenolic compounds identified were confirmed based on their molecular mass and fragmentation pattern. From PLJ characterization, seven phenolic compounds were identified, namely meliadanoside A, feralolide, kukoamine A, methyl-5-O-caffeoylquininate, stilbostemin D, octahydrocurcumin and agrimol C. As the time increased from 0 to 60 min, the phenolic compounds concentration-absorbance (Au) value decreased, indicating a decrease in the phenolic concentration. This is due to the inhibition of microbes by phenolic compounds in PLJ. The inhibition data obtained in microbial inhibition assay were plotted according to the Hill equation, where the kinetic constants (Vmax, K0.5, and n) were estimated. Meliadanoside A has the highest K0.5 value followed by feralolide, kukoamine A, methyl-5-O-caffeoylquininate, and stilbostemin D. Meliadanoside A displayed a sigmoidal behaviour with a Hill coefficient (n) greater than 1. Feralolide, kukoamine A, methyl-5-O-caffeoylquininate, and stilbostemin D corresponded to negative cooperativity with n values lower than 1. This study demonstrated that PLJ could be exploited as a natural plant source that acts as an effective microbial growth inhibitor. Thus, it becomes one of the greener alternatives MGI compared to other synthetic agents.

Keywords: Pineapple leaf juice, phenolic compounds, microbial growth inhibition, UPLC-QTOF-MS, kinetic study

Abstrak

Perencat pertumbuhan mikrob sangat penting dalam mencegah penyebaran jangkitan. Jus daun nanas (Ananas comosus) dipilih sebagai agen alternatif perencat pertumbuhan mikrob, kerana kandungan sebatian fenoliknya. Sebatian fenolik dalam jus daun nanas ditentukan dengan analisis kromatografi cecair berprestasi tinggi dan spektrometri massa masa penerbangan empat kali ganda (UPLC-QTOF-MS). Sebatian fenolik yang dikenal pasti disahkan berdasarkan jisim molekul dan corak pemecahan. Dari pencirian jus daun nanas, terdapat tujuh sebatian fenolik yang telah dikenal pasti, iaitu meliadanoside A, feralolide, kukoamine A, methyl-5-O-caffeoylquininate, stilbostemin D, octahydrocurcumin dan agrimol C. Seiring meningkatnya waktu dari 0 hingga 60 min, kepekatan fenolik-penyerapan (Nilai Au) menurun, menunjukkan penurunan kepekatan fenolik. Ini disebabkan oleh perencatan mikrob oleh sebatian fenolik dalam jus daun nanas. Data perencatan yang...
Microbial growth inhibitor (MGI) is an agent that inhibits and prevents the growth of cells. A wide range of chemical agents are commercially available which have been used to combat the existing infection. However, they may pose a high risk to humans, plants and the environment. Finding a new alternative MGI agent from natural plant sources will be favorable. Pineapple (Ananas comosus) leaf is one of the potential natural products that contain MGI properties due to its high phenolic compounds and it is higher than other fruits [1]. These phenolic compounds are vital to supply a protective component against disease and harm. Therefore, utilization of pineapple leaf juice (PLJ) as a natural MGI will directly benefit the communities since they are one of the accessible waste materials in Malaysia and has not yet been utilized [2].

It is therefore important to identify and characterize individual phenolic compounds in PLJ which can act as an effective MGI. A research done by Vaquero et al. [3] found seven phenolic compounds from pineapple leaf, namely β-sitosterol, ananasate, caffeic acid, 1-O-cafeoylglycerol, p-coumaric acid, 1-O-p-coumaroylglycerol, and daucosterol. High-performance liquid chromatography (HPLC) has been the most commonly used separation technique to measure compounds. According to Steingass et al. [4], HPLC detected more than 60 phenolic compounds in methanol extracts from various tissues of pineapple infructescence. However, it has the disadvantage of having a low resolution, which leads to inaccuracy [5]. Therefore, in this study, ultra-high-performance liquid chromatography-quadrupole time of flight-mass spectroscopy (UPLC-QTOF-MS) analysis was used to discover phenolic compounds [6]. UPLC analysis has been utilized in separation and identification of plant chromatographic and resulted in better efficiency with speedy analysis [7]. In order to understand the inhibition process, kinetic study was performed to determine the reaction rate model. The aim was to obtain the experimental reaction rate and the product formation rate with the best fit. Time-inhibit kinetic assay is required to examine the inhibition of microbe growth [8]. However, there are currently various studies involving time-kill kinetic compared to time-inhibit kinetic studies. A research by Boakye et al. [9] on time-kill kinetic illustrated the microbiostatic agents of Phyllanthus muellerianus (PLE) and geraniin extract. The time-kill kinetics of PLE against E. coli and P. aeruginosa show a reduction in the cell number over the first 3 h, while 4 h for geraniin against C. albicans. A similar study by Appiah et al. [10] supports the claim, where the time-kill kinetic study showed that methanol extracts of T. gibbosa, T. elegans, V. volvacea and S. commune displayed bacteriostatic activities. The present study involved the kinetic of microbial inhibition activities using the Hill equation. This research focuses on PLJ characterization and time-inhibit kinetic study on microbial growth inhibition by using PLJ.

