Potential of *Curcuma xanthorrhiza* ethanol extract in inhibiting the growth of T47D breast cancer cell line: *In vitro* and bioinformatic approach

[Potencial del extracto de etanol de *Curcuma xanthorrhiza* para inhibir el crecimiento de la línea celular de cáncer de mama T47D: enfoque *in vitro* y bioinformático]

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Abstract

Context: Breast cancer most commonly occurs in women globally and has the highest mortality rate in Asia. Therefore, a safe and prominent drug to cure the disease needs to be urgently developed.

Aims: To investigate the molecular mechanism of the ethanol extract of *Curcuma xanthorrhiza* (ECx) in inducing apoptosis in breast cancer cell line T47D.

Methods: The research was started by extracting *Curcuma xanthorrhiza* using ethanol as solvent. The anticancer research was carried out by cell toxicity assay and apoptosis assay. This study also observed changes in cell morphology and protein expression levels that can induce cell apoptosis. The bioinformatic approach was carried out to determine the activity of the active compound in inhibiting AKT-1, which plays an important role in the development of cancer cells. TIG-1 cells were used as controls in toxicity assays.

Results: ECx showed antioxidant and nitric oxide scavenging activity, which is beneficial for human health, and exhibited selective toxicity in T47D breast cancer cells compared to TIG-1 normal cells. ECx increased the expression of p53, Bax, caspase-3, and caspase-9, which induces apoptosis. Further analysis showed that ECx contained at least eight active compounds: curcumin, curcumin II (desmethoxycurcumin), curcumen, camphor, 1,8-cineole, p-cymene, ar-turmerone, and carophyllene oxide. Bioinformatics studies suggest that active compounds may be involved in apoptosis via the PI3K/AKT signaling pathway.

Conclusions: ECx significantly acts as an anticancer agent by inhibiting the growth of T47D cells. This research proves that the bioinformatics approach shows that curcumin can inhibit the expression of AKT-1.

Keywords: AKT1; apoptosis; bioinformatics; *Curcuma xanthorrhiza*.

Resumen

Contexto: El cáncer de mama ocurre con mayor frecuencia en mujeres a nivel mundial y tiene la tasa de mortalidad más alta en Asia. Por lo tanto, es necesario desarrollar urgentemente un fármaco seguro y destacado para curar la enfermedad.

Objetivos: Investigar el mecanismo molecular del extracto etánolico de *Curcuma xanthorrhiza* (ECx) en la inducción de apoptosis en la línea celular de cáncer de mama T47D.

Métodos: La investigación se inició extrayendo *Curcuma xanthorrhiza* usando etanol como solvente. La investigación contra el cáncer se llevó a cabo mediante un ensayo de toxicidad celular y un ensayo de apoptosis. Este estudio también observó cambios en la morfología celular y los niveles de expresión de proteínas que pueden inducir la apoptosis celular. El enfoque bioinformático se llevó a cabo para determinar la actividad del compuesto activo en la inhibición de AKT-1, que juega un papel importante en el desarrollo de células cancerosas. Se usaron células TIG-1 como controles en los ensayos de toxicidad.

Resultados: ECx mostró actividad antioxidante y de eliminación de óxido nítrico, que es beneficiosa para la salud humana, y exhibió toxicidad selectiva en células de cáncer de mama T47D en comparación con células normales TIG-1. ECx aumentó la expresión de p53, Bax, caspasa-3 y caspasa-9, lo que induce la apoptosis. Un análisis posterior mostró que ECx contenía al menos ocho compuestos activos: curcumin, curcumin II (desmethoxycurcumin), curcumen, alcanfor, 1,8-cineol, p-cimeno, ar-turmerona y óxido de caroiflono. Los estudios de bioinformática sugieren que los compuestos activos pueden estar involucrados en la apoptosis a través de la vía de señalización PI3K/AKT.

Conclusiones: ECx actúa significativamente como un agente anticancerígeno al inhibir el crecimiento de las células T47D. Esta investigación demuestra que el enfoque bioinformático muestra que la curcumina puede inhibir la expresión de AKT-1.

