

***Ficus septica* Burm. f. Leaves Ethanolic Extract Triggered Apoptosis on 7,12-Dimethylbenz[*a*]anthracene-Induced Rat Mammary Carcinogenesis Qualitatively**

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Abstract

Ficus septica Burm. f. ethanolic extract (FEE) shows cytotoxic effects on several cancer cell lines. Our research aimed to investigate the effect of FEE on apoptosis induction and p53 expression against carcinogenesis of 7,12-Dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary. The research was conducted by comparing both apoptosis induction and p53 expression in DMBA-induced rats that were treated with FEE against control groups. Cells that undergo apoptosis were visualized by Double Staining method with acridine orange and ethidium bromide, while p53 expression was detected by IHC staining. Double staining results showed increased occurrence of apoptotic cells compared to the control groups. IHC staining of p53 did not show significant difference between treatment and control groups. However, FEE was able to repair morphology of cells undergoing carcinogenesis. Thus, we conclude that FEE has an anti-carcinogenic activity on DMBA-induced rat mammary through apoptosis induction without affecting p53 expression. Therefore, the ethanolic extract of *Ficus Septica* leaves is a potential chemo-preventive agent on breast cancer. Further study on its molecular mechanism needs to be explored.

Keywords: *Ficus septica*, breast cancer, 7,12-Dimethylbenz[*a*]anthracene, carcinogenesis, apoptosis, p53

INTRODUCTION

Nowadays, the incidence of cancer as a disease with a complex cellular pathophysiology is increasing (King, 2000). In the United States, there were 1,400,000 incidences of cancer, 26% were breast cancer (Jemal, *et al.*, 2008). The transformation of normal cell into malignant state is called carcinogenesis, which consists of four phases: initiation, promotion, progression, and metastases (Hanahan and Weinberg, 2011).

Cancer cells are able to avoid apoptosis, a programmed-cell-death, due to mutation of p53 (Hanahan and Weinberg, 2011). In order to suppress cancer growth, one existing mechanism of carcinogenesis inhibition is through apoptosis induction. This evidence may occur by the regulation of Bcl-2 family proteins, both pro-apoptotic and anti-apoptotic (Wyllie, *et al.*, 2000). p53 induces the expression of pro-apoptotic proteins, which are

Bax and Bak, resulting in the release of cytochrome C, leading cell to undergo apoptosis (Nakamura, 2003).

Ficus septica Burm. f. has been reported as a potential chemo-preventive agent. Phenantroindolicidine, an alkaloid found in *Ficus septica*, was proven to be potential against cancer (Staerk, *et al.*, 2002). Chemo-preventive agents are compounds that have the ability to prevent, to slow down, or to stop carcinogenesis (Tamimi, *et al.*, 2005). *Ficus septica* Burm. f. ethanolic extract (FEE) exhibited the ability to trigger apoptosis and performed cytotoxic activity on T47D (breast cancer) cells with the IC₅₀ value of 58 µg/mL (Nugroho, *et al.*, 2011).

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Mubarok, *et al.* (2008) also figured out that *Ficus septica* was synergist with doxorubicin against MCF7 (breast cancer) cell line with the IC₅₀ value of 6 µg/mL and was able to induce apoptosis by suppressing the expression of Bcl-2.

Several *in vitro* studies on *Ficus septica* as a chemo-preventive agent have been conducted. This study aimed to observe FEE's ability to trigger apoptosis *in vivo* by increasing p53 expression. Observation was done on apoptosis and p53 expression of DMBA-induced rat mammary.

MATERIALS AND METHODS

Ficus septica Extraction

Ficus septica leaves were collected from Sleman, Yogyakarta, and determination was conducted in Laboratory of Pharmacognosy, Faculty of Pharmacy, Universitas Gadjah Mada. Fresh leaves were rinsed, dried, and powdered. Extraction was done by maceration with 70% ethanol (1:10). Filtrate was then concentrated using vacuum Rotary Evaporator.

Animals

Female Sprague Dawley Rats, aged 30 days, weighed from 86 to 112 g were purchased from Unit Pengembangan Hewan Percobaan Universitas Gadjah Mada. Animals were raised in ambient temperature (25-30°C), fed with pellet and tap water.

Chemicals

7,12-Dimethylbenz[*a*]anthracene (DMBA) (Sigma Chem. CO, St. Louis, MO), corn oil, 0.9% NaCl, anti p53 Ab-1/Clone Pab 240 (Neomarkers), acridine orange (Sigma), ethidium bromide (Fluka-Biochemika).

Treatment

There were 4 groups containing 5 rats in each group. Before being given to the test animals orally, DMBA and concentrated extract were dissolved in corn oil and 0.5% CMC-Na, respectively. DMBA with the dosage of 20 mg/kgBW were given in the first 5 weeks, twice a week, while FEE was given every day for 2 weeks, during the 9th and 10th week.

