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Cytotoxic Activity Test of Kepel Leaf Extract and Fraction (Stelechocarpus burahol Hook. f. and Thomson) Against T47D Breast Cancer Cells and P53 and BCL-2 Protein Expression

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A B S T R A C T

This study aims to determine the cytotoxic activity of ethanol extract, n-hexane fraction, ethyl acetate fraction, and water fraction of kepel leaves (Stelechocarpus burahol Hook. Data were analyzed using anova test. The results showed that ethanol extract has cytotoxic activity against T47D breast cancer cells with IC50 value of 53.766 µg/ml, while the results of n-hexane fraction, ethyl acetate fraction and water fraction have IC50 values of 178.184 µg/ml, 52.766 µg/ml and 120.754 µg/ml. The most active fraction according to the IC50 value is the ethyl acetate fraction, although there is no significant difference with the ethanol extract. Extracts and ethyl acetate fractions have selectivity values against T47D breast cancer cells. Protein expression results that the extract and ethyl acetate fraction were able to increase p53 protein expression with a value of 50.743 ± 4.65 µg/ml and 49.869 ± 1.67 µg/ml and were able to inhibit bcl-2 protein expression with a value of 53.428 ± 0.822 µg/ml and 52.511 ± 0.437 µg/ml.

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INTRODUCTION

Cancer is the rapid growth of abnormal cells in the body that can cause disease, can attack surrounding tissues to other organs. Cancer is currently a health problem in the world including Indonesia. The type of cancer that many women suffer and fear is breast cancer. Based on data from the World Health Organization (2020), deaths from breast cancer in Indonesia are 1,008 or 12.9% of total deaths, beating cervical cancer which has an incidence rate of 631 or 8.1% (Prihantono, 2023). Cancer is caused by mutations in tumor suppressors, namely mutations in p53 and Bcl-2. The p53 protein is a tumor suppressor protein that acts as a cell cycle regulator. Mutations in p53 cause a decrease in cell apoptosis mechanisms. This can lead to cancer in the body and cause cells to become uncontrollable. Mutation of bcl-2 can lead to increased expression that can suppress the normal function of pro-apoptotic proteins. If the protein is downregulated, the cell will lose the ability to regulate apoptosis, which can lead to cancer (saraswati, 2020). Kepel leaves have potential as a cytotoxic substance. Kepel leaves contain antioxidant activity related to anticancer potential so that they can inhibit the acceleration of growth in cancer cells. Based on the results of the phytochemical study of kepel leaves, it shows the content of alkaloid compounds, flavonoids, polyphenols, saponins, triterpenoids, terpenoids and quinones. Kepel leaves show potential as anticancer, with research indicating the presence of cytotoxic substances that can fight cancer cells, including colorectal carcinoma. Kepel leaf extracts have IC50 values that indicate effectiveness in inhibiting cancer cell growth. The study explained that the in vitro potential of cytotoxic substances of kepel leaves against colorectal carcinoma. The results showed that kepel leaf extract can inhibit the growth of cancer cells and can be used as a development of cancer therapy. Flavonoid compounds as free radical-capturing antioxidants produced 5 active isolates capturing free radicals and the highest antioxidant activity was found in isolate B4b with EC50 6.34 µg/mL whose identification showed 3,7,3',4'- tetrahydroxy-5methyl flavone (sunarni, 2007).Research on the activity of kepel leaf extracts and fractions and p53 protein expression and bcl-2 protein expression against T47D breast cancer cells has never been done. Based on the results of phytochemical studies of kepel plants, it shows the content of phenolics, flavonoids, tannins, alkaloids, saponins and triterpenoids/steroids (Rondevaldova, 2024) Flavonoid compounds have potential as anti-cancer through the mechanism of inhibition of the cancer cell cycle, induction of apoptosis and inhibition of angiogenesis. Flavonoid compounds can have anticancer activity because they have Reactive Oxygen Species (ROS) enzyme activity in stopping the cell cycle, inducing apoptosis, suppressing cancer cell proliferation and invasion. Isoflavones can arrest breast cancer cells in the G2/M phase and apoptotic ROS. Antioxidant and pro-oxidant activities in anti-cancer involve flavonoid compounds. Isoflavones can arrest breast cells in G2/M phase and ROS apoptosis. Flavanones induce apoptosis of carcinoma, esophageal cancer, hepatocellular carcinoma and breast cancer cells through activating the mitochondrial apoptotic pathway by increasing ROS production (Deluna, 2023).

