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A Cytotoxic Activity of Ethanol Extract of Yellow Root Stem (*Arcangelisia flava* (L.) Merr.) and Its Effect on Expression of Bcl-2 and Bcl-xL Genes in MCF-7 Cells.

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ABSTRACT

Breast cancer was the main cause of death in women on the world, the percentage of breast cancer deaths in Indonesia reaching 9.6% with a prevalence of 201,143 cases of total cancer cases in 2020. The increasing incidence of breast cancer caused the use of breast cancer drugs to increase, especially natural ingredients that could be used as an alternative treatment for breast cancer with minimal side effects. Yellow root (*Arcangelisia flava* (L.) Merr.) contained berberine compounds which was known to have cytotoxic activity. he aim of this study was to determine the cytotoxic activity of the yellow root stem extract (*Arcangelisia flava* (L.) Merr.) and its effect on the expression of Bcl-2 and Bcl-xL in MCF-7 breast cancer cells. Extraction was obtained by maceration method with 70% ethanol as solvent. The method of testing cytotoxic activity using the MTT method assay at the concentration series (2800; 1400; 700; 350; 175; 87.5; 43.75) µg/ml then calculated the IC_{50} value using linear regression. The narrative review was conducted by collecting data from various online research journals using the keywords cytotoxicity of extracts of yellow root (*Arcangelisia flava* (L.) Merr.) AND the effect on the expression of Bcl-2 and Bcl-xL genes in MCF-7 cells. The results of the cytotoxic test showed that the extract had cytotoxic activity against MCF-7 breast cancer cells with an IC50 of 838.67 µg/ml so that the cytotoxic effect was moderate (100-1000 µg/ml) and the results of the study of the effect of protein expression on cancer cells showed that berberine contained in yellow root can reduce the expression of Bcl-2 and Bcl-xL through the mitochondrial intrinsic pathway in MCF-7 breast cancer cells so that it can induce apoptosis induction.

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INTRODUCTION

Cancer is a condition where body cells mutate and grow uncontrollably, dividing faster than normal cells (Pusat Data dan Informasi Kemenkes RI, 2015). Breast cancer is a leading cause of death among women worldwide, with breast cancer mortality in Indonesia reaching 9.6%, and a prevalence of 201,143 cases out of 273,523,621 total cancer cases in 2020 (IARC, 2020). Breast cancer is a heterogeneous malignancy with varying clinical and molecular presentations, often showing a different prognosis for each individual (Han, Wang, and Xu, 2020).

Over the past 10-15 years, treatment concepts have evolved to account for this heterogeneity, emphasizing more biologically targeted therapies and reducing treatment to minimize side effects. The side effects of various treatments, especially non-specific mechanism anticancer chemotherapy drugs, can damage normal cells and have not provided optimal results. This is why many researchers are conducting studies to find ideal developmental therapies using natural materials. Traditional medicine using herbs through medicinal plants in the form of plant extracts or single active compounds isolated from plants has become a new product for the renewal of treatment and disease prevention. These are believed to have minimal side effects and are now familiar worldwide due to increasing demand. Bioactive phytochemicals in plants can inhibit cancer cell cytogenesis by inhibiting or modifying epigenetic processes that suppress gene initiation and development (Lee et al., 2020).

The yellow root plant (*Arcangelisia flava* (L.) Merr) is known to have various pharmacological activities, one of which is anticancer. The secondary metabolite of yellow root known to exhibit anticancer activity is the alkaloid compound berberine. Docking results show that the berberine content in yellow root provides the most negative free binding energy and the smallest inhibition constant on all EGFRs, with the highest affinity shown for EGFR-2, making it a potential candidate for developing HER2-positive breast cancer therapy (Pratama, Mulyani, and Suratno, 2019). Berberine can also decrease the expression of anti-apoptotic genes such as Bcl-2, Bid, and Bcl-xL in cell lines. Berberine-induced apoptosis can be dose-dependent or dose-independent (Jabbarzadeh Kaboli et al., 2014).

Basic research is needed involving cell lines, as they have been widely used in many aspects, especially as in vitro models for cancer research. MCF-7 cells are estrogen receptor (ER) positive breast cancer cell lines commonly used for over 40 years in various studies. MCF-7 cells allow researchers to use them to shed more light on breast cancer pathogenesis and treatment protocols through reliable in vitro tests. MCF-7 cells are highly advantageous due to their characteristics of overexpressing P-glycoprotein, being resistant to the chemotherapeutic agent doxorubicin, overexpressing Bcl-2, and not expressing caspase-3, thus being able to avoid apoptosis (Comşa, Cîmpean, and Raica, 2015). Based on the given explanation, this study aims to determine the cytotoxic activity of the yellow root (*Arcangelisia flava* (L.) Merr.) stem extract and its effect on the expression of Bcl-2 and Bcl-xL in MCF-7 breast cancer cells.

MATERIALS AND METHODS

The research was conducted from April to August 2020 and continued from March to June 2021. The research locations were the Phytochemistry Laboratory, Faculty of Pharmacy, Universitas Setia Budi Surakarta, and the Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada Yogyakarta. This study was approved by the Health Research Ethics Committee of RSUD Dr. Moewardi, Surakarta, Indonesia.

Materials and Equipment

The sample material used was the stem of yellow root (*Arcangelisia flava* (L.) Merr.), which was

dried and powdered. For extraction, 70% ethanol was used. Materials for the cytotoxic test included MCF-7 breast cancer cells, stock media: DMEM (Dulbecco's Modified Eagle Medium), M199, Cisplatin, Sodium bicarbonate, and HEPES (Sigma); cell culture media: DMEM, M199 media, 10% v/v Fetal Bovine Serum (FBS) (Gibco), 1% v/v Penicillin-Streptomycin (Gibco), 0.5% v/v Fungizone (Amphotericin B) (Gibco), Dimethyl sulfoxide (DMSO), 0.5% Trypsin, 5 mg/ml MTT in PBS; cell washing media: PBS solution pH 7.2; 10% Sodium Dodecyl Sulfate (SDS) in 0.1N HCl as a stopper. Literature used included accredited national and international journals (non-predatory), proceedings, theses, WHO data, and national and international cancer therapy guidelines published in the last 10 years.

