

Andrographis paniculata extract induced apoptosis of adenocarcinoma mammae in C3H mice

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ABSTRACT

BACKGROUND

Apoptosis plays an important role in tumorigenesis. Induction of apoptosis is a strategy for developing cancer therapy. In vitro study found that andrographolide isolated from *Andrographis paniculata* has anticancer activity by an apoptotic mechanism in cancer cell lines. The aim of the present study was to prove the effect of *Andrographis paniculata* extract administered orally on apoptosis of mammary adenocarcinoma in C3H mice.

METHODS

This study was of post test randomized control group design. Twenty four C3H mice with transplanted mammary adenocarcinomas were divided into four groups. To three groups *Andrographis paniculata* extract was administered orally for 14 days, at doses of 5, 10 and 15 mg/day, respectively, whereas to the control group no *Andrographis paniculata* extract was administered. On day 15 the mice were terminated. The mammary adenocarcinomas were examined by the terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) method. The values of the apoptotic index were expressed as mean \pm SD and analyzed using ANOVA and Pearson's correlation test.

RESULTS

The mean apoptotic index values differed significantly among the experimental groups ($p=0.001$). The highest value was found in the group receiving *Andrographis paniculata* extract 15 mg/day, while the lowest was in the control group, the values being significantly correlated ($r=0.974$).

CONCLUSIONS

Oral administration of *Andrographis paniculata* extract induced apoptosis in C3H mice with mammary adenocarcinoma

Key words: *Andrographis paniculata*, apoptosis, mammary adenocarcinoma, mice

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Ekstrak *Andrographis paniculata* menginduksi apoptosis adenokarsinoma mamma pada mencit C3H

ABSTRAK

LATAR BELAKANG

Apoptosis mempunyai peran penting pada tumorigenesis. Induksi apoptosis merupakan strategi yang dikembangkan untuk terapi kanker. Andrografolid yang diisolasi dari tanaman *Andrographis paniculata* memiliki aktivitas antikanker melalui mekanisme apoptosis sel line kanker pada penelitian in vitro. Penelitian ini bertujuan untuk menilai pengaruh ekstrak *Andrographis paniculata* yang diberikan secara oral terhadap apoptosis sel adenokarsinoma mamma mencit C3H.

METODE

Penelitian dilakukan dengan desain Post test Randomized Control Group Design. Sebanyak 24 mencit yang telah tumbuh adenokarsinoma mamma dibagi menjadi 4 kelompok. Diberikan ekstrak *Andrographis paniculata* secara oral dengan variasi dosis 5, 10 dan 15 mg/hari selama 14 hari. Satu kelompok sebagai kontrol tidak mendapat ekstrak. Hari ke 15 mencit diterminasi, jaringan kanker diambil untuk duperiksa apoptosisnya dengan metode terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL). Data dihitung rerata dan penyimpangannya dan dianalisis dengan uji beda ANOVA serta uji korelasi Person.

HASIL

Terdapat perbedaan rerata indeks apoptosis antara keempat kelompok perlakuan ($p=0,001$). Apoptosis terbanyak didapatkan pada mencit yang mendapat ekstrak 15 mg/hari dan paling sedikit pada mencit kontrol dan berhubungan secara bermakna ($r=0,974$).

KESIMPULAN

Pemberian ekstrak *Andrographis paniculata* secara oral dapat menginduksi apoptosis sel adenokarsinoma mamma pada mencit C3H.

Kata kunci: *Andrographis paniculata*, apoptosis, adenocarcinoma mammae, mencit

INTRODUCTION

Apoptosis is a mechanism of cell death for the purpose of maintaining a stable cell population and plays an important role in tumorigenesis. Inhibition of apoptosis results in uncontrolled growth, such as occurs in the development of malignancy. Apart from influencing growth, apoptosis also facilitates cancer metastasis. This is through the inhibition of a type of apoptosis called anoikis [cell-detachment-induced apoptosis], which

normally eliminates cells that are not in their appropriate environment/extracellular matrix. Inhibition of the apoptotic process is also connected with resistance to both chemotherapy and radiotherapy.⁽¹⁾

