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Pineapple Leaves Juice Characterization for Microbial Growth Inhibition

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Abstract. Since pineapple leaves are one of the lavishly organic waste materials which contain phenolic compounds with antimicrobial properties, it was desired to gain deeper knowledge on its potential as microbial growth inhibitor (MGI). In this research, the pineapple leaves were extracted using an electrical sugarcane pressing machine to acquire the pineapple leaves juice (PLJ). The PLJ was autoclaved at 121 °C for 15 min. Ultra-high-performance liquid chromatography-quadrupole time of flight mass spectroscopy (UPLC-QTOF-MS) analysis was used to examine the existence of different bioactive components in PLJ. Based on the molecular mass and its fragmentation pattern, the identification of the phenolic compounds was confirmed. From the characterization of PLJ using UPLC-QTOF-MS, seven types of the phenolic compound has been identified namely Octahydrocurcumin, Meliadoside A, Kukoamine A, Stilbostemin D, Agrimol C, Feralolide and Methyl-5-O-caffeoylquininate. The effect of microbial inhibition time on the colony forming unit (CFU) between the mixtures of PLJ and microbe was studied. CFU/mL decreased with increasing microbial inhibition time from 0 to 1.50 h. Meanwhile, CFU/mL was increased again after increasing the microbial inhibition period from 1.50 to 3 h. This suggests that 1.50 h was necessary for the process of microbial growth inhibition. This research shows that pineapple leaves could be manipulated and used as an MGI agent as useful sources of natural products. Furthermore, it can become one of the cheaper and greener MGI alternatives compared to available synthetic antimicrobial agent.

Keywords: pineapple leaves juice; phenolic compounds; microbial growth inhibitor; characterization; UPLC-QTOF-MS

1. Introduction

Pineapple (*Ananas comosus*) with outstanding consistency, unique taste and nutritious richness, is the favourite of consumers all over the world. It is the third most commercially valuable tropical fruit [1]. However, microbial diseases have resulted in a low quality of pineapple fruit that is less marketable and wasted on farms [2]. A wide range of formulations were used to control existing crop diseases by converting a chemical with anti-pathogenic properties. However because of its toxicity content, it can pose dangerous problems to other living organisms. Pineapple leaves were thought to have antimicrobial properties due to their phenolic compounds. Phytosterol, beta-sitosterol, stigmasterol and campesterol, which contribute to antioxidant activity, were the major phenolic contents found in pineapple leaves [2]. The total phenolic content (TPC) of pineapple has been reported to be higher than other fruits [3, 4]. In placing more emphasis, Rodriguez et al. [5] discovered seven phenolic compounds derived from pineapple leaves, namely ananasate, 1-O-caffeoylglycerol, 1-O-p-coumaroylglycerol, caffeic acid, p-coumaric acid, β -sitosterol and daucosterol. A research by Sen et al. [6] and Joy et al. [7] also found that β -sitosterol inhibits the development of *S.aureus* and *E.coli*. These phenolic compounds were beneficial



to plant growth in providing a defense system against infectious disease [8]. Aromatic plants with phenolic compounds have received attention in the management of crop disease [9, 10]. Therefore, as they were abundantly available after pineapple processing, pineapple leaves were seen as potential useful items, offering a safer environmental effect.

According to Mursyid [11], screening was the way of evaluating which variables returned the best desired effect. One of them was the one-factor-at-a-time (OFAT) method that requires only one variant at a time by fixing the other variables [12]. The OFAT method, also known as one-variable-at-a-time was a method for designing experiments involving the testing of factors one at a time rather than several factors at a time. Engineers and scientists also conduct OFAT tests, which vary from one factor to another at a time while keeping others constant. Given the above, this research aimed to characterize the pineapple leaves juice (PLJ) and study the microbial growth inhibition using PLJ by analyzing the effect of microbial inhibition time on microbial colony count. Therefore the OFAT method was conducted in the present study using Design Expert software to evaluate the microbial growth inhibition response factor.

2. Materials and methods

2.1. Chemicals and Reagents

Reagents and chemicals used were Potato Dextrose Agar (PDA) powder (<1mm; 99%), Gallic acid (66.66 µm; 99%), methanol (99.8%), Folin-Ciocalteu reagent (99%) and sodium carbonate (Na₂CO₃, 99%). All were graded analytically and had high purity.

2.2. Materials and PLJ Extraction Preparation

Pineapple leaves and the microbe analyzed were collected from the pineapple plantation in Pekan Pina, Pahang. The pineapple leaves (average length of 60 cm) were extracted using an electrical sugarcane press machine to produce pineapple leaves juice (PLJ). The PLJ was then autoclaved at 121 °C for 15 min. Approximately 60 mL of PLJ was obtained with 0.1 kg of pineapples leaves.

