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Anticancer Activity of Ethyl Acetate Extract of Water Guava Leaves (*Syzygium aqueum*) Against Cancer Cells Huh7it

Siti Nur Asiyah[1], Eva Agustina[2*], Nova Lusiana[1], Risa Purnamasari[2*], Lina Afiifatul Abroroh[3]**

[1]Faculty of Psychology and Health, Sunan Ampel State Islamic University, Jl. Dr.Ir. H Soekarno No 682, Gunung Anyar, Surabaya, Jawa Timur 60294, Indonesia
[2] Faculty of Science and Technology, Sunan Ampel State Islamic University, Jl. Dr.Ir. H Soekarno No 682, Gunung Anyar, Surabaya, Jawa Timur 60294, Indonesia
[3]Faculty of Science and Technology, Airlangga University, Jl. Dr. Ir. H. Soekarno, Mulyorejo, Surabaya, Jawa Timur 60115, Indonesia
*__corresponding author__: risap1989@gmail.com
**editor and proofreading

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ABSTRACT
Hepatocellular carcinoma (HCC) is a primary malignant liver tumor with a general prognosis of death. This encourages the search for anticancer candidates against HCC cytotoxicity using natural ingredients such as plants. The water guava plant (*Syzygium aqueum*) is a medicinal plant that is widely grown in tropical areas. This study aims to determine the anticancer activity of ethyl acetate extract of *Syzygium aqueum* leaves by investigating its impact on Huh7it cell proliferation. The leaves of the water guava plant (*Syzygium aqueum*) were extracted using ethyl acetate. Anticancer activity was examined via MTT (3-(4,5-dimethylthiazol-2yl)-5-(3 carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H tetrazolium) on Huh7it liver cancer cells. The MTT test results showed that water guava leaves (*Syzygium aqueum*) had an IC50 value of 163.3 µg/ml.

Keywords: (Hepatocellular carcinoma, *Syzygium aqueum*, anticancer, MTT Assay)

Introduction

Hepatocellular Carcinoma (HCC) is a primary malignant tumor in hepatocyte cells with a general prognosis of death. This disease is one of the leading causes of death due to cancer in the world (Llovet et al., 2022). Around 70-90% of HCC sufferers have a history of chronic hepatitis and cirrhosis which can be caused by infection with Hepatitis B Virus, Hepatitis C Virus, alcoholic liver disease, and nonalcoholic steatohepatitis (NASH). Other risk factors in

the development of HCC include diabetes, obesity, aflatoxin contamination of food (Balogh et al., 2016).

Currently, HCC therapy has a low cure rate, especially in patients who do not qualify for surgical methods (resection). Resection can be chosen in patients with a liver without cirrhosis, but most HCC liver conditions are in an advanced phase, only <30% of patients can be treated with resection and transplantation. Other options without resection include percutaneous ethanol injection, radiofrequency ablation and transarterial chemoembolization. Successful resection reaches 35% of HCC sufferers who survive for 5 years, whereas without resection it is <10%. This encourages the search for anticancer candidates against HCC cytotoxicity using natural ingredients such as plants because they are considered to have no side effects (Liste, 2020).

Indonesia has great biological riches, especially medicinal plants which have been used as traditional medicine for generations. Based on the results of Basic Health Research (Riskesdas) in 2010, the percentage of the population aged ≥ 15 years who chose traditional medicine was 45.17%. One of the potential medicinal plants widely planted in Indonesia is water guava (*Syzygium aqueum*). *S. aqueum* from the Myrtaceae family is native to Indonesia and is known as water guava. The research results of Thamilvaani et al., (2012) stated that *S. aqueum* leaf extract contains six types of flavonoids and flagonoids so it has the potential to inhibit the growth of cancer cells (Hariyanti et al., 2015).

