

Antibacterial activity of taro (*Colocasia esculenta* L.) leaves extracts against *Staphylococcus aureus* and *Vibrio parahaemolyticus* and its effect on microbial population in sardine (*Sardinella longiceps* Val.)

¹Padzil, K.N.M, ²Ayob, N.M, ²Alzabt, A.M. and ^{1,2,*}Rukayadi, Y.

¹Laboratory of Natural Medicine, Institute of Bioscience, Universiti Putra Malaysia, 43400, Serdang, Selangor Darul Ehsan, Malaysia

²Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400, Serdang, Selangor Darul Ehsan, Malaysia

Article history:

Received: 12 August 2020

Received in revised form: 17 September 2020

Accepted: 9 November 2020

Available Online: 27

February 2021

Keywords:

Colocasia esculenta L.,
Staphylococcus aureus,
Vibrio parahaemolyticus,
Microbial population,
Sardine

DOI:

[https://doi.org/10.26656/fr.2017.5\(2\).431](https://doi.org/10.26656/fr.2017.5(2).431)

Abstract

Colocasia esculenta L. which is popularly known in Malaysia as “keladi” has extensive traditional uses for certain of the plant part such as the leaves, corm and stem. An earlier study has reported on the analysis of its pharmacological properties such as anti-inflammatory and antibacterial activity. This study aimed to determine the antibacterial activity of *C. esculenta* L. leaves extracts against *Staphylococcus aureus* ATCC 29737 and *Vibrio parahaemolyticus* ATCC 17802 and its effect on the microbial population of sardine. The extract was tested for antibacterial activity against *S. aureus* and *V. parahaemolyticus* in term of disc diffusion assay (DDA), minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and killing time curve by using Clinical and Laboratory Standard Institute (CLSI) methods. DDA assay of the extract resulted in 8.40±0.10 mm and 8.20±0.16 mm of inhibition zone of the extract on *S. aureus* and *V. parahaemolyticus*, respectively. The extract can inhibit the growth of *S. aureus* and *V. parahaemolyticus* with MICs values of 1.25±0.00 and 0.31±0.00 mg/mL and can kill the bacteria with MBCs values of 2.50±0.00 and 0.63±0.00 mg/mL, respectively. The killing time analyses showed that the *S. aureus* and *V. parahaemolyticus* can be killed completely for 4 hrs at 2 × MIC (25.0 mg/mL) and 2 × MIC (6.25 mg/mL), respectively. Application of the extract on sardine samples shows significant reduction in total plate count (TPC), *S. aureus* and *V. parahaemolyticus* count after treated with 5.00% of the extract and stored at 4°C for 30 mins. Based on the findings, it indicates that *C. esculenta* L. can be listed as one of the alternatives natural sanitiser or preservative agent to reduce the bacterial loading of raw sardine prior to cooking.

1. Introduction

An illness caused by the consumption of food contaminated with pathogens has a wide economic and public health impact worldwide (Gandhi and Chikindas, 2007). Symptoms for foodborne illness include diarrhoea, abdominal pain, vomit, nausea and fever. Microorganisms such as *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus* and *Staphylococcus aureus* are often reported as causative agents of foodborne diseases. The uses of food preservative or food sanitiser in the food industry exist to combat the growth of microorganism as well as to extend the shelf life of food. However, some of the chemicals preservative or sanitiser are harmful for human consumption and increase the safety issues.

Therefore, the medicinal plant has the potential as an alternative and safe antibacterial agent for human and environment to reduce the occurrence of food spoilage caused by foodborne pathogens and spoilage microorganisms. *C. esculenta* L. also called “keladi” in Malaysia is one of the potential natural plants that have been traditionally used worldwide as treatment of various ailments, flavouring in cooking and food products. Over 255 genetic diversities of *C. esculenta* L. was studied by Kreike *et al.* (2004) and has been taken from various countries including Malaysia, Vietnam, Thailand, Indonesia, Philippines, Papua New Guinea and Vanuatu. According to Kubde *et al.* (2010), the young leaves of *C. esculenta* L. are rich in vitamin C while the roots are rich in starch-containing thiamine, riboflavin,

*Corresponding author.

Email: yaya_rukayadi@upm.edu.my

niacin, oxalic acid, calcium oxalate, sapotoxin and flavones, apigenin and luteolin. Anti-inflammatory, antibacterial and antifungal activities of *C. esculenta* L. was proven by several researchers which have contributed to its medicinal value and potential in the treatment of foodborne. Hence, the study is to determine the antibacterial activity of *C. esculenta* L. leaves extracts against *Staphylococcus aureus* ATCC 29737 and *Vibrio parahaemolyticus* ATCC 17802 in terms of disc diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and the time-kill assay and to evaluate the effect of ethanolic *C. esculenta* L. leaves extract on the number of microorganism of raw sardine at different concentration, exposure time and storage temperature.

2. Materials and methods

2.1 Plant extracts and sardine sample

Colocasia esculenta L. extraction procedure was conducted according to the methods by Metwally *et al.* (2010). An organic solvent which is absolute ethanol (R & M Chemicals, 99.8%) are used in the extraction of *C. esculenta* L. leaves. *C. esculenta* L. leaves (2 kg) were washed under running tap water, oven-dried at 40°C for 24 hrs and was stored at ambient temperature away from the light. Briefly, 100 g of dried leaves of *C. esculenta* L. was pulverized to a coarse powder. The samples were extracted with 400 mL of ethanol solvent for 48 hrs at room temperature with occasional shaking. Whatman filter paper No. 2 (Whatman International Ltd., Middlesex, England) was used to filter the plant extracts. Rotary vacuum evaporator (BUCHI Rotavapor R-200, Switzerland) used to concentrate the extracts at 40°C for three to four hrs whereby yielded ethanolic crude extract of *C. esculenta* L. The extract was diluted into a final concentration of 1% (w/v). All the crude extracts were subjected to freeze-drying for 48 hrs. Initially, 100 mg of ethanolic crude extract was diluted in 1 mL of 100% dimethyl sulfoxide (DMSO) (R & M Marketing, Essex, UK) to obtain 100 mg/mL and then further diluted in 1:10 (v/v) distilled water to get 10 mg/mL (10 000 µg/mL) stock solutions. *C. esculenta* L. leaves crude extract are collected and stored in the fridge (- 20°C) for use in further studies. While the raw sardine samples from groceries were bought at Seri Kembangan, Selangor. The samples were stored in the icebox at 4±2°C during transportation. The sardine samples were processed immediately in less than 1 hour at Food Microbiology Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia, Selangor, Malaysia upon arrival.