THEORETICAL REVIEW

A frequently used method for traditional drug discovery is the extraction method. The choice of extraction method depends on the nature of the material and the compound to be isolated. Extraction can be done by maceration, percolation, digestion, reflux. In addition, the maceration method can be modified using an extractor equipped with a heating mantle. Maceration is commonly used in the preparation of homemade tonics for a long time and is a popular and inexpensive way to obtain bioactive compounds (Senapati & Behera, 2023). Maceration is an old technique used for drug preparation. Maceration is a solid-liquid extraction method in which the bioactive compounds (solutes) in the plant material are extracted by soaking the plant material in a specific solvent for a certain period of time. Maceration is a simple method using uncomplicated equipment and for this reason is a popular choice for researchers. Unfortunately, the duration of the extraction time is long and sometimes time-consuming (Njila et al., 2017).

When large quantities of extractions are performed, it is not possible to extract every plant sample with a customized solvent system. A common procedure should be developed and validated, taking into account the detection rates of active extracts ("hits") obtained using multiple extraction methods, and followed by an analysis of false positive response rates. This approach has been used to develop common extraction protocols to be used in extracting plant constituents used to perform in vivo or in vitro biological screening (Sabotič et al., 2024). Fractionation is the process of separating a plant extract into various fractions. It further separates the fractions into parts consisting of a number of compounds. The process continues until the pure compound is isolated. When multiple solvents are required for fractionation, they should be added according to the order of increasing polarity (Abubakar & Haque, 2020).

Plant secondary metabolites play an important role in determining the biological activity of medicinal plants used in traditional medicine. Therefore, identification and isolation of secondary metabolites are important for standardization and quality improvement of plant extracts (Sholikhah, 2016). Flavonoids are secondary metabolites of polyphenols found widely in plants as well as foods and have various bioactive effects including one as an antioxidant. Flavonoid compounds are polyphenolic compounds that have 15 carbon atoms arranged in the C6-C3-C6 configuration, meaning that the carbon skeleton consists of two C6 groups (substituted benzene rings) connected by a three-carbon aliphatic chain. Flavonoids are significantly found in several parts of plants such as fruits and vegetables which play a role as neurotrophins in mammals, reduce angiogenesis, antioxidant substances, resistance to aging morphological changes (Arifin et al., 2018).

In vitro cytotoxicity testing using cell cultures is used in the safety evaluation of drugs, cosmetics, food additives, pesticides, and to detect the antineoplastic activity of a compound. One of the developments in this method is to predict the presence of compounds that are toxic to cells, which can be used to detect the anticancer activity of a compound. Antineoplastic activity indicates

that there is a greater difference in the response given by cancer cells than by normal cells (Dona, 2019). Cytotoxicity testing using the MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is based on enzyme activity that can be measured colorimetrically. The MTT method is fast, sensitive, accurate, and a large number of samples can be tested automatically using a spectrophotometer. The MTT method measures living cells (whether still dividing or not) and metabolic activation or cell inhibition (Amir & Murcitro, 2017). Testing of T47D breast cancer cells and Vero cells. T47D breast cancer cells were grown in RPMI 1640 culture medium, while Vero cells were grown in M199 culture medium. The selectivity used as an indication of cytotoxic selectivity (safety level) of kepel plant extract against normal cells (Vero) was toxic to cancer cells but not toxic to normal cells. An extract of the kepel plant is said to have high selectivity if the SI value is less than 3.

