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Original Article

THE ROLE OF ETHYL ACETATE FRACTION OF ANDROGRAPHIS PANICULATA AND DOXORUBICIN COMBINATION TOWARD THE INCREASE OF APOPTOSIS AND DECREASE OF VEGF PROTEIN EXPRESSION OF MICE FIBROSARCOMA CELLS

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ABSTRACT

Objective: The objective of this study is to prove the role of an ethyl acetate fraction of *Andrographis paniculata* and doxorubicin combination toward the increase of apoptosis and decrease of VEGF protein expression of mice fibrosarcoma cells.

Methods: The mice were injected subcutaneously on the scapular with benzo(a)pyrene once every 2 days for 10 days to induce fibrosarcoma and after 3 months they were randomly divided into four groups, consisting of: negative control, ethyl acetate fraction of *A. paniculata* group, doxorubicin group, and combination of ethyl acetate fraction of *A. paniculata* and doxorubicin group. By immunohistochemistry preparation, fibrosarcoma cells were scored for apoptosis and VEGF protein expression.

Results: Ethyl acetate fraction of *A. paniculata* and doxorubicin combination significantly ($p<0.05$) increase apoptosis. Ethyl acetate fraction of *A. paniculata* and doxorubicin combination also significantly ($p<0.05$) decrease VEGF protein expression.

Conclusion: It can be concluded that ethyl acetate fraction of *A. paniculata* and doxorubicin combination increase apoptosis and decrease VEGF protein expression of mice fibrosarcoma cells. Decrease of VEGF protein expression led to an inhibition of tumor growth, tumor neovascularization, decrease in proliferation of tumor cells and the formation of necrosis.

Keywords: *Andrographis paniculata*, Doxorubicin, Apoptosis, VEGF, Fibrosarcoma, Benzo(a)pyrene.

INTRODUCTION

Conventional cancer therapies, including surgery, chemotherapy, and radiotherapy, as single modalities have a limited but important role in the overall treatment of most solid tumors. Thus, the strategies of cancer treatment using combined therapies or combined agents with distinct molecular mechanisms are considered more promising for higher efficacy, resulting in a better survival.

Andrographolide is an active compound from *A. paniculata* (King of Bitters) [1] an important herbal medicine used in Asia to treat a range of diseases, such as respiratory infection, fever, bacterial dysentery and diarrhea [2-4]. It also has been studied in patients with HIV [5]. The major bioactive component extracted from *A. paniculata* is andrographolide and the three hydroxyls at C-3, C-19 and C-14 are responsible for its biological activity [6].

Andrographolide has been recognized as a cancer chemopreventive agents because of their anticarcinogenic activity [7]. Moreover, these compounds also exert the antitumor activities through regulation of different cell signaling pathways. Therefore, common cancer therapies combined with these dietary compounds may exert enhanced antitumor activity through synergic action. Andrographolide enhances the sensitivity of cancer cells to doxorubicin mainly via STAT3 suppression [8]. Andrographolide and Doxorubicin combination has anticancer activity in HeLa cell lines [9]. The combination treatment may also decrease the systemic toxicity caused by chemotherapies or radiotherapies because lower doses could be used.

Those mechanism, gave an idea of using these compounds as a combination for mice fibrosarcoma treatment. We used apoptosis examination and VEGF immunohistochemical to prove the role of ethyl acetate fraction of *A. paniculata* and doxorubicin combination toward the increase of apoptosis and decrease of VEGF protein expression of mice fibrosarcoma cells, to give a brief view on the new and emerging field for optimal treatment of cancer with better survival.

MATERIALS AND METHODS

Plant Material

A. paniculata herbs was collected from Mojokerto, East Java area, and was authenticated by the Pharmacognocy and Phytochemistry Department of Faculty of Pharmacy, Airlangga University Surabaya, Indonesia.

Preparation of Ethyl acetic fraction of *A. paniculata* herbs

500 g of the dried and powdered herbs of *A. paniculata* was extracted in 2L of 96% (v/v) ethanol for 24 h and the extract was centrifuged for 15 min at 4000 rpm. Supernatant was taken as ethanolic extract *A. paniculata* herbs and was separated using ethyl acetate and water. The ethyl acetate was removed with a rotary evaporator at reduce temperature to obtain a solid mass of fraction. The concentrated fraction was then added with Avicel: Cab-O-Sil (4:1) for treatment. *A. paniculata* fraction were lyophilized at 40-50 ^{o}C and kept at room temperature for further use.

Drug

Doxorubicin from Ebewe (vial 10 mg/5 ml) purchased from PT. Kalbe Farma (Surabaya, Indonesia) was diluted directly with Normal Saline.

