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THE EFFECT OF ADMINISTRATION OF ETHANOL EXTRACT OF RED GALANGA (*Alpinia purpurata K. Schum*) RHIZOMES ON THE HISTOPATHOLOGY OF WISTAR RATS BREAST CANCER CELLS INDUCED BY DMBA (7,12-dimetilbenz[a]antrasena)

S. Wahjuni*, I. G. A. G. Bawa, and N. M. N. Sawitri

Department of Chemistry Faculty of Mathematics and Natural Sciences Udayana University, Jalan Raya Kampus Unud, Jimbaran, Badung, Bali, Indonesia
****Email: sri_wahjuni@unud.ac.id***

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ABSTRAK

Rimpang lengkuas merah (*Alpinia purpurata K.Schum*) merupakan tanaman yang berpotensi sebagai produk alami. Produk alami ini memiliki aktivitas yang bergantung pada jumlah senyawa aktif yang diekstraksi dan metode ekstraksi. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian suplementasi etanol rimpang lengkuas merah terhadap perubahan histologis kanker payudara. Metode yang digunakan adalah metode skrining fitokimia, identifikasi menggunakan instrumen LC-MS/MS, pemeriksaan histopatologi jaringan kelenjar payudara, DMBA, rotary evaporator vakum, dan seperangkat alat LCMS/MS ACQUITY UPLC®H-Class System (Water, AS). Rimpang lengkuas merah (Alpinia purpurata K.Schum) diperoleh dari Desa Badung dan diidentifikasi di Pusat Konservasi Tumbuhan LIPI-UPT Kebun Raya "Eka Karya" Bali. Rimpang lengkuas merah dengan induksi DMBA pada berbagai dosis menunjukkan bahwa ekstrak etanol rimpang lengkuas merah berpengaruh dalam memperbaiki gambaran histopatologi sel kanker payudara mencit pada dosis 70 mg/kgBB/hari dan 90 mg/kgBB/hari.

Kata kunci: Antikanker, kanker payudara, DMBA, histopatologi, rimpang lengkuas merah

ABSTRACT

The red galangal rhizomes (*Alpinia purpurata K.Schum*) plant has natural product potential. The Natural product has the activity of a plant, which depends on the amount of active compound extracted and the extraction method. This study aimed to determine the effect of the administration of ethanol on supplementing red galanga rhizomes on histological changes in breast cancer. Using phytochemical screening methods, identification using LC-MS/MS instruments, histopathological examination of breast glandular tissue, DMBA, a Vacum rotary evaporator, a set of tools, and an LC-MS/MS ACQUITY UPLC®H-Class System (Waters, USA). The red galangal rhizome (*Alpinia purpurata* K.Schum) was obtained from Badung village and determined at the LIPI-UPT Center for Plant Conservation of the Bali "Eka Karya" Botanical Garden. The red galanga rhizomes reported that the higher the ethanol concentration, the higher the perhexiline content was thought to be anti-breast cancer. Phytosphingosine had the anticancer activity of red galanga rhizomes, with DMBA induction at various doses showing that the ethanol extract of red galangal rhizomes had an effect in improving the histopathological picture of mouse breast cancer cells at doses of 70 mg/kgBW/day and 90 mg/kgBW/day.

Keywords: Anticancer, breast cancer, DMBA,histopathology,red galanga rhizomes

INTRODUCTION

Cancer is a disease associated with abnormal cell growth and division. Cancer is the second biggest cause of death in the world after heart or cardiovascular disease. According to the World Health Organization (WHO), in 2018 there were around 18.1 million cases of cancer worldwide and it is estimated that this will increase in 2040 to 29.4 million cases. This disease is known to cause 1 in 6 people to die (WHO, 2020). Meanwhile in Indonesia, according to the Basic Health Research Agency (RISKESDAS), it was reported that there were 1.4% of cancer cases in 2013 and this increased in 2018 to around 1.8% (RISKESDAS, 2018).

