THESIS

THE EFFECTS OF PROPOLISON HISTOLOGICALFEATURESONSMALL INTESTINE OF MICE (*Musmusculus*)

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THE EFFECTS OF PROPOLIS ON HISTOLOGICAL FEATURES OF SMALL INTESTINE OF MICE (*Mus musculus*)

Thesis

Submitted in partial fulfillment of the requirement for the degree of Bachelor of Veterinary Medicine

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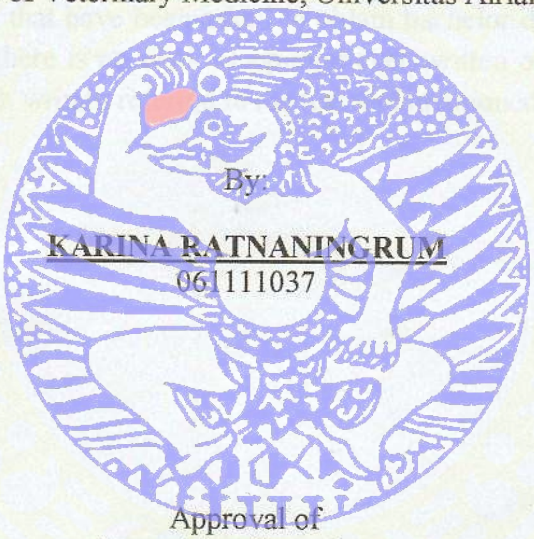

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THE EFFECT OF PROPOLIS ON HISTOLOGICAL FEATURES OF SMALL INTESTINE OF MICE (*Mus musculus*)

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ABSTRACT

The purpose of this research was to determine the side effect of propolis on histology of small intestine of mice (*Musmusculus*). 25 male mice (*Musmusculus*) aged 12-week-old with average 25-30 gram were used. Mice divided randomly into five groups; each group consisted of five mice. P0 as control group, and other groups; P1, P2, P3 and P4 were given propolisethanolic extract orally with doses as follows; 1.6 mg/0.5ml/day, 3.2 mg/0.5ml/day, 6.4 mg/0.5ml/day and 12.8 mg/0.5/day. Treatment were given for two weeks, animal were euthanized and the organs (small intestine) were taken for making the histological specimen using H.E staining. Objects observed were epithelial villi damage, congestion &oedema and neutrophil infiltration. Data were analyzed using Kruskall-Wallis method, continued using Mann-Whitney test if there's significant difference. The result showed that treatment using different dose of ethanol extractedpropolis did not give any significant difference effect toward histological features of small intestine of mice.

Key words: propolis ethanolic extract, small intestine specimens, epithelial villi damage, congestion, oedema, neutrophil infiltration

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I, as the author, understand that this writing is still lacking in several parts and far from perfection. However, I sincerely hope that this research will be useful for the advancement of science and may contribute to the veterinary medicine world and the society.

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Author

CONTENTS

COVER ……i
ENDORSEMENT FORM ……...ii
DECLARATION ……iii
ABSTRACT……iv
ACKNOWLEDGEMENT ……...……v
CONTENTS……...……vii
LIST OF TABLES ……ix
LIST OF FIGURES ……...x
LIST OF APPENDICES ……xi
ABBREVIATIONS AND SYMBOLS ……xii
CHAPTER 1 INTRODUCTION ……..……1
1.1 Background of Problem……..1
1.2 Identification of Problem……2
1.3 Theoritical Base ……3
1.4 Objectives of Research……..……5
1.4.1 Spesific objectives……5
1.4.2 General objectives ……5
1.5 Outcomes of Research……6
1.6 Hypothesis of Research ……..6
CHAPTER 2 LITERATURE REVIEW ……..……7
2.1 Characteristic of Propolis……...……6
2.2 The advantage of Propolis ……9
2.3 Histological Features of Small Intestine……..……10
2.4 Mice (*Musmusculus*) as Experimental Animal ……..……13
CHAPTER 3 MATHERIAL AND METHODS ……16
3.1 Place and Period of Research ……..……16
3.2 Design of Research ……...16
3.3 Research Material ……..…..17
3.4 Research Method……..18
3.4.1 Preparing extract of propolis ……..…18
3.4.2 Treatment in experimental animal ……..…18
3.5 Preparing Histological Specimen of Small Intestine…..……..…20
3.5.1 Organs collection ……...……20
3.5.2 Histological specimenmaking process ……..…….20
3.6 Examination of Histological Specimen of Small Intestine ……..20
3.7 Data analysis ……..……21
3.8 Research Framework……22
CHAPTER 4 RESULT OF RESEARCH ……...……23
4.1 Epithelial Villi Damage……25
4.2 Congestion and Oedema ……..26
4.3 Neutrophil Infiltration ……..27
CHAPTER 5 DISCUSSION ……..……28
5.1 Epithelial Villi Damage ……...……28
5.2 Congestion and Oedema ……..29

5.3 Neutrophil Infiltration ……30
5.4 General Discussion ……..30
CHAPTER 6 CONCLUSION AND SUGGESTION ……..…….33
6.1 Conclusion……...…….33
6.2 Suggestion ……33
SUMMARY ……...……34
REFERENCES ……..…….37
APPENDIX ……40

LIST OF TABLE

Table Page

2.1 Chemical Composition of Propolis 9

4.1 Scoring of epithelial damage, congestion & oedema, and neutrophil infiltration (Mean±SD) of small intestine of mice (*Mus musculus*)..... 23

LIST OF FIGURES

Figure		Page
2.1	*Apismellifera*	7
2.2	Histological picture of small intestine	12
2.3	*Musmusculus*	14
4.1	Villi of small intestine intended to visualize epithelial damage	25
4.2	Red blood cells accumulation in venule and oedema in muscular layer of the small intestine	26
4.3	Neutrophil infiltration in lamina propia of small intestine	27

LIST OF APPENDICES

Appendix Page

1. Propolis Extraction Process 40
2. Propolis Dilution Process 42
3. Dosage Convertion Table 43
4. Determination of the Number of Minimal Replications 45
5. Determination of the Dose of Propolis 46
6. Dosage Calculation 47
7. Procedure of Making Histological Specimen 49
8. Scoring Result 52
9. SPSS Windows Statistic Result 43
10. Documentation 55

ABBREVIATION AND SYMBOL INTEPRETATION

Ca : Calcium

CMCna :Carboxymethyle Cellulose Natrium

CRD : Completely Randomized Design

Cu : Cuprum

et al : et alli

Fe :Ferum

GDC :Gedung Diagnostic Center

H^+ : Ion Hidrogen

HE :*Hematoxilin Eosin*

I :Iodin

K^+ : Ion Kalium

Mg :Magnesium

Mn : Manganese

Na^+ : Ion Natrium

NaCl :*NatriumClorida*

pH : potential of Hidrogen

SPSS : *Statistical Product and Service Solutions*

Zn : Zinc

CHAPTER 1

INTRODUCTION

1.1 Background of Problem

It has been known that Indonesia has many kinds of local bees. One of the local bee propolis producer is *Trigona sp.* and *Apis sp.* (Angraini, 2006). The genuses of Apis has several species, namely *Apis cerana, Apis koschevnikovi, Apis nigrocincta,* and *Apis mellifera*. *Apis mellifera* honeybees are the most productive and able to withstand all weather, makes this species became the most widely farmed bees (Suranto, 2010).

Bee propolis is a mixture of compounds collected by honey bees from various plant sources and used by bees to seal holes in their honey combs, smooth out the internal walls and protect by entrance against intruders (Kamel, 2007). Propolis is not only a building material, it is the most important "chemical weapon" of bees against pathogen microorganisms and has been used as a remedy by humans since ancient times (Bankova, 2005). Etymologically, the Greek word propolis means "*pro*", means indefence, and "*polis*" means the city, so that the meaning is "defence of the hive" (Sforcin and Bankova, 2007).

In general, propolis is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and other substances (Burdock, 1998). Propolis contains some minerals such as Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe as well as some vitamins like B1, B2, B6, C and E, and a number of fatty acids (Thikonov, 1987 in Lotfy, 2006). Halim et al. (2012) in their study

comparing propolis products from Indonesia (*Apis Melifera*) and propolis products from Brazil and the result said that Indonesian Propolis has a content of vitamins and mineral nutrients (vitamin B1, B2, B6, C, and E and minerals Na, Ca, Mg, Cu, Zn, Mn and Fe) is higher than the Brazilian propolis, but its levels of vitamin A are lower.

There are so many benefits of propolis. In 1963, a rat was found dead for 5 years inside a bee hive and does not rot. This happens because the rat carcass wrapped by propolis which proved that propolis contains an antimicrobial agent. In addition of its antibacterial effect, some research indicates that effective propolis as anticancer, antiviral, antifungal, antioxidant, immune-boosting, strengthen, anti-inflammatory, and accelerate cell regeneration (Angraini, 2006).

Plants and plant extracts are effective mainly on the digestive system of animals. Their function either by wiping out the pathogenic microflora in the digestive system or increasing the concentration of microbial population in the digestive system that contributes to improved digestion and absorption of nutrients (Wenk, 2000 in Tekeli et al., 2010).

1.2 Identification of Problem

Based on the proposed formulation of the problem has been described as follows:

1. Does propolis administration affect on the histological features of small intestine of male mice (*Mus musculus*)?
2. Does different dose of propolis affect on the histological features of small intestine of male mice (*Mus musculus*)?

1.3 Theoritical Base

Small intestine of mice consisting of the duodenum, jejunum and ileum. Small intestine has functions such as doing food digestion and absorption (Samuelson, 2007). Villi is the most responsible part of nutrition absorbtion because it has absortive cell in its mucosa which is a single layer of columnar epithelial cells with striated border (Leeson, 2012).

