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## "Industry Based On Knowledges"

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**Organized by:**

**Animal Science Faculty of Andalas University**
**and**
**Alumbi Center of Universiti Putra Malaysia**

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# Contents

# Preface

# List Paper of Oral Presentation

# List of Poster

# KEYNOTE LECTURER

# Pharmacodynamic Evaluation of the Additive Combination of Pterostilbene and Oxacillin against Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591

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## Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) was initially limited to hospital and healthcare facilities but has gradually become a growing problem in healthy children and adult. Pterostilbene belongs to the phenylpropanoid phytoalexin which is involved in plant response to various pathogen and herbivores attack. The aim of this study was to evaluate the anti-MRSA action of pterostilbene in combination with selected antibiotics such as vancomycin, linezolid and oxacillin against MRSA ATCC 33591. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of individual antimicrobial agents were determined using microbroth dilution technique whereas the microdilution checkerboard (MDC) assay was employed to verify the type of interaction of the combined agents from the fractional inhibitory concentration (FIC) index values. Time- kill assay (TKA) analysis and post-antibiotic effect (PAE) time were determined only on the combination which showed synergistic interaction. Cell morphology and ultrastructural changes of the treated and untreated strains were also observed. The MIC and MBC of pterostilbene against ATCC 33591 were 31.25µg/ml and 62.50µg/ml, respectively. This indicated that pterostilbene was bacteriostatic against ATCC 33591. MDC results showed that pterostilbene-oxacillin combination exhibited lowest FIC value (0.56) for ATCC 33591 which implies partial synergistic interaction. On the other hand, combination of pterostilbene and vancomycin generated an additive effect (FIC 1.00) whereas pterostilbene-linezolid combination displayed indifference effects with FIC of 1.25 against MRSA ATCC 33591. Despite the partial synergism, TKA proved an additive effect for the combination of pterostilbene and oxacillin against ATCC 33591 with concentration-dependent bactericidal action within 24 hour. After one hour exposure at 10X-MIC, prolonged PAE time of 2.6 ± 1.48 hour against ATCC 33591 was demonstrated by pterostilbene-oxacillin combination treatment compared to pterostilbene (2.02 ± 0.36 hour) and oxacillin (0.53 ± 0.28 hour) alone. Scanning and transmission electron microscopic observations revealed that pterostilbene targeted the cell wall which is the same site of action as oxacillin hence additive effects by the combination treatment. In conclusion, pterostilbene in combination with oxacillin showed partial synergism with bactericidal and persistent antimicrobial effect against MRSA ATCC 33591. Therefore, pterostilbene has the potential to be developed as an alternative phytotherapeutic agent against MRSA infections.

**Keywords**: Pterostilbene, MRSA, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Fractional Inhibitory Concentration (FIC), Bactericidal, Time-Kill Assay (TKA), Post-Antibiotic Effect (PAE).

## 1. Introduction

*Staphylococcus aureus* is a normal microflora that can be found in the skin and nose of healthy people. However, these bacteria will become opportunistic pathogens when these bacteria enter the human body through small surgical wound or trauma on someone who has weakened immune system causing skin and soft tissue infections [1]. Infections caused by *S. aureus* have been treated with various antibiotics such as penicillin, macrolides and aminoglycosides. However, these bacteria have managed to grow and resistant against various type of antibiotic [2]. The emergence of MRSA not only give special attention because of resistance against methicillin but also due to resistant against almost all kinds of existing antibiotics even though there are new antibacterial drugs on the market [3,4]. These bacteria caused many infections in communities such as endemic and epidemic nosocomial infections [5].

Therefore, to find ways and strategies to prevent or delay the development of resistance of MRSA, various studies have been conducted to find an alternative treatment to overcome this phenomenon from becoming more serious. The step is to use a combination of antimicrobial therapy that consists of existing antibiotics and plant extracts [6]. Natural products and their derivatives have been identified over the years as a source of therapeutic agent [7,8]. Natural products derived from plants, animals and minerals has been the basis for the treatment of humans since long ago [9,10,11]. Natural product is an organic or chemical compounds produced by living organisms in nature that usually have a pharmacological or biological activity that are valuable and can be used in the invention and design of drugs [12].

Phytochemicals are bioactive compounds derived from plants and the term is often used to describe a large number of secondary metabolites [13]. Pterostilbene is a component that belongs to a phenolic group known as stilbene [14]. Stilbene was secondary metabolites of the plants that formed from flavonoid biosynthesis pathway and belongs to the family of phenylpropanoid [15]. Pterostilbene found in sandalwood *Pterocarpus santalinus* by [16], hardwood of *Pterocarpus marsupium* by [17], leaves of *Vitis vinifera* by [18] and also found in some species of *Vaccinium* berries [19]. Pterostilbene also is an analog of resveratrol found in grapes and blueberries [20]. This component is biologically classified as a phytoalexin. Antimicrobial phytoalexin is a plants defense system which is involved in plant response to various pathogen and herbivores attack [15].

In addition, antibiotics used in this study were vancomycin, oxacillin and linezolid. Vancomycin and oxacillin known as agents that target cell wall while lonezolid targeting protein synthesis [21,22,23]. Evaluation on the morphology and ultrastructure of cells after treatment studies using electron microscopy. Transmission electron microscope (TEM) is typically used by microbiologists to study on intracellular or cell wall structure [24,25,26,27]. Scanning electron microscope (SEM) was used to study the surface of the cell and its features [28,29,30,31].

## 2. Results and Discussion

### *2.1 Determination of MIC and MBC value*.

MIC and MBC of pterostilbene for MRSA ATCC 33591 was 31.25 µg / ml and 62.50 µg / ml (Table 1). The MBC is two-fold higher than the MIC for both strains. Therefore, this indicated that pterostilbene has potential as an antimicrobial agent that produce bacteriostatic effects. MIC and MBC values of oxacillin against ATCC 33591 was same, each value 62.50 µg/ ml (Table 2). Therefore, oxacillin exhibit antimicrobial bactericidal agent. The MIC and MBC of vancomycin against ATCC 33591 was same, 0.98µg / ml (Table 3). Therefore, this suggests that vancomycin exhibits bactericidal activity

against ATCC 33591. MIC values of linezolid against MRSA ATCC 33591 was 1.56 µg / ml (Table 4). MBC values for linezolid shows that linezolid displayed bacteriostatic activity against MRSA strain which is four times higher than the MIC of ATCC 33591.

This study showed that pterostilbene exhibit stronger antibacterial properties than oxacillin on MRSA ATCC 33591. Pterostilbene is a phytoalexin which belongs to the phenylpropanoid family that involved in plant response to various pathogens and herbivores attack [15]. Comparison with antibiotics that are used in this study as current therapy against MRSA infection, suggesting that the antimicrobial activity of pterostilbene is lower than linezolid and vancomycin on ATCC 33591. It is supported by [32] which states that phytochemicals produce lower activity compared to standard antibiotics.

Pterostilbene may be able to reduce the problem of microbial resistance that was supported by [33] reported that phytochemicals capable of exhibiting significant potential for changing the resistivity of an antibiotic. Although the antimicrobial effectiveness of pterostilbene stronger than oxacillin but pterostilbene showed bacteriostatic effect same like linezolid with MBC value exceeds the MIC.

This corresponds to [34] reported that stilbenoid compound which belongs to the phenylpropanoid family also exhibit bacteriostatic action against MRSA strains. Generally, phytochemicals or secondary plant metabolites can prevent and slow the growth of bacteria compared than kill the pathogens [35]. In this study, oxacillin and vancomycin exhibits bactericidal action against ATCC 33591 with the same value of MIC and MBC and supported by [36].

*2.2 Determination of FIC value*

The combination of pterostilbene and oxacillin exhibit partial synergistic activity against MRSA strain ATCC 33591 of the FIC values is greater than 0.5 but less than 1 which is 0.56. Pterostilbene was capable of lowering the MIC of oxacillin doubled from 62.50 µg / ml to 31.25 µg / ml against ATCC 33591. Pterostilbene in combination with vancomycin exhibit additives activity (FIC 1.00) against ATCC 33591. This suggests that pterostilbene can also act at the same site of action with vancomycin. Linezolid showed indifference activity against ATCC 33591 in the FIC of 1.25. This shows that the combined action of pterostilbene-linezolid is the same as action of pterostilbene or linezolid singly. Therefore, pterostilbene could potentially targeting different cell wall of oxacillin actions but more specifically to the site of action of vancomycin. The result of the combination between pterostilbene and three kinds of antibiotics against ATCC 33591 was shown in Tables 5.