Palabras Clave: AKT1; apoptosis; bioinformática; *Curcuma xanthorrhiza*. 
INTRODUCTION

Breast cancer is a significant global health problem and most commonly occurs in women (Akram et al., 2017; Sun et al., 2017). The International Agency for Research on Cancer shows that in 2020, more than 2 million breast cancer cases occurred, with >600,000 breast cancer-associated mortalities (DeSantis et al., 2015). Asia has the highest incidence and mortality rates of breast cancer, with more than 45% and 50%, respectively (Barbieri, 2019). Moreover, no prominent drug is safe and effective in curing all cancers.

Curcuma xanthorrhiza is a member of the Zingiberacea family and is empirically trusted for traditional medicine for its anti-inflammatory, antioxidant, and anticancer activities (Rajkumari and Sanatombi, 2018). A previous study has shown that the ethanolic extract of the herb has cytotoxic activity on the 4T1 mouse breast cancer cell line (Sutejo et al., 2019). Apoptosis, known as cell suicide, is a series of events that lead to cell death. Apoptosis is characterized by cellular, morphological, and biochemical changes in cells. These include cell shrinkage, nuclear fragmentation, chromatin condensation, caspase activation, membrane blebbing, and apoptotic bodies (Balachandran et al., 2014).

The PI3K/AKT signaling pathway is one of the signaling pathways that regulate the apoptotic process. A previous study has shown that protein kinase B (AKT) has been proven to regulate apoptosis-related proteins, such as Bax and caspase (Bak et al., 2011). The regulation of these proteins is very important in the development of anticancer drugs with the target of inducing the apoptosis process in cancer cells (Pfeffer and Singh, 2018). In this study, we investigated the molecular mechanism of the ethanol extract of C. xanthorrhiza (ECx) in inducing apoptosis in breast cancer cell line T47D. The research is expected to provide more complete information on the benefits of Curcuma xanthorrhiza in inhibiting the growth of breast cancer cells.

MATERIAL AND METHODS

Plant material and extraction

Rhizome of C. xanthorrhiza was collected from UPT. Balai Materia Medica, Batu, East Java, Indonesia (7°52’01.2”S and 112°31’13.2”E). Taxonomic identification was confirmed and deposited by UPT. Balai Materia Medika, Batu, East Java, Indonesia. The dried powder of rhizome was macerated using 96% ethanol (1:2, w/v) for three days at room temperature. The extracted compound was then filtered using a Whatman No. 41 filter paper. The filtrate was concentrated by a rotary evaporator (Buchi R-114) at 55°C. The crude extract was used for further analysis.

Cell line, cell culture, and preparation of the drug

T47D and 4T1 breast cancer cell lines were cultured in Roswell Park Memorial Institute medium (Gibco) and Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a 5% CO2 incubator. In addition, TIG-1 was used as a control to determine the toxicity level of ECx in a normal cell. The stock solution of C. xanthorrhiza was prepared by dissolving 64 mg/mL in dimethyl sulfoxide. The stock solution was stored at -20°C.

Cell viability assay

The cytotoxic property of ECx was measured by WST-1 assay based on a previous study with modification (Scarcello et al., 2020). Cells were seeded in 96-well plates with a density of 7.5 x 103 cells/well and incubated for 24 h at 37°C in a 5% CO2 incubator. Cells were treated with different concentrations (untreated, 20, 40, and 80 µg/mL) of C. xanthorrhiza and incubated under the same condition. The treatment medium was replaced with a medium containing WST-1 (Roche, Sigma-Aldrich, USA) and incubated for 30 min. Absorbance was measured at 450 nm by Elx808™ (BioTek Instrument, USA).

