CHAPTER V

BIOASSAY STUDY ON SYNTHESIZED FERULATE ESTERS

5.1 Introduction

Today, there has been an emerging interest for ethnopharmacologists, botanists, microbiologists and natural-product chemists in investigating biologically active compounds isolated from plant species for a fundamental source of antimicrobial agents (Ncube et al., 2008). The variety of secondary metabolites presence in the plant species such as flavonoids, alkaloids, terpenes, coumarins, phenolics and polyphenols have been found to offer a broad-spectrum of antimicrobial properties for therapeutic effects (Cowan, 1999; Savoia, 2012).

Since both of ethyl ferulate and olive oil are derived from plant species, therefore, the presence study was aimed at determining the in-vitro antimicrobial activity of their combined effects with regards to any possible boosting outcome. The targets of antimicrobial action were studied using different bacterial physiological indices: agar well diffusion, minimum inhibitory effect (MIC) through MTT assay and spectrophotometric assay and minimum bactericidal concentration (MBC).

According to Cloete (2003), different types of bacteria may react differently to a tested antimicrobial agent, either due to inherent differences such as unique cell envelope composition and non-susceptible proteins, or to the development of resistance, either by adaptation or by genetic exchange. Therefore, different types of enteric bacterial pathogens were selected based on the previous study (Tambekar & Dahikar, 2011) for their resistance towards ferulate esters synthesized.

5.2 Materials and Methods

5.2.1 Materials

Substrates (ethyl ferulate and olive oil) were obtained from Sigma-Aldrich (St. Louis, USA) and solvent (toluene) was purchased from Merck, Germany. Commercial lipases of Novozym 435 (immobilized lipase B from *Candida antarctica*) and Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*), streptomycin sulfate powder and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma-Aldrich (St. Louis, USA). All chemicals were commercially available and of analytical grade unless otherwise specified.

5.2.2 Media

Mualler-Hinton broth and Mualler-Hinton agar were obtained from Merck, Germany.

5.2.3 Microorganisms

Gram-positive bacteria (*Bacillus subtilis* ATCC 11774, *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus aureus* ATCC 29213) and Gram-negative bacteria (*Salmonella typhimurium* ATCC 13311, *Klabsiella pneumoniae* ATCC 10031 and *Escherichia coli* ATCC 25922) were obtained from Microbiology Laboratory, Faculty of Science and Technology, Universiti Sains Islam Malaysia.

5.2.4 Preparation of Test Microorganisms

All the bacteria strains were cultured at 37 °C on Mueller-Hinton broth (MHB) medium for overnight. The optimal density (OD) of bacterial growth was then determined at 600 nm by BioPhotometer (Eppendorf AG, Hamburg, Germany). The concentration of each bacterial inoculum was determined by referring to McFarland Standard Formula (Appendix D). The bacterial inoculum was diluted to the concentration of 10^7 cell/ mL for further experiment.

5.2.5 Preparation of Test Samples

The esters were prepared by adding substrates ethyl ferulate and olive oil (1: 2 g/g ratio) and 80 mg of Novozym 435-Lipozyme RM IM (1: 9 w/w ratio) in 25 mL of screw capped vials containing 5 mL of toluene. The vials were placed in a controlled water-bath shaker at 60 °C and shaken at 200 rpm. The mixture was continuously reacted for 12 hrs.

In the bioassay study, the samples were diluted in Mueller-Hinton broth (MHB) to give a range of concentration. The series of serial dilution is summarized in Table 5.1. In this experiment, antimicrobial agent or antibiotic (Streptomycin Sulfate) was used as positive control. Antibiotic solution (at a concentration of 10 mg/mL) was prepared by dissolving about 10 g of streptomycin sulfate powder in 1 mL of sterilized distilled water. The antibiotic solution was further sterilized by filtration technique using membrane filter with pore size of 0.2 μm.

TABLE 5.1: Preparation of Serial Dilution of Ferulate Esters

Concentration (%)	Ferulate esters (mL)	Mueller-Hinton broth (mL)
100	2	
50	2	2
33.3	2	4
25	2	6
20	2	8
16.7	1	5
14.3	1	6
12.5	1	7
11.1	1	8
10	1	9

[a] Concentration of ferulate esters at 100, 50, 33.3, 25 and 20 % were prepared by adding 2 mL of pure ferulate esters with 2, 4, 6 and 8 mL of MHB subsequently

[b] Concentration of ferulate esters at 16.7, 14.3, 12.5, 11.1 and 10 % were prepared by adding 1 mL of pure ferulate esters with 5, 6, 7, 8 and 9 mL of MHB respectively