2.2 Bacterial strains and inoculum preparation

The antibacterial activity of *C. esculenta* L. leaves extract was evaluated against two bacterial species. They were *Staphylococcus aureus* and *Vibrio parahaemolyticus*, which were obtained from Bacteriology Food Safety Laboratory, Universiti Putra Malaysia, Selangor, Malaysia. The references control used throughout the study was *S. aureus* ATCC 29737 and *V. parahaemolyticus* ATCC 17802 from the American Type Culture Collection (Rockville, MD, USA). All the test strains were sub-cultured on Muller Hinton Agar (MHA) (Difco Becton Dickinson, Sparks, MD, USA), incubated at 37°C for 24 hrs and stored at 4°C in the refrigerator to maintain stock culture.

2.3 Preparation of Mueller Hinton agar/ broth media

Media preparation was conducted according to the manufacturer instructions provided on the media bottle (Oxoid Ltd., United Kingdom). Mueller-Hinton agar (MHA) was prepared by dissolving 10.5 g of Mueller-Hinton broth powder (Oxoid Ltd., United Kingdom) with 8.5 g of agar powder into 500 mL deionized water in 500 mL Schott's bottle. The solution sterilized using autoclave for 15 mins at 121°C. Then, the sterilized agar was poured until it covers the surface of sterile petri plates. The petri plates were left at room temperature until solidified. The plates were then kept at 4°C until further use. Mueller-Hinton broth (MHB) was prepared by dissolving 5.25 g of Mueller-Hinton broth powder (Oxoid Ltd., United Kingdom) with 250 mL deionized water in 250 mL Schott's bottle. The solution sterilized using autoclave for 15 mins at 121°C. Then, the sterilized solution was kept at 4°C until further use.

2.4 Disc diffusion assay

The initial determination of the antibacterial activity of the ethanolic extract of *C. esculenta* L. leaves was conducted by screening the susceptibility of the bacterial isolates to the plant extract and chlorhexidine (CHX) was used as a control by following the Kirby-Bauer diffusion method (Bauer *et al.*, 1966). An overnight culture of each strain was spread on the Muller Hinton agar (Difco Becton Dickinson, Sparks, MD, USA) using a sterile cotton swab. Sterile paper disc (6 mm) was impregnated with 10 µL of 5% plant extract. A 0.1% of chlorhexidine (CHX) was used as positive control and was prepared by dissolving 10 mg of CHX (Sigma Aldrich, USA) sample in 1 mL of distilled water to make it 1% of CHX (stock solution). Then 100 µL from stock solution was dissolved in 900 µL of distilled water to make the final concentration of chlorhexidine into 0.1%. The 0.1% of chlorhexidine was included as positive control while

10% of DMSO was used as the negative control. The discs were placed with equal distance between them on the agar plates. All the plates were then incubated at 37°C for 24 hrs. The experiment was carried in three times in duplicates and the diameter of zones of inhibition formed was measured and their average determined using the method as reported by Clinical and Laboratory Standards Institute (CLSI, 2012). A clear inhibition zone around the impregnated disc was the indication of the presence of antibacterial.

2.5 Determination of minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of *C. esculenta* L. leaves extract for each isolate was performed on MHB according to Clinical and Laboratory Standard Institute M7-A9 (CLSI, 2012) with a slight modification. This test was performed in 96-well microtiter plate using a two-fold standard broth microdilution method. A two-fold dilution of *C. esculenta* L. leaves extracts stock solution mixed with the bacterial isolates in MHB was filled in the 96-well microtiter. The first columns of the wells were filled with 200 µL of MHB which served as negative control growth. The second columns, positive control growth columns were filled with 200 µL of the bacterial suspension. The microdilution was performed at concentration ranged from 5 mg/mL in column twelve to 0.01 mg/mL in column three. The microtiter plate was then incubated at 37°C for 24 hrs. According to Talaro and Chess (2012), the MIC can be interpreted as the lowest concentration of the antibacterial agent that visually inhibits the growth of microorganisms after overnight incubation. The first well with no visible growth after the incubation period was taken as the MIC.

2.6 Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) is the minimal concentration of antibacterial that kills the inoculums and can be determined from broth dilution MIC test by subculturing into MHA medium (approximately of 10 µL) without any antibacterial agents (French, 2006; Rukayadi et al., 2009). A volume of 10 µL from all the wells from column one to twelve was pipetted onto the agar plates. The plate was then incubated at 37°C for 24 hrs. MBC is defined as the lowest concentration with no visible growth on MHA plates or the lowest concentration of antibacterial agent whereby the *S. aureus* and *V. parahaemolyticus* in the culture were completely killed.

2.7 Time-kill assay

The time-kill assay study of *C. esculenta* L. leaves extract was carried out to assess the killing rate of the extract within a given contact time. In this study, the assay was done according to the CLSI reference method, with slight modification. The extract of *C. esculenta* L. leaves with different concentration (0 × MIC, 0.5 × MIC, 1 × MIC, 2 × MIC, 4 × MIC) were mixed with inoculum. All isolates were incubated at 37°C with 150 rpm agitation at predetermined time points (0, 15 and 30 mins as well 1, 2 and 4 hrs). Aliquots of 100 µL were removed from each concentration at each of the time points and spread on the MHA medium. The plate was then incubated at 37°C for 24 hrs. After incubation, the agar plates were observed visually for the presence of growth. The number of colonies formed on the plate was counted and the number was recorded as log₁₀ (CFU/mL). Assays were carried out two times in duplicate. Time-kill curves were constructed by plotting the log₁₀ CFU/mL versus time (Rukayadi et al., 2009).

2.8 Effect of *Colocasia esculenta* L. leaves extract on the number of microorganisms of raw sardine at different concentration, exposure time and storage temperature

The raw sardine sample was treated to the ethanolic extract of *C. esculenta* L. leaves at different concentration of 5.00%, 0.50%, 0.05%, 0.00% and sterile tap water. These treatments were exposed in 0 min, 15 mins and 30 mins at room temperature (22±2°C) and chiller (4±2°C).

2.8.1 Preparation of ethanolic *Colocasia esculenta* L. leaves extract at different concentration

Preparation of *C. esculenta* L. extract for raw sardine treatment was conducted by using the method reported by Yusoff et al. (2015) with some modification. In this treatment, the sterile de-ionized water (DIW) (B Braun Medical Industries, Penang, Malaysia) was used to prepare different concentration of *C. esculenta* L. extract (5.00%, 0.50% and 0.05%) to be used later, in the application to raw sardine samples. A 5 g of *C. esculenta* L. leaves extract was diluted in 50.0 mL of DIW to make 10% concentration of *C. esculenta* L. extract. The 5.0% concentration was prepared by pipetted 12.5 mL of the 10% extract concentration and diluted with 12.5 mL of DIW. A 22.5 mL DIW was added into 2.5 mL of 5% extract concentration to make the extract concentration of 0.50%. For final concentration 0.05% preparation, 2.5 mL of 0.5% extract concentration was diluted to 22.5 mL of DIW. Each concentration solution was prepared in a total of 25 mL.