*2.3 Result of Time-Kill Assay (TKA)*

Result of TKA for ATCC 33591, showed that growth curve of pterostilbene singly about the same as growth curve for positive control, which showed an increase in the number of bacterial colonies. Pterostilbene showed no effect either bacteriostatic or bactericidal. However, the impressive results produced when pterostilbene combined with oxacillin. This is because, although pterostilbene singly not produce a decrease in the number of bacterial colonies but when combined, this combination resulting in a decrease of bacterial colonies. Results of pterostilbene-oxacillin combination for 0.5XKS produce bacteriostatic effect (mean inhibition <3 $\log_{10}$ CFU / ml) at 4$^{th}$ hour with reduction of bacterial colonies of 2.29 $\log_{10}$ CFU / ml. While for concentration of 1.0XKS exhibit bactericidal effect at 16$^{th}$ hour with reduction of 2.13 $\log_{10}$ CFU / ml. For 2XKS, combination treatment showed bactericidal effect at 11$^{th}$ hour with decrease in the bacterial colonies of 1.58 $\log_{10}$ CFU / ml.

In addition, through the observation of the relationship between the rate of growth of

bacterial colonies with increasing concentrations of the treatment given showed that combination treatment of pterostilbene and oxacillin was concentration-dependent action.

Pterostilbene and oxacillin combination also produces additive interaction for all three concentrations against MRSA ATCC 33591. For oxacillin, growth curve graph showed bactericidal activity with decreasing of more than 3 $\log_{10}$ CFU / ml at the $8.5^{th}$, $16^{th}$ and $8^{th}$ hour at 0.5X, 1.0X and 2.0XMIC. Figure 1 (a, b, c) shows the growth curve graph for strain ATCC 33591.

Dilution of checkerboard and time-kill assay used in this study was to evaluate the antimicrobial effect of pterostilbene in combination with antibiotics. This is because, this technique provides detailed information about the type of interactions and bactericidal activity [37]. Checkerboard assay was used to determine the inhibitory effect of the combination while time-kill study was used to evaluate the bactericidal activities which rely either on time or concentration [37]. Furthermore, time-kill study gives an overview of the dynamics of antimicrobial action and interaction from time to time compared to checkerboard assay that used only once [38]. Individually, pterostilbene and linezolid displayed bacteriostatic action but pterostilbene in combination with linezolid produced indifference effect. This is consistent with studies from [32] reported that when eugenol combined with bacteriostatic antibiotic minocycline, indifference interaction effects are obtained. According to [39], indifference effect was produced when the combination action is the same as action by itself for a single component.

Pterostilbene exhibit partial synergistic effects when combined with bactericidal agents such as oxacillin against MRSA despite having same potential of anti-MRSA. Synergistic interactions showed that mechanism of action may be different [40]. This study also indicated that the interaction between pterostilbene and oxacillin can enhance partial activity of oxacillin by reducing MIC of oxacillin two-fold. In other words, an important finding in this study is pterostilbene increasing anti-MRSA activity of oxacillin. This is supported by [41] that pterostilbene act on the part of different targets of oxacillin action which is at the cell wall of bacteria and at the sites that were not involved with linezolid action on protein levels. It is also supported by the FIC study shows that pterostilbene with vancomycin against MRSA produce additive effects. Previous studies by [42] reported that combination of ellagic acid and gallic acid with β-lactam antibiotics produces additive effect. The combination of pterostilbene-vancomycin which produces an additive effect disputed by studies [43] states that the synergies was produced by the combination of ε-viniferin and vancomycin. It is also likely due to the ε-viniferin and pterostilbene has a different chemical structure. ε-viniferin do not have two groups metoxyl on benzene ring which may lead to the same site of action of pterostilbene and vancomycin.

However, there showed contradiction in the value of this two techniques, namely checkerboard technique and time-kill study. This is because, result from checkerboard technique showed partial synergistic interaction against MRSA ATCC 33591 for combination pterostilbene-oxacillin but for time-kill study found that the additive interaction was produced against MRSA ATCC 33591. It is similar to a study by [44] that showed additives activity was produced by acetone extract of *Garcinia kola* seed and methanol extracts of *Helichrysum pedunculatum* in combination with antibiotics against *Staphylococcus aureus* as a result of time-kill study [45]. Active component of the essential oil of *Thymus vulgaris* also produce additive antimicrobial activity when time-kill method was used to validate synergistic interaction that produced by FIC test [46]. The contradiction in this two technique also

supported by [47] that concluded most of the studies show contradict results for both of these techniques.

*2.4 Results of Post-Antibiotic Effect (PAE)*

The time value of PAE for the combination treatment was 2.6 hours (156 minutes) and 2.02 hours (121.2 minutes) for the treatment of pterostilbene in single and followed with the time value of PAE oxacillin singly, 0.53 hours (31.8 minutes) against MRSA ATCC 33591 (Table 6). Figure 2 shows a graph for growth rate of post-antibiotic effect (PAE) against strain ATCC 33591 of pterostilbene in combination with oxacillin, oxacillin singly, pterostilbene singly at concentrations 10XMIC for treatment singly and 10XKS for combination treatment for 24 hours.

In this study suggests that pterostilbene and oxacillin combination generates a longer time of PAE for the MRSA strain ATCC 33591. The results shown by the combination therapy against MRSA ATCC 33591 was also supported by [48,49] which demonstrated the effectiveness of antimicrobial agents can be enhanced by combining crude plant extracts with antibiotics against many pathogens, including *S. aureus*. Results from previous studies also shown that combination treatment of extracts from gall *Q. infectoria* with vancomycin exhibit longer PAE time compared to single agent against MRSA [50]. In addition, there are reports stating that aminoglycoside antibiotics produce longer PAE time on Gram-positive and Gram-negative bacteria [51]. However, this is in contrast to a study was carried out because longer PAE time was shown by combination treatment against MRSA ATCC 33591 compared to oxacillin singly.

The study by [52] reported that the beta-lactam antimicrobial produce short PAE time same as a result of this study that demonstrate short time of PAE for oxacillin singly against ATCC 33591. In addition, longer PAE time by an antimicrobial agent can be beneficial in terms of faster in killing the bacteria and likely can produce longer regimen therapeutic [53]. Studies by [54] showed that different strains exhibit different PAE time for a single treatment. PAE time for all tested strains of *S. aureus* produce a different time and length of PAE time except *S. aureus* PAE 352-3028 that showed short PAE time.

In addition, interesting discoveries from the PAE time was combination treatment in this study resulted in a longer time compared to PAE of oxacillin singly against the ATCC 33591 thus increase its potential against MRSA resistance through similar mechanism of action to oxacillin. This means that pterostilbene is synergistic to the effects of persistent antimicrobial of oxacillin against MRSA ATCC 33591. In addition, the advantage of the combination treatment was the use of a smaller dose than the dose used singly. Thus, it can reduce the toxic effect of certain medications or antibiotics [55,56,57]. TKA technique simply showed bacteriostatic effect for combination treatment and the site of action as a result of additive effect from pterostilbene and oxacillin combination.

*2.5 Observation on Morphology and Ultrastructure*

Observations of pterostilbene treatment singly using SEM against ATCC 33591 indicates the presence of protuberance or swollen on the surface of cells such as bacteria, rough surface of the cell, the cell shrinks and looks a little deformed shape of the cell (Figure 3a) compared to cells bacteria without treatment that looks smooth and rounded (Figure 5a). Observations by TEM (Figure 3b) shows the accumulation of black granules (white arrows), which are mostly located on the edge of the cell wall caused by accumulation of nucleotides, abnormal proteins or membranes that have been denatured. In addition, the cell wall also seems thicker, loss of membrane integrity and there is damage to the membrane. Cell shape also looks irregular, deformed and elongated.

The presences of *ghost* cells or cell lysates that have been observed are caused by loss of cytoplasm contents, including genetic material. In addition, the observed DNA is still clearly visible in the cell.

SEM observation for the treatment of oxacillin singly against ATCC 33591 exhibited morphological changes at the cellular level such as the presence of protuberance, swollen and rough on the bacterial cell surface (Figure 4a) compared to untreated bacteria cells that seem to exhibit a smooth cell surface (Figure 5a). In addition, bacterial cells has been shrinking and fragmented and lost its original circular structure. Observations by TEM (Figure 4b) showed that there were accumulation of black granules (white arrows), which are mostly located on the edge of the cell walls caused by nucleotide, abnormal proteins or membranes that have been denatured. Cell shape also changed, look like rods compared to the control cells (Figure 5b). In addition, the cell wall also looks thicker, loss of membrane integrity and there is damage to the membrane. *Ghost* cells or bacterial cell lyses, which was characterized by loss of cytoplasm contents including genetic material can also be observed. In addition, from observation, DNA was still clearly visible in the bacterial cell.