2.0 METHODOLOGY

2.1 Chemicals and Reagents

Potato dextrose agar (PDA) powder (<1 mm; 99%), gallic acid (66.66 μm; 99%), methanol (99.8%), Folin-Ciocalteu reagent (99%) and sodium carbonate (Na2CO3; 99%) were purchased from Sigma Aldrich Co. (M) Sdn Bhd, Selangor, Malaysia. All reagents and chemicals used were analytical grade with high purity.

2.2 Material and PLJ Extraction

Pineapple leaf and infected pineapple leaf which contain microbe were collected from a pineapple plantation in Pekan Pina, Pahang. Pineapple leaves were collected after harvested, takes up to 12 months to harvest. After collected, the leaves were clean first and stored in chiller at 4–7 °C before
extract. The same variant of leaf was used as it was collected in one batch. The pineapple leaf juice (PLJ) with an average length of 60 cm was extracted using an electrical sugarcane press machine (Himitzu, Malaysia), prior to autoclave for 15 min at 121 °C. Approximately 60 mL of PLJ was collected from 0.1 kg of pineapple leaf.

### 2.3 Total Phenolic Content (TPC) Analysis

A Folin-Ciocalteu assay was used to evaluate the total phenolic content (TPC) with Gallic acid as standard. Initially, 10 mL of PLJ was centrifuged for 15 min at 5000 rpm. 2.5 mL of tenfold-diluted Folin-Ciocalteu reagent was mixed with 0.5 mL of the supernatant. The prepared mixture was stored at normal temperature for 5 min. Subsequently, an additional 2 mL of Na$_2$CO$_3$ (7.5%) was added to the mixture before it was allowed to rest for another 1 h. Finally, a spectrophotometer was used to measure the absorbance at a wavelength of 450 nm. Gallic acid with a concentration range of 0.1–1.0 mg/mL was prepared in an 80% methanol solution for use as a standard curve. Similar treatment was also applied to the solution by mixing it with Folin-Ciocalteu reagent and 7.5% Na$_2$CO$_3$. TPC was presented as mg of gallic acid equivalent per gram of PLJ extract (mg GAE/mL) [11].

### 2.4 UPLC-QTOF-MS Analysis of the Extract

UPLC-QTOF-MS was applied to obtain chromatographic profiles for the phenolic compounds present in PLJ [12]. The samples were analyzed using electrospray ionization (ESI) interface comprising both positive and negative ion modes. A total of 10 µL of the sample was injected into ACQUITY UPLC BEH C18 1.7 µm (2.1×100 mm) column, with column temperature of 30 °C. The separation was accomplished by gradient elution using 2.0% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.6 mL/min (0–30 min, 2%–40% B) and proceeded until 45 min for column reconditioning. The MS/MS data were collected from 100–1700 m/z with a scan rate of 1 spectra/scan. The spectra obtained by a collision energy ramp of 30–35 eV. Data analysis was done by PDA and MASS SPECT.QTOF. Then, proceeded with pre-processed using the MZmine software for peak filtering, peak identification, peak matching, peak filing retention and time correction [13]. The identified peak list was produced using the corrected retention time, average area, and mass over charge (m/z) data as the identifiers.

### 2.5 Culture Medium

Potato dextrose agar (PDA) was evenly mixed with distilled water and then autoclaved for sterilization. The cold solution was then poured into the Petri plates. The solidified agar plates were then placed in the refrigerator until used.