The occurrence of apoptosis, which is not accompanied by an inflammatory reaction, is an important part of cancer therapy. Apoptosis is responsible for a reduction in the mass of solid tumors. Induction of apoptosis is one of the strategies has been developed for cancer therapy through manipulation of the

genes and proteins responsible for apoptosis, as well as through manipulation of inhibitory apoptosis protein (IAP).⁽²⁾

Breast cancer is a type of malignancy frequently found in women throughout the world, and ranks second as a cause of death from cancer. Worldwide, more than 1.2 million women are diagnosed with breast cancer annually.⁽³⁾ Each year 250,000 new cancer cases are found in Europe and around 175,000 in America. In 2005 there were 40,870 deaths from breast cancer in America, comprising 40,410 women and 460 men. According to data from the WHO in 2008, 460,000 of deaths are caused by breast cancer.⁽⁴⁾

The Department of Health of the Republic of Indonesia stated that the number of breast cancer patients in Indonesia ranks first among malignancies. In the year 2004, a total of 29,740 breast cancer cases were reported from hospitals, while in 2005 16,671 cases were reported, consisting of 8,829 cases on ambulatory care and 7,844 hospitalized ones. In 2006 the number of hospitalized cases increased to 41,736.⁽⁵⁾

Standard treatments of breast cancer according to WHO guidelines⁽³⁾ are surgery, radiotherapy, cytostatic drugs and hormonal therapy. These treatments do not yet yield satisfactory results, as indicated by the high mortality rate among breast cancer patients. In order to obtain better therapeutic results, herbal preparations have been used.

Sambiloto (*Andrographis paniculata*) is one of the traditional medications widely used in Asian countries. Aqueous extracts of *Andrographis paniculata* contain alkaloids, amino acids, flavonoids, glycosides, saponins and tannins.⁽⁶⁾ *Andrographis paniculata* has various activities, viz. gastroprotective,⁽⁷⁾ hepatoprotective,^(8,9) antiviral, antipyretic, immuno-stimulatory and anticancer.⁽¹⁰⁾ Isoandrographolide, 3,19-isopropylidene-andrographolide and 14-acetylandrographolide isolated from *Andrographis paniculata* have tumor suppressant effects.⁽¹¹⁾ Andrographolide

possesses anticancer activity through an apoptotic mechanism on HeLa cancer cells with an IC_{50} of 109.90 $\mu\text{g/mL}$,⁽¹²⁾ and can also induce apoptosis of the TD-47 human breast cancer cell line.⁽¹³⁾ An aqueous extract of *Andrographis paniculata* applied to cell cultures of mammary adenocarcinoma from C3H mice showed increased apoptosis starting from a concentration of 1 mg/L.⁽¹⁴⁾ All three of the aforementioned studies used acridine orange staining to identify apoptotic cells, which is however less sensitive than the terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) method.⁽¹⁵⁾

The previous studies were in vitro studies, therefore oral administration of *Andrographis paniculata* extract may yield differing results, because of the roles of the microenvironment and the processes of drug absorption, distribution, and metabolism. The microenvironment influences the growth of cancer tissue and is associated with therapeutic success.⁽¹⁶⁾ Fibronectins in the extracellular matrix can induce apoptosis, thus the different result may found between in vitro and in vivo study.⁽¹⁷⁾ The in vitro studies demonstrating the potential of *Andrographis paniculata* extract as an anticancer drug need to be proven by in vivo studies. The objective of the present study was to evaluate the effect of orally administered *Andrographis paniculata* extract on apoptosis in C3H mouse mammary adenocarcinoma cells.

METHODS

Design of study

This study was of posttest randomized control group design. The experimental animals were kept at the Experimental Laboratory, Department of Pathologic Anatomy, Faculty of Medicine, University of Indonesia. The assays for apoptosis were performed at the Pathologic Anatomy Laboratory, Dr.Sardjito Hospital/ Faculty of Medicine, Gadjah Mada University. The study was conducted from April to December 2012.

