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White Turmeric Acetone Extract (*Curcuma zedoaria*) Effectivity on 4T1 Breast Cancer Cell Line Proliferation

Gregorius William Thongiratama[1], Ni Wayan Tianing[2], I Wayan Surudarma[2], Ida Ayu Dewi Wiryanthini[2]

[1]Undergraduate Medical School, Faculty of Medicine, Udayana University, Denpasar, Indonesia,
[2]Department of Biochemistry, Faculty of Medicine, Udayana University, Denpasar, Indonesia.

Corresponding Author: Gregorius William Thongiratama

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ABSTRACT

The development of breast cancer therapy has now reached the utilization of complementary alternative medicine (CAM) as an adjuvant to breast cancer therapy. One of the natural ingredients that has the potential for further research is white turmeric (Curcuma zedoaria). The purpose of the research is to determine the effects of white turmeric extract on breast cancer cell line 4T1. The research involved extraction by maceration and evaporation methods. White turmeric extract with acetone solvent was tested at 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml compared to the control to see the most potent concentration in providing therapeutic effects. The results of the test analysis of water content and ash content of acetone extract of white turmeric meet the standards as standardized herbal ingredients with a percentage of 15.935% and 5.523%. Higher concentrations specifically showed a better breast cancer proliferation inhibition effect (mean cell absorbance proportion of 0.096 ± 0.0008). White turmeric extract has the potential to be developed as an alternative therapy for 4T1 breast cancer. Higher concentrations showed a better breast cancer proliferation inhibition effect. White turmeric extract has the potential to be developed as an alternative therapy for 4T1 breast cancer.

Keywords: acetone extract, breast cancer, cell proliferation, white turmeric

INTRODUCTION

Breast cancer is one of the types of cancer with the highest number of cases in the world. Based on data from Globocan, breast cancer is at a percentage of 11.7% of 9,292,789 cancer cases in the world.[1] The number of breast cancer cases in Indonesia in 2020 ranged from 65,858 cases and was the cancer with the highest number of sufferers in Indonesia.[2]

The mesenchymal type of triple-negative breast cancer subtype has more influence related to EMT, often associated with a worse prognosis in patients, so therapy will be more effective for patients if using therapy that can reduce EMT activity. LAR type has high luminal cytokeratin and low basal cytokeratin, characterized by cancer cells with a consistent luminal state and often correlated with a higher prevalence of old age. A good therapy for this type of triple-negative breast cancer is hormonal therapy that impacts the LAR receptor.[3] Meanwhile, based on histopathological observations, 4T1 breast cancer cells can be classified as mesenchymal (M) type so that

therapies that can provide good effectiveness are therapies that can inhibit EMT to reduce cancer cell motility and adhesion.[4]

The 4T1 breast cancer cell line is a triple-negative breast cancer cell type that can be cultured in vitro and in vivo. In vitro culture can use ethanol or acetone-based media. Meanwhile, in vivo, culture generally uses BALB/C Wistar rats. The culture method used in this study is in vitro culture. It utilizes an appropriate environment for optimal cancer cell growth, such as hypoxic conditions and the necessary nutrients in RPMI 1460. Cells that have been grown will then be applied criteria in the form of an adequate number of cells in each microplate.[5,6]

4T1 breast cancer cells are classified as an adherent continuous cell line isolated from the mammary gland of mice (Mus musculus) of the BABL/cfC3H strain. 4T1 breast cancer cells were grown on Dulbecco's Modified Eagle's Medium (DMEM) growth medium with the addition of 10% v/v Fetal Bovine Serum (FBS) and 1x antibiotic-antimycotic diluted from 100 times stock.[7] Breast cancer cell line 4T1 has a doubling time of 22.9 hours. 4T1 cells are one of the best syngenetic xenograft mouse models for human cancer research in vitro.[8] The transplantation for tumor tissue is from similar species of syngenetic xenograft mice model.[9]