Apoptosis assay

Apoptotic cells were studied by Annexin V-FITC/PI binding assay that was performed based on a previous study (Miao et al., 2013). Cells were seeded in 24-well plates (7.5 x 104 cells/well) and treated with different concentrations of C. xanthorrhiza for 24 h at 37°C in a 5% CO2 incubator. Cells were harvested, trypsinized, resuspended, and washed with PBS. Cells were centrifuged for 5 min at 10°C at 2,500 rpm. The supernatant was discarded, resuspended with 50 µL Annexin V-FITC/PI (1:2; Invitrogen, USA), and incubated for 30 min at dark and cold conditions. Then, 400 µL of PBS was added and analyzed by flow cytometry (BD Biosciences, USA). Data were analyzed by CellQuest software (BD Biosciences).

Mitochondrial membrane potential assay

The effect of ECx on the mitochondrial apoptotic pathway was evaluated by Rhodamine 123 with modification (Kars et al., 2006). Then, 7.5 x 104 cells/well were seeded in 24-well plates and treated with different concentrations of C. xanthorrhiza for 24 h. Rhodamine 123 (2 µM) was added to each well and incubated for 1 h at 37°C in a 5% CO2 incubator. Cells were
harvested and centrifuged for 5 min at 10°C at 2500 rpm. Cells were washed with PBS, resuspended with basal medium, and incubated for 30 min at room temperature. Cells were analyzed by flow cytometry (BD Biosciences). Data were analyzed by CellQuest software (BD Biosciences).

Morphological changes using phase contrast inverted microscope

Morphological changes observation of apoptotic cells was performed based on a previous study with slight modification (Syed Abdul Rahman et al., 2013). Cells 8 x 10^5 were seeded in 60 mm dishes and incubated for 24 and 48 h with different concentrations of *C. xanthorrhiza* ethanol extract. The treatment medium was discarded, and cells were washed with PBS. The morphological changes of apoptotic cells were observed using a phase contrast inverted microscope at 100x magnification.

Apoptosis-related protein assay

Cells were seeded in 24-well plates and treated with 55 µg/mL of ECx. After 24 h, cells were harvested, trypsinized, and centrifuged for 5 min at 10°C at 2500 rpm. A 500 µL of fixation buffer was added and incubated on ice for 30 min. Cells were centrifuged and resuspended with 50 µL of anti-p53 antibody (sc-126), anti-caspase-3 antibody (sc-7272), anti-caspase-9 antibody (sc-17784), and anti-Bax antibody (sc-20067). Cells were incubated for 30 min in dark and cold conditions. A 400 µL of PBS was added and analyzed by flow cytometry (BD Biosciences). Data were analyzed by CellQuest software (BD Biosciences).

2,2-Diphenyl-1-picrylhydrazyl scavenging activity assay

Antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay method. A 100 µL of extract of each concentration and standard was added to 100 µL of DPPH in ethanol solution (0.4 mM) in 96-well plates. The plate was incubated for 30 min at room temperature, and its absorbance was measured at 490 nm by an ELISA reader (Elx808™, BioTek Instrument, USA) (Badami et al., 2003). Ascorbic acid was used as a positive standard.

Nitric oxide scavenging assays

Nitric oxide (NO) scavenging assay was performed according to a previous method with slight modification (Tsai et al., 2007). A 60 µL of sodium nitroprusside (SNP) with 10 mM concentration in each well containing 60 µL of the extract was added. SNP was dissolved in phosphate-buffered saline (PBS). The mixture of samples and PBS was incubated at room temperature and under light for approximately 150 min. An equal volume of Griess reagent (5% phosphoric acid, 1% sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride) was added for the sample and control. NO scavenging level was measured at an absorbance of 570 nm by an ELISA reader. The double volume of SNP was used as a control.

Determination of total flavonoid

Total flavonoid was measured by aluminum chloride colorimetric assay based on a previous method with slight modification (Chatatikun and Chibchialard, 2013). A 50 µL of extract or standard was added to 10 µL of AlCl₃ (10%, w/v), followed by 150 µL of 96% ethanol solution. A 10 µL of CH₃COONa (1 M) was added to the mixture in 96-well plates. The mixture was incubated for 40 min at room temperature and under dark conditions. The absorbance was measured at 405 nm by an ELISA reader (Elx808™, BioTek Instrument, USA). Quercetin was used as standard in 96% ethanol solution.

