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Research Article

Effect of Cowpea Extract on PCNA Expression and BAX/BCL2 Ratio in Mice Epithelial Cells Menopause Model

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Abstract

Objective: In biology, breast cancer has two main biological factors - proliferation and apoptosis. Currently, therapeutic agents focus on the regulation of these factors that can manage and prevent progression of the cancer. However, current drugs provide not only therapeutic benefits but also serious side effects that eventually adds burden to the patient. With this, medicinal plants were explored as a potential source of therapeutic agents such as Cowpea that are abundant in Indonesia that showed a potential regulation of the progression of breast cancer. To evaluate the effects of Cowpea extract in the two biological factors of breast cancer, PCNA expression and ratio of BAX/BCL2 were measured in mice epithelial cells menopause model.

Design: Prospective controlled experimental trial.

Setting: University medical center.

Animal: *Rattus norvegicus* Strain Wistar.

Intervention: The experimental mice oovorectomy were divided into 3 groups: Group I: the control group; no treatment was given and experimental mice Group II: experimental mice given Cowpea extract (15 mg/kgBW/day), Group III: experimental mice given cowpea extract (60 mg/kgBW/day).

Main Outcome Measurement: Histopathological assessment.

Result: The effect of Cowpea extract with two dose-response showed a significant difference between the control group (85 ± 13.87a) and the test groups, group 2 (61 ± 6.67b) and group 3 (47.8 ± 6.14c) (p = 0.00) in PCNA expression. This indicates that the test groups, both the two different doses significantly decrease the PCNA expression in female rats. In BAX/BCL2 expression the control group (0.32 ± 0.18a and group 2 (0.56 ± 0.50a), group 3 (1.33 ± 0.78b). This indicates that the test groups, both the two different doses significantly decreases the BAC/BCLA2 expression in female rats.

Conclusion: The remarkable effects of Cowpea extract on the inhibition of PCNA expression and upregulation of BAX/BCL2 will served as a good platform for pharmaceutical and medical industries to explore Cowpea which is abundant in Indonesia as a source of effective therapeutic treatment for breast cancer.

Keywords: *Cowpea Extract; PCNA; BAX/BCL2 Ratio; Mice Epithelial Cells*

Introduction

Breast cancer is the most frequent cancer among women with a mortality rate 22.9% worldwide. In Indonesia, breast cancer ranks second after cervical cancer. Moreover, the Dharmais cancer hospital reports that there is a 2.3% increase/year of breast cancer morbidity.

Breast cancer, in biology, have two main biological factors that influence its occurrence - proliferation and apoptosis. In diagnosis, proliferation is often done by measuring Proliferating Cell Nuclear Antigen (PCNA) which is a cell nucleus protein. The PCNA measurement of proliferation has an inverse relationship to apoptosis; the lower the PCNA score the higher the apoptosis will occur. On the other hand, the most commonly used apoptosis marker is the ratio of pro-apoptotic members BAX/B-Cell Lymphoma2 (BAX/Bcl2). BAX is a factor that triggers BCL2 as an inhibiting factor, the more it increases, the more apoptosis will occur [1,2].

Notably, physiological condition such as menopause also influences the occurrence of breast cancer; as menopause women have more pronounced incidence of breast cancer [3]. At menopausal stage various short-term and long-term problems will emerge as a result of decrease in estrogen termed as hypo estrogenemia. This condition can be managed by administering hormone replacement therapy (HRT). Moreover, the hormone replacement therapy has remarkable benefits in decreasing the risk of colorectal cancer and osteoporosis fractures. However, prolonged use HRTs increases the risk of cardiovascular disease with and breast cancer [4]. With this serious side effects of HRTs, ethnobotanical resources such as plants are explored as a potent source of estrogen or its derivatives. There were existing studies that provide evidence of high content of estrogen and its derivatives in Bitok (*Pueraria lobata*) and Cowpea (*Vigna Unguiculata*) which are abundantly found in Indonesia. However, there were no dose-response studies were made using animal models to screen the potential effects of these plants as a natural source of estrogen. With this, the study utilized Cowpea as a source of phytoestrogen and to possibly elucidate its mechanism of action on proliferation and apoptosis pathways. The goal of the study is to determine the potential of Cowpea as an alternative natural source of phytoestrogen and to explore its possible anti-cancer activity using PCNA expression and BAX/BCL-2 ration in mice menopause model.