The T47D cell line (human ductal breast epithelial tumor cell line) is often used in in vitro cancer research because it is easy to handle, has unlimited replication capacity or rapid growth, has high homogeneity, and can be easily replaced with new frozen cells in case of contamination (Abcam, 2007). T47D cells can express estrogen receptors, commonly referred to as ER-positive, and express mutated p53. Molecularly, the cells undergo p53 mutations, causing them to lose control over cell cycle regulation (Tusanti, 2014).

The cytotoxicity testing method uses the MTT analysis method, the principle of which is the conversion of tetrazolium salt (3-(4,5-dimethylazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan in active mitochondria in living cells. In addition to testing cytotoxicity, this study also calculated the selectivity index value, which indicates the selectivity (safety) of a test solution against normal cells. In this study, Vero cells were used as normal cells. Active extracts and fractions will be further tested using immunocytochemistry to determine the effect of p53 and Bcl-2 protein expression on T47D breast cancer cells.

METHODOLOGY

Tools

Sentrifuge, autoklaf, sentrifuge sigma 3K12 (B, Braun Biotech Internasional), Laminar Air Flow kelas II (Labconco), ELISA reader (SLT 240 ATC), Nebauer Haemocytometer (BDH Merck) tabung konikal steril, microplate 96 sumuran (Biologix) lampu ultra violet, neraca elektrik, mikropipet (pipetman), mesin vortex, mikroskop terbalik (Axiovert-25), labu kultur jaringan (Nunclone), inkubator 37^0C (Merck), pengaduk magnetik, dan kamera digital.

Materials

The materials used in this study include kepel leaf (*Stelecocharpus burahol*) leaves are dry pollinated and for extraction using 96% ethanol. Materials for cytotoxic and immunocytochemical tests are T47D cell line, Vero cells, p53 and bcl-2, RPMI 1640 media (Gibco), sodium bicarbonate, vortex machine, tissue culture flask (Nunclone), HEPES (Sigma), penicillin-streptomycin (Gibco), DMSO (Dimethyl Sulfoxide), Functional 0.5% MTT 5 mg/ml, Trypsin 0.5%, M199

Medium, PBS solution, in PBS, 10% Sodium Dodecyl Sulfate in 0.1 N HCl as a stopper for cell washing media, PBS solution pH 7.2.

Procedure

The extraction process of kepel leaf powder was carried out by maceration method using 96% ethanol. The filtrate was then evaporated using an evaporator (temperature maintained at 40-50^{0}C) until a thick extract was obtained. The cytotoxic test was carried out by inserting 104 T47D cells into a 96 microplate of 100 µL each and then the cells were incubated for 24 hours to reattach after harvesting. Stock solution of ethanol extract and kepel leaf fraction with 10 mg/100 µL in DMSO. Then from the stock solution a concentration series was made in the culture medium with variations of 500; 250; 125; 62.5; 31.25; 15.625; 7.81 µg/mL. After that, the culture medium was discarded and 10 µg MTT was added to each well. Living cells will react with MTT and form purple formazan crystals. After that, incubate for 4 hours in a 5% CO_2 incubator at 37^{0}C after incubation, add 10% stopper into each well and incubate in a dark place for 24 hours at room temperature.

Experimental Procedure

Sample Preparation

Fresh kepel leaves taken from Mukiran, Kaliwungu, Semarang were identified to justify sample credibility. Leaves were taken that were dark green in color, the leaves were taken when the plant was flowering. The leaves were oven dried at 40 □C to dry. Kepel leaves that have dried are then pulverized into powder and sieved on sieve no. 40. Kepel leaf powder is then macerated with 70% ethanol solvent to obtain kepel leaf extract. The extract obtained was then fractionated through liquid-liquid technique using n-hexane, ethyl acetate and water solvents.

Characteristics of simplisia and extracts

Knowing the drying shrinkage of simplisia and kepel leaf extracts is done through the Moisture Analyzer tool, namely by weighing simplisia and extracts of each as much as + 2 grams. Then the Moisture Analyzer is closed and waited until it gives a sign and sound, the determination of drying shrinkage is repeated 3 times. Determination of water content of kepel leaf extract was carried out by means of toluene distillation, the determination of water content was repeated 3 times.