The first group (as positive control), treated with DMBA (DMBA control group). The second group was treated with DMBA and

FEE with the dosage of 750 mg/kgBW (treatment group). The third group was treated with FEE 750 mg/kgBW (FEE control), and the fourth group was treated only with CMC-Na (solvent extract control). Mammary sampling was done in the 10th week.

Observation of Apoptosis

Apoptosis was detected with double staining method. Preparat of mammary tissue on poly-L-Lysine slide was incubated with acridin orange-ethidium bromide solution for 10 minutes in ambient temperature. Observation was done using fluorescence microscope (Zeiss MC 80). Cells that were viable will be observed as green fluorescence, while cells undergoing apoptosis appeared to be reddish-orange.

Observation of p53 Expression

Apoptosis was detected with IHC staining. Preparat of mammary tissue on poly-L-Lysine slide was incubated with normal mouse serum for 5 minutes, followed by addition of primary antibody (monoclonal antibody anti-p53) for a night in 8°C. After being washed with PBS for 3 times, biotinylated secondary antibody were given (incubation for 5 minutes) and the preparate was rewashed with PBS. DAB (3, 3'-diaminobenzidine) was used as substrate for biotinylated secondary antibody. Counterstaining with hematoxylin (3-4 minutes) was followed by rehydration and mounting. Observation was done microscopically. Positive p53 expression will appear to be brown.

RESULTS AND DISCUSSION

Apoptosis Induction and p53 Expression

Apoptosis is a programmed cell death resulting in morphological and biochemistry changes of a cell. One of the detection method provided is by double staining using acridine orange-ethidium bromide, that later will let us differ DNA fluorescence in viable cells and cells undergoing apoptosis. Analysis was done qualitatively by using a fluorescence microscope. As shown in Fig.1, apoptosis was detected in FEE-treated group and was negative in DMBA control group. Therefore, FEE with the dosage of 750 mg/kgBW triggered apoptosis *in vivo*.

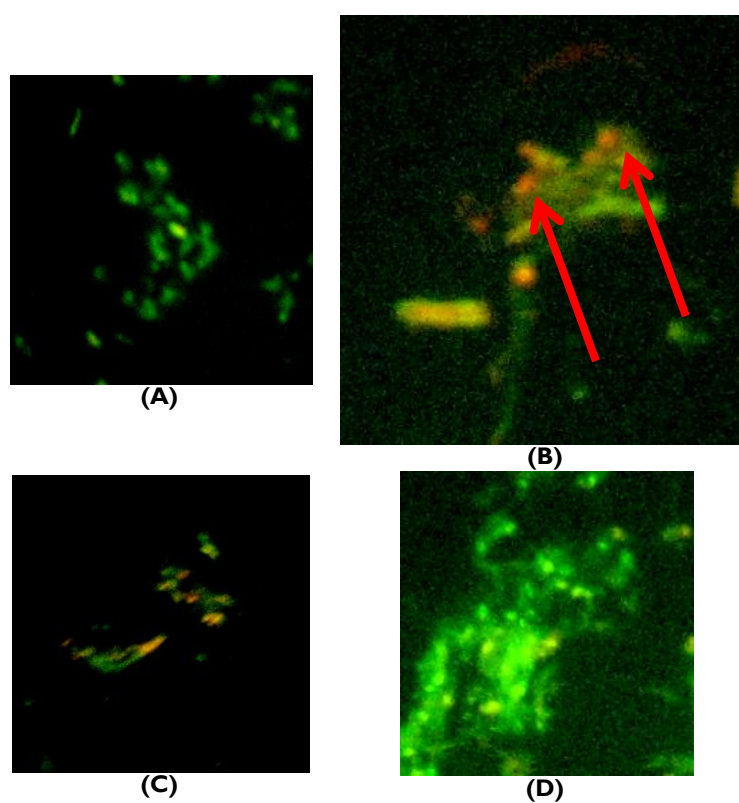


Figure 1. Apoptosis detection in rats' mammary by Double Staining method (A) DMBA control group: apoptosis was negative in DMBA control group; (B) treatment group (DMBA and FEE): apoptosis was detected (orange fluorescence pointed with red arrows); (C) FEE control group; (D) CMC-Na control group