Determination of total phenolics

Total phenolic content was measured by Folin Ciocalteu assay, and the method was performed according to a previous study with slight modification (Jing et al., 2015). A 100 µL of extract or standard was added to 1.0 mL Folin Ciocalteu reagent (Merck: 1090010500) (a ready to use reagent diluted tenfold with distilled water). The mixture was incubated for 5 min. A 1.0 mL of Na₂CO₃ (7.5%, w/v) was added and incubated for 90 min at room temperature and in dark conditions. The total phenolic content was measured at 725 nm by spectrophotometry.

Liquid chromatography–high resolution mass spectrometry analysis

The extract was diluted by adding 0.1% ethanol solvent with a final volume of 1500 µL. The sample was mixed, vortexed, and centrifuged for 2 min at 6000 rpm. The sample was filtered by a 0.22 µm syringe filter. The sample was processed into an autosampler (Thermo Fisher Scientific Inc., USA) and injected in liquid chromatography–high-resolution mass spectrometry (LC–HRMS, Thermo Scientific Dionex Ultimate™ 3000 RSLCnano with microflow meter; Thermo Fisher Scientific Inc.).

The analysis of LC–HRMS, the column oven maintained at 30°C, 100 mL of volume was injected, the analytical column was Hypersil GOLD aQ (Hypersil GOLD™ aQ C18 Polar Endcapped HPLC Columns; Thermo Fisher Scientific Inc.) 50 x 1 mm x 1.9 µm particle size and solvent A = 0.1% formic acid in water and solvent B = 0.1% formic acid in acetonitrile

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were used. The analytical flow rate was 40 mL/min with a run time of 30 min. The positive ion mode detection was performed using a mass spectrometer (Q Exactive™ Orbitrap Mass Spectrometers; Thermo Fisher Scientific Inc.).

Statistical analysis

Statistical analysis of the cell viability and apoptosis assays results using One Way ANOVA with the Tukey HSD method (p<0.05 and p<0.01). Statistical analysis of the potential mitochondrial membrane and apoptosis-related protein assay using independent sample T-test (p<0.05). Statistical analysis was conducted using SPSS version 22 software.

In silico analysis

Analysis of biological activity and ligand–protein interaction

The biological activity of eight bioactive compounds of ECx were analyzed by PASS server (http://www.pharmexpert.ru/passonline). The bioactive compounds were investigated based on canonical smile retrieved from PubChem server (https://pubchem.ncbi.nlm.nih.gov/). The probability activity of bioactive compounds was indicated as the Pa value. In this study, anticancer properties of ECx were predicted based on PASS server results. Ligand–protein interaction was evaluated by STITCH database integrated into the Cytoscape.

Molecular docking analysis

Molecular docking was conducted to determine the interaction of ligand–protein based on STITCH results. Molecular docking was performed by Auto-dock Vina software integrated with PyRx 0.8 (Trott and Olson, 2009). A lower binding affinity of ligand than the inhibitor was taken and continued with molecular dynamics (MD) simulation. The docking results were visualized by BIVOIA Discovery Studio 2019 to determine the position of the protein–ligand interaction.

MD simulation

Yet Another Scientific Artificial Reality Application was used for MD simulation. This simulation compared the ligand with the lowest binding affinity value and the inhibitor as a positive control. The parameter used according to cell physiologist condition at 37°C, 1 atm, pH 7.4, and 0.9% salt content for 50 ns with autosaved every 25 ps. The simulation was run by the md_run macro program, and the results were displayed by the md_analyze and md_analyze-bindenergy macro programs (Bagheri and Fatemi, 2018; Deeba et al., 2017; Krieger and Vriend, 2015).