Extraction equipment included a maceration vessel, flannel cloth, sieve no. 40, stirring rod, blender, O'haus MB23 moisture balance, filter paper, desiccator, electric scale, Buchner funnel, oven, evaporator, and glassware. Cytotoxic test equipment included a liquid nitrogen tank, centrifuge (B. Braun Biotech International), autoclave, 37ºC incubator with 5% CO2 flow model 6200 (Napco), class II laminar air flow (Labconco), spectrophotometer on an ELISA reader (SLT 240 ATC), Nebauer hemocytometer (Olympus CKX41), sterile conical tubes (Nunclone), ultraviolet lamp, electronic balance (Sartorius), micropipettes 20-200 µL and 200-1000 µL (Pipetman), vortex machine, inverted microscope (Axiovert-25), magnetic stirrer, and digital camera. Tools for narrative review composition included Google, Google Scholar, PubMed, NCBI, Elsevier, DOAJ, Science Direct, SINTA, and the reference manager Mendeley.

Study Population and Design

The population used in the study for the cytotoxic test was the yellow root stem obtained from the Research and Development Center for Natural Resource Conservation Technology Samboja, East Kalimantan, and the population for the narrative review data was journal articles meeting the inclusion and exclusion criteria with the theme of the effect of berberine alkaloid compound from yellow root stem extract (*Arcangelisia flava* (L.) Merr.) on Bcl-2 and Bcl-xL gene expression in MCF-7 cells using immunocytochemistry. The type of research used was observational with a cross-sectional approach aimed at determining the cytotoxic activity of the ethanol extract of the yellow root stem (*Arcangelisia flava* (L.) Merr.) on MCF-7 breast cancer cells, followed by a narrative review with descriptive research through comprehensive study and deductive methods by collecting secondary data discussing the molecular mechanism of berberine cytotoxicity contained in the yellow root plant (*Arcangelisia flava* (L.) Merr.) on Bcl-2 and Bcl-xL gene expression in MCF-7 breast cancer cells.

Yellow Root Stem Extraction (*Arcangelisia flava* (L.) Merr)

Yellow root plants obtained from the Research and Development Center for Natural Resource Conservation Technology Samboja, East Kalimantan, were weighed and cleaned with water, then chopped into smaller sizes and dried in an oven at 40°C. Once dried, they were powdered and sieved with sieve number 40. The extraction process involved weighing 200 grams of yellow root stem powder, soaking it in 2 liters of 70% ethanol, stirring every 6 hours, letting it stand for 24 hours, and then filtering with filter paper. The filtrate was collected, and the process repeated until a clear filtrate was obtained. The filtrate was concentrated using a rotary evaporator to produce an extract, and the yield was calculated (BPOM RI, 2012).

Phytochemical Screening Test

The compounds in the ethanol extract of the yellow root stem were identified using a color test with specific reagents for each compound group as a phytochemical screening test. The results showed the presence of flavonoid, alkaloid, saponin, and tannin compounds. The method is described as follows:

- Flavonoid Test: Identification was carried out by dissolving the yellow root stem extract in hot methanol and adding 0.1 gram of Mg powder and 5 drops of concentrated HCl. The formation of orange or red color indicated the presence of flavonoids (Muflihah, Rusli, and Febrina, 2015).
- Alkaloid Test: 0.5 grams of yellow root stem extract was added with 1 ml of 2M HCl and 9 ml of distilled water, heated for 2 minutes, cooled, and then filtered. The filtrate was divided into 3 parts, each added with Dragendorff, Bouchardat, and concentrated HNO3 reagents. The formation of an orange precipitate in the Dragendorff test indicated positive alkaloids, a brown precipitate in the Bouchardat test indicated positive alkaloids, and a red color in the concentrated HNO3 test indicated positive alkaloids (Endarini, 2016).
- Saponin Test: 2-3 ml of yellow root stem extract was placed in a test tube, 10 ml of hot water added, cooled, and shaken vigorously for 10 seconds, then 1 drop of 2N HCl added. A positive test was indicated by the formation of stable foam 1-10 cm high for no less than 10 minutes (Muflihah, Rusli, and Febrina, 2015).
- Tannin Test: Identification was carried out by dissolving the yellow root stem extract in 10 ml of distilled water, filtering, and adding 3 drops of 1% FeCl3. A positive result was indicated by the formation of a greenish-black color (Muflihah, Rusli, and Febrina, 2015).

Cytotoxic Test on MCF-7 Breast Cancer Cells with MTT-Assay Method

The cytotoxic test was performed using the MTT assay method. Confluent MCF-7 cells were harvested and distributed into 96-well microplates at a density of 1x10^4 cells/well. Cells were incubated for 24 hours in a CO2 incubator for adaptation, preparing them for treatment. Culture media containing the sample was added after 24 hours in various concentration series (2800; 1400; 700; 350; 175; 87.5; 43.75) µg/ml with several replications and re-incubated for 24 hours. Solvent control (DMSO), cell control, and DMEM culture media control were used as controls. In the solvent control, MCF-7 cells were given DMSO in the same amount as in the sample. The culture media in the wells at the end of the incubation was removed by inverting the plate, then washed with 100 µL PBS for each well. Each well was then added with 100 µL MTT reagent (5 mg/ml stock). Incubation continued for 3-6 hours at 37°C until formazan formed. Living cells converted MTT to dark blue/purple formazan. Stopper reagent was then added to dissolve the formazan crystals, and the cells were incubated overnight at room temperature, protected from light. The microplate was shaken horizontally with a rotator for 10 minutes and then read with an ELISA reader at a wavelength of 595 nm. The absorbance results were converted to cell viability percentages (Sari and Handayani, 2020).