Identification of chemical compounds

The content of chemical compounds in yakon leaves was identified by tube reaction and KLT. Chemical compounds identified include flavonoids, saponins, alkaloids, tannins and triterpenoids / steroids. Flavonoid tube reaction identified with cyroborate reagent comparator quersetin and rutin, saponins identified anisaldehyde reagent concentrated sulfuric acid, alkaloids identified with dragendorf perection, tannins identified with FeCl3 5% reagent and triterpenoids / steroids identified with stigma sterol comparator. On the

stationary phase KLT used is silica gel GF254, on the mobile phase flavonoids motion n-hexan: ethyl acetate: formic acid (6:4:0.2), saponins chloroform: methanol: water (60:30:10), alkaloids ethyl acetate: methanol: water (90:9:1), tannins methanol: water (9:1) and triterpenoids / steroids n-hexan, ethyl acetate (7:3).

Cytotoxic test

Harvested T47D cells and vero cells were counted using a Haemocytometer to calculate the cells needed for the cytotoxic test, after obtaining the required volume ad 10 ml of complete medium. Cells were placed into a 96-well microplate at a concentration of 2 x 10^4 cells / 100 µl well, then incubated for 24 hours to adjust and adhere to the wells until the cells were ready for treatment. Preparation of stock solutions with a series of test concentrations on cells in culture medium through dose variance of 500 µg/ml; 250 µg/ml; 125 µg/ml; 62.5 µg/ml; 31.25 µg/ml; 15.625 µg/ml: 7.81 µg/ml. The samples were put into each microplate according to the concentration series, incubated for 12 hours, after which the MTT assay was carried out and the absorbance was read with an ELISA reader at 595 nm and the IC50 value was calculated. In this study, doxorubicin was used as positive control.

Immunocytochemical assay

Transferring cell cultures of up to 5 x 104 cells/well to 24 wells filled through glass coverslips, then incubating the cells in a 5% CO2 incubator at 37°C for 12 hours. After cell recovery, the cells were treated with extracts and fractions at concentrations of ½ IC50, 1 IC50 and 2 IC50, then re-incubated for 15 hours. Furthermore, the extracts and active fractions were subjected to immunocytochemical assay through indirect method using primary antibodies, namely anti p53 antibody and anti bcl-2 antibody.

Positive control doxorubicin was used as a comparison. By using an inverted microscope, the expression of p53 and Bcl-2 is known through the brown color in the cell nucleus and cytoplasm. Immunocytochemical observation using primary monoclonal antibodies, if the cell nucleus produces p53 and Bcl-2 proteins in brown/dark color, it is positive, and if the cell nucleus is fixed through hematoxylin to produce purple color, the result is negative. The expression of p53 and Bcl-2 protein was quantitatively calculated using image J software application.

RESULTS

Characteristics of the extract

The drying shrinkage rate of kepel leaves was 4.16 ± 0.174. This is based on the drying shrinkage requirement which is <10% (Ministry of Health, 2008). The moisture content of kepel leaf extract is obtained 6.833 ± 2.466 v/b or <10%, it can be concluded that kepel leaves are in accordance with the standard moisture content of less than 10% to minimize the presence of contamination such as fungi (Ministry of Health, 2000).

Phytochemical screening

Phytochemical screening was carried out by tube reaction and KLT. The tube reaction showed positive results of extracts containing flavonoids, polyphenols, triterpenoids/steroids. From the KLT results, the ethanol extract and ethyl acetate fraction were positive for flavonoids.

MTT assay results and selectivity index

The IC50 value represents the concentration that can inhibit 50% of cells from proliferating. An IC50 value of 10–20 µg/ml is considered highly active, while an IC50 value >20 µg/ml is considered less active. If the IC50 value is <100 µg/ml, the compound still has cytotoxic properties against cancer cells. The study results showed that the ethanol extract of kepel leaves and the ethyl acetate fraction of kepel leaves exhibited cytotoxic activity with IC50 values of 53.76 µg/ml and 52.82 µg/ml, respectively, while the water fraction and n-hexane fraction had IC50 values exceeding 100 µg/ml, indicating no cytotoxic activity. The selectivity index values of the extracts and fractions of Kepel leaves were calculated, with the selectivity index obtained by dividing the IC50 value of Vero cells by the IC50 value of T47D cells. Selectivity indicates that the extracts and fractions only target cancer cells while leaving normal cells unaffected. A selectivity index value greater than 3 indicates selectivity, targeting only cancer cells while not affecting normal cells (Rahmawati et al., 2016). The selectivity index value indicates safety for normal cells (Alali et al., 1999).