The active compounds found in many *Syzygium aqueum* leaves are flavonoids, phenolics and tannins which can be used as antimicrobials. Apart from that, S. aqueum leaves also contain active compounds that have the potential to act as anti-oxidants, anti-cancer, anti-diabetic and anti-hyperglycemic compounds, namely hexahydroxyflavone, Myricetin, vitamin C, 2',4'-dihydroxy-6-methoxy-3, 5–dimethylchalcone, compounds 4-Hydroxybenzaldehyde, myricetin-3-O-ramnoside, europetin-3-O-ramnoside, floretin, myrigalon-G and myrigalon-B (AnggrawatiP.S., Ramadhania Z.M., 2016). Water guava leaves have potential as an anticancer with an LC50 value of 170.01 ppm in the category of potential toxicity to shrimp larvae through the BSLT test because they contain flavonoids, tannins and saponins (Ningsih S and Misgiati, 2017). Therefore, this study aims to determine the anticancer activity of ethyl acetate extract of *Syzygium aqueum* leaves by investigating its impact on Huh7it cell proliferation in liver cancer.

Material and Methods

Materials

Syzygium aqueum leaves, Ethyl Acetate (Sigma-Aldrich, St. Louis, MO, USA), ultraviolet-visible (UV-vis) spectrophotometers, Quvetes Glass, 2,2-diphenyl-1-picrylhydrazyl (DPPH), MTT (3- (4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution (Sigma-Aldrich, St. Louis, MO, USA), dimethyl sulfoxide (DMSO), GloMax-Multi Microplate Multimode Reader (Promega Corp., WI, USA), Huh7it Cancer Cells, phosphate-buffered saline (PBS), Annexin-PI, BD Biosciences FACS CaliburTM flow cytometry, DMEM (Gibco BRL, Grand Island, NY , USA), t-EDTA, Dulbecco PBS (D-PBS, Gibco BRL, Grand Island, NY, USA), nonessential amino acids, fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA), Dulbecco's modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA), kanamycin (Sigma-Aldrich, St. Louis, MO, USA), and nonessential amino acids (Invitrogen).

Methods

Sample preparation

Sample preparation was carried out by separating water guava leaves from the main leaf veins and cutting them into small pieces. Drying was carried out using an oven at a temperature of 60°C for 24 hours. After the sample in the form of chopped water guava leaves was dry, it was destructed or crushed into powder using a blender. Sample extraction uses the cold method, namely maceration. This type of maceration method is used for the extraction of compounds that are less heat resistant. Extraction was carried out by weighing a 15 gram sample of powdered water guava leaves, then soaking in 100 mL of ethyl acetate solvent and homogenizing. Samples that have been mixed homogeneously with solvent are covered with plastic wrap and waited for 3 x 24 hours or 3 days with observations carried out every day to determine the evaporation of the solvent that occurs. After the sample solution is produced, it is filtered. The filtrate from the filter was transferred to another Erlenmeyer flask and covered with plastic wrap. Meanwhile, the residue is soaked again for the remaceration process. Evaporation is carried out on the filtrate until it becomes a paste, then put into a vial and covered using aluminum foil to speed up evaporation and produce a thicker paste. Next, the thick extract of water guava leaves was subjected to a phytochemical test according to the procedure carried out by Agustina (2018).

Toxicity Test using the MTT Method

Toxicity tests are carried out according to established procedures in the laboratory

Huh7it cell culture

Huh7it hepatocyte cells were cultured in Dulbecco's modified Eangle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biowest. Nuaille, France), 150 µg/ml kanamycin (SigmaAldrich, St. Louis, MO, USA) and non-essential amino acids (Invitrogen). Every time the cell growth in the petri dish reaches >80%, cell passage is carried out.

Test Sample Preparation

A total of 2 mg of water guava thick extract was dissolved in 20 µg DMSO (stock solution, concentration 100,000 µg/ml). Next, serial dilutions were made from the stock solution to obtain final concentrations of 2000 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml, and 12.5 µg/ml.