Cytotoxic Test Data Analysis

Absorbance data from each well was converted into cell viability percentages using the following equation:

$$\%\ \text{Viability} = \frac{\text{absorbance of treated cells} - \text{absorbance of media control}}{\text{absorbance of cell control} - \text{absorbance of media control}} \times 100\%$$

Analysis was continued to determine the linear regression between the log concentration of the test sample (ethanol extract of yellow root stem) and the probit of cell viability percentage using Microsoft Excel 2010, resulting in the following equation:

$y = a + bx$

x: log concentration of the test sample

y: probit of cell viability percentage

The antilog of x from the above equation represents IC50.

Literature Review on Bcl-2 and Bcl-xL Gene Expression

The type of literature review used was a narrative review, presented to examine and summarize previously published scientific literature to obtain a summary and identify gaps in the manuscript relevant to the research topic and questions. The results of the review on Bcl-2 and Bcl-xL gene expression activity in MCF-7 cells obtained from various valid sources were analyzed to gather data on the expression of Bcl-2 and Bcl-xL genes under the influence of berberine compounds found in yellow root plants (*Arcangelisia flava* (L.) Merr.). The analysis included the plant part used, extraction method, solvent, immunocytochemistry test method, cell culture used, and research results based on the interpretation of specific protein expression data.

RESULTS AND DISCUSSION

Description of the Characteristics of the Ethanol Extract of Yellow Root Stem

The yellow root plant (*Arcangelisia flava* (L.) Merr.) used in this research consisted of stem parts collected from the Samboja Research and Development Center for Natural Resource Conservation Technology, East Kalimantan, totaling 316 grams in coarse powder form. A total of 253 grams of dried yellow root stem powder was obtained. The yield of dried yellow root stem in relation to the wet weight was 80%. The coarse dried yellow root stem powder was then ground and finely milled using a blender and sieved with a number 40 sieve to reduce the particle size, thereby increasing the surface area, which maximizes the extraction process by enhancing the contact between the solvent and the powder (BPOM RI, 2014). The results of the organoleptic examination of the yellow root stem powder are shown in Table 1.

Based on the obtained organoleptic data, it can be used as a standard for good quality yellow root stem powder before further testing (Putra et al., 2018). The next step was to prepare the yellow root stem extract using the maceration method with 70% ethanol as the solvent. The maceration method was chosen for its simplicity, cost-effectiveness, ease of execution, and to prevent the degradation of plant compounds due to excessive heating. The 70% ethanol solvent was selected for the maceration process as it is expected to extract most of the active compounds from the yellow root stem. Ethanol has both polar (-OH group) and non-polar (CH2CH3 group) properties, allowing it to dissolve active compounds such as alkaloids, glycosides, flavonoids, and steroids. Ethanol is also more selective against mold and bacterial growth, non-toxic, neutral, has good absorption, can mix with water in any proportion, requires relatively less heat for concentration, and does not cause cell membrane swelling, thereby improving the stability of the dissolved medicinal compounds. The Indonesian Herbal Pharmacopoeia recommends using 70% ethanol for the maceration extraction method if no other solvent is available (Azis, Febrizky, and Mario, 2014; Putra et al., 2018). In this study, 29 grams of yellow root stem ethanol extract was obtained with a yield of 14.5%, indicating that the yellow root stem maceration extraction process with ethanol solvent was successful, as the yield percentage falls within the 10%-15% range as per the existing requirements (Hasan and Moo, 2014). The results of the organoleptic examination of the yellow root stem ethanol extract are shown in Table 2.

Based on the organoleptic examination results of the yellow root stem ethanol extract, they are consistent with previous research conducted by Umayah & Rachmawati (2016). This data can be used to determine any physical changes in the extract during storage. Physical changes are generally followed by chemical changes that can affect the efficacy of the extract. The characteristics of the yellow root stem ethanol extract in this study, based on literature reviews of previous research, are shown in Table 3.

Table 1. Organoleptic Examination Results of Yellow Root Stem Powder

Organoleptic	Result
Form	Slightly coarse powder
Color	Yellow
Odor	Characteristic yellow root
Taste	Bitter

Table 2. Organoleptic Examination Results of Yellow Root Stem Extract

Organoleptic	Result
Form	Thick
Color	Brown
Odor	Characteristic yellow root
Taste	Bitter

Table 3. Review of Standardization Results of Yellow Root Stem Ethanol Extract

Characteristic Type	Research Results	Reference
Water Content	8.3% ± 0.577%	Frihani *et al.,* (2019)
Drying Shrinkage	1.6% ± 0.07% b/b	Umayah and Rachmawati (2016)
Ash Content	Total ash ≤ 9.9% Acid insoluble ash ≤ 3.7%	Kemenkes RI (2017)