Propolis contains of many nutrition values, like vitamin A, B, C, minerals (Ca, Mg, Na, Fe, Mn, Cu, Zn). Active substances known in propolis are poliferol (flavonoid, fenolat acid, and its esters), terpenoid, steroid, and amino acid. Flavonoids are substances found in many plants and has an antioxidant effect on decreasing free radical (Halim *et al*., 2012). Flavonoids and phenolic acids is known as a substance that is capable of enhance the immune system. Propolis as an immunomodulator is increasing the number of macrophage cells as a cellular immune response when administered in a short period of time (Mustafiah *et al.*, 2011).

Propolis extract and other substance Z. officinale supplemented in the ration both separately and in combination proved to stimulate lactic acid bacteria and significantly decrease pathogenic bacteria such as total mesophilic aerobicm coliform and E. Coli (Tekeli *et al.*, 2010). Propolis showed a significant protective effect on ileal mucosa and reduced bacterial translocation by enhancing mucosal barrier function, supporting generalized immune function, and reducing bacterial overgrowth (Sabuncuoglu, 2007). Modern herbalists recommended it for its

antibacterial, antifungal, antiviral, hepatoprotective, immunomodulatory anti-inflammatory, and anti-ulcer properties (Castaldo, 2002 in de Barros *et al*., 2007).

Naturally, propolis contains of wood chips, sand and leaves. To separate propolis from those material, the used method is using ethanol solvent extraction. Ethanolic extract of *Apis mellifera* propolis had a potent antioxidant activity (Radiati *et al.*, 2008).

The excess of propolis as a natural antibiotic compared with synthetic material is safer and side effects are minor. Previous studies stated the only side effects occured and rarely happened was allergic reaction when it used locally, whereas given orally did not cause resistance. (Winingsih, 2004 in Angraini, 2006).

Research about the side effect of propolis were still minimum thus propolis aministration to mice or to humans does not seem to have side effects (Sforcin and Bankova, 2007). The knowledge of propolis volatile oils is far from being exhaustive. The flora that bee used to collected porpolis determines the chemical composition of propolis, including volatile compounds. Volatile compound, which is the secondary plant metabolites are produced by different plant species and are not the same in all over the countries. The term “propolis” does not have any specific chemical connotation, unlike the scientific name of a plant species (Bankova[c], 2014). The research are going to determine whether the area of local *Apis mellifera* to collecting propolis will give the side effect the histological features of small intestine.

In acute inflammation of small intestine, there was transient vasoconstriction followed by vasodilatation increased capillary permeability and decrease in blood flow. Thus happened congestion of blood vessels, oedema plus the presence of fibrin network, and also infiltration of leucocytes such as neutrophils, lymphocytes, macrophages, eosinophils (Chauhan, 2010). Ulcer are the loss of mucosa and deeper tissue. It happened when cells repaired from acute inflammation (Kemp *et al.*, 2008).

Therefore, the research would examine the effect of propolis ethanolic extract based on three objects, which were: villi epithelial damage, congestion and oedema, and neutrophil infiltration.

1.4 Objectives of Research

1.4.1 Specific objectives

1. To observe the effect of propolis on the histological features of small intestine of male mice (*Mus musculus*).
2. To observe the effect of different dose of propolis on the histological features of small intestine of male mice (*Mus musculus*).

1.4.2 General objective

This study aimed to determine the provision of propolis can be used as natural remedy to improve animal and human health.

1.5 Outcomes of Research

The results of this study were expected to provide information to the public that propolis is a natural remedy that is safe for the intestinal organs. Propolis can be useful for human and veterinary medicine, and also to contribute knowledge to medical and veterinary science.

1.6 Hypothesis of Research

Hypothesis proposed in this study are:

1. Administration of propolis affect on the histological features of small intestine of male mice (*Mus musculus*).
2. The different dose of propolis affect on the histological features of small intestine of male mice (*Mus musculus*).

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristic of Propolis

Bees have been in existence for 125 million years and their evolutionary success has allowed them to become perennial species that can exploit virtually all habitats on earth. This success was largely because of the chemistry and application of the specific products that bees manufacture: honey, beeswax, venom, pollen, royal jelly and propolis. (Wollenweber, 1990 in Bankova, 2005). *Apis mellifera* (Figure 2.1) is one of the most productive bees which widely farmed in Europe, Africa, Asian, and American (Suranto, 2010).

Figure 2.1 *Apis mellifera* (Suranto, 2010)

Propolis is not only a building material, it is the most important "chemical weapon" of bees against pathogen microorganisms and has been used as a remedy

by humans since ancient times. It was applied for wounds and burns treatment, soar throat, and stomach ulcer (Wollenweber, 1990 in Bankova, 2005).

Percentage of workers who are in charge of collecting bee propolis is very low but it is done all the time. At the source location, bee propolis collector bite the material with their mandibulae (lower jaw) and with the help of a pair of first leg. Propolis is transferred to the pollen basket. This activity takes 15-60 minutes. In the hive, hive guards bee move propolis from collector bee's arm and dispensing propolis with their mandibulae, sometimes add a little wax. Then propolis transfered to where it's needed or stored as a backup. (Gojcmerac, 1993 in Angraini, 2006).

Honeybees collect propolis from plants and use it in their hive. They apply it to seal the walls, to strengthen the borders of combs, to embalm dead invaders. (Wollenber, 1990 in Bankova, 2005). It is dense, sticky, blackish brown, has a typical smell, and a bitter taste (Radiati et al., 2008). Propolis has a characteristic smell and shows adhesive properties because it strongly interacts with oils and proteins of the skin (Burdock, 1998 in Sforcin and Bankova, 2007).

Propolis in natura is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and 5% other substances (Sforcin and Bankova, 2007). Krell (1996) in Radiati et al. (2008) describes the chemical composition of propolis (Table 2.1):

Table 2.1 Chemical Composition of Propolis (Source: Krell, 1996).

Component	Percentage (%)	Component group
Resin	45-55	Flavonoid, phenolic acid, ester
Wax	25-53	Most of bee glue, and some from plants
Essential and aromatic oils	10	Volatil compound
Protein	5	Protein probably from pollen and free amino
Other substances	5	Minerals: Fe, Zn, Au, Ag, Cs, Hg, La, Sb; other organic compunds ketones, laktan, quinones, benzoic acid and esters, sugar, vitamins (B3) and sugar.

Propolis chemical composition depend on the phytogeographic characteristics of the site of collection, since bees choose different plants as source of propolis in different habitats. This aspect made propolis standardization more difficult, and different solvents (ethanol, methanol and water) might extract different compounds, influencing its activity (Bankova, 2010).

Propolis is non-toxic, and its LD50 ranges from 2 to 7.3 g/kg in mice. It is suggested that the safe concentration for humans could be 1.4 mg/kg and day, or approximately 70 mg/day (Burdock, 1998 in Sforcin and Bankova, 2007).

2.2 The advantage of Propolis

The ethanolic extract of propolis has been reported to possess various biological activities, such as antibacterial, antiviral, antiinflammatory, local-anesthetic, antioxidant, immunostimulating and cytostatic (Silici and Kutluca et al., 2005).

The pharmacologically active molecules are flavonoids, phenolic acids, and their esters. These components have multiple effects on bacteria, fungi and viruses. In addition, propolis and its components have anti-inflammatory,

immunomodulatory activities, and antitumor activity. Moreover, propolis has been shown to lower blood pressure and cholesterol levels (Lotfy, 2006).

Tekeli et al. (2010) found that propolis extract and other substance Z. officinale supplemented in the ration both separately and in combination proved to stimulate lactic acid bacteria and significantly decrease pathogenic bacteria such as total mesophilic aerobicm coliform and E. coli. This effect might be due to the the ability of essential oils in terms of inactivication of extracellular enzymes and their antibacterial properties which lead to death of bacteria by decreased pH of the medium and damage to cell wall structure.

Phenolic structures denature the proteins in the cell wall of the bacteria and increase cell wall permeability. The corrupted permeability of the cell wall induces the release of the intracellular fluid, which consequently kills the bacteria. Phenolic substances that include cinnamic acid derivatives and some flavonoids are detected in honey and propolis (Kutlu, 1999 in Tekeli et al., 2010).

2.3 Histological Features of Small Intestine

There are three parts of small intestine: duodenum, jejunum and ileum, while the caecum, colon and rectum are part of the large intestine. Intestinal epithelium covered by one layer of columnar epithellium with numerous goblet cells. (Leeson et al., 2012). Though the submucosal Brunner's glands are ussually confirmed that a section were taken from duodenum parts (Scudamore, 2014).

The small intestine has features that enhance food digestion and absorption, such as its extended length, mucosal plicae and villi. All these

modifications plus the development of microvilli along the apical surface of the luminal cells are designed to greatly expand the surface area for absorption (Samuelson, 2007).

The mucosal folds or plicae, are prominent semicircular extensions project into the lumen and as a result more than double the surface area of the epithelial lining. Along the entire inner mucosa, extensions smaller than the plicae project into the lumen forming the villi. The villi tend to be more developed within the proximal portion (duodenum) than those located distally (ileum). The shape of the villi in animals vary: short and conical in rodents. The presence of villi easily increases the surface area by 10 times or more. (Samuelson, 2007).

The mucosa of small intestine is thrown up into finger like projecting villi separated by crypts and confined by single layer of columnar epithelial cells with goblet cells (Figure 2.2) Goblet cells are found along the small intestine but tend to be more numerous in the ileum (Scudamore, 2014). Although their heights exceeds their widths, these secretory cells become round as their cytoplasm becomes filled with mucigen. When mucigen is released as mucin into the intestinal lumen, it becomes hydrated and better known as mucus. Mucus protects the mucosal epithelium as a whole and facilitates the movement of nonabsorbed ingesta and excrement toward the rectum and anus (Samuelson, 2007).