According to GLOBOCAN's 2018 forecast status report regarding events and cancer deaths, breast cancer is the second most frequently diagnosed malignancy, accounting for more than 11.6% of all cancers in women and causing 6.6% of all cancer deaths worldwide. Breast cancer remains the main cause of cancer-related morbidity and death

throughout the world. So studying the development of breast cancer epidemiology is very valuable because prevention can be carried out through a combination of primary and secondary prevention strategies (Huang *et al*., 2021)

One factor that can increase the prevalence of cancer is the uncontrolled development of carcinogenic substances. Thousands of toxic chemicals can become common ingredients that can contaminate the

This compound has hydrophobic and lipophilic properties so it is easily deposited in the food chain system. This compound is a carcinogenic compound with activity that depends on the presence of certain enzymes (Abbass *et al*., 2021). As the number of cancer cases increases, the costs and duration of treatment also increase (Das *et al*., 2020). In recent years, cancer sufferers have begun to combine medical and non-medical (herbal) treatments from natural ingredients. Drug

One of the herbal plant research that has been widely developed is red galanga. Red galanga (*Alpinia purpurata* K. Schum) rhizomes has various pharmacological activities, such as antiviral, antifungal, antioxidant, antibacterial, anti-inflammatory and anticancer (Pangestuti *et al*., 2020). The content of flavonoid derivatives (galangin) and phenol derivatives (ACA(1-Acetoxychavicol Acetate)) in red galangal rhizomes (*Alpinia purpurata* K. Schum) is thought to have anticancer potential (Liu *et al*., 2018). According to research by Pramushinta and Ajiningrum (2017), the content of flavonoid compounds in galangal methanol extract of 16.76 µg/mL was able to inhibit P388 murine leukemia cancer cells with a percentage of 50% ($P < 0.05$) at very low extract concentrations. Inhibition is influenced by amide (-$CONH_2$), hydroxyl (-OH) and dilactone groups. Considering the potential of ethanol extract of red galangal rhizomes against breast cancer based on a literature review, it is important to carry out further research as an innovation in preventing breast cancer.

MATERIAL AND METHOD

Materials

Red galangal rhizomes was purchased from Badung village Indonesia. The following

environment. DMBA or 7,12-dimethylbenz(α)antrhacene is a carcinogen that is classified as a polycyclic aromatic hydrocarbon (PAH) and has a structure similar to estrogen (Wuyung, 2016). Polycyclic aromatic hydrocarbons (PAHs) are a class of environmental chemicals, common in both rodent and human models. PAH exposure most often occurs in air pollution, car exhaust, cigarette smoke, diesel fuel, and grilled or smoked foods (Ledet *et al*., 2018).

combinations that include dietary supplements and natural products have been postulated to achieve similar effects to conventional chemotherapy drugs but with fewer side effects (Lin *et al*., 2020). This natural (herbal) treatment is supported by Indonesia's potential as a country with millions of biodiversity. Research on the potential content of these metabolites is very much needed to guarantee the efficacy and level of safety of herbal plants that have this potential (Chan., 2017).

chemical were used: 96 % ethanol (Sigma Aldrich), distilled water, DMBA. Method of extracting active components from natural ingredients, male Wistar rats aged 2-3 months weighting 150-200 g were used this experiment breast cancer.

Equiptments

Laboratorium glasswear, rotary vacuum evaporator, histopathological picture of mouse breast cancer, LCMS/MS Xevo type G2TOF, light microscope, sonde, automatic tissue processor, tissue embedding console, rotary microtome and rotary vacuum evaporator.

Procedure

Preration of red galanga rhizomes ethanol extract

Dry red galanga rhizomes (*Alpinia purpurata* K. Schum) Rhizomes with water content of 5.26% was extracted with 96% at room temperatur for 48 h,the solvent was the evaporatorat 45^0C.This extract was to LCMS/MS.

Extraction

A total of 1.0 kg of red galangal rhizome simplicia was extracted by maceration using 96% technical ethanol until all the powder was submerged in the solvent for ± 24

hours. Then, the filtrate is separated from the residue by filtration. The filtrate was concentrated using a rotary vacuum evaporator until a thick ethanol extract of red galangal rhizomes (*Alpinia purpurata* K. Schum) was produced.