Based on a literature review from previous research, Frihani et al. (2019) tested the water content of the yellow root stem ethanol extract using the Sterling-Bidwell apparatus. The water content of the yellow root stem extract obtained in the study was 8.3% ± 0.577%, which meets the requirements set by BPOM RI (2019), where the acceptable water content is less than 10%. The principle of the method used involves evaporating the water from the material by distillation using a solvent with a boiling point higher than water but with a lower density than water, allowing the water and solvent to separate, with water being in the (Legowo, Nurwantoro, and Sutaryo, 2007). The drying shrinkage characteristics found by Umayah & Rachmawati (2016) showed a drying shrinkage of the yellow root stem extract of 1.6% ± 0.07% w/w, meaning that from 100 grams of extract, 1.6 grams of compounds were lost during heating. This result is in accordance with Kemenkes RI (2017), which states that it should be less than 10%. Generally, the lost compounds are the solvents used in the extraction and other volatile compounds in the yellow root extract (*Arcangelisia flava* (L.) Merr). Another characteristic is the ash content, which provides an overview of the internal and external mineral content from the initial process to the formation of the extract (Emilan et al., 2011). The total ash content of the thick yellow root stem extract recorded in the second edition of the Indonesian Herbal Pharmacopoeia monograph is no more than 9.9% (Kemenkes RI, 2017). High ash content in the extract indicates a high internal mineral content in the yellow root stem, where the higher the obtained ash content, the higher the mineral content in the material (Utami et al., 2017). The second edition of the Indonesian Herbal Pharmacopoeia monograph also notes that the acid-insoluble ash content in the yellow root stem ethanol extract is no more than 3.7% (Kemenkes RI, 2017). The acid-insoluble ash content indicates the presence of mineral or metal contamination that is insoluble in acid within a product. High acid-insoluble ash content suggests the presence of silicates from soil or sand, as well as metal elements such as silver, lead, and mercury (Utami et al., 2017).

Identification of Chemical Compounds in Yellow Root Stem Ethanol Extract

The identification of compounds in the yellow root stem ethanol extract was carried out qualitatively to determine the presence of secondary metabolites in the extract. Testing was conducted by observing color changes, foam formation, or precipitate formation after adding specific reagents to the extract, then comparing the results based on the reference literature. The identification results are shown in Table 4. Based on the results of the qualitative chemical tests, the ethanol extract of yellow root stem contains flavonoids, alkaloids, saponins, and tannins. To further confirm the presence of these compound groups, results from previous studies using Thin Layer Chromatography (TLC) analysis on yellow root stem extracts were reviewed. The findings of the compound identification using the TLC method are shown in Table 5.

Based on the identification results by Frihani et al. (2019), it can be discussed that the ethanol extract of the yellow root stem is positively identified to contain flavonoid compounds, which are likely not quercetin because the Rf value produced differs from the quercetin standard. A similar situation was observed for the identification of other alkaloids, which were not papaverine compounds as the correct standard used should have been berberine. For the identification of tannin compounds, it is likely the same as the standard used, gallic acid, indicated by the Rf value with a negligible difference.

The study by Mutiah et al. (2020), which identified the alkaloid compounds in the ethanol extract of the yellow root stem from samples taken from different regions, showed that all *Arcangelisia flava* samples produced orange spots, indicating all samples were positive for alkaloid compounds with nearly identical Rf values. The TLC profile of the ethanol extract of the yellow root stem from Pratama et al. (2018) revealed the separation profile of the ethanol extract of the yellow root stem with various solvents used. The TLC method on the extract used two solvent systems: n-hexane:ethyl acetate as a non-polar combination and chloroform:methanol:water as a polar solvent combination.

Table 4. Results of Qualitative Testing of Chemical Compounds in the Ethanol Extract of Yellow Root Stem

Compound	Result	Conclusion	Reference
Flavonoid	Orange	(+)	Formation of orange or red color indicates the presence of flavonoids (Muflihah, Rusli, and Febrina, 2015).
Alkaloid	Orange	(+)	Formation of orange precipitate in Dragendorff's test indicates positive alkaloid presence (Endarini, 2016).
	Brown ppt	(+)	Formation of brown precipitate in Bouchardat's test (Endarini, 2016).
	Red	(+)	Formation of red color in concentrated HNO3 test (Endarini, 2016).
Saponin	Stable foam	(+)	Formation of stable foam 1-10 cm high for at least 10 minutes (Muflihah, Rusli, and Febrina, 2015).
Tannin	Greenish-black	(+)	Formation of greenish-black color (Muflihah, Rusli, and Febrina, 2015).

Note: + (contains compound group); - (does not contain compound group)

Table 5. Review of Compound Identification Results Using TLC Method

Sample Identity	Extraction Method	Identification Results	Reference
Yellow root stem (*Arcangelisia flava* (L.) Merr.)	Remaceration method (70% ethanol solvent)	a. Flavonoid (Rf 0.29); Quercetin standard (Rf 0.93); Sitoborat reagent, UV254 nm (Yellow), UV 366 nm (Bright yellow) b. Alkaloid (Rf 0.12); Papaverine standard (Rf 0.66); Dragendorff reagent, UV254 nm (Orange), UV 366 nm (Yellow-orange) c. Tannin (Rf 0.84); Gallic acid standard (Rf 0.82); FeCl3 reagent, UV254 nm (Black), UV 366 nm (Black)	Frihani et al., (2019)
Yellow root stem (*Arcangelisia flava* (L.) Merr.) Samarinda (AfSR)	Maceration with Ultrasound-Assisted Extraction (UAE) method (80% ethanol solvent)	Positive alkaloid component with orange color using Dragendorff reagent	Mutiah et al., (2020)
Yellow root stem (*Arcangelisia flava* (L.) Merr.) around Palangka Raya, Central Kalimantan	Maceration method (96% ethanol solvent)	a. AfSR: UV254 nm (Rf 0.36) and UV 366 nm (Rf 0.46) b. AfBM: UV254 nm (Rf 0.32) and UV 366 nm (Rf 0.2) c. AfBT: UV254 nm (Rf 0.32) and UV 366 nm (Rf 0.4) d. AfMN: UV254 nm (Rf 0.32) and UV 366 nm (Rf 0.4) e. AfBP: UV254 nm (Rf 0.32) and UV 366 nm (Rf 0.4) Using mobile phase solvent combination: a. Rf n-hexane: Ethyl acetate 8:2 = -; 7:3 = -; 6:4 = - b. Rf Chloroform: Methanol: Water 15:2:1 = 0.52; 0.37; 0.11; 8:2:1 = 0.93; 0.65; 0.16; 5:2:1 = 0.98; 0.64; 0.26	Pratama et al., (2018)
Yellow root stem (*Arcangelisia flava* (L.) Merr.) from Meru Betiri National Park, Andongrejo Village, Tempurejo Subdistrict, Jember Regency	Ultrasonic extraction for 1 hour (methanol p.a solvent)	Positive berberine alkaloid based on berberine standard curve with a content of 0.31 ± 0.04% w/w	Umayah & Rachmawati (2016)
Arcangelisia flava stem from Suban Jeriji village, Rambang Dangku, Muara Enim, South Sumatra	Extraction with distilled water	Positive berberine alkaloid based on berberine standard curve with a content of 1.55 ± 0.12% w/w	Setyowati et al., (2014)