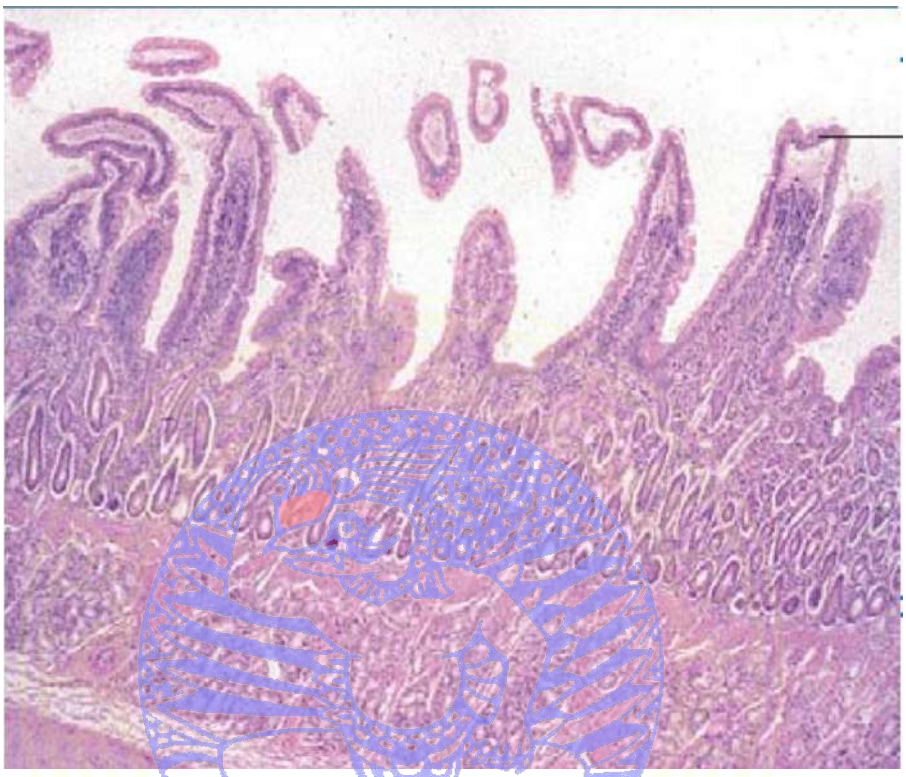


Figure 2.2 Histological picture of small intestine (Kierszenbaum, 2012)

Between the base of adjacent villi the epithelium invaginates into the adjacent lamina propia, forming simple tubular glands, called intestinal crypts or crypts of Lieberkuhn. These glands composed of a absorptive and goblet cells in the upper half. Regenerative cells which provided the replenishment of the absorptive and goblet cells lie more within the lower halves of the glands. Toward the bottom of each are found the acidophilic granule cells or Paneth cells. Those cells are typically pyramidal and have been shown to have antimicrobial capabilities (Samuelson, 2007).

The form of lamina propria is a loose connective tissue, which is the center of the villi and surrounding intestinal glands, composed of collagen and elastic fibers in the tangle of reticular fibers. Inside the tangle there are blood vessels,

lymph vessels, leukocytes, fibrocyte, smooth muscle, plasma cells and mast cells (Eurell et al., 2007). Intestinal glands extend from the base of the villi into the underlying lamina propia. Undifferentiated epithelial cells located in the glands divide and migrate up to renew the glandular and surface epithelium every 4-5 days (Leeson, 2012).

Tunica submucosa consists of loose connective tissue containing collagen and elastic fibers, located between the lamina muscularis mucosa and tunica muscularis (Eurell et al., 2007). The myenteric plexus is located between the Submucosal glands or Brunner's glands and Aggregated lymphatic nodules (GALT) are present in submucosa layer. The nodules are particularly numerous in the ileal region. Specialized M cells which present antigens to the lymphoid tissue are present in the epithelium which overlies the nodules (Leeson, 2012).

Tunica muscularis is consists of two layer of smooth muscle, the inner layer of circular muscle and outer layer of longitudinal muscle. The myenteric plexus (Auerbach) is located between the inner and outer layers of the tunica muscularis and functions to direct the outer longitudinal muscle. (Samuelson, 2007). Tunica serosa lies outside the tunica muscularis as the outermost layer of the small intestine (Leeson, 2012).

2.4 Mice (*Mus musculus*) as Experimental Animal

Mice (*Mus Musculus*) are rodentia animal which easy to breed, also easy to mantain in large scale, have many various genetics and have good anatomic and physiological character (Akbar, 2010).

Mice or white mouse are frequently used as a experimental animals for research. This laboratory mice strains have many good inbread (DDY, Balb / c, DBA, and B6) and outbred like webster. Ballenger (1999) classified the laboratory mice are as follows:

Kingdom	: Animalia
Phylum	: Chordata
Subphylum	: Vertebrata
Class	: Mammalia
Order	: Rodentia
Family	: Muridae
Subfamily	: Murinae
Genus	: *Mus*
Species	: *Mus musculus*

Mice (*Mus musculus*) have characteristics such as small and white body. (Figure 2.1). Cage condition for maintenance of mice (*Mus musculus*) should always be clean, dry and away from the noise. Room temperature should be maintained range between 18-19ºC and relative humidity between 30-70%.

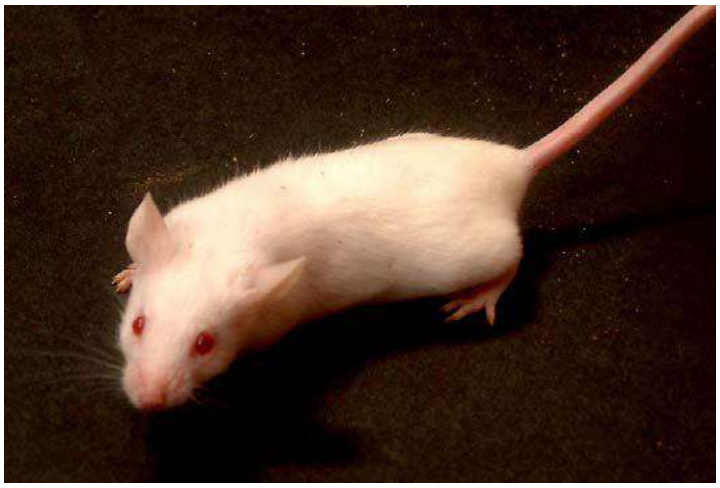


Figure 2.3 *Mus musculus* (Sancheti & Goyal, 2007)

Adult mice long life of 1-2 years, can reach 3 years. Male or female mice can be bred at the age of 8 weeks. Long gestation 19-20 days. The average number of giving birth was 6-15 mice, with birth weight between 0.5- 1.5 g (Akbar, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Place and Period of Research

This study were held at the Laboratory of Experimental Animal, Faculty of Medicine, Universitas Airlangga for the treatment of experimental animals and organs collection. The extraction were carried out in the Laboratory of Phytochemistry, Faculty of Pharmacy, Universitas Surabaya. The making of histological specimens were performed at the Gedung Diagnostic Center (GDC) Dr. Soetomo Hospital. The examination of histological specimens of small intestine were held at Laboratory of Histology, Veterinary Medicine Faculty, Universitas Airlangga. Implementation of this research are performed from November 2014 to January 2015.

3.2 Design of Research

The research of effect of propolis ethanolic extract on small intestine (*Mus musculus*) mice changes was using experimental with CRD (Completely Randomized Design) patterns. The type of research is experimental research with 25 male mice (Mus musculus) used as the experimental animal. Experimental animals divided into five group with five repetition, P0 as control group (0.5 ml Tween80/head/day), P1 (1.6 mg propolis/kgBW/head/day), P2 (3.2 mg propolis/kgBW/head/day), P3 (6.4 mg propolis/kgBW/head/day), and P4 (12.8 mg propolis/kgBW/head/day). Examination were carry out by looking at the changes occured in the small intestine organ with post-test design.

The independent variable of this research is propolis dose given to experimental animals, while the dependent variable is the histological features of small intestine after administration of propolis ethanolic extract. The control variable are the type of mice, feed, age of mice, and tools for research.

3.3 Reasearch Material

Experimental animals used in this study were 25 of 12-week-old male mice (*Mus musculus*) with an average weight of 25-35 grams. Mice (*Mus musculus*) were obtained from PUSVETMA Surabaya and developed in Laboratory of Experimental Animal of Medicine Faculty, Universitas Airlangga.

The equipment used in this study include stomach tube (1 ml), water and feed box, plastic cage with length 50 cm, width 50 cm, height 40 cm, and husk. The other tools are digital scales, plastic bags, scrap paper, scissors, pipette, bottles, knives, syringes, forceps, scalpel, cutting tools, plastic pot, measuring tube, object glasses and cover glasses, microtome, hot plate, microscope, and lens micrometer.

Propolis that used in this research was the result of extraction and dilution in Phytochemistry Laboratory of the Faculty of Pharmacy, Universitas Surabaya. Material used for extraction is ethanol 70%. Materials used for dilution are Tween80 and E-pure. Materials required for making the histological specimens are formalin 10%, alcohol, or ethanol (70%, 80%, 90%, absolute), xylol, Hematoxylin solution, eosin solution, glyserin, ewith, lithium carbonat, Canada

balsam, and paraffin block. (Muntiha, 2001). Mice were given shaped pellet feed and drinking water.

3.4 Research Methods

Implementation of this research was conducted in two phases: the first phase was the extraction of propolis, and the second phase is the treatment of experimental animals.

3.4.1 Preparing extract of propolis

The basic ingredients of propolis is a mixture of beeswax, resin and soil attached to the honeycomb Apis mellifera. Material samples used are raw propolis from local *Apis mellifera* obtained from Ranch Bees Rimba Raya, Lawang, Malang, East Java, which was extracted using the method of maceration with 70% ethanol. Extraction was carried out in the Laboratory of Phytochemistry, Faculty of Pharmacy, Universitas Surabaya. The extraction process desribed in Appendix 1.

3.4.2 Treatment in experimental animals

The second stage of research, the treatment on mice (*Mus musculus*) males held in cage experiments. Mice (*Mus musculus*) males (age 12 weeks) with a weight of 25-35 grams, was developed in the Laboratory of Experimental Animal, Medicine Faculty of Airlangga University and randomized by table.