2.3. Total Phenolic Content (TPC) Analysis

The determination of the total phenolic content (TPC) was formed using the Folin-Ciocalteu reagent. Initially, 10 mL of PLJ extract was centrifuged at 5000 rpm for 15 min. 0.5 mL of the supernatant was then injected into the test tubes and combined with 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent. The mixture was placed in a dark place at normal temperature for 5 min. After that, 2 mL of 7.5% Na₂CO₃ was added to the mixture and left to rest for another 1 h. The mixture was eventually measured at 450 nm using the UV-Vis spectrophotometer. The compositions of TPC were compared to the Gallic acid standard curve. The standard concentration of Gallic acid was prepared at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/mL in 80% methanol solution and mixed with Folin-Ciocalteu reagent and 7.5% Na₂CO₃. TPC was presented as mg Gallic acid equivalent per gram of PLJ extract (mg GAE/mL) [13].

2.4. UPLC-QTOF-MS Analysis of the Extract

An ultra-high-performance liquid chromatography-quadrupole flight mass spectroscopy time (UPLC-QTOF-MS) method was used to obtain chromatographic profiles for phenolic compounds present in PLJ. It was used to identify metabolites existing in the PLJ as defined by Lawal et al [14] with slight changes. Chromatographic separation and mass spectrometry detection were performed on the Waters Acquity UPLC system. Samples were analyzed with an electrospray (ESI) interface consisting of both positive and negative ion modes. Approximately 10 µL of injection volume was inserted into the ACQUITY UPLC BEH C18 1.7 µm (2.1 x 100 mm) column previously flushed with water.

The temperature of the column was set at 30°C. Separation was accomplished by gradient elution of 2.0 per cent formic acid solution (solvent A and acetonitrile (solvent B) at a flow rate of 0.6 mL/min: 0–30 min, 2–40 per cent B; and identification of up to 45 min column. Full screen mass spectra detection was performed in positive mode. The MS/MS data were collected in a range of 100–1700 m/z with a scanning rate of 1 spectra/scan. The MS/MS spectra were created by a collision energy ramp of 30–35eV. Data obtained was analyzed using PDA & MASS SPECT (QTOF). The data were then pre-processed to obtain the peak filtering, peak detection, peak matching, time retention and peak filing [15]. The peak list was built using the average area, corrected retention time and overload mass (m/z) data.

2.5. Culture Medium

39 gram of Potato Dextrose Agar (PDA) was put into 1000 mL distilled water, stirred until the powder was completely dissolved prior to autoclave at 121°C for 15 min. Then the solution was cooled and poured approximately 20 mL portions into the petri plates. After the agar solidified, the plates were labelled and flipped to avoid condensation of water and kept in a chiller at 4°C until further use.

2.6. Cultivation of the Microbe

Microbe growing on the contaminated pineapple leaves obtained from Pekan Pina pineapple plantation was used as the tested microorganism. The microbe was streaked on the solid agar surface of the petri plate using a sterile loop across the quadrant one until the quadrant four and incubated at 37 °C for 24 h. [16].

2.7. Experimental Set-up for Microbial Growth Inhibition using OFAT

The experiment was started by re-culturing the microbe as mentioned in Section 2.6 into the new PDA plate at at 37 °C for 24 h. The re-cultivated microbe was scraped out of the PDA using an inoculation loop and mixed with a nutrient broth to produce microbe broth (MB). The MB was then agitated in the incubator shaker for 1 h at 37 °C and 100 rpm. Then, the MB was mixed with PLJ at microbe to PLJ ratio of 1:1 (20 mL of MB and 20 mL of PLJ). The selected range of microbial inhibition time in this study was between 0 and 3 h. The mixture was agitated in the incubator shaker at 100 rpm and 37 °C in accordance with the OFAT specification (Table 1). Then colony count analysis was conducted to all samples run.

Table 1. Experimental design set-up of microbial inhibition time for OFAT.