Test solutions that exhibit cytotoxic activity and have a selectivity index greater than 3 are safe for normal cells and cytotoxic to cancer cells, so they are followed up with immunocytochemistry testing to observe the expression of p53 and bcl-2 proteins, which influence apoptosis. The results of the study showed that the extract and ethyl acetate fraction exhibit cytotoxic activity and have a selectivity index greater than 3. The extract and ethyl acetate fraction were then subjected to immunocytochemistry testing.

Table 1. Results of the MTT assay and selectivity index values of kepel leaf extract, n-hexane fraction, ethyl acetate fraction, and water fraction on T47D breast cancer cells

Sampel	IC50 Sel Vero (µg/ml)	IC50 Sel T47D (µg/ml)	Nilai indeks selektivitas
Ekstrak	276,90 ± 3,60	53,76 ± 3,98	5,2424
Fraksi N-Heksan	266,36 ± 7,93	178,19 ± 14,56	1,4948
Fraksi Etil Asetat	565,98 ± 5,20	52,82 ± 4,42	10,5259
Fraksi Air	774,97 ± 2,98	120,76 ± 5,53	6,4178
Kontrol Positif	1,28 ± 2,54	1,24 ± 2,18	1,0322

Immunocytochemistry results

Immunocytochemistry testing was performed on four treatment groups, namely the untreated group (cell control), positive control, and groups given kepel leaf extract with each concentration of ½ IC50 (26.883 µg/ml), 1 IC50

(53.766 µg/ml), 2 IC50 (107.532 µg/ml), ethyl acetate fraction with each concentration of ½ IC50 (26.411 µg/ml), 1 IC50 (52,822 µg/ml), 2 IC50 (105,644 µg/ml).

Table 2. Results of increased expression of p53 extract and ethyl acetate fraction of kepel leaves in T47D breast cancer cells

Perlakuan	Konsentrasi (µg/ml)	Konsentrasi (µg/ml)	Rata-rata Ekspresi p53 ± SD	Peningkatan Ekspresi p53 (%) ± SD	EC50 ± SD (µg/ml)
Ekstrak	½ IC50	26,883	7,958	6,958	50,734 ± 4,65
	1 IC50	53,766	13,348	12,348	
	2 IC50	107,532	17,224	16,224	
Etil Asetat	½ IC50	26,411	8,374	7,374	49,869 ± 1,67
	1 IC50	52,822	10,532	9,392	
	2 IC50	105,644	11,675	10,675	
Kontrol Sel		0	6,323		

Table 3. Results of increased expression of bcl-2 extract and ethyl acetate fraction of kepel leaves in T47D breast cancer cells

Perlakuan	Konsentrasi (µg/ml)	Konsentrasi (µg/ml)	Rata-rata Ekspresi p53 ± SD	Peningkatan Ekspresi p53 (%) ± SD	EC50 ± SD (µg/ml)
Ekstrak	½ IC50	26,883	28,950	27,950	53,428 ± 0,822
	1 IC50	53,766	22,407	21,470	
	2 IC50	107,532	20,384	19,384	
Etil Asetat	½ IC50	26,411	21,163	20,163	52,511 ± 0,437
	1 IC50	52,822	14,384	13,384	
	2 IC50	105,644	9,633	8,633	
Kontrol Sel		0	25,06		

DISCUSSION

From the research concluded that there is qualitative testing of kepel leaves, extracts, and fractions aimed to determine the presence of secondary metabolite compounds in kepel leaves and could also be used as a qualitative description of the content of the crude drug. The test results are shown in Table 1. From the qualitative testing table, flavonoids showed positive results due to a chemical reaction that caused a color change in the test solution. Flavonoids are known to have anticancer activity that can protect against cell damage. When compared with the qualitative test using TLC, the results can be seen in Figure 1. This indicates that the kepel leaf extract sample tested positive for flavonoids and terpenoids.

