INDONESIA UNIVERSITY

THE EFFECT OF TETRAGONULA AFF TOLERANT PROPOLIS ETHANOL EXTRACT. BUREAU OF ENDOMETRIOSIS LESI GROWTH, APOPTOSIS ACTIVITY CHANGE AND NETWORK ENDOMETRIOSIS ACTIVITIES

STUDY PROTOCOL

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MEDICAL SCIENCE DOCTORAL PROGRAM

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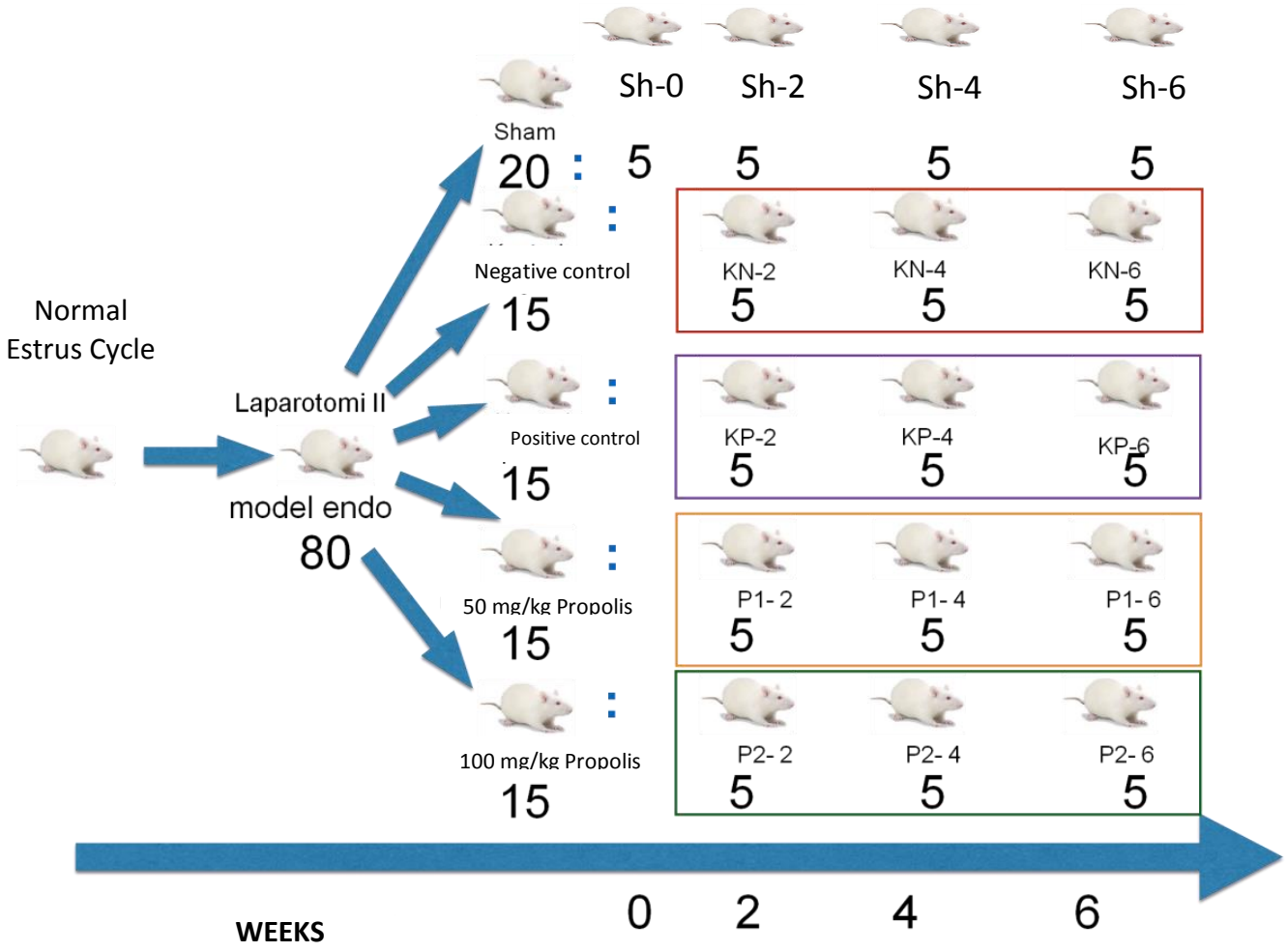
STUDY PLANNING



