CHAPTER 3

RESEARCH METHODOLOGY

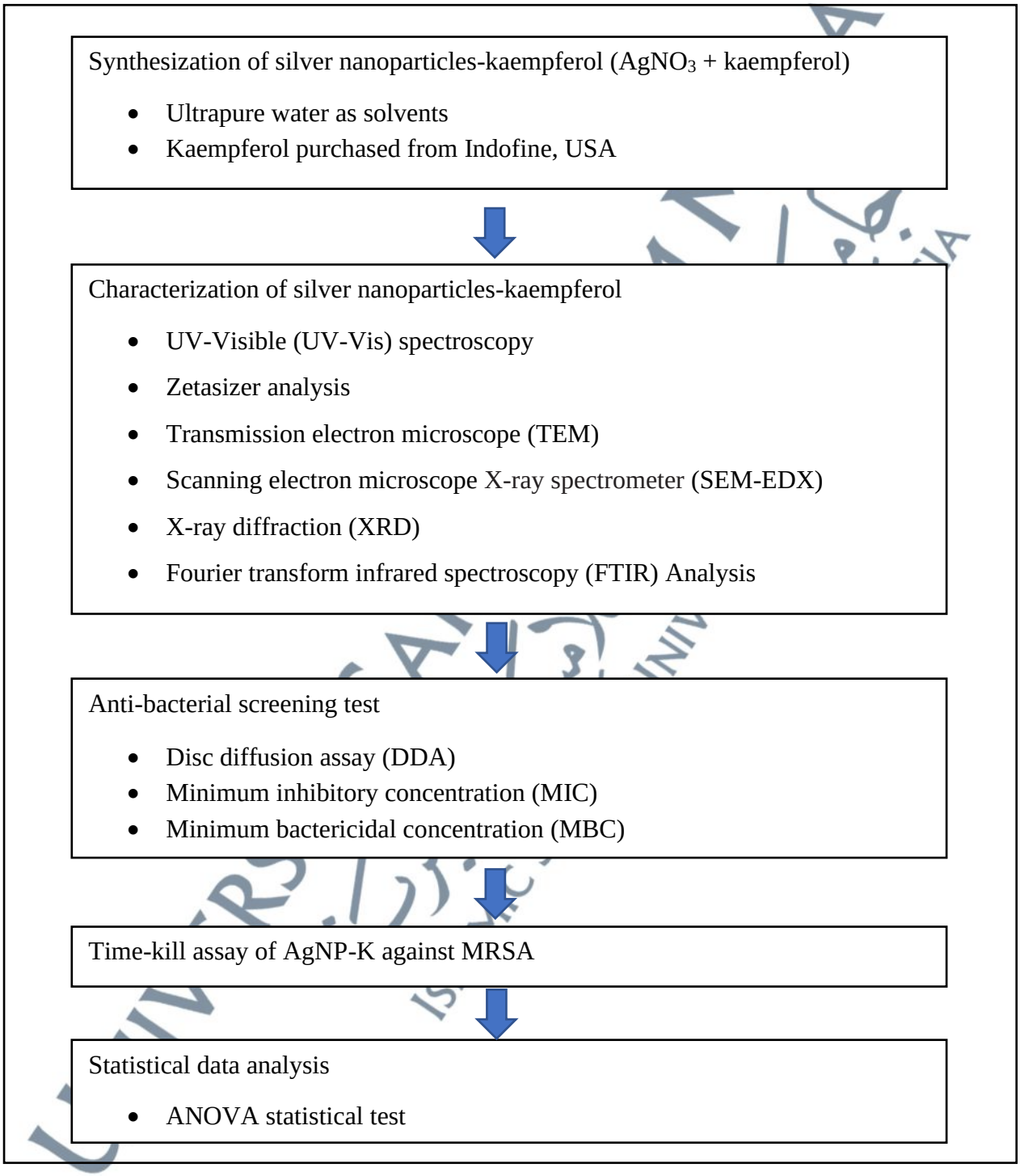

Figure 3.1: Flowchart of research methodology.