### 2.6 Cultivation of the Microbe

The rotting pineapple leaf that was infected with microbes obtained from Pekan Pina pineapple plantation was used as a tested microbe in this study. The microbe was streaked on the agar plate in quadrant one until quadrant four using a sterile loop before incubating at 37°C for 24 h [14].

### 2.7 Experimental Set-up for Kinetic Study

The kinetic experiments were conducted according to the experimental design table (Table 1). The experiment was performed by re-culturing the microbe onto a new PDA in Petri plate. Microbe broth (MB) was prepared by mixing the re-cultured microbes into nutrient broth. The MB was agitated in the incubator shaker at 100 rpm for 1 h at 37 °C. Next, a 1:1 ratio mixture of MB and PLJ was then mixed and agitated at optimum condition (37 °C) with an agitation speed of 100 rpm [15]. Samples were taken at specified time intervals during the experiments.

### 2.8 Colony Forming Unit (CFU) Analysis

The microbe and PLJ mixture was widely spread on PDA evenly using a triangular cell spreader. The plate was incubated for 24 h at 37 °C. The plate count was calculated using the CFU/mL formula:

\[
\text{CFU/mL} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of culture plate}}
\]

### 2.9 Kinetic Study

The phenolic compound concentration of PLJ in terms of response-absorbance (Au) and microbial concentration (CFU/mL) were measured. Control sets containing PLJ only were prepared for concentration of phenolic compounds in PLJ, whereas control sets containing microbe only were prepared for microbe concentration. Inhibition data of microbe obtained in microbial inhibition assay was plotted according to Hill plot (Equation 2) by Rana et al. [16] in which $G_0$ is the microbial count in the absence of phenolic compounds, $G_i$ is the microbial count in the presence of phenolic compounds, $V_i$ is the microbial count in response (Au) and $V_{\text{max}}$ is the maximum reaction rate (or reaction velocity). $K_{0.5}$, the half-maximal concentration constant, is the substrate concentration (phenolic compounds) at which the reaction rate was precisely half of $V_{\text{max}}$. $K_{0.5}$ is inversely related to the apparent affinity of the transporter for its substrate (phenolic compounds). Therefore, a very high affinity of the interaction between the protein and its substrate
indicates a low numerical value of $K_{0.5}$ (for this study, it was the interaction between the microbes and the phenolic compounds). Usually, it takes a minimal amount (i.e., low concentration) of the substrate to reach 50% of the saturating concentration. The Hill coefficient ($n$) is used to measure the degree of cooperativity in kinetics and binding systems. It was evaluated from the slope of Hill plot (polynomial equation) of the log $I$ vs. CFU/mL. It offers an assessment of substrate binding cooperativity. Positive cooperative binding alludes to a situation when the binding of one substrate enables the other substrate to bind to the protein. The $n > 1$ proposes that two or more binding sites exist within the protein which there is positive cooperativity for substrate binding. If $n = 1$, there is no substrate binding cooperativity and is indicative of either a single substrate binding site in the protein or multiple binding sites that do not interact cooperatively. Finally, if $n < 1$, the reaction exhibits negative cooperative binding for substrate binding. According to Karekar et al. [17], the effect of substrate (phenolic compounds) inhibition was studied using Hill equation (2), where the three parameters ($V_{\text{max}}$, $K_{0.5}$, and $n$) were estimated by plotting a graph. Furthermore, the kinetic parameters can be determined based on Hill Equation-interactive graph, a plot of the reaction velocity as a function of the substrate concentration as described by Equations (2) and (3) [18].

\[
V = \frac{V_{\text{max}}[S]^n}{(K_{0.5})^n + [S]^n}
\]

### Table 1
Experimental design setup of kinetics inhibition

| Sample | Time (min) |
|--------|------------|
| 1      | 0          |
| 2      | 10         |
| 3      | 20         |
| 4      | 30         |
| 5      | 40         |
| 6      | 50         |
| 7      | 60         |

