EFFECTIVITY OF AVOCADO SKIN ETHANOL EXTRACT (*Persea americana* Mill.) ON HISTOPATHOLOGICAL IMAGE OF MIDDLE EAR MUCOSA OF WHITE RATS (*Wistar* Rattus norvegicus) INFECTED BY *Staphylococcus aureus*

Ricard Daniel Manurung¹, Andre Jonathan Samuel Gultom², Yuliani Mardianti Lubis³

¹Medicine Study Program, Faculty of Medicine, Prima Indonesia University, Medan

*Corresponding Author: ricarddaniel16@gmail.com*

**ABSTRACT**

This study was conducted with the aim to determine the effectivity of avocado skin ethanol extract with 50% and 100% concentrations on histopathological image of middle ear mucosa infected by *Staphylococcus aureus* in white rats. *Staphylococcus aureus* are bacteria that can cause infection to the middle ear mucosa, or usually known as otitis media. This study used posttest only control group design. Effectivity was assessed using embedding technique, i.e. by dipping the tissue to paraffin liquid. The results were analyzed using Post-hoc Tukey HSD statistical test, where the results found significant difference between treatments. Avocado skin ethanol extract with 100% concentration was better, with PMN cell count of 40.25 per HPF, compared to avocado skin ethanol extract with 50% concentration with only 71 PMN cells per HPF, while treatment using ofloxacin antibiotic only found 22 PMN cells per HPF. The most effective concentration of avocado skin ethanol extract was 100%.

Keywords: Avocado skin, histopathological image, otitis media, *Staphylococcus aureus*

**INTRODUCTION**
Infectious diseases are the primary cause of high morbidity and mortality rate, especially in developing countries, such as Indonesia. Infectious disease is a disease caused by pathogenic microbes (Darmadi, 2008). One of the causes of infection is bacteria (Radji, 2011).

Inflammation of the middle ear, or usually known as otitis media, is an inflammation that occurs in all or part of the middle ear mucosa, Eustachian tube, mastoid antrum, and mastoid cells. Otitis media can be divided into suppurative and non-suppurative otitis media, whereas both have acute and chronic condition (Yuspita, 2018).

Acute otitis media has local or system symptoms and clinical signs which occurs in full or in part, such as otalgia, fever, anxious, nausea, vomiting, and diarrhea. The most common cause of otitis media is pyogenic bacteria. These bacteria are Streptococcus pneumoniae (40%), Haemophilus influenzae (25-30%) and Moraxella catarrhalis (10-15%), and the rest 5% was found in cases with other pathogens, such as Streptococcus pyogenes, Staphylococcus aureus and gram-negative organisms. Streptococcus aureus is usually found in children and neonates. Meanwhile, Haemophilus influenzae is commonly found in toddlers (Kerschner, 2007).

In 2006, according to the data obtained from the ENT clinic, H. Adam Malik General Hospital, Medan, 26% of all visits were chronic suppurative otitis media, while in 2007 and 2008 the case reached 28 and 29%, respectively. In 2009, there were 130 cases of chronic suppurative otitis media and 65 cases with cholesteatoma, and 35 cases with complications in H. Adam Malik General Hospital, Medan (Rambe, 2009).

Antibiotic resistance is possibly caused by frequent administration of antibiotic, characterized by increasing antibiotic sensitivity each year. Sensitivity of Staphylococcus aureus on tetracycline was 53.3%, chloramphenicol was 23.6%, ampicillin was 18.1%, cefotaxime was 6.6% and gentamycin 4.2%. This condition showed that most microorganisms were resistance (Refdanita et al., 2001).