5.2.6 Screening Test via Agar Diffusion Method

The agar diffusion test was done according to Smith-Palmer et al. (1998). Each bacteria culture at a concentration of 10^7 cell/ mL was spread onto different Mueller-Hinton agar plates by using sterilized cotton-swabs. All experiments were performed in triplicate. Using a sterile cork-borer of 6 mm diameter, two wells per plate were made in the set agar containing the bacterial culture. A total of 10 μL of 100 % concentration of ferulate esters and streptomycin sulfate solution were loaded into different wells per plate. The inhibition zone was observed at the inoculation points after incubation at 37 °C for overnight. The diameter of inhibition zone in three different directions was measured and the mean was recorded if greater than 6 mm.

5.2.7 Determination of Minimum Inhibitory Concentration (MIC) by MTT Assay

The bacteria which showed potent inhibition zone in the agar diffusion method were selected for MIC determination using the standard broth dilution assay. The method was modified based on Eloff (1998), performed in 96-well round-bottomed microtitre plate (Costar, USA). A 50 μL of an overnight bacterial inoculum was added into each well in the microtitre plate containing 50 μL of the test sample solutions (either ferulate esters at different concentrations or streptomycin sulfate solution). The microtitre plate was covered with a lid and then incubated overnight at 37 °C.

The bacterial viability following the treatment with test substances was identified by using MTT solution. MTT solution (at a concentration of 0.3 mg/mL) was prepared by dissolving about 0.3 mg MTT powder in 1 mL of sterilized distilled water. About 40 μL of MTT was added into each test well and the microtitre plate was further incubated for 5 min at 37 °C. The lowest concentration without visible formation of formazon blue was defined as MICs. All experiments were performed in duplicate.

5.2.8 Determination of Minimum Inhibitory Concentration (MIC) by Spectrophotometric Assay

The quantitative MIC value were measured based on spectrophotometric determination as adapted from Tan et al. (2009) with some modifications. A 50 μL of an overnight bacterial inoculum was added into each well in the microtitre plate containing 50 μL of different concentrations ferulate esters. For each assay, control wells included: 1) wells containing inoculum alone as positive control and 2) wells containing different concentration of ferulate esters (without inoculum) as negative control. The microtitre plate was covered with a lid and then incubated overnight at 37 °C. The OD reading of each test well was measured immediately at a wavelength 630 nm by microtitre plate reader (BioTek ELx800, USA) and compared with the control wells. All experiments were performed in duplicate. The OD value of positive control was assigned a value of 100 % growth. The results were presented (Equation 5.1) as percentage of growth inhibition.

Percentage inhibition (%) =

$$\frac{1 - (\text{OD test well} - \text{OD corresponding negative control well}) \times 100}{\text{OD corresponding positive control well}}$$

(Equation 5.1)

5.2.9 Determination of Minimum Bactericidal Concentration (MBC) Value

The MBC was determined by taking a loop of the culture medium from each test well (from the broth MIC assay) that showed no apparent growth and sub-culturing on fresh Mueller-Hinton agar plates. Examination of the bacterial regrowth was done after incubation overnight at 37 °C. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5 % killing of the original inoculum. All experiments were performed in duplicate.

5.3 Results and Discussion

5.3.1 Agar Well Diffusion Assay

The potential antimicrobial activity of synthesized ferulate esters was evaluated based on the formation of inhibition zone against various Gram-positive (*Bacillus subtilis*, *Staphylococcus epidermidis* and *Staphylococcus aureus*) and Gram-negative (*Salmonella typhimurium*, *Klabsiella pneumoniae* and *Escherichia coli*) bacteria. The results of inhibition zone were compared with the activity of the standard antibiotic, streptomycin sulfate solution. When a sample of ferulate esters is dropped into a well on agar, the chemical will diffuse from the well into the agar containing the bacterial culture. The inhibition zone will be appeared, assigns there is no growth of bacteria around the well, if the sample has the antimicrobial activity towards that bacteria.

In this study, ferulate esters at concentration 100 % were used as a preliminary check for the antimicrobial activity. Various sizes of inhibition zone observed as depicted in Figure 5.1 and the average mean of inhibition zones are summarized in Table 5.2. Due to the hydrophobicity, the samples did not diffuse well in the agar compared to the streptomycin sulfate solution. Alternatively, Parveen et al. (2010) have classified zone of inhibition into five classes: no activity (-), low activity (1-6 mm), moderate activity (7-10 mm), high activity (11-15 mm) and very high activity (≥16 mm).