### Table 2
Phenolic compounds identified in PLJ by using UPLC-QTOF-MS

| No | Component name | Molecular formula | Neutral mass (Da) | $m/z$ | Rt (min) | Response (Au) | Total fragment found |
|----|----------------|-------------------|-------------------|------|---------|--------------|---------------------|
| 1  | Octahydrocurcumin | C21H28O6         | 376.19            | 377.19 | 1.08    | 9899         | 0                   |
| 2  | Meliadanoside A  | C16H24O10        | 376.14            | 377.15 | 5.99    | 21557        | 2                   |
| 3  | Kukoamine A      | C28H42N4O6       | 530.31            | 531.32 | 3.38    | 7389         | 8                   |
| 4  | Stilbostemin D   | C16H12O3         | 258.12            | 281.11 | 7.00    | 12111        | 1                   |
| 5  | Agrimol C        | C36H44O12        | 668.29            | 669.29 | 9.17    | 8158         | 2                   |
| 6  | Feralolide       | C18H18O7         | 344.09            | 345.09 | 10.41   | 4973         | 3                   |
| 7  | Methyl-5-O-caffeoylquinlate | C17H20O6 | 368.11            | 369.12 | 12.36   | 5896         | 0                   |

UPLC-QTOF-MS method is commonly used for structural characterization of phenolic compounds [19]. Phenolic compounds were separated from the PLJ by chromatographic methods, and spectroscopic methods were used to identify the structures of the isolated compounds. The UPLC-QTOF-MS chromatogram of the peaks detected is shown in Figure 1 and 2. These compounds were identified by their total ion chromatogram (TIC) and intensity as shown in Figure 1 and Figure 2, respectively. Chromatogram UPLC-QTOF-MS analysis of PLJ showed the different peaks and the components corresponding to the peaks were determined as shown in Figure 2.
Peak 1 ([M + H]+ at m/z 377.19) was distinguished as octahydrocurcumin. It is necessary to emphasize that this seems to be the first report of the presence of octahydrocurcumin in the pineapple leaf. UPLC-QTOF-MS methods have previously been employed for the determination of curcuminoids and curcumin metabolites in human blood plasma [20]. The complete turmeric matrix improves the octahydrocurcumin (OHC) concentration within the blood plasma once the product is administrated. Peak 2, meliadanoside A with [M + H]+ at m/z 377.15 was the highest response of phenolic compound. Peak 3 produced a fragmentation ion at m/z 531.32, suggesting the presence of kukoamine A with [M + H]+. Kukoamines are a series of bioactive phytochemicals conjugated by a polyamine spine and phenolic moieties [21]. Stilbostemin D was assigned to Peak 4. This compound with [M + Na]+ exhibited an MS spectrum that produced a fragmentation ion at m/z 281.11. Peak 5 presented a [M + H]+ at m/z 669.29, suggesting that it could be agrimol C. Peak 6 displayed a fragmentation pattern with [M + H]+ at m/z 345.09 that could be identified as feralolide. Finally, the presence of methyl-5-O-caffeoylquinic (Peak 7) was confirmed by its fragmentation pattern m/z 369.11 with [M + H]+. The phytochemical screening of natural products of Gynura divaricata led to the segregation of methyl-5-O-caffeoylquinic [22]. The findings demonstrated that L. japonica contains caffeoylquinic acids that could be used agents for medicinal purpose or functional food [23]. In this work, seven phenolic compounds detected by UPLC-QTOF-MS analysis were obtained more quickly.
compared with other analytical methods [20]. Kinetic studies were proceeded in order to examine the time-inhibit inhibition of microbial growth by these characterized phenolic compounds.

3.2 Kinetic Study

3.2.1 Phenolic Compounds Concentration-Absorbance (Au) against Time

Generally, phenolic compounds have one or more aromatic rings with one or more hydroxyl groups. It is widely assumed that with the free hydroxyl groups and the conjugation of side chains with aromatic rings, the anti-oxidant capacity of phenolics increases. The antioxidant properties of phenolic compounds are determined by the presence and number of hydroxyl groups. Changes in the position of the hydroxyl group play an important role in inhibition activity. The antioxidant capacity of phenolics is commonly assumed to increase with the number of free hydroxyls and the conjugation of side chains. Table 3 and Figure 3 present the phenolic compounds concentration-absorbance (Au) in PLJ against time. The experimental results were analyzed according to the highest absorbance (Au) of phenolic compounds present in the PLJ (inhibitor). Kinetic studies of PLJ was evaluated on five phenolic compounds identified by UPLC-QTOF-MS analysis. Figure 5 shows the concentration of meliadanoside A, feralolide, kukoamine A, methyl-5-O-caffeoylquininate, and stilbostemin D decreased as the time increased. From 0 to 60 min, the microbes (CFU/mL) were inhibited by the phenolic compounds. These phenolic compounds have been utilized as MGI and led to a decrease in the phenolic compounds concentration-absorbance (Au). This can be supported by Hammerbacher et al. [24] who found that fungal metabolism was responsible for a decrease in some phenolic compound concentrations, as reported in Ceratocystis polonica against declination of stilbenes concentration. C. polonica converted stilbenes to open-ring lactones, aglycones, and dimeric products, which make it diffuse more gradually into fungal cells.

