Synergistic effect of *Alocasia longiloba* fruit's extract with ampicilin and tetracycline against bacteria

Synergistic effect of *Alocasia longiloba* fruit's extract with ampicilin and tetracycline against bacteria

I S Nur-Alya[1], M Aurifullah[2], A A Nazahatul[1], T Srisawat[3], P Permpoonpattana[3], M Z Norhazlini[1], O Suhaimi[4] and H Zulhazman[1*]

[1]Department of Natural Resources and Sustainability Science, Faculty of Earth Science, Campus Jeli, Universiti Malaysia Kelantan, Locked Bag 100, 17600 Jeli, Kelantan
[2]Faculty of Agro-Based Industry, Campus Jeli Universiti Malaysia Kelantan, Locked Bag 100, 17600 Jeli, Kelantan
[3]Faculty of Science & Industrial Technology, Prince of Songkla University, Suratthani Campus, Suratthani 84000, Thailand
[4]FMC Agrochemicals Malaysia, 82, Persiaran Cinta Sayang, 08000 Sg. Petani, Kedah, Malaysia
*Corresponding author: zulhazman@umk.edu.my

Abstract. The inappropriate usage of antibiotic is one of the factors of the emergence of the antibiotic resistance bacteria that limit the effectiveness of the current antibiotic and lead to the treatment failure. The combination of plant extract with antibiotic approach may lead to the new ways in the treatment of the infectious diseases and this combination may reduce of bacterial resistance toward antibiotics. The objective of this study was to determine the synergistic effect of *Alocasia longiloba* fruit extract with Ampicillin and Tetracycline against *Staphylococcus aureus* and *Escherichia coli.* The synergistic effect of *A. longiloba* fruit extract and antibiotics was determined by using agar well diffusion and minimal inhibitory concentration (MIC) Resazurin 96-well micro-dilution methods. The results of this study showed the increasing in the inhibition zone when the plant extract was combined with Ampicillin against *E. coli*. The value of MIC only showed by Ampicillin on *E. coli* which was 12.5 µg/ml, and the combination of plant extract and Ampicillin (2000+12.5 µg/ml). These results indicated that the fruit extract of *A. longiloba* showed low antibacterial activity against *E. coli* and *S. aureus* and this plant extract may show the inhibition if the concentration is increase and test against the different microorganisms.

1. Introduction

Araceae or locally known as *keladi hutan* is the fourth largest family after orchids, grasses and sedges. The Araceae family comprises of 125 genera and 3,750 species includes the genus of Alocasia which widely distributed in the humid tropics [1, 2, 3, 4].

The resistance of the pathogenic bacteria towards the antimicrobial agent is increasing due to the genetically ability of the bacteria to transmit and acquire resistance to the antibiotic. This will be a concern because the patient will suppress immunity due to this new bacteria strain which are multi-resistance and will cause new infection that will lead to mortality [5]. Also, use of antibiotics can cause some unpleasant side effects, such as yeast overgrowth and gastrointestinal trouble. On the other hand, medicinal plant can promote beneficial effects on killing of bacteria with safer than that of antibiotics. To reduce the risk of side effect when use antibiotics, the combination of antibiotic at less than

commonly use and the plant extract may increase the inhibition of the antimicrobial agents against the bacteria. A synergy effect can occur when the antibiotic is combined with an agent that antagonizes bacterial resistance mechanism [6].

This study focused on the synergetic effect of *Alocasia longiloba* fruit extract with Ampicillin and Tetracycline against gram positive and gram negative bacteria. *A. longiloba* is the species from Araceae family are recommended as a plant of pharmaceutical importance based on its antioxidant potential and the properties bioactive compound present in it [7, 8]. *A. longiloba* is believed to have antibacterial properties, because the stem part of this plant had been used for treating pus in cattle and abdominal disease [9]. This plant has been used by the locals especially the Malay in Peninsular Malaysia as staple food [10] and as the natural remedies for treating of wound healing (stem) [11] and the effect of gout (fruit) [12, 13]. However, there was no study regarding the antibacterial activity and synergetic effect of *A. longiloba* fruits.