2.8.2 Media enumeration for total plate count (TPC), *Staphylococcus aureus* and *Vibrio parahaemolyticus*

Total plate count (TPC) is considered as a gold standard method permits the bacterial growth to form a colony (Davey, 2011). In preparation of media enumeration, for TPC, a 17.5 g of Plate Count Agar (PCA) (OXOID, Hampshire, United Kingdom) was added to 1 L of distilled water for, for *S. aureus*, 108 g Mannitol Salt Agar (MSA) (OXOID, Hampshire, United Kingdom) was suspended in 1 L of distilled water, meanwhile for *V. parahaemolyticus* of 88.1 g of Thiosulfate Citrate Bile Salts Sucrose Agar (TCBSA) (OXOID, Hampshire, United Kingdom) was suspended in 1 L of distilled water. PCA and MSA was sterilized by autoclaving at 121°C for 15 mins. TCBSA was slowly bringing to boiling, stirring, with constant agitation until complete dissolution. TCBSA was transferred immediately to a water bath at 50°C. The sterilized solution was left to be cooled down before being poured into sterile Petri dish. An aliquot (0.1 mL) of each dilution was spread on TPC agar. The logarithm numbers of colony forming unit per grams (\log_{10} CFU/g) of samples was calculated by observing and enumerating the total colonies (for TPC), yellowish colonies with yellow zones (for *S. aureus*) and colonies with blue to green centers (for *V. parahaemolyticus*) formed after incubation at 35°C for 48 hrs. After incubation, the macroscopic colonies grow on the plate were counted and calculated into CFU/g of the sample, by dividing the number of colonies with the amount of plated sample volume that has been multiplied with the dilution factor. The calculated amounts were then converted to \log_{10} CFU/g for the easiness of comparative purpose. All analysis was performed in two times in duplicate for data verification.

2.8.3 Treatment of sardine sample with *Colocasia esculenta* L. leaves extract

The raw sardine sample was cut and weighed for 5 g. Each samples were treated with different concentrations of *C. esculenta* L. leaves extract (0.00%, 0.05%, 0.50% and 5.00%) and deionized water (DIW) in 4 mL volume at different exposure time of 0, 15 and 30 mins. The treated samples were kept individually in sterile universal bottles as in Figure 3.6. All the samples were then stored at two different temperatures of 22±2°C and 4±2°C. Next, the serial dilutions was done by pipetted 10 µL from each treatment and diluted into 990 µL of phosphate buffer saline (0.1%) to create 10⁻² and 10⁻⁴ dilution. A 10 µL from each dilution series were spread on the selective agar as previously mentioned. The agar plates were incubated as according to the requirement of respective media used. The logarithm numbers of colony forming unit per grams (\log_{10} CFU/g) of samples were

calculated by observing and enumerating the presumptive colonies formed after incubation. Referring to Yusoff *et al.* (2015), the experiments were done two times with duplicate ($n = 2 \times 2$).

2.9 Statistical data analysis

The means value of microbial populations (\log_{10} CFU/g) from each treatment series were calculated for total plate count, *S. aureus* and *V. parahaemolyticus* count in a duplicate manner on each experiment. Minitab ® Version 16 for Windows (Minitab Inc.) application software was used to analyze the data for variance (One-Way ANOVA), to find out the significant differences among the means for each treatment. The Tukey's test was used to determine the significant effect at 95% confidence level ($p < 0.05$) between the factors; treatment group, exposure time and storage temperature. The results were expressed as mean±standard deviation (SD) of duplicate analyses.

3. Results and discussion

3.1 Antibacterial activities

According to the principle of DDA, a large inhibition zone or ring will form around the disc after incubation indicating great of antibacterial activity. The effect of the ethanolic extract of *C. esculenta* L. leaves and stem against *S. aureus* and *V. parahaemolyticus* were identified and the results the inhibition zone were summarized in Table 1.

Table 1. DDA of *C. esculenta* L. extracts against *S. aureus* and *V. parahaemolyticus*

Testing Organisms	Inhibition Zone (mm)	
	<i>S. aureus</i> ATCC 297370	<i>V. parahaemolyticus</i> ATCC 17802
Control (Chlorhexidine)	10.8±0.6 ^a	10.1±0.2 ^a
<i>C. esculenta</i> L. stem extract	8.1±0.1 ^b	7.4±0.1 ^{bc}
<i>C. esculenta</i> L. leaf extract	8.4±0.1 ^b	7.7±0.1 ^b

Values are expressed as mean±standard deviation. Values with the different superscript within the row are significantly different at $P < 0.05$.

The result from Table 1 shows that the ethanolic extract of *C. esculenta* L. leaves was potent compared to stem extract with an inhibition zone of 8.4±0.1 and 7.7±0.1 against *S. aureus* and *V. parahaemolyticus*, respectively. The principle of the disc-diffusion method is the larger the inhibition zone meaning has a greater antibacterial activity. The result for *C. esculenta* L. leaves extract was found to be statistically significant when compared with the standard control, chlorhexidine where the P-value is 0.00 which is ($p < 0.05$).

S. aureus is one of the most encountered pathogens

in health concern and is known to have strong resistance against many conventional antibiotics. In a previous study by Dutta *et al.* (2017) reported that the antibacterial activity of ethanolic extract of *C. esculenta* against *S. aureus* showed the inhibition zone of 8.24 ± 0.66 mm. A research done by Ehiobu *et al.* (2018), they analyzed quantitatively for selected phytochemicals, such as tannins, flavonoids, alkaloids, saponins, steroids, phenols and cardiac glycosides and found that the flavonoids and tannins extracts have also been reported to possess antioxidants and antibacterial properties, at which its antifungal activities is the highest. This was supported by Wafa *et al.* (2016), reported that alkaloids present in most stem barks, leaves and roots of plants have long been recorded to possess potent antibacterial properties.