Run	Microbial inhibition time (h)
1	0
2	0
3	0.75
4	1.50
5	2.25
6	3.00
7	3.00

2.8. Colony Forming Unit (CFU) Analysis

The microbe and PLJ mixture from Section 2.7 was spread on PDA plate evenly using a triangle stick and incubated at 37 °C for 24 h. After 24 h, colony count was carried out. The CFU/plate was read from the plate in the linear range, and then the CFU/mL of the control was deducted mathematically, factoring

in the quantity plated and its dilution factor. The plate count was linear for microbe in the range of 30 to 300 CFU on a standard sized petri plate [17]. Microbial growth inhibition (%) was estimated by CFU/mL. The inhibition of CFU/mL and microbial growth was determined using equations (1) and (2), respectively.

$$\text{CFU/mL} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of culture plate}} \quad (1)$$

$$\text{Microbial growth inhibition (\%)} = \frac{\left(\frac{\text{CFU}}{\text{mL}} \text{ of control} - \frac{\text{CFU}}{\text{mL}} \text{ of mixture}\right)}{\frac{\text{CFU}}{\text{mL}} \text{ of control}} \times 100 \quad (2)$$

3. Results and Discussion

3.1. UPLC-QTOF-MS Analysis

Specific and sensitive UPLC-QTOF-MS methods for phenolic compounds determination in PLJ explored the existence of various bioactive components. The detection of phenolic compounds was confirmed on the basis of the molecular mass and its pattern of fragmentation. These phenolic compounds were then separated using the chromatographic conditions indicated in the experimental section. Table 2 shows that PLJ contain seven bioactive compounds.

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

No	Compound name	Molecular formula	Neutral mass (Da)	m/z	Rt (min)	Response (Au)	Total fragment found
1	Octahydrocurcumin	C ₂₁ H ₂₈ O ₆	376.19	377.19	1.08	9899	0
2	Meliadoside A	C ₁₆ H ₂₄ O ₁₀	376.14	377.15	5.99	21557	2
3	Kukoamine A	C ₂₈ H ₄₂ N ₄ O ₆	530.31	531.32	3.38	7389	8
4	Stilbostemin D	C ₁₆ H ₁₈ O ₃	258.12	281.11	7.00	12111	1
5	Agrimol C	C ₃₆ H ₄₄ O ₁₂	668.29	669.29	9.17	8158	2
6	Feralolide	C ₁₈ H ₁₆ O ₇	344.09	345.09	10.41	4973	3
7	Methyl-5-O-caffeoylquininate	C ₁₇ H ₂₀ O ₉	368.11	369.12	12.36	5896	0

UPLC-QTOF-MS method had commonly been used for structural characterization of phenolic compounds [18]. Phenolic compounds have been extracted from pineapple leaves by chromatographic methods and spectroscopic methods have been used to classify isolated compound structures [19]. The UPLC-QTOF-MS chromatography of the peaks observed was shown in Figures 1 and 2. These compounds were identified by their total ion chromatogram (TIC) and intensity as shown in Figure 1 and Figure 2 respectively. UPLC-QTOF-MS analysis showed the presence of different peaks and the components corresponding to the peaks as shown in Figure 2.

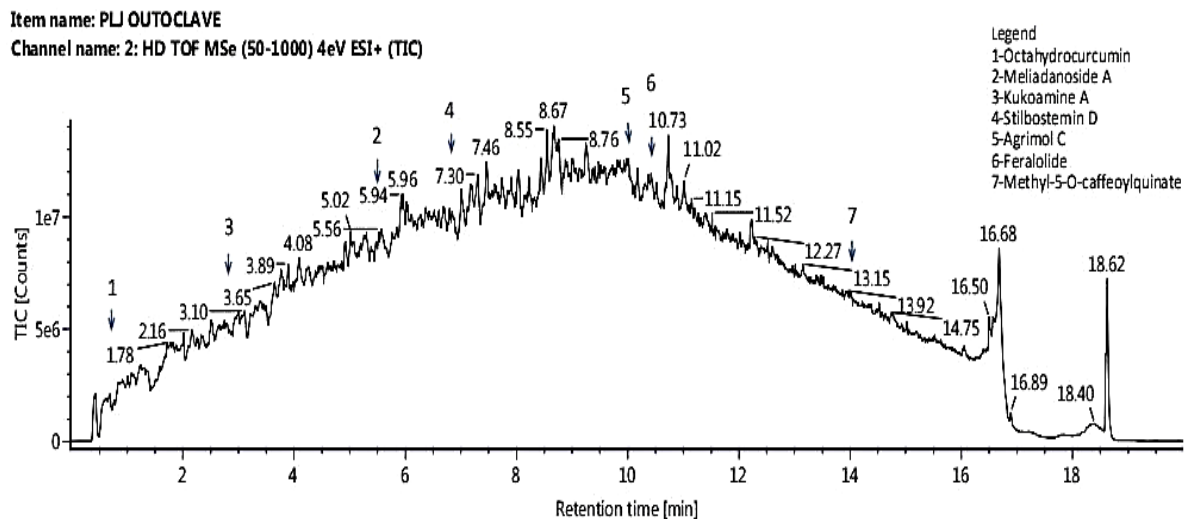


Figure 1. Base-peak chromatogram of phenolic compounds in PLJ by using UPLC-QTOF-MS (TIC plot against retention time).

Identified Compounds

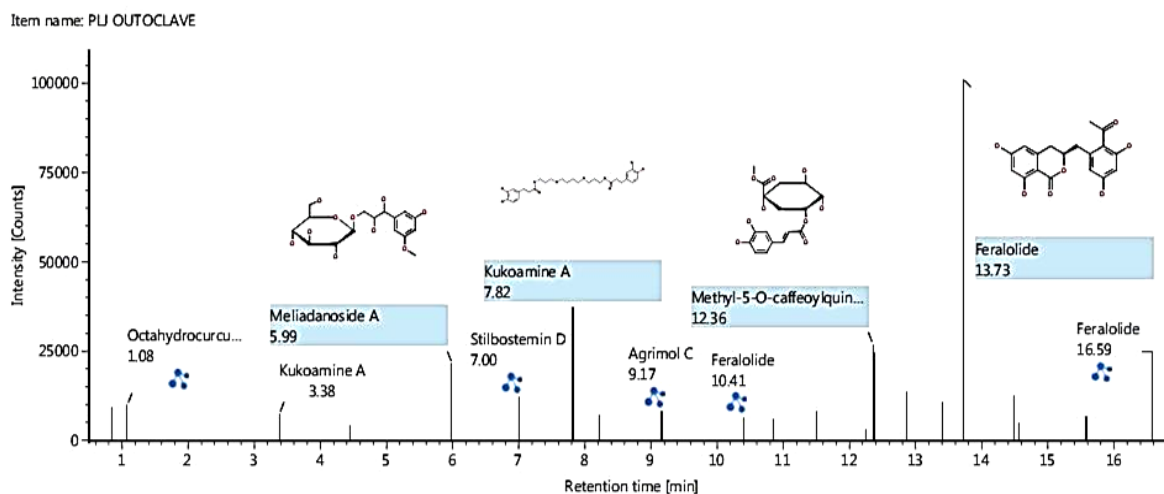


Figure 2. Base-peak chromatogram of phenolic compounds (Intensity against retention time).

Peak 1 ($[M + H]^+$ -at m/z 377.19) was examined as octahydrocurcumin. It was important to emphasize that it appears to be the first report of the presence of octahydrocurcumin in the pineapple leaves. A study conducted by Jude et al [20], UPLC-QTOF-MS was used for the determination of curcumin to octahydrocurcumin (reduction) metabolites in human blood plasma. Peak 2 was the meliadanoside A, $[M + H]^+$ at m/z 377.15 with the highest response of phenolic compound detected. Peak 3 produced a fragmentation ion at m/z 531.32, suggesting the presence of kukoamine A with $[M + H]^+$. Kukoamines was a series of bioactive phytochemicals conjugated by polyamine backbone and phenolic moieties [21]. Stilbostemin D was assigned to Peak 4. This compound with $[M + Na]^+$ exhibited a MS spectrum

formed a fragmentation ion at m/z 281.11. Stilbostemin D was isolated from the roots of *Stemona japonica* [22]. Their structures were elucidated by spectroscopic analysis and exhibited significant antifungal activities against *Candida albicans*. Peak 5 presented a $[M + H]^+$ at m/z 669.29, suggesting that it could be an agrimol C. Peak 6 displayed a (fragmentation pattern with $[M + H]^+$ at m/z 345.09 that could be identified as feralolide derivative. Research conducted by Kurizaki et al [23] found that feralolide was isolated from the 70% EtOH extract *Aloe arborescens*. The leaves of *A. arborescens* were reported to have antioxidant and antifungal activities. Finally, the presence of methyl-5-O-caffeoylquininate (Peak 7) was confirmed by its fragmentation pattern m/z 369.11 with $[M + H]^+$. The phytochemical investigation of natural products of *Gynura divaricata* led to the isolation of methyl-5-O-caffeoylquininate [24]. The results showed that caffeoylquinic acid derived from *L. japonica* may act as agents for medicinal use or functional food [25]. In this study, seven phenolic compounds were characterized in PLJ. The possible active substances obtained by the UPLC-QTOF-MS system were faster than other analytical methods [26].

3.2. Microbial Inhibition Time based on OFAT Experiment

The effect of microbial inhibition time between the PLJ and microbe was studied. To perform OFAT, only one factor was varied, and other variables were fixed. Thus, the experiment was based on the different microbial inhibition time and two fixed factors which were ratio of PLJ to microbe (1:1) and temperature (37 °C). OFAT was a widely used tool for determining the influence of a set of parameter value in an experiment. It simplifies the selection of the most productive ranges in the experiment by reducing experimental time, expense and complexity. Table 3 shows the experimental results of OFAT. The effect of microbial growth inhibition on the colony count (CFU/mL) is shown in Figure 3. CFU/mL decreased from 0 to 0.75 h with increasing microbial inhibition time. In the meantime, after rising the microbial inhibition time from 1.50 to 3 h, CFU/mL was slightly increased again.

The results of this study therefore suggested that increasing microbial inhibition time had a negative effect on the value of CFU/mL. One of the explanations by increasing back of CFU/mL from 1.50 to 3 h was due to the phenolic compounds may not effective much, therefore limited in time. According to Mouton et al. (2007) as time increased above a certain value, microbe already started to regrow. However, it seems at 2.25 h gave the higher inhibition with lesser CFU/mL. This result might be happen because of there are some phenolic compounds that might be actively inhibit at that time. An experiment conducted by Leite et al [27], microbial inhibition time of 2 to 3 h between citral and *C. albicans* could kill 99.9% of the inoculum as well. Therefore, 0.75 h was adequate enough for microbial growth inhibition process to occur. It can achieve a minimum CFU/mL which indicates the higher microbial growth inhibition.

Table 3. Experimental results of OFAT

Run	Microbial inhibition time (h)	Response 1: CFU/mL	Response 2: Microbial growth inhibition (%)
1	0	1.66E+06	-84.94
2	0	1.66E+06	-84.94
3	0.75	6.39E+05	29.16
4	1.50	6.65E+05	26.29
5	2.25	1.70E+05	81.09
6	3.00	7.02E+05	22.14
7	3.00	7.02E+05	22.14

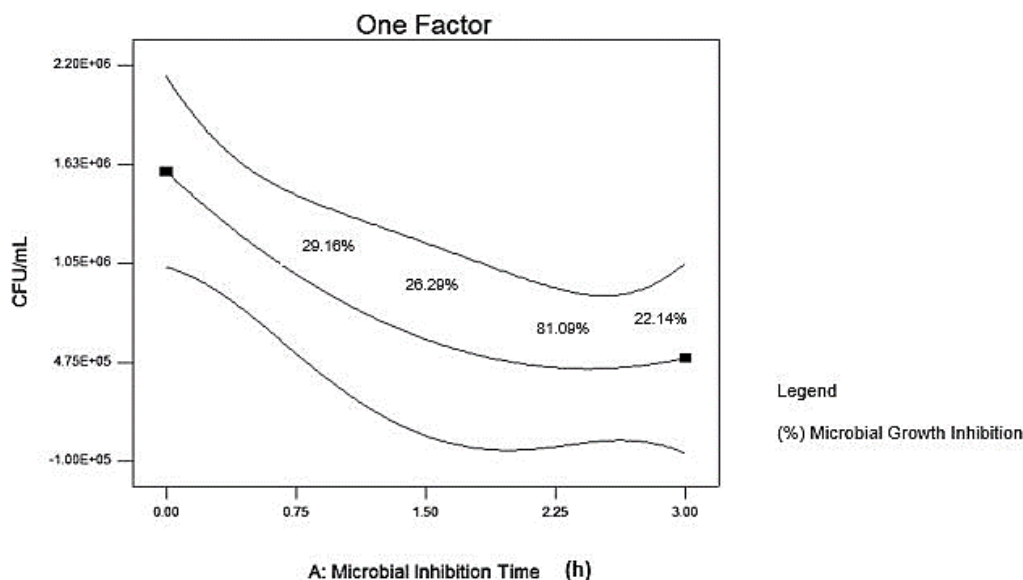


Figure 3. Effect of microbial inhibition time on CFU/mL.

4. Conclusion

The UPLC-QTOF-MS analysis of phenolic compounds in pineapple leaves detected the composition of different bioactive components. The detection of phenolic compounds was confirmed based on the analysis of the molecular mass and its fragmentation pattern. The impact of microbial inhibition time on OFAT-based colony count (CFU/mL) was studied. Microbe CFU/mL decreases with increasing microbial inhibition time from 0 to 0.75 h. A minimum CFU/mL that suggests higher inhibition of microbial growth can be achieved. The findings of this study indicate that PLJ has a good potential as MGI indicating the presence of phenolic compounds with antimicrobial properties. Further analysis is recommended to determine the time-inhibit kinetic assay for the study of microbial activity over time.

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