Staphylococcus aureus is a spherical gram-positive bacterium with 0.7-1.2 μm diameter, divided into grape-shaped irregular parts, facultative anaerobe, did not produce spores, and immobile. Around 90% clinical isolate produced Staphylococcus aureus with polysaccharide capsules or thin membrane which plays a role in bacterial virulence (Yulina, 2015).
*Staphylococcus aureus* is non-motile, non-spore, facultative anaerobe, positive catalase, and negative oxidase. *Staphylococcus* can grow within 6.5-46°C under 4.2-9.3 pH (Paryati, 2002). The colony can grow 4 mm diameter within 24 hours. The colony is solid, round smooth, prominent and shiny. The colony of *Staphylococcus aureus* has grey to dark gold-yellow color. Lipochrome pigment will form on *Staphylococcus aureus*, which lead to golden-yellow and orange-yellow color (Todar, 2002).

Avocado (*Persea americana* Mill.) is a plant that thrives in tropical region such as Indonesia. Avocado is a fruit favored by many Indonesian, due to its delicious flavor and high antioxidant content (Afrianti, 2010). However, many people had not utilized the seed optimally, and most people just throw it out and let it become a waste.

According to Vinha et al., (2013), avocado seed and skin have similar benefit, which is as antioxidant. Compared to its flesh, avocado seed and skin have higher antioxidant content. Other than antioxidant, avocado skin also has flavonoid, which is an anti-inflammatory substance. Adeyemi et al. (2002) mentioned flavonoid as a substance contained in avocado leaf extract, which had analgesic and anti-inflammatory effects. In the medicine field, flavonoid has been widely used, e.g. for inflammatory disease (Zuhrotun, 2007).

With abundant nutrition, avocado can be used for various needs, including:

1. Avocado skin has many benefits which can be used for medicine. One of the substances contained in the skin is flavonoid. Flavonoid can be found in many parts of plants, such as the fruit, root, leaf, and skin. Flavonoid is a natural substance with a potential as antioxidant, which can deflect free radicals that acts in degenerative disease through damaging the body immune system, lipid and protein oxidation (Rais, LR, 2015).

2. Tannin is an active secondary metabolite known to have several benefits, such as astringent, antidiarrhea, anti-bacteria and antioxidant. Tannin is a highly complex organic component, which comprises of phenolic substance, which is difficult to separate and crystalize, depositing protein from its solution and compound with the protein (Desmiaty et al., 2008). Tannin is divided into two groups, i.e. hydrolyzed tannin and condensed tannin. Tannin has a complex biological role, starts from protein settler and metal chelator. Tannin can also act as biological antioxidant (Hagerman, 2002).
Based on the statements above, the author would like to conduct a study on whether avocado skin extract has the potential as antimicrobe by investigating the middle ear Histopathologically. Therefore, we conduct a study to provide results comparable to antibiotic, which is an alternative in the form of avocado skin extract. Many studies stated that avocado skin had many benefits as an alternative medicine, due to its flavonoid content which is highly beneficial for the human body.

METHODS

This study is experimental with posttest only control group design. The study was conducted for 2 months, from September 2019 to October 2019 in Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, Sumatera Utara University, and Histology Department, Faculty of Medicine, Sumatera Utara University, Medan.

The tools used in this study include 1 ml 30G injection syringe (Terumo®, Japan), 1 ml 26G injection syringe (Terumo®, Japan), per oral syringe, measuring cup, volume pipette, scalpel and bladder, surgical scissors, tweezers, wax board, and fixation tool, ointment bottle, mortar and pestle, Erlenmeyer flask, beaker glass, micro pipette, stirrer, micro pipette (Socorex®, Switzerland), analytical balance (Chyo® Jupiter C3, 100 MD), electrical gram balance (PJ, Precisia® Junior, Switzerland), binocular microscope (Olympus®, Japan), camera (Olympus®, Japan), gloves, DA-5 OptiVisor, nasogastric tube, forceps and documentation equipment.

The materials used in this study include avocado skin, divided into two concentrations, i.e. 50% and 100% and ofloxacin tablets, 50 mg/kg body weight ketamine hydrochloride and phenobarbital inj. 80 mg/kg body weight and 20 white wistar rats (*Rattus norvegicus*) with average body weight of 200 mg and average age of 6-8 weeks. The sample size in this study was determined according to Federer formula for experimental study, i.e. $(t-1)(n-1) > 15$. This *in vitro* study used 5 groups; thus, each group should contain 4 rats.

