The Ethanol Extract of *Physalis angulata* Linn Inhibits COX-2 Activity in MCF-7 Cell *In Vitro*

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Abstract

Some studies showed that ceplukan *(*Physalis angulata *Linn) have cytotoxic effects toward HeLa (cervical cancer), KB (Nasopharyngeal), Colo 205 (colon), Calu (Lung) and MCF-7 cells* in vitro*. This plant has cytotoxic effects toward rat P388 lymphocytic leukemia* in vivo*. One of the mechanisms of cytotoxicity is the inhibition of Cyclooxygenase-2 (COX-2) pathway. The purpose of this study was to determine if 70% ethanol extract of* Physalis angulata *Linn inhibited COX-2 activity in MCF-7 cell. The cytotoxic effect of ethanol extract of* Physalis angulata *Linn toward MCF-7 cell was examined at concentration 20, 40, 80 and 160 µg/mL. Its estimated IC_{50} was used to test inhibition of COX-2 activity. After 24 hours of incubation, the inhibition of COX-2 activity was assayed by imunohistochemical staining with a monoclonal anti COX-2 antibody. COX-2 positive cells were counted using binocular microscope and IC_{50} for inhibition of COX-2 activity was calculated. The inhibition of COX-2 activity at 10, 20 and 40 µg/mL of the extract was 39.3%, 42.06% and 51.73%, respectively. The IC_{50} value of the ethanol extract of* P. angulata *Linn. for the inhibition of COX-2 activity was 37.57 ± 3.11 µg/mL, while the IC_{50} of celecoxib was 5.31 ± 0.27 µg/mL. The ethanol extract of* Physalis angulata *Linn. have an inhibitory effect on COX-2 activity in MCF-7 cell with IC_{50} 37.57 ± 3.11 µg/mL*

Key words: Physalis angulata *Linn, cyclooxygenase-2 (COX-2), MCF-7 cell line*

INTRODUCTION

In Indonesia, cancer is the sixth leading cause of death after infectious diseases, cardiovascular diseases, traffic accidents, malnutrition and congenital abnormalities (Tjindarbumi & Mangunkusumo, 2002). According to WHO report in 1998, the most frequent type of cancer found in women are cervix (25.3%) and breast cancer (18.4%) (Anonim[1], 2005).

Cancer is usually treated by surgery, radiotherapy, chemotherapy and immunotherapy (Van de Velde *et al*., 1999). Those treatments cost highly and bring many side effects. Due to those reasons, many researchers hold studies to find new more effective and selective drugs.

Indonesia is a country that has great biodiversity. There are about 30.000 plant species found in Indonesia, and approximately 1260 species can be used to cure diseases. Several plants are understood to have anti-cancer effect (Mangan, 2003). One of them is *ceplukan* (*Physalis angulata* Linn. and *Physalis minima* Linn.). A study done by Chiang[2] *et al*. (1992), suggests that ethanol extracts of plants (whole plant) *P. angulata* Linn. have cytotoxic activity *in vitro* in several human cell lines, i.e. HA22T (hepatoma), HeLa (cervical cancer), KB (Nasopharyngeal cancer), Colo 205 (colon) and Calu (lung). When it was tested to animals, the plants have the cytotoxic activity *in*

vitro against H1477 (melanoma), Hep-2 (laryngeal) and 8401 (glioma) and they also showed antitumor effect against P388 lymphocytic leukemia in mice in vivo (Chiang[2] *et al*., 1992).

Other study conducted by Choi and Hwang (2003), showed *P.angulata* Linn. could inhibit inflammation in mice carrageenan (Choi & Hwang, 2003). According to Davies *et al*. (2002), the main mechanism of action of compounds/drugs that have anti-inflammatory effect is through inhibition of the cyclooxygenase (COX). Cyclooxygenase also is known as prostaglandins endoxyperoxide synthase. This enzyme is a catalyst for the transformation of prostaglandin endoxyperoxide (prostaglandin H2) from arachidonic acid. Two identified isoforms of prostaglandin synthase are COX-1 and COX-2. COX-1 is expressed in most tissues and thought to be involved in the process of cellular homeostasis, while COX-2 is often not detected in normal tissue, but it will show up quickly in response to various stimuli including mitogen, hormones, cytokines, growth factors and factors. The excessive expression of COX-2 and the high concentrations of prostaglandins are often supposed to have correlation with chronic inflammatory diseases such as rheumatoid arthritis, and several human cancers including colon, lung, bladder, prostate, stomach and breast cancer.