Material and Methods

Cowpea Extract preparation and Administration

The cowpea extract is dissolved with aquabidest to a total volume of 5 mL. Given only once a day. Special foods for mice are given as much as 40 grams/day/1 mouse. The cowpea extract was given a gastric dose (inserted), carried out for 21 days at a dose according to the group.

Mice menopause model

The rat model of *Rattus norvegicus* Strain Wistar hypoestrogen were house at the Pharmacology Laboratory of the Faculty of Medicine, Universitas Brawijaya. The study followed a strict inclusion and exclusion criteria as follows: Adult (8 - 9 weeks), with a weight between 300-350 grams, female mice, not pregnant, and apparently healthy (active, pure white fur, bright eyes and not deformed). Experimental mice died during the experiment were excluded.

Briefly, the experimental mice were divided into 3 groups:

- **Group I:** The control group; no treatment was given and experimental mice.
- **Group II:** Experimental mice given Cowpea extract (15 mg/kgBW/day).
- **Group III:** Experimental mice given cowpea extract (60 mg/kgBW/day).

Mouse ovariectomy

The body weight of the mice was weighed, anesthetized with an i.m meter dose of 10 mg/kg Fur. The experimental rats were laid on the operating table, sterilization of the operating area with betadine and 70% alcohol, then covered with sterile duk. The operating area (abdomen) is shaved, and a trans-abdominal incision of 1.5 - 2 cm is made above the uterus. Binding is done in 2 places, which are proximal and distal to the ovary, then followed by removal of the left and right ovary. During the search process and removal of the ovary the humidity of other organs must be maintained by dripping with physiological fluids. Before suturing, nebacetin powder is spread into the abdominal cavity. After the wound is sewn and closed, the mice are inserted into the cage. Each cage contains only one mouse. Days I,

II, and III after ovariectomy, Gentamicin i.m was injected at a dose of 60 - 80 mg/kgBW/day. During maintenance, adequate drinking and food are given, light is set light/dark alternately for 12 hours and at room temperature.

Sample preparation

Rat breast tissue was soaked in fixative solution in the form of formalin or PFA (1 - 7 days), then immersed in 70% ethanol at least 24 hours and continued with 80% ethanol for 2 hours. Standard tissue processing was done to all rat breast tissue samples.

Immunohistochemistry

Deparaffinization and Rehydration were performed in preparation of immunohistochemistry using xylol and multilevel alcohol (100%, 90%, 80%, 70%, 30%), and distilled water in sequence. After, cell blocking of the tissues were made. For immunohistochemistry Primary Antibodies in Serum Goat/FBS/BSA to desired concentration and volume (Leptin Antibody 1: 500 in serum Goat or FBS) were used. Cell incubation in primary antibodies at 4°C for 12 hours or at room temperature for 2 hours. Secondary Antibodies (Gout-Anti Rabbit IgG Biotin Labeled) were then utilized labeled biotin in PBS to the desired concentration and volume (Anti Rabbit IgG Labeled Biotin 1: 500 in PBS). Counterstaining using with Mayer's hematoxylin for 10 minutes were used for visualization.

Ethical considerations

This research was approved by the research ethics committee in the health field of the Faculty of Medicine, University of Brawijaya Malang.

Statistical analysis

Data were analyzed using one way ANOVA and MANOVA tests, to test the relationship of cowpea extract and the number of post-menopausal β estrogen receptors in the five treatments. The calculation process is carried out with the help of the SPSS computer software program with the alpha of 0.05.

Result and Discussion

Cowpea extract effect on PCNA expression

The effect of Cowpea extract with two dose-response showed a significant difference between the control group and the test groups ($p = 0.00$) in PCNA expression. This indicates that the test groups, both the two different doses significantly decrease the PCNA expression in female rats (Table 1).