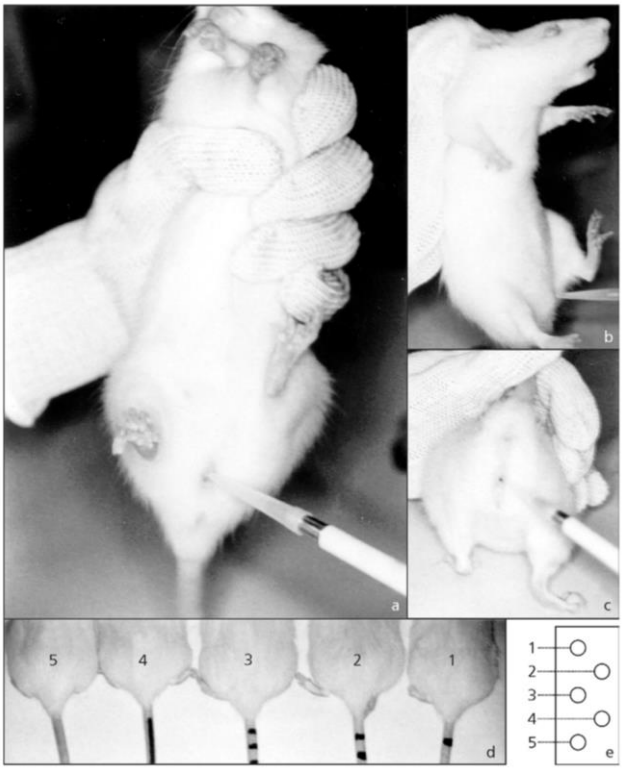
The study involved 80 endometriosis mice which were randomly divided into 16 distinct groups: the sham (SH) -0, SH-2, SH-4, SH-6, negative control (KN) -2, KN-4, KN groups -6, positive control (KP) -2, KP-4, KP-6, Dosage of Propolis 1 (P50) -2, P50-4, P50-6, Dosage Propolis 2 (P100) -2, P100-4, P100 -6. All mice that will be made endometriosis models are certain to have a normal estrous cycle. The first laparotomy was performed to make an endometriosis model according to the Vivian technique, which was confirmed to be successful 30 days later with a second laparotomy. Subsequently given different treatment according to the study group and carried out a third laparotomy at the appropriate time (2, 4, and 6 weeks after the second laparotomy).

TREATMENT OF RAT

All rats were treated in the ARF IMERI FKUI laboratory. Standard care in research rate follows standard maintenance protocols according to Bauman135 to provide optimal well-being for these rats, including: the need for social contact, a place to rest, a place to hide, exploration needs, the need for nutrition and free from environmental disturbances (e.g temperature, sound, etc.)

ESTRUS CYCLE EXAMINATION

The estrous cycle of rats was examined by taking vaginal lavage. Each rat in one cage was marked to distinguish the first to fifth mice. Vaginal discharge is taken by inserting the tip of a plastic pipette containing 10 µL NaCl 0.9% into the rat's vagina, but not too deep. If the rat is considered aggressive, sampling can be carried out. The vaginal discharge is then placed on a glass slide. Glass slide that is used is different for each cage.

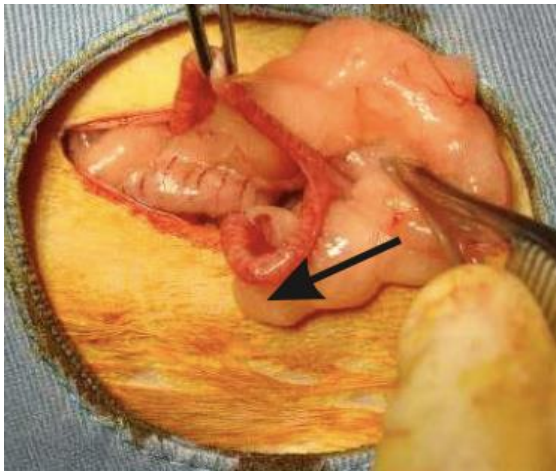


PROPOLIS ETANOL EXCRETION

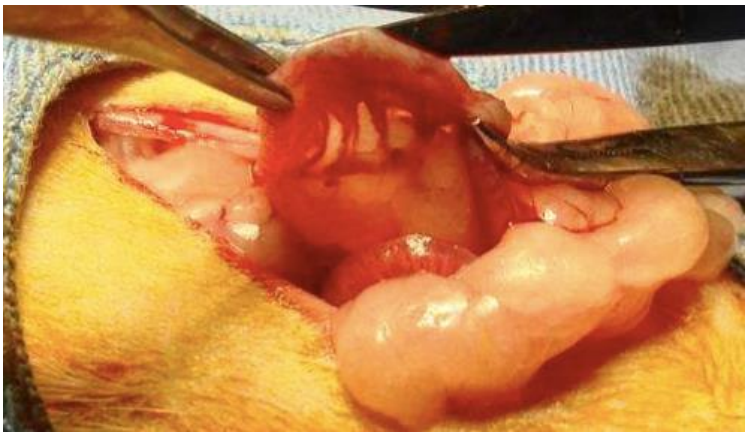
Ethanol Propolis extract (EEP) is made from fine Propolis extracted with 96% ethanol using the method described in Pratami. EEP powder according to the treatment dose was dissolved in 10 cc of aqua before it was given to rat endometriosis models