Phytochemical screening

The phytochemical screening method is an analysis to initially identify the chemical components contained in plant extracts. This method uses various chemical detection reagents for secondary metabolite compounds such as alkaloids, flavonoids, tannins, terpenoids, saponins, and others. The chemical changes that occur indicate the types of compounds contained in the extract (Putri and Lubis, 2020). This analysis specifically aims to determine the biosynthesis mechanism, chemical structure, distribution of compounds, isolation and chemical composition of a compound (Agustina, 2021).This method is based on geographical location, climate, fertility and temperature which are different for each test plant. The test plants used can be parts of leaves, flowers, fruit or stems which have properties as traditional medicine. Screening is carried out based on three methods, namely qualitative, quantitative, or semi-quantitative according to the research objectives. The choice of solvent and extraction technique is an important factor in this analysis method. This screening is basically a qualitative analysis by placing the sample in a test tube and adding reagents according to the compound being analyzed (Marjoni and Ismail, 2016).

Identification of active compounds through LCMS/MS

The active compounds of the ethanol extract of red galangan rhizomes (*Alpinia purpurata* K. Schum) Rhizomes were identified through phytochemical test and LC-MS/MS by comparing the spectrum of standard compounds in the database A chromatogram was obtained and examined using Massynx V4 to determine the mass spectrum.

Haematoxylin-eosin staining

Histology is a branch of science regarding the tissues in the body that make up various organs. Histological examination using preparations of the body part to be studied (Soesilawati, 2020). Histotechnics is a method of making samples from the parts of body tissue being analyzed (Ravindran *et al*., 2018). This analysis method can make it easier to make thin slices of the preparation because the texture produced by this method will make it easier to diagnose the sample (Sofyanita *et al*., 2022). The Haematoxylin-Eosin (HE) staining method is a tissue staining technique in histology. Haematoxylin causes a bluish color in the nucleus (cell nucleus) and Eosin gives a reddish color in the cytoplasm (Mamay *et al*., 2022).

Staining (HE) is very sensitive and influenced by several factors. These factors can include appropriate cutting techniques (not too thin or thick) and accurate coloring duration so that the color is absorbed perfectly and does not blur (Chlipala *et al*., 2020). The HE staining mechanism is related to the acid-base reaction mechanism of the Haematoxylin-Eosin dye. Cell nuclei with acidic properties will interact with Haematoxylin which is alkaline. Meanwhile, eosin will bind proteins in connective tissue and cytoplasm with a positive charge because of its acidic nature (Halim, 2018). Histological testing through a series of procedures, namely (Soesilawati, 2020):

1. Preparation of materials, body part samples used must be fast and not less than 4 hours so that the samples are not damaged. The samples were then sliced into 2 - 5 mm sizes using a knife without pressure.
2. Fixation, this stage functions so that the sample structure is intact and hard by absorbing paint so that it is easy to cut. Fixation is classified into chemical and physical.
3. Soaking (dehydration, embedding, clearing and sectioning), dehydration functions to remove water components by immersing in ethanol solutions of various percentages. Then the samples were fixed in paraffin with xylene solution. The resulting sample then goes through a process of inserting it into wax (embedding) to minimize shrinkage caused by high temperature changes and which will affect the results. The finished preparations are then cleaned with alcohol, water and xylol so that the paraffin is removed.
4. Daubing, functions to differentiate between the tissues in the sample. Daubing is also able to clarify tissue components on a microscope.
5. Mounting, is the process of drying the sample on the glass deck.

Haematoxylin is colorless in its pure state.

RESULTS AND DISCUSSION

Ethanol extract Red galangan rhizomes (*Alpinia purpurata* K. Schum) Rhizomes

The thick ethanol 96 % with 5 kg washed using clean water and drained. Samples of red galanga rhizomes were cut into smaller sizes. Red galangal is then dried by air-drying, without being exposed to direct sunlight. Samples of dried red galangal rhizomes were then ground again using a blender and weighed on an analytical balance. concentrated using a rotary vacuum evaporator until a thick ethanol extract of red galanga rhizome (*Alpinia purpurata* K. Schum) was produced and then determination of Water Content of Red Galangal Rhizome Powder.