Groups	n	Mean±SD	*p* value
Group I: Control (OVX)	5	85 ± 13.87^{a}	0.000
Group II: OVX + Cowpea ext 15 mg/kg/body/day	5	61 ± 6.67^{b}	
Group III: OVX + Cowpea ext 60 mg/kg/body/day	5	47.8 ± 6.14^{c}	

Table 1: *Testing the significant difference of control group and test groups in PCNA expression in female rats*

Description: The mean ± SD column containing the same letters indicates none significant difference ($p > 0.05$), when loading different letters indicates there is a significant difference ($p \leq 0.05$).

Remarkably, the cowpea dosage 15 mg/kg/body/day and dosage 60 mg/kg/body/day showed a significant difference; this shows a decrease in PCNA expression depending on the dosage of cowpea. Therefore, the dose-response relationship of the Cowpea extract and the expression of PCNA has positive inverse relationship. The higher the dosage of cowpea given, the lower the PCNA expression.

In clinical interpretation, it may decrease the proliferation of the tumor size. The mechanism behind this relationship can be explained with the study of Basu and colleagues [5]. In their study, the phytoestrogen showed a potent anti-proliferative effect [5]. However, this claim is still not well-established, leaving phytoestrogen as a potential chemopreventive agent for breast cancer [6], or trigger [7]. Thus, with the result of this study, this good be a good platform to pharmaceutical industries to explore the potentials of Cowpea as a potent chemoprotective agent.

Histologic effect of cowpea extract on PCNA mice epithelial cells

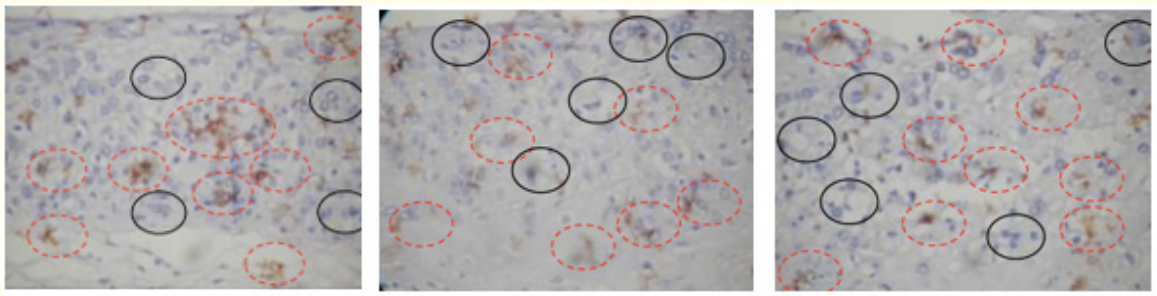

Plate 1: *Control Group.* ***Plate 2:*** *Group II (15 mg/kgbw).* ***Plate 3:*** *Group III (60 mg/kgbw).*

**Legend: Red-broken line: Cell target; Black-solid line: Normal cells.*

Cowpea extract effect on BAX/BCL2 expression

The effect of Cowpea extract with two dose-response showed a significant difference between the control group and the test groups ($p = 0.03$) in BAX/BCLA2 expression. This indicates that the test groups, both the two different doses significantly decrease the BAC/BCLA2 expression in female rats (Table 2).

Groups	N	Means ± stand.devs	*p*-value
Control (OVX)	5	0.32 ± 0.18^{a}	0.039
Group II: OVX + Cowpea ext 15 mg/kg/body/day	5	0.56 ± 0.50^{a}	
Group III: OVX + Cowpea ext 60 mg/kg/body/day	5	1.33 ± 0.78^{b}	

Table 2: *Testing the significant difference of control group and test groups in BAX/BCL2 expression in female rats.*

Description: The mean ± stand.devs column containing the same letters indicates none significant difference (p> 0.05), when loading different letters indicates there is a significant difference (p ≤ 0.05).