Calculation of sample size

The sample size was calculated according to Federer's formula, resulting in 6 animals per group or 24 animals for 4 groups.

The Federer's formula:

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

k = number of experimental group (4)

n = number of sample

Induction of mammary adenocarcinomas in C3H mice

The C3H mice were obtained from the Department of Pathologic Anatomy, Faculty of Medicine, University of Indonesia. A total of 24 tumor-free C3H mice aged 2-3 months and weighing 17-25 grams were prepared for transplantation of mammary adenocarcinoma. Three C3H mice with established mammary adenocarcinoma tumors around 1.5 cm in diameter were prepared as donors. The donor mice were terminated with ether, then dissected for collection of cancer tissue. The cancer tissue was minced to pulp in a petri dish, 5 mL of normal saline was added, and 0.2 mL of the cancer suspension was injected subcutaneously into each of the experimental animals. The site of injection was palpated daily to determine tumor growth. On the seventh day the tumors were about the size of a pea, when the experimental animals were ready to receive interventions.

Preparation of *Andrographis paniculata* extract

This extract made from leaves of *Andrographis paniculata* plant processing in Lansida Laboratory Yogyakarta. The fresh leaves was dried in the fresh dryer at 30^o C. The dried leaves was blended in hammer mills, and then extracted by distilled water for about 24 hours. The filtrat of extract was evaporated in rotavapor at 40^oC to get powder form. The aqueous *Andrographis paniculata* leaf extract (AAPLE) powder was prepared in solution by added aquades as solvent.

Intervention

The 24 C3H mice with transplanted tumors were assigned by simple random sampling into 4 groups of 6 animals. One group as the control group did not receive *Andrographis paniculata* extract. The other three groups as the intervention groups were given *Andrographis paniculata* extract at doses of 5, 10 and 15 mg per animal per day. The oral dose was based on the study by Sheeja et al., who used *Andrographis paniculata* extract at 10 mg/animal/day to determine its antiangiogenic effects.⁽¹⁸⁾ Administration of the *Andrographis paniculata* extract was performed by means of a gavage tube. The *Andrographis paniculata* extract was given in the form of a solution in distilled water, once daily for 14 days. On the fifteenth day the mice were terminated with ether. The cancer tissue specimens were formed into paraffin blocks for apoptosis testing by means of the terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) method. During experimental periodes the mice were given animal feed pellets and drinking water *ad libitum*.

Apoptosis assay by the TUNEL method

TUNEL is one of the methods for detecting apoptosis by examining for DNA fragmentation. It is based on the presence of nicks in DNA, which can be identified by terminal deoxyribonucleotidyl transferase. This is an enzyme catalyzing the addition of labelled dUTP, which will label cells with damaged DNA.

In this study, apoptosis in cancer tissue was detected by means of the Apo-BrdU-IHC in situ DNA fragmentation assay kit (BioVision K403). The paraffin sections were deparaffinized and rehydrated. Proteins were digested with 100 μ l proteinase K solution for 20 minutes at room temperature. Proteinase activity is blocked with 100 μ l of 3% H₂O₂, then incubated at room temperature for 5 minutes. After a rinse in phosphate buffered saline (PBS), the specimens were flooded with 100 μ l of dH₂O reagent buffer, then incubated for 10-30 minutes at room

temperature. The reagent buffer is carefully blotted once, then 50 μ l of labelling reagent mixture is added. The specimens were covered with thin paraffin sheets of wider dimension than the specimens and incubated at 37°C for 1 to 1.5 hour. The paraffin cover was removed and the slides were rinsed in PBS. All specimens were covered with 100 μ l blocking buffer then incubated at room temperature for 10 minutes. After blotting the blocking buffer, the specimens were covered with 100 μ l antibody solution. The antibody solution was prepared for 25 slides by dissolving 125 μ l Anti-BrdU-Biotin in 2,375 μ l of blocking buffer. The slides were covered with aluminum foil then incubated in a dark room for 1-5 hours at room temperature, after which the slides were rinsed in PBS. All specimens were then covered with 100 μ l blocking buffer, counterstained with 100 μ l methyl green, and incubated at room temperature for 3 minutes. The slides were twice immersed in 100% ethanol, blotted with absorbent tissue and immersed in xylene. The excess xylene under the slides and around the specimens was removed, then the slides were mounted and covered with a cover glass.