Evaluation of cell morphology and ultrastructure provide an overview of site of actions for each treatment which are pterostilbene, oxacillin, vancomycin, linezolid, gentamycin and ciprofloxacin singly. The bacteria were exposed to five different types of antibiotics. Antibiotics are chosen based on how their site of actions. Antibacterial agents inhibit bacterial growth through a variety of complex mechanisms, including inhibition of cell wall synthesis, cell membrane disruption, inhibition of the synthesis of nucleic acid and protein synthesis and inhibition of nucleic acid metabolism [58]. The purpose of the use of various antibiotics was to determine the site of action of pterostilbene same as the site of action of these antibiotics. Therefore, the use of various antibiotics targeting various sites on bacteria cells was used. Vancomycin and oxacillin were an antibiotic that targets cell membrane, gentamycin and ciprofloxacin are also agents that target RNA and DNA, whereas linezolid is an antibiotic that inhibits protein synthesis of the cell bacterial [21,22,23,59]. Observation of morphology and ultrastructural of the this untreated MRSA strain exhibit a spherical shape, the cell surface is smooth and like clusters of grapes. However, exposure to a variety of antibiotics and pterostilbene has changed the shape of the cell significantly compared to the untreated control cells.

The most significant morphological changes were observed in treated MRSA ATCC 33591 with pterostilbene which is the formation of protuberance and swollen on the cell surface that makes the bacterial cell looks rough. Similar results were also reported from previous studies that used extract from grape seeds. It produced effect in disrupting and damaging the cell wall that observed using transmission and scanning electron microscope [60]. Studies by [61] confirmed that the presence of protuberance because of the cell resistance to cell lysis. Increase in protuberance most likely because of bacteria mechanism to prevent damage to the plasmalemmal. Plasmalemmal damage was either one of cycle that is commonly occurred in most cells that lead to premature and necrotic death. From the observation using electron microscope showed that pterostilbene may affect the cell wall or cell membrane. Similar results were also reported by previous studies that show that the morphology of the cell wall uneven after treated of *S. aureus* with vancomycin [62].

TEM analysis with pterostilbene resulted in the presence of intracytoplasmic black granules. This can be attributed to the results of previous studies that demonstrate the presence of intracytoplasmic black

granules on treated MRSA strain with thioridazine after cultured for 18 hours [63].

This phenomenon is also likely that the presence of this structure was to prevent cells from lyses or defence mechanism of the bacterial cell. Additionally, small molecule of natural product has potential to give impact on structure of the bacterial cell. For example, natural products such as flavonoids and polyphenolic phytochemicals having the ability to inhibit the growth of bacteria by disrupting the cell membrane stability [64].

Results from observations of SEM and TEM show that changes in morphology and ultrastructure of pterostilbene is approximately the same as the changes by oxacillin. Therefore, we can conclude that the actions of pterostilbene same as action of oxacillin singly rather than other antibiotics. It is also consistent with the results of TKA and PAE which confirm additive action of pterostilbene-oxacillin combination with oxacillin singly against MRSA ATCC 33591. Next, it showed that pterostilbene act as oxacillin, which inhibits the synthesis of bacterial cell walls. This is a new discovery in identifying the site of action of pterostilbene against MRSA ATCC 33591.

## 3. Experimental Section

### *3.1 Stock Plant Compounds*

Compounds used in this study is pterostilbene that purchased commercially from EMD Biosciences / Calbiochem (USA).

### *3.2 Bacteria Study*

Bacteria used were methicillin-resistant *Staphylococcus aureus* (MRSA) which is ATCC 33591.

### *3.3 Preparation of Antimicrobial Agents*

Phytochemicals used in this study is pterostilbene that purchased commercially from EMD Biosciences / Calbiochem (USA). Stock solutions of antibiotics and phytochemicals prepared in accordance with the manufacturers recommendations. Stock phytochemicals and antibiotics were dissolved in its solvent and shaken using autovortex until the solution is dissolve completely.

### *3.4 Preparation of Bacteria Inoculum*

Bacteria inoculum preparation process begins with culturing bacteria stock obtained from the collection of Novel Antibiotic Laboratory Faculty of Health Sciences, UKM on Muller-Hinton agar (MHA) plates and incubated overnight at 37 $^0$C to obtain isolated colony. Then, three to five colonies of bacteria that grow on the agar plate were transferred into Muller-Hinton broth (MHB) using a sterile wire loop before being incubated for 24 hours at 37°C. After incubation, the turbidity of the bacterial inoculum was adjusted between 0.08 to 0.10 that equivalent to the concentration of $10^8$ CFU / ml using a spectrophotometer at a wavelength of 625nm. To achieve the desired absorption, MHB will be added to dilute the bacterial suspension. Then, the inoculation was dilute by dilution of 1: 100 to obtain inoculation size $10^6$ CFU/ml.

### *3.5 Determination of Minimum Inhibitory Concentration (MIC)*

MIC was determined using sterile 96-well microtiter plate with microbroth serial dilution method. MIC is the lowest concentration of the extract to inhibit the growth of bacteria after incubation overnight at a temperature of 37 $^0$C [65]. To facilitate observation of the growth of bacteria in each well, 2, 3, 5 - triphenyltetrazolium chloride (TTC) (2mg / ml) is dripped into each well. Bacterial growth is indicated when there is a colour change in the wells while clear or no changes in colour indicate inhibition of bacterial growth for the antimicrobial agent. This test is carried out in triplicate.

### *3.6 Minium Bactericidal Concentration Determination Test (MBC)*

MBC value was determined by culturing each clear well on MHA plates.

MBC value is determined based on the observation of the plates for the lowest concentration which shows no bacterial growth on the agar plate. This test is carried out in triplicate. The MBC is defined as the lowest concentration of an antimicrobial agent in killing 99% of microorganisms in which no bacterial growth was shown in the MHA after incubation [66].

*3.7 Determination of Fractional Inhibitory Concentration (FIC)*

Combination effect of pterostilbene and three types of antibiotics were assessed using a checkerboard method to obtain fractional inhibitory concentration index value (FIC) is either synergistic, additive, indifference and antagonistic. Both pterostilbene and selected antibiotics were prepared in five different concentrations of 1XMIC, 1 / 2XMIC, 1 / 4XMIC, 1 / 8XMIC, 1 / 16XMIC. Then, along the x-axis in 96-well microtiter plates, added 5 µl solution of pterostilbene into each well from 1 / 16XMIC, 1 / 8XMIC, 1 / 4XMIC, 1 / 2XMIC and 1XMIC. For the y-axis, 5 µl solution of antibiotics was added to each well in the same order as pterostilbene [34]. However, 40 µl of MHB were prepared in each well before pterostilbene and antibiotic solution is inserted into each well. Then, 50 µl of bacterial inoculum was added to each well and the final volume of each well was 100 µl. Positive control contain MHB and bacteria inoculum while negative control consist of MHB and distilled water or antimicrobial agents. This test is carried out in triplicate.

After that, the plates were incubated at 37 ° C for 24 hours. Turbidity or the presence of pellets in the bottom of the well indicates the presence of bacteria tested. In addition, the TTC was added to each well and incubated for 20 minutes up to two hours in a dark place. A positive result is the presence of bacterial growth that produces a colour change to pink while for negative results produced no colour change. FIC index was calculated according to the equation as follows: FIC Index = FIC A + FIC B = (MIC of drug A in combination / MIC of drug A only) + (MIC of drug B in combination / MIC of drug B only) [67]. Synergy is defined as an FIC index $\leq 0.5$, partial synergy is FIC value> 0.5 <1, additive such as FIC = 1, indifference is the FIC> 1 $\leq$ 4 and antagonistic as FIC index of more than 4.0 [68].

*3.8 Time-Kill Assay (TKA)*

Time-kill was assessed using microbroth dilution technique by [69] which use for confirmatory test of synergistic effect between pterostilbene and antibiotics. 40 µl MHB was inserted in the well of microtiter plate and then 10 µl combination agents (in ratio 1:1) with a concentration of synergistic respectively were added. Next, 50 µl of bacterial inoculum was added and make the final volume of each well is about 100 µl. Growth control only contains 50 µl of bacteria inoculum and 50 µl of MHB. This test is carried out in triplicate. Then, 96-well microtiter plate was incubated at 37 ° C and counting of bacterial colonies was performed at 0, 4, 8 and 24 hours.

At each of these times, 10 µl of samples are taken and two-fold dilution was conducted using normal saline (0.9% NaCl). After that, spread evenly over MHA using the L-shaped rod or wire loop and incubated for 24 hours at 37 $^{0}$C. Bacteria colony count was done to determine CFU / ml based on each plate consisting of 10-500 colonies. Certain dilution factors used to determine the range of the number of colonies that can be counted on petri plates. Curve of time-kill was plotted which y axis represents $\log_{10}$ CFU / ml and the x axis represents time (hour). Time-kill assay was performed using three different concentrations of 0.5X, 1.0X, 2.0X MIC and synergistic concentration (KS).