From the results, *Staphylococcus aureus* displayed very high activity by ferulate esters followed by *Staphylococcus epidermidis* and *Escherichia coli*. *Salmonella typhimurium*, on the other hand, demonstrated partial inhibitory effect by ferulate esters as there was no clear bacteria growth inhibition but the bacteria surface appearance was changed (Yuen et al., 2011). However, there were no activity recorded for *Bacillus subtilis* and *Klabsiella pneumoniae*. Similarly, Sellami et al. (2013) also reported insensitivity of both *Bacillus subtilis* and *Klabsiella pneumoniae* towards their synthesized dopamine esters.

Silhavy et al. (2010) has explained that although both Gram-positive and Gram-negative bacteria have a layer of peptidoglycan that gives the cell rigidity and strength, the peptidoglycan layer in Gram-positive bacteria tends to be thicker. This fact may clarify the results obtained for *Bacillus subtilis* in this study. Due to the diameter of inhibition zones, Gram-negative bacteria (<10 mm) exhibited more resistant effect compared to the Gram-positive bacteria (>10mm), which is related to lipopolysaccharides outer membrane presence in Gram-negative bacteria (Silhavy et al., 2010).

Indeed, there are many factors may affect the results such as agar depth, diffusion ability of the sample, growth rate of the specific bacteria, incubation time and temperature (Friedman et al., 2002). Regarding the screening test, *Staphylococcus aureus, Staphylococcus epidermidis*, *Escherichia coli* and *Salmonella typhimurium* were selected for subsequent experiments in order to measure the strength of antimicrobial activity of ferulate esters towards them.

FIGURE 5.1: Typical Agar Plates Showing the Inhibition Zone of Ferulate Esters Synthesized (Left-Side of Plate) and Positive Control (Right-Side of Plate) Against Bacterial Strains

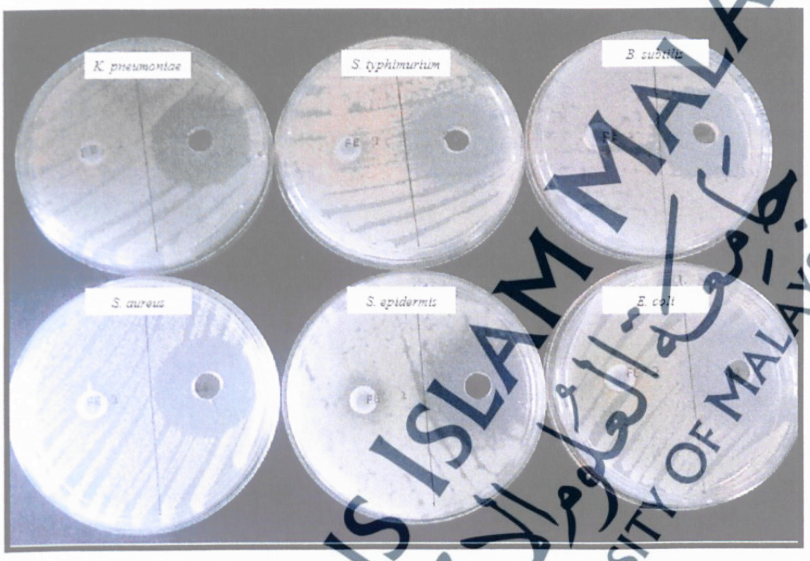


TABLE 5.2: Antimicrobial Activity of Ferulates Esters Synthesized

Test Bacteria	Diameter of inhibition zone (mm)	
	Ferulate esters (100%)	Streptomycin sulfate (10 mg/mL)
Bacillus subtilis	-	32 ± 0.4
Staphylococcus epidermidis	13 ± 0.1	31 ± 0.2
Staphylococcus aureus	16 ± 0.2	34 ± 0.2
Salmonella typhimurium	P	35 ± 0.0
Klabsiella pneumoniae	-	31 ± 0.1
Escherichia coli	8 ± 0.0	33 ± 0.0

Partial inhibitory effect (P); no inhibitory effect (-)

5.3.2 Minimum Inhibitory Concentration (MIC)

The strength of the antimicrobial activity of ferulate esters can be determined by serial dilution in Mueller-Hinton broth at different concentration. Again, streptomycin sulfate was used as positive control. After overnight incubation, the growth of bacteria in each well can be easier to visualize and detect by the formation of formazon blue by MTT indicator (thiazolyl blue). Determination of the MIC is important because it helps in confirming resistance of tested bacteria to the potential antimicrobial agent. The MIC values of ferulate esters towards both selected Gram-positive and Gram-negative bacteria are shown in Table 5.3.