1st LAPAROTOMY

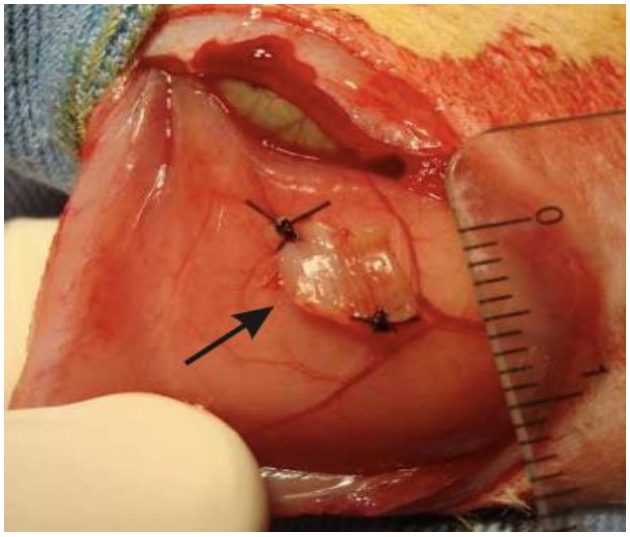
1. Anaesthesia of rats (pentobarbital sodium, 50 mg / kg BW i.m.)
2. Perform Asepsis and antisepsis procedures in the abdomen of the rat
3. Incision mediana below the center along the 4 cm
4. Treat bleeding
5. Identification of bikornu uterus
6. Resection of rat uterus by 0.5 x 0.5 cm with regard to the endometrium and serous sides
7. Suturing the rat's uterus in the right anterior peritoneum 3 cm from the line
8. incision with the endometrial side facing the peritoneum
9. After there was no bleeding, the abdominal wall was covered in one layer with PGA thread number 2.0
10. Rat returned to the cage, then complete the research form



Bikornu Uterus Identification



Incision in the uterus



Stitching the uterus into peritoneum

2nd LAPAROTOMY

1. Anesthesia of rats
2. Perform Asepsis and antisepsis procedures in the abdomen of the rat
3. The median incision is below the center 4 cm long
4. Treat bleeding
5. After the peritoneum is opened, display the endometriosis lesions that have formed
6. Do lesion documentation
7. Excision of the formed lesion, then insert the tissue into the anatomic pathology tube
8. Cover the entire abdomen using a thread and complete the research form

THE TREATMENT

The treatment is given by sonde orally

PROPOLIS

Each mililiters contains 20mg of propolis. The propolis dosage that we use is 50mg/kg BW. So, we give 0,2ml for propolis 50 treatment, and 0,4ml for propolis 100 treatment

DIENOGEST

We use 0,1mg/Kg BW/day dienogest

WATER

We give 0,2 ml every day for each rat

SPECIMEN COLLECTION

After the treatment is done (for 2, 4, and 6 weeks), we do the necropsy. We take a picture of the lesion (to compare with the beginning lesion), then we take the lesion and peritoneal rinses and blood for the protein level examinations. We also take the ovarium, uterus and peritoneum for pathologic anatomy examination for each treatment.

SPECIMEN EXAMINATION

Protein Levels of Bax, Bcl, Cleaved Caspase 3, IL-1B, and PGE 2

Protein samples were obtained from endometriosis tissue taken by the treatment group. The lysis network uses a lysis buffer solution at 4 ° C for 30 minutes. The total protein is then broken down based on its molecular weight by the SDS-PAGE (Sodium Docecyl Sulphate-Polycramide Gel Electrophoresis) method and transferred to the PVDF (Polyvinylidene Difluoride) membrane. The membrane is then incubated with primary antibodies (Anti-Bax antibody [Abcam], Anti-Bcl-2 antibody [Abcam], Anti-Cleaved Caspase-3 antibody [Abcam], Anti-IL-1B antibody [Abcam]), followed by incubation with secondary antibodies (Horse-Radish Peroxidase [Santa Cruz Biotechnology]). Proteins that have been bound to antibodies are then viewed using enhanced chemiluminescent reagent kits [Boster Bio]

Mollecular docking

Twenty-three compounds contained in Propolis were tested by screening using rule 5 (Lipinski's rule) consisting of: molecular weight <500 Dalton, <10 hydrogen donor bonds, <10 hydrogen acceptor bonds, partition coefficient values between -5 to 5. The screening results determine which compounds can enter the body and can work in endometriosis tissue. Furthermore, the three-dimensional shape is made of compounds which have passed the first stage. This process was created using the Marvin Sketch program. Furthermore, the compound that has been formed is stored as a ligand with extension pdb (protein data bank). Look for NFκB receptor proteins, estrogen A, estrogen B, progesterone A and progesterone B in the protein data bank, which will be stored as protein. Ligands and proteins are tested using standard Lamarckian algorithms in the Autodock program. The results obtained are in the form of bond energy (ΔG), where smaller results indicate a higher likelihood of interaction.