Bactericidal effect was defined as a decrease of $\geq$ 3 $\log_{10}$ CFU / ml in colony count compared to original inoculum after 24hours of incubation [70]. Synergy was

defined as a decrease of $\geq 2$ $\log_{10}$ CFU / ml in colony count between the combination agents with the most active single agent after 24 hours of incubation [37]. Additive effect was defined as a reduction of <2 $\log_{10}$ CFU / ml in colony count after 24 hours of incubation for the combination agents with the most active single agent [71]. Antagonistic was defined as an increase in the number of colonies of $\geq 2$ log10 CFU / mL between combination treatments with single agent that is most active after incubated for 24 hours [72].

*3.9 Determination of Post-Antibiotic Effect (PAE)*

PAE for MRSA strain ATCC 33591 was determined using colony count method that introduced by [56,73]. Treatment group is provided using pterostilbene or antibiotics singly and combination of pterostilbene-antibiotic at concentration of 10XMIC. Growth control was prepared with MHB and bacteria suspension that already dilute to $10^6$ bakteria/ml. PAE value can be generated through a comparison between the growth of bacteria with treatment and without treatment. This test was carried out by added 40 µl MHB in appendorf tube. Then, 10 µl stock solution of pterostilbene-antibiotic combination (ratio 1: 1) is inserted into appendorf tube and followed by 50 µl of bacterial inoculum. Then, this makes the final volume in the appendorf tubes 100 µl with a concentration of 10XMIC mg / ml. The same steps were used for pterostilbene and antibiotic singly.

At the same time, growth control were prepared using 50 µl MHB and 50 µl of bacteria inoculum in appendorf tube. After that, all appendorf tubes were immersed in the water bath at 37 °C for one hour. After incubation for one hour, dilution of 1: 1000 was used to remove the effect of pterostilbene or antibiotics. Similar measures were also taken to control growth. Then, 2 µl of diluted sample was spread on MHA at 0, 2, 4, 6, 8, 10 and 24 hours to allow the calculation of the number of colonies that appear after incubation for 24 hours at 37 ° C. This test is carried out in triplicate. Graf $\log_{10}$ CFU / ml were plotted against time where the PAE time can be obtained from the graph. Calculation of PAE was defined as PAE = T-C where T is the time required for bacteria of the treatment group to increase by 1 $\log_{10}$ CFU / ml after dilution. C is the time required for the control group increased by 1 log 10 CFU / ml after dilution was carried out at 1: 1000 [73]. PAE results were presented in the form of mean ± SD.

*3.10 Analysis of Electron Microscope*

This analysis was conducted to identify the site of action of pterostilbene treatment with the site of action for five types of antibiotics on changes in morphology and ultrastructural for ATCC 33591. Bacteria are exposed to five different types of antibiotics. Antibiotics are chosen based on their site of actions. Five types of antibiotics used were linezolid, gentamycin, oxacillin, ciprofloxacin and vancomycin. The purpose of the use of various antibiotics was to determine which antibiotics have the same site of action with pterostilbene. Samples for electron microscope analysis were determined using disc diffusion method [59]. Bacterial around rim of inhibition zone for each disc that will be used for electron microscope analysis.

*3.10.1 Scanning Electron Microscope (SEM)*

Bacterial cells were treated with 10% DMSO and used as a control, while the bacterial cells that treated with antibiotics used as a positive control. Bacterial cells are collected from the centrifuge and washed with distilled water. Then, binding process is done using 2% glutaraldehyde in 0.1 M phosphate buffer solution (PBS) and pH 7.4 for 15 minutes. Next, the cells are washed with distilled water for three times. After that, the binding process is carried out using 1% osmium tetroxide in distilled water for five minutes at room temperature. Samples were dehydrated using a graded ethanol series (70,

90% and 100% ethanol) for five minutes for each one. Then, coated with a thickness of 42nm gold and analyzed using a Philips XL30 ESEM (FEI Company, Oregon, USA) at 28-30 kV.

*3.10.2 Transmission Electron Microscope (TEM)*

Bacterial cells are also provided for the TEM to perform binding process using 2% glutaraldehyde in 0.1M PBS and washed with distilled water [59]. Then, staining was performed with 2% uranyl acetate for five minutes. Thereafter, bacterial cells exposed to 1% osmium tetraoxide for five minutes and the dehydration process is carried out using a series of acetone (70, 90% and 100% acetone) for five minutes for each one. Polymerization was carried out using pure epoxy resin and embedding it in the oven at 90 ° C for two hours after the bacterial cell added into a mixture of acetone and epoxy resin (1: 1) for 5 minutes. Blocks that have been trimmed will be cut into 90 nm ultrathin sections and coloured with Reynold's staining for a minute. Each specimen would be analyzed using Tecnai G2 TEM at a voltage of 100 kV.

## 4. Conclusion

Results of a study conducted by microdilution checkerboard method found that pterostilbene in combination with oxacillin produce a partial synergistic effect against tested MRSA strains. However, follow-up pharmacodynamic study showed that combination treatment produced an additive interaction and bacterisidal effect through time-kill assay against tested MRSA strains. This study also demonstrated that pterostilbene was capable in prolonging PAE time of oxacillin on both MRSA strains. This shows that test of PAE support that pterostilbene in combination with oxacillin produce a synergistic effect. The use of combination treatment can reduce the risk of toxicity, side effects from the use of antibiotics and the use of a smaller dose than the dose used singly. Moreover, the observations in the cell morphology and ultrastructural using SEM and TEM exhibit same site of action of pterostilbene with oxacillin supported by TKA test results that showed an additive effect for the combination treatment with oxacillin.

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**Conflicts of Interests**

The authors declare no conflict of interest.

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# The Development of Goat Meat Production in Thailand

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## Abstract

Goat meat has recently become an important aspect in the meat markets due to its containing of low fat and cholesterol contents that may benefit to human health as compared to mutton, pork and beef. Although the goat meat consumption is less as compares to chicken, pork, and beef, but the amount of world's goat meat consumption trend to be increased. In Thailand, the number of the goat population was about 26.2% increase from the year 2011 to 2015, (427,567 vs. 539,583 heads). The largest distribution of the goat population is within the Southern region (39.1%; 271,730 heads) followed by the Central region (36.6%; 209,155 heads), Northern region (19.4%; 8,876 heads) and Northeastern region (4.8%; 19,822 heads), respectively. However, goat production in this country was primarily raised for the meat purpose (95.5%; 515,093 heads). More acceptability of goat products, especially the meat was reflected a growth of Thai consumer attitude which concern more about the nutritional quality. This was due to the hard research working, and strong knowledge transferring by many organizations such as universities, private companies, Ministry of Agriculture, and the Royal projects. Nevertheless, the demand of goat meat in the market was still slowly increased probably due to the high price of meat(350 to 380 Baht or 10.9 to 11.9 $US per kg) that limit the consumer decision compared to beef (280 to 300 Baht or 8.75 to 9.38 $US per kg), and chicken (61 to 75 Baht or 1.9 to 2.34 $US per kg of chicken breast meat). In addition, goaty odour was a negative preference that consumer concern. To cope with these two main limitations, research works which had been done in Thailand within two decades were concerned more about the improvement of production and nutritional systems to increase the meat yield, whereas breeding improvement program, slaughterhouse and slaughtering protocol, goat meat quality and consumer acceptability of goat meat were also studied. Thus, studies to develop strategies for improving production efficiency of goat with minimum cost may need to be undertaken. Moreover, in order to export goat products to the halal food markets, goat products development and consumer behaviour may also need to be considered. In conclusion, it could be emphasized that the Thai goat meat industry has more opportunity in development as indicated by the increasing trend in production and consumption.

**Keywords**: Thailand, Goat meat production, Thai goat meat industry

# PRODUK HALAL MENGIKUT HUKUM DI MALAYSIA: FOKUS TERHADAP PRODUK MAKANAN, ISU KEPENGGUNAAN DAN UNDANG-UNDANG DI MALAYSIA

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## 1. Pendahuluan

Produk yang bersih dan suci yang secara langsung dianggap halal perlu memenuhi aspek lahir dan batin yang merupakan faktur terpenting dalam penghidupan muslimin dan muslimah di dunia. Ini adalah selari dengan firman Allah SWT di dalam Surah Al Baqarah, ayat 22 yang bermaksud, "Sesungguhnya Allah mengasihi mereka yang banyak bertaubat dan mengasihi mereka yang mensucikan diri". Manakala Profesor Dr. Yusof Al Qardawi di dalam bukunya bertajuk, the lawful and the prohibited in Islam mengatakan bahawa, memakan makanan yang boleh menyebabkan mudarat kepada diri manusia dianggap haram. (CAP, 2006). Adalah menjadi pegangan orang Islam bahawa faktur halal dan haram sesuatu makanan itu merupakan sesuatu yang sangat penting. Ini adalah sapertimana firman Allah di dalam Surah Al Maidah ayat 5 yang membawa pengertian yakni, "makanlah sesuatu yang di rezekikan oleh Allah kepada kamu dari yang halal lagi baik".(Jurnal Syariah, 12: 2, 2004).