Tetracycline is the family antibiotic that can inhibit the synthesis of bacteria protein by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. It has been widely used in the treatment of human and animal infection due to the antimicrobial properties and the absence of the major side effect. However, in the recent year, the emergence of the microbial resistance has limited their effectiveness [14]. Ampicillin is the antibiotic from penicillin group and it is responsible in the inhibition of the cell wall synthesis by interacting with the penicillin- binding proteins that are responsible in the synthesis of the peptidoglycan in the cell wall. The interruption of peptidoglycan will cause the cell lysis and cell death [15].

This study investigated the synergistic effect of ethanol extract from the fruits of *A. longiloba* with Tetracycline and Ampicillin against the gram positive bacterium *Staphylococcus aureus* and gram negative bacterium which is *Escherichia coli*.

2. Materials and methods

2.1. Plant materials and extraction

The samples of *A. longiloba* were collected from the village areas in the District of Pasir Mas, Kelantan, Peninsular Malaysia. The fruits of *A. longiloba* were collected, cleaned, dried and grind to powder form. Then the dried sample was suspended in 95 % ethanol for 5 days and filtered by using two cotton layers. The ethanol filtrate was collected and concentrated by using rotary evaporator (Buchi R-100) at 70 ℃. The extract was kept in dark glass bottle and stored in dark cold storage at 4 ℃ for further analysis.

2.2. Microorganisms tested

The bacteria strains were used are: *S. aureus* (ATCC25923) and *E. coli* (ATCC25922) are maintained at the Scientific Laboratory and Equipment Centre (SLEC), Prince of Songkla University, Surat Thani Campus, Thailand.

2.3. Chemicals

Nutrient agar (Oxoid), nutrient broth (Oxoid), Mueller-Hilton agar (Oxoid) and Mueller-Hinto broth (Oxoid), 95 % ethanol (Fisher Chemical), Resazurin, dimethyl sulfoxide (DMSO) (Fisher Chemical), Tetracycline antibiotic and Ampicillin antibiotic.

2.4. Determination of the combined activity using agar well diffusion method

The antibacterial activity was measured by using the agar-well diffusion method on Mueller-Hinton Agar. The bacteria isolates were sub-cultured for 18 hours in the nutrient broth before transfer it into the MHA medium. The sterile cottons swab was dipped briefly in the bacteria strain suspension (1× 107 CFU/mL) and the whole surface of the agar plate was inoculated (25mL medium). A 6 mm diameter hole was punched aseptically by using sterile cork-borer and 50 µL of the extract solution at concentration (3000, 4000, 5000 µg/well) and antibiotic which were tetracycline (3.75, 7.5 and 15 µg/well) and ampicillin (12.5, 25 and 50 µg/well). In case of the synergism effect 25 µL of each was

introduced into the well. For 24 hours, the bacteria cultured plates was incubated at 37ºC and the antibacterial effect of the ethanol extract on bacteria was evaluated using Tetracycline (15 µg/well) and Ampicillin (50 µg/well) as the positive control. The antibacterial activity was accessed by measuring the inhibition zone around the well. Synergism effect was considered when the combination exhibited with enlargement of the combined inhibition zone more than 5mm [16].

2.5. Determination of minimum inhibitory concentration (MIC) using Resazurin-based 96-well micro-dilution

The determination of minimum inhibitory concentration (MIC) was carried out based on method described by [17] with modifications. Bacterial suspension at mid-log phase was used.

Briefly, for the plant extract, 280 µL of mixed solution of Mueller Hinton Broth (MHB) and 2-fold extract with 8000 µg/mL concentration was filled in the first well and 140µL of MHB was filled in the wells 2 until 7. The preparation of the extract concentration from 8000-125 µg/mL was done by pipetting 140 µL aliquot from the first well into the next well to make the 2-fold serial micro-dilution in the 96-well plate. Each well was added with 50 µL of bacteria suspension except for well 8. Well 8 was the negative control which was without the bacteria suspension. The 96-wells was incubated for 24 hours at 37ºC, then 10 µL of 0.015% Resazurin was added and the plate was incubated for 1-2 hours before the evaluation of colour.