Determination of the number of minimal replications are listed in Appendix 3. Experimental animals were divided into five groups, P0 as control group (0.5 ml Tween80/head/day), P1 (1.6 mg propolis/kgBW/head/day), P2 (3.2 mg propolis/kgBW/head/day), P3 (6.4 mg propolis/kgBW/head/day), and P4 (12.8

mg propolis/kgBW/head/day), given environment adaptation for one week. Determination of the dose of propolis are listed in Appendix 4. The treatment of each group is given in the second week. Feeding and drinking is given *ad libitum*.

3.5 Preparing Histological Specimen of Small Intestine

3.5.1 Organs collection

Collection of the small intestine will be start by anasthesized the male mice using chloroform and then dissect the body to get the small intestine.

3.5.2 Histological specimen making process

The dissected small intestine will be kept in a pot which filled with formaline. One specimen of small intestine was made with horizontal slice from every male mice. The making of histological specimen and staining will be done at GDC Dr. Soetomo Hospital, Surabaya. The procedure of histological specimen will be explained in Appendix 4.

3.6 Examination of Histological Specimen of Small Intestine

Examination of intestine specimens that have been stained by HE performed under a light microscope with a magnification of 400x to see epithelial tissue damage, hemorrhagic congestion and mucosal oedema, and neutrophil infiltration. Each specimens were observed in five field. The results of each field are summed and then average were calculated.

The assessment of the level of damage in a single field can be seen below (Pothoulakis et al., 1994):

1. Epithelial damage

   Score 0: None of the villus are damaged.

   Score 1: Destruction of tips of villi.

   Score 2: Destruction of distal half of villi.

Score 3: Complete destruction of villi.

2. Congestion and Oedema

   Score 0: None.

   Score 1: Rare venules with Red Blood Cells .

   Score 2: Up to half of venules with Red Blood Cells.

   Score 3: More than half of venules with Red Blood Cells.

3. Neutrophil Infiltration

   Score 0: None

   Score 1: Rare of Polymorphonuclear cells which mainly marginated.

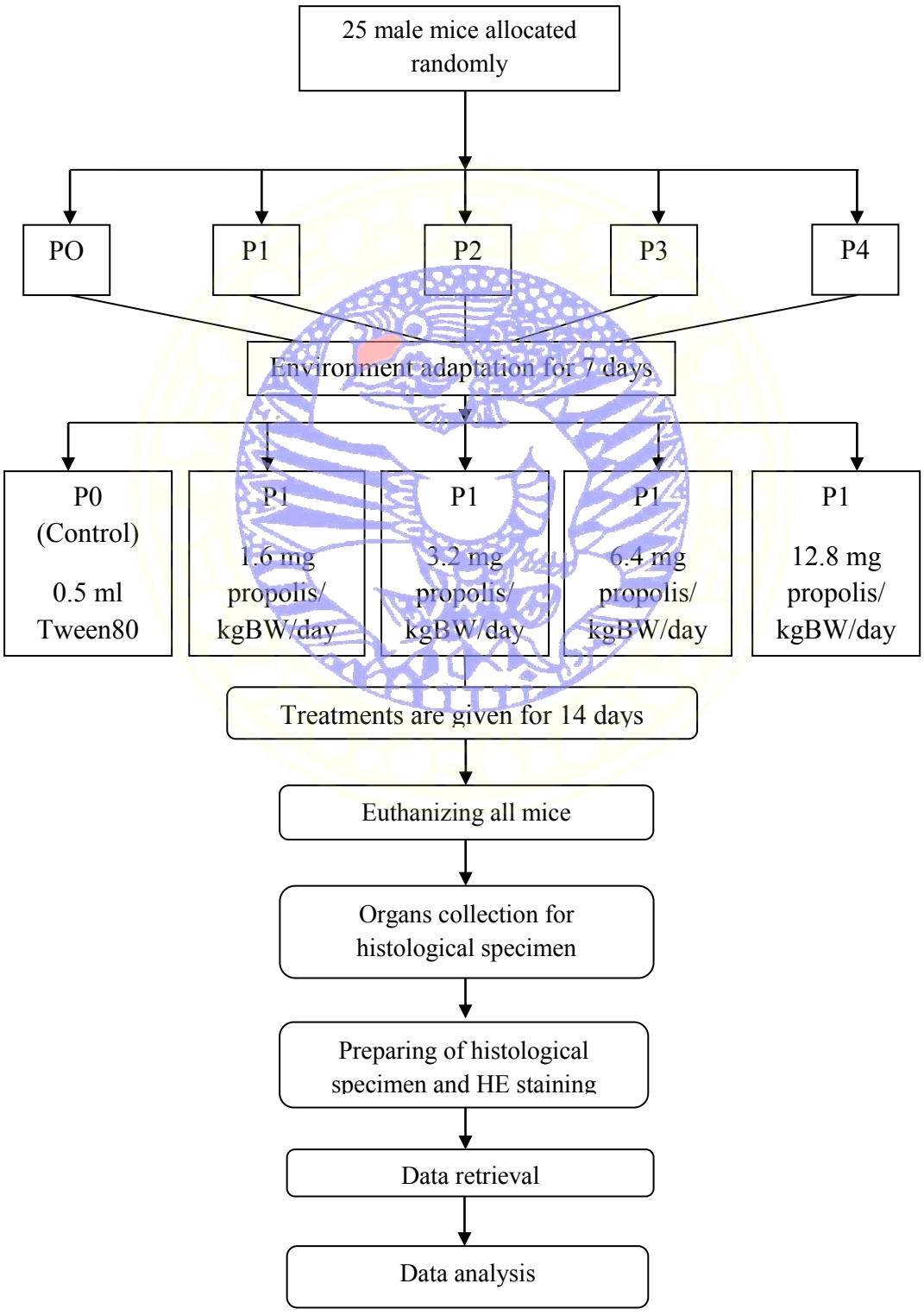
   Score 2: Marginated and extravasated Polymorphonuclear cells (up to 5 each vessels).

   Score 3: Marginated and extravasated Polymorphonuclear cells throughout lamina propia and epithelium.

3.7 Data analysis

The form of data obtained stated in scores of histopathology changes level in the small intestine of mice that arranged in table for later statistically analyzed using the *Kruskal-Wallis* test. If there is a real difference, then the analyzing using *Mann-Whitney* test (Mehotcheva, 2008). The whole process of analysis done with SPSS 16 for Windows.

3.8 Research Framework



CHAPTER 4

RESULTS OF RESEARCH

Histological speciments of small intestine of mice (*Mus musculus*) of control or P0 (Tween80 at a dose of 0.5 ml/day), P1 (propolis dose of 1.6 mg/kgBW/day), P2 (propolis dose of 3.2 mg/kgBW/day), P3 (propolis dose 6.4 mg/kgBW/day), P4 (propolis dose of 12.8 mg/kgBW/day) were stained by HE and observed under a light microscope with a magnification of 400x to see epithelial tissue damage, hemorrhagic congestion and mucosal oedema, and neutrophil infiltration (Figure 4.1). The specimenswere observed in fivefield each.Epithelial damage, congestion andoedema, and neutrophil infiltration of small intestine of mice (*Mus musculus*) were scored using Pothoulakis method (Pothoulakis, 1994). (Table 4.1).

Table 4.1 Scoring of epithelial damage, congestion and oedema, and neutrophil infiltration (Mean ± SD) of small intestine of mice (*Musmusculus*).

Treatment Groups	Mean ± SD		
	Epithelial Damage	Congesti andOedema	Neutrophil Infiltration
P0	7.50 ± 0.45	13.20 ± 1.52	7.80 ± 0.84
P1	12.50 ± 0.55	12.40± 1.52	13.10 ± 0.45
P2	12.50 ± 0.55	15.70 ± 0.41	15.50 ± 0.00
P3	15.00 ± 0.45	13.20 ± 0.52	13.10 ± 0.45
P4	17.50 ± 0.00	10.50 ± 0.71	15.50 ± 0.00

Description: P0=Tween 0.5ml/kgBW/head/day; P1=Propolis 1.6 mg/0.5ml/day; P2=Propolis 3.2 mg/0.5ml/day; P3=Propolis 6.4 mg/0.5ml/day; P4=Propolis 12.8 mg/0.5ml/day.

Data analyzing using Kruskal-Wallis test resulted p=0.119 (>0.05) for epithelial damage, meanwhile the result for congestion and oedemawas p=0.842 (>0.05) andfor neutrophil infiltration was p=0.110 (>0.05).

4.1 Villi Epithelial Damage

The histological features of epithelial damage of small intestine of mice (*Mus musculus*) can be seen in Figure 4.1.