Manakala pengguna pula merupakan suatu golongan yang mempunyai hak kepenggunaan yakni setidaknya ada lapan hak pengguna yang diisytiharkan di dunia yang pada awalnya di masyurkan oleh Presiden Reagan, Amerika Syarikat. Antaranya adalah hak untuk mendapat makanan dan pakaian sapertimana selari dengan teori Masslow; hak untuk mendapatkan pendidikan; hak untuk bersuara atau hak untuk didengar; hak untuk mendapatkan udara yang bersih; hak untuk hidup dan sebagainya. Hak untuk hidup ini adalah sapertimana tertera di dalam kes Rural Litigation and Entitlement Kendra Dehradun and Others Lwn State of U.P. and Others (1985, 2 SCC431) dan Devaki Nandan Pandey Lwn Union of India and Others(1985, 3 SCC 614)(Mohd. Bakri Ishak dan Azmi Sharom, Sistem perundangan di Malaysia:Undang-undang alam sekitar, Jilid 8, DBP, 2007). Daripada aspek perundangan atau hukum pula di Malaysia terdapat beberapa Akta yang berkaitan secara tersirat dengan isu produk halal inter alia:

Akta Makanan 1983; Akta Kerajaan Tempatan 1976; Akta Perlindungan Pengguna 1999; Akta Perihal Dagangan 2011; Akta rumah penyembelihan (Penswastaan) 1993 dan sebagainya. Oleh yang demikian konsep alam sekitar yang bersih yang turut merangkumi aspek makanan dan produk halal boleh dikategorikan sebagai suatu hak asasi manusia kerana ia

merupakan satu locus standi rakyat untuk membawa kes seumpamanya ke mahkamah dalam menuntut hak untuk mengambil bahagian dalam proses pentadbiran yang lebih mantap (Mohd Bakri lshak dan Azrni Sharom, 2007).

2. Terminologi khusus

Halal boleh di definasikan sebagai dibenarkan atau tidak dilarang pengambilannya didalam Islam. Mengikut Al Quran semua makanan yang baik dan bersih adalah dianggap halal.Oleh yang demikian boleh dikatakan hampir keseluruhan sumber makanan daripada laut, tumbuhan dan binatang dianggap sebagai halal kecuali mana-mana yang telah diharamkan untuk mengambilnya atau memakannya.(CAP, 2006). Haram pula bermaksud tidak dibolehkan atau dihalang pengambilannya menurut Islam. Bahkan diantara makanan yang diharamkan oleh Islam inter alia: Darah, bangkai, babi, minuman yang membawa kepada mabuk saperti minuman yang mengandungi alkohol. Ini juga termasuk produk yang telah tercemar oleh sumber yang haram dimana diharamkan menurut Islam. (CAP, 2006).

Manakala makruh pula membawa maksud dibenci atau tidak digalakkan sapertimana yang di tetapkan oleh Allah S.W.T dan Nabi Muhammad S.A.W yakni merupakan benda yang boleh menjejaskan jiwa dan memudaratkan manusia. (CAP, 2006). Mubah atau juga dikenali sebagai harus pula membawa maksud tidak digalakkan atau tidak dilarang. Walau bagaimanapun dalam beberapa keadaan status mubah boleh berubah. Sebagai contoh, mubah akan berubah menjadi haram sekiranya terbukti bahawa ianya memudaratkan kesihatan manusia.(CAP, 2006).

3. Antara isu produk makanan dan produk halal di Malaysia

Persatuan Pengguna Pulau Pinang (CAP) telah mengenalpasti beberapa isu yang berkaitan dengan perkara diatas inter alia: keraguan tentang isu logo halal di pasaran; kes logo halal palsu yang tertera di akhbar-akhbar; status halal di hotel dan di restoran; kandungan bahan kimia di dalam makanan; isu ramuan dan penambah makanan yang bersumberkan binatang samada halal atau sebaliknya; gelatin yang meragukan dilabel halal; isu keju haram atau yang diragukan; isu lemak babi didapati di dalam susu rumusan untuk bayi; lelemak dalam makanan; Gliserin atau monogliserida di dalam makanan dan kosmetik serta isu alkohol yang terdapat di dalam makanan dan minuman.

Sebagai contoh pada tahun 1994, CAP melalui kajiannya mendapati bahawa Coca - cola, minuman ringan yang dipasarkan di lebih kurang 185 buah negara di dunia termasuk Malaysia mungkin mengandungi bahan alkohol.

CAP telah banyak mendedahkan tentang isu halal dan haram di Malaysia, inter alia:

1977: sosej daging lembu daripada Australia didapati mengandungi daging babi
1981: terdapat alcohol di dalam minuman tonik untuk bayi

1982: CAP mengesahkan bahawa gelatin dibuat daripada sumber babi

1984: susu rumusan bayi mengandungi lemak binatang 1985: ujian terhadap pelbagai makanan yang diproses didapati mengandungi gelatin

1986: lelemak daripada sumber binatang dikesan di dalam kek dan biskut

1986:berus diperbuat daripada bulu babi

1986: gelatin dan gliserin digunakan di dalam ubatan dan produk perubatan

1986:1emak babi digunakan di dalam penghasilan minyak wangi

1994: Buku bertajuk For God, Country and Coca-cola Oleh Mark Pendergrast yang menyatakan rumusan rahsia coca-cola menggunakan alcohol.

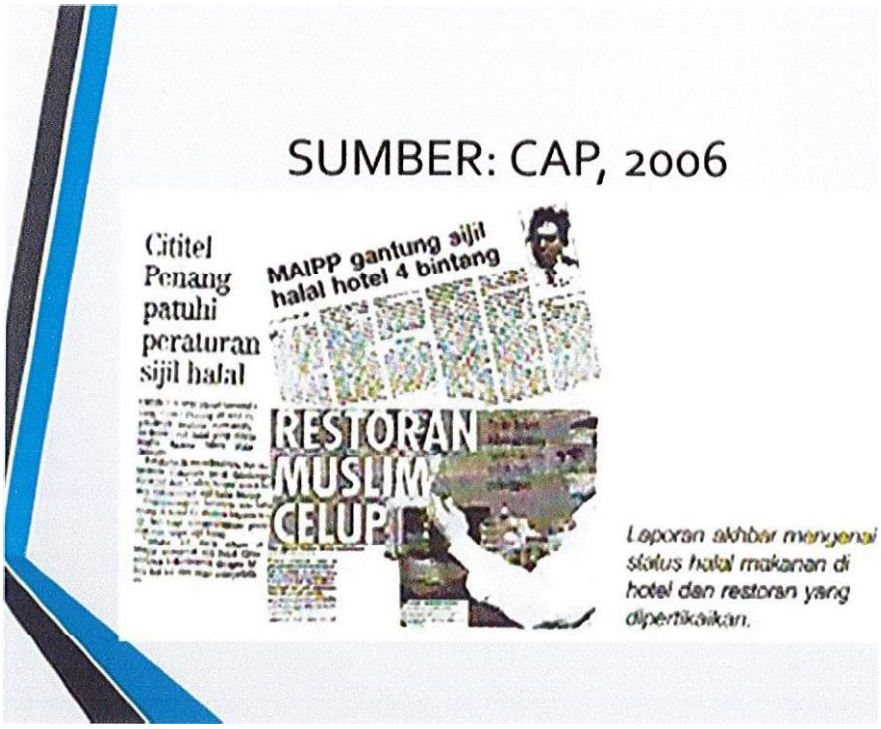
1995: Wei terkandung dalam susu rumusan bayi dan makanan yang diproses.