RESULTS

Cell viability, phytochemical screening, and antioxidant activity

The viability of T47D, 4T1, and TIG-1 cells was decreased in line with the increasing concentration of ECx administered. T47D cells had the highest sensitivity to ECx compared to other cells (Fig. 1A), with an IC50 value of 54.5 ± 5.9 µg/mL (Fig. 1B). The effectiveness of ECx in inhibiting cell growth is supported by the presence of phenolic and flavonoid contents contained in ECx (Fig. 1C). The extract also has antioxidant activity with an IC50 value of 179 ± 1.1 and 245 ± 8.2 ppm, respectively, for DPPH and NO (Fig. 1C). Based on the literature ECx has low antioxidant activity because the IC50 value is more than 150 ppm (Priska et al., 2019). However, the anticancer activity is quite strong, with an IC50 value of less than 100 µg/mL.

T47D cell apoptosis-induced ECx

ECx induces T47D cell death, which is the highest concentration of 110 µg/mL that causes >98% of cells to undergo apoptosis (Fig. 2A-C). This study found that cisplatin used as a control caused 17% of cells to undergo apoptosis. However, this treatment had a less significant effect than the treatment with 55 µg/mL, which caused 74.5% of cells to undergo apoptosis (Fig. 2A-C). The apoptotic cells were shown by round cell shape, cell shrinkage, and loss of cell contact with other surrounding cells. In addition, an increase in ECx concentration administered causes cells undergoing apoptosis to lose their ability to adhere to the cell culture dish (Fig. 3). The extract also stimulated mitochondrial depolarization, with a maximum concentration of 55 µg/mL after 24 h of treatment (Fig. 2D-F). It is known that treatment with cisplatin and ECx induces mitochondrial membrane potential loss. These results indicate that ECx has the potential as an anticancer agent in T47D breast cancer cells.

ECx effect on the apoptosis-related protein activation

Several key proteins for apoptosis such as p53, Bax, caspase-9, and caspase-3 were detected in this study. ECx (55 µg/mL) could significantly induce the activation of p53, Bax, caspase-9, and caspase-3. The activation of p53 in treated cells was 24.6%, whereas that in untreated cells was 5.5% of the total cell population. The expression of Bax, caspase-9, and caspase-3 was increased in almost all of the cell populations (>95%) (Fig. 4A-H). The expression of p53 and Bax

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Figure 1. Anticancer and antioxidant activities of ECx.
(A) Percentage of cell viability treated with various concentrations of ECx. (B) The IC_{50} value of ECx on breast cancer cells and normal diploid fibroblast cells. (C) Total phenolic and flavonoid contents of ECx, percentage of inhibition of antioxidant activity by DPPH and NO methods. Data are presented as mean ± SD of triplicate independent experiments. *p<0.05, **p<0.01 respect control group.

Figure 2. ECx induces apoptosis in T47D breast cancer cells as indicated by plasma membrane damage and loss of mitochondrial membrane potential.
(A) population of T47D cells undergoing apoptosis by Annexin V-FITC/PI staining.
(B-C) Percentage of viable cells and cells undergoing apoptosis by Annexin V-FITC/PI staining.
(D) Gating results of a cell population that undergoes mitochondrial depolarization by Rho-123 staining.
(E-F) Percentage of viable cells between untreated and ECx-treated cells after 24 h and 48 h. Data are presented as mean ± SD of triplicate independent experiments. **p<0.01 respect control group.
Figure 3. Morphological changes of T47D breast cancer cells after ECx treatment.

Figure 4. Apoptotic and non-apoptotic T47D cell populations for apoptosis-related protein activation after treatment with ECx. (A, C, E, and G) Gating results of cell population in which apoptosis-related protein activation is analyzed by flow cytometry. Black peaks indicate untreated cells, pink peaks indicate cisplatin-treated cells, and red peaks indicate 55 µg/mL-treated cells. (B, D, F, and H) Percentage of a cell population that expresses apoptosis-related protein. Data are presented as mean ± SD of triplicate experiments. *p<0.01 respect control group.
Increased by more than 60% in cisplatin-treated cells (Fig. 4A-D). The percentage of cells expressing caspase-9 between cisplatin-treated and ECx-treated cells was not significantly different (Fig. 4E-F), but as seen in Annexin V-FITC/PI staining (Fig. 2A-C), only a few cells undergo apoptosis. These results were possible due to the low expression of caspase-3 in cisplatin-treated cells, which was 6.2% (Fig. 4G-H), so the apoptotic process could not be executed optimally.