Animals and study design

Thirty two Balb/c mice (*Mus musculus*), (4-6 weeks old, 20-30 g) were obtained from the animal house of Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia. The animals were housed in a temperature-controlled environment (23-25°C), kept under laboratory conditions in plastic cage (47×34×18 cm^3) with sawdust (renewed every 48 h) and free acces to food and water. All the animal experiments were conducted according to the rules and regulations of Animals Care and Use Committee, Faculty of Veterinary Medicine, Airlangga University, Indonesia.

The animals were quarantined for one week before starting the experiment. Benzo(a) pyrene (B [a]P) as carcinogen was obtained

from Sigma Aldrich Chemical Company. It was dissolved in oleum olivarum (0.3%) and was prepared just before the use. Fibrosarcoma's carcinogenesis was produced by the induction of 0, 3% B [a]P subcutaneously into mice once in every two concecutive days. The carcinogenesis was observed for three months. Then, they were randomized and divided into four groups and continually treated with 0.5% CMC-Na suspension p. o (negative control group); ethyl acetate fraction of *A. paniculata* (594.80 mg/kg bw/day p. o) for 15 days (FDLS group); doxorubicin (1.2 mg /kg bw i. p) in day 1 (DOX group); combination of ethyl acetate fraction of *A. paniculata* (594.80 mg/kg bw/day) for 15 days and doxorubicin i. p (0.6 mg /kg bw i. p) in day 1 (KOMB group). At the end of the experiment, all mices were sacrificed by cervical dislocation. Fibrosarcomas tissue were collected for further immunohistochemistry analysis. Samples were then fixed in neutral buffered 10% formalin, processed for apoptosis examination using Apo-BrdU-IHC DNA Fragmentation Assay Kit (BioVision) and immunohistochemical analysis using VEGF immunolabeling.

TUNEL assay

The apoptotic index was determined by counting 200 cells in 10 selected areas and averaging numbers of positive cells (brown staining in nucleus) using the TUNEL assay in ten 400× fields.

Immunohistochemical staining for VEGF

The immunostaining for VEGF was performed using *Vascular Endothelial Growth Factor* antibody MAB293 (Santa Cruz). Positive cells expressing VEGF were identified by a brown stain on cytoplasmic of fibrosarcomas cell. Expression was evaluated according to a modified semiquantitative IRS scale of Remmele [10]. The method takes into account both percentage of positive cells and intensiveness of colour and the final score represents product of the parameters and ranges from 0 to 12 pts (no reaction-0 pts (-); poor reaction-1-2 pts (+), moderate reaction-3-4 pts (++), intense reaction-6-12 pts (+++).

Statistical analysis

Values were expressed as mean±SD. Apoptotic index was analyzed using ANOVA followed with Tukey test. Evaluation of VEGF protein expression were analyzed with Kruskall-Wallis followed with Mann-Whitney test using the SPSS 16.0 software. The accepted level of significance was $p<0.05$.

RESULTS

Apoptotic index

The result of apoptosis examination showed positive apoptotic cells (brown colour in the nuclear) of mice fibrosarcoma cells (fig. 1). Administration of an ethyl acetate fraction of *A. paniculata* and doxorubicin combination increase apoptosis (fig. 2).

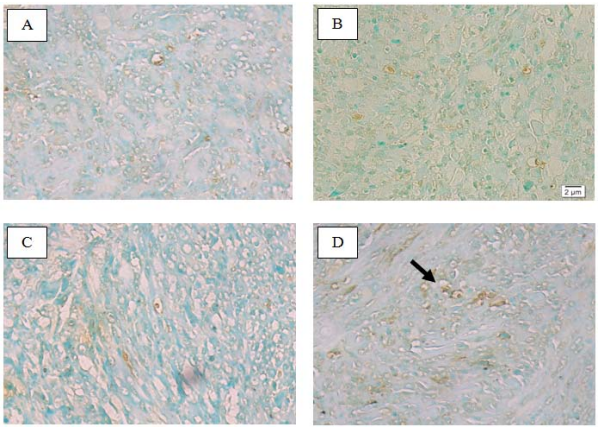

Fig. 1: These shows immunohistochemical images (x400) of apoptosis (Black arrow) in mice fibrosarcoma cells (A) Negative control group; (B) Ethyl Acetate Fraction of *A. paniculata* group; (C) Doxorubicin group; (D) Ethyl Acetate Fraction of *A. paniculata* and Doxorubicin combination group

There is a statistically significant difference (P<0.05) present between combination group (7.38±1.42) and the other groups regarding the apoptotic index score, but there is no significant difference (P>0.05) between negative control group (5.12±0.87) and DOX group (5.63±1.04). Scores mean of apoptotic index of mice fibrosarcoma cells is shown in table 1.