White turmeric is rich in anticancer agents. The well-researched content of white turmeric is stigmasterol, which can reduce the proliferation rate of cancer cells and the risk of angiogenesis. In addition, the active ingredients of white turmeric can induce apoptosis. White turmeric can be utilized as a complementary alternative medicine (CAM) supporting therapy related to its role in reducing hypoxia-inducible factor-1α (HIF-1α) and vascular epidermal growth factor (VEGF). Decreased HIF-1α can increase CD4+ and CD3+ infiltration, stimulating breast cancer cells' cytotoxic effect.[10] VEGF reduction may reduce breast cancer metastasis.[11]

White turmeric extract could increase the methylation of gamma synuclein and decrease the expression of gamma synuclein, which plays a role in cancer progression in people with hereditary cancer.[12] The proliferation inhibition potentially demonstrated by the presence of curcumin compounds occurs by inhibiting the activity of MMP-9.[13] In addition, the presence of curcumin compounds in white turmeric inhibited TGF-β activity which plays a role in the development of breast cancer and oxidative metabolism of breast cancer cells.[14]

MATERIALS & METHODS

The materials used in the study included 96% acetone, white turmeric, 4T1 breast cancer cell line Kit, distilled water, culture medium kit: FBS, DMEM medium, amphotericin B (fungizone), paraben, NaOH, cera alba, span 80, methylparaben, glacial stearate acid, tween 80, carbopol 940, DPPH. Research instruments used in this study are a spatula, blender, glassware, serum tube, centrifugation test tube, centrifuge, funnel, vacuum rotary evaporator, gauze, aluminium foil, and water bath.

The white turmeric (Curcuma zedoaria Rosc.) rhizome samples were initially cleaned with water and then cut into thin pieces, oven-dried, and blended until smooth. The white turmeric rhizome was then macerated using 96% acetone for 24 hours, stirring every 6 hours. The sample was filtered on filter paper. The supernatant was then evaporated with a pressure of 100 bar at 40^{o}C using a vacuum rotary evaporator until no solvent evaporated, and the extract was collected in an evaporator tube.

The pulverized sample weighed as much as 3 grams in a porcelain cup with a known constant weight. The sample was ignited in an oven at 550^{o}C for 3-5 hours. The samples were then cooled and put in a desiccator. The result was then weighed until it reached a constant weight. The weight of the ash content divided by the weight of the sample

multiplied by 100% will produce a percentage value of ash content.[15]

A sample of 3 grams mashed was put in a porcelain cup; the constant weight had already been known. The sample was then dried in an oven at 100-105^{o}C for 3-5 hours and cooled in a desiccator. Afterwards, the sample was weighed. The water content's weight divided by the sample's weight multiplied by 100% will produce a percentage value of water content.[15]

The number of samples in this study met the requirements based on Federer's formula, with 28 samples measured in three repetitions. The samples were measured after the culture lasted for one night or one breast cancer cell proliferation cycle of 22.9 hours had passed. 4T1 breast cancer cells were taken from the nitrogen tank, thawed at 37°C until liquefied, then sprayed with 80% alcohol.

Cells were placed in a centrifuge tube containing 10 mL of RPMI 1640-non-serum medium in a laminar airflow chamber and centrifuged at 1200 rpm for 3 minutes. The supernatant was discarded, and the precipitate formed was added to the RPMI 1640 serum. After standing for 20 minutes, the cells were centrifuged at 1200 rpm for 5 minutes. The supernatant was left with 1 mL for further resuspension. The cell suspension was placed in a tissue culture flask (TCF) with a growth medium containing 20% FBS, and the cells were observed using an inverted microscope. Tissue culture flasks containing cells were incubated in a CO_2 incubator at 37^{o}C with the lid loosened facing inward.

