



***Elephantopus scaber* Linn Extract Inducing Apoptosis and Activate Caspase Cascade in T47D Cancer Cell Line**

Mahfudh Nurkhasanah, Nanik Sulistyani*, Raga Revamia Anugrah Putri

Faculty of Pharmacy, Universitas Ahmad Dahlan, Jalan Prof Soepomo, Janturan, Yogyakarta, Indonesia, 55161

Abstract

Elephantopus scaber Linn. was used traditionally for treating various diseases. Previous studies found the cytotoxicity of this herb against different cell lines. This study aimed to determine the effectiveness of the ethanolic extract of *E.scaber* in inducing apoptosis by observing its effect on caspase cascade. This ethanolic extract was obtained by maceration method using 96% ethanol. It was fractionated with petroleum ether to discard compounds with very low polarity. This step was followed by the fractionation with chloroform to isolate compounds with optimum polarity. The soluble part in chloroform was dried and used for the assay. The immunocytochemistry method used specific antibodies for caspase-8, caspase-3, and caspase-9 to observe the expressions of each caspase. T47D cell line was treated with the chloroform fraction of *E. scaber* with the concentrations of 7.06 µg/ml and 3.53 µg/ml. The results of the immunocytochemistry showed that this chloroform fraction increased the expressions of caspase-8, caspase-3, and caspase-9 protein significantly. Such increase led to apoptotic cells. This finding supported the development of *E.scaber* extract as an anticancer agent.

Keywords: *Elephantopus scaber*, apoptosis, caspase-8, caspase-3, caspase-9, immunocytochemistry.

1. Introduction

Corresponding Authors: N. Sulistyani, Faculty of Pharmacy, Universitas Ahmad Dahlan, Jalan Prof Soepomo, Janturan, Yogyakarta, Indonesia, 55161

Tel: +6285878322867

E-Mail: naniksulistyani@gmail.com

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Cancer is a major health problem that causes death after cardiovascular disease. Breast cancer is the first ranked most common malignancy in female population [1]. Although

cancer treatment has currently used different methods, it still does not provide any satisfying results. Also, cancer therapy has many side effects and damages the normal cells.

Cancer progresses due to an imbalance between cell proliferation and cell death. The process of programmed cell death, or apoptosis, is considered vital in normal homeostatic settings. This process produces a balance in the number of cells

by eliminating damaged cells and physiological proliferation. The defects in the mechanisms of apoptosis play essential roles in tumor development as they allow neoplastic cells to survive and reproduce uncontrollably [2].

Elephantopus scaber L. has been used traditionally to treat various disease. It has been reported to have a cytotoxic effect and induce the apoptotic death of HeLa cancer cells [5]. Some of its active compounds have been successfully isolated, particularly deoxyelephantopin and isodeoxyelephantopin of the sesquiterpene lactone class [3]. Deoxyelephantopin inhibits the growth of cancer cells by an apoptotic mechanism in which the caspase cascade, i.e., caspases-8, -9, -3, and -7, are activated [4]. This finding affirms the potential of *E. scaber* L. as an anticancer [7].

The fractions obtained from the extract of *E. scaber* L. are empirically found to exhibit cytotoxic activities, which are indicated by IC_{50} value. The chloroform fraction of the leaves of *E. scaber* L. shows cytotoxic activity against T47D breast cancer cell line with IC_{50} of 7.06 μ g/ml [6]. The understanding of the mechanism by which a compound works is fundamental to drug development. This study aimed to identify the efficacy of the active fraction of the ethanolic extract of *E. scaber* L. in inducing apoptosis by the activation of caspase cascade.

2. Materials and Methods

2.1. Materials

The leaves of *E. scaber* L. were obtained in Yogyakarta, Indonesia. The plant was

identified as *Elephantopus scaber* Linn. from the family Compositae in Laboratory of Biology, Universitas Ahmad Dahlan under the supervision of Assoc. Prof. Hadi Sasongko, and the number of the herbarium specimen is 073/Lab.Bio/B/VII/2016.

2.2. Extraction and Fractionation

The leaves of *E. scaber* L. were dried in an oven at 50°C. The dried leaves were powdered and sieved with 20/40 mesh. The powder that passed through the 20 mesh but was retained by the 40 mesh was used for extraction. The extraction was performed by maceration with 96% ethanol solvent. The maceration was replicated three times to maximize the collection of the compounds. The macerate was evaporated with a vacuum evaporator to obtain a viscous extract.