Prostaglandins mediate tumor formation through several mechanisms such as cell proliferation, apoptosis, modulation of the immune system and angiogenesis. In chronic inflammation, angiogenesis can help preventing the inflammatory process. (Davies *et al*. 2002). Angiogenesis is an important factor in tumor growth and metastasis (Costa *et al*., 2002). In experiment done on animals, inhibition of COX-2 expression by COX-2 inhibitors can inhibit the angiogenesis process. COX-2 inhibitors may also prevent the development of breast tumors in mice (Davies *et al*., 2003). Non-steroidal anti-inflammatory drugs administration, for at least 3 times a week in at least a year, will reduce the risk of breast cancer (Haris *et al*., 1996). The administration of non-steroidal anti-inflammatory drugs for 13-36 months will reduce the risk of breast cancer (Langman, *et al.,*2000).

The present study was aimed at examining the inhibition of COX-2 activity of *P angulata* L. for MCF-7cells.

METHODS

Tools

Glassware (Pyrex), analytical balance, beaker glass (Pyrex), mixer, filter 0.2 um, micropipette, liquid-nitrogen tank, refrigerator, CO_2 incubator (Nuaire), laminar air flow cabinet (Nuaire), microplate 96 (Nuclone) wells, pipette ependorf, inverted mycroscope, hemocytometer (New Bouer), pH meter, glass objects, light microscopes, centrifuges, tissue culture flasks (Olympus).

Materials

Test substance. ethanol extracts of ceplukan (*Physalis angulata* Linn.), Positive controle tamoxiphen (Tamoplex® Combiphar), and the positive control celecoxib (Celebrex® Pharmacia). MCF-7 cell line containing estrogen receptor alpha and beta isoforms (Anonim[2], 1998), Medium RPMI 1640 (GIBCO), fetal bovine serum/FBS 10%, 3% penicillin-streptomycin, 1% fungison, gentamicin (Merck), aquabidest, 70% ethanol, 0.5% trypsin and thrypan blue, monoclonal antibody COX-2 (NovoCastra), PBS pH (7.4), avidin-biotinylated antibody IgG secondary biotinilated (Novo Castra), haematoxylin eosin (Dako), 3% H_2O_2 and methanol.

Extraction

Ceplukan plants (roots, stems, leaves and fruit) which has been milled ,weighed about 50 g, wrapped with filter paper. The extraction was done by Soxhlet with 70% ethanol up to two times the circulation. This extract was evaporated by evaporator (MOH, 1986).

MCF-7 cell propagation

MCF-7 cells taken from nitrogen tank were heated in 37° C immediately and then sprayed with 80% alcohol. The cells were transferred in a sterile centrifuge tube containing 10 ml RPMI 1640-serum medium. This suspension then was centrifuged on 1200 rpm for 5 minutes. Supernatant liquids were removed and replaced with RPMI 1640 medium. After 20 minutes, the cells were centrifuged at 1200 rpm for 5 minutes. Supernatant fluids were discarded, left 1 ml of suspension to be done again. Once homogenized, the cells were included in the tissue culture flask with medium containing 20% fetal bovine serum (FBS). The cells were observed by inverted microscope. After the cells grew confluent, cells were harvested and then centrifuged at 2000 rpm for 5 minutes. Supernatant fluids were removed and left for approximately 1 ml for re-suspension until homogeneous. The cells were added by medium containing 10% FBS afterwards (Bakhriansyah, 2004)

Cytotoxic test

Micro cultures with 96 wells were prepared. MCF-7 cells with density of 1.5 x 10^4/mL were included in the 96 wells plate dissolved in 100 µl RPMI 1640 culture medium. The MCF-7 cells were incubated for 24 hours. Then 100 µl of ethanol extract of *Physalis angulata* Linn and tamoxiphen were added. Each concentration was done three times. The micro cultures were incubated using CO2 incubator 37°C for 24 hours. The MCF-7 cells then were washed using 100 µl PBS 0.25% 2 times. To release the live cancer cells attached to the wells, samples were given with100 µl of 0.25% trypsin. 10 µl of cells were discarded and then were added with 10 µl tryphan blue on these wells. 10 µl of mixtures was taken, and placed on a haemocytometer chamber and the number of the living cells were counted. The percentage inhibition of the ethanol extract of *Physalis angulata* Linn, and tamoxiphen and negative control were calculated by the following formula (Bakhriansyah, 2004):

$$\frac{\text{Live cells} - \text{control} - \text{live cells test material}}{\text{Live cells - control}} \times 100\%$$