The antibacterial activity of *C. esculenta* L. leaves extract on *V. parahaemolyticus* has been reported in several studies. In one study by Wei *et al.* (2008), the ability of *C. esculenta* L. leaves extract to inhibit the growth of all tested *Vibrio* spp. and *C. freundii* was found. In this study, the largest inhibition zone with a diameter of 12mm was recorded against *Vibrio harveyi*, and 9 mm *V. parahaemolyticus*. Among all the five *Vibrio* spp. used (*V. cholera*, *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi* and *V. vulnificus*). *V. vulnificus* showed the least inhibition zone diameter which is 8 mm when treated with the *C. esculenta* L. leaves extract, while the stem and legume extract shows no inhibition zone in the DDA test.

It is proved that the medicinal plant like *C. esculenta* L. leaves possesses strong antibacterial properties, which very potential to be listed as one of the alternatives natural sanitiser or preservative agent to reduce the bacterial loading of raw sardine prior cooking. Othman *et al.* (2011) claimed that the DDA test is not so optimal for fully access the antibacterial activity since there was a restriction problem. This is because the data obtained from DDA is inadequate as the assay provides only qualitative data and acts as preliminary screening (Smith-Palmer *et al.*, 1998). Therefore, through the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values, the quantitative analyses of the *C. esculenta* L. leave extract on the antibacterial activity were obtained.

3.2 Minimum inhibitory concentration and Minimum bactericidal concentration of *Colocasia esculenta* L. leaves extract against *Staphylococcus aureus* and *Vibrio parahaemolyticus*

The antibacterial activity of *C. esculenta* L. leaves extract was further confirmed by the determination of MIC and MBC. As mentioned by Talaro and Chess

(2012), MIC is the lowest concentration of the antibacterial agent that visually inhibits the growth of microorganisms after overnight incubation. Meanwhile, MBC is standing for the lowest concentration of antibacterial agent that completely killed the growth of culture after endpoint (12 – 24 hrs).

Table 2 represents the comparative MIC and MBC assessment of *C. esculenta* L. leaves extract, positive and negative control against *S. aureus* and *V. parahaemolyticus*. The results in Table 2 revealed that ethanolic extract of *C. esculenta* L. leaves exhibit antibacterial activity against all the tested microorganisms. The extract was found to inhibit the growth of *S. aureus* and *V. parahaemolyticus* at MIC value of 12.50 mg/mL and 3.13 mg/mL, respectively. The MBC values also have been obtained in this study with 25.00 mg/mL and 6.25 mg/mL for *S. aureus* and *V. parahaemolyticus*, respectively. According to Nduche *et al.* (2016), there were variations in the activity of MIC of ethanolic *C. esculenta* L. extract against *S. aureus* at a different concentration ranging from 50 mg/mL to 200 mg/mL. In addition, Ramli *et al.* (2017) reported that *L. monocytogenes* and *S. aureus* were found to be the most susceptible pathogen against *S. polyanthum* L. extract with the MIC value of 0.63 mg/mL.

Table 2. MIC and MBC of *C. esculenta* L. leaves extract against *S. aureus* and *V. parahaemolyticus*

	Concentration (mg/mL)			
	<i>S. aureus</i> ATCC 29737		<i>V. parahaemolyticus</i> ATCC 17802	
	MIC	MBC	MIC	MBC
Positive Control (Chlorhexidine)	0.098	0.195	0.195	0.39
Negative Control (DMSO)	0	0	0	0
<i>C. esculenta</i> L. leaves extract	12.5	25	3.13	6.25

In the previous study by Ramli *et al.* (2017) showed that the *V. cholerae* and *V. parahaemolyticus* had the same MIC and MBC values (1.25 mg/mL), meaning these bacteria can be inhibited and killed at the same concentration of *S. polyanthum* L. extract. This result showed that Gram-positive bacteria were easier to inhibit compared to Gram-negative ones. Gram-negative bacteria have a hydrophilic outer membrane rich in lipopolysaccharide molecules. Therefore, it serves as a penetration barrier towards macromolecules (Witkowska *et al.*, 2013). Analysis of the phytochemical constituents for *C. esculenta* L. leaves extract was confirmed in the presence of tannins, flavonoids, alkaloids, saponins, steroids, phenols and cardiac glycosides. Ehiobu *et al.* (2018) reported that folic acid alkaloids, alkylresorcinols, glycosides, phenolics, and resins (Chandra *et al.*, 2012)

are considering the reports of previous research, they could probably be the bioactive ingredients responsible for the observed antibacterial activities (Saad *et al.*, 2014; Agyare *et al.*, 2016; Adeosun *et al.*, 2016; Gwa *et al.*, 2017; Anyanwu *et al.*, 2017; Ehiobu *et al.*, 2018).

3.3 Time-kill assay

Generally, to evaluate the effect of antibacterial activities over time, the time-kill curves that monitor bacterial growth and death over a wide range of antibacterial concentrations has been frequently used. Figures 1 and 2 are the representative of the time-kill curve graph for the bacteria *S. aureus* and *V. parahaemolyticus*, respectively. In the time-kill assay, the results were presented in terms of the changes in the \log_{10} CFU/mL of viable colonies which indicated a significant bactericidal activity, exhibited by the extract. According to Olajuyigbe and Afolayan (2012), once the viable colony counts were equal to $3 \log_{10}$ CFU/mL or have a greater reduction, it was deliberated to have bactericidal activity.

The result for the time-kill curve of the *C. esculenta* L. leaves extract against *S. aureus* is plotted and presented as in Figure 1. At $2 \times$ MIC (25.0 mg/mL) and $4 \times$ MIC (50.0 mg/mL), *S. aureus* was completely killed, after 4 hrs and 2 hrs of incubation time, respectively. From the results at $4 \times$ MIC concentrations, the bacterial activity of *S. aureus* decreases 99.9% ($\geq 3 \log_{10}$) of the total CFU/mL in the original inoculum after 2 hrs incubation. The results showed that *C. esculenta* L. leaves extract can kill the strain completely after 4 hrs of incubation time at a concentration of $2 \times$ MIC (25.0 mg/mL). While Ramli *et al.* (2017) had reported the bactericidal activity of *S. polyanthum* leaves to extract on *S. aureus* at $4 \times$ MIC (2.5 mg/mL) after 4 hrs of incubation time.

