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KETERANGAN LOLOS KAJI ETIK

ETHICAL APPROVAL

Komite Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Indonesia dalam upaya melindungi hak asasi dan kesejahteraan subyek penelitian kedokteran, telah mengkaji dengan teliti protokol berjudul:

The Ethics Committee of the Faculty of Medicine, University of Indonesia, with regards of the Protection of human rights and welfare in medical research, has carefully reviewed the research protocol entitled:

"Potensi Ekstrak Kedelai Kaya Lunasin pada Penghambatan Karsinogenesis Kanker Payudara Tikus *Sprague Dawley* yang diinduksi DMBA (Studi Daya Sitotoksik Limfosit T/CD8+, Inhibitor PD-1, Ekspresi ER, HER2 dan EGFR)".
No. protokol: 19-06-0682

Peneliti Utama / *Principal Investigator* : **Dr. Drs. Kusmardi, M.S.**

Nama Institusi / *Name of the Institution* : **Patologi Anatomik FKUI-RSCM**

dan telah menyetujui protokol tersebut di atas.
and approves the above mentioned protocol.

Jakarta, 10 JUN 2019

Ketua
Chair

Prof. dr. Rita Sita Sitorus, PhD, SpM(K)

* *Ethical approval* berlaku satu tahun dari tanggal persetujuan.
** Peneliti berkewajiban
1. Menjaga kerahasiaan identitas subyek penelitian.
2. Memberitahukan status penelitian apabila
   a. Setelah masa berlakunya keterangan lolos kaji etik, penelitian masih belum selesai, dalam hal ini *ethical approval* harus diperpanjang.
   b. Penelitian berhenti di tengah jalan.
3. Melaporkan kejadian serius yang tidak diinginkan (*serious adverse events*).
4. Peneliti tidak boleh melakukan tindakan apapun pada subyek sebelum protokol penelitian mendapat lolos kaji etik dan sebelum memperoleh *informed consent* dari subjek penelitian.
5. Menyampaikan laporan akhir, bila penelitian sudah selesai.
6. Cantumkan nomor protokol ID pada setiap komunikasi dengan KEPK FKUI-RSCM.

RESEARCH PROTOCOL

1. Research Design

The type of research carried out is a true-experimental laboratory study in vivo on Sprague-Dawley (SD) rats induced by 7,12-Dimethylbenz[a]anthracene (DMBA), with a completely randomized design and controlled and measured treatment.

2. Location and Setting of Research

Making Lunasin Extract (LE) at the Drug Development Research Center Laboratory, Indonesia Medical Education and Research Institute, Faculty of Medicine, University of Indonesia and Molecular Biology and Proteomics Core Facilities Laboratory, Indonesia Medical Education and Research Institute, Faculty of Medicine, University of Indonesia. Hematoxylin Eosin, ICAM-1 and E-cadherin expression were examined at the Department of Anatomic Pathology, Faculty of Medicine, University of Indonesia from December 2020 to June 2020.

3. Material

3.1. Soybean Extract

Equipment: non-heated soybean grinder (manual grinder), 40 mesh sieve, freeze drying, cold room, Whatman™ 54, syringe filter 0.22, 50 mL falcon tube, mini rocker-shaker (BioSan®), Centrifugator (Eppendorf Centrifuge 5404R), analytical balance (Kern®), Erlenmeyer, and measuring cup.

Ingredients: Soybean seeds (Glycine max (L.) Merr) of Grobogan, East Java variety obtained from the Research Institute for Various Nuts and Tubers (Balitkabi) Malang, PBS, and distilled water, Tamoxifen (Sigma®) for positive control treatment.

3.2. Induction of Breast Cancer in Experimental Animals

The experimental animals used in this study were female white rats of the Sprague-Dawley (SD) strain obtained from the Indonesian Food and Drug Administration (BPOM), aged 4-6 weeks, body weight ranging from 60-80 grams. Breast cancer induction using DMBA (Sigma®) at a dose of 20mg/kg BW rats.

Making DMBA detoxifier, by dissolving 2% sodium thiosulfate in phosphate buffer pH 8.0. Making phosphate buffer pH 8.0 is by mixing 16.73 grams of dipotassium hydrogen phosphate (K2HPO4) and 0.525 grams of potassium dihydrogen phosphate (KH2PO4) in 1 liter of distilled water.

4. Method

4.1. Induction of Experimental Animals with DMBA

Female SD rats aged 6 weeks were given standard feed and water *ad libitum*. DMBA dissolved in corn oil 2 mg/ml. DMBA induction was administered intragastrically at a dose of 20 mg/kg BW, performed 11 times, twice a week. The incidence of tumors in experimental animals was observed, then rat with a tumor volume of 1-2 cm^3 were given treatment according to their respective groups.

4.2. Sample Calculation

There were 6 treatment groups with each group consisting of 4 SD rats. To determine the number of repetitions in each group, the Federer formula is used.

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

t = the number of treatment groups, in this study there were 6 groups.

n = number of repetitions in each group.

$(6-1)(n-1) \geq 15$

$5n-5 \geq 15$

$5n \geq 20$

$n \geq 4$

Based on the calculation of the formula above, it was obtained that $n \geq 4$ so that the minimum number of replications for each group was 4 individuals. In this study, 4 SD rats/group will be used. The groups and number of SD rats used and the treatment in each group can be seen in Table 1.