Table 1 shows the results of water content analysis. Based on Table 1, the average water content of red galanga rhizomes is 5.26 % classified as good because it meets the quality standard requirements set by Wijaya and Noviana (2022), The standard water content value set to maintain the quality of simplicia is ≤ 10%.

Phytochemical Screening of Ethanol Extract of Red Galangal Rhizomes

A natural experimental method was performed with the randomized pottest-only control group design. 25 male Wistar rats aged ± 3 months and weighing 150-200 grams were aclimated for 1 week and then divided into five groups. Figure hystopatologi I control positif, figure II control negatif, figure III consumption ethanol extract of red galanga rhizomes 50mg/kgBW/day, figure IV consumption ethanol extract of red galanga rhizomes 70mg/kg BW/day and figure V consumption ethanol extract re galanga rhizomes 90 mg/kgBW/day

Table 1. Results of water content analysis

Test	Blank filter paper (g)	Filter paper+sample before healting (g)	Filter paper + sample after healting(g)	Mass Sample (g)	% water contant
1	1.82	6.82	6.57	5.10	5.00 %
2	1.57	6.58	6.44	5.01	2.79 %
3	1.62	5.62	6.22	5.00	8.00 %
Average water content			5.26 %		

Table 2. Results of phytochemical screening of ethanol extract of red galanga rhizomes

Phytochemical test	Reagen	Result	Description
Alkaloid	Mayer and Wagner reagent	A cloudy precipitate appears	+
Flavonoid	Mg powder and HCl concentrated	Red solution	+
Polifenol	$FeCl_3$	Blackish brown solution	+
Steroid and triterpenoid	Asam asetat Anhidrat. $(CH_2CO)_2O+H_2SO_4$ pekat	A green solution and a red brown solution	+
Saponin	Mg powder and HCl	Constant foam	+

Compound Identification

Compound identification was carried out using the LC-MS/MS method. In principle, this method consists of separating compounds based on relative interaction with the chemical layer of the particles (stationary phase) and solvent elution through the column (mobile phase). The results of LC/MS-MS data analysis will produce a chromatogram in the form of a peak height plot and the molecular weight of the compounds contained in the extract will be obtained so that you can know the number of compounds contained in each sample (Mangurana *et al*., 2019). The results of compound analysis are in the form of chromatograms with several different peaks and retention times. Analysis was carried out by comparing the mass spectrum of each peak with the database to obtain the metabolite compounds contained in the ethanol extract of red galanga (*Alpinia purpurata* K. Schum) rhizomes.

The results of compound identification in samples of thick red galanga rhizome extract are shown in Table 3. Its table 3 Different retention times for the same compound can be caused by factors such as the column not having perfect balance before injection or imperfect sample preparation. Spectrum of ethanol extract of red galangal rhizomes at low and high energy positive ionization showing fragment ions. Based on a retention time of 12.75 minutes, positive ionized m/z data was obtained with a molecular mass of 519.3400 g/mol ($C_{26}H_{39}N$.) and the closeness to the Masslynx software reading (%iFit) was 67.80% and Perhexiline is an anti-breast cancer agent. Considering that testing of the anticancer activity of red galanga rhizomes (*Alpinia purpurata* K. Schum) used various variations in doses tested on Wistar rats induced by DMBA. So that data results were obtained regarding the effect of giving ethanol extract of red galangal rhizomes. Variations in doses used were the negative control group (Figure I) was not given any treatment, the positive control (Figure II) was only induced by DMBA and was not given ethanol extract of red galangal rhizome, the first treatment group (Figure III) was given ethanol extract of red galangal rhizome orally. with a dose of 50 mg/kgBW/day, the second treatment group (Figure IV) was given ethanol extract of red galangal rhizome orally at a dose of 70 mg/kgBW/day, and the group (Figure V) was given ethanol extract of galangal rhizome red orally at a dose of 90 mg/kgBW/day. Test results at a dose of 90 mg/kgBW/day can effectively prevent cancer cell proliferation.