Overall, the MIC results revealed that ferulate esters synthesized exhibited antimicrobial effect in concentration-dependent manner on different type of bacteria tested. *Escherichia coli* demonstrated the most resistance bacteria with MIC value of 50 %. *Salmonella typhimurium* exhibited medium MIC value of 33.3 %. While, Gram-positive bacteria of *Staphylococcus epidermidis* and *Staphylococcus aureus* were very sensitive and most susceptible to the ferulate esters as they still can be inhibited with only 20 % of the sample. In a study by Ergun et al. (2011) found that simple aromatic esters of ferulic acid may inhibit biofilm formation by *Staphylococcus aureus* at concentrations lower than 8 μg/mL.

TABLE 5.3: The Minimum Inhibitory Concentration (MIC) Values of Ferulate Esters

Concentration of test samples (%)	Test bacteria			
	Staphylococcus epidermidis	*Staphylococcus aureus*	*Salmonella typhimurium*	*Escherichia coli*
100.0	-	-	-	-
50.0	-	-	-	-
33.3	-	-	-	+
25.0	-	-	+	+
20.0	-	-	+	+
16.7	+	+	+	+
14.3	+	+	+	+
12.5	+	+	+	+
11.1	+	+	+	+
10.0	+	+	+	+
S10	-	-	-	-

No bacterial growth (-); bacterial growth (+); S10 streptomycin sulfate (10 mg/ mL)

5.3.3 Spectrophotometric Assay

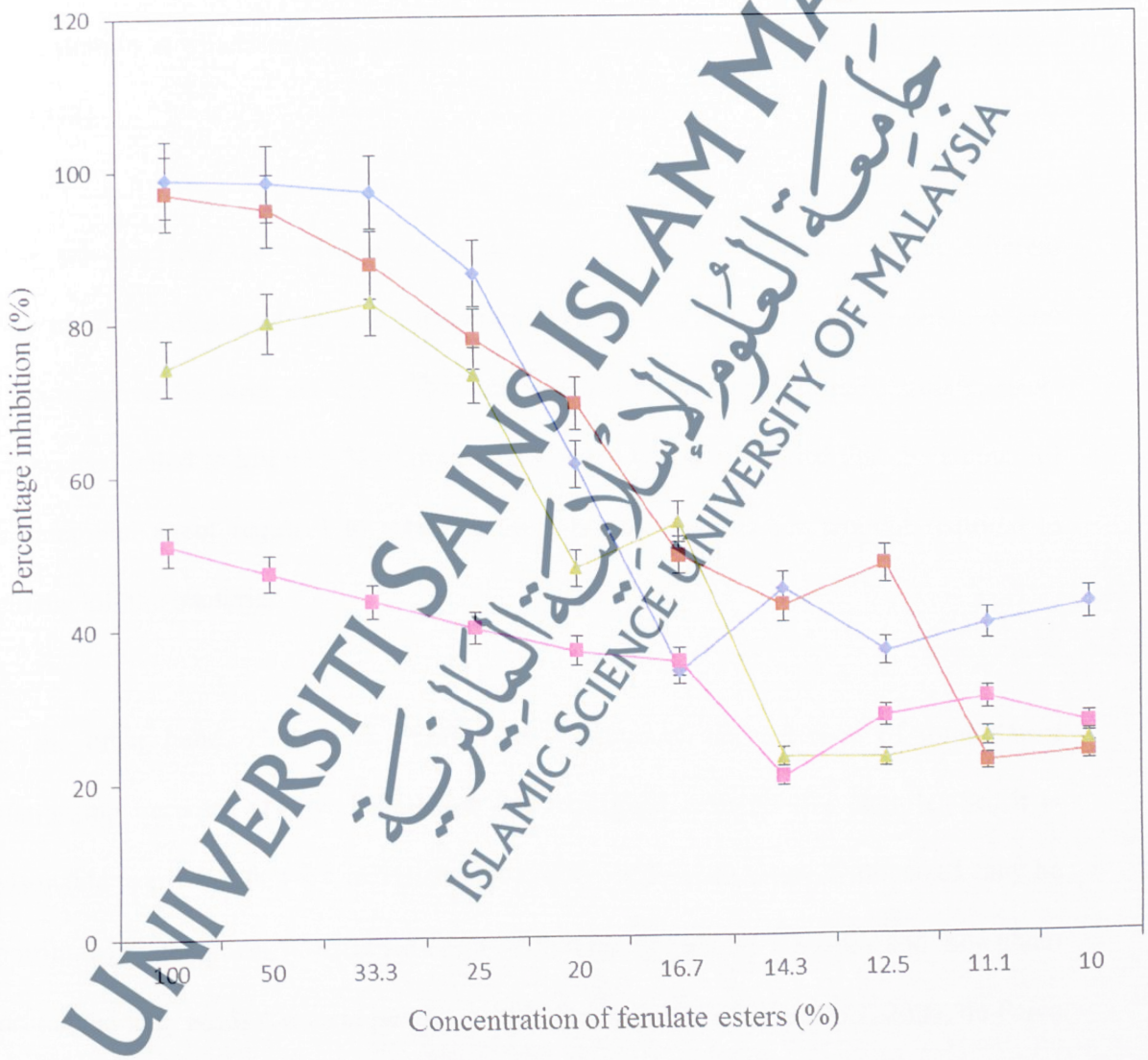
The determination of MIC was further supported by quantitative values which were based on OD reading. Several studies have successfully used this spectrophotometric method to perform further calculations rather than directly stating the MIC values (Koczon et al., 2005; Patton et al., 2006). This method was proved to offer