The result for the time-kill curve of *C. esculenta* L. leaves extract against *V. parahaemolyticus* ATCC 17802 is plotted and presented in Figure 2. At $2 \times$ MIC (6.25

mg/mL) and $4 \times$ MIC (12.5 mg/mL) of concentration, all *V. parahaemolyticus* were completely killed, after 4 hrs and 2 hrs of incubation time, respectively. From the results at $4 \times$ MIC concentrations, the bacterial activity of *V. parahaemolyticus* decreases 99.9% ($\geq 3 \log_{10}$) of the total CFU/mL in the original inoculum after 2 hrs incubation. The results show that *C. esculenta* L. leaves extract can kill the strain completely after 2 hrs of incubation time at a concentration of $4 \times$ MIC (12.5 mg/mL). In contrast, Ramli *et al.* (2017) had reported the bactericidal activity of *S. polyanthum* leaves extract on *V. parahaemolyticus* at $4 \times$ MIC (5.0 mg/mL) for 4 hrs of incubation time. In addition, Penduka and Okoh (2012) reported that 69% of *V. parahaemolyticus* was killed at 5 mg/mL after 2-hour incubation using Garcia kola seed methanol extract.

The results showed that *C. esculenta* L. leaves extract can kill *S. aureus* and *V. parahaemolyticus* at concentration $2 \times$ MIC (25.0 mg/mL) and $2 \times$ MIC (6.25 mg/mL) after 4 hrs of incubation time, respectively. Therefore, this study revealed that the bactericidal effect of the plant extract was less effective to kill *S. aureus* compared to *V. parahaemolyticus*, as it required higher extract concentration. Furthermore, *S. aureus* was Gram-positive bacteria where the membrane structure was easier to disrupt compared to Gram-negative bacteria. Increasing of plant extract's concentration will lead to diffusion into membrane cell thus causing membrane destruction (Thangiah *et al.*, 2013).

3.4 Effect of ethanolic *Colocasia esculenta* L. leaves extract on raw sardine samples

The most important part of any food safety plan and quality control are the detection and enumeration of microorganisms in the food. These conventional test methods are simple, very practical, easily adaptable, and generally inexpensive. However, they can be difficult and depend on the growth of the microorganisms in different culture media (pre-enrichment, selective

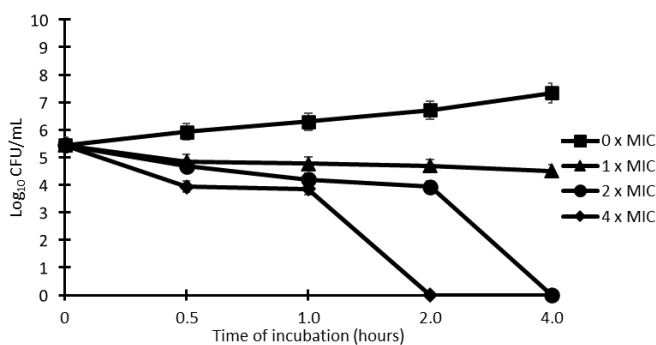


Figure 1. Time-kill curve plot of *S. aureus* ATCC 29737 for $0 \times$ MIC, $0.5 \times$ MIC, $1.0 \times$ MIC, $2.0 \times$ MIC and $4.0 \times$ MIC of ethanolic *C. esculenta* L. leaves extracts at 0 to 4 hrs incubation time

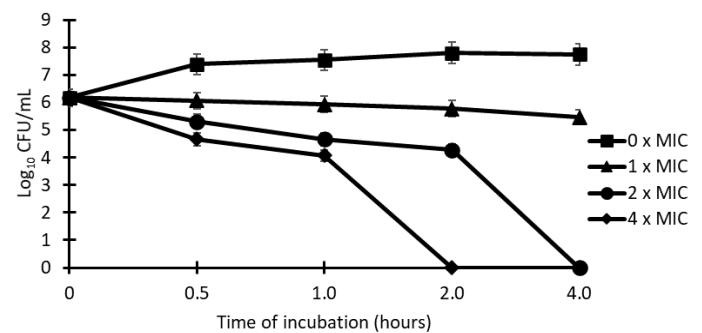


Figure 2. Time-kill curve plot of *V. parahaemolyticus* ATCC 17802 for $0 \times$ MIC, $0.5 \times$ MIC, $1.0 \times$ MIC, $2.0 \times$ MIC and $4.0 \times$ MIC of ethanolic *C. esculenta* L. leaves extracts at 0 to 4 hrs incubation time

enrichment, selective plating, identification), which may require several days before results are known even not lacking sensitivity. Generally, cooling or refrigeration can only maintain the fish products freshness, but the microorganisms will not be eliminated, killed or stopped any enzymatic activity. This fact was supported by Ashie *et al.* (1996), which most biochemical spoilage process can be slow down at a lower temperature. Recently, antibacterial agents are frequently used in perishable products such as raw fish for the inhibition of growth of bacteria. In this study, *C. esculenta* L. leaves extract was used as an antibacterial agent to investigate the correlation between different concentrations of the extract with the storage of raw sardine samples.

3.5 Total plate count (TPC) of sardine sample

The result for TPC of sardine samples stored at room temperature and chiller were presented in Table 3. The findings show significant reduction of TPC (\log_{10} CFU/g) for the treated samples from 0.05%, 0.50% to 5.00% concentrations of *C. esculenta* L. leaves extract accordingly, after 15 and 30 minute exposure time, while the TPC significantly increased when treated in 0.00% concentration. From Table 3, the TPC for samples treated at 0 min with all the treatment exceeded 10^6 CFU/g in both storage temperature (room temperature and chiller). When stored in the chiller, the microbial counts were significantly lower in each treatment and every exposure time, compared when stored at room temperature.

At high concentration, the extract has higher chances to express its antibacterial activity towards the bacterial loading on the sardine samples. Thus, fewer bacteria were survived at higher extract concentration (5.00%) compared to a lower concentration (0.05%). According to Unklesbay *et al.* (1992), due to the enzymatic and microbial spoilage caused by improper onsite storage, around 4-5 million tons of trawled and shrimp fish are lost every year. Thus, in preventing the spoilage of fish product, one of the most important factors is by

controlling the storage temperature (Jay, 2000). Briefly, spoilage can be prevented by storing foods at or below 4°C, using them immediately or storing them for longer periods at -18°C or below. Chilling was recognized as one of the cold preservation that preserves the nutritional and sensory value of the food, but the temperature does not eliminate the harmful microorganism (Hiramatsu *et al.*, 2005). According to Speck and Ray (1977), the lower temperature is able to control the microbiological activity during frozen by limiting the microbial growth and water activity (Aw). In this study, with the addition of antibacterial activity of *C. esculenta* L. leaves extract at 5.00% concentration, the bacterial count were decreasing significantly upon storage at 4°C, compared with 0.05% extract concentration.