Table 1: It shows scores mean of apoptotic index of mice fibrosarcoma cells

Groups	Scores mean±SD
Negative control	5.12±0.87
Ethyl acetate fraction of *A. paniculata*	6.47±1.00
Doxorubicin	5.63±1.04
Ethyl acetate fraction of *A. paniculata* and doxorubicin combination	7.38±1.42

n = 6, *p<0.05

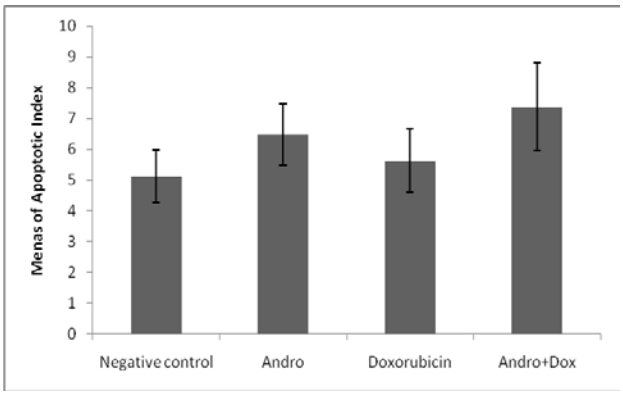

Fig. 2: It shows scores mean of apoptotic index of mice fibrosarcoma cells. Data were expressed as mean±SD of each group (n = 6 per group)

Immunohistochemical of VEGF Protein expression

The result of VEGF protein immunostaining showed positive immunoreaction (brown colour on the cytoplasmic) of mice fibrosarcoma cells (fig. 3). Administration of ethyl acetate fraction of *A. paniculata* and doxorubicin combination decrease VEGF protein expression (fig. 4). There is statistically significant difference (P<0.05) present between negative control group (5.83±1.67) and the other groups regarding the VEGF protein expression, but there is no significant difference (P>0.05) between FDLS group (4.65±1.36) and DOX group (5.15±1.42).). Scores mean of VEGF protein expression of mice fibrosarcoma cells is shown in table 2.

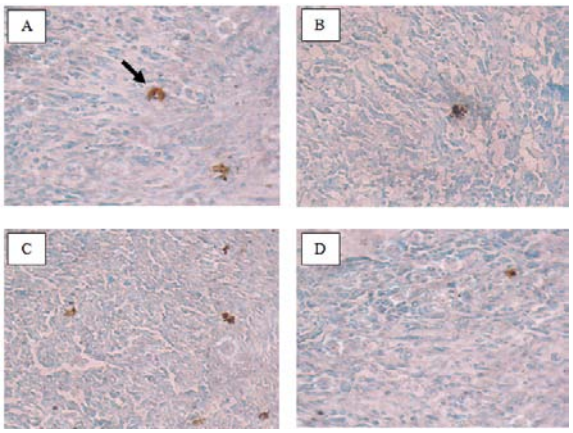

Fig. 3: These shows immunohistochemical images (x400) of VEGF protein staining (Black arrow) in mice fibrosarcoma cells (A) Negative control group; (B) Ethyl Acetate Fraction of *A. paniculata* group; (C) Doxorubicin group; (D) Ethyl Acetate Fraction of *A. paniculata* and Doxorubicin combination group

Table 2: It shows scores mean of VEGF protein expression of mice fibrosarcoma cells

Groups	Scores mean±SD
Negative control	5.83±1.67
Ethyl acetate fraction of *A. paniculata*	4.65±1.36
Doxorubicin	5.15±1.42
Ethyl acetate fraction of *A. paniculata* and doxorubicin combination	3.90±1.23

n = 6, *p<0.05

The result showed that administration of ethyl acetate fraction of *A. paniculata* and doxorubicin combination decrease VEGF protein expression of mice fibrosarcoma cells.