Table 1. Research Groups

Group	Treatment	N
Normal	Group without treatment	4
Negative Control	The DMBA-induced group, after a tumor volume of 1-2cm^3, was left for 8 weeks without treatment.	4
Positive Control	The DMBA-induced group, after a tumor volume of 1-2 cm^3, was given Tamoxifen for 8 weeks.	4
Adjuvant	The DMBA-induced group, after 1–2 cm^3 tumor volume, were given LE and Tamoxifen for 8 weeks.	4
Curative	The DMBA-induced group, after a tumor volume of 1-2 cm^3, was given LE for 8 weeks	4
Preventive	The group that was given LE 1 week before DMBA induction, during induction and continued until the end of the treatment.	4

4.3. Animal Handling

1. Prior to the experiment, the experimental animals were acclimatized for 1 week in cages at the Animal Science Laboratory of the Faculty of Medicine.
2. Induction of DMBA 2 times a week for 11 times. Tumor incidence was observed for 24 weeks. During the observation, palpation and measurements of rat body weight, tumor diameter, and tumor volume were performed once a week. Tumor volume was calculated using the formula: (length) x (width2)/2. If the tumor volume reaches 1-2 cm3, treatment is started.
3. The treatment for each group is in accordance with table 1.
4. During the treatment, the rats' body weight, tumor diameter, and tumor volume were measured once a week. The body weight of the rats used digital scales from the Ohaus adventurer pro brand and the tumor volume was measured by electronic digital calipers.
5. After treatment, the animals were sacrificed and then the tumor mass, liver, kidney, lung, spleen and brain, as well as blood from the heart were collected. Mammary tumor tissue and normal mammary tissue were partially frozen and stored in paraffin blocks at room temperature for CPI examination.
6. Then the ICAM-1 and E-Cadherin expression tests were performed.

4.4. ICAM-1 and E-Cadherin Expression Analysis

Immunohistochemistry (IHC) procedure with the following steps:

a. Slide preparation

1. Fixation
    a) Pieces of breast cancer tissue are put in a formalin buffer solution (made from a 10% formalin solution in a Sodium Acetate Buffer until it reaches a pH of 7.0)
    b) The tissue is cut to a thickness of 3-5 mm with a knife (scalpel).
2. Dehydration

    The tissue pieces were immersed in 96% ethanol for 30 minutes, repeated 5 times with the ethanol always replaced.
3. Clearing
    a) The tissue is immersed in xylol I solution for 15 minutes.
    b) Then the tissue is put in xylol II solution for 15 minutes.
4. Impregnation and Embedding

    The tissue was planted in solid paraffin having a melting point of 60-70°C for 30 minutes, waiting for the paraffin to solidify. Then put it in the freezer for 10-15 minutes.
5. Tissues in paraffin blocks were stored at room temperature.

b. Tissue Staining with Hematoxylin and Eosin (HE)

HE staining aims to confirm the incidence of breast cancer in DMBA-induced rat. Positive results on tissue staining with HE, then will be continued with IHC examination. The procedure for staining tissue with HE is that a paraffin block is cut into slices of 4 m thick and glued to a glass object for HE staining with the following steps. The preparations were deparaffinized using xylol I, II, and III for 5 minutes each, then the preparations were rehydrated using absolute, 96%, and 70% alcohol for 5 minutes, and washed in running water for 5 minutes and then the preparations were added. into hematoxylin for 7 minutes and rinsed in running water for 10 minutes. After that, the preparation was immersed in 2-3 dips of saturated lithium carbonate or 1-2 minutes of soaking and rinsed with running water for 5 minutes. The preparation was controlled whether the blue color was sufficient, if it had not been put back into hematoxylin for 2 minutes then rinsed in running water, immersed in eosin for 1-2 minutes,

dehydrated with 70%, 80%, 96% and absolute alcohol, respectively. for 3 minutes, then clearing with xylol I, II, and III, finally dripped with entelan and covered with a cover slip. The preparations were viewed using a light microscope with a magnification of 400x.

c. IHC Staining

The IHC staining procedure refers to the IHC-Paraffin Protocol from Abcam®. The tissue in the paraffin block was cut using a microtome with a thickness of 3 m and placed on a glass slide that had been coated with poly-L-lysine and then heated in an oven at 60°C overnight. The tissue was deparaffinized with xylene 3 times for 3 minutes each and rehydrated with 100%, 95% and 70% ethanol for two minutes, two minutes, and one minute, respectively. The preparations were then immersed in 0.01 M citrate buffer (pH 6.0) in the microwave for 5 minutes. The preparation was dripped with 3% hydrogen peroxide to remove endogenous peroxide for 5 minutes at room temperature. Each preparation was incubated with ICAM-1 and E-Cadherin antibodies in PBS for 2 hours at room temperature in a humidity chamber followed by overnight incubation at 4°C. As a negative control, the N-Universal negative control was used. The preparations were then incubated with the appropriate secondary antibody for 1 hour at room temperature followed by incubation for 30 minutes with HRP-conjugated streptavidin. Proteins were visualized using 3, 3'-diaminobenzidine (DAB) for 10 min at room temperature. The preparation was added counterstain with Harris Hematoxylin, dehydrated and mounted.

d. Observation of IHC Test Results

After IHC staining, all preparations were photographed using a light microscope equipped with a camera. Photos were obtained at 400x magnification with a light microscope. Each preparation was taken randomly for 10 fields of view, then the total number of positive (brown) and negative (blue) cells was counted from the ten photos. The calculation is done with the help of imageJ application and IHC Profiler. The percentage of cells expressing protein was presented in tabular form for data analysis.

e. Expression Analysis

Assessment of the expression of immunohistochemical staining results using a weighted histoscore, which is based on the percentage of stained cells and staining intensity based

on IHC profiler analysis. Cell calculations were performed using ImageJ and IHC Profiler software and then quantified using histoscore (H-score). The H-score is calculated by multiplying the percentage value by the intensity score.

H-score = (% of tumor cell with low intensity x 1) + (% of tumor cell with moderate intensity x 2) + (% of tumor cell with strong intensity x 3).