The apoptotic index was counted under a binocular microscope. The slides were examined at 40 x magnification for selection of 5 areas for reading of the results. Each area was examined up to a total of 200 cancer cells at 400 x magnification. Examination of the apoptotic index was done by counting positive cells among 1000 cancer cells in the 5 reading areas.⁽¹⁹⁾

Ethical clearance

Ethical clearance was obtained from the Commission on Medical and Health Research Ethics, Medical Faculty Dr.Kariadi General Hc

Data analysis

For each group, deviation of apoptotic

Test of normality of the data was performed with the Shapiro-Wilk test and homogeneity of data was by means of the Lavene test. Differences in apoptotic index in each study group was evaluated by ANOVA, followed by an LSD post hoc to test for differences in between-group means. The level of significance was set at 0.05.

RESULTS

The apoptotic cells were identified by their brown-colored nuclear region (Figure 1). The lowest number of apoptotic cells was in the control group (A) and the highest in the group receiving 15 mg/day of *Andrographis paniculata* extract (D). The test of normality and homogeneity showed that the data were normally distributed and homogenous. The collected apoptotic indices are presented in Table 1. The highest apoptotic index was obtained for the group receiving *Andrographis paniculata* extract at 15 mg/day, and the lowest for the control group. The analysis was then followed by ANOVA to test for differences between groups.

The results of ANOVA showed significant differences in the means of the apoptotic indices between the four groups ($p=0.001$). The results of LSD post hoc shows significant between-group differences in apoptotic indices for all study groups. The Pearson's correlation test showed a very strong correlation between dose of *Andrographis paniculata* extract and apoptotic index ($r=0.974$) (data not shown).

Tabel 1. Mean apoptotic indices and results of ANOVA

Group	Mean \pm SD	ANOVA *
Control	0.88 \pm 0.183	p=0.000
AP 5 mg	1.37 \pm 0.150	
AP 10 mg	2.03 \pm 0.121	
AP 15 mg	2.53 \pm 0.150	