From Figure 3, meliadanoside A shows the highest phenolic concentrations-absorbance (Au) that was identified in the PLJ, followed by feralolide, kukoamine A, methyl-5-O-caffeoylquininate, and stilbostemin D. However, meliadanoside A concentration decreased from 0 to 60 min. Kuang et al. [25] reported that meliadanoside A was isolated from Sambucus williamsii Hance fruit extract. Hwang et al. [26] found that the phytochemicals in S. williamsii exhibited antifungal effects. The phenolic compounds concentration for feralolide decreased from 10 to 60 min. Kurizaki et al. [27] isolated feralolide from 70% ethanol (EtOH) extract of Aloe arborescens flowers. The A. arborescens leaves have chromone or anthrone derivatives, antioxidant, antitumor, and antifungal activities. Kukoamine A (KuKA) started to show changes in concentration value from 10 to 60 min. KuKA is a spermine (SPM) conjugate with dihydrocaffeic acid (DHCA), with interesting biological activities. According to Litina et al. [28], four possible regioisomers and a series of KuKA analogs incorporated changes in either the SPM or the DHCA structural units. They were assessed for their antioxidant and inhibitory action on soybean lipoxigenase (LOX) and lipid peroxidation. Yuan et al. [29] reported that KuKA is a series of bioactive phytochemicals conjugated by a polyamine backbone and phenolic moieties.

The phenolic compounds concentration-absorbance value of methyl-5-O-caffeoylquininate decreased throughout 0–60 min. Chen et al. [28] studied the phytochemical contents of Gynura divaricata which driven to methyl-5-O-caffeoylquininate isolation. According to Zhou et al. [30], methyl-5-O-caffeoylquininate is a strong antioxidant candidate. This claim was supported by a research done by Wong et al. [31], which stated that methyl-5-O-caffeoylquininate isolated from methanol (MeOH) leaf extract of Vallaris glabra (Apocynaceae) has antioxidant and antimicrobial properties. The concentration of phenolic compound in stilbostemin D decreased from 0 to 60 min. Yang et al. [32] observed strong antibacterial activity against Staphylococcus epidermidis and Staphylococcus aureus with stilbostemin D isolated from the roots of Stemona japonica. Zhang et al. [33] elucidated the structure of stilbostemin D by spectroscopic analysis and showed that it exhibited strong antifungal activities. In general, the results showed that phenolic compounds concentration-absorbance value in PLJ decreased as time increased. This is because the phenolic compounds have been used to inhibit microbes.

Table 3 Phenolic Compounds Concentration-Absorbance (Au) experimental data

| No. | Phenolic Compounds (Au) | 0    | 10   | 20   | 30   | 40   | 50   | 60   |
|-----|-------------------------|------|------|------|------|------|------|------|
| 1   | Meliadanoside A         | 62938| 56374| 55579| 52685| 49079| 57218| 27008|
| 2   | Feralolide              | 34794| 51345| 48903| 48185| 42979| 45436| 24229|
| 3   | Kukoamine A             | 39517| 41870| 39451| 32680| 39313| 28854| 17572|
| 4   | Methyl-5-O-caffeoylquininate | 17957| 16212| 14612| 13978| 13115| 11213| 10577|
| 5   | Stilbostemin D          | 14690| 14560| 14350| 12075| 13020| 11397| 8265 |

*aControl (Au): Meliadanoside A=30743, Feralolide=63378, Kukoamine A=52252, Methyl-5-O-caffeoylquininate=25516, Stilbostemin D=15911*
3.2.2 **Kinetic of Microbial Inhibition**