Immunohistochemistry test

The 70% ethanol extract of *P. angulata* Linn. at concentration 10, 20 and 40 µg/mL and celecoxib at 5, 10 and 20 µg/ml which had been incubated 24 hours were made on a glass object, then was soaked using 3% H_2O_2 in methanol for 20 minutes. The sample was washed using water flowing then followed by distilled water for 15 minutes. This glass object was placed in EDTA (pH 8.0) and then put in the microwave. After that, the glass object was placed in normal horse serum (1:50) for 15 minutes, then was removed and etched with the primary monoclonal anti-COX2 antibody (1:50) for 60 minutes. Then, this glass object was washed with PBS pH 7.4 for 3 times. This glass object was incubated in secondary antibody for 15 minutes (1:2) in PBS plus 5% serum antibodies, then was washed 3 times using PBS pH 7.4. This glass object was incubated in streptavidin-biotin complex and PBS with 5% antibodies (1:2) for 15 minutes, then was washed in PBS pH 7.4 three times. This glass object was incubated in diaminobenzidin for 3-8 minutes then was washed using water flowing and followed by distilled water. This glass object was immersed in haematoxylin-eosin for 3-4 minutes then washed using water flowing. COX 2 activity was observed using a binocular microscope. The cytoplasm of cells that express COX 2 was dark brown, while the cytoplasmof cells that do not express COX 2 tends clear. The inhibition of COX 2 activity was calculated based on the percentage of MCF-7 cells that inhibited COX-2 expression (Bakhriansyah,2004).

DATA ANALYSIS

The inhibition of COX 2 activity was calculated by formula:

$$\frac{\text{COX2 - control} - \text{COX2 test material}}{\text{COX2 - control}} \times 100\%$$

IC_{50} was determined based on the inhibitory activity of COX-2 expression. Differences in IC_{50}values were analyzed by independent T-test(95%)

RESULTS

Immunohistochemistry test

On visual observation, the cells that indicate COX-2 expression have dark brown -colored cytoplasm, while the cells that do not expressCOX-2 have light-colored cytoplasm. (figure 1).

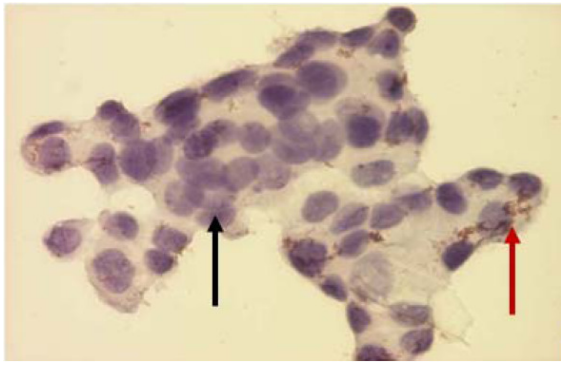

Figure 1. MCF-7 cells that express COX-2 (red arrows) and did not express COX-2 (black arrows).

The calculation results from visual observation of cells with COX-2 expression can be seen in table 1.

From Table 1, the percentage inhibition of ethanol extract of *P angulata* Linn. can be calculated. The percentage inhibition of the ethanol extract of *P angulata* Linn on 10µg/mL, 20 µg/mL and 40 µg/mL were 39.3%, 42.06% and 51.73% respectively. From the results, IC_{50} can be determined by linear regression.

Table 2 shows that the IC_{50} value of the ethanol extracts of *P. angulata* Linn. on the inhibition of COX-2 activity 37.57 is ± 3.11 µg/mL, while the IC_{50} for celecoxib 5.31 is ± 0.27 µg/mL. It means that although *P. angulata* Linn. is capable of inhibiting COX-2 activity but the potential of ethanol extracts *P. angulata* Linn is lower than celexocib. The independent T-test result shows that there are significant differences on IC_{50} values between *P. angulata* Linn. and celexocib p. 0.000 (95% CI)

DISCUSSION

Many studies claim that the inhibition of COX-2 has effect on tumor formation. In an experiment using mice, excessive expression of COX-2 can cause mamae tumor formation, but COX-2 selective inhibitors can suppress the tumor mamae (Sivula*et al*., 2005). To investigate whether the cytotoxic effect of ethanol extract *P. angulata* Linn. through inhibition of COX-2 activity, the cells are stained by antibody of COX-2. From table 2, it is known that ethanol extract of *P. angulata* Linn can deter COX-2 activity with IC_{50} 37.57 ± 3.11 µg / mL. This happens because celecoxib is COX-2 selective medicine.