Test Procedure

Testing of the samples was carried out using the MTT (3-(4,5-dimethylthiazol-2yl)-5-(3 carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H tetrazolium) assay (Sigma-Aldrich). Before testing, the day before Huh7it cells were seeded in 96 well plates with a density of 2.4 x 10^4 per well. The test was carried out by adding water guava ethyl acetate extract to the cells and then incubating them for 48 hours in a 37ºC incubator with a CO_2 level of 5%. After incubation, the old medium was discarded and 150 µg of medium containing MTT (15 µL) was added and then incubated again for 4 hours. Next, 100 µL of DMSO was added to dissolve the precipitate formed as a result of the MTT reaction. Measurement of the absorbance of the MTT reaction results was carried out at wavelengths of 560 nm and 750 nm using the GloMax Microplate Multidetection Reader (Promega).

Data analysis

In this study, data on the percentage of hepatocyte cell viability was obtained by comparing the absorbance results of samples and controls. Inhibitory activity on 50% of cells (IC50) inhibited cell growth was determined by creating a relationship curve between the percentage of inhibition and the log dose.

Result and Discussions

The results of the phytochemical test of water guava leaf extract show that it contains alkaloid, flavonoid and terpenoid compounds. Testing for alkaloid compounds showed positive

results because a precipitate was formed after adding Mayer and Wagner reagents. The white precipitate resulting from the reaction is a potassium-alkaloid complex. Water guava leaf extract also contains flavonoid compounds because the color changes to dark red due to the reaction with FeCl3. Testing for terpenoid compounds also showed positive results because the color changed to orange and a greenish ring was detected.

The cytotoxicity test in this study was carried out to determine the cytotoxic effect of water guava leaf ethyl acetate extract on huh7it liver cancer cells in vitro. The test method used is the MTT (Microculture Tetrazolium Technique) method. The MTT reagent used is tetrazolium salt which can be broken down into formazan crystals by the succinate tetrazolium reductase system found in the respiration pathway in active mitochondria in living cells (Hughes & Mehmet, 2003). These formazan crystals can give a purple color and are not soluble in water, the absorbance of which can be read using a microplate spectrophotometer at a wavelength of 540 nm (CCRC, 2012). If the absorbance is greater, the number of living cells will increase (Fajarningsih et al., 2018).

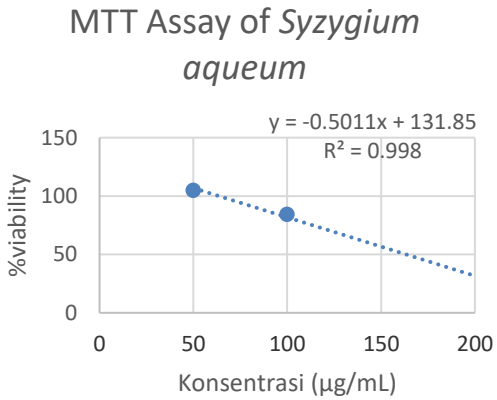

Figure 1. Huh71it Liver Cell Viability Curve after administration of water guava leaf ethyl acetate extract (*Syzygium aqueum)*

The aim of the MTT test for ethyl acetate extract of water guava leaves (*Syzygium aqueum*) on Huh7it liver cancer cells is to obtain the IC50 value. The IC50 value is a concentration value that is capable of inhibiting cells from 50% of the population, in this case Huh7it liver cancer cells (Dewi et al., 2019).Toxicity tests using the MTT method were carried out using serial concentrations, namely 2000; 1000; 500; 250; 125; 62.5; 31.5; and 15.625 µg/ml. The MTT assay technique is a test that is commonly used to determine the toxicity of extracts to cells. The MTT test works on the principle of reducing tetrazolium salts through mitochondrial succinate dehydrogenation in cells (Houdkova et al., 2017; Stockert et al., 2012;

Yang et al., 2015). Huh7it cells are a line of hepatocyte carcinoma cells taken in 1982 from the liver tumor of a 57 year old Japanese man. This cell is a discovery by Nakabayshi, H and Sato J. This cell is immortal or can continue to divide. These cells were grown in DMEM media given 10% FBS at a temperature of 37 degrees Celsius and a CO2 level of 5% (Darmanto, 2018).