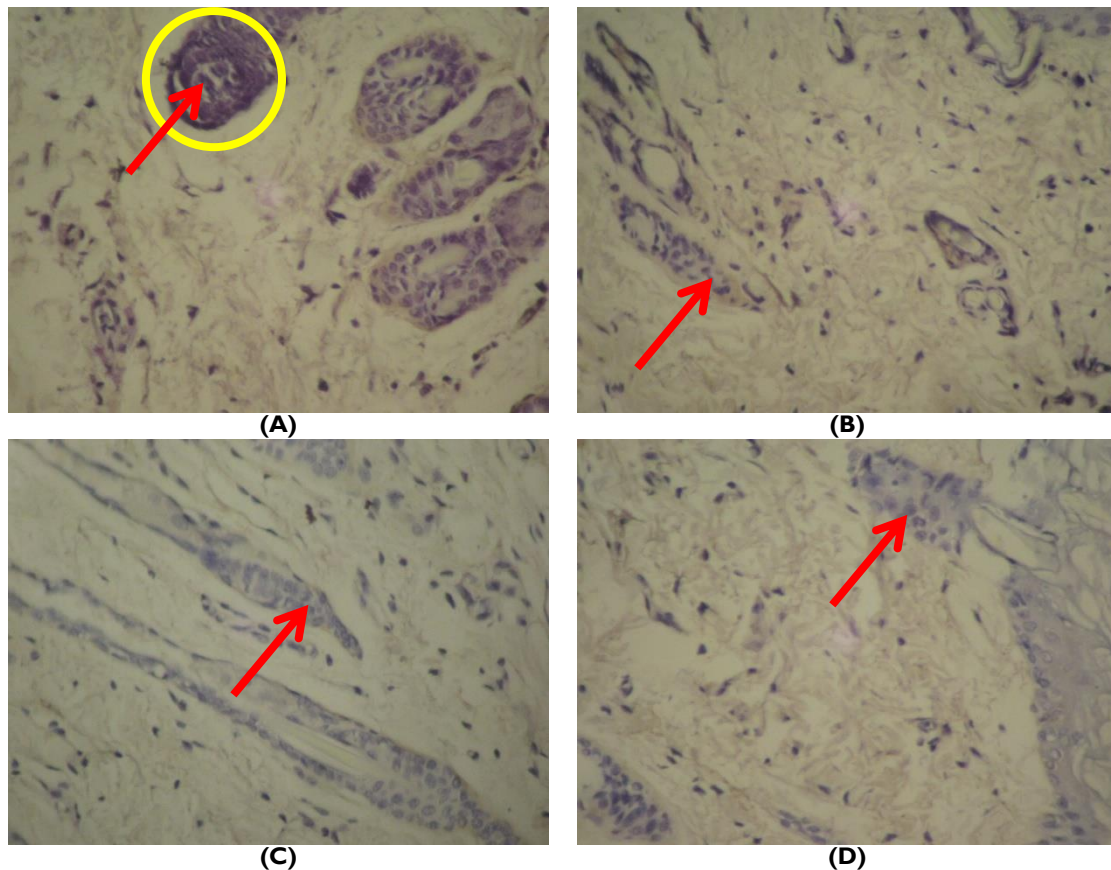


Figure 2. Expression of p53 in rats' mammary by IHC staining (a) DMBA control group: hyper-proliferation in DMBA control groups is shown by yellow circle does not appear in control groups; (b) treated group (DMBA and FEE); (c) FEE control group; (d) CMC-Na control group; There was no significant difference of p53 expression between control and treated group (cytoplasm pointed with red arrows).

The second observation was done to know whether or not apoptosis induced by FEE is occurring following the increase of p53 expression, rendering to the fact that p53 takes an important role in apoptosis induction via p53-dependent pathway. This p53 protein also plays a role as a checkpoint control in both G1/S and G2/M phase. The detection was done by IHC staining which may identify specific protein in the cell or tissue by antibody-labeling. Fig.2 shows that there was no significant differences of p53 expression between control and treated group. Hence, FEE did not increase p53 expression in rats' mammary *in vivo*. However, repair of cell's morphology undergoing carcinogenesis by FEE was observed (hyper-proliferation in DMBA control groups is shown by green circle).

Since FEE triggered apoptosis without affecting p53 expression, the apoptosis induction was not via the p53-dependent

pathway. The p53-independent pathway, known as the extrinsic pathway which is induced by activation of death receptors such as TNFR and FAS by proper ligands (TNF, FAS ligand). This pathway passes through the activation of caspase 8 which then cleaves Bid into tBid. Truncated Bid induces pores formation in the outer membrane of mitochondrion, causing conformational change. This pore formation will lead to the accumulation of Bax in the outer layer of mitochondrion, inducing the release of apoptogenic proteins by mitochondrion such as Cytochrome C (Sun, *et al.*, 2004). tBid is the active form of Bid that is in charge of turning the caspase pathway on leading the cell to undergo apoptosis.

This research concludes that *Ficus septica* Burm. f. ethanolic extract was able to trigger apoptosis without affecting the expression of p53 in rat mammary tissue induced by DMBA. Since this treatment did not

affect p53 expression, the mechanism could be predicted that the apoptosis induction maybe through p53-independent pathway.

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