Next, morphological observations of the cells were conducted under a microscope. Living cells react with MTT, which is broken down by dehydrogenase enzymes into insoluble formazan. The number of living cells is proportional to the formation of formazan crystals. Testing with the MTT method is based on the conversion of yellow tetrazolium salt by succinate dehydrogenase enzymes with NaDPH into a blue-purple color called formazan. The principle of the MTT assay

method measures the activity of mitochondrial enzymes that reduce MTT into formazan crystals. The MTT assay works by measuring the activity of mitochondrial dehydrogenase in living cells, which converts the yellow tetrazolium salt MTT into a purple-colored form. This process reflects cell viability, where the more live cells there are, the higher the absorbance produced. Live T47D cells are oval-shaped, transparent, and slightly brighter because live cells have cytoplasm that can transmit light from the microscope.

Dead cells will be discarded along with the medium in the microplate, leaving live cells attached to the bottom of the microplate. MTT is then added to each well, incubated at 37°C with 5% CO2 flow for 4 hours. The difference between dead and live cells becomes apparent after the addition of MTT salt. Live cells form formazan crystals, as shown in Figure 2. The reaction is then stopped by adding a 10% SDS stopper reagent in 0.1 N HCl. The function of SDS is to denature proteins in the polypeptide chain units, forming SDS-polypeptide complexes and dissolving formazan. The purple color of formazan can be measured using an ELISA reader at a wavelength of 595 nm, as formazan has maximum absorbance at this wavelength. This allows for accurate detection of formazan concentration, which correlates with the number of live cells in the sample. Absorbance indicates the number of live cells; the stronger the intensity of the color formed, the higher the absorbance. This is influenced by the amount of MTT absorbed by the cells and broken down. Absorbance is used to calculate the percentage of viability, which can be seen in Figure 3. The figure shows the percentage of viability of the extract and fraction of kepel leaves against T47D cells, indicating that as the concentration of the extract and fraction increases, the lower the percentage of viability of T47D breast cancer cells and the higher the percentage of inhibition of T47D breast cancer cells.

The intensity of the color formed is proportional to the number of living cells, so that the absorbance value obtained can be converted into a percentage viability vs. log concentration equation and the equation y = ax+b is obtained. The IC50 value can be seen in Table 3. The IC50 (Inhibitory Concentration 50) value indicates the concentration that can inhibit 50% of cells from proliferating and the toxicity potential of a compound toward cells. The IC50 value is considered to have potential as an anticancer compound if the value is < 10 µg/mL (very active), 10–100 µg/mL (active), and > 100 µg/mL (less active or inactive).

Doxorubicin is a single compound that has a broad spectrum of anticancer effects and is sensitive to breast cancer cells. Doxorubicin is able to form a tripartite complex with topoisomerase II and DNA. Topoisomerase II is an ATP-dependent enzyme that binds to DNA and causes double-strand breaks at the 3-phosphate end, allowing strand exchange and DNA straightening. This strand unwinding is followed by DNA strand ligation by topoisomerase II. This topoisomerase is crucial for DNA replication and repair. The formation of this tripartite complex inhibits the rejoining of DNA strands, causing cell cycle arrest in the G1 and G2 phases and inducing apoptosis11. The extract, compared to the fraction, exhibits low cytotoxic effects because the extract still contains compounds polar, semipolar, nonpolar, and other impurities. The ethyl acetate fraction has a better IC50 value than the n-hexane fraction and the water fraction. This indicates that the ethyl