**Compound identification and in silico analysis**

Identification of the ECx active compound was conducted using LC–HRMS as described in a previous method. Eight active compounds were identified and selected for further analysis, namely, curcumin, curcumin II known as desmethoxycurcumin, curcumene, camphor, 1,8-cineole, p-cymene, ar-turmerone, and caryophyllene oxide (Table 1). The eight active compounds then predicted the biological function by using PASS server. The results showed that curcumin and desmethoxycurcumin had strong activity to induce apoptosis by stimulating caspase-3 and p53 (Fig. 5A).

Further analysis using STITCH database suggested that the ECx active compound is involved in the process of apoptosis through the PI3K/AKT signaling pathway (Fig. 5B). Then, we performed molecular docking to determine the binding affinity between curcumin and AKT1. The docking results show that curcumin is able to bind to the phosphorylation site of AKT1 with a binding energy of −9.1 kcal/mol, which is better than its inhibitor. Curcumin has a hydrogen bond to AKT1 on Asn54, Trp80, Leu264, Lys268, Val270, and Val271 (Fig. 5C–D, Table 2).

**Results of MD simulation**

The stability complex of AKT1–curcumin was examined by MD simulation. Data of MD analysis showed that the AKT1–curcumin complex was stable up to 50 ns with root-mean-square deviation (RMSD) value <3 Å. Some amino acid residues Gln113, Lys112, and Tyr350 fluctuate movement but are still comparable with the control (AKT1 inhibitor) (Fig. 6A–B). The number of hydrogen bonds and binding energy of the AKT1–curcumin complex was not significantly different compared to the AKT1–inhibitor complex (Fig. 6C–D). The stability of the protein–ligand complex interaction during the simulation process was demonstrated by the MD binding energy (Chen et al., 2015).
Table 1. The active compounds in ECx.

| Compound            | Formula  | Molecular weight (g/mol) | RT (min) | Area (max.) | mzCloud |
|---------------------|----------|--------------------------|----------|-------------|---------|
| p-Cymene            | C_{10}H_{14} | 134.10927                | 5.314    | 614,662.81  | 80.4    |
| 1,8-Cineole         | C_{10}H_{18}O | 136.12500                | 9.511    | 303,150.13  | 70.5    |
| Camphor             | C_{10}H_{16}O | 152.11991                | 8.942    | 1,318,214.61| 89.8    |
| Curcumene           | C_{12}H_{12} | 202.17180                | 12.069   | 30,462,961.47| 95.9    |
| Ar-turmerone        | C_{10}H_{18}O | 216.15094                | 12.317   | 171,522,210.02| 92.3    |
| Caryophyllene oxide | C_{12}H_{18}O | 220.18230                | 12.939   | 3,139,408.81| 88.5    |
| Desmethoxycurcumine | C_{20}H_{20}O | 338.11479                | 10.412   | 9,838,583.60| 61.3    |
| Curcumin            | C_{21}H_{20}O | 368.12520                | 0.863    | 13,116,889.51| 92.0    |

Tables 2. Molecular docking results and the interaction of ligands-AKT1 protein.

| Ligand               | Binding affinity (kcal/mol) | Chemical bonds position | Hydrogen bond      | Hydrophobic interaction |
|----------------------|----------------------------|-------------------------|-------------------|-------------------------|
| Curcumin             | -9.1                       | Tyr272                  | Asn53, Asn54, Gln79, Trp80, Leu210, Thr211, Tyr263, Leu264, Lys268, Val270, Val271, Arg273, Asp279 |
| AZD5363 (Inhibitor)  | -9.8                       | Gln79, Asp292           | Asn54, Trp80, Thr81, Thr82, Ile84, Leu264, Lys268, Val270, Val271, Tyr272, Asp274, Asn279, Gly294 |

Figure 6. Molecular dynamics simulation.