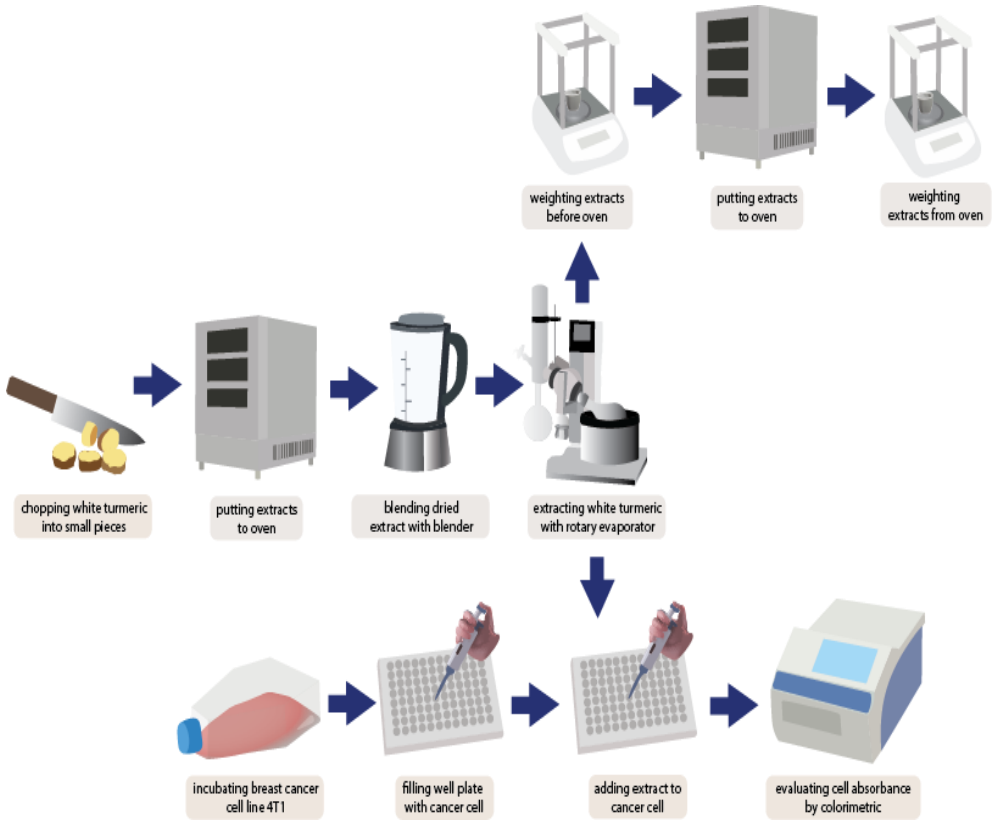

Figure 1. Research Methods Observation of the Effect of White Turmeric Extract on the Absorbance of Breast Cancer Cells

Cells were observed daily with an inverted microscope, and the growth medium was changed periodically daily. The indicator used was when the TCF medium was clear yellow, the cells had filled the TCF, and the cells were harvested and distributed into several TCFs. The old medium was discarded in a laminar airflow, and the adherent cells were gently sprayed with the new medium. The obtained cell suspension

was put into several TFs and stored in a CO_2 incubator at 37°C.

Cell density was calculated by taking 20 µL of cell suspension. Cells were counted with the help of a hemocytometer on a light microscope. The total cell number was obtained by flowing the dilution factor with 10^4/mL. The apoptosis assay requires a minimum number of 5 x 10^5/mL to 1 x 10^6/mL of culture medium. Therefore, samples with fewer cells than the minimum criteria were excluded. The Federer formula was utilized to determine the sample size for in vitro research, which results in 3,4 (rounded to 4) as a minimum for every control group. Thus, the total sample required for the research is 28 samples.

4T1 breast cancer cells were then given white turmeric extract with concentration variations of 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml. The variation was then analyzed to conclude whether concentration differences (higher or lower) signified any diverse effect on inhibiting breast cancer cell proliferation. Using a micropipette, a group without treatment (control) was used as a comparison for breast cancer cell growth.

The 4T1 cancer cell suspension was cultured in a 24-well microplate with 5x104 cells/microplate and incubated for 24 hours at 37°C. Cancer cell lines were incubated with one concentration series of white turmeric acetone extract in the culture medium for 24-48 hours. At the end of incubation, the culture medium was discarded and washed with PBS once. Then, 100 microliter MTT was added, including for media control. Cells given MTT were incubated for 2-4 hours until formazan salts are formed. Cell culture was added to a 10% SDS stopper in 0.1N HCl and incubated in the dark overnight. After approximately 24 hours, cells were examined on an ELISA reader with a wavelength of 550 nm. Living cells will react with MTT to form a purple color, where the intensity of the purple color formed will be inversely proportional to the number of dead cells.[16]

Observation of the antioxidant content of white turmeric was carried out by DPPH test and particle number calculation utilizing spectrophotometry with settings at a wavelength of 517 nm (violet) as a color change that occurs due to the chemical reaction of free radical DPPH with antioxidants from white turmeric extract. We further analyzed the results using IBM SPSS Statistics 26 to determine the relationship between variables.