3.6 *Staphylococcus aureus* count of sardine sample

S. aureus is commonly found in the environment (soil, water and air) and is also found in the nose and on the skin of humans. According to Argudin *et al.* (2010), the main sources of food contaminations are from the food handlers which carrying enterotoxin-producing *S. aureus* in their noses or on their hands via direct contact or through respiratory secretions. Even though *S. aureus* is a poor competitor, but it is able to grow under osmotic and pH stress, meaning that it is capable of thriving in a wide variety of foods. The result for *S. aureus* count of raw sardine samples treated with different concentration of *C. esculenta* L. leaves extract, stored at room temperature and chiller were presented in Table 4. From the result, the sample treated with extract concentration of 0.05%, 0.50% and 5.00%, showed a significant reduction of *S. aureus* (\log_{10} CFU/mL) after 30 mins exposure in the chiller.

From Table 4, upon chilling at 30 min exposure time, no *S. aureus* was able to survive when treated with 0.50% and 5.00% concentration of *C. esculenta* L. extract. In this study, *C. esculenta* L. leaves extract express their antibacterial activity on the treated samples at an exposure time of 15 min, where 0.50% and 5.00%

Table 3. Total plate count (\log_{10} CFU/g) of sardine sample treated with *C. esculenta* L. leaves extract stored at room temperature and chiller for 0, 15 and 30 minutes exposure time

Temperature Storage	Exposure Time	Extract Concentration (%)				
		Tap Water	0	0.05	0.5	5
Room Temperature	0 min	6.56±0.04 ^{Aa}	6.46±0.05 ^{aA}	6.50±0.11 ^{aA}	6.26±0.008 ^{aA}	6.37±0.07 ^{aA}
	15 min	6.37±0.10 ^{abAB}	6.59±0.19 ^{aA}	6.42±0.08 ^{abA}	6.12±0.11 ^{bcA}	5.92 ±0.16 ^{cB}
	30 min	6.34±0.01 ^{Ab}	6.64±0.05 ^{aA}	6.37 ±0.06 ^{aA}	5.91±0.17 ^{bA}	5.79±0.02 ^{bB}
Chiller	0 min	6.56±0.04 ^{Aa}	6.46±0.05 ^{aA}	6.50±0.11 ^{aA}	6.26±0.008 ^{aA}	6.37±0.07 ^{aA}
	15 min	6.30±0.09 ^{Aa}	6.53±0.07 ^{aA}	6.37±0.05 ^{aA}	5.92±0.11 ^{bAB}	5.72 ±0.02 ^{bB}
	30 min	6.27±0.06 ^{Ba}	6.60±0.05 ^{aA}	6.12 ±0.10 ^{bA}	5.77±0.02 ^{cB}	5.44±0.05 ^{dC}

Values are expressed as mean±standard deviation. Values with different lowercase superscript within the row are significantly different at P<0.05 while value with different uppercase superscript within the column are significantly different at P<0.05.

Table 4. *S. aureus* count (log₁₀ CFU/g) of sardine sample treated with *C. esculenta* L. leaves extract stored at room temperature and chiller for 0, 15 and 30 minute exposure time

Temperature Storage	Exposure Time	Extract Concentration (%)				
		Tap Water	0	0.05	0.5	5
Room Temperature	0 min	6.10±0.12 ^{Aa}	6.12±0.09 ^{aA}	6.02±0.00 ^{aA}	6.10±0.12 ^{aA}	6.12±0.11 ^{aA}
	15 min	5.90±0.21 ^{Aa}	6.14±0.09 ^{aA}	5.89±0.20 ^{aA}	5.83±0.07 ^{abB}	5.87 ±0.19 ^{aA}
	30 min	5.77±0.01 ^{Aa}	6.26±0.07 ^{aA}	5.89 ±0.16 ^{aA}	5.78±0.03 ^{abB}	5.40±0.56 ^{aA}
Chiller	0 min	6.10±0.12 ^{Aa}	6.12±0.09 ^{aA}	6.02±0.00 ^{aA}	6.10±0.12 ^{aA}	6.12±0.11 ^{aA}
	15 min	5.86±0.20 ^{aA}	6.14±0.11 ^{Aa}	5.89±0.19 ^{aA}	5.74±0.01 ^{abB}	5.82 ±0.05 ^{abB}
	30 min	5.74±0.02 ^{baA}	6.21±0.00 ^{Aa}	5.86±0.19 ^{abA}	ND ^{cC}	ND ^{cC}

Values are expressed as mean±standard deviation. Values with different lowercase superscript within the row are significantly different at P<0.05 while value with different uppercase superscript within the column are significantly different at P<0.05. ND, not detected.

extract concentration showed a reduction in the microbiological count, less than 106 CFU/g which were 5.74±0.01 CFU/g and 5.82±0.05 CFU/g, respectively when stored in chiller. According to Hiramatsu *et al.* (2005), the chilling temperature does not eliminate the harmful microorganism, because as soon as the temperature becomes favourable, the spoilage microorganism is able to survive and resumed their activity. According to Casarin *et al.* (2009), the temperature is able to inactivate part of the bacterial population, and the magnitude of its effect will vary according to the type of food. In this study, the antibacterial effect of *C. esculenta* L. leaves extract was enhanced with the cold storage temperature (4°C).

3.7 *Vibrio parahaemolyticus* count of sardine sample

In the study by Liu *et al.* (2009) summarized that the number of reported *V. parahaemolyticus* infections in Asia has increased significantly by year. This was supported by Harth *et al.* (2009) and Chang *et al.* (2011) which reported the most common *Vibrio* species associated with illnesses were *V. parahaemolyticus* and *V. vulnificus* that resulted from consumption of raw or partially cooked seafood. Table 5 shows the details for *V. parahaemolyticus* count for the treated samples with

different concentration of *C. esculenta* L. leaves extract, stored at room temperature and chiller. Samples treated with tap water, 0.05%, 0.50% to 5.00% of extract concentration showed a significant reduction of *V. parahaemolyticus* (log₁₀ CFU/mL) after 30-minute exposure, stored in the chiller.

Samples that stored both at room temperature and chiller, the *V. parahaemolyticus* count showed reduction accordingly with increasing of the extract concentration (0.05%, 0.50% and 5.00%). The most significant decrease in bacterial count occurred in the sample treated with 0.50% and 5.0% of extract concentration that can be seen when stored in the chiller, which able to kill *V. parahaemolyticus* as *V. parahaemolyticus* colonies did not grow over TCBS agar with a decrease of 5.0 log₁₀ CFU/mL for both concentrations. In this study, application of the extract on the raw sardine samples significantly reduce the *V. parahaemolyticus* count through the antibacterial activity from the *C. esculenta* L. leaves extract that enhance by low storage temperature.