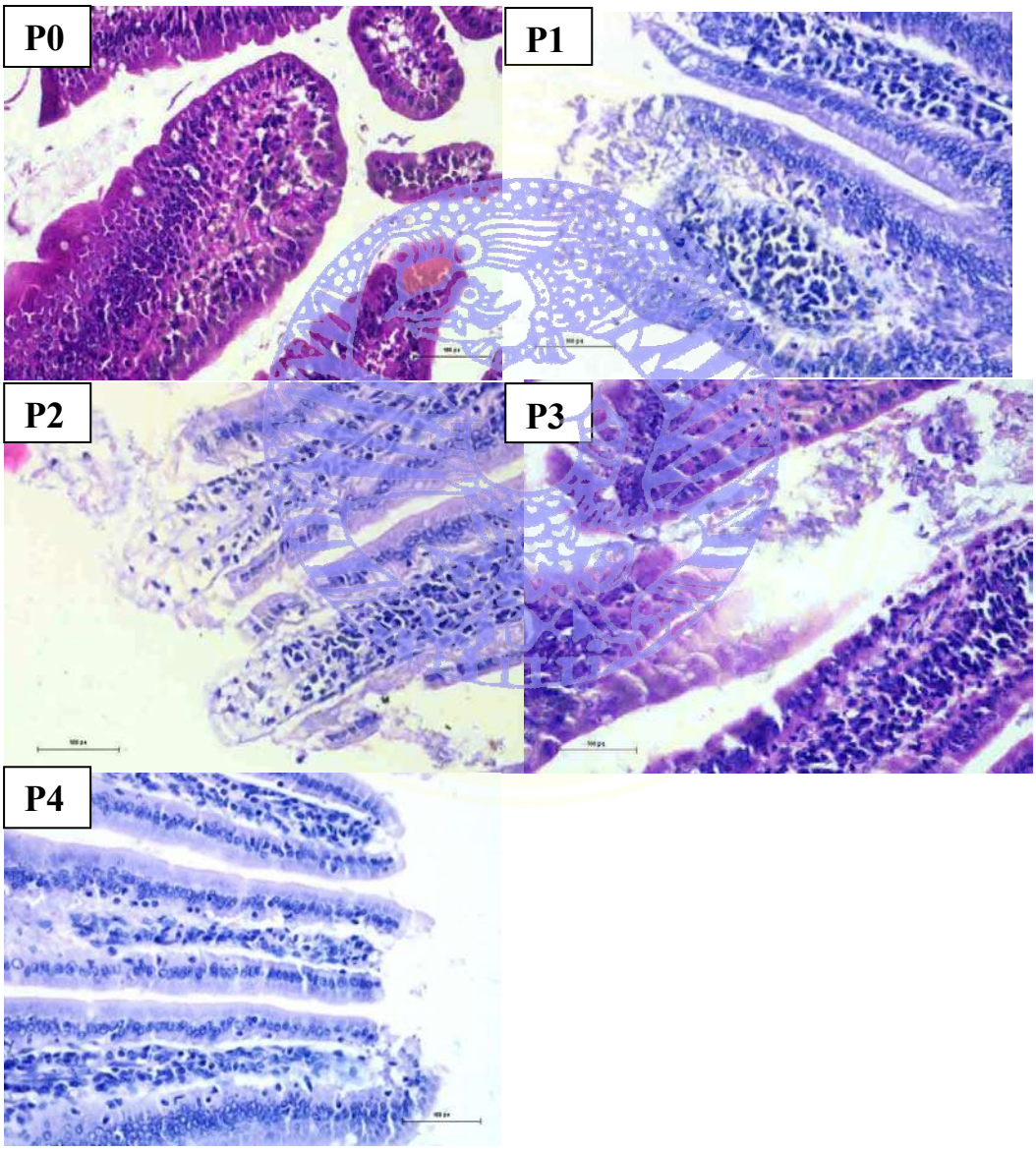


Figure 4.1 Villi of small intestine intended to visualize epithelial damage (HE staining; 400x magnification; P0=Tween 0.5ml/kgBW/head/day; P1=Propolis 1.6 mg/0.5ml/day; P2=Propolis 3.2 mg/0.5ml/day; P3=Propolis 6.4 mg/0.5ml/day; P4=Propolis 12.8 mg/0.5ml/day).

4.2 Congestion andOedema

The histological features of congestion and oedema of small intestine of mice (*Mus musculus*) can be seen in Figure 4.2.

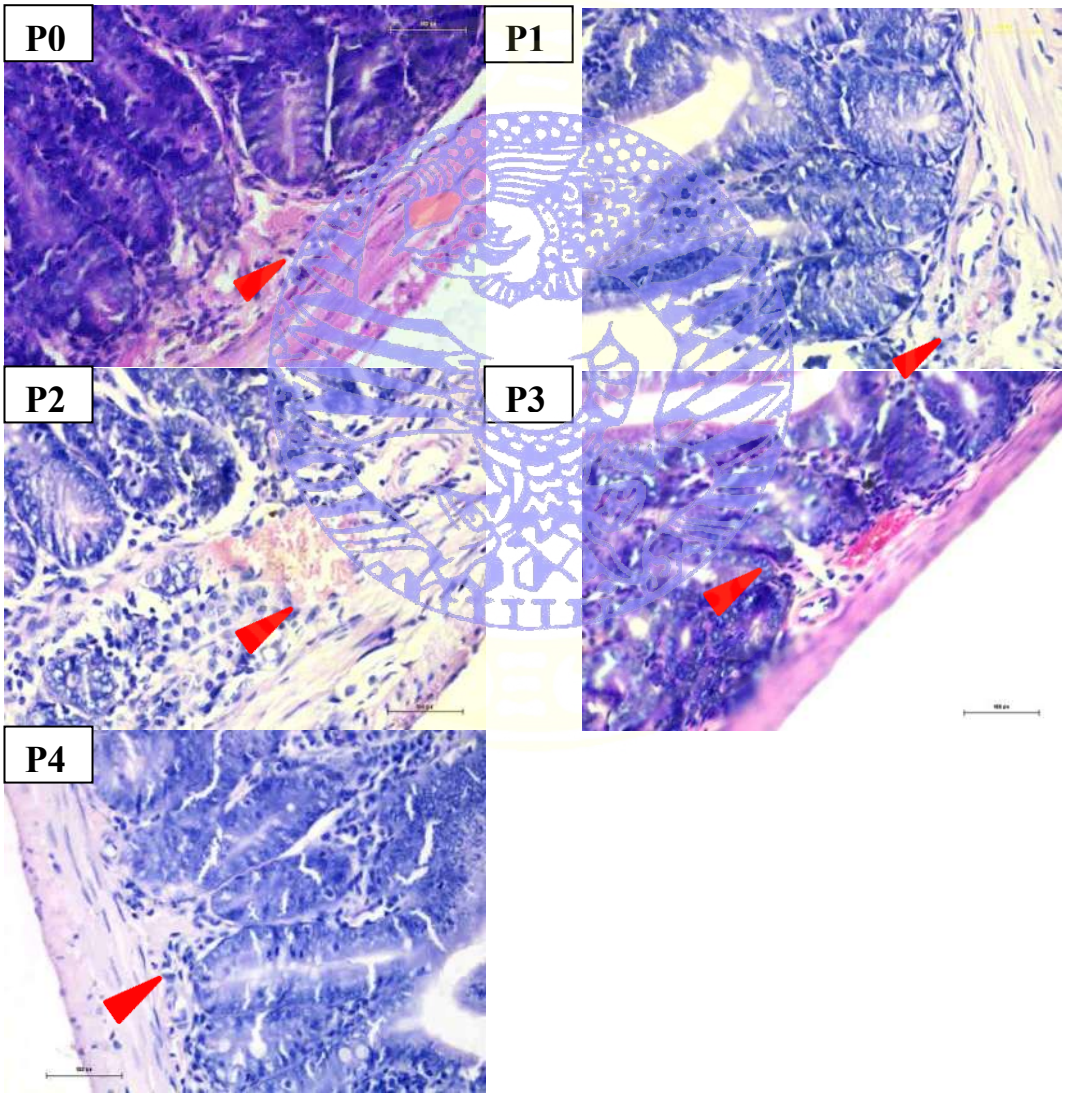


Figure 4.2 Red blood cells accumulation in venule and oedema in lamina propia and submucosa layer of small intestine. Red arrows shows red blood cells accumulation. (HE staining; 400x magnification; P0=Tween 0.5ml/kgBW/head/day; P1=Propolis 1.6 mg/0.5ml/day; P2=Propolis 3.2 mg/0.5ml/day; P3=Propolis 6.4 mg/0.5ml/day; P4=Propolis 12.8 mg/0.5ml/day).

4.3 Neutrophil Infiltration

The histological features of neutrophil infiltration of small intestine of mice (*Mus musculus*) can be seen in Figure 4.3.

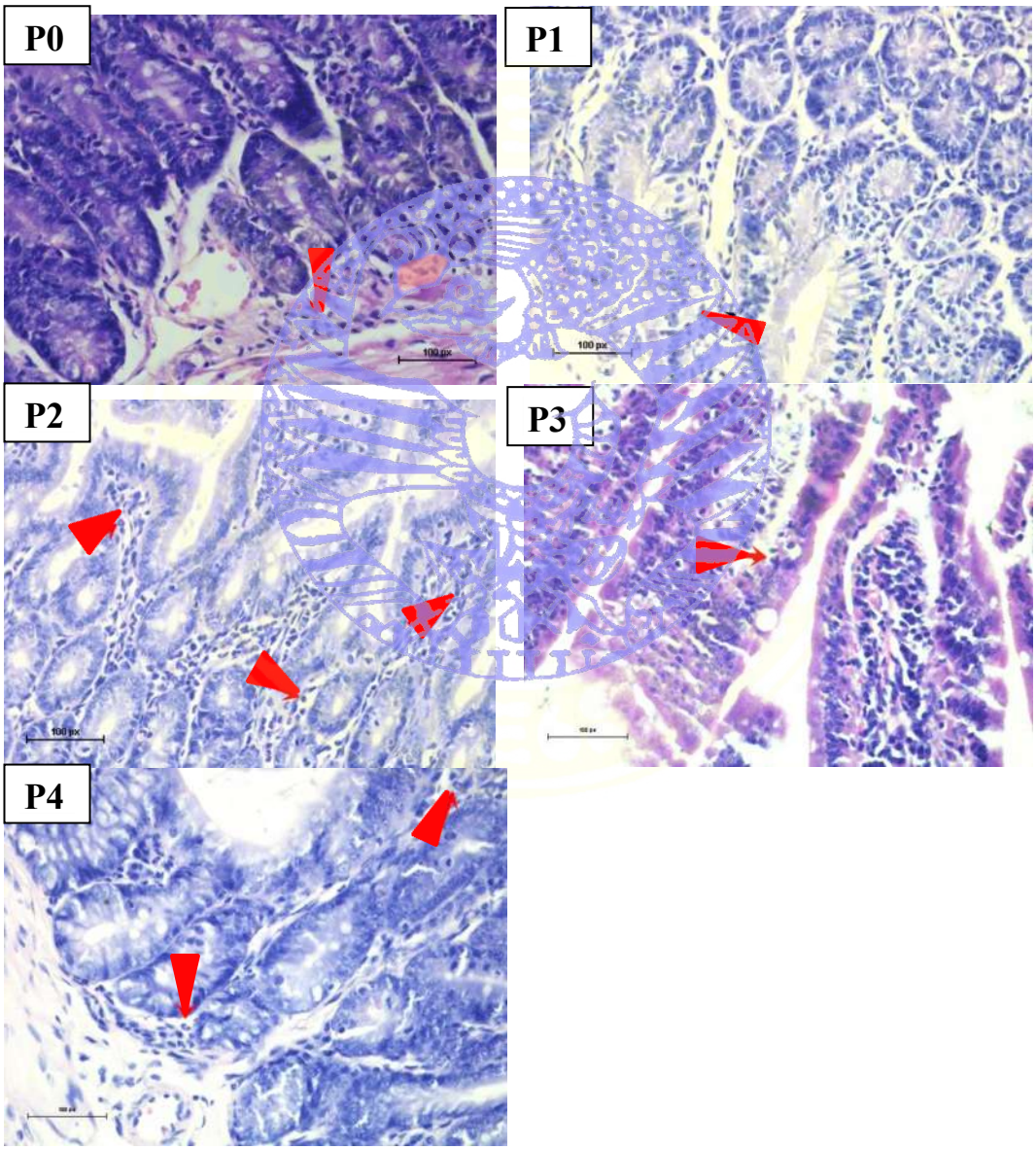


Figure 4.3 Neutrophil infiltration in lamina propia and submucosa layer of small intestine. Red arrows shows PMN infiltration. (HE staining; 400x magnification; P0=Tween 0.5ml/kgBW/head/day; P1=Propolis 1.6 mg/0.5ml/day; P2=Propolis 3.2 mg/0.5ml/day; P3=Propolis 6.4 mg/0.5ml/day; P4=Propolis 12.8 mg/0.5ml/day).