The TLC results showed that no spots appeared in the non-polar solvent combination for all solvent ratios. However, in the polar solvent combination, three spots appeared in all solvent combinations with different Rf values. The spots also formed 'tailing,' which complicated the observation process, although they could still be observed. Based on the presented Rf values, the

best combination of polar solvents to separate the spots was the ratio 5:2:1 due to the highest eluent resolution compared to other eluents. This is consistent with Rafi et al. (2017), who stated that compounds with greater affinity for the mobile phase or less affinity for the stationary phase will move faster than compounds with the opposite affinity. The study by Pratama et al. (2018) also confirmed that the resulting peak profile was very similar to that shown by the berberine compound standard.

The determination of the berberine alkaloid compound can be carried out using the densitometric TLC method with slight modifications. The stationary phase used was silica gel 60 F245 plates, and the mobile phase was a chloroform:methanol mixture (6:1). The presence of berberine was detected by observing spots under UV light and comparing them with the standard. The berberine standard was used as a reference based on the Rf value, while the berberine content in the *Arcangelisia flava* extract was calculated based on the berberine standard curve. Berberine was chosen because its structure is well known and is an active compound in *Arcangelisia flava* extract. According to the Indonesian Ministry of Health (2017), the berberine content in the thick extract of the yellow root stem should not be less than 18.60%. However, the berberine content found by Umayah & Rachmawati (2016) was 0.31 ± 0.04% w/w, and Setyowati et al. (2014) found only 1.55 ± 0.12% w/w. According to Rafi, Heriyanto, and Septaningsih (2017), technical factors influencing TLC separation, such as the stationary phase, sample application, mobile phase, chromatography vessel, and derivatization (coloring), may account for these differences.

Cytotoxic Activity of Ethanol Extract of Yellow Root (*Arcangelisia flava* (L.) Merr.) Stem

The cytotoxic activity test in this study was conducted to determine the toxic effects of the ethanol extract of yellow root stem (*Arcangelisia flava*) on MCF-7 cell cultures as an initial screening to assess its impact on inhibiting cancer cell proliferation. Cytotoxic activity is determined by the inhibition concentration 50% (IC50) parameter, which is the concentration that results in 50% inhibition of cell population proliferation (Ismaryani et al., 2018). The concentrations of ethanol extract used in the treatment were 2800; 1400; 700; 350; 175; 87.5; 43.75 μg/ml. Cisplatin was used as a positive control at concentrations of 100; 50; 25; 12.5; 6.25 μg/ml. Cisplatin was chosen as the positive control because it is one of the most potent chemotherapy drugs and a primary antineoplastic in cancer treatment, essential in this study to analyze and study the product or candidate compounds with cytotoxic activity against MCF-7 cells. The morphology of MCF-7 cells after 24-hour incubation with the ethanol extract and subsequent addition of MTT reagent, observed under an inverted microscope at 100x magnification after 4-hour incubation, is shown in Figure 1.

Based on Figure 1, normal MCF-7 cells appear in the control group as small, oval, clustered, and luminescent. A concentration of 2800 μg/ml of yellow root ethanol extract affects the morphological changes of the cells to become rounder and smaller with a lower cell density compared to the control. Dead cells are dark-colored and do not adhere to the tissue culture base because they lose their ability to maintain and provide energy for metabolic functions and cell growth. These changes in MCF-7 cell morphology support cytotoxic test data showing a decrease in the number of live cells with increasing concentrations of yellow root ethanol extract.

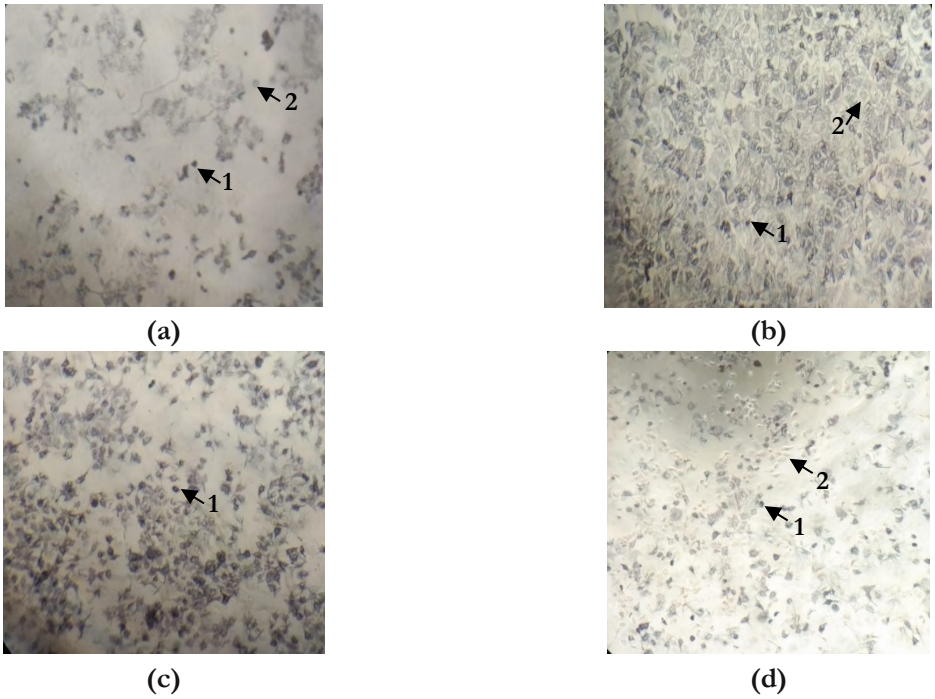


Figure 1. Morphology of MCF-7 cells at 100x magnification after the addition of yellow root ethanol extract and MTT reagent in treatments of (a) 2800 µg/ml concentration (b) 43.75 µg/ml concentration (c) cell control (d) Cisplatin 100 µg/ml concentration. Note: (1) Live cells (2) Dead cells.