*Significance level 0.05

AP = *Andrographis paniculata*

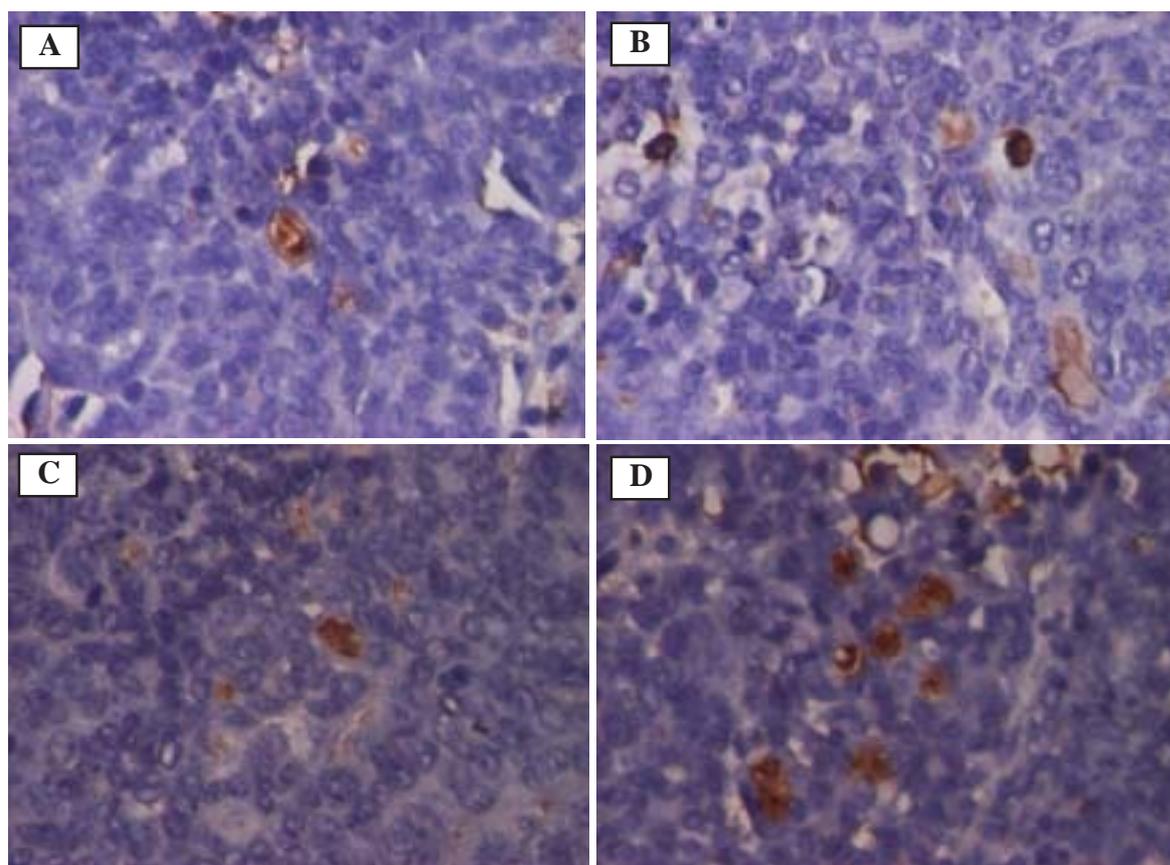


Figure 1. Apoptosis in mammary adenocarcinoma cells without *Andrographis paniculata* extract (A) and after administration of *Andrographis paniculata* extract at doses of 5 mg/day (B), 10 mg/day (C), dan 15 mg/day (D) in the TUNEL assay

DISCUSSION

The results of this study indicated that administration of *Andrographis paniculata* extract to C3H mice was able to induce apoptosis in cancer cells. The effect of apoptosis was already apparent in the group on *Andrographis paniculata* extract of 5 mg/day, showing a significant difference with the control group not receiving the extract. There was more apoptosis in the group receiving the extract at 10 mg/day in comparison with the group receiving the extract at 5 mg/day. Similarly there was more apoptosis in the group receiving the extract at 15 mg/day in comparison with the groups receiving 5 and 10 mg/day. This indicates that apoptosis increased proportionally with increasing dose of *Andrographis paniculata*

extract. Apoptosis can be detected by various methods, such as by identification of proteins involved in apoptosis, identification of apoptosis-regulating genes, or identification of apoptotic cells.⁽²⁰⁾

These results are consistent with those of previous in vitro studies, which demonstrated that *Andrographis paniculata* extract increased apoptosis of C3H mouse mammary adenocarcinoma cells cultured in RPMI-1640 medium⁽¹⁴⁾ and that of HeLa cells.⁽¹²⁾ The results of the present study show that oral administration of *Andrographis paniculata* extract is capable of increasing apoptosis of mammary adenocarcinoma cells after undergoing the processes of absorption, distribution, metabolism, and excretion (ADME). Systemic absorption of a preparation or a drug

from the gastrointestinal tract or other absorption sites is dependent on the physicochemical characteristics and form of the preparation, and on the anatomical and physiological features of the absorption site. Factors such as surface area of the gastrointestinal tract, gastric emptying time, gastrointestinal motility, drug metabolism by the intestinal microflora, and blood supply of the absorption site, may all influence the absorption rate and the amounts absorbed.^(21,22) Absorption and distribution of a preparation or a drug throughout the body takes place via several mechanisms, via passive diffusion, transport by convection, active transport, facilitated transport, ion pair transport, and pinocytosis. On arriving in the blood stream, a large fraction of the preparation is bound to plasma proteins and only a small fraction remains unbound and is called the free drug fraction, the form in which the drug is distributed to all tissues. Drug distribution occurs by diffusion or transport from the blood stream into the interstitial spaces and the cells in tissues and organs. Drug distribution is not always uniform throughout the tissues, because it depends on several factors, such as the physicochemical characteristics of the drug, the ratio of the protein- or biomaterial-bound fraction of the drug in the blood and that in the tissues, vascularization and blood velocity in the tissues, the chemical characteristics of the tissues, and the presence of carrier proteins (transporter) in the tissues. Another factor affecting dose variations in the blood and tissues is the quality of the subject, which in turn is affected by intrinsic and extrinsic factors. Therefore, administration of the same preparation or substance to an individual, using an identical dose and identical route of administration, may at different time points result in different drug concentration profiles in the blood.⁽²¹⁾