3.1 Synthesizing and Optimization of silver nanoparticles kaempferol

In this study, green synthesis method was applied with some modifications from Yakop et al., (2018) and Mat Yusuf et al., (2020) with different concentrations of kaempferol (Indofine, US) by 1.0 mMol and 2.0 mMol in 1 litre of solution (H_20). Briefly, 1.0 mMol of kaempferol was added dropwisely (titration) into continuously stirred 1.0 mMol of silver nitrate ($AgNO_3$) (Nacalai Tesque Inc, Japan) as showed in Figure 3.2. Certain amount of heat (RT, 40 ºC, 60 ºC and 80 ºC) was applied in oven and kept for 168 hours.

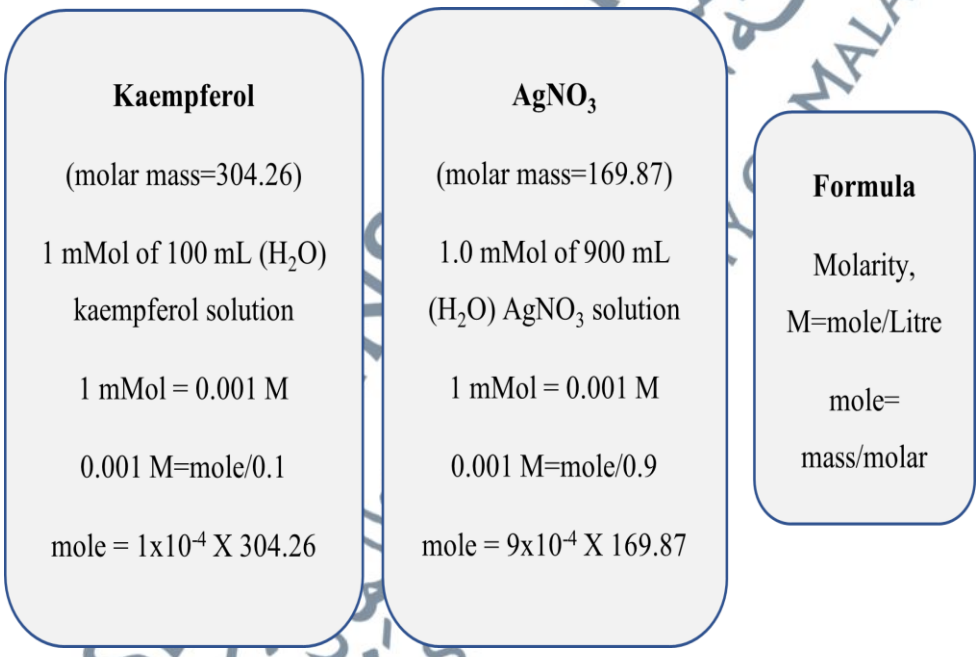

Figure 3.2: Calculation for kaempferol and $AgNO_3$ needed for 1 mMol concentration.

In the beginning, 1.0 mMol (100 mL H_20) of kaempferol was prepared by weighing 30.43 mg of kaempferol powder and added with 100 mL of H_2O, continuously

stirred. Both $AgNO_3$ powder and kaempferol were as shown in Figure 3.3. Then 153.00 mg of $AgNO_3$ powder was weighed and added into 900 mL of H_2O.

First of all, 1.0 mMol (100 mL H_20) of kaempferol was added dropwisely into continuously stirred 1.0 mMol (900 mL H_20) of silver nitrate ($AgNO_3$) and kept at RT until 168 hours as shown in Figure 3.4. Every 24 hours, the sample was analyzed by UV-Vis to observe the formation of AgNP-K. The steps were repeated with heat at 40 ºC, 60 ºC and 80 ºC and changes the concentration of kaempferol to 2.0 mMol in 1 litre of solution (H_20). Colour changes to brown solution was observed.

(a) (b) (c)

Figure 3.3: Materials used in synthesis of AgNP-K. Figure 3.3 **(a)** showed silver nitrate ($AgNO_3$) (Nacalai Tesque Inc, Japan) powder used: Figure 3.3 **(b)** and Figure 3.3 **(c)** showed kaempferol powder (Indofine, US).

