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50% ethanol extract cytotoxic test of temulawak on MCF-7 breast cancer cells

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ABSTRACT

According to WHO, the number of breast cancer sufferers always increases from year to year. One of the medicinal plants used is temulawak. The levels of active compounds in plants can differ depending on several factors, one of which is the planting location, therefore research was conducted on the cytotoxicity of 50% ethanol extract of temulawak rhizomes from 5 regions on MCF-7 breast cancer cells in vitro using the MTT-Assay method. Temulawak used in this research comes from 5 regions in Indonesia, namely Tembalang, Wonogiri, Jambi, Sumba and Sukabumi. Cytotoxic testing of 50% ethanol extract of temulawak rhizomes used 6 concentrations, namely 500; 250; 125; 62.5; 31.25; and 15,625 ppm. The IC50 value obtained from 50% ethanol extract of temulawak rhizomes from Tembalang, Wonogiri, Jambi, Sumba and Sukabumi at MCF-7 was 109; 550.9; 622.3; 341.6; and 116.5 ppm. The results of the cytotoxic test showed that the 50% ethanol extract of temulawak rhizomes from Tembalang obtained the best IC50 value compared to temulawak rhizome extracts from other areas.

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INTRODUCTION

Curcuma xanthorrhiza Roxb. is a high-value native Indonesian plant known as "Temulawak" or Javanese Turmeric. This plant is widely cultivated in Indonesia and Southeast Asian countries such as Malaysia, Thailand, Vietnam and the Philippines. Traditionally, temulawak is widely used as a herbal herb (Indonesian supplements and herbal medicines), for the treatment and control of various diseases and disorders since ancient times such as lack of appetite, stomach disease, liver disease, constipation, bloody diarrhea, dysentery, arthritis, children's fever, hypotriglyceridemia, hemorrhoids, vaginal discharge, rheumatism, and skin eruptions. The efficacy of ginger to treat various diseases is proven to have pharmacological properties such as anti-inflammatory, antibacterial, antioxidant, neuroprotective, nephroprotective, antitumor, and hepatoprotector activities. Scientific studies show that the most abundant essential phytochemicals obtained by ginger are terpenoids and curcuminoids. Therefore, the medicinal activity of Temulawak is largely due to these two main groups of compounds. The main medicinal part of ginger that contains many sesquiterpenoids and curcuminoids is the rhizome (Rahmat et al., 2021).

The chemical study of ginger is the foundation of pharmacological research. Curcuminoids and terpenoids make up the majority and both have important biological properties (Rahmat et al., 2021). According to the European Medicines Agency Science Medicines Health (2014) ginger root consists of *curcuminoids* by 1-2%, namely a mixture of dicinnamoylmethane such as curcumin *(diferuloylmethane)*, monodemethoxycurcumin *(feruloyl-p-hydroxycinnamoylmethane)* and bisdesmethoxycurcumin (bis-(p-hydroxycinnamoyl)methane) and phenolic and non-phenolic diarylheptanoids and essential oils by 3-12% (containing sesquiterpenes such as *β-curcumene* and *arcurcumene*, *xanthorrhizol* by 44.5% and small amounts of camphor by 1.39%) (Mukti & Hermady, 2020). Based on various studies, most of the quantity of secondary metabolites obtained from the essential oil of temulawak rhizomes is *xanthorrhizol*. *Xanthorrhizol* in ginger depends on the species and distinguishes between curcuma and turmeric (Rahmat et al., 2021). *Xanthorrhizol* has the activity of suppressing or reducing inflammation (anti-inflammatory). Research conducted by Itokawa

et al. (2008) showed that α-curcumen, ar-turmerone, and *xanthorrhizol* have antitumor activity (Khaerana et al., 2008).