Extract was formed by the following procedure: The cleaned avocado skin was cut thinly and dried under the lamp until the water content reduced and the avocado skin
can be easily broken. Afterwards, the dried avocado skin was grinded using a blender and extracted with 96% ethanol solvent (Yusra, 2012).

Avocado skin ethanol extract was divided into two concentrations by dilution with DMSO. Treatment group 1 (P1) with 50% concentration and treatment group 2 (P2) with 100% concentration. The concentration was made using the following formula (Faradiba, 2014):

\[
\% = \frac{\text{Solute volume}}{\text{Total volume}} = \frac{\text{Solute volume}}{\text{Solute volume} + \text{Solvent volume}}
\]

Preparation of Animal Model and Treatment

1. Male wistar rats were cared for in Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, USU and given code 1 to 20
2. Rats that were used in this study were placed individually in a box cage made from plastic with surface area of 150 cm² and 15 cm height.

Animal Treatment

This study was conducted using male wistar rats weighing in average of 220 g and none of them had outer and middle ear infection without underlying tympanic membrane disorder under DA-5 OptiVisor. There were 4 rats included in group I that were not given any treatment. Afterwards, 16 rats were anesthetized with ketamine hydrochloride (50 mg/kg body weight intramuscularly). The animals were assessed using optivisor for 48 hours after inoculation and acute otitis media was verified and 4 rats were included in group II. Afterwards, group III was given avocado skin extract treatment with 50% concentration (4 rats), and group IV was given avocado skin extract with 100% concentration (4 rats), and group V was given 15-20 mg/kg body weight/day ofloxacin tablet (4 rats).

The Making and Observation of Middle Ear Mucosa Histology

The process of histopathology preparation followed Sari (2015), i.e.:

1. Fixation
The middle ear mucosa was immersed in 10% formalin solution, preceded by rinsing with 0.9% physiological NaCl to fixate the tissue to prevent damage and stored at 25°C.

2. Dehydration and Infiltration
   Dehydration was conducted using alcohol with the aim to release water contained in tissue.

3. Clearing
   Clearing aims to release alcohol in the tissue which will bond with paraffin.

4. Paraffin Infiltration
   Tissue was immersed in liquid paraffin I, II, and III.

**Tissue Embedding and Sectioning**

The embedding technique comprised of tissue dipped into liquid paraffin previously poured into a block-shaped mold which will solidify.

1. **Hematoxylin-Eosin Staining**
   Hematoxylin staining will give blue color to the nucleus and eosin will give pink color to the cytoplasm.

**Observation of Histopathological Slide of Middle Ear Mucosa**

Histopathological observation was conducted using Olympus BX51 light microscope. The histopathological damage was observed by scoring method using five different fields of view with 400x magnification concordant to histopathological scoring method by Manja Roenigk (Sutrisna et al., 2013).

**Study Variables**

Independent variable : Avocado skin ethanol extract with administration pattern

Dependent variable : Histopathological image of middle ear mucosa of wistar rats

Control variable : Age of rats, application method of testing materials, cage and animal control.
Data Analysis

If the data were normally distributed (P > 0.05), then variance analysis (ANOVA) was conducted to determine the effect of avocado skin extract with concentration variance on histopathological image of middle ear mucosa. If variance analysis showed significant difference (P < 0.05), then Post-hoc LSD multiple range test was conducted to determine the mean difference of observation between each group using SPSS (statistical package for the social sciences) program version 25.0 for Windows.

RESULTS AND DISCUSSION

Results

In this study, the rats were divided into five groups, whereas each group was given different treatment in order to count PMN cells in middle ear mucosa induced by S. aureus.