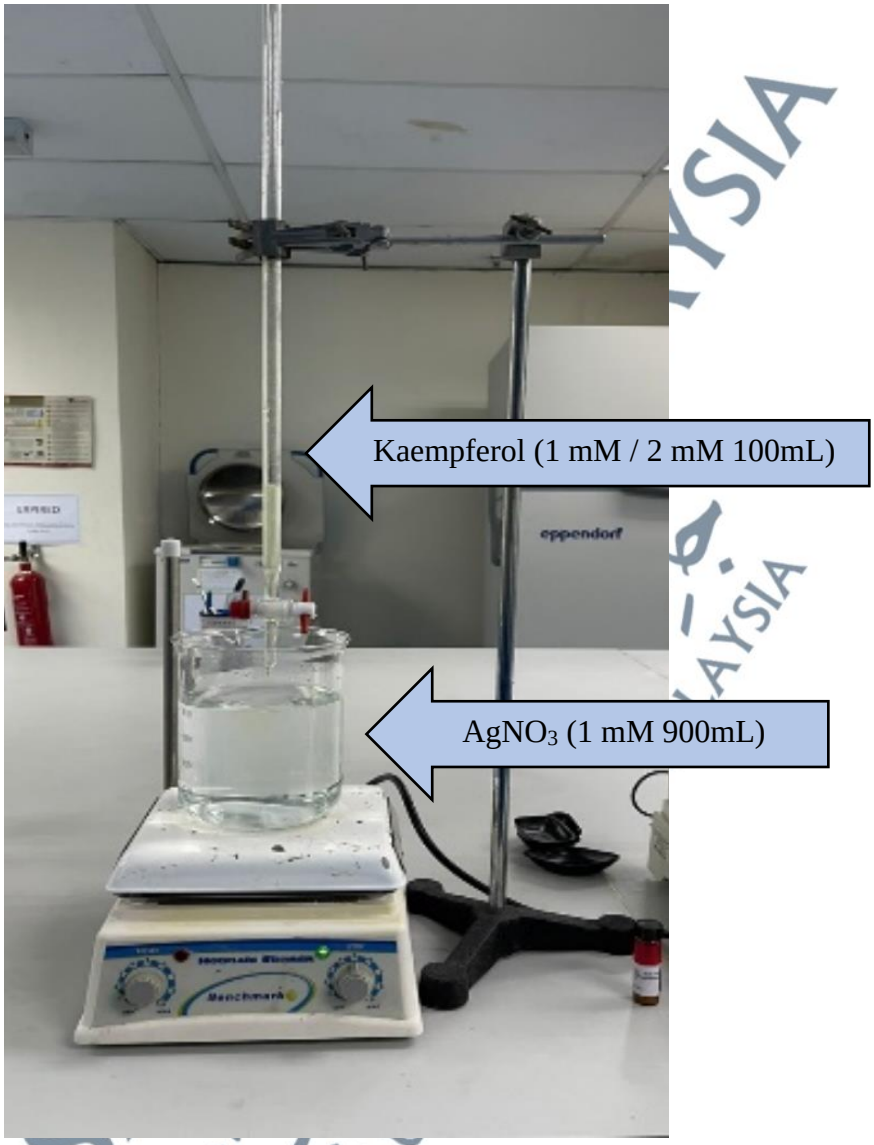

Figure 3.4: Kaempferol was added dropwisely into $AgNO_3$ solution by titration method.

The optimization methods were obtained from applying heat and period during the synthesis of AgNP-K in order to obtain the highest amount of AgNP-K synthesized with green synthesis technique. Optimization on synthesis AgNP-K was done in accordance with the UV-Vis spectroscopy analysis. Table 3.1 showed all of the samples synthesized with set parameters.

Table 3.1: List of samples synthesized with selected parameters. Samples with yellow highlight were chosen to further testing and analysis.

Parameters			Sample specs	Sample name
Ratio concentration Of $AgNO_3$ and kaempferol	Temperature (ºC)	Time (hours)		
1:1	RT	168	AgNP-K 1:1 RT/168	Sample A
1:1	40	168	AgNP-K 1:1 40/168	Sample B
1:1	60	168	AgNP-K 1:1 60/168	Sample C
1:1	80	168	AgNP-K 1:1 80/168	Sample D
1:2	RT	168	AgNP-K 1:2 RT/168	Sample W
1:2	40	168	AgNP-K 1:2 40/168	Sample X
1:2	60	168	AgNP-K 1:2 60/168	Sample Y
1:2	80	168	AgNP-K 1:2 80/168	Sample Z

The parameters of applying heat at 60 ºC for 168 hours was determined as the optimum conditions to synthesis AgNP-K under this study. Lastly, the sample will be freeze-dried with a freeze dryer machine (Christ, Alpha 1-2 LDPlus, Germany) shown in Figure 4.2 to obtain the AgNP-K powder.