(A-D) The stability of complex protein-ligand interaction during simulation indicated with RMSD value, RMSF value based on amino acid residues fluctuation, the number of hydrogen bonds, and the molecular dynamic binding energy, respectively.
DISCUSSION

C. xanthorrhiza is one of the ethnomedicinal plants empirically used to treat various diseases, such as fever, hypertension, inflammation, and cancer, in the Asian region. The herb contains phenolics, flavonoids, alkaloids, terpenoids, and other active compounds that have antioxidant activity. In accordance with the results obtained, it was shown that ECx had high total phenolic and total flavonoid contents with 23.3 ± 1.4 mg GAE/g sample and 18.4 ± 0.2 mg QE/g sample, respectively. Previous studies show that curcumin is the main compound in the Zingiberaceae family that contributes to the largest antioxidant activity (Alafiatayo et al., 2014; Ismail et al., 2017; Muflihah et al., 2021).

The extract also has anticancer activity on T47D breast cancer cells with the smallest IC50 value of 54.5 ± 5.9 µg/mL. Data correspond with previous studies that C. xanthorrhiza has toxicity on MCF-7, HT-29, SF 3169, 4T1, and Vero cells (Kirana et al., 2003; Sutejo et al., 2019). ECx inhibits cell growth and drives cancer cells to apoptosis in T47D cells in a dose-dependent manner. Moreover, the extract induces the depolarization of the mitochondrial membrane and significantly increases the level of apoptosis signature proteins p53, Bax, and caspase-3/9.

Further analysis by bioinformatics suggested that curcumin as one of the ECx active compounds might inhibit AKT1 that plays a significant role in breast cancer process. Phosphorylation of the PIP3-binding PH domain by PI3K causes the opening of ATP-binding pocket of AKT1 and activates the downstream signaling pathway (Hein et al., 2014; Lin et al., 2019; Nitulescu et al., 2016) for activating cell proliferation processes and inhibiting apoptotic signaling pathways. Molecular docking simulations show that curcumin could bind with AKT on the ATP-binding site, similar to the AKT1 inhibitor binding site (Fig. 6C, Table 2) that might inhibit mdm2 activation and result in p53 upregulation. Furthermore, its condition stimulated Bax expression that causes mitochondrial outer membrane permeability and release of cytochrome C, activating the caspase cascade (caspase-9 and caspase-3) for driving cell apoptosis (Haupt et al., 2003; Rahmanian et al., 2016). In addition, MD simulations indicated that the interaction of the AKT1-curcumin complex was stable and similar to the AKT1-inhibitor complex. The stability of the complex was indicated by an RMSD value < 3 Å, in which an RMSD value >3 Å indicates that the protein structure has changed during the simulation process (Martinez, 2015).

CONCLUSION

Research shows that ECx significantly acts as an antioxidant and anticancer agent. ECx could inhibit T47D cell growth and induce cell apoptosis through the upregulation of p53, Bax, and caspase-3/9. Curcumin is one of the extract active compounds that might inhibit AKT1 comparable with the AKT1 inhibitor. Further study to validate the curcumin activity for inhibiting AKT1 in vitro is important to understand the ECx anticancer mechanism.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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|--------------|-----------|----------|---------|--------------|----------|
| Concepts or ideas | x         | x        | x       |               | x        |
| Design       | x         | x        | x       |               | x        |
| Definition of intellectual content | x         | x        | x       | x            |          |
| Literature search | x         | x        | x       | x            | x        |
| Experimental studies | x         | x        | x       | x            |          |
| Data acquisition | x         | x        | x       | x            | x        |
| Data analysis | x         | x        | x       | x            | x        |
| Statistical analysis | x         | x        | x       | x            |          |
| Manuscript preparation | x         | x        | x       | x            |          |
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| Manuscript review | x         | x        | x       | x            | x        |

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