On oral administration of *Andrographis paniculata* extract to rats, the preparation reaches its peak plasma concentration in the relatively short time of around 2 hours and starts to diminish after three hours.⁽²³⁾ Orally

consumed andrographolide accumulates in visceral organs, including the brain, kidneys, heart and lungs. A major fraction of andrographolide is bound to plasma proteins and only a small fraction enters the cells. Around 80% of the absorbed andrographolide is rapidly excreted from the body in 8 hours, and 90% in 48 hours.⁽²⁴⁾

The diterpenoids from *Andrographis paniculata* increase apoptosis through induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is one of the important types of tumor necrosis factor (TNF) having a high potential in cancer therapy. *Andrographis paniculata* extract sensitizes cancer cells to TRAIL-induced apoptosis and increases TRAIL-induced apoptosis mainly by promoting caspase 8 activation. The activated caspase 8 promotes FLICE-like inhibitory protein (FLIP) cleavage and downregulates inhibitor of apoptosis proteins (IAP).⁽²⁵⁾ The abovementioned sensitization of cancer cells to TRAIL-induced apoptosis occurs through p53-dependent transcriptional regulation resulting in upregulation of death receptors 4 (DR4). *Andrographis paniculata* extract stabilizes and activates p53 functions through activation of reactive oxygen species (ROS)-dependent cJun N-terminal kinase (JNK).

Apoptosis occurs because of the presence of extrinsic and intrinsic signals. Apoptosis in cancer cells does not proceed as in normal cells, because of systemic abnormalities resulting in inhibition of proapoptotic gene expression. The apoptotic process requires adenosine triphosphate (ATP) as cofactor. ATP breakdown produces energy that is used for releasing cytochrome-c from the mitochondria. ATP is obtained from nutrients that are transported through blood vessels in cancer tissue. If no ATP is available, the cell may proceed to necrosis, inducing an inflammatory reaction.

The andrographolide contained in *Andrographis paniculata* extract may induce apoptosis through intrinsic as well as extrinsic

pathways. It can initiate apoptosis through p53, which functions primarily in the intrinsic pathway.^(13,25) The presence of an intrinsic signal releases bound apoptosis activating factor 1 (Apaf-1) from Bcl-2. Apaf-1 then complexes with cytochrome-c and forms an apoptosome with caspase 9 and ATP. This apoptosome complex activates pro-caspase 3 to become caspase 3, which is the effector of the caspase cascade.

Andrographolide may also act on the extrinsic apoptotic pathway, thereby activating caspase 8, which in turn activates caspase 3 as the effector caspase. The extrinsic signal is formed by complementary binding of apoptosis stimulating fragment (Fas) and TNF which form the death activator.⁽²⁶⁾ The signal from the death activator is transmitted to the cytoplasm, where it activates caspase 8. The latter initiates the caspase pathway, resulting in the caspase cascade and apoptosis. Activation of caspase 8 also causes Bcl-2-interacting domain (Bid) to become active and initiate the apoptotic program through increased activity of the pro-apoptotic proteins Bcl-2-associated X-protein (Bax) and Bak. One limitation of this study was the absence of a positive control group receiving a cytostatic drug.

CONCLUSIONS

Oral administration of *Andrographis paniculata* extract induces apoptosis of mammary adenocarcinoma cells in C3H mice. A dose of 15 mg/day yielded the best results in this study, but it cannot as yet be determined if this is the optimal dose.

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