Temulawak (Curcuma xanthorrhiza) Extract as a Cancer Chemopreventive Agent Via Up-Regulation p53 and Caspase-3 Gene

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Abstract. The development of biomaterials from herbs needs to be done as a chemopreventive compound to inhibit cervical cancer. Curcuma xanthorrhiza (CX) contains curcumin and xanthorrhizol, which are the main active substances for inhibiting carcinogenesis. Experimental research was carried out on cervical cancer cells from ethanol extracts of CX/EECX. Testing for proapoptosis potential by observing p53 gene expression in the HeLa cell was conducted by the flow cytometry method. Cytotoxic tests were carried out to obtain IC50, the results of 36.12 ± 6.66μg ml⁻¹. The IC50 value is then made into three concentrations, namely, ½ IC50, IC50, 2xIC50. The ICC test results showed an increase in the p53 gene expression index in the 1/2IC50 and IC50 concentration groups. The expression of p53 in the IC50 concentration group was higher than that in the 1/2IC50 group. There were no living cells in the 2xIC50 concentration group, so p53 expression could not be seen. EECX has potential as a chemopreventive biomaterial to prevent cervical cancer because it increases apoptotic activity in HeLa cells. The possibility of cell apoptosis, which is influenced by EECX with the optimal concentration that can be used, is IC50, which is 36.12 ± 6.66 μg ml⁻¹.

Keywords: curcuminoid, xanthorrhizole, apoptosis, chemopreventive, immunocytochemistry, p53, caspase-3

1. Introduction

Cervical cancer is a health problem in Indonesia and the world due to its high incidence and mortality rate[1]. Strategies and therapies for cervical cancer patients already exist, but cervical cancer deaths are still high at 250,000 / year. This condition requires a new approach to handling cervical cancer problems in Indonesia, so that cervical cancer incidence decreases[2][3]. Increasingly high prices for modern medicines and drug side effects have driven the movement rate back to nature. Developing a new cancer prevention strategy is developing biomaterials from herbs as chemopreventive compounds[4]. As the second-largest country in terms of diversity and number of medicinal plants, Indonesia has an excellent opportunity to develop biomaterials from medicinal plants/herbs as an alternative treatment for cervical cancer[5] [2]. Cervical cancer is a primary malignant tumor derived from squamous epithelial cells. Carcinogenesis is a multi-stage process that
genetically modified a normal cell to a progressive form and eventually became malignant (malignant)[2]. Understanding of carcinogenesis initiated efforts to develop a promising new strategy for cancer prevention[6]. The chemopreventive agents are more effective in precancerous lesions than in the cancerous stage.[7][8][3]. In Indonesia, Temulawak (Curcuma xanthorrhiza) (CX) is a medicinal plant that has been empirically used as an immune-booster[9] and is assumed as a potential chemoprevention agent. Indonesian people have long known CX in traditional or complementary medicine[10]. The biological activity of CX is for appetite enhancement, antioxidants, anti-cancer, hepatoprotective, and body immunity[11][12][13]. Curcumin and xanthorrhizole are the main active ingredients of ginger[13]. The laboratory has been proven that xanthorrhizole effect as anti-inflammation, anti-cancer, antioxidant, anti-hyperglycemia, antibacterial, and antihypertension [14][15]. The CX has been shown proapoptosis and antiproliferation in an in vitro laboratory. Xanthorrhizole is cytotoxic to MCF7 mammary cancer cells by inducing apoptosis. The proapoptotic mechanism of the xanthorrhizole compound was through the regulation of p53, Bax, and Bcl2 gene activity [16]. The p53 gene is a chemopreventive proapoptosis gene[17]. Based on data from this research, we assumed that the ethanolic extract of CX (EECX) is adequate to prevent carcinogenesis of cervical cancer through the p53 gene [18][19]. Apoptosis is an exciting phenomenon since apoptosis through caspase-9 activation usually involves p53, while T47D cells have lost their p53 function [20][21]. There are three pathways of apoptosis include caspase regulators activation, either Caspase-8 or Caspase-9 [22]. The p53 gene mutations caused p53 protein not to be able to regulate proapoptotic[23]. To be thought that apoptosis by analog curcumin PGV-0 in T47D cells through cytochrome c release from mitochondria[24]. The same activity is also suspected by curcumin in Hela cells [25]. The research objective is to determine the impact of the EECX on p53 and caspase-3 expression in HeLa cells.

2. Methods and materials

2.1. Curcuma xanthorrhiza rhizome extraction

Samples were placed in a 25 ml bottle; 10 ml of ethanol is added. Samples are kept in the darkroom for 24 hours, 1 ml of supernatant then centrifuged for 5 minutes at 10,000 rpm.