4. Conclusion

C. esculenta L. leaves extract scientifically reported with numerous beneficial phytochemicals that proved to have high antibacterial activity against *S. aureus* and *V.*

Table 5. *V. parahaemolyticus* count (log₁₀ CFU/g) of sardine sample treated with *C. esculenta* L. leaves extract stored at room temperature and chiller for 0, 15 and 30 minute exposure time

Temperature Storage	Exposure Time	Extract Concentration (%)				
		Tap Water	0	0.05	0.5	5
Room Temperature	0 min	5.36±0.53 ^{aA}	5.30±0.60 ^{Aa}	5.27±0.65 ^{aA}	5.30±0.60 ^{aA}	5.28±0.61 ^{aA}
	15 min	4.81±0.16 ^{baA}	5.73±0.01 ^{Aa}	4.86±0.02 ^{baA}	4.87±0.08 ^{baA}	4.74±0.27 ^{baA}
	30 min	4.62±0.11 ^{abA}	5.74±0.00 ^{Aa}	4.62 ±0.11 ^{abA}	4.30±0.42 ^{baA}	4.04±0.50 ^{baA}
Chiller	0 min	5.36±0.53 ^{aA}	5.30±0.60 ^{Aa}	5.27±0.65 ^{aA}	5.30±0.60 ^{aA}	5.28±0.61 ^{aA}
	15 min	4.50±0.14 ^{baA}	5.72±0.00 ^{Aa}	4.57±0.04 ^{baA}	4.20±0.28 ^{baA}	4.09±0.12 ^{baA}
	30 min	4.09±0.13 ^{baA}	5.73±0.01 ^{Aa}	3.85 ±0.21 ^{baA}	ND ^{cB}	ND ^{cB}

Values are expressed as mean±standard deviation. Values with different lowercase superscript within the row are significantly different at P<0.05 while value with different uppercase superscript within the column are significantly different at P<0.05. ND, not detected.

parahaemolyticus. Sardine samples treated with the extract stored at room temperature and chiller resulted in the continuous reduction of the bacterial count for both *S. aureus* and *V. parahaemolyticus* and showed bacterial count less than 10⁶ CFU/g; which is the safe microbial limits for fish and fish product. Among all the treatment, the optimum condition for sample storage can be achieved by using 5.00% extract concentration stored at 4°C for 30 mins. From the findings, it showed that *C. esculenta* L. leaves extract exhibits the potential natural antibacterial candidate as a food natural sanitiser or preservative agent to reduce the bacterial loading of raw sardine prior to cooking. Therefore, the reduction of microbial loading is very important in ensuring the food safeness as well as reducing the occurrence of foodborne poisoning associated with perishable product such as fish. The antibacterial mechanisms of the compound in the *C. esculenta* L. leaves extract can be further studied by referring to the results obtained in this study so that the benefits of the plants can be exploited.

Conflict of interest

The authors declare no conflict of interest.

References

- Adeosun, O.M. (2016). Antibacterial activities and phytochemical properties of extracts of *Dioscorea bulbifera* Linn (Air Potato) tubers and peels against some pathogenic bacteria. *Journal of Phytopharmacology*, 5(1), 20-26.
- Agyare, C. (2016). Antimicrobial and anti-inflammatory properties of *Anchomanes difformis* (Bl.) Engl. and *Colocasia esculenta* (L.) Schott. *Biochemical Pharmacology*, 5, 201. <https://doi.org/10.4172/2167-0501.1000201>
- Anyanwu, M.U. and Okoye, R.C. (2017). Antimicrobial activity of Nigerian medicinal plants. *Journal of Intercultural Ethnopharmacology*, 6(2), 240-259. <https://doi.org/10.5455/jice.20170106073231>
- Argudin, M.A., Mendoza, M.C. and Rodicio, M.R. (2010). Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins*, 2(7), 1751–1773. <https://doi.org/10.3390/toxins2071751>
- Ashie, I.N.A., Smith, J.P. and Simpson, B.K. (1996). Spoilage and shelf-life extension of fresh fish and shellfish. *Critical Reviews in Food Science and Nutrition*, 36(1-2), 87–121. <https://doi.org/10.1080/10408399609527720>
- Bauer, A.W., Kirby, W.M.M., Sherris, J.C.T. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 45(4), 493-496. https://doi.org/10.1093/ajcp/45.4_ts.493
- Casarin, L.S., Tondo, E.C., Klein, M.P. and Brandelli, A. (2009). Survival of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella Enteritidis* in frozen chicken hamburger. *Journal of Muscle Foods*, 20(4), 478-488. <https://doi.org/10.1111/j.1745-4573.2009.00162.x>
- Chandra, S., Saklani, S. and Singh, J. 2012. Phytochemical screening of Garhwal Himalaya wild edible tuber *Colocasia Esculenta*. *International Research Journal of Pharmacy*, 3(3), 181-196.
- Chang, H.C., Chen, M.L., Su, Y.C., Pai, J.Y. and Chiu, T.H. (2011). Molecular characterizations of pathogenic *Vibrio parahaemolyticus* isolated from Southern Taiwan oyster-growing environment. *Food Control Journal*, 22(2), 245–251. <https://doi.org/10.1016/j.foodcont.2010.07.004>
- Clinical and Laboratory Standards Institute, (2012). Approved guidelines M100. Performance standard for antimicrobial susceptibility testing, 27th ed. Wayne, Philadelphia, USA: CLSI.
- Davey, H.M. (2011). Life, death, and in-between: meanings and methods in microbiology. *Applied and Environmental Microbiology*, 77, 5571–5576. <https://doi.org/10.1128/AEM.00744-11>
- Dutta, S. and Aich, B. (2017). Study of antibacterial and antifungal activity of the leaves of *C. esculenta* L. *International Journal of Pharmaceutical Science and Research*, 8(3), 1184-1187.
- Ehiobu, J.M. and Ogu, G.I. 2018. Phytochemical content and *in vitro* antimycelial efficacy of *Colocasia esculenta* (L), *Manihot esculenta* (Crantz) and *Dioscorea rotundata* (Poir) leaf extracts on *Aspergillus niger* and *Botryodiplodia theobromae*. *Journal of Horticulture and Plant Research*, 1, 9-18. <https://doi.org/10.18052/www.scipress.com/JHPR.1.9>
- French, G.L. (2006). Bactericidal agents in the treatment of MRSA infections—the potential role of daptomycin. *Journal of Antimicrobial Chemotherapy*, 58(6), 1107-1117. <https://doi.org/10.1093/jac/dkl393>
- Gandhi, M. and Chikindas, M.L. (2007). *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology*, 113(1), 1-15. <https://doi.org/10.1016/j.ijfoodmicro.2006.07.008>
- Gwa, V.I., Ekefan, E. and Nwankiti. (2017). Antifungal potency of some plant extracts in the control of white yam (*Dioscorea rotundata* Poir) tuber rots. *Advance Biotechnology and Microbiology*, 7(1), 555703. <https://doi.org/10.19080/AIBM.2017.07.555703>
- Harth, E., Matsuda, L., Hernandez, C., Rioseco, M.L., Romero, J., Gonzalez-Escalona, N., Martinez-Urtaza, J. and Espejo, R.T. (2009). Epidemiology of