The same procedure was applied to antibiotic with the concentration of 15-0.234 µg/mL for tetracycline and 50-0.781 µg/mL for Ampicillin. The concentration for the combination of plant extract with Tetracycline was 8000:15 µg/mL until 125:0.234 µg/mL (plant extract concentration: antibiotic concentration) and 8000:50 µg/mL until 125:0.781 µg/mL for Ampicillin. This procedure was done by using two antibiotics which were Tetracycline and Ampicillin against *E. coli* and *S. aureus*.

2.6. Fractional inhibitory concentration (FIC)

Fractional inhibitory concentration (FIC) is the lowest concentration of the extract and the antibiotic in combination giving no detectable bacterial growth after incubation. FIC index value was calculated using formula:

$$\text{FIC index} = \frac{MIC\ extract\ in\ combination}{MIC\ of\ extract\ alone} + \frac{MIC\ antibiotic\ in\ combination}{MIC\ of\ antibiotic\ alone} \quad (1)$$

The combination defined synergy if ΣFIC ≤ 0.5, addictive if 0.5 < ΣFIC ≤1, indifference if 1< ΣFIC ≤ 4 and antagonism if ΣFIC > 4 (Noor, 2016).

3. Results and discussion

3.1. Agar well diffusion method

The result obtained (Table 1) showed the inhibition zone of the plant extract alone, Tetracycline alone, Ampicillin alone, the combination of plant extract with Tetracycline and the combination of plant extract with Ampicillin against *E. coli* and *S. aureus*. The *A. longiloba* extract showed no inhibition against both bacteria at the concentration of 3000, 4000 and 5000 µg/well as shown in Figure 1 (A & B).

For the combination of plant extract and Tetracycline against *S. aureus*, the highest inhibition shown by the concentration of 4000+15 µg/well and 5000+15 µg/well which were 26.33 mm. The inhibition zone of Tetracycline without extract at the same combination as the combination which was 15 µg/well, showed the same diameter of inhibition zone indicated that there was no synergistic effect between the combination of Tetracycline and plant extract against *S. aureus*. The combination of antibiotic and the plant extract with the concentration of 4000+15 µg/well showed the reduction in the inhibition zone. The inhibition zone of the combination of Tetracycline and plant extract as well as Tetracycline alone on *S. aureus* shown in Figure 1 (C & D).

Table 1. The inhibition zone of *A. longiloba*, antibiotics and the combination against *E. coli* and *S. aureus*.

Treatment	Inhibition Zone (mm)	
	S. aureus	*E. coli*
A. longiloba extract		
3000	0.00±0.00^{b}	0.00±0.00^{b}
4000	0.00±0.00^{b}	0.00±0.00^{b}
5000	0.00±0.00^{b}	0.00±0.00^{b}
10 % DMSO	0.00±0.00^{b}	0.00±0.00^{b}
Tetracycline	25.33±0.58^{a}	25.33±0.58^{a}
A. longiloba + Tetracycline		
3000 + 15	25.67±0.58^{ab}	19.00±0.00^{a}
4000 + 15	26.33±0.58^{a}	19.33±0.58^{a}
5000 + 15	26.33±0.58^{a}	19.67±0.58^{a}
10 % DMSO	0.00±0.00^{c}	0.00±0.00^{c}
Tetracycline (15)	25.00±0.00^{b}	17.67±0.58^{b}
A. longiloba + Ampicillin		
3000 + 50	34.33±3.79^{ab}	27.33±0.58^{ab}
4000 + 50	35.67±4.16^{a}	26.67±0.58^{a}
5000 + 50	35.67±3.21^{a}	28.00±0.00^{a}
10 % DMSO	0.00±0.00^{c}	0.00±0.00^{d}
Ampicillin (50)	32.00±3.61^{b}	24.33±0.58c
Tetracycline		
3.75	22.00±1.00^{d}	17.33±0.58^{c}
7.50	24.67±0.58^{c}	18.67±0.58^{b}
15.00	26.33±1.15^{b}	19.67±0.58^{b}
10 % DMSO	0.00±0.00^{e}	0.00±0.00^{d}
Ampicillin (50)	36.33±1.53^{a}	24.67±0.58^{a}
Ampicillin		
12.5	34.67±0.58^{b}	19.00±0.00^{c}
25	35.67±1.15^{ab}	21.33±0.58^{b}
50	36.33±1.15^{a}	24.33±0.58^{a}
10 % DMSO	0.00±0.00^{d}	0.00±0.00^{e}
Tetracycline (15)	25.00±0.00^{c}	17.67±0.58^{d}