Table 2 shows the higher the Cowpea extract dose will significantly increase the BAX/BCL2 expression. Based on the average Bax/BCl2 ratio, it appears that the treatment effect shows an increase in the Bax/BCl2 ratio compared to the control group. This signifies that the dose concentration of the Cowpea extract influences the apoptosis pathway, therefore, inhibits progression of malignancies. The results indicated that the Cowpea extract-treated mice have upregulation effects to BAX/BCL2 ratio. This can be supported with the study of Tang and colleagues [8] were phytoestrogen induces apoptosis with increasing expression of BAX and decreasing expression of BCL2; the BAX/BCL2 ratio will increase accelerating the apoptosis process [8-10]. Although, in the current study, it was not elucidated the upregulation properties of Cowpea however, the results suggest that the Cowpea extract is a good candidate for an effective therapeutic agent for breast cancer with which to induce apoptosis in epithelial cells [11-13].

Histologic effect of cowpea extract on BAX/BCL2 mice epithelial cells

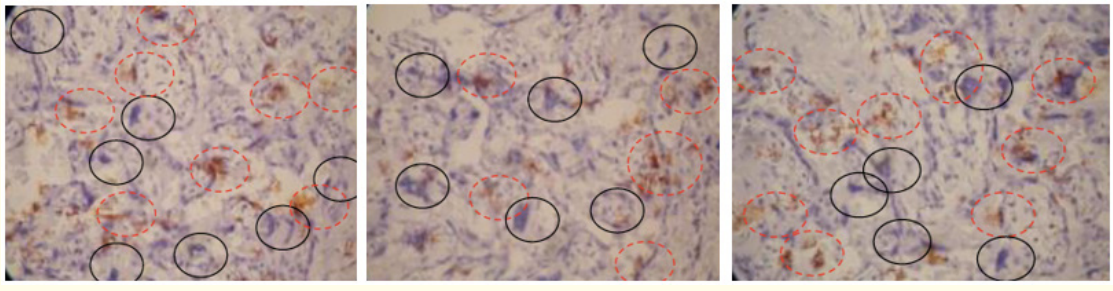

Plate 4: *Control Group.* ***Plate 5:*** *Group II (15 mg/kgbw).* ***Plate 6:*** *Group III (60 mg/kgbw).*

**Legend: Red-broken line: Cell target; Black-solid line: Normal cells.*

Histologic effect of cowpea extract on BCL2 mice epithelial cells

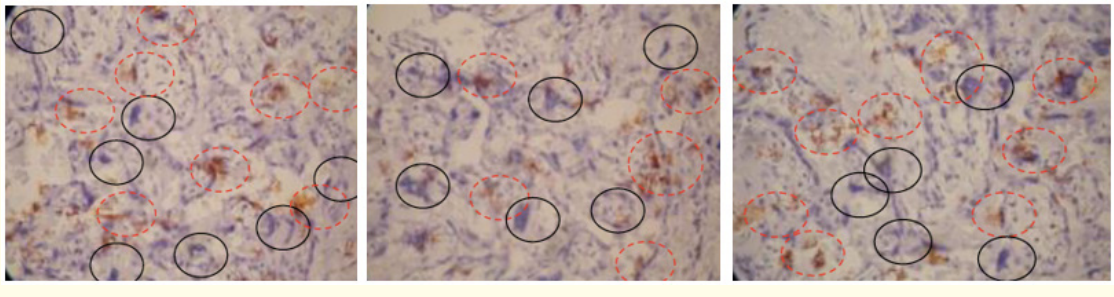

Plate 7: *Control Group.* ***Plate 8:*** *Group II (15 mg/kgbw).* ***Plate 9:*** *Group III (60 mg/kgbw).*

**Legend: Red-broken line: Cell target; Black-solid line: Normal cells.*

Conclusion

The remarkable effects of Cowpea extract on the inhibition of PCNA expression and upregulation of BAX/BCL2 will served as a good platform for pharmaceutical and medical industries to explore Cowpea which is abundant in Indonesia as a source of effective therapeutic treatment for breast cancer.

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