The antimetastatic and antitumor potential of *xanthorrhizol* was further assessed in *vivo* using mouse lung metastatic specimens and tumor cell development assays. Results showed *xanthorrhizol* significantly suppressed spot tumor induction in lung tissue and intra-abdominal tumor mass development. Further assessment of *xanthorrhizol* isolated from ginger against cancer cell proliferation was done in combination with curcumin in MDA-MB-231 cells (breast cancer cells). The experiment proved that the application of *xanthorrhizol* and curcumin showed synergistic growth inhibition in MDA-MB-231 cells through apoptosis activation. However, the above study is not the first study to report the ability of *xanthorrhizol* to induce cell apoptosis. Previous studies have also shown that *xanthorrhizol* is able to activate apoptosis through the induction of mitochondrial pathways (p53-dependent) in HeLa cervical cancer and HepG2 liver cancer. In HeLa cervical cancer cells, *xanthorrhizol* increased p53 and Bax regulation but had no effect on Bcl-2 (antiapoptic protein). Different results also found that increased p53 regulation did not affect Bax expression but decreased Bcl-2 levels in HepG2 liver cancer cells and MCF-7 breast cancer (Rahmat et al., 2021).

The composition of active compounds in plants can differ depending on several factors, one of which is the location of planting. Differences in the composition of active compounds in a plant cause their biological activity to also change. Widyastuti et al. (2021) examined the antioxidant activity of temulawak harvested from different locations, the results showed that temulawak has strong antioxidant activity and its levels are influenced by different harvest areas. Rahman et al. (2022) examined the antibacterial activity of temulawak harvested from different locations, the results showed that the content of compounds in temulawak as antibacterial based on where it grows in Indonesia has a variety of effects because it is influenced by geographical factors in each place of growth, namely climate, soil pH, rainfall, air humidity, and altitude (Rahman et al., 2022). Based on the results of the two journals, it can be concluded that the anticancer activity of temulawak harvested from different locations can also show variations in effects, because geographical factors such as weather, altitude, soil pH, temperature, and humidity can cause changes in plant metabolite content so that it affects its pharmacological effects (Rahman et al., 2022).

Breast cancer is the most common cancer in women, with an estimated incidence of 2.3 million new cases in 2020. According to the latest statistics of 2020, breast cancer is the fifth leading cause of cancer death worldwide, mainly occurring in developing countries in Melanesia, the Caribbean and sub-Saharan Africa although an 88% higher incidence rate is found in developed countries in Australia, New York. Zealand, and Western Europe (Aniogo et al., 2022). Breast cancer is one of the highest number of sufferers in Indonesia, every year the number of breast cancer patients tends to rise. Cancer is an abnormal cell growth where cells continue to grow and uncontrollably damage the organs where they grow. Breast cancer can grow in the mammary glands, milk ducts, fat tissue, or breast connective tissue. Cancer cells in the breast can spread through the bloodstream throughout the body if the condition has worsened. Therapy to overcome breast cancer is needed to reduce the number of deaths of sufferers. Standard treatment procedures for breast cancer include surgery, chemotherapy, immunotherapy, and radiation therapy. In general, chemotherapy as a treatment option is often opposed by responsive tumor recurrence and developing resistance, a significant setback in current treatment (Aniogo et al., 2022). Chemotherapy also has a large toxicity and side effects, besides that chemotherapy also requires a fairly high cost so that many people prefer traditional treatment from natural ingredients that have been empirically believed to have anticancer activity. One of the natural ingredients studied for anticancer activity is Temulawak (Putra, 2015; Udin, 2013).

Most published *in vitro* studies focusing on breast cancer and/or estrogen receptor biology have used the MCF-7 human breast cancer cell line. MCF-7 cells are responsive to estrogen, and are often used in *vitro* to study estrogen receptor-positive breast cancer. Although the genome is unstable, the vast literature available makes MCF-7 cells a useful model for understanding estrogen receptors and breast cancer biology (Vantangoli et al., 2015). In most cases of breast cancer, the level of estrogen receptor alpha (Erα) expression is directly proportional to tumor growth. Therefore, MCF-7 cell models have been extensively examined to determine the mechanism of estrogen-stimulated tumor growth (Razak et al., 2019). One of the *in vitro* studies that focused on breast cancer and used the MCF-7 breast cancer cell line, namely, with the MTT-Assay method.