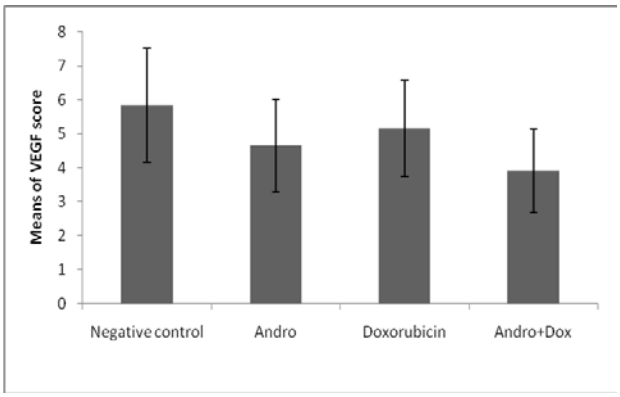

Fig. 4: It shows scores mean of VEGF protein expression of mice fibrosarcoma cells. Data were expressed as mean±SD of each group (n = 6 per group)

DISCUSSION

Apoptosis is an evolutionarily conserved mechanism for the selective removal of unwanted cells [11]. Regulation of apoptosis is critical for tissue homeostasis, therefore, its deregulation can lead to a variety of pathological conditions, including cancer. Apoptosis is primarily mediated thorugh the activation of specific proteases called caspases [12]. Cells undergoing apoptosis undergo cell membrane remodeling and blebbing, cell shrinkage with cytoskeletal rearrangement, nuclear condensation and DNA fragmentation [13].

In the present study, fibrosarcoma cells were treated with ethyl acetate fraction of *A. paniculata* and doxorubicin combination and apoptosis were measured to investigate the effects of this combination. The results demonstrated that the rate of apoptosis was increased in fibrosarcoma cells treated with ethyl acetate fraction of *A. paniculata* and doxorubicin combination, indicating that ethyl acetate fraction of *A. paniculata* and doxorubicin combination was able to induce apoptosis.

A research conducted by [14] showed that doxorubicin induced cell death of HT1080 fibrosarcoma cells mainly by regulating the abundance of factors mediating the mitochondrial (intrinsic) apoptosis pathway. Furthermore doxorubicin influences other pathways and crosstalk to other pathways (including to the death receptor pathway) at multiple levels. Doxorubicin increase levels of cytochrome c, APAF-1 and members of the STAT-family (STAT1, STAT3), while Bcl-2 expression was decreased. Caspase-1, -3, -6, -8, and-9 were increase indicating that these proteases are the key factors in the execution of doxorubicin mediated apoptosis.

Andrographolide activate the extrinsic death receptor pathway (including caspase-3 and caspase-8) and induce apoptotic cell death in certain human cancer cell types [15] (including cervical, breast and hepatoma cell lines) [16]. A recent work demonstrates that tumor necrosis factor-α (TNF-α) related apoptosis inducing ligand (TRAIL-an important member of extrinsic apoptosis pathway) was significantly enhanced in various human cancer cell lines after treatment with andrographolide [17]. Studies have demonstrated that andrographolide is also effective in combination therapy. Andrographolide increased the apoptosis rate in multidrug resistant cancer cells (colorectal cancer cell line), when used in combination treatment along with other anticancer agents like 5-florouracil (5-FU), adriamycin (doxorubicin) and cisplatin [18].

The results above showed that administration of ethyl acetate fraction of *A. paniculata* and doxorubicin combination increase apoptosis of mice fibrosarcoma cells.

Tumors require angiogenesis to grow beyond 1 to 2 mm^3 [19] in size and to facilitate metastasis [20]. VEGF is expressed in most tumours and its expression correlates with tumour progression. The expression of VEGF mRNA is highest in hypoxic tumour cells adjacent to necrotic areas [16], indicating that the induction of VEGF by hypoxia in growing tumours can change the balance of inhibitors and activators of angiogenesis, leading to the growth of new blood vessels into tumour [21-22].

The results demonstrated that the VEGF protein expression was decreased in fiborsarcoma cells treated with ethyl acetate fraction of *A. paniculata* and doxorubicin combination, indicating that the combination was able to decrease VEGF protein expression. Doxorubicin blocked HIF-1α-mediated upregulation of target genes, and when combined with anti-VEGF therapy has a synergistic effect in blocking sarcoma tumor growth [23]. Andrographolide inhibited tumor specific angiogenesis by down regulating various proangiogenic molecules such as VEGF, NO and proinflammatory cytokines and upregulating antiangiogenic molecules like IL-2 and TIMP-1 and may be exploited to prevent tumor growth and metastasis [24].

Decrease of VEGF protein expression led to an inhibition of tumor growth, tumor neovascularization, decrease in proliferation of tumor cells and the formation of necrosis [25].

CONCLUSION

Ethyl acetate fraction of *A. paniculata* and doxorubicin combination increase apoptosis and decrease VEGF protein expression of mice fibrosarcoma cells. These results suggest that the decrease of VEGF protein expression inhibits tumor neovascularization, which is then follow by the reduction of tumor cell proliferation due to the starving conditions and result in an increase of tumor cell apoptosis and tumor necrosis.

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CONFLICT OF INTERESTS

Declared None

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