The viscous extract of 20 grams of *E. scaber* L. was dissolved and shaken in 100 ml of petroleum ether to discard compounds with very low polarity. The soluble fraction of the petroleum ether was separated from the insoluble matter, which was later dissolved in 100 ml of chloroform. The chloroform fraction was evaporated until a solid chloroform fraction was formed. The fractionation of the extract with chloroform was performed three times to get the maximum active fraction.

2.3. Sample Preparation

A sample of 10 mg was dissolved in 1 ml of dimethyl sulfoxide (DMSO). Then, it was diluted with RPMI to achieve the concentrations of 7.06 μ g/ml and 3.53 μ g/ml by gradual dilution. The treatment used these

two concentrations because the IC_{50} of chloroform fraction was 7.06 $\mu\text{g/ml}$ [8]. At this concentration, the cell growth and protein expression were easily observable due to the presence of adequate viable cells. The final concentration of DMSO in the sample was 0.007%. Exposure to 1% DMSO is empirically proven to inhibit cell survival insignificantly [9]. Therefore, this concentration is not toxic to cell growth.

2.4. Immunocytochemistry

The expressions of the caspases were observed with immunocytochemistry technique [10]. T47D cells were grown in a 24-well microplate. They were left to attach and grow in the bottom of the plate after overnight incubation. The microplate was taken from the incubator, and the culture medium was then removed from each well using a micropipette. A solution of 1 ml was sampled from the extract with concentrations of 7.06 $\mu\text{g/ml}$ and 3.53 $\mu\text{g/ml}$, transferred into the well, and then incubated for 24 hours. After the incubation, all culture mediums were removed from the well, added with 300 μl of PBS, and then left for 5 minutes. The PBS solution was discarded. The culture mediums were added with 300 μl of distilled water, left for 5 minutes, and then discarded. The cells were fixed with 300 μl of methanol and left for 10 minutes before the methanol was discarded. After the fixation, the cells were washed two times with 300 μl PBS, added with 100 μl of hydrogen peroxide solution, and left for 5-10 minutes. The solution was removed and washed with 300 μl of PBS two times.

Afterward, the cells were added with 100 μl of prediluted blocking serum and left for 10-15 minutes. They were then removed, added with 100 μl of primary anti-caspase-8, anti-caspase-3, and anti-caspase-9, and incubated for 24 hours. After the incubation, they were washed two times using 300 μl of PBS, added with 100 μl of secondary antibodies, and left for 20 minutes. Afterward, the cells were washed two times using 300 μl of PBS, added with 100 μl of HRP solution, left for 10 minutes, and then washed with PBS.

DAB solution were added to the cells and left for 2 minutes. After washing them with distilled water, the microplate was added with Mayer Hematoxylin solution and left for 5 minutes. The last step was washing the cells with 500 μl of distilled water and left them to dry. The expressions of caspase-8, caspase-3, and caspase-9 were observed under a light microscope.

2.5. Analysis

The cells were observed under a light microscope with 100x magnification. The expressions of caspase 8, caspase-3, and caspase-9 were characterized by the color of the cell. The positive caspases appear in brown or dark color, while the negative ones have blue or purple color. The expressions were observed on six fields of view for every sample and presented as the percentage of positive expression compared to the total area of the cells.

3. Results and Discussion

3.1. Extraction and Fractionation

The extraction of the leaves of *E. scaber* L. using 96% ethanol produced a concentrated extract with adark color. The yield of the extraction was 8.5%, which was in line with the standard, i.e., higher than 2.7% [11]. Fat and other compounds with very low polarity were then removed with petroleum ether. Fractionation with chloroform aimed to isolate active compounds with optimum polarity. The result was 20.5% compared to the crude extract. The high cytotoxicity of chloroform fraction was indicated by IC_{50} of 7.06 $\mu\text{g/ml}$ [6].

3.2. The Increased Expression of Caspase-8 by the *Elephantopus scaber* Extract

The activation of apoptosis-signaling pathways by anticancer drugs is frequently formed during the activation of caspases, a family of cysteine proteases that act as common death-effector molecules. Caspases can trigger apoptosis by cleaving various cytoplasmic or nuclear substrates, which are the morphologic features of apoptotic cells. The activation of caspase can be initiated in the plasma membrane with different

mechanisms, by either death receptor-mediated signaling (receptor pathway) or mitochondrial pathway [12].

Elephantopus scaber was reported as a promising anticancer agent. Some active compounds that are isolated from *E. scaber* exhibit cytotoxic activity against some cell lines [4,13]. This study observed the expression of caspases after the addition of the fraction of *E. scaber* as a potential treatment for cancer. The expression of caspases involved a series of complex processes and many factors. The gene expression system, including initiation, transcription, translation, and other concomitant processes, was carefully controlled.