2.2. Cytotoxic test on hela cell

We weighed 10 mg of Samples from ethanol extract CX and it was placed in 100 µl DMSO, then obtained sample solution of 100,000 µg/ml. SubsequentlyWe make a series of concentrations of 2000 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62,5 µg/ml, 31,25 µg/ml, 15,625 µg/ml. The culture medium was made from 20 ml of Fetal Bovine Serum (FBS) 10% poured into a sterile bottle and added 2 ml of penicillin-streptomycin 1%, 1 ml fungizone 0.5%, and 200 ml of RPMI 1640. The culture medium is made in LAF and stored in the refrigerator at ± 4°C 20. Next, the cell moved from the vial to a sterile conical tube with 10 ml of RPMI 1640 in it, and this suspension was centrifuged at 1500 rpm for 5 minutes. After that, the supernatant is disposed of, and 1 ml of RPMI 1640 containing FBS 10% added to the pellet, then the cell suspense until it becomes homogenous. Cells were plated in 2-3 small tissue culture flask then maintained at 37°C in a humidified atmosphere (90%) containing 5% CO2 for 24 hours. After the number of Hela cells are enough or confluent (70%), the medium discarded. Cells washed with PBS twice, and 300 µl trypsin 0.25% was added then placed in an incubator for 3 minutes. 5 ml medium and suspend can be combined with a pipette, and cells are ready for use.

The cells were seeded at a density of 1x106 cell/ml. After 24 hours incubation, the cells were treated with various concentrations, with culture medium disposed of first, on 96 well-plate given 100µl concentration serial sample—the cell control, including just the cell with no extract. At the end of incubation, 100µl of MTT 5 mg/ml was added to each well and incubated for 4 hours. After that, 100µl of a stopper, SDS 10% reagent on 0,1N HCL, was added. The plate was wrapped in aluminum foil and incubated in a dark room overnight. Next, using ELISA to read absorbance at a wavelength of 595nm.
2.3. Proapoptotic and antioxidants test of EECX on hela cell

The Proapoptotic test of p53 and caspase-3 EECX on Hela cell was performed with flow cytometry. The cells were seeded at a density of 1x10^6 cell/ml on six-well, and after 24 hours of incubation, they were given with ½ IC50 and incubated overnight. After they were hatched, cells were moved to a sterile conical tube. A well plate washed by 1ml PBS then moved to the conical tube; the well plate was given 1 ml of trypsin 0.025%, incubated for 3 minutes, then collected again with a conical tube. PBS was then added to 10ml, and a conical tube was centrifuged for 10 minutes. The supernatant disposed of, and the remaining sediment then combined with 1 ml of cold PBS. Suspend it and move 1 ml from conical tube to microtube 1ml, centrifuge for 5 minutes at 5000 rpm. Supernatant disposed of again, add 100µl of p53 and caspase-3 reagent to remaining sediment. Incubate for 10 minutes in a dark room, 300µl buffer, then added and read using flow cytometry.

2.4. Data analysis

We determined the cytotoxicity activity of EECX as IC50. The mean difference of proapoptosis and antioxidant activity of EECX was analyzed One-way Annova with a 95% significance value.

3. Result and discussion

3.1. Test of chemopreventive potential of the EECX on hela cell culture

The potential chemopreventive of EECX was tested on Hela cell culture using the MTT method by determining IC50 value. IC50 is a concentration of EECX that can inhibit 50% proliferation activity of Hela cells. Based on the research results can be proved that CX potentially as a chemopreventive agent on cervical cancer cells. Data on the potency test result of chemopreventive of EECX on Hela cell culture is presented in Table 1.

| Group | Series dilution of the EECX | The value of R2 probit series dilution of the EECX | IC50 (µg/ml) |
|-------|-----------------------------|---------------------------------------------------|-------------|
| I     | 91.06                       | 43.22                                             |
| II    | 92.24                       | 34.94                                             |
| III   | 97.46                       | 30.20                                             |
| Total |                             |                                                   | 36.12 ± 6.66|

Note: IC50= inhibition concentration 50%; EECX= ethanolic extract of Curcuma xanthorrhiza

MTT assay of the chemopreventive test potency showed that IC50 EECX on Hela cell-cell was 36.12 ± 6.66 µg / ml. The results showed that the potential EECX as a material for chemopreventive on cervical cancer. The active compound of the CX, curcumin, has several biological activities [26-28]. Curcumin can be proved to affect the inflammatory reaction by regulating NFκ-b via a toll-like receptor 4 (TLR-4) receptor [29]. Curcumin has been shown to play an essential role in the inhibition of cancer cell proliferation, increased apoptosis, and inhibition of carcinogenesis. Curcumin is a ligand for Aryl Hydrocarbon Receptor (AHR)[27] and a scavenger for free radicals and increases the expression of hepatic S-transferase (GST) glutathione[30]. Curcumin may also inhibit the activity of the NF-kB transcription factor and proliferation activity [10][31] [32]. Inhibition of proliferation associated with inhibition of cell cycle progression.[33].