Table 4 and Figure 4 show the kinetics of microbial inhibition that describe changes in CFU/mL at different times. The kinetics is used to study the activity of an inhibitor agent against microbes over time. It can monitor the effect of various concentrations of PLJ over time concerning the stages of the growth of the microbes. Five phenolic compounds were identified in PLJ, namely meliadanoside A, feralolide, kukoamine A, methyl-5-O-caffeoylquinate, and stilbostemin D. The CFU/mL was evaluated for the period of 0–60 min. CFU/mL decreased from 10 to 40 min and slightly increased from 50 to 60 min. The highest CFU was 2.62×10⁶ CFU/mL at 0 min. At 0 min, phenolic compounds might not be active yet, so the inhibition process did not occur. Meanwhile the lowest CFU was 1.21×10⁵ at 40 min. This could be explained by the optimum conditions in this study, which required 34.25 min of inhibition time. It means that 30–40 min was adequate for the inhibition process to take place. Longer than that, the phenolic compounds may not be effective much, as they are limited by time [34]. In general, the reduced microbial growth rate was influenced by phenolic compounds in PLJ. At this rate, microbes may be inhibited at certain time and cause the population to decrease [35].

**Table 4** CFU/mL at different times (min) experimental data

| Time (min) | CFU (CFU/mL) |
|------------|--------------|
| 0          | 2.62×10⁶     |
| 10         | 1.02×10⁶     |
| 20         | 2.16×10⁵     |
| 30         | 1.26×10⁵     |
| 40         | 1.21×10⁵     |
| 50         | 1.65×10⁵     |
| 60         | 1.80×10⁵     |
Kinetic of microbial inhibition in terms of CFU/mL obtained from microbial inhibition assay was plotted according to Hill equation [18]. CFU/mL decreased over the time for 0-40 min but increased at 50-60 min. This indicates the decrease in phenolic compounds-response (phenolic compounds concentration) (log I) after a certain time (Table 5) as it was utilized in microbe inhibition process. Figure 5a, 5b, 5c, 5d, and 5e represent the plot of CFU/mL against log I (Au) of five phenolic compounds (Au), namely meliadanoside A, feralolide, kukoamine A, methyl-5-O-cafeoylquinimate, and stilbostemin D, respectively. Figure 5 shows that as the concentration of phenolic compounds (log I) increased, more of the microbes (CFU/mL) were inhibited. Table 6 shows the three parametric ($V_{max}$, $K_{0.5}$, and n) forms of the Hill equation. In this study, the maximum rate of microbial inhibition reaction ($V_{max}$) can be achieved by progressively increasing the phenolic concentration. It indicates that the rate of inhibition reaction approaches at very high phenolic concentrations (saturated). Figure 5a, 5c, 5d, and 5e show that the $V_{max}$ for meliadanoside A, kukoamine A, methyl-5-O-cafeoylquinimate, and stilbostemin D, respectively was higher than the $V_{max}$ for feralolide (Figure 5b). Feralolide may be effective only to inhibit microbes up to 0.0493 CFU/mL; more than that, feralolide is no longer effective. Furthermore, feralolide concentration was much higher at 0.1373 CFU/mL compared to at 0.1373 CFU/mL as shown in Table 5.

$K_{0.5}$ is the concentration of phenolic compounds required to achieve half $V_{max}$. Meliadanoside A has the highest $K_{0.5}$ value, followed by feralolide, kukoamine A, methyl-5-O-cafeoylquinimate, and stilbostemin D (Table 6). Phenolic compounds with a high $K_{0.5}$ value have a low affinity and require a greater phenolic concentration to achieve half $V_{max}$ [36]. Table 5 shows that the phenolic concentration (log I) for meliadanoside A is the highest, followed by feralolide, kukoamine A, methyl-5-O-cafeoylquinimate, and stilbostemin D. Furthermore, it can be seen from Figure 5, which shows that the log (I) value is proportional to CFU/mL. A high CFU/mL value corresponded to a high phenolic concentration for microbial inhibition and effecting $K_{0.5}$ value, which is required to achieve half $V_{max}$.