acetate fraction has higher cytotoxic activity compared to the n-hexane fraction and the water fraction based on the phytochemical screening results of the kepel leaf extract and the ethyl acetate fraction positively contains flavonoid compounds12. The mechanism by which flavonoids induce apoptosis involves inhibiting the activity of topoisomerase I/II, where the enzyme functions to control DNA topology. The presence of inhibitors causes DNA damage in cancer cells, affecting cell replication and ultimately leading to cell death. Other detected compounds include polyphenols such as carnosic acid, rosmarinic acid, and carnosol. Carnosic acid induces apoptosis by increasing the expression of pro-apoptotic protein Bax and decreasing anti-apoptotic protein Bcl-2. Rosmarinic acid is a flavonoid compound that has the ability to inhibit the growth of cancer cells by inhibiting the formation of the enzyme Nitric Oxide Synthase (NOS). Inhibition of this process will trigger apoptosis of cells through the p5313 pathway. The ethyl acetate fraction has a better IC50 value than the extract because it contains terpenoid compounds such as ursolic acid, betulinic acid, and oleanolic acid. Betulinic acid can inhibit the formation of the topoisomerase I-DNA apoptosis complex and prevent topoisomerase. I from inducing the apoptosis process. Ursolic acid can inhibit the activity of DNA polymerase and DNA topoisomerase and reduce the rate of cell proliferation. Ursolic acid can also induce apoptosis through TNF-related apoptosis-inducing ligand in cancer cells and JNK-mediated upregulation of death receptor (DR) and downregulation of decoy receptor-2 (DcR2), thereby affecting cell survival. Breast cancer cells treated with ursolic acid in vitro demonstrated cytostatic activity in the G1 phase. Known mechanisms that affect intracellular Ca2+ signals to reduce cell proliferation 15. Oleanolic acid suppresses aerobic glycolysis.

Disruption of nucleotide and amino acid biosynthesis contributes to the growth of proliferating cells such as tumor cells. Betulinic acid shows significant inhibition of tumor formation in tumor size. Histopathological analysis of tumors also showed reduced angiogenesis, proliferation, and invasion of cancer cells in treated animals. Phytochemical screening results indicated that the ethyl acetate fraction of the extract contained flavonoids and polyphenols. Chemopreventive agents must have selectivity for cancer cells over normal vero cells, meaning that only cancer cells are killed. Currently, chemotherapy drugs have a mechanism of action that can attack both cancer cells and normal cells, so normal cells can also be damaged and various side effects can occur. Extracts and fractions exhibit cytotoxic activity against both cancer cells and normal cells. The IC50 values for Vero cells are shown in Table 4. The IC50 values for Vero cells indicate that the extract, ethyl acetate fraction, water fraction, and n-hexane fraction do not exhibit active cytotoxic activity, with IC50 values >100 µg/ml. However, doxorubicin exhibits active cytotoxic activity with IC50 values < 10 µg/ml. This means that doxorubicin is a chemotherapy drug with strong cytotoxic activity against various cell types, not only cancer cells but also normal cells (Vero), which can be affected by cytotoxic activity.

The cytotoxic activity of doxorubicin on Vero cells may occur because doxorubicin binds to DNA and interferes with replication and transcription, causing cell death. Doxorubicin can also inhibit topoisomerase II enzymes for the