The expression of caspase-8 in T47D breast cancer cell line after treatment with *E. scaber* extract is presented in Figure 1. Treatment with 3.53 $\mu\text{g/ml}$ increased the expression of caspase-8 (Figure 1B). The dark brown cells were identified in nearly all culture cells. This expression was significantly different from the morphology of the control sample (Figure 1A). The dark brown color indicates high expression of caspase-8 following the treatment with *E. scaber*. A higher dose of the fraction of *E. scaber* (7.06 $\mu\text{g/ml}$) resulted in

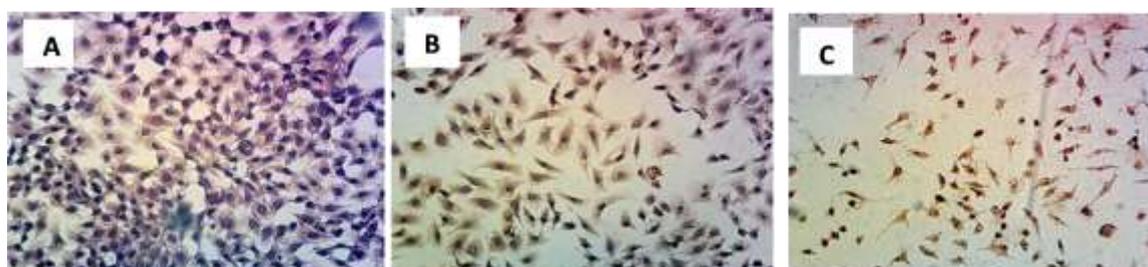


Figure 1. The caspase-8 expression after ES treatment on T47D cells A. Control cells B. 3.53 ug/ml, C. 7.06 ug/ml.

damaged cells (Figure 1C). Subsequently, the T47D cells entered the late stage of apoptosis and, then, necrosis. The morphology of the T47D cells was characterized by cellular shrinkage and apoptotic bodies in their surrounding. The morphological changes, signifying apoptosis, were observed in most cell types. These changes started with a reduction in cell volume and followed by the condensation of the nucleus[14].

At a concentration of 3.53 µg/ml (Figure 1B), treatment with *E. scaber* produced brown cells, indicating the expression of caspase-8 in the cytoplasm. This expression led to apoptotic cells. The calculation of the expression is summarized in Table 1.

The activation of caspase-8 after the treatment indicates the activation of the extrinsic pathway. Caspase-8 also has a significant role in the transcription of p53 tumor suppressor protein [15].

3.3. the Increased Expression of Caspase-9

Caspase-9 is an initiator caspase that regulates the occurrence of apoptotic processes through the internal pathway. It is activated by binding cytochrome c to Apaf-1, which forms a complex known as apoptosome. This complex activates the caspase-9 zymogen (pro-caspase-9). Once activated, caspase-9 will trigger the activation of the effector caspase and cause apoptosis[16].

This research showed that the treatment of T47D cells with *E.scaber* increased the expression of caspase-9 (Figure 2). The calculation of the expression of caspase-9 after the treatment is presented in Table 2.

This study found that the expression of caspase-9 significantly increased after treatment with 3.53 µg/ml of *E. scaber* extract. In a normal condition (the control sample), the expression of caspase-9 in T47D cell lines was low (Figure 2A). After the treatment, it started

Table 1. The quantification of caspase-8 expression on T47D cells after *Elephantopus scaber* extract treatment.

Treatment	Percentage of caspase-8 expression ± SD
control	0 ± 0%
3.53 µg/ml	96.62 ± 2.69%
7.06 µg/ml	100 ± 0%

Table 2. The quantification of caspase-9 expression on T47D cells after *Elephantopus scaber* extract treatment.

Treatment	Percentage of <i>caspase-9</i> expression ± SD
Control	18.89 % ± 0.03
3.53 µg/ml	45.23 % ± 0.02

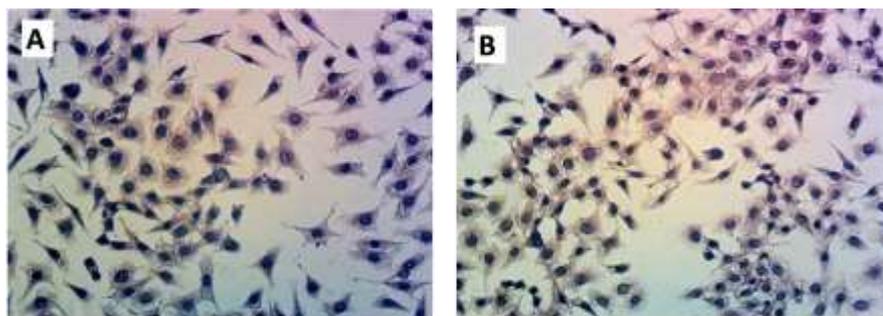


Figure 2. Caspase-9 expression after *Elephantopus scaber* treatment (A) control, (B) treatment with 3.53 µg/ml.