The condition of the Huh7it cells after treatment with the extract showed that some were not dead or were still intact. The results of the test on Huh7it cells showed that the IC50 value of ethyl acetate extract of water guava leaves (*Syzygium aqueum*) was 163.3 µg/ml. The IC50 value is used to determine the toxic properties that ethyl acetate extract of water guava leaves (*Syzygium aqueum*) can cause on Huh7it cells. An extract declared active has a very strong cytotoxic effect, 50 > IC50 < 200 µgmL–1 has a moderate cytotoxic effect and 200 > IC50 < 1000 µgmL has a weak cytotoxic effect. IC50 value> 1000 µgmL–1 is stated to have no cytotoxic activity (Fatmawati 2018). Based on these results, it shows that the ethyl cetate extract of water guava leaves (*Syzygium aqueum*) has moderate toxic properties against Huh7it cancer cells. The MTT test is a sensitive, quantitative and reliable test. The MTT reaction is a cellular reduction reaction based on the breakdown of the yellow MTT tetrazolium salt into purplish blue formazan crystals. The succinate dehydrogenase enzyme in the mitochondria of living cells is able to break down MTT into formazan crystals (Hughes & Mehmet, 2003).

The MTT compound causes color changes in Huh7it liver cancer cells that have been given water guava (*Syzygium aqueum*) ethyl acetate extract. Cells that experience proliferation after being given a sample of water guava leaf ethyl acetate extract (*Syzygium aqueum*) will change color to purplish blue. Judging from the research results, the higher the sample concentration, the fewer cells will change color to blue. This shows that the higher the sample concentration, the more toxic the compound is to cells. The color change occurs due to the breakdown of the yellow MTT tetrazolium salt into purplish blue formazan crystals (Bahuguna et al., 2017).

The mechanism for this color change is that when cells undergo proliferation, the mitochondria will absorb MTT so that the cells will turn purple due to the formation of tetrazolium (formazan) crystals (Depa-mede et al. 2009: 97). The principle of the MTT test is to measure cellular activity based on the ability of the mitochondrial reductase enzyme in mitochondria to reduce Methylthiazole Tetrazolium (MTT) salt. When metabolizing, living cells will produce the enzyme mitochondrial reductase. This enzyme reacts with MTT and forms purple formazan crystals. The intensity of the purple color formed is proportional to the number of living cells, so that the more live cells there are, the more formazan crystals are

formed, the higher the absorbance value obtained and this indicates low mortality (Arifah et al., 2015).

The potential of water guava leaves as an anticancer is influenced by secondary metabolite compounds, especially flavonoids (Agustina et al., 2018). The research results of Arullappan et al. (2017) stated that flavonoid compounds are therapeutic agents to prevent cancer by reducing the proliferation of cancer cells. Flavonoids have been proven to inhibit cell proliferation through the regulatory mechanism of cyclindependent kinase 1 (CDK1) and cyclin B, as well as the tumor suppressor gene which plays an important role in restraining the p53 cell cycle in cancer cells.

Apart from that, water guava ethyl acetate extract also contains phenolic compounds. Phenolic compounds are known for their activity as antioxidants. Phenolics act as antioxidants because they can capture free radicals by releasing hydrogen atoms from their hydroxyl groups. The provision of hydrogen atoms will cause free radicals to become stable and stop carrying out extreme movements, so that they do not damage lipids, proteins and DNA (genetic material) which are targets of cellular damage. With such a mechanism, free radicals can be destroyed or stabilized, which in the end can suppress the occurrence of cancer (Shahidi, et al., 1995; Ilhami et al., 2013).

Conclusion

Ethyl acetate extract of guava leaves (*Syzygium aqueum*) has an IC50 value of 163.3 µg/ml using the MTT Assay method which has been proven to have moderate potential as an anticancer against Huh7it liver cancer cells.

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