The MTT reagent (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a mono-tetrazolium salt consisting of a positively charged quaternary tetrazole ring core containing four nitrogen atoms surrounded by three aromatic rings including two phenyl groups and one thiazyl ring. The reduction of MTT results in disruption of the tetrazole core ring and the formation of a purple-blue water-insoluble molecule called formazan. MTT reagents can pass through cell membranes as well as inner membranes of living cell mitochondria, possibly due to their positive charge as well as their lipophilic structure and are reduced to formazan by metabolically active cells. The chromogenic properties of this redox chemical reaction provided colorimetry-based measurements of intracellular formazan production on which the MTT test developed by Mosmann et al. (1983). As a result, this test has wide uses as a test of cellular metabolic activity. However, its usefulness is increasingly being applied to infer secondary processes or cell states, such as viability, which are

often not substantiated. The MTT test is usually performed after several hours of cell incubation with MTT. The resulting water-insoluble formazan is then dissolved with a solvent such as Dimethyl sulfoxide (DMSO). Furthermore, the decrease in light transmission through absorption and other mechanisms by homogenized MTT-formazan solutions is measured with a microplate reader in terms of optical density (OD) at the wavelengths most absorbed by MTT-derived formazan (approximately 570 nm). The measured OD value is assumed to be a representation of formazan concentration and consequently a reduction in intracellular MTT. This has been the basis for the application of the MTT assay for nearly four decades as a common tool for measuring cell proliferation/viability, drug cytotoxicity, and mitochondria/metabolic activity of cells (Ghasemi et al., 2021).

Based on this, this study will be conducted cytotoxic test of 50% ethanol extract of Temulawak rhizomes (*Curcuma xanthorrhiza* Roxb.) from 5 regions against MCF-7 breast cancer cells. The purpose of this study was to conduct phytochemical screening of simplisia powder and 50% ethanol extract of temulawak rhizomes from 5 regions, and conduct cytotoxic tests of 50% ethanol extract of temulawak rhizomes from 5 regions against MCF-7 breast cancer cells *in vitro using the* MTT-Assay method. This study provided information on 50% ethanol extract of temulawak rhizomes (*Curcuma xanthorrhiza* Roxb.) from 5 regions in inhibiting the growth of MCF-7 breast cancer cells.

METHOD

The research method used is experimental *in vitro*. Temulawak rhizomes from 5 different regions, namely Tembalang, Sukabumi, Jambi, Wonogiri, and Sumba, were used to be made into simplisia powder, then extracted with 50% ethanol using the kinetic maceration method, then concentrated using an evaporator until a thick extract was obtained. After that, phytochemical screening is carried out. Furthermore, temulawak extract was tested for cytotoxic activity by *MTT-Assay* method (3-(4,5-dimethylthiazolil-2)-2,5-diphenyl tetrazolium bromide) against MCF-7 cells, to determine cytotoxic activity against MCF-7 cells using the comparative method of temulawak ethanol extract with concentrations of 500, 250, 125, 62.5, 31.25, and 15,625 ppm (each was done triplo).

The research was conducted at the mammalian cell culture laboratory of the Center for Pharmaceutical and Medical Technology, Building No. 611 LAPTIAB-BPPT, BRIN puspiptek Serpong Area, South Tangerang. The ingredients used in this study were 50% ethanol extract of Temulawak rhizomes obtained from the areas of Tembalang, Sukabumi, Jambi, Wonogiri and Sumba, and MCF-7 cell culture.