3.2. Test of p53 expression of EECX on hela cell culture

The result of the influence of EECX on the expression of p53 on Hela cell culture is presented in Table 2.

| Table 2. The result of the influence of EECX on the expression of p53 on hela cell culture (p53 expression index) |
|-----------------------------------------------------------|-----------------------------------------------------------|


The result showed that EECX increased expression of p53 in HeLa cell culture both in ½ IC50% and IC50% groups, as evidenced by the increase of p53 expression index (p <0.05). The expression of p53 in the concentration group = IC50 was higher than in the concentration group ½IC50. There was no cell visible in the 2xIC50 concentric group, so it could not be assessed for p53 expression. Curcumin increases p53 expression to a lower extent throughout the cell cycle in non-malignant cells. In these cells, curcumin reversibly up-regulates Cip1 expressions and inactivates pRB and thus arrests them in the G0 phase of the cell cycle. Therefore, these cells escape from curcumin-induced apoptosis at the G2 stage. Works from other laboratories also suggest that curcumin induces p53 expression in colon, breast, and other cancer cells. Curcumin is even thought to induce the expression of p53 in Hela cells, thus inhibiting proliferative activity by resting Hela cells to G0[2][34]. The p53 gene is a multifunctional tumor suppressor gene and often suffers from an alteration in cervical cancer and other cancers [35]. The various roles of p53 associated with cancer continue to be investigated; so far, known functions include regulation of cell cycle, cell aging, cell death or apoptosis, repair of DNA damage caused by agents genotoxic, angiogenesis, and control of oxidative stress[36] [37]. Therefore, the p53 protein, as a guardian of the genome, is an essential inhibitor of tumor progression[17].

3.3. Effect of EECX to the caspase-3 expression on hela cell culture

The EECX effect on the caspase-3 expression on Hela cell culture is presented in Table 3.

| Groups               | Expression (+) | Expression (-) | Index of p53 expression (expression p53+/total cell) |
|----------------------|----------------|----------------|------------------------------------------------------|
| 1/2 IC50 group (n=3) | 118,33±30,56   | 21,00±7,00*    | 0.81±0.05*                                           |
| IC50 group (n=3)     | 156,00±32,00*  | 23,00±1,00*    | 0.88±0.03*                                           |
| 2xIC50 group (n=3)   | N.A.           | N.A.           | N.A.                                                 |
| Media group (n=3)    | 77,33±7,50     | 55,67±10,01    | 0.58±0.02                                            |

Note : * = P < 0.05 to a media group
IC50 = inhibition concentration 50%

Curcumin has been shown to play a role in enhancing apoptosis. The presence of resistance to NF-kB due to curcumin administration causes galectin-3 to be inhibited, resulting in increased apoptosis[10]. Curcumin also induces increased apoptosis by decreasing Bel-xL, which inhibits apoptosis by binding and alienating bax. Besides, curcumin can also inhibit Akt, a protein kinase that causes cell resistance through resistance to apoptosis, i.e., by phosphorylation of the bad resulting in the release of BCL-2 and inhibit apoptotic processes. Also, the mechanism of curcumin in increasing apoptosis is through decreased expression of IAP. IAP is a protein that can inhibit apoptosis through its function as a direct inhibitor of activated caspase effector, caspase 3, and caspase-5, as well as cytochrome-induced inhibitors caspase-9 activation[9]. The EECX Show increased expression of p53, thus increasing p53 protein activity in inhibiting Hela cell proliferation activity. Previous in vivo research has proved CX as chemopreventive, the action of CX rhizome tablet to prevent mouth cancer in female Sprague Dawley strain-induced SP C1 cell line. The study provides positive test results against the reduction of
nodule or tumor size[13]. Protein p53 is modulated by several critical biological processes, such as phosphorylation, acetylation, sumoylation, and ubiquitination[27][38].

4. Conclusion
EECX has the potential as a chemopreventive agent for the prevention of cervical cancer. The mechanism of EECX in inhibiting carcinogenesis in cervical cancer is through increased expression of p53 and caspase-3.

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