Nonlinear regression has multiple slopes that depend on the point at which it is determined. The Hill coefficient (slope) is best thought of as an interaction coefficient, illustrating the degree of cooperativity among multiple binding sites [37]. From Table 6, the Hill coefficient (n) obtained for meliadanoside A is greater than 1, which shows the positive cooperative binding between phenolic compounds and microbe (CFU/mL). Meliadanoside A was similar to the sigmoidal behavior (S-shaped curve) [Figure 5a]. Feralolide, kukoamine A, methyl-5-O-cafeoylquinimate, and stilbostemin D showed negative cooperative binding, in which their Hill coefficients (n) are smaller than 1. This means that the phenolic compounds’ affinity for the microbe decreased, making them less likely to bind with each other. It can be said that feralolide, kukoamine A, methyl-5-O-cafeoylquinimate, and stilbostemin D might be less effective in inhibiting microbes over time. A study by Decker and Nadja [39] used negative cooperative term as defined by a Hill coefficient when the yielding oxygen binding curves with Hill coefficient were smaller than unity. However, this claim is contended by Weber [40], who indicated that the condition n < 1 is not sufficient to demonstrate the presence of negative cooperativity since microbes with multiple binding sites and different phenolic concentration affinities will too represent a lower n values.

### Table 5 Microbes in absence and present of phenolic compounds (I) vs. log I data

| Time (min) | [GI/(G0-GI)] (CFU/mL) | log I (Au) |
|------------|------------------------|------------|
|            | (Meliadanoside A)      | (Feralolide) | (Kukoamine A) | (Methyl-5-O-cafeoylquininate) | (Stilbostemin D) |
| 0          | 0.1373                 | 4.80       | 4.54       | 4.60       | 4.25       | 4.17       |
| 10         | 0.0493                 | 4.75       | 4.71       | 4.62       | 4.21       | 4.16       |
| 20         | 0.0101                 | 4.74       | 4.69       | 4.60       | 4.16       | 4.16       |
| 30         | 0.0058                 | 4.72       | 4.68       | 4.51       | 4.15       | 4.08       |
| 40         | 0.0056                 | 4.69       | 4.63       | 4.53       | 4.12       | 4.11       |
| 50         | 0.0077                 | 4.72       | 4.66       | 4.46       | 4.05       | 4.06       |
| 60         | 0.0084                 | 4.43       | 4.38       | 4.24       | 4.02       | 3.92       |

### Table 6 Kinetic parameters ($V_{max}$, $K_{0.5}$, and n) of phenolic compounds

| Phenolic compounds   | $V_{max}$ (CFU/mL) | $K_{0.5}$ (Au) | Hill coefficient (n) |
|----------------------|-------------------|---------------|----------------------|
| Meliadanoside A      | 0.1373            | 4.7659        | 1.30                 |
| Feralolide           | 0.0493            | 4.6982        | 0.43                 |
| Kukoamine A          | 0.1373            | 4.2553        | 0.22                 |
| Methyl-5-O-cafeoylquininate | 0.1373 | 4.2288        | 0.44                 |
| Stilbostemin D       | 0.1373            | 4.1480        | 0.38                 |
(a) CFU/mL vs log I

\[ y = 2.4024x^3 - 22.54x^2 + 52.87x \]
\[ R^2 = 0.9907 \]

(b) CFU/mL vs log I

\[ y = 90.48x^4 - 1264.6x^3 + 5892x^2 - 9150.2x \]
\[ R^2 = 0.999 \]
(c) $y = 7.6947x^4 - 101.79x^3 + 448.67x^2 - 658.94x$

$R^2 = 0.9962$

(d) $y = 8.8364x^4 - 108.41x^3 + 443.29x^2 - 604.17x$

$R^2 = 0.9974$
4.0 CONCLUSION

The UPLC-QTOF-MS analysis explored the presence of phenolic compounds in PLJ. The phenolic compounds identified were confirmed based on their molecular mass and fragmentation pattern. These phenolic compounds were separated using the chromatographic conditions indicated in the experimental section. Kinetics study on microbial inhibition activity by phenolic compounds was performed using the Hill equation. Each kinetics constant ($K_{0.5}$) and Hill coefficient ($n$) were estimated from the Hill equation. The result shows that meliadanoside A is associated with positive cooperative inhibition ($n>1$) while feralolide, kukoamine A, methyl-5-O-cafeoylquinicate, and stilbostemin D show negative cooperativity ($n<1$). Meliadanoside A has the highest $K_{0.5}$ value followed by feralolide, kukoamine A, methyl-5-O-cafeoylquinicate, and stilbostemin D. The Hill equation was applicable to the kinetics analysis of microbial inhibition.

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