The stages of research are:

1) Preparation of research materials: The material that will be used in this research is Temulawak rhizomes from 5 different regions, namely Tembalang, Sukabumi, Jambi, Wonogiri, and Sumba.
2) Manufacture of simplisia powder: Temulawak rhizomes are washed first until clean and then dried. Then the temulawak is cut into pieces, after that the temulawak slices are put into the 500C oven for 24 hours. After temulawak dries, temulawak is ground to become simplisia powder.
3) Making 50% ethanol extract of temulawak rhizomes: The extract is made by means of temulawak rhizome powder extracted by kinetic maceration using 50% ethanol solvent.
4) Phytochemical filtration of ethanol extract 50% of the rhizome: In phytochemical screening, powders and extracts are identified, flavonoids, alkaloids, tannins, saponins, steroids, quinones, essential oils, and coumarins.
5) MCF-7 breast cancer cell culture preparation:
    a) Creation of culture media
    b) Cell Thawing (growing cells from liquid nitrogen tanks)
    c) Subculture

Cell subculture is the process of moving cells from a confluent state to an empty growing place. This process is important so that the cells to be used for testing can grow optimally.

1) Cytotoxic test of breast cancer cells (MCF-7) by MTT method: Stages of implementation:
    a) Cell harvest: Cells that have reached the growth rate are made permanent by trypsination.
    b) Cell count: The number of cells is calculated by giving color with *tryphan blue* and a *Haemocytometer device* to determine the number of cells / mL culture
    c) Plating: Plating is the implantation of cells into a well (using a 96-well *plate*), after which the cells are used for treatment using test samples
    d) Sample preparation and extract of Temulawak: Cells that have been attached to the base of the *plate and* reached a confluence of 70% are immediately added to the concentration series of test samples, for Temulawak extract prepared 6 concentrations, namely 500; 250; 125; 62.5; 31.25; and 15.625 ppm.
    e) MTT Awarding: At the end of incubation, the culture medium is removed and the cells are washed with PBS (*Phosphate Buffer Saline*). After that, culture media (without cells) and MTT

reagents were added to each contribution. Then the cells are incubated for 4 hours. The cell condition was examined through an *inverted* microscope and the MTT reaction was stopped by adding *a stopper reagent* (sodium dodecyl sulfate), then *the plate* was incubated at room temperature in a dark room overnight.

The analysis techniques used are qualitative analysis looking at cell morphology images on *an inverted microscope* and quantitative analysis looking at IC50 values with *GraphPad Prism 5 software*.

RESULTS AND DISCUSSION

Based on the results obtained cytotoxicity test of 50% ethanol extract of Temulawak rhizomes from 5 regions against MCF-7 breast cancer cells gave different IC50 values. MTT-Assay test results IC50 values obtained from temulawak extracts from Tembalang, Wonogiri, Jambi, Sumba, and Sukabumi on MCF7 were 109; 550.9; 62.3; 341.6; and 116.5 ppm. The difference in IC_{50} results obtained shows that differences in planting locations affect the secondary metabolites contained. Of the five regions, it shows that temulawak extract from Tembalang obtained the best IC50 value compared to temulawak extract from other regions. Temulawak extract from Tembalang has the potential as the best cytotoxic agent against MCF-7 breast cancer cells.

The cytotoxic activity of a compound that attacks cancer cells can be classified into three categories, namely very active if IC50 < 10 µg/ml, active if IC50 10-100 µg/ml, and moderately active if IC50 100-500 µg/ml. A compound is said to have no cytotoxic activity if the IC50 value > 500 µg/ml (26).

The results of cytotoxicity test of temulawak rhizome extract from 5 different regions against MCF-7 breast cancer cells can be seen in the graphic image V.6, V.7, V.8, V.9, and V.10 produced from *software GraphPad Prism* 5 shows an increase in cell death as the concentration of the test sample given increases. IC50 results on *software GraphPad Prism* 5 is automatically read. Cytotoxic testing of temulawak extract against MCF-7 cells using the MTT method using 6 concentrations, namely 500; 250; 125; 62,5; 31,25; and 15,625 ppm.