- Vibrio parahaemolyticus* outbreaks, southern Chile. *Emerging Infectious Diseases Journal*, 15, 163–168. <https://doi.org/10.3201/eid1502.071269>
- Hiramatsu, R., Matsumoto, M., Sakae, K. and Miyazaki, Y. (2005). Ability of Shiga toxin-producing *Escherichia coli* and *Salmonella* spp. to survive in a desiccation model system and in dry foods. *Applied and Environmental Microbiology*, 71, 6657–6663. <https://doi.org/10.1128/AEM.71.11.6657-6663.2005>
- Jay, J.M. (2000). *Modern Food Microbiology*, 6th ed., p. 283-295. USA: Aspen Publishers Inc. <https://doi.org/10.1007/978-1-4615-4427-2>
- Kreike, C.M., Van Eck, H.J. and Lebot, V. (2004). Genetic diversity of taro, *Colocasia esculenta* (L.) Schott, in Southeast Asia and the Pacific. *Theoretical and Applied Genetics*, 109, 761-768. <https://doi.org/10.1007/s00122-004-1691-z>
- Kubde, M.S., Khadabadi, S.S., Saboo, S.S., Ghorpade, D.S. and Modi, A.J. (2010). *In vitro* antimicrobial activity of the crude extracts of *Colocasia esculenta* leaves (araceae). *International Journal of Pharmaceutical Sciences and Research*, 1(8), 88-91.
- Liu, F., Guan, W., Alam, M.J., Shen, Z., Zhang, S., Li, L., Shinoda, S. and Shi, L. (2009). Pulsed-field gel electrophoresis typing of multidrug-resistant *Vibrio parahaemolyticus* isolated from various sources of seafood. *Journal of Health Science*, 55(5), 783–789. <https://doi.org/10.1248/jhs.55.783>
- Metwally, A.M., Omar, A.A., Harraz, F.M. and Sohafy, S.M. (2010). Phytochemical investigation and antimicrobial activity of *Psidium guajava* Linn. leaves. *Pharmacognosy Magazine*, 6(23), 212-218. <https://doi.org/10.4103/0973-1296.66939>
- Olajuyigbe, O.O. and Afolayan, A.J. (2012). *In vitro* pharmacological activity of the crude acetone extract of *Erythrina caffra* Thunb: antibacterial and antifungal assessment. *Journal of Medicinal Plants Research*, 6(9), 1713-1720. <https://doi.org/10.5897/JMPR11.1517>
- Othman, M., Loh, H.S., Wiart, C., Khoo, T.J., Lim, K.H. and Ting, K.N. (2011). Optimal methods for evaluating antimicrobial activities from plant extracts. *Journal of Microbiological Methods*, 84(2), 161–166. <https://doi.org/10.1016/j.mimet.2010.11.008>
- Penduka, D. and Okoh, A.I. (2012). *In vitro* antilisterial properties of crude methanol extracts of *Garcinia kola* (Heckel) seeds. *The Scientific World Journal*, 2012, 694828. <https://doi.org/10.1100/2012/694828>
- Ramli, S., Radu, S., Shaari, K. and Rukayadi, Y. (2017). Antibacterial Activity of Ethanolic Extract of *Syzygium polyanthum* L. (Salam) Leaves against Foodborne Pathogens and Application as Food Sanitiser. *BioMed Research International*, 2017, 9024246. <https://doi.org/10.1155/2017/9024246>
- Rukayadi, Y., Lee, K., Han, S., Yong, D. and Hwang, J.K. (2009). *In vitro* activities of panduratin A against clinical *Staphylococcus* strains. *Antimicrobial Agents and Chemotherapy*, 53(10), 4529-4532. <https://doi.org/10.1128/AAC.00624-09>
- Saad, R. (2014). Phytochemical screening and antibacterial activity of five Malaysian medicinal plants. *British Journal of Pharmaceutical Research*, 4(17), 2019-2032. <https://doi.org/10.9734/BJPR/2014/12233>
- Smith-Palmer, A., Stewart, J. and Fyfe, L. (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Applied Microbiology*, 26(2), 118-122. <https://doi.org/10.1046/j.1472-765X.1998.00303.x>
- Speck, M.L. and Ray, B. (1977). Effects of freezing and storage on microorganisms in frozen foods: a review. *Journal of Food Protection*, 40(5), 333-336. <https://doi.org/10.4315/0362-028X-40.5.333>
- Talaro, K.P. and Chess, B. (2012). *Foundations in microbiology*, 8th Ed. USA, New York: McGraw-Hill.
- Thangiah, A.S., Obey, J. and Mutuku, N.C. (2013). Phytochemical analysis of *Vernonia adoensis* leaves and roots used as a traditional medicinal plant in Kenya. *International Journal of Pharmaceutical and Biological*, 3(3), 46–52.
- Unklesbay, N. (1992). *World Food and You*, p. 251. New York, USA: Food Product Press.
- Wafa, N., Sofiane, G. and Mouhamed, K. (2016). The antioxidant and antimicrobial activities of flavonoids and tannins extracted from *Phlomis bovei* De Noé. *European Journal of Experimental Biology*, 6(3), 55-61.
- Wei, L.S, Musa, N. and Wee, W. (2010). *In vitro* antimicrobial activities of *Colocasia esculenta* extract against *Vibrio* spp. *Journal of Agricultural*, 7, 3-7.
- Witkowska, A.M., Hickey, D.K., Alonso-Gomez, M. and Wilkinson, M. (2013). Evaluation of antimicrobial activities of commercial herb and spice extracts against selected food-borne-bacteria. *Journal of Food Research*, 2(4), 37-41. <https://doi.org/10.5539/jfr.v2n4p37>
- Yusoff, N.A.H., Noor, N.F. and Rukayadi, Y. (2015). Effects of *Cosmos caudatus* Kunth. (ulam raja) extract on microflora in raw chicken meat. *International Journal of Current Microbiology and Applied Sciences*, 4, 426-435.