Several studies on COX-2 states that COX-2 inhibitors affect cell proliferation. Celecoxib (selective COX-2 inhibitor) and the SC560 (COX-1 inhibitor) can induce cells to rest in G0/G1 phase in three different colon cancer cells regardless of whether these cells express COX-2 or not (Grosch*et al*., 2001). A study conducted by Hu *et al*. (2003) showed that the administration of selective COX-2 inhibitors, Ns-398 in Hep G2 cells in humans will lead to inhibition of proliferation which correlates with the reduction of 5-bromo-2-deoxy-uridin (BrdU) obtaining. This causes the reduction of cell cycle progression in the G1-S transition phase.

The provision of nimesulide, a selective inhibitor of COX-2, in gastric adenocarcinoma SGC7901 cells increases the proportion of cells in G0/G1 phase, but

Table 1. Quantitative results of visual observation of COX-2 expression cells

BU(µg/mL)	CI	∑1	%I	probit	C2	∑2	%I	probit	C3	∑3	%I	probit
PA 10	96	60	37,50	4,68	96	58	39,58	4,73	98	58	40,82	4,77
PA20	96	57	40,63	4,78	96	56	41,67	4,79	98	55	43,88	4,84
PA40	96	45	53,13	5,08	96	47	51,04	5	98	48	51,02	5
Cel 5	96	51	46,88	4,92	96	49	48,96	4,98	98	51	47,96	4,95
Cel10	96	38	60,42	5,26	96	39	59,38	5,23	98	36	63,27	5,34
Cel20	96	29	69,79	5,52	96	33	65,63	5,4	98	33	66,33	5,42

Notes: PA (*P. angulata* L.), Cel (Celexocib), % I: % inhibition, C1: control on replication1, ∑I: number cells on replication 1

Table 2. The IC_{50} of ethanol extract of *Physalisangulata* Linn and celecoxibin MCF-7 cells

Test material(µg/mL)	IC_{50}			mean IC_{50}(µg/mL±SEM)
	I	II	III	
Pa	34.82	34.11	43.77	37.57±3.11
Cel	5.83	5.11	4.98	5.31±0.27

Notes : Pa: *P. angulata* Linn, Cel: celecoxib

decreases the proportion of cells in S phase and G2/M on the cell cycle (Li *et al*., 2003). COX-2 selective inhibitor, SC'236, given to mice suffering from sarcoma 6mg/bw inhibits tumor growth and neo-angiogenesis (Kishi*et al*., 2000). The provision of COX-2 inhibitors reduce cancer through the reduction of blood vessel formation (Davies *et al*., 2002). COX-2 expression inhibits angiogenesis and apoptosis in breast cancer (Costa, *et al*., 2002). The study by Chiang[1] *et al*. (1992), suggests that the extract of *P. angulata* Linn. inhibits protein synthesis in leukemia cells. The ethanol extract of Physalis peruviana (EEPP) triggers apoptosis through the release of cytochrome c, SMAC / DIABLO and Om/HtrA2 from mitochondria to cytosol that may lead caspase 3 activation (Wu, *et al*., 2004). Apoptosis occurs when cells are treated by 50µg / mL EEPP. After 48 hours of treatment with EEPP, apoptosis of Hep G2 cells is correlated with increased expression of p53, CD95 and CD95L. The administration of this EEPP also causes down-regulation of Bcl-2, Bcl-xl and XIAP and up-regulation of Bax. The results also indicate that apoptosis triggered by EEPP in Hep G2 cells is possibly through CD95 and CD95L system and the mitochondrial signaling trans-duction pathway. Other study claimed that the EEPP has strong antihepatoma activity. Besides, it has apoptosis effects that are correlated with mitochondrial dysfunction (Wu *et al*., 2004)

CONCLUSION

The ethanol extract of Ceplukan (*P. angulata* Linn.) inhibits COX-2 activity in MCF-7 cells with IC_{50}37.57±3.11 µg / mL

SUGGESTION

Further investigation is needed to find out active compounds that have effect on inhibition of COX-2 expression.

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