CHAPTER 5

DISCUSSION

Bee propolis is a mixture of compounds collected by honey bees from various plant sources and used by bees to seal holes in their honey combs, smooth out the internal walls and protect by entrance against intruders (Kamel, 2007). Plants and plant extracts are effective mainly on the digestive system of animals. Their function either by wiping out the pathogenic microflora in the digestive system or increasing the concentration of microbial population in the digestive system that contributes to improved digestion and absorption of nutrients (Wenk, 2000 in Tekeli et al., 2010).

The observed effects were the epithelial damage, congestion and oedema, and neutrophil infiltration in small intestine of mice.

5.1 Epithelial Villi Damage

The loss of the mucosa and deeper tissues are called ulcer. If only the mucosa is lost, the correct term is erosion. The observation of small intestine specimens, it was suspected that mice were suffered from erosion. Erosion happened in several pathologic condition in duodenal, such as exudative diarrhea, mucosal exposure to gastric acid, bacterial invasion. Ulcer and erosion happened when body cannot riditself of the inciting agent.Macrophages, mast cells, dendritic cells, granulocytes (neutrophils, eosinophils and basophils) and the innate lymphocytes are the cells of the innate immune system converge at the site of damage (Kemp et al., 2008).

The result told that propolis ethanolic extract treatment gave no significant effect on epithelial damage of small intestine of mice. Control group had the lowest mean rank which is 7.50 meant propolis could not act as remedy to epithelial damage of small intestine of mice (*Mus musculus*) which in this research, erosion were happened in every treatment group.

5.2 Congestion and Oedema

Congestion is passive accumulation of blood within vessels, where the blood vessels are dilated by red blood cells. Due to multiple episodes of acute passive congestion, red blood cells break down, leaving hemosiderin and stimulate mild inflammation (Kempet al., 2008). There were production of chemical factor in inflammation (histamine, serotonin, arachidonic acid derivatives, quinine) which caused vasodilation and increased capillary permeability, thus edema were formed (Jankowski, 2011).

The scoring results of treatment using propolis ethanolic extract gave no significant effect on congestion and oedema of small intestine of mice. All treatment showed the presence of congestion and oedema in similar condition. P4 treatment group (Propolis 12.8 mg/0.5ml/day) showed lowest mean rank. From that result could be concluded that propolis ethanolic extract could act as a remedy for congestion and oedema, but still couldn't give a significant difference.

5.3 Neutrophil Infiltration

In inflammation, there is transient vasoconstriction followed by vasodilatation increased capillary permeability and decrease in blood flow. Circulatory changes are more pronounced in acute inflammation. Thus happened congestion of blood vessels, oedema plus the presence of fibrin network, and also infiltration of leucocytes such as neutrophils, lymphocytes, macrophages, eosinophils (Chauhan, 2010). Since it was the acute symptom that we were looking for, the observation were focused on neutrophil infiltration.

The scoring result of treatment using propolis ethanolic extract gave no significant effect on neutrophil infiltration of small intestine of mice. All treatment showed the presence of neutrophil infiltration in similar condition.Control group had the lowest mean rank which is 7.80 meant propolis ethanolic extract could not act as remedy to neutrophil infiltration of small intestine of mice (*Mus musculus*).

5.4 General Discussion

As it was already seen, there was erosion occurred in mice's histology picture. Erosion and ulcer is acute inflammation form. (Kemp, 2008).The acute inflammatory response could be stimulated by exogenous and endogenous agent which resulted to injury in vascularized tissue. The response began as active hyperemia, facilitated by various chemical mediator such as prostaglandins, leuktrienes, nitric oxide caused dilation of arterioles and capillaries. Active hyperemia is rapidly followed by changes in junctional complexes of endothelial cells resulting in destruction of individual endothelial cells, hemorrhage occurs

and plasma and plasma proteins can leak directly through breach in the wall of the capillary or venule. Once activated, endothelial and perivascular cells such as mast cells, dendritic cells, fibroblasts, and pericytes can produce cytokines and chemokines that regulate the expression of receptors for inflammatory mediators and adhesion molecules.

The plasma proteins and fluid that initially accumulate in the extracellular space in response to injury form transudate. The most common the formation of a transudate is due to hypertension in veins and capillaries or hypoproteinemia resulting in oedema; however, transudates occur in the early stages of the acute inflammatory response when intercellular gaps that open between endothelial cells are so small that only water and electrolytes can pass through them. In time, neutrophils and additional protein can enter injured areas resulting in the formation of exudate (McGavin, 2007).

Propolis ethanolic extract gave no significant effect on histological features of mice small intestine, specifically on epithelial damage, congestion and oedema, and neutrophil infiltration. This is contrary to statement that propolis were active as anti-inflammatory, strengthen and accelerate cell regeneration (Gojmerac, 1983 in Angraini, 2006). Although higher dose, especially the highest (Propolis 12.8 mg/0.5ml/day) showed better results in congestion and oedema, significant differences was still could not be achieved.

It was probably because of mice used were still not SPF (Spesific-Pathogen Free). According to International Committee of Laboratory Animal, Spesific-Pathogen-Free Animal are defined as „animals that are free of specific

micro-organisms and parasites, but not necessarily free of others not specific'. The nature of animal facility and diets might modulate the outcome of study (Reliene, 2006). So the mice were probably suffered with unknown pathogen agent which lead to mild inflammation occurred in every treatment, include the control group (P0) and propolis ethanolic extract dose groups (P1, P2, P3, P4).

CHAPTER 6

CONCLUSION AND SUGGESTIONS

6.1 Conclusion

Based on the present research that had already been conducted, it showed:

1. Propolis ethanolic extract gave no significant effect on the histological features on small intestine of mice.
2. Different level of propolis ethanolic extract dose did not affect histological features of small intestine of mice.
3. Propolis ethanolic extract were safe when given orally to intestinal organ of mice.

6.2 Suggestion

Based on the research results, the writer suggest:

1. Necessary to conduct a research on the effect of propolis extract using different dose.
2. Necessary to conduct a research on the effect of propolis extract in other experimental animal.
3. Necessary to conduct a research using older experimental animal.
4. Necessary to conduct a research using unhealthy animal.

SUMMARY

Karina Ratnaningrum. The Effect of Propolis On Histological Features of Small Intestine of Mice (*Mus musculus*). This present research was conducted under the guidance of Dr. Eka Pramytha H., M.Kes.,drh, as the creator of research, Suzanita Utama, drh., MP.Phill., Ph.D as the main supervisor and Prof. Hj. Romziah Sidik, drh., Ph.D as the co-supervisor.

Indonesia has many kinds oflocal bees *Apis mellifera* which became the most widely farmed bees. Bee propolis is a mixture of compounds collected by honey bees from various plant sources and used by bees to seal holes in their honey combs, smooth out the internal walls and protect by entrance against intruders. propolis is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and other substances.Active substances known in propolis are poliferol (flavonoid, fenolat acid, and its esters), terpenoid, steroid, and amino acid.

Researchs suggest that propolis has many effects such as antibacterial, anticancer, antiviral, antifungal, antioxidant, immune-boosting, strengthen, anti-inflammatory, and accelerate cell regeneration. Small intestine has functions such as doing food digestion and absorption. Villi is the most responsible part of nutrition absorbtion because of its absortive cell in mucosa with a single layer of columnar epithelial cells with striated border.

In acute inflammation, there was transient vasoconstriction followed by vasodilatation increased capillary permeability and decrease in blood flow. Thus

happened congestion of blood vessels, oedema plus the presence of fibrin network, and also infiltration of leucocytes such as neutrophils, lymphocytes, macrophages, eosinophils.

This study consisted of two stages: the propolis extraction process and propolis treatment to experimental animals. Basic material used was raw propolis from Rimba Raya Lawang bees ranch, as much as 500g and extracted using ethanol 70%. Propolis extraction was conducted in the Phytochemistry Laboratory Faculty of Pharmacy, Universitas Surabaya in November to December2014. The second stage is propolis extract administration to experimental animals using five treatments with five replications. The treatment given to the inclusion criteria of male mice, aged 12 weeks, weigh 25-35g strain Balb/c. Provision of propolis was held for 14 days preceded by a period of adaptation for seven days. Experimental were using 25 mice (*Mus musculus*). Plastic enclosure cages were used for maintenance of experimental animals at Experimental Animal Laboratory, Faculty of Medicine, Universitas Airlangga in December 2014 to January 2015. The observed parameters in this research were epithelial tissue damage, hemorrhagic congestion and mucosal oedema, and neutrophil infiltration. Experimental animals grouping of the present study used Completely Randomized Design. Data were analyzed with Kruskall-Wallis Test.