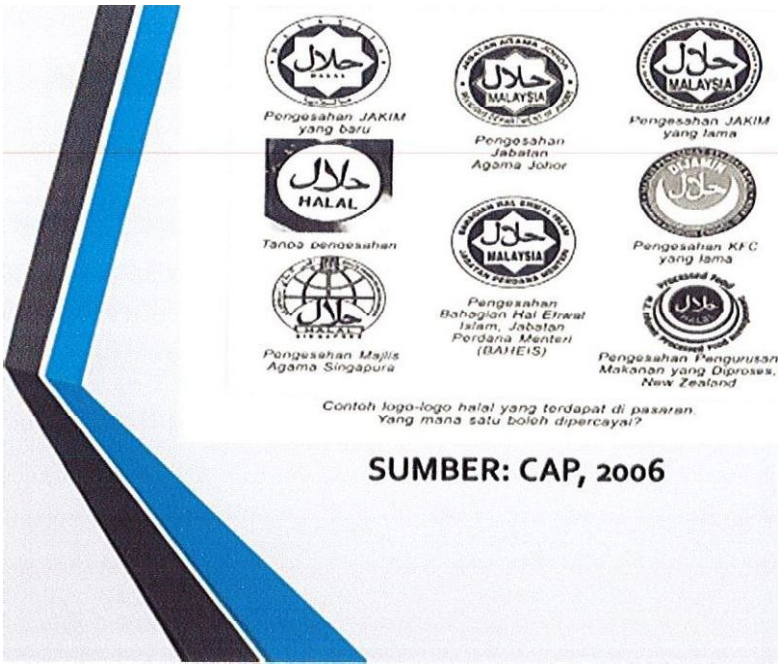
1996: Makanan yang diproses dan agar-agar diuji didapati mengandungi gelatin

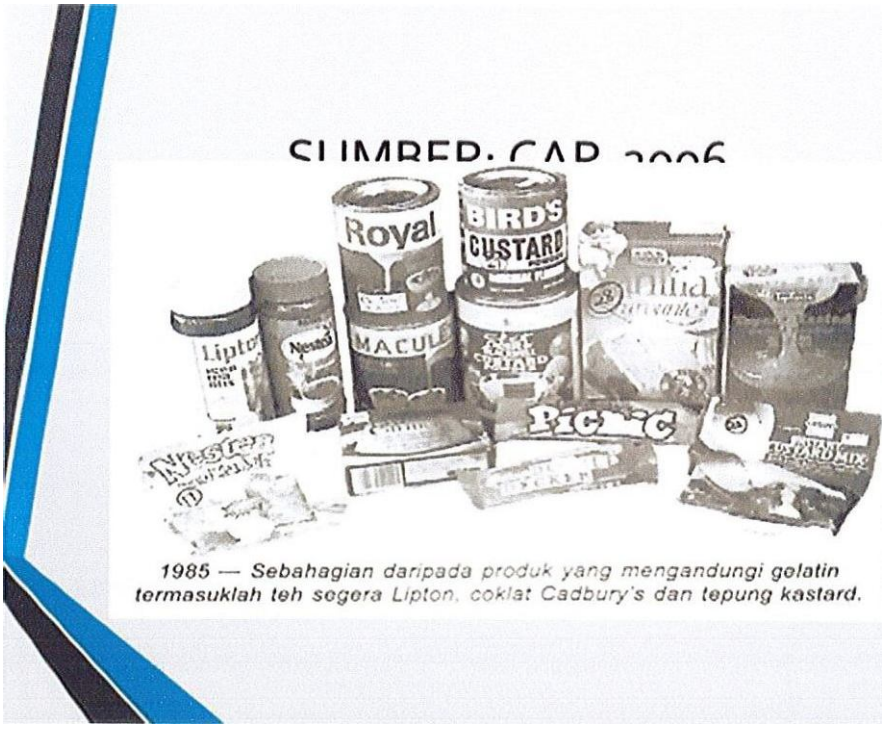
1998: Plasma darah ditemui digunakan dalam produk makanan. 2000: Kolagen, bahan bergelatin daripada sumber binatang atau janin digunakan dalam kosmetik.

2005: perisa makanan didapati mengandungi alcohol.

2005: L-cysteine, bahan yang diperolehi daripada rambut manusia digunakan sebagai penambah makanan saperti didalam roti, Pizza dan perisa makanan yang tidak manis.




Pengesahan JAKIM yang baru
Pengesahan Jabatan Agama Johor
Pengesahan JAKIM yang lama
HALAL
Tanpa pengesahan
Pengesahan KFC yang lama
Pengesahan Majlis Agama Singapura
Pengesahan Bahagian Hal Ehwal Islam, Jabatan Perdana Menteri (BAHEIS)
Pengesahan Pengurusan Makanan yang Diproses, New Zealand
Contoh logo-logo halal yang terdapat di pasaran. Yang mana satu boleh dipercayai?
SUMBER: CAP, 2006


Royal
BIRD'S CUSTARD
1985 — Sebahagian daripada produk yang mengandungi gelatin termasuklah teh segera Lipton, coklat Cadbury's dan tepung kastard.

4. ELEMEN PERUNDANGAN DAN ADMINISTRASI Dl MALAYSIA

Terdapat beberapa statut yang berkaitan samada secara langsung mahupun tidak langsung dengan produk halal di Malaysia. Sebagai contoh seksyen 2, Akta Perihal Dagangan 1972 (Kini telah digantikan dengan Akta Perihal Dagangan, 2011), memperuntukkan penalti terhadap sebarang kesalahan yang dilakukan oleh individu dimana jika sabit kesalahan pada kali pertama maka pesalah boleh didenda sehingga RMIOO,OOO.OO atau dipenjarakan selama tidak lebih daripada 3 tahun atau kedua-duanya sekali. Manakala bagi kesalahan kali kedua dan seterusnya maka pesalah boleh didenda sekiranya sabit kesalahan sehingga RM 200, 000 dan penjara tidak melebihi 6 tahun atau kedua-duanya sekali.(Jurnal Syariah, 12:2, 2004). Malahan di bawah seksyen 10 dan 11 Akta ini maka dua peraturan telah telahpun di bentuk iaitu, Perintah perihak dagangan (Penggunaan perbahasan halal) 1975 dan Perintah dagangan (Penandaan makanan) 1975.(Zalina Z, 2004).

Bagi syarikat atau perusahaan yang pertama kali melakukan kesalahan dan sabit kesalahannya maka mengikut seksyen 18 (2) ia boleh didenda sehingga RM 250,000.00 dan tidak lebih daripada RM 500,000.00 jika sabit kesalahan buat kali kedua dan seterusnya. Manakala seksyen 29 (1) pula memperuntukkan denda bagi kesalahan menghalang pegawai atau penjawat awam atau bagi mereka yang gagal mengikut keperluan yang diminta oleh pegawai atau penjawat awam dan jika sabit kesalahan maka boleh dikenakan denda sebanyak RM 2,000.00. atau boleh dipenjarakan selama tidak lebih daripada 1 tahun atau kedua-duanya sekali. Seksyen 29 (2) pula memperuntukkan penalti atau denda bagi mereka yang memberikan pernyataan salah. (Zalina Z, 2004).

Regulasi Makanan 1985 tidak memperuntukkan secara nyata tentang keperluan halal. Walau bagaimanapun Seksyen 11 (1) (c ), mengatakan bahawa makanan yang terkandung daging lembu, daging babi, atau apa juga yang berasal daripadanya, lemak babi maka perlu dibuat pernyataan tentang penggunaan atau kewujudan bahan tersebut yakni hendaklah tertera pada label produk tersebut. Manakala seksyen 22 (d) menyatakan tentang kewujudan alkohol di dalam apa juga bahan makanan dan minuman yang juga perlu dinyatakan pada label sesuatu produk. (Zalina Z, 2004)

Manakala di bawah Akta Kerajaan Tempatan 1976, seksyen 20 (D) (1) (a) dan (b), Perlesenan Pembuatan Makanan Wilayah Persekutuan dan Kuala Lumpur (Undang-undang Kecil 1995) dimana telah mensyaratkan supaya pemegang lesen mencuci peralatan yang digunakan untuk makanan berstatus tidak halal secara berasingan dengan peralatan yang digunakan untuk makanan yang halał. Sub seksyen 3 pula adalah berkaitan dengan premis yang menyediakan makanan tidak halal supaya mempamirkan perkataan tidak halal sahaja pada menu makanannya. (Zalina Z, 2004).

Peraturan Haiwan 1962 pula telah memberi kuasa kepada pihak JAKIM dan Jabatan Perkhidmatan Haiwan untuk membuat pemeriksaan terlebih dahulu terhadap daging yang hendak di import ke Malaysia. Juga dimestikan untuk proses re- auditing yakni membuat pemeriksaan semula pada setiap dua tahun sekali dimana kos lawatan perlu ditanggung oleh pihak penganjur. Akta tersebut mensyaratkan supaya semua daging yang diimport mestilah berstatus halał, selamat dan tidak menyebabkan timbulnya sebarang penyakit. (Zalina Z, 2004).

Dengan adanya Akta Perlindungan Pengguna, 1999 pula maka seksyen 8 dan 10 Akta tersebut menyatakan bahawa sebarang perbuatan yang boleh menyebabkan kekeliruan kepada para pengguna samada dengan cara tipu atau pernyataan salah terhadap sijil atau logo halal pada apa juga produk maka ia merupakan satu kesalahan yang boleh dihukum denda yakni tidak lebih daripada RM 250,000.00 bagi kesalahan kali pertama dan RM 500,000.00 bagi kesalahan untuk kali kedua. ( Zulkifli, 2007).