According to the MTT method, live cells will form formazan crystals as seen in Figure 1. The reduction of MTT to formazan salts occurs if the mitochondrial reductase enzyme is active. Cell reduction involves an enzymatic reaction with NADH or NADPH produced by live cells to generate insoluble deposits. The breakdown of MTT occurs in the mitochondria of living cells by succinate dehydrogenase enzymes. The absorbance produced is proportional to the concentration of the purple formazan dissolved in SDS. The reduction of tetrazolium salts is a reliable way to determine cell proliferation. Yellow MTT tetrazolium salts decrease due to cell metabolic activity primarily by the action of succinate dehydrogenase enzymes (Nirwana, 2015; Sari and Handayani, 2020).

Yellow root ethanol extract can inhibit MCF-7 cell growth but not as significantly as the positive control (cisplatin). This can be seen from the number of formazan crystals produced and the cell death percentage; the higher the test compound content, the higher the cell death percentage, and the more formazan crystals produced, indicating a higher number of live cells, thus the resulting color intensity will be more purple. Data on treatment concentrations with both yellow root ethanol extract and the positive control against the percentage of MCF-7 cell viability and IC50 values are shown in Table 6.

Based on Table 6, it is clear that yellow root ethanol extract has a higher IC50 value than cisplatin as the positive control, with the IC50 value for cisplatin being 63.94 µg/ml, making cisplatin more toxic than yellow root ethanol extract. Cisplatin is a highly effective cytotoxic drug that works non-selectively because it is toxic to both cancer and normal cells, particularly those with high proliferation rates such as bone marrow. The high effectiveness of cisplatin also means it has a high nephrotoxicity side effect rate, with an occurrence rate of 20-30%. Potential side effects of cisplatin use include ototoxicity, gastotoxicity, bone marrow suppression, and allergic reactions, with the main side effect being nephrotoxicity

(Kurniandari, Susantiningsih and Berawi, 2015; Sari and Handayani, 2020).

Despite the higher IC50 value of yellow root ethanol extract compared to cisplatin, it can be seen from Table 6 that yellow root ethanol extract at doses ranging from 43.75 µg/ml to 2800 µg/ml can inhibit or reduce the proliferative properties of MCF-7 cells. The relationship graph between cell viability percentage and yellow root ethanol extract concentration is shown in Figure 2.

The cytotoxic test results of yellow root ethanol extract show a decrease in the percentage of live MCF-7 cells with increasing treatment concentrations (Figure 2). From the curve, a linear regression equation was derived, and the equation y=-26.529x+127.56 was obtained with a correlation coefficient (r) = 0.9855. The IC50 value was calculated using this equation, resulting in an antilog x value of 838.67 µg/ml. This indicates that yellow root ethanol extract has moderate cytotoxicity (100-1000 µg/ml) against MCF-7 cells. The cytotoxic activity of a compound is categorized into three types: potential cytotoxic if IC50 <100 µg/ml, moderate cytotoxic if IC50 is 100-1000 µg/ml, and less active if IC50 >1000 µg/ml (Widyanto et al., 2020). Based on previous studies on the cytotoxic activity of various components of the yellow root plant (*Arcangelisia flava* (L.) Merr.) against MCF-7 cells, it has moderate cytotoxic effects under three different treatments despite varying IC50 values (Table 7).

Based on the study of cytotoxic activity from various components of yellow root plant extracts against MCF-7 cells in previous research, it is known that various factors can cause significant differences in IC50 values, especially in yellow root ethanol extract, which has a relatively high IC50 value in this study. One of the causes is presumed to be the complexity of compounds contained in the extract. The low levels of berberine alkaloid, which has cytotoxic activity in several plant components tested, may also lead to a high IC50 value, and the reduction in the quality of the alkaloid compound in the extract can be influenced by various external factors such as the duration of concentration and storage, affecting the stability of the compound in the extract. The practitioner's precision and other variables during the cytotoxic test also significantly impact the optimal results in this study.

Table 6. Average absorbance, percentage of MCF-7 cell viability, and IC50 values for treatments with yellow root ethanol extract and cisplatin

Test Sample	Concentration (µg/ml)	Average Absorbance	Average % Viability	IC50 (µg/ml)
Yellow root ethanol extract	2800	0.359	40.80	838.67
	1400	0.372	42.78	
	700	0.438	53.21	
	350	0.477	59.20	
	175	0.545	69.88	
	87.5	0.574	74.47	
	43.75	0.581	75.46	
Positive control (cisplatin)	100	0.371	42.73	63.94
	50	0.406	48.20	
	25	0.563	72.69	
	12.5	0.672	89.79	
	6.25	0.695	93.33	