3.2 Characterization of silver nanoparticles-kaempferol (AgNP-K)

The formation of AgNP-K was monitored from the reduction process of (Ag^{+} - Ag^{0}) using UV-Visible spectroscopy. UV-Vis observed the formation of AgNP-K through the synthesization process every 24 hours. This was measured from the absorbance peak of AgNP-K in the range of 300 nm to 900 nm of wavelength using Spectramax ID3 (Molecular Device, USA). About 150 µL of AgNP-K solution was aliquoted out to 96 well-plate (Biologix Plastic, Changzhou) to observe the absorbance nanoparticles with the machine. The absorbance parameter was set from 300 nm to 900 nm of its wavelength. The absorbance peak for nanoparticles was usually formed in range 390 nm - 530 nm (Deng et al., 2021; Yakop et al., 2018). UV-Vis acted as the first screening method to determine whether AgNP-K was successfully synthesized or vice versa.

The sample was diluted with ultrapure water (H_2O) for the measurement of average size, zeta potential, and polydispersity index (PDI) using zetasizer analyzer Malvern Zetasizer Nano ZS (Nano-ZS90, Malvern, UK). Sample was diluted and tested until the lowest size of the AgNP-K was obtained. A few parameters such as the refractive index of water (1.330), viscosity (0.8872 cP) and temperature (25 ºC) were set before proceeding with the analysis.

Meanwhile, transmission electron microscope (TEM) was used to determine the shapes, sizes, and morphologies of the nanoparticles through the cross-section of each nanoparticle, thus giving the actual size of the AgNP-K. The sample was diluted with distilled water and dropped on a copper grid. Usually, TEM gave a different result from zetasizer due to the procedure of the instruments itself.

The surface morphology of the nanoparticles was measured by scanning electron microscope with a dispersive X-ray spectrometer (SEM; Quanta Feg 650 attached with X-Max 50 energy dispersive X-ray spectrophotometer) (EDX; Oxford Instrument, Abingdon, UK) by placing the sample on the holder then loaded the sample onto the chamber. The images shown were adjusted by increasing the magnification to get clearer images in the area of interest. This analysis also showed the composition percentages of silver contained in the AgNP-K from EDX analysis.

AgNP-K was placed on the glass sample plate holder by flattening and compressing the sample as the same height as the holder. The holder was mounted onto standard sample stages in the sample chamber. The X-ray wave was beamed, and the scattered intensity was measured by the instruments. X-Ray diffraction (XRD machine Rigaku Miniflex 600) was used to measure its crystallinity and phases to a certain angular degree. XRD also detected the existence of silver in the compound by referring to the silver database for XRD analysis.

Fourier transform infrared (FTIR) analysis was conducted using Thermo Scientific™ Nicolet™ iS50 FTIR Spectrometer to observe the presence of a functional group in the nanoparticle which is the hydroxyl group (OH). AgNP-K was placed on the diamond crystal and touched with ATR touchpoint probe. This was to ensure the synthesized silver nanoparticles were successfully incorporated with kaempferol.

3.3 Antibacterial activities of silver nanoparticles-kaempferol (AgNP-K) against MRSA

3.3.1 Disc diffusion assay (DDA)

MRSA (ATCC, 33591) bacteria showed in Figure 3.5 (b) were cultured in BHI broth (Oxoid, USA) and incubated in an incubator at 37 °C overnight. Approximately 0.5 Mc Farland (Oxoid, USA) shown in Figure 3.5 (a) turbidity standard of MRSA bacteria was used in this analysis.

A sterilized cotton swab was used to spread the diluted MRSA (0.5 McFarland) on MH (Mueller Hinton) agar (Oxoid, USA) before the application of 5 x 6 mm paper discs (Whatmann No. 1) impregnated with 10 µL of samples (AgNP-K 1:1, AgNP-K 1:2, commercial AgNP, and kaempferol) at concentrations between 10 mg/mL – 1.25 mg/mL. Sterilized water was used as negative control and vancomycin (30 U) (Sigma, US) was used as a positive control.