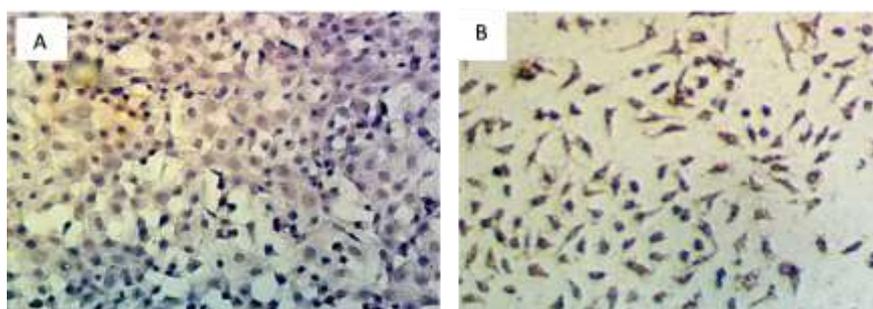


Figure 3. Caspase-3 expression after *Elephantopus scaber* treatment (A) control, (B) treatment with 3.53 µg/ml.

to increase, proving the role of *E. scaber* in inducing apoptosis through mitochondrial pathway.

3.4. The Increased Expression of Caspase-3

Caspase-3 is a proapoptotic agent that acts as a major effector caspase (executioner) in the process of apoptosis. It plays an essential role in breaking the apoptotic substrate and activating other effect or caspases, including caspase-6 and caspase-7[17]. Treatment with *E. scaber* extract was able to increase the expression of caspase-3, as presented in Figure 3 and Table 3.

During the research, caspase-3 seemed to be downstream of caspase-8. The increased expression of caspase-3 after treatment with *E. scaber* was most likely caused by the

increased expression of caspase-8. Caspase-8 is an initiator caspase that cleaves pro-caspase-3 into activated caspase-3. Upon the activation, caspase-3 becomes capable of cleaving many cellular substrates and induces morphological changes like chromatin condensation, membrane blebbing and DNA fragmentation, indicating the process of apoptosis[18].

3.5. *Elephantopus Scaber-Induced Caspase Cascade*

The activation of caspase-3 involved the intrinsic and extrinsic pathways. Caspase-3 was activated via the extrinsic pathway (death ligand) where the death signal was caused by the compound of *E. scaber* bound to the death receptor. This bond formed a trimer with

Tabel 3. The level of *caspase-3* expression on T47D cells after *Elephantopus scaber* extract treatment.

Treatment	Percentage of <i>caspase-3</i> expression \pm SD
Control	2.13 \pm 0.028
7.06 μ g/ml	17.65 \pm 0.018

FADD (Fas-Associated Death Domain) and activated pro-caspase-8. The active caspase-8 activated caspase-3 as an effector caspase.

Meanwhile, in the intrinsic pathway (mitochondria), treatment with *E. scaber* extract induced the release of cytochrome c, which later formed a complex with Apaf-1 and pro-caspase-9 known as apoptosome. The active caspase-9 activated caspase-3 as an effector caspase. This study found that after the treatment with *E. scaber* extract, the expressions of caspase-8, caspase-9, and caspase-3 were increased, suggesting that this extract induces the apoptosis through intrinsic and extrinsic pathways.

The results of this study were in line with the previous research, which reported that caspase-3 induced apoptosis and mediated cell cycle arrest in T47D cells by isodeoxyelephantopin. *E. scaber* was also reported to induce cell cycle arrest at G2/M phase [4].

This study proved the potential of *E. scaber* as an anticancer agent. The extract of *E. scaber* exhibits cytotoxicity against various cancer cell lines, including MCF-7 breast cancer cell lines [4,13], A549 lung carcinoma cells [4], Hela cervical cancer cell lines [19], HCT human colon cancer cell lines, and

Dalton's Lymphoma Ascites (DLA) tumor cells [3].

This study also confirmed that the mode of death induced by *E. scaber* was apoptosis. The ability to induce apoptosis is an essential requisite of anticancer agents, including chemotherapeutic agents, hormones, and various biological compounds [16]. This study found that *E. scaber* induced the apoptosis of T47D breast cancer cell lines by activating caspase cascade. The expression of caspase-9, caspase-8, and caspase-3 increased significantly. Therefore, *E. scaber* is potentially developed as an anticancer agent.

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