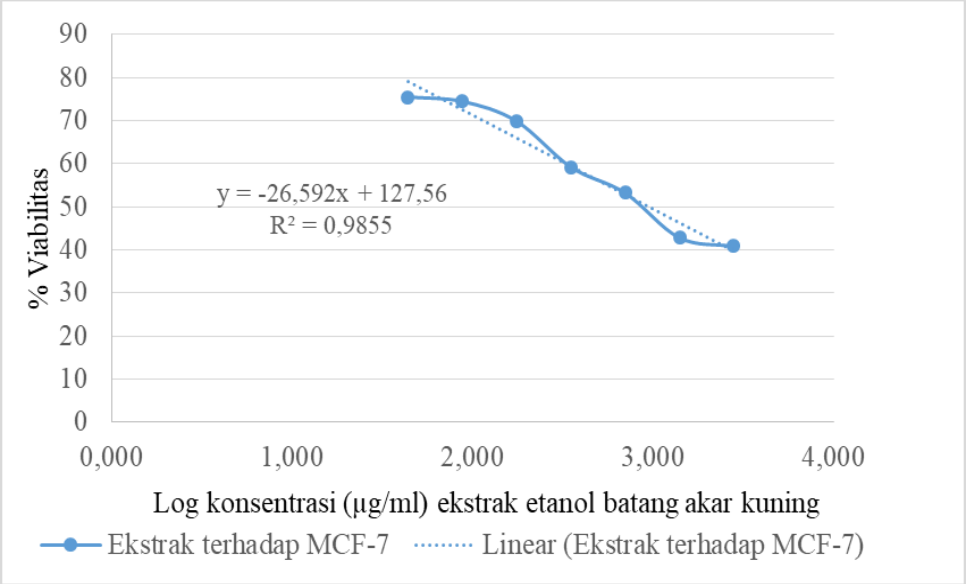


Figure 2. Graph of the relationship between cell viability percentage and yellow root ethanol extract concentration

Table 7. Review of the cytotoxic activity of yellow root (*Arcangelisia flava* (L.) Merr.) extract components against MCF-7 cells

Extract Component of *Arcangelisia flava*	Cytotoxicity against MCF-7 Cells	Reference
Ethanol Extract of *Arcangelisia flava* Leaves	136 + 17b µg/ml	(Puspitasari et al., 2015)
Ethanol Extract of *Arcangelisia flava* Leaves	135.74 ± 16.82 µg/ml	(Isparnaning et al., 2015)
Purified Leaf Extract of *Arcangelisia flava*	1829.84 ± 288.2 µg/ml	(Utami, Puspitasari and Pangaribowo, 2015)
Water Extract of *Arcangelisia flava* Roots	216.3 µg/ml	(Haryanti and Widiyastuti, 2017)

Expression of Bcl-2 and Bcl-xL Genes in MCF-7 Cells

The compounds found in yellow root (*Arcangelisia flava* (L.) Merr.) with anticancer activity include berberine. The anticancer mechanism of berberine in MCF-7 cells showing its influence on the expression of Bcl-2 and Bcl-xL genes from various studies can be seen in Table 8. Patil et al., (2010) in their study tested berberine compound isolated from purified berberine chloride using chromatography techniques. The results described berberine-induced apoptosis through cell cycle distribution and DNA fragmentation with agarose gel electrophoresis, having a significant impact as an anticancer with apoptosis effects tested on breast cancer cells, including MCF-7, due to its ability to reduce Bcl-2 and Bcl-xL gene expression through the mitochondrial pathway by increasing cytochrome c levels, caspase-9 activity, and PARP cleavage, leading to apoptosis.

According to Xie et al., (2015), in their study, berberine purchased from Wuhan Fortuna Chemical CO. Ltd. (China) was induced in MCF-7 and MDA-MB-231 breast cancer cells developed from Prof. Sun Y (Key Laboratory of Human Functional Genomics of Jiangsu Province, Nanjing Medical University, Nanjing, People's

Republic of China). The treatment of MCF-7 and MDA-MB-231 cells with berberine induction inhibition showed that berberine increases ROS (reactive oxygen species) production, which activates the pro-apoptotic JNK protein signaling. Phosphorylated JNK triggers mitochondrial membrane potential depolarization (ΔΨm) and decreases the expression of anti-apoptosis proteins Bcl-2 and Bcl-xL, along with increased expression of pro-apoptosis Bax protein. The downregulation of Bcl-2 anti-apoptosis family proteins in parallel with the loss of ΔΨm causes an increase in the release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria, ultimately leading to caspase-dependent and caspase-independent apoptosis. Overall, this study revealed that berberine provides antitumor activity in breast cancer cells by producing reactive oxygen species and mitochondria-associated apoptosis pathways.

Table 8. Literature Review of Bcl-2 and Bcl-xL Expression in MCF-7 Breast Cancer Cells by Berberine

Sample Identity	Method	Mechanism Result	Reference
Purified berberine chloride (90%) (Cat. No. 14050)	Western blotting	Decreased Bcl-2 and Bcl-xL gene expression through the mitochondrial pathway by increasing cytochrome c levels, caspase-9 activity, and PARP cleavage	(Patil, Kim, and Jayaprakasha, 2010)
Berberine from Wuhan Fortuna Chemical CO. Ltd. (China)	Western blotting	Increase (ROS, cytochrome C, JNK, AIF, and caspase 3), Decrease in Bcl-2 anti-apoptosis family including Bcl-xL and loss of ΔΨm	(Xie et al., 2015)
Berberine hydrochloride from Shanghai Jinhe Biological Technology Co., Ltd. prepared using the film dispersion method into targeted berberine liposomes	ELISA double-antibody	Decreased ABCC1, ABCC2, ABCC3, ABCG2, Bcl-2, mitochondrial permeability, increased cytochrome C release, Caspase 9/3	(Ma et al., 2013)
Berberine with 98% purity from Sigma Inc.	Western blotting	Decreased expression of anti-apoptosis Bcl-2 protein including Bcl-xL protein expression, and increased expression of pro-apoptosis Bax, caspase-3, and cleaved caspase-3 proteins	(Chen and Zhang, 2018)