Zones of inhibition were derived from the inhibition zone diameter around the disk after incubating the agar plate under 37 °C for 24 hours.

(a) (b)

Figure 3.5: Figure 3.5 **(a)** 0.5 McFarland (Oxoid, USA) used for standard turbidity and Figure 3.5 **(b)** ATCC MRSA bacteria obtained from Hospital Tuanku Ja'afar Seremban (ATCC, 33591).

3.3.2 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

MRSA (ATCC, 33591) bacteria showed in Figure 3.5 (b) were cultured in BHI broth (Oxoid, USA) and incubated in an incubator at 37 °C overnight. Approximately 0.5 Mc Farland (Oxoid, USA) shown in Figure 3.5 (a) turbidity standard of MRSA bacteria was used in this analysis.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was performed using the two-fold serial dilution method described by (Hanafiah et al., 2019). MIC was performed using sterile 96-well plate. MIC test was conducted to determine the lowest concentration of test samples (AgNP-

K, commercial AgNP, and kaempferol) to prevent bacterial growth or known bacteriostatic.

Diluted 0.5 McFarland test bacteria (100 µL) was added into each well plate containing diluted samples (AgNP-K, AgNP, and kaempferol) with sterile MH broth to reach a final volume of 100 µL/well. Ultrapure water was used as negative control and vancomycin 10 mg/mL with serial dilution was used as positive control.

Then the plate was incubated at 37 °C for 24 hours. An aliquot of 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Oxoid, USA) was added and the colour changes to purple was observed. MIC value was determined as the lowest concentration that inhibits the visible growth of bacteria. MTT assay was added as indicator for bacterial growth. MTT colour changed from yellow to purple indicated the presence of bacteria growth due to reduction process by cellular redox enzymes while the remain yellow colour implies no bacterial growth. (Oh and Hong, 2022).

Minimum bactericidal concentration (MBC) was done to determine the lowest concentration that killed the MRSA or know bactericidal. The method was determined by culturing 10 µL aliquoted from wells that exhibited no bacterial growth in MIC wells onto MH agar and incubated overnight at 37 °C. MBC values was defined as the lowest concentration that prevent bacterial growth.

3.3.3 Time kill assay

Time-kill assay of the AgNP-K, AgNP, and kaempferol were assessed referred to the method described by Hanafiah et al., (2015) and Abd Ghafar et al., (2022) with modification. MRSA bacteria was grown in BHI broth at 37 °C for 24 hours. The turbidity of the bacteria culture was adjusted to 0.5 McFarland standard (≈ 1.5×10^8 CFU/mL) in sterile fresh MH broth. AgNP-K sample with concentration of MIC and MBC value was prepared.

An aliquot of 100 µL fresh sterile MH broth was added into 96 well plate and followed by 100 µL of treatment for performing serial dilution. After that, MRSA bacteria with the amount of 100 µL was added into that well.

Next, 10 µL of treated sample from the plate was aliquot out, then added to second 96 well plate filled with 90 µL of sterile MH broth to perform 10^1 ,10^2, 10^3, 10^4 dilution factors. The dilution factors were done by streaking on MH agar in every four (4) hours interval (0, 4, 8, 12, 16, 20, 24 hours). The agar was incubated at 37˚C for 24 hours. Colonies on individual plates were counted and expressed as number of colony forming units/ml (CFU/mL).

The killing rate was determined by plotting logarithm of the viable colony counts (CFU/mL) against time (Rani et al., 2017). From the observation, the characteristics of the AgNP-K towards MRSA could be determined either it was bacteriostatic or bactericidal.

3.4 Statistical analysis

The data was analysed by using Statistical Package for the Social Sciences (SPSS) Statistics. All three antibacterial activities assays were done in triplicate. The diameter of the inhibition zone with different concentrations (1.25, 2.5, 5.0 and 10.0 mg/mL) of AgNP-K and control against MRSA were determined using One-Way ANOVA. The statistical test was used to find the significant difference in study parameters between the groups, where there was a significant difference if the *p*-value is less than 0.05 ($p < 0.05$). The significance was acknowledged at $p < 0.05$. Data presented were analyzed using ANOVA and values were given as mean ± SD and means were separated using Tukey's HSD post hoc analysis.