Examination of PGE2 receptor mRNA expression

Isolation of RNA in Endometriosis Tissue Samples

The RNA isolation process is carried out by first preparing a mixture consisting of 400 µL RB Buffer and 4 µL Beta-Mercaptoethanol. Then, the tissue is removed and placed in a later RNA, then the later RNA is removed and mixed with RB buffer and beta mercaptooethanol

RNA spectrophotometry

The instrument used to measure the concentration and purity of RNA is the Nano Drop Spectrophotometer. The device is turned on then the sample place is cleaned with tissue and distilled water. After that, blank measurements were taken by dripping RNAse-free water as much as 2 µL in the sample place. Next, concentration and purity (A260 / A280) measurements were taken in each sample. After the measurement is complete, the sample is again stored at -20 ° C.

Synthesis of cDNA

CDNA synthesis was carried out using the ReverTra Ace® qPCR RT Master Mix kit with gDNA Remover [Toyobo] based on the ReverTra Ace® qPCR RT Master Mix Instruction Manual protocol with gDNA Remover (Toyobo 2017: 2). The first thing to do is calculate the ratio of the volume of RNA template and free water in order to obtain a mixture with a final concentration of RNA 25 ng / µL with the formula:

$$\text{Volume RNA} = \frac{25 \text{ ng } x \text{ 6 } \mu L}{\text{Initial RNA concentration}}$$

$$\text{Volume } \textit{nuclease free water} = 6 \text{ µl} - \text{RNA Volume Used}$$

RNA templates and reagents to be used are placed on ice and then thawed. After that, Genomic DNAse I reaction solution is prepared according to the table
Composition of Genomic DNAse I solution

Materials	Volume
4x DN Master Mix (50 µl 4X DN Master Mix dan 1 µl g DNA Remover)	2 µL
RNA template	Variabel
Nuclease free water	Variabel
Total Volume	8 µL

Genomic DNAse I reaction solution was then incubated at 37 ° C for 5 minutes then immediately placed on ice. Then, the Reverse transcription solution is made according to the following table with the sample remaining on the ice:

Materials	Volume
RNA template	8 µL
5x R Master Mix II	2 µL
Total Volume	10 µL

Realtime PCR
Determination of Melting Temperature (Tm)
The primary melting temperature (Tm) is determined by the formula:

$$Tm = 2°C\ (A+T) + 4°C\ (G+C)$$

A = Adenin, T = Thymine, G = Guanin, C = Sitosi

Quantitative Real-time PCR (Q-PCR) samples
Real-time PCR is done using the QuantBR SYBR Green PCR kit
Master Mix [Qiagen] is based on the QuantiTech® SYBR® Green PCR Handbook protocol (Qiagen 2011: 11). Samples and reagents to be used are prepared and then homogenized by spin down. After that, the correction mix is made according to the following table:
Mix-real time PCR reaction composition

Components	Volume
Nuclease free water	4,6 µL
2x QuantiTect SYBR Green PCR Master Mix	7,5µL
Primer *forward*	0,45 µL
Primer *reverse*	0,45µL
Template cDNA	2µL
Total Volume	15µL

The reaction mix is then put into a 0.2 ml tube and homogenized by spin down for a few seconds then put into the Techne Prime Pro 48 [Techne] PCR machine and set according to the following table:

Real-time PCR cycle

Steps	Time	Temperature
PCR *initial activation step*	15 menit	95°C
Denaturasi	15 detik	95°C
Annealing	30 detik	57°C
Ekstensi	30 detik	72°C
Jumlah siklus	40	

The results are then compared with standard curves and analyzed

DATA ANALYSIS

Data on lesion area of endometriosis tissue models, Bax / Bcl-2, Caspase 3, IL-1B, and PGE2 ratios obtained from In Vivo research are numerical data that will be processed using SPSS 20. The data will be presented in the average form if normal distribution or median if not normally distributed. The normality of distribution will be known through the Shapiro Wilk Test. Differences in endometriosis responses between dose and duration groups were performed with the Anova test (normal distribution) or Kruskal Wallis (abnormal distribution). Changes in estrous cycles between groups are known through the chi square test.

To find out the binding of EEP active compounds, it was carried out using the in silico test. Compounds are said to bind when the value of ΔG is less than zero or negative. From the binding compounds, path analysis will be carried out to determine the relationship of the bonds with receptors related to the pathophysiology of endometriosis.