*values are expressed as mean of the three replicates. Values with different letters in the same column indicated significantly difference.

The highest inhibition of the combination between plant extract with Tetracycline against *E. coli* showed at the concentration of 5000+15 µg/well which was 19.67 mm. The same diameter of inhibition zone shown by the treatment of Tetracycline alone against *E. coli* indicated the combination of Tetracycline and plant extract against *E. coli* showed no synergistic effect as the inhibition zone of the combination same with the individual effects. The inhibition zone was slightly decrease when the combination of Tetracycline and plant extract with the concentration of 3000+15 µg/well and 4000+15 µg/well were tested against *E. coli* as shown in Figure 1 (E & F).

For the combination of the plant extract and Ampicillin against *S. aureus*, the highest inhibition shown by the concentration same as combination with Tetracycline, 4000+50 µg/well and 5000+50 µg/well with the inhibition zone of 35.67 mm. The inhibition zone of Ampicillin against *S. aureus* was 36.33 mm showed that the inhibition zone of the combination of Ampicillin and plant extract was lower than the inhibition of Ampicillin alone. The inhibition zone of the combination of Ampicillin and plant extract with the concentration of 3000+50 µg/well and 4000+50 µg/well also slightly decreased which

were 34.33 mm and 35.67 mm respectively. The inhibition zone of the combination of Ampicillin with plant extract and Ampicillin alone shown by Figure 1 (G & H).

The highest inhibition of the combination of Ampicillin against *E. coli* was 28.00 mm, shown by the highest concentration of the combination which was 5000+50 µg/well. The inhibition zone 3000+50 µg/well and 4000+50 µg/well were 27.33 mm and 26.67 mm respectively. The inhibition zone of the combination of plant extract with Ampicillin against *E. coli* showed the increasing in the inhibition compared to the inhibition by Ampicillin alone which was 24.33 mm as shown in Figure 1 (I & J). The increasing of the inhibition zone of the combination of plant extract with Ampicillin compared to Ampicillin alone indicated there was no synergistic effect of the combination of *A. longiloba* and Ampicillin against *E. coli*.