The administration of propolis ethanolic extract show the result to epithelial tissue damage ($p=0.119$), congestion and oedema ($p=0.842$), and neutrophil infiltration ($p=0.110$), mean the administration of propolis ethanolic extract give no significant effect on histological features of mice small intestine,

specifically on epithelial damage, congestion and oedema, and neutrophil infiltration. Suggestions for this research are conduct a research on the effect of propolis extract using different dose, other experimental animal, the older experimental animal, and unhealthy animal.

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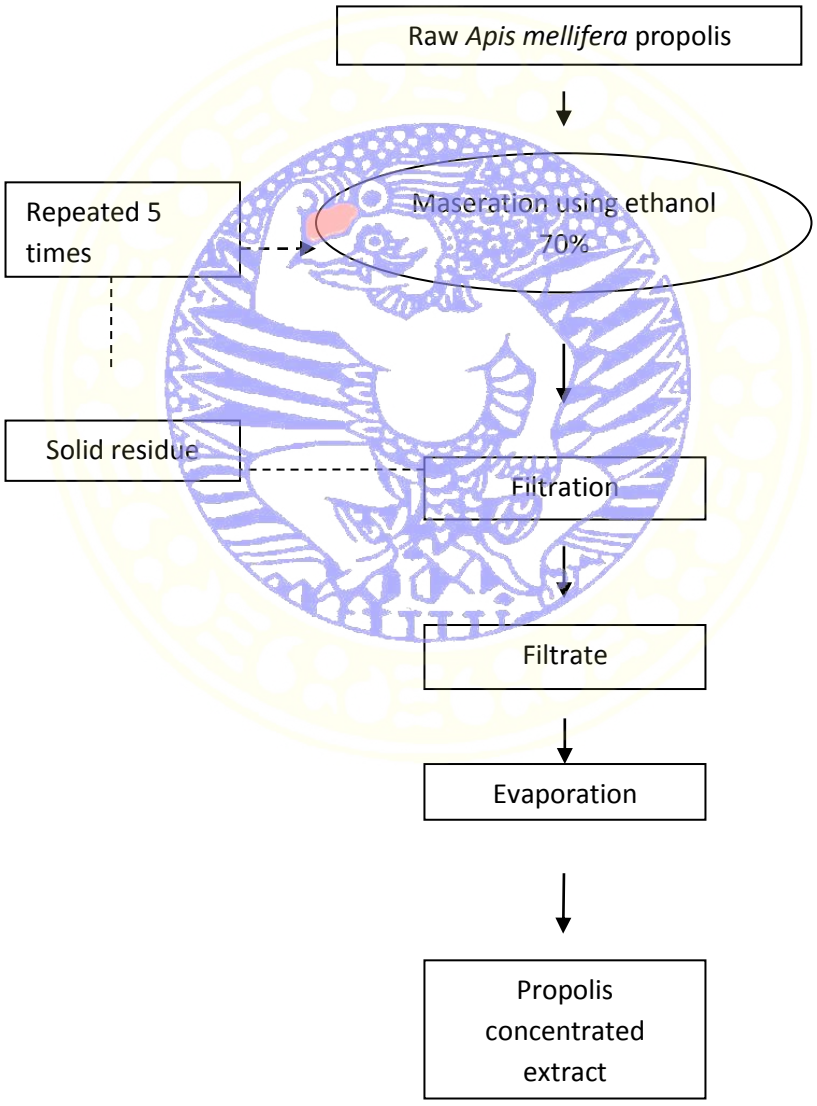
Appendix 1

Propolis Extraction Process



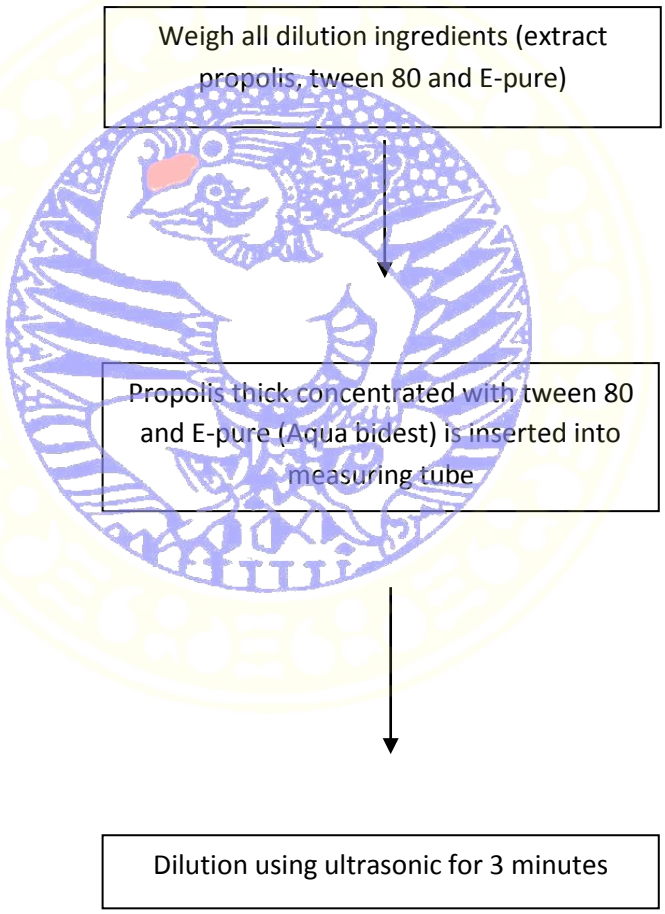
Propolis was extracted by soaking 300 grams of raw propolis using 850 ml of 70% ethanol for 4 days, with shaking for 1 hour and conducted a three-day immersion, the filtrate was decanted, the remaining residue was extracted again

with 850 ml of 70% ethanol, shaken 1 hour at 120 rpm, and the filtrate was decanted. Extraction of the residue was repeated up to five times, for a total of 4250 ml of solvent used, and the total time of maceration 7 days. The filtrate is collected in a container, the filtrate was concentrated using a rotary evaporator, the extract forms a paste that is ready for further testing (EEP: ethanol extract of propolis).

Propolis maceration result is a dark red filtrate (red-brown). The amount of yield obtained is closely related to the intensity of the color of the solution extract. Propolis extract solution with a darker color, indicating a higher yield obtained compared with a brighter color. Dark color is due to the high content of flavonoids it contains. Removal of the solvent using a freeze dryer is done to minimize heating. Evaporation of the solvent using a vacuum evaporator, still requires heating to a temperature of about 60^0C. Removal of the solvent using a spray dryer also need heating. Heat kept to a minimum in the extraction of propolis, as it can alter or damage the structure of the main bioactive namely propolis bioflavonoids.

Appendix 2

Propolis Dilution



Appendix 3

Dosage Convertion and Maximum Volum of Solution Administered Table

1) Human and Animals Dosage Convertion Table

	Mice 20 gr	Rat 200 gr	Guinea Pig 400 gr	Rabbit 1,5 kg	Cat 2 kg	Ape 4 kg	Dog 12 kg	Human 70 kg
Mice 20 gr	1.0	7.0	12.25	27.8	29.7	64.1	124.2	387.9
Rat 200 gr	0.14	1.0	1.74	3.9	4.2	9.2	17.8	56.0
Guinea Pig 400 gr	0.08	0.57	1.0	2.25	2.4	5.2	10.2	31.5
Rabbit 1,5 kg	0.04	0.25	0.44	1.0	1.08	2.4	4.5	14.2
Cat 2 kg	0.03	0.23	0.41	0.92	1.0	2.2	4.1	13.0
Ape 4 kg	0.016	0.11	0.19	0.42	0.45	1.0	1.9	6.1
Dog 12 kg	0.008	0.06	0.10	0.22	0.24	0.52	1.0	3.1
Human 70 kg	**0.0026**	0.018	0.031	0.07	0.076	0.16	0.32	1.0

(Suhardjono, D. 1995).

2) Table of Maximum Volume of Solution Administered into Various Animals

	Maximum Volume (ml) according the pathway				
	i.v.	**i.m.**	**i.p.**	**s.c.**	**p.o.**
Mice (20-30 gr)	0.5	0.05	1.0	0.5-10	1.0
Rat (100 gr)	1.0	0.1	2.5	2.5	5.0
Hamster (50 gr)	-	0.1	1-2	2.5	2.5
Guinea Pig (250 gr)	-	0.25	2-5	5.0	10.0
Dove (300 gr)	2.0	0.5	2.0	2.0	10.0
Rabbit (2,5 kg)	5-10	0.5	10-20	5-10	20.0
Cat (3 kg)	5-10	1.0	10-20	5-10	50.0
Dog (5 kg)	10-20	5.0	20-50	10.0	100.0

(Suhardjono ,D. 1995).

Explanation

i.v. : intravena

i.m. : intramuscular

i.p. : intraperitoneal

s.c. : subcutan

p.o. : peroral

Appendix 4

Determination of the number of minimal replicates

Each treatment was repeated five times so that there are 25 experimental units. Then randomization were performed in 25 experimental units for placement of experimental animals. Determination of the number of minimal replications is as follows :

$$t(n-1) \geq 15$$

$$5(n-1) \geq 15$$

$$5n - 5 \geq 15$$

$$5n \geq 20$$

$$n \geq 4$$ (Steel and Torrie, 1993).

Explanation :

t = number of treatment (1, 2, 3, 4,5)

n = treatment repeating

so the minimum number of repeating which can be done is more than or equal to four.

Appendix 5

Determination of the dose of propolis

Propolis dosage used in this study was developed from previous studies about propolis effect toward the amount of lymphoblast and spleen diameter. The research examined smaller dose of propolis, which were: 0.4 mg/0.5ml/head/day, 0.8 mg/0,5ml/head/day, 1.6 mg/0,5ml/head/day and 3.2 mg/0.5ml/head/day (Adhityananda, 2014) and showed good results, where the amount of lymphoblast and spleen diameter increased in every higher dose, but both showed decreased results in third dose (1.6 mg/0.5ml/head/day). Therefore, doses used in this study started from 1.6 mg/0.5ml/head/day.