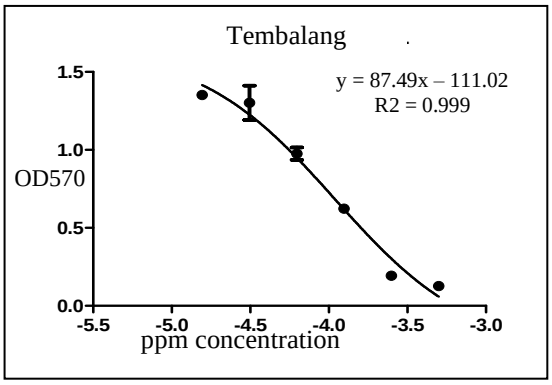


Figure 1. Cytotoxic test graph on Temulawak Tembalang

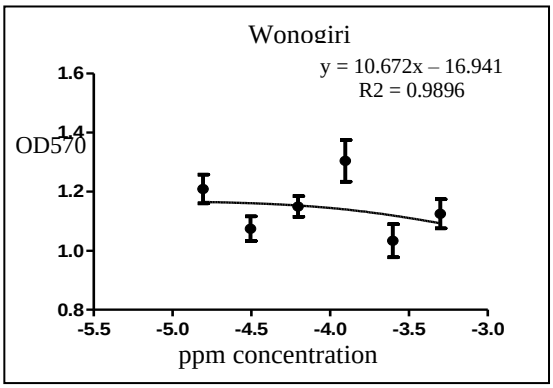


Figure 2. Cytotoxic test graph on Wonogiri temulawak

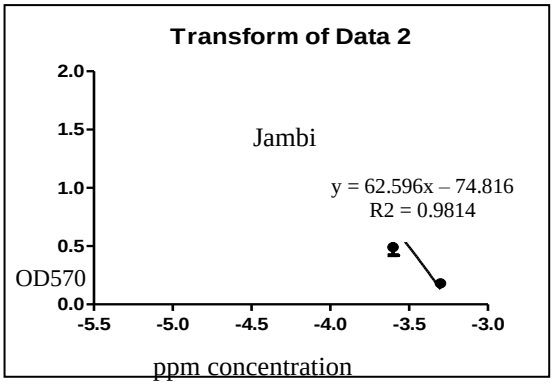


Figure 3. Cytotoxic test graph on Jambi temulawak

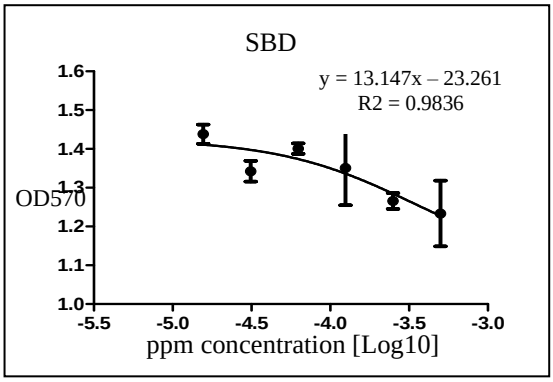


Figure 4. Cytotoxic test graph on Temulawak South West Sumba

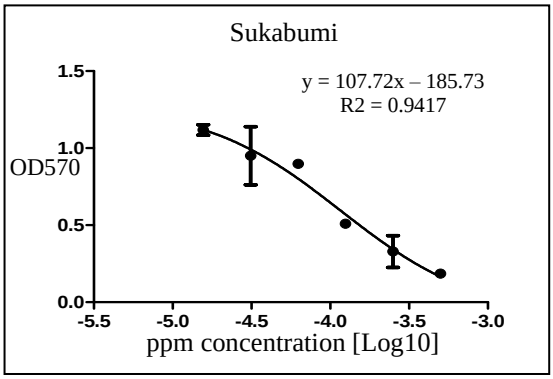


Figure 5. Cytotoxic test graph on Sukabumi temulawak

CONCLUSION

From the results of the MTT-Assay test of 50 % ethanol extract of temulawak rhizomes from Tembalang, Wonogiri, Jambi, Sumba and Sukabumi against MCF-7 breast cancer cells, showed cytotoxic effects with IC50 values of 109; 550.9; 622.3; 341.6; and 116.5 ppm. The cytotoxic test results showed that temulawak rhizome extract from Tembalang had the best cytotoxic effect compared to temulawak rhizome extract from other regions. Further research needs to be done with variations in solvents to determine the active compound content of ginger rhizome extract. Further research needs to be done on the antiproliferative activity of other cancer cells.

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