maintenance of DNA structure during cell replication. Doxorubicin can produce reactive oxygen species (ROS) that can damage lipid, protein, and DNA components, contributing to cell death. Vero cells are not cancer cells, yet they can still exhibit a cytotoxic response to doxorubicin, with a different level of sensitivity compared to cancer cells. Immunocytochemistry (ICC) is a method used to detect the presence of specific protein expression or antigens in cells using specific antibodies that bind to proteins or antigens. Antibodies that do not bind are separated by washing, and detection is carried out continuously with labeled primary antibodies labeled primary antibodies, while indirect detection is performed with enzyme-labeled or fluorescent secondary antibodies for bound antibodies17. The expression of p53 and bcl-2 proteins is indicated by the binding of the proteins to p53 and bcl-2 monoclonal antibodies, which are detected as brown coloration in the cytoplasm and cell membrane of T47D cells. Data analysis using ImageJ software was performed to semi-quantitatively calculate Bcl-2 protein levels by counting the number of threshold pixels (area) and the percentage of threshold pixels (% area). The immunocytochemistry results of T47D cells for p53 expression can be seen in Figure 3, and Bcl-2 expression can be seen in Figure 4. The average percentage of T47D cancer cells expressing p53 and Bcl-2 after immunocytochemistry staining for all treatment groups can be seen in
The EC50 (Effective Concentration 50) value is the concentration of a compound required to achieve 50% of the maximum effect that can be achieved by that compound. This measure is crucial in determining the potential of a drug or active compound. A percentage increase in p53 protein expression is considered active if the EC50 value is < 1 µM, indicating a highly active compound; a value of 1 µM–10 µM indicates an active compound; and a value > 10 µM indicates an active compound but with low potency (Muna, 2022). Based on the results in Table 13, the percentage increase in p53 protein expression for the extract was 50.734 ± 4.65 and for the ethyl acetate fraction was 49.869 ± 1.67. This indicates that the extract and ethyl acetate fraction contain active compounds in p53 protein expression but with low potential. Administration of the extract and ethyl acetate fraction of kepel leaf extract with various treatment groups can increase p53 protein expression.

The average percentage increase in p53 protein expression is proportional to the concentration administered to T47D cells. The EC50 value indicates the concentration of the extract or ethyl acetate fraction that can increase p53 protein expression by 50%. Table 6 shows that as the EC50 value decreases, p53 protein expression increases. Based on the results of the percentage decrease in bcl-2 protein expression in the extract was 53,428 ± 0.822 and in the ethyl acetate fraction was 52,511 ± 0.437. This indicates that the extract and ethyl acetate fraction contain active compounds that reduce bcl-2 protein expression but have low potential.

The results of the study show that the extract and ethyl acetate fraction of kepel leaves are capable of increasing p53 protein. The p53 protein is a tumor suppressor; if it is affected by mutation, it will cause the loss of cell regulation mechanisms. The main function of the p53 is to regulate and control the cell cycle and cellular responses to DNA damage, initiate DNA replication and repair, induce apoptosis, and promote cellular differentiation. The p53 protein can induce the activity of enzymes that function to repair DNA. DNA damage that cannot be

repaired will cause the cell to be directed by the p53 protein to undergo apoptosis. Thus, the expression of the p53 protein plays a role in responding to DNA damage DNA damage, namely inhibiting cell cycle progression and directing the cell toward the apoptosis pathway if DNA damage cannot be repaired. The B-lymphoma 2 (bcl-2) protein acts as the primary regulator of the intrinsic or mitochondrial pathway that causes apoptosis. The bcl-2 protein is a strong anti-apoptotic agent, and overexpression of the bcl-2 protein increases cellular survival. In cancer cells, increased expression that suppresses the normal function of pro-apoptotic proteins is caused by mutations in the bcl-2 protein. If this occurs in pro-apoptotic proteins, it can reduce regulation and cause cells to lose their ability to regulate apoptosis, which can lead to cancer.

CONCLUSIONS AND RECOMMENDATIONS

The extract, n-hexane fraction, ethyl acetate fraction, and water fraction exhibit cytotoxic activity against T47D breast cancer cells using the MTT assay, with IC50 values of 53,766 µg/ml, 178,184 µg/ml, 52,822 µg/ml, and 120,754 µg/ml, respectively. The extract, n-hexane fraction, ethyl acetate fraction, and water fraction exhibit selectivity indices for kepel leaves with values of 5.24 µg/ml, 1.49 µg/ml, 10.52 µg/ml, and 6.41 µg/ml. Selectivity was observed in the extract, ethyl acetate fraction, and water fraction because the selectivity index values were greater than. The extract and ethyl acetate fraction of kepel leaves were able to increase p53 protein with EC50 values of 50.828 µg/mL and 49.678 µg/mL. The extract and ethyl acetate fraction of kepel leaves inhibit bcl-2 protein with EC50 values of 54.793 µg/mL and 52.986 µg/mL.

FURTHER STUDY

From the research, researchers hope to further expand research on the benefits of the malay apple plant and research hopes that there will be more extensive research on antioxidants in various plants.

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