<table>
<thead>
<tr>
<th>Histopathological Parameter</th>
<th>Normal (without treatment)</th>
<th>Induced by S. aureus</th>
<th>Injected by S. aureus + 50% avocado skin extract</th>
<th>Injected by S. aureus + 100% avocado skin extract</th>
<th>Injected by S. aureus + ofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN count</td>
<td>0 0 0 0</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>Mean</td>
<td>0</td>
<td>157.25</td>
<td>71</td>
<td>40.25</td>
<td>22</td>
</tr>
</tbody>
</table>

In this table, the group that was not given treatment showed mean PMN count per field of view of 0 per HPF. The group that was induced with S. aureus without treatment showed 157.25 per HPF, while the group that was induced by S. aureus and given avocado skin extract with 50% concentration showed 71 per HPF and the group induced by S. aureus and given avocado skin extract with 100% concentration showed
40.25 per HPF. Meanwhile, the group induced by *S. aureus* and given ofloxacin showed 22 per HPF.

The results of ANOVA parametric test showed significant, thus can be continued with Post-hoc Tukey HSD.

From ANOVA, we obtained p value of histopathological score of each treatment was 0.005 (< 0.05), which means there was a significant difference from each treatment. Afterwards, the analysis was continued with Post-hoc Tukey HSD to determine correlation between treatments in each group.

The results of Post-hoc Tukey HSD for histopathological score of middle ear mucosa showed group 1 had significant difference with group 2 and 3 (p < 0.05). Group 2 had significant difference with all other treatments (p < 0.05). Group 4 and 5 had significant difference with group 2 (p < 0.05).

![Histopathological Results](image)

**Figure 3.1** Mean PMN cell count

**Discussion**

In rats induced by *S. aureus*, the mean PMN cell count was 157.25 per HPF. This showed that *S. aureus* infection can increase PMNs in middle ear mucosa. The body has non-specific defense mechanism which can prevent the entry and spread of microorganisms, and prevent tissue damage, and eliminate intracellular bacteria by
polymorphonuclear cells (PMNs) and macrophages, which is called innate immunity (Zakiudin Munasir, 2001).

The group that was given avocado skin extract with 50% and 100% concentrations showed PMN decrease. This showed that avocado skin extract had the potential as anti-inflammation by suppressing inflammation in the middle ear caused by infection.

The skin and seed extract of *Persea americana* Mill. with 80% methanol contain a lot of phenolic substances such as flavonoid, procyanidin, and hydroxycinnamic acid. The phenolic content was higher in the skin compared to the seed. Procyanidin, catechin, quercetin, and 5-O-caffeoylquinic acid are several substances that are found both in the skin and seed of *Persea americana* Mill. (Kosinska et al., 2012).

Phenolic substance contained in avocado has high antioxidant activity shown in various *in vitro* studies. It has several abilities as anti-inflammatory, anticoagulant, antioxidant, and immune system improvement (Arukwe et al., 2012).

The pharmacological activity of flavonoid substance is as antiallergy, antivirus, antiinflammation, hepatoprotective, antioxidant, antithrombotic, vasodilator and anticarcinogenic (Seyoum et al., 2006).

Phenolic and flavonoid substances can reduce and bond free radicals in human body (Hidalgo et al., 2010).

In this study, the administration of avocado skin extract with 100% can reduce PMN count better than avocado skin extract with 50% concentration. This showed that the higher the concentration of the extract, the higher the potential as antiinflammation.

Avocado skin contains flavonoid, which also acts as antimicrobe, which are triterpenoid, flavonoid, tannin, and polyphenol (Zuhrotun, 2007). Flavonoid has 3 working mechanisms as antimicrobe, i.e. inhibiting the synthesis of nucleic acid, inhibiting the function of cell membrane, and inhibiting the function of energy metabolism (Hendra R., 2001).