Appendix 6

Dosage Calculation

Propolis doses for human = 100 mg (Krell, 1966).

Dosage convertion from human (70 kg) to mice (20 g) in Appendix 2 tabel is 0,0026

- Dosage of propolis Extract for 20 g weight mice

= 100 mg × 0.0026

= 0.26 mg

- Dosage for 30 g weight mice

$= \frac{30\ g}{20\ g} \times 0{,}26\ mg$

= 0.39 mg ~ (integration to be 0.4)

- Consider the dose range of propolis extract for mice are 1,6 mg for minimum dosage and 16 mg for maximum dosage.
- The formulation to determine the dosage constanta

$$X = \sqrt[n-1]{\%\text{maximum dose range}}$$

Explanation : n = treatment (in this calculation the control group are not include, so there are 4 propolis dose treatments)

$$X = \sqrt[4-1]{\frac{16\%}{1{,}6\%}}$$

$$X = \sqrt[3]{\frac{16}{1{,}6}}$$

$X = \sqrt[3]{10}$

X= 2,15 ~ (constanta integration to be 2)

Therefore, the dosage calculation is

P1 = 1,6 mg (minimum dose)

P2 = 1,6 X = 1,6×2 = 3,2 mg

P3 = 1,6 X^2 = 1,6×2^2 = 1,6 × 4 = 6,4 mg

P4 = 1,6 X^3 = 1,6×2^3 = 1,6 × 8 = 12,8 mg

- Maximum dose volume that can be given to the mice per oral is 1 ml. In this research the maximum dose which used is 0.5 ml

Appendix 7

Procedure of Histological Specimen Preparation

1. Organ Cutting

The first thing to do after the experimental animals euthanized is tissue immersion in preservative agent. Preservatives used are Neutral Buffered Formalin solution (BNF) 10% with a pH 7.0. Comparison between the organ and the solution is 1: 10 so that the fixation of tissue with the solution is complete, while the length of fixation of at least 2 days.

2. Dehydration and Clearing

Drained the organ using filter then cut it using a scalpel blade with a thickness of 0.3 - 0.5 mm and arranged into a tissue cassette, then a tissue cassette is inserted into a special basket. Basket that contains the organ tissue are placed in an automatic processor machine. Furthermore, the network experienced a gradual dehydration process with a lap time as follows: ethanol 70% (2 hours) ethanol 80% (2 hours), 90% ethanol (2 hours) 96% absolute ethanol (2 hours) xylol (2 hours) of liquid paraffin (2 hours). Furthermore, the basket which contain tissue cassette are needed to do the next process.

3. Vacuum

Dehydration process carried out, followed by removal of air from the tissue using a vacuum machine that includes the tube to store basket filled with liquid paraffin with temperature (59-60 ° C) in a vacuum for 30 minutes. Basket removed, the tissue cassette removed and stored at 60 ° C for a while before molding process done with liquid paraffin.

4. Paraffin Block Production

Iron mold that has been smeared with glycerin are prepared in order to prevent the paraffin sticking inside the mold, then a tissue that has been cut using tweezers and wait until the paraffin became solid. Paraffin blocks removed from

the mold and stored in a freezer (-20 ° C) before cutting. Parafn block containing the tissue will be cut using a microtome machine with thicknesses ranging from 3-4 µm.

5. Tissue Block Slicing

The tissue blocks are carefully placed on the surface of water in a water bath at temperature 46 ° C. On this occasion the tissue sliced orderly then placed on a microscope slide smeared with ewith as an adhesive. Slide with tissue on it are arranged in a rack and put in an incubator at 60 ° C until the specimen is ready for staining process.

6. Hematoxilin – Eosin Staining

Specimen which will be stained are placed in a special rack then dipped in a series into some solutions with time as follows

1. Xylol 3 minutes
2. Xylol 3 minutes
3. Absolute ethanol 3 minutes
4. Absolute ethanol 3 minutes
5. Ethanol 90% 3 minutes
6. Ethanol 80% 3 minutes
7. Rinse with tap water 1 minute
8. Hematoxilin solution 7 minutes
9. Rinse with tap water 1 minute
10. Blue solution 1 minute
11. Tap water 1 minute
12. Eosin solution 5 minutes
13. Rinse with tap water 1 minute
14. Ethanol 80% 10 times dip
15. Ethanol 90% 10 times dip
16. Absolute ethanol 10 times dip
17. Absolute ethanol 1 minute

18. Xylol 3 minute
19. Xylol 3 minute
20. Xylol 3 minute

Specimens removed one by one from xylol solution in wet condition, then give one drop of liquid adhesive and then covered it with cover glass. Staining results are seen under a microscope using oil emersion to clarify the examination (Muntiha, 2001).

Appendix 8

Scoring Result

Number	Epithelial Damage	Congestion and Oedema	Neutrophil Infiltration
P0(1)	0	0	1
P0(2)	0	0	3
P0(3)	1	3	3
P0(4)	0	2	2
P0(5)	0	3	2
P1(1)	0	0	2
P1(3)	1	3	3
P1(4)	0	3	3
P1(5)	1	0	3
P1(6)	1	1	3
P2(1)	0	3	3
P2(2)	1	3	3
P2(3)	0	0	3
P2(4)	1	1	3
P2(5)	1	3	3
P3(1)	1	3	3
P3(2)	1	2	3
P3(3)	1	0	2
P3(4)	1	0	3

P3(6)	0	3	3
P4(1)	1	2	3
P4(2)	1	1	3
P4(3)	1	1	3
P4(4)	1	1	3
P4(5)	1	0	3

Appendix 9

SPSS Windows Statistic Result

Ranks

	perlakuan	N	Mean Rank
kerusakan_epitel	P0	5	7.50
	P1	5	12.50
	P2	5	12.50
	P3	5	15.00
	P4	5	17.50
	Total	25	
congesti_edema	P0	5	13.20
	P1	5	12.40
	P2	5	15.70
	P3	5	13.20
	P4	5	10.50
	Total	25	
infiltrasi_neurofil	P0	5	7.80
	P1	5	13.10
	P2	5	15.50
	P3	5	13.10

	P4	5	15.50
	Total	25	

Test Statistics[a,b]

	kerusakan_epitel	congesti_edema	infiltrasi_neurofil
Chi-Square	7.333	1.415	7.535
df	4	4	4
Asymp. Sig.	.119	.842	.110

a. Kruskal Wallis Test

b. Grouping Variable: perlakuan

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
kerusakan_epitel	P0	5	.20	.447	.200	-.36	.76	0	1
	P1	5	.60	.548	.245	-.08	1.28	0	1
	P2	5	.60	.548	.245	-.08	1.28	0	1
	P3	5	.80	.447	.200	.24	1.36	0	1
	P4	5	1.00	.000	.000	1.00	1.00	1	1
	Total	25	.64	.490	.098	.44	.84	0	1
congesti_edema	P0	5	1.60	1.517	.678	-.28	3.48	0	3
	P1	5	1.40	1.517	.678	-.48	3.28	0	3
	P2	5	2.00	1.414	.632	.24	3.76	0	3
	P3	5	1.60	1.517	.678	-.28	3.48	0	3
	P4	5	1.00	.707	.316	.12	1.88	0	2
	Total	25	1.52	1.295	.259	.99	2.05	0	3
infiltrasi_	P0	5	2.20	.837	.374	1.16	3.24	1	3
	P1	5	2.80	.447	.200	2.24	3.36	2	3

neurofil	P2	5	3.00	.000	.000	3.00	3.00	3	3
	P3	5	2.80	.447	.200	2.24	3.36	2	3
	P4	5	3.00	.000	.000	3.00	3.00	3	3
	Total	25	2.76	.523	.105	2.54	2.98	1	3

Appendix 10

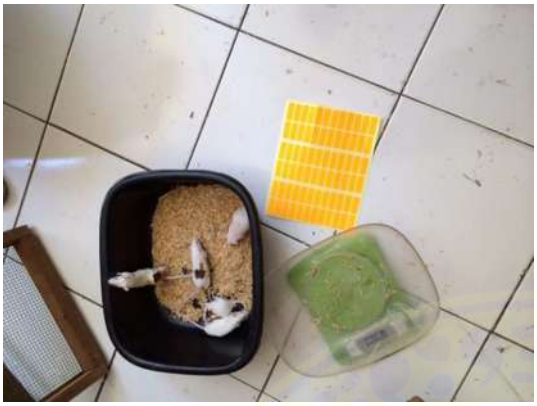
Research Documentation

Picture 1: Propolis Extract

Picture 2: Propolis dilution

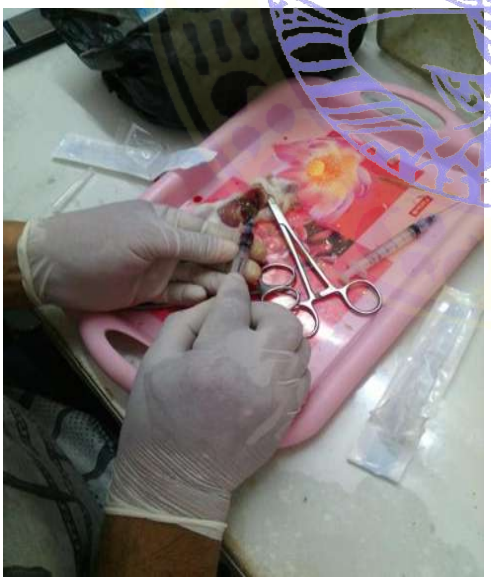
Picture 3: Propolis extract after dilution

Picture 4: Propolis dose (P1=Propolis 1.6 mg/0.5ml/day; P2=Propolis 3.2 mg/0.5ml/day; P3=Propolis 6.4 mg/0.5ml/day; P4=Propolis 12.8 mg/0.5ml/day).



Picture 5: Mice weighing

Picture 6: Mice cages



Picture 7: Mice necropsy collection

Picture 8: Small intestine