STATISTICAL ANALYSIS

Each group intervention was primarily normally distributed based on Shapiro-Wilk Analysis, apart from P3 (white turmeric extract 62.5 µg/ml) and P5 (white turmeric extract 250 µg/ml), which showed a significance value of 0.001. Therefore, One-Way ANOVA was implemented for normally distributed group intervention and abnormally distributed data were analyzed separately using Spearman's Rho non-parametric test. Based on the observation of the analysis using the One-Way ANOVA method, the P value was less than 0.001, which means that the hypothesis regarding the significance of the role of white turmeric in overcoming breast cancer proliferation was categorized as showing strong evidence. However, the ANOVA analysis's high F value means that the group variation occurred by chance. Moreover, there is a significant correlation according to the non-parametric test with a bootstrap of 1000 times with moderate-high significance (P=0.002; 95% CI, -0.630 to -1,000).

The interpretation of the P value shown from the Post-Hoc analysis indicated that the difference is less significant if it shows a value of less than 0.005. Therefore, based on the Post-Hoc analysis, it was found that each control (dependent) variable showed a significant difference, except for the comparison between P1 (white turmeric Extract 15.625 µg/ml) and P2 (white turmeric extract 31.25 µg/ml).

Relationship between variables were explored statistically with Pearson Correlation analysis and multiple linear

regression. Based on the results of Pearson Correlation analysis, it was found that there is a significant relationship between the variation of concentration of white turmeric acetone extract and the absorbance of breast cancer cell proliferation (P<0.001). In addition, the effect between variables were -0.882, considered as high. Based on multiple linear regression analysis, we obtained P<0.05 so that there is a relationship between concentration and proliferation rate of 4T1 breast cancer. The results of the multiple linear regression analysis represented constant value is 0.541, meaning that without intervention in the form of acetone extract of white turmeric, the proliferation rate of breast cancer is 0.541.

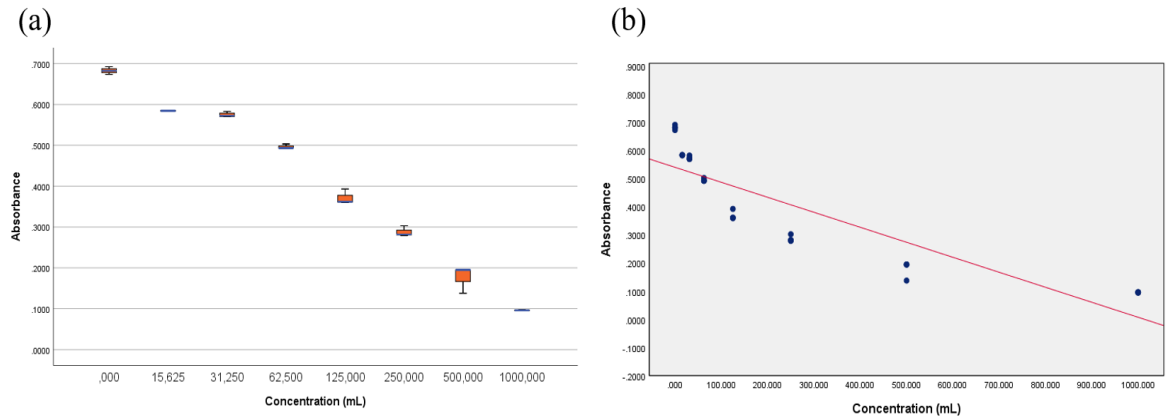

Figure 2. (a) Weight-Plot of Absorbance Relation to Concentration (b) Dot and Plot Absorbance Relation to Concentration

RESULT

The white turmeric extract was evaluated by testing its purity, ash and moisture content. The extract quality that provided potential cytotoxic effects was tested in the IC50 parameter. The IC50 value of white turmeric extract with acetone solvent was measured 101.28 µg/ml. Assessment of water content and ash content by the extract with acetone solvent was measured 15.935% and 5.523%. The evaluation results of white turmeric extract can be summarized in **Table 1**.