Manakala seksyen 42 Akta Kesalahan Jenayah Syariah (Wilayah Persekutuan) 1997, telah memperuntukkan hukuman kepada sebarang individu yang mempamerkan apa-apa makanan dan minuman yang tidak halal dengan apa apa label yang menunjukkan bahawa makanan atau minuman tersebut berstatus halał. Jika sabit kesalahan maka individu tersebut boleh dikenakan denda tidak melebihi RM 5,000.00 atau penjara tidak lebih enam bulan atau kedua-duanya sekali. (Zulkifli, 2007). Seksyen 59 (6) Enakmen Kesalahan Jenayah Syariah (Negeri Sabah) 1995, memperuntukkan bahawa bilamana menyembelih binatang yang halal tanpa mendapat kebenaran daripada Majlis Agama Islam Negeri Sabah maka seseorang iłu jika sabit kesalahan boleh didenda tidak lebih RM 1,000.00 atau dipenjara selama tidak melebihi enam bulan atau keduaduanya sekali. (Zulkifli, 2007).

Akta Binatang 1953 (Semakan 2006), beserta dengan Akta Rumah Penyembelihan (Penswastaan) 1993, dan juga Perintah (Pengimportan) Haiwan 1962 telah memperuntukkan kuasa kepada Kementerian Kesihatan Malaysia (KKM) dan Jabatan Perkhidmatan Veterinar (JPV) berkaitan dengan penternakan dan kawalan penyakit haiwan terutama berkaitan proses permohonan sijil halal untuk daging yang diimport. (Norazla A.W., 2015).

Penggunaan sijil atau logo halal di Malaysia telah melalui beberapa fasa. Pada suatu masa sijil halał dikeluarkan oleh banyak pihak antaranya JAKIM dan juga jabatan-jabatan agama negeri-negeri di Malaysia yang mana jika di kumpulkan melebihi 10 agensi. lni telah menimbulkan pelbagai isu dan masalah kepada pengguna di Malaysia. Oleh yang demikian pada April, 2004, Kabinet di Malaysia telah membuat keputusan bahawa hanya JAKIM sahaja yang mempunyai locus standi dalam pengeluaran sijil halal di Malaysia. Akhirnya ini telah memberikan suatu impak positif kepada pembangunan industri makanan di Malaysia.

Dalam aspek penguatkuasaan Undang-undang halal pula, sebelum berlakunya pindaan terhadap APD 1972, elemen penguatkuasaan undangundang berkaitan kesalahan produk halał dianggap kurang berkesan dan mempunyai banyak kekangan untuk dikuatkuasakan kerana ianya dilaksanakan oleh dua agensi berbeda. Didapati JAKIM hanya bertindak sebagai badan yang memantau sahaja termasuk sebagai pengesah kesalahan dan saksi manakala elemen penting saperti pendakwaan dijalankan oleh KPDNKK. lni kerana soal pendakwaan adalah bidangkuasa KPDNKK dan bukan bidangkuasa JAKIM mahupun JAIN. (Zulkifli, 2007). API, 2011 pula telah memberikan locus standi kepada JAKIM dan MAIN yang mana para pegawai JAKIM telah dilantik sebagai Penolong Pegawai Perihal Dagangan dan diberikan kuasa untuk memberikan amaran, menggantung atau menarek balik lesen perniagaan. Bidangkuasa mereka telahpun di sama tarafkan dengan pegawai penguatkuasa KPDNKK. (Tun Abdul Hamid, 2015).

## 5. KESIMPULAN

Perbincangan telah menyerlahkan bahawa terdapat beberapa Undang-undang khusus samada secara terus mahupun tersirat berkaitan dengan isu dan hukum produk halal di Malaysia sepertimana yang tertera di para 4.0 diatas. Namun rakyat Malaysia secara positifnya sangat-sangat mengharapkan wujudnya Akta Produk Halal Malaysia yang berbentuk berintegrasi dalam semua aspek penting terhadap kelestarian produk halal di Malaysia.

# Determinants of Birth Weight at Various Quantiles in West Sumatra

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## Abstract

Covariates could affect the responses differently at various points of the response distribution. Many covariates might have higher impact on conditional mean of the response than on conditional 10th percentile, for example. These effect can be analyzed directly by using quantile regression. This paper aims to implement the use of quantile regression to identify the determinants of birth weight at various quantiles. A cross-sectional study was conducted in March to June 2016 by distributing questonnaires to mothers who gave birth at any selected hospital in West Sumatra. This research proves that determinants of birth weight at low quantile are education level, problems during pregnancy and prenatal care. Meanwhile parity, pragnancy spacing, problems during pregnancy and gender are associated with higher birth weights. All proposed model could be accepted based on goodness of fit test.

Keywords: quantile regression, cross-sectional study, birth weight

## 1. Introduction

Birth weight has served as leading indicator of infant health, with low birth weight infants classified as those weighing less than 2500 grams at birth. Obsevable measures of mother weight gain, education level, problems while pregnant, age, parity, prenatal care, mother weight gain, hemoglobin (Hb) and spacing pregnancies (Abrevaya& Dahl, 2008; Burgette & Reiter, 2012) were assummed had strong associations with birth weight. For instance, according to a report by Burgette & Reiter (2012), mothers who had problems during pregnancy would have babies with birth weight less than 2500 grams.

Many researches have examined that the low birth weight will cause many problems. Abrevaya in his article wrote that the infant mortality rate increases at lower birth weights. The direct medical costs for babies with low birth weight are quite high as well. The babies with low birth weight have the long term effects on their cognitive development, educational outcomes and labor market outcomes. The babies would have development problems in cognition, attention and neuromotor functioning that persist until adolescence (Hack et. Al., 1995). The babies with low birth weight are more likely to attend special class, delay entry into kindergarden or repeat a grade in school(Corman and Chaikind, 1998). Those babiesare also more likely to have inferior labor-market outcomes, being more likely to be unemployed andearn lower wages (Behrman and Rosenzweig (2004); Case et. al. (2005); Currie and Hyson (1999)).

Although it has received less attention in the economics literature, highbirthweight outcomes can also represent adverse outcomes. For instance, babies weighing more than 4000 grams, classified as high birthweight (HBW) and especially those weighing more than 4500 grams, classified as very high birthweight (VHBW) are more likely to require cesarean-section births, havehigher infant mortality rates, and develop

health problems later in their life.

A difficulty in evaluating initiatives aimed at improving birth outcomes is to accurately estimate the causal effects of prenatal activities on these birth outcomes. Unobserved heterogeneity among childbearing women makes it difficult to isolate causal effects of various determinants of birth outcomes. Whether or not a mother's age affect the infant weight, for instance, is likely to be correlated with unobserved characteristics of the mother. To deal with this difficulty, various studies have used an instrumental-variable approach to estimate the effects of prenatal care (Currie and Gruber (1996); Evans and Lien (2005); oyce (1999)), and air pollution (Chay and Greenstone (2003a, 2003b)) on birth outcomes.

Another approach has been to utilize panel data (i.e., several births for each mother) to identify these effects from changes in prenatal behavior or maternal characteristics between pregnancies (Abrevaya (2006); Currie and Moretti (2002); Rosenzweig and Wolpin (1991); Royer (2004)). One concern with the panel-data identification strategy is the presence of "feedback effects," specifically that prenatal care and mother's habbits in later pregnancies may be correlated with birth outcomes in earlier pregnancies. Royer (2004) provides an explicit estimation strategy to deal with such feedback effects (using data on at least three births per mother). Since the costs associated with birthweight have been found to exist primarily at the low end of the birthweight distribution (with costs increasing significantly at the very low end), any studies have estimated the effects of birth inputs on the fraction of births below various intervals. This present study conside a quantile regression approach to estimate the effects of birth inputs on birthweight.Quantile approach provides a method for determining how birth inputs affect birthweight at different parts of the distribution. The birth inputs involved in this study are 8 indicators consists of continuous and categorical types, they are education level, problems while pregnant, age, parity, prenatal care, mother weight gain, hemoglobin (Hb) and spacing pregnancies (Abrevaya& Dahl, 2008; Burgette & Reiter, 2012).

There are any advantages using quantiles regression then analysis of variance or classical regression. Any reasons why we better use quantile regression are :

- Analysis of variance (ANOVA) and regression provide information only about the conditional mean.
- More knowledge about the distribution of the statistic may be important.
- The distribution of , the dependent variable,conditional on covariate X, may have thick tails.
- The conditional distribution of Y may be asymmetric.
- The conditional distribution of Y may not be unimodal.
- Neither regression nor ANOVA (analysis of variance) will give the robustresults, especially if theoutlier existinside the data.

## 2. Material and Methods

In this present study, we used primary data collected by distributing the questionnaires to mother who just have baby, live, single and stay in West Sumatra. The questionnaires were distributed from March to July 2016. There are 93 respondents with complete information that involved in this study.

The response variable is birth weight, recorded in kilograms. Meanwhile the birth inputs are assummed affeced by eight indicators consists of continuous and categorical types. There are education level, problems while pregnant, age, parity, prenatal care, mother weight gain, hemoglobin (Hb) and spacing pregnancies.The following Table 1 presents the summary statistics of Birthweight data.