Flavonoid has A and B rings to inhibit the synthesis of nucleic acid by its role in intercalating process or hydrogen bond by stacking nucleic acid to inhibit the process of DNA and RNA formation. Interaction between flavonoid and bacterial DNA will cause damage to the permeability of bacterial cell wall, microsome, and lysosome (Chusnie TP, 2005). Flavonoid will create a complex substance with protein to inhibit cell
membrane function, thus damaging it and release intracellular substance (Li, H. Wang, 2003).

Flavonoid will inhibit the use of oxygen by bacteria, thus inhibiting energy metabolism. The metabolism is inhibited because flavonoid inhibit cytochrome C reductase (Chusnie TP, 2005).

Meanwhile, phenol can be denatured by cell protein, which is antibacterial mechanism to eliminate microorganism. When a hydrogen bond form between phenol and protein, it will damage protein structure. Cell wall and cytoplasm membrane are composed by protein; thus, hydrogen bond will affect permeability of cell wall and cytoplasm membrane and misbalancing macromolecules and cell ions, thus causing lysis (Placzar JM, 1998).

Other content of avocado skin is tannin, which acts as antibacterial by precipitating protein. Tannin has antibacterial reaction toward the cell membrane, enzyme inactivation and genetic material function inactivation. As antibacterial, tannin works by inhibiting the reverse transcriptase and DNA topoisomerase enzymes, thus bacteria cells fail to form (Nuria, 2009).

This was in accordance with the results of this study, in which the administration of avocado skin with 50% and 100% concentrations can suppress inflammation caused by *S. aureus* infection. We can assume that the active substance in avocado skin can suppress *S. aureus* infection, seen by the reduction of PMN cell count caused by the infection.

In this study, ofloxacin reduced more PMN cells in middle ear mucosa of rats with 22/HPF, compared to avocado skin with 50% concentration with 71/HPF and 100% with 40.25/HPF. This showed that ofloxacin has the potential to reduce inflammation.

By using the system to provide assessment for examination materials other than sputum and vaginal secretions, the level of PMN cells produced from the administration of avocado skin extract with 50% and 100% concentrations was considered high. Meanwhile, ofloxacin was in moderate category. This study showed that ofloxacin was more effective as an anti-inflammatory agent compared to avocado skin extract. The administration of avocado skin extract and ofloxacin in this study was performed in 8
days. Longer duration and continuous appropriate dose might show better effectivity of avocado skin extract compared to ofloxacin, thus requiring further studies.

CONCLUSION

The results of Post-hoc Tukey HSD for histopathological score of middle ear mucosa showed that group 1 had significant difference with group 2 and 3 (p < 0.05). Group 2 had significant difference with all other treatment groups (p < 0.05). Group 4 and group 5 had significant difference with group 2 (p < 0.05).

The rats induced by *S. aureus* and given avocado skin extract with 50% concentration showed 71 PMNs per HPF. Rats induced by *S. aureus* and given avocado skin extract with 100% concentration showed 40.25 PMNs per HPF. The administration of ofloxacin reduced PMN count in middle ear mucosa of rats with 22 PMNs per HPF, higher than 50% avocado skin extract with 71/HPF and 100% with 40.25/HPF. This showed that ofloxacin had the potential to reduce inflammation.

ACKNOWLEDGEMENT

The author and team acknowledge Dr. dr. I Nyoman Erich Lister, M.Kes., AIFM as the head of Universitas Prima Indonesia, dr. Yuliani Mardiati Lubis, Sp. THT-KL as advisor, Dr. Chrismis Novalinda Ginting, M.Kes as the rector of Universitas Prima Indonesia, and dr. Linda Chiuman, M.K.M., AIFO-K as the Dean of Faculty of Medicine, who provided facilitations for the completion of this study.

REFERENCES


Chua ML, Barez MYC, Santos M, et al. The Value of Prompt Culture of an Adequate Sputum Specimen in predicting the Potential Etiology of


Clinical Microbiology Proficiency Testing Guideline Agustus 2003. 50 Indonesian Journal of Clinical Pathology and Medical Laboratory, Vol. 17, No. 1, November 2010: 44-43