Table 1. Content evaluation of White Turmeric Acetone Extract

Parameter	Result
IC_{50} Value	101.28 µg/ml
Water Content Percentage	15.935%
Ash Content Percentage	5.523%

Breast cancer cell growth was measured by repeating three times using acetone-solubilized extracts varied in seven concentrations, with a negative control as a comparison, as illustrated in Error! Reference source not found. and

Table 2. In the intervention group, white turmeric with a concentration of 1000 µg/ml, showed the highest inhibition effect (95% CI, 0.0952 to 0.0968). The study also indicated that a higher concentration of an extract as an intervention associated with a more potent inhibition effect. The effect furthermore could be related to the cytotoxic effect. The extract has the lowest value of 0.5849 in measurements with a concentration of 16.25 µg/ml in the second repetition or expressed in the average calculation of three times the absorbance measurement of 0.5843.

Table 2. Proportion of Absorbance of 4T1 Breast Cancer Cell Proliferation

No.	Treatment Group	N	95% CI
1	P0 (negative control)	4	0,683 ± 0,0075
2	P1 (white turmeric extract 15.625 µg/ml)	4	0,584 ± 0,0004
3	P2 (white turmeric extract 31.25 µg/ml)	4	0,576 ± 0,0051
4	P3 (white turmeric extract 62.5 µg/ml)	4	0,496 ± 0,0047
5	P4 (white turmeric extract 125 µg/ml)	4	0,372 ± 0,0151
6	P5 (white turmeric extract 250 µg/ml)	4	0,288 ± 0,0106
7	P6 (white turmeric extract 500 µg/ml)	4	0,176 ± 0,0268
8	P7 (white turmeric extract 1000 µg/ml)	4	0,096 ± 0,0008

DISCUSSION

The white turmeric extract utilization as intervention initially tested for the quality with ash and water content test. The purity of the extract in testing ash and water content tests the ratio of the composition of organic and inorganic materials in providing effects as breast cancer therapy. The results of the water content test show a good value because it meets the standards set by the Indonesian Food and Drug Administration (BPOM) and the Ministry of Health of the Republic of Indonesia, which is 5-30%. In addition, the ash content value of 5.523% is also considered good because it meets the standards set by BPOM and the Indonesian Ministry of Health, which does not exceed 16%.[17] Thus, suggesting the exclusion of the quality of extract from the factors that potentially confound the results.

The potential cytotoxic effect of white turmeric extract with acetone solvent is indicated by the finding of an IC_{50} value classified as medium in the range of 21-200 µg/ml (101.28 µg/ml). Based on the low IC_{50} value, according to the AJCC assessment, white turmeric extract with acetone solvent has high potential of cytotoxic effect because only a low concentration of extracts can show the significance of the expected effect.

The results can be further clarified by observing the percentage of breast cancer cell viability after the intervention, which showed that the white turmeric extract with acetone solvent showed the highest inhibition value at 85.6662, while the white turmeric extract with acetone solvent concentration of 15.625. In contrast, acetone solvent showed poor significance in affecting breast cancer cell viability, with the lowest value in inhibition at 13.9140 percent at a solvent concentration of 1000.

This study has similar results to its predecessor research conducted by Borges et al. (2020) with extract materials of acacia (Acacia dealbata) and olive (Olea europaea), resulting in the conclusion that acetone provides a better effect, mainly when utilized as a solvent for active ingredients that can have therapeutic effects related to the IC_{50} value as shown in the white turmeric extract used in the study.[18]

CONCLUSION

The effectiveness of white turmeric acetone extract in suppressing the proliferation process has an intermediate level effect with an IC_{50} value of 101.28 µg/ml. The results showed that white turmeric with acetone solvent has moderate effect on the proliferation process of cancer cells. However, other factors, such as relating to drug form and administration, can be further evaluated to increase the effectiveness of using white turmeric extract as a breast cancer therapy.

Declaration by Authors

Ethical Approval: All the experimental procedures in this study were approved by the Research Ethics Committee of Faculty of Medicine Udayana University, Denpasar, Bali (ethical code: 2024.01.1.0102).

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Conflict of Interest: The authors declare no conflict of interest.

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