Table 1. Descriptive of Birth Weight Data

| | | |
|---|---|---|
| Mean | | 3,063 |
| Median | | 3,100 |
| Mode | | 3,2 |
| Skewness | | -0,592 |
| Kurtosis | | 0,578 |
| Minimum | | 1,1 |
| Maximum | | 4,5 |
| Percentiles | 25 | 2,700 |
| | 50 | 3,100 |
| | 75 | 3,500 |

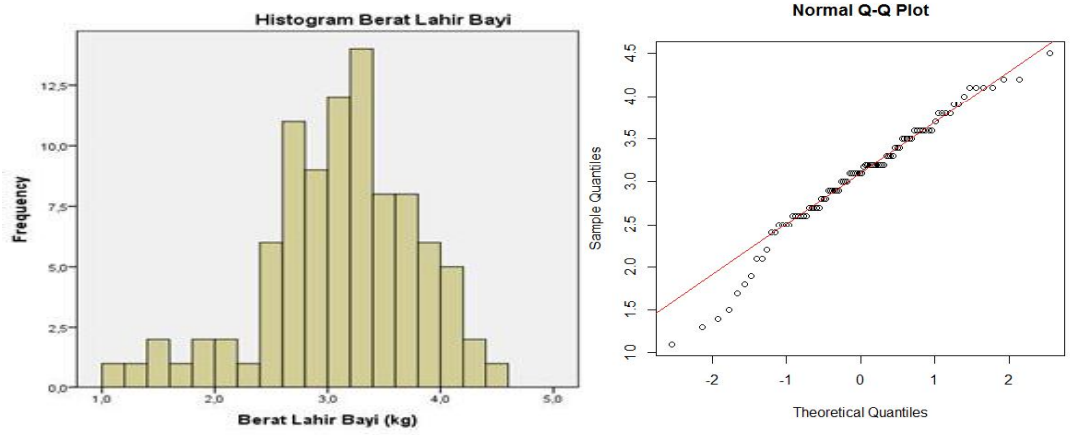
Based on the description in Table 1 we are informed that the mean of birthweigth data is 3,063. The highest value of the data is 4,5 and the lowest value is 1,1. We also see that the distribution of the data is skewed to the left since its skewness is - 0,592, as presented in Figure 1.(a).

For construction of Birthweight model, this present study apply the quantile regression approch, since we purpose to identify the Birthweigth model for any quantiles (low quantile, middle quantile and high quantile). The following is the general explanation regard quantile procedure used in this research.

As described by Gilchrist (2000), quantile is defined as the value that corresponds to a specified proportion of a sample or population. Thus, we may defined $\tau$ thquantile as the value which divide the data into two parts, the $\tau$ fraction of the data below it and 1-$\tau$ fraction of the data above it, and $0<\tau<1$. Median is a very commonly used quantile, which is aqual to a proportion of 0.5 is the ordered data. Regression analysis is used to quantify the relationship between a responce variable and one or several of free covariates (Yanuar, 2013).

Quantiles regression as an introduced by Koenker and Basset (1978), is a statistical method used to estimate models for conditional quantile functions. Unlike the classical linear regression methods that are based on minimizing sums of squared residuals and to estimate models for conditional mean functions, quantile regression methods are based on minimizing absolute residuals, and intended to estimate conditional median functions and a full range of other conditional quantile functions. Quantile regression also provides a more complete graph of the conditional distribution of variable ot interest $Y$ given $X=x$.

For a random sample $\{y_1,\ldots,y_n\}$, the classical linear regression can be estimated by the well-known method, which minimizes the sum of squared residuals:




(a) (b)

Figure 1.(a) Histogram of Birthweight data.
(b) Empirical quantile plot and Normal plot of Birthweight data.

$$\min \sum_{i=1}^{n}(y_i - \hat{y}_i)^2 \qquad (1)$$

For the special case of estimation the conditional median of function, we can define the solution as problem to minimization a sum of absolute residuals, where there are the same numbers of observations above and below the median, which can be calculated by:

$$\min \sum_{i=1}^{n}|y_i - \hat{y}_i| \qquad (2)$$

Analogous to the concept of median, Koenker and Basset (1978) proposed a complete and different method for estimation of an unknown value, say a, for any $\tau$ in the interval (0,1), which may be defined as any solution to the minimization problem of the equation:

$$\min_{a\in\Re}\left\{\sum_{i=1}^{n}\ \tau\ |y_i\ - a| + \sum_{i=1}^{n}(1-\tau)\,|y_i - a|\right\} \qquad 0<\tau<1 \qquad (3)$$

Consider a classical linear regression model $y_i = \boldsymbol{x}_i'\boldsymbol{\beta} + e_i$, we defined a linear model for the $\tau$ -thquantile as:

$$y_i = \boldsymbol{x}_i'\boldsymbol{\beta}_\tau + e_i \qquad i = 1, \ldots, n \qquad (4)$$

In estimating models for conditional quantile function, we minimize a sum of asymmetrically weighted absolute residuals. This will contribute to different weights to positive and negative residuals. The general $\tau$ -th sample quantile, which is the analogue to equation (3) can be formulated as:

$$\hat{\beta}(\tau) = \min_{\beta\in\Re}\left\{\sum_{i=1}^{n}\ \tau\ |y_i - \boldsymbol{x}_i'\boldsymbol{\beta}_\tau| + \sum_{i=1}^{n}(1-\tau)\,|y_i - \boldsymbol{x}_i'\boldsymbol{\beta}_\tau|\right\}$$

And equivalently written as:

$$\hat{\beta}(\tau) = \min_{\beta\in\Re}\sum_{i=1}^{n}\ \rho_\tau\ (y_i - \boldsymbol{x}_i'\boldsymbol{\beta}_\tau) \qquad 0<\tau<1 \qquad (5)$$

Several software packages can be used to implement the quantile regression method, such as S-plus, R-program and Stata. In this research, R software was used to analyze data of the Birthweight.

## 3. Result and Discussion

In this analysis, quantile regression approach is used to examine the relationships between the Birthweight and some potential explanatory variables. Table 2 provides a summary of describe the explanatory variables which are found to have a significant relationships with Birthweight for various conditional quantile function, particularly for τ equals 0.10, 0.25, 0.50, 0.75 and 0.90. In the last column are the estimated of ordinary least square approach and its corresponding standard errors in the brackets.

Table 2 informs us that mother with middle or high education level tent to have havier baby than mother with low education level. Mother with more parities tent to have havier baby than less parities. Mother with longer space of pregnancies tent to have ..... baby. The heavier of mother's weight gain the heavier of birthweight of her baby. The higher of mother's hemoglobin, the heavier of her baby's birthweight. Thus this study found that significant variables which effect the baby's birthweight are education level, parity, spacing pregnancies, mother's weight gain and mother's hemoglobin.

Table 3 presents the goodness of fit for all model, indicated by *PseudoR*$^2$ value. Based on the result of this study, it proved that all model at any selected quantiles are acceptable since all *PseudoR*$^2$ values more than 0.7. The best model is at middle quantile (*PseudoR*$^2$equals 0.910).

Table 2. Coefficient Estimated for Birth Weight Model Using Quantile Regression (QR) and OLS

| Indicator Variable | Estimate of QR (Standard Error) | | | | | Estimate of OLS (Standard Error) |
|---|---|---|---|---|---|---|
| | $\tau = 0.10$ | $\tau = 0.25$ | $\tau = 0.50$ | $\tau = 0.75$ | $\tau = 0.90$ | |
| (Middle) | 0.700 (0.224)** | 0.501 (0.243)** | 0.378 (0.173)** | 0.421 (0.262) | 0.203 (0.228) | 0.364 (0.174)** |
| (High) | 0.662 (0.345)** | 0.433 (0.258)* | 0.378 (0.184)** | 0.613 (0.279)** | 0.149 (0.243) | 0.420 (0.178)** |
| Parity) | 0.331 (0.137)** | 0.235 (0.102)** | 0.106 (0.073) | 0.216 (0.111)** | 0.279 (0.096)** | 0.242 (0.073)** |
| Spacing pregnancies) | -0.089 (0.057) | -0.070 (0.042)* | 0.014 (0.030) | -0.039 (0.046) | -0.074 (0.040) | -0.052 (0.030)* |
| Weight gain) | 0.038 (0.025) | 0.023 (0.019) | 0.033 (0.013)** | 0.006 (0.020) | 0.015 (0.017) | 0.021 (0.013) |
| Hb) | 0.108 (0.099) | 0.130 (0.074)* | 0.097 (0.052)* | 0.062 (0.080) | 0.126 (0.069)* | 0.077 (0.053) |

* Significant at 10% level
**Significant at 5% level

Table 3. *PseudoR*$^2$ for Selected Quantile for Low Birth weight Cases

| Quantiles | *PseudoR*$^2$ |
|---|---|
| 0.10 | 0.736 |
| 0.25 | 0.877 |
| 0.50 | 0.910 |
| 0.75 | 0.909 |
| 0.90 | 0.856 |

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