several advantages over current bioassays such as rapid, more sensitive, more amenable to analysis and able to determine the inhibition index (Akujobi & Njoku, 2010).

Figure 5.2 illustrates the patterns of bacterial growth inhibition caused by exposure to different concentration of ferulate esters synthesized. Basically, from the profile, percentage inhibitions of bacteria tested were decreased by decreasing the concentration of ferulate esters; which are conformed to the results obtained in MTT assay before. *Escherichia coli* was the only species that demonstrated a near-linear relationship between ferulate esters concentration and percentage inhibition.

From concentration of 100 % to 25 %, the following general ranking (in decreasing order of percentage inhibition) of *Staphylococcus epidermidis* > *Staphylococcus aureus* > *Salmonella typhimurium* > *Escherichia coli* was observed. According to Patton et al. (2006), a negative inhibition value indicates growth promotion of inoculum in the test well. However, our results only display positive inhibition values for both Gram-positive and Gram-negative bacteria.

FIGURE 5.2: The Degree of Growth Inhibition *Staphylococcus epidermidis* (◆), *Staphylococcus aureus* (■), *Salmonella typhimurium* (▲), and *Escherichia coli* (■) by Different Concentration of Ferulate Esters after 24 hrs of Incubation



5.3.4 Minimum Bactericidal Concentration (MBC)

Further, MBC value is essential to indicate either the antimicrobial agent tested exhibits bacteriostatic (bacteria-inhibiting) or bactericidal (bacteria-killing) effects. Bacteriostatic refers to the lowest concentration at which bacteria fail to grow in broth but grow when the broth is plated onto agar. Whilst bactericidal concentration is the lowest concentration at which bacteria fail to grow both in broth and agar plate (Smith-Palmer *et al.*, 1998).

After sub-culturing the test dilution in this part, however, ferulate esters at different concentrations displayed only bacteriostatic effects towards both Gram-positive and Gram-negative bacteria selected. This observation suggested that the ferulate esters synthesized failed to kill 99.5 % of the original inoculum and revealed that the amount of antimicrobial agent required to inhibit growth is not same as the amount required to actually kill the bacteria.

On the other hand, Hansch & Fujita (1964) proposed an existence of quantitative relationship between chemical structure and biological activity of a sample. So, it is reasonable to predict that the antimicrobial actions of ferulate esters synthesized may be contributed by the phenolic rings (of ethyl ferulate) to destabilize electrons and long chain unsaturated fatty acids (donated by olive oil) presence in the sample (Burt, 2004; de Paiva et al., 2013). The hydrophobicity characteristic of the sample also enables them to partition in the lipids of the bacterial cell membrane thus, disturbing the structures and

rendering them more permeable (Burt, 2004). Though, the antimicrobial activities obtained in this study may differ from other synthesized ferulate esters considerably due to selection of substrates, route of synthesis and purification procedures.

5.4 Conclusion

Synthesized ferulate esters originated from plant-derived molecules, exhibited low antimicrobial properties towards *Bacillus subtilis* and *Klabsiella pneumoniae* and moderate to good antimicrobial properties against *Staphylococcus aureus, Staphylococcus epidermidis*, *Escherichia coli* and *Salmonella typhimurium*. The serial dilution technique offered more effective results compared to the agar diffusion method, where by using spectrophotometric it showed an improvement over current methods.