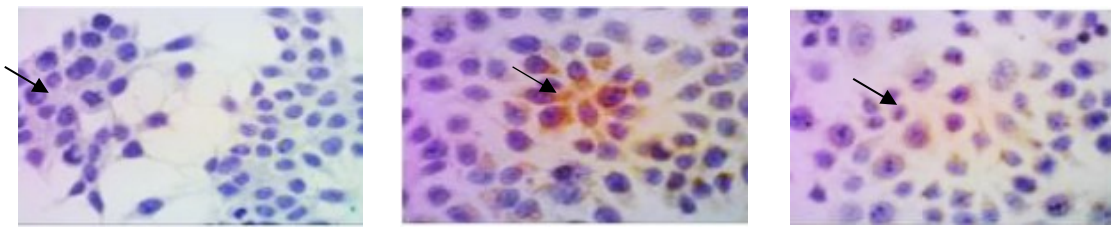
Based on the research conducted by Ma et al., (2013), one of the objectives was to determine the underlying mechanism of apoptosis protein from targeted berberine liposomes, prepared using the film dispersion method followed by membrane extrusion in MCF-7 breast cancer cell cultures by evaluating the expression of Bcl-2 family proteins (Bax, Bcl-2) and ABC transporters (ABCC1, ABCC2, ABCC3, ABCG2, ABCB1) from MCF-7 sourced from Macgene Biotech Co., Ltd., Beijing, China. The double-antibody ELISA method was conducted using an ELISA kit (Cusabio Biotech, Co. Ltd, Wuhan, China). In short, MCF-7 cell cultures were enzymatically separated by trypsin (0.25% EDTA) and cultured with serum-containing media for 24 hours under 5% CO2 at 37°C, followed by the addition of targeted blank liposomes (20 mM), free berberine (20 mM), berberine liposomes (20 mM), and targeted berberine liposomes (20 mM). The results showed that the rank of activity ratio for anti-apoptosis Bcl-2 protein from MCF-7 cell cultures was 0.33 ± 0.11 in targeted berberine liposomes; $<0.39 \pm 0.06$ in targeted blank liposomes; $<0.46 \pm 0.01$ in berberine liposomes; and $<0.47 \pm 0.08$ in free berberine. Targeted berberine liposomes mediated

the most efficient activation of pro-apoptosis Bax protein and suppression of anti-apoptosis Bcl-2 protein. The mitochondrial signaling pathway mechanism involved in the apoptosis process showed that targeted berberine liposomes activated pro-apoptosis Bax protein, suppressed anti-apoptosis Bcl-2 protein including Bcl-xL, and opened mitochondrial permeability transition pores, causing the release of cytochrome c from mitochondria into the cytosol, and activated apoptosis enzymes, caspase-9, and caspase-3.

Additionally, according to Chen & Zhang (2018) in their study, one of the aims was to investigate the molecular mechanism of the combination therapy of berberine and cisplatin for breast cancer in vitro on MCF-7 cells (ATCC® HTB-22™) and MDA-MB-231 cells (ATCC® HTB-26™). The western blotting method was used to detect Bax, Bcl-2, caspase-3, and cleaved caspase-3 levels in this study. The results showed a decrease in the expression of anti-apoptosis Bcl-2 protein, including Bcl-xL protein expression, whereas the expression of pro-apoptosis proteins increased in Bax, caspase-3, and cleaved caspase-3 in MCF-7 cell cultures. The molecular mechanism of each treatment, whether with berberine alone, cisplatin alone, or a combination of both, had the same mechanism, but the combination of cisplatin with berberine showed better results than monotherapy alone.

Based on the four reviewed studies, it can be concluded that the methods that can be used to determine the expression of anti-apoptosis proteins, especially for the Bcl-2 family including Bcl-xL, are the western blotting and double-antibody ELISA methods. According to the research by Arianingrum et al., (2016), on the observation of Bcl-2 apoptosis regulator protein expression in MCF-7 cells with treatments such as the addition of certain compounds like berberine, induced as in the four previous studies, or treatment with yellow root ethanol extract using immunocytochemistry methods with the principle of specific antibody binding. The research results visually, if showing that the extract can reduce Bcl-2 expression in MCF-7 cells, then the cytoplasm of the treated test cells has a blue color significantly different from the control cells with specific Bcl-2 antibodies (anti Bcl-2) which are brown. The study used control cells without specific antibodies that were blue as a comparison because Bcl-2 expression was not detected due to the absence of specific Bcl-2 antibodies recognizing it (Figure 2).

The berberine compound contained in yellow root ethanol extract has cytotoxic activity with its molecular mechanism capable of reducing the overexpression of anti-apoptosis Bcl-2 proteins including Bcl-xL, indicating that MCF-7 breast cancer cells are not resistant to yellow root ethanol extract. Suppression or reduction of Bcl-2 protein expression can cause apoptosis induction because the release of cytochrome c by mitochondria is not hindered, which can subsequently activate the caspase pathway.



(a) **(b)** **(c)**

Figure 2. Observation of Bcl-2 protein expression in MCF-7 cells using an inverted microscope (a) Control cells without specific antibodies (b) Control cells with specific antibodies (c) Cells treated with optimal concentration extract (Arianingrum et al., 2016)

CONCLUSION

Yellow root ethanol extract (*Arcangelisia flava* (L.) Merr.) has potential as a chemopreventive agent with moderate effects based on its cytotoxic activity against MCF-7 breast cancer cells with an IC50 value of 838.67 µg/ml. The berberine alkaloid compound contained in the yellow root plant (*Arcangelisia flava* (L.) Merr.) affects the expression of Bcl-2 and Bcl-xL by decreasing it through the mitochondrial pathway in MCF-7 breast cancer cells. The impact on Bcl-2 and Bcl-xL expression in MCF-7 cells necessitates direct research using immunocytochemistry methods by isolating the berberine alkaloid compound from the yellow root plant extract (*Arcangelisia flava* (L.) Merr.).

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