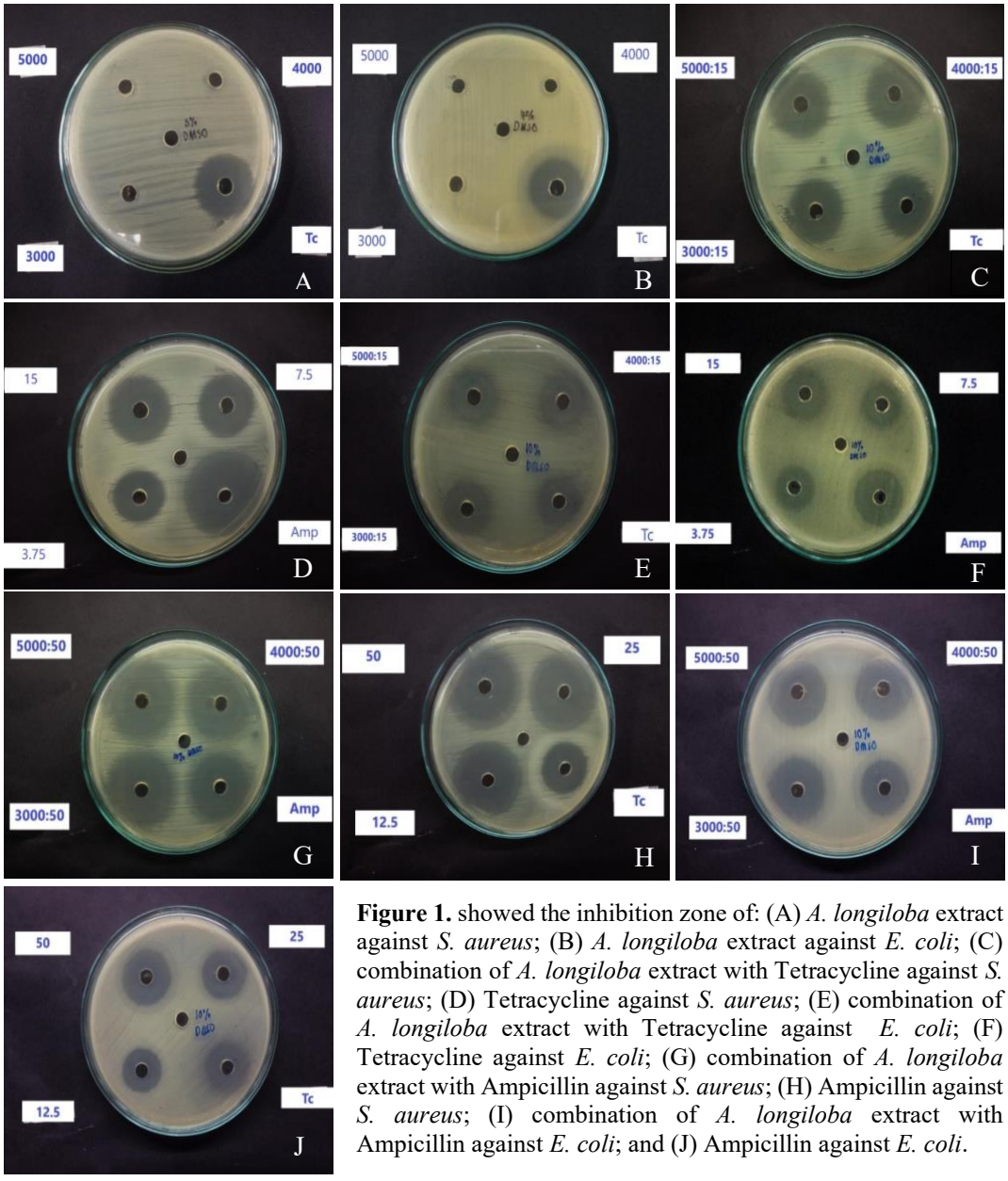

Figure 1. showed the inhibition zone of: (A) *A. longiloba* extract against *S. aureus*; (B) *A. longiloba* extract against *E. coli*; (C) combination of *A. longiloba* extract with Tetracycline against *S. aureus*; (D) Tetracycline against *S. aureus*; (E) combination of *A. longiloba* extract with Tetracycline against *E. coli*; (F) Tetracycline against *E. coli*; (G) combination of *A. longiloba* extract with Ampicillin against *S. aureus*; (H) Ampicillin against *S. aureus*; (I) combination of *A. longiloba* extract with Ampicillin against *E. coli*; and (J) Ampicillin against *E. coli*.

From the results, it can be concluded that the synergistic effect only happened with the combination of the Ampicillin and plant extract against *E. coli*. The other combination showed the same inhibition

as the antibiotic alone and some of the combination showed the decreasing of the inhibition zone when it was combined compared to the individual effects of the antibiotics.

The results also indicated that *S. aureus* are more sensitive to antibiotics compared to *E. coli*. This is because of gram positive bacteria does not have the outer membrane unlike the gram negative bacteria that will prevent certain antibiotic and drug from penetrating bacterial cell and make gram negative bacteria more resistant to antibiotic compared to gram positive bacteria [18, 19].

3.2. Minimal inhibitory concentration (MIC)

From the studied, the data obtained presented in Table 2 shows the MIC values are not detected for any treatments on *S. aureus*. Whereas, on *E. coli*, the MIC values only were detected for the treatments with Ampicillin (12.5 µg/ml) and combination of *A. longiloba* with Ampicillin (2000+12.5 µg/ml).

Table 2. The MIC of *A. longiloba,* antibiotics and their combination on bacteria.

Treatment	The Minimal Inhibitory Concentration (MIC) (µg/ml)	
	S. aureus	*E. coli*
A. longiloba extract	nd	nd
Tetracycline	nd	nd
Ampicillin	nd	12.5
A. longiloba + Tetracycline	nd	nd
A. longiloba + Ampicillin	nd	2000 + 12.5

*nd = not detected.

The MIC values on *S. aureus* cannot be detected for all treatments which were plant extract alone, Tetracycline alone, Ampicillin alone, the combination of plant extract and Tetracycline and lastly the combination of plant extract with Ampicillin.

The MIC values of the treatments on *E. coli*, only for the treatment Ampicillin alone and the combination of Ampicillin with plant extract showed the MIC values which were 12.5 µg/ml and 2000+12.5 µg/ml respectively. The other treatments which were plant extract, Tetracycline and combination of Tetracycline and plant extract showed no inhibition against the bacteria

The MIC value of treatment on *S. aureus* should be lower than *E. coli*, however in this study, the MIC value on *S. aureus* cannot be detected and there was MIC value on *E. coli*. *S. aureus* should be more susceptible to antibiotics compared to *E. coli* and showed lower resistance toward bacteria. The synergistic effect cannot be shown by both of the combination with Ampicillin and Tetracycline. The combination of the plant extract with antibiotic showed no reduction in the MIC value compared to the individual effects. Thus, no synergistic effect shown by the combination with both antibiotic against *S. aureus* and *E. coli*.

3.3. Fractional inhibitory concentration (FIC)

The FIC index for this study cannot be determined due to insufficient of MIC values. The only available MIC values were for Ampicillin and the combination of Ampicillin with *A. longiloba*. Thus, there is no synergistic effect of *A. longiloba* with Ampicillin and Tetracycline against *S. aureus* and *E. coli.*

4. Conclusions

In conclusion, medicinal plant has been used for century as the natural to the antibiotic remedy for the treatment for many diseases and found safe for human consumption and give less side effects compared to the antibiotic. The combination of plant extract and antibiotic is one of the approaches in combating the antibiotic resistance that will cause the difficult to treat or untreatable disease and cause mortality.

The combination of these two substances will decrease the effectiveness dosses of antibiotic and reduce the side effect to the consumers.

In this study, the synergistic effect of *A. longiloba* fruit extract were studied with the combination of Tetracycline and Ampicillin. *A. longiloba* is the medicinal important plant. However, in this study, it showed low inhibition against gram positive and gram negative bacteria. This plant extract showed no inhibition against *E. coli* and *S. aureus* when it tested alone and tested together with the antibiotics except for the combination of Ampicillin and plant extract against *E. coli*. The MIC value of this plant also cannot be detected against both of the bacteria and only the combination with Ampicillin against *E. coli* showed the MIC value.

References

[1] Zulhazman H, Hafzan-Eva M, Elvaene J, Muhammad-Firdaus A K and Aweng E R 2021 Phytogeographic study of araceae obligate to limestone hill forest in Kelantan, Malaysia *Malay. Nat. J.* **73**(2) 199-211.

[2] Zulhazman H, Aweng E R, Mohamad-Faiz M A, Muhamad-Azahar A, Kamarul-Ariffin H, Nor-Hizami H, Mohammad-Firdaus A K, Fiffy H S, Norhazlini M Z and Norzielawati S 2021 Diversity and ecology of araceae in the water catchment area of Ulu Sat, Kelantan, Peninsular Malaysia *IOP Conf. Ser.: Earth Environ. Sci.* **756** 012087.

[3] Zulhazman H, Norhazlini M Z and Boyce P C 2019 Notes on araceae in Pulau Pangkor, Perak, Peninsular Malaysia *Malaysian For.* **82**(1) 161-71.

[4] Zulhazman H, Norzielawati S and Boyce P C 2017 Studies on the Alocasia clade of Peninsular Malaya I: *Alocasia farisii*, sp. nov from Limestone in Kelantan *Nord. J. Bot.* **35** 300-304.

[5] Nascimento G G, Locatelli J, Freitas P C and Silva G L 2000 Antibacterial activity of plant extracts and phytochemical on antibiotic resistance bacteria *Braz. J. Microbiol.* **31** 247-256.

[6] Wagner H and Ulrich-Merzenich G 2009 Synergy research: Approaching a new generation of phytopharmaceuticals *Phytomedicine* **16** 97-110.

[7] Nur-Izzati M, Arifullah M, Nazahatul A A, Klaiklay S, Chumkaew P, Norhazlini M Z, Abdulhafiz F and Zulhazman H 2021 Elucidation of total phenolic content and antioxidant activity in medicinal aroid, *Alocasia longiloba* Miq. *IOP Conf. Ser.: Earth Environ. Sci.* **756** 012043.

[8] Gengaderan H 2016 *Phytochemical analysis and antioxidant properties of Keladi Candik (Alocasia longiloba) plant* BSc Project Report Universiti Malaysia Kelantan p 64.

[9] Williams C 2012 *Medicinal Plants in Australia: Plants, Potions and Poisons* (Vol. III, p. 118)(Online). EBL eBooks online p 472.

[10] Zulhazman H, Asraf Fizree M, Muhamad Azahar A, Mohd Fadzelly A B and Nazahatul Anis A 2021 A survey on edible aroids consumed by locals in Kelantan, Peninsular Malaysia *IOP Conf. Ser.: Earth Environ. Sci.* **736** 012076.

[11] Hamzah N H C, Mohammed A, Sirajudeen K N S, Asari M A, Hamzah Z and Shaik I K 2019 Keladi candik (*Alocasia longiloba* Miq.) petiole extracts promote wound healing in a full thickness excision wound model in rats *Asian Pac. J. Trop. Biomed.* **9**(4) 140.

[12] Abdulhafiz F, Mohammed A, Kayat F, Bhaskar M, Hamzah Z, Podapati S K and Reddy L V 2020 Xanthine oxidase inhibitory activity, chemical composition, antioxidant properties and GC-MS analysis of *Keladi Candik* (*Alocasia longiloba* Miq) *Molecules* **25**(11) 2658.

[13] Abdulhafiz F, Mohammed A, Kayat F, Zakaria S, Hamzah Z, Reddy Pamuru R and Reduan M F H 2020 Micropropagation of *Alocasia longiloba* Miq and comparative antioxidant properties of ethanolic extracts of the field-grown plant, in vitro propagated and in vitro-derived callus *Plants* **9**(7) 816.

[14] Chopra I and Roberts M 2001 Tetracycline antibiotics: Mode of action, applications molecular biology and epidemiology of bacterial resistance *Microbiol. Mol. Biol. Rev.* **65**(2) 232-260.

[15] Peechakara B V and Gupta M 2018 *Ampicillin/Sulbactam* StatPearls Publishing p 205.

[16] Adwan G and Mhanna M 2008 Synergistic effects of plant extracts and antibiotics on *Staphylococcus aureus* stains isolated from clinical specimens *Middle East J. Sci. Res.* **3**(3)

134-139.
[17] Elshikh M, Ahmed S, Funston S, McGaw M, Marchant R and Banat I M 2016 Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants *Biotechnol. Lett.* **38** 1015-1019.
[18] Delcour A H 2009 Outer membrane permeability and antibiotic resistance *Biochim. Biophys. Acta* **1794**(5) 808-816.
[19] Abas, M.A., Hambali, K.A., Hassin, N.H., Karim, M.F.A, Ismail, L., and Rosli, H. 2020 Antifungal Activity of Selected Malaysia's Local Medicinal Plants Against Sick Building Syndrome (SBS) Fungi. *Asian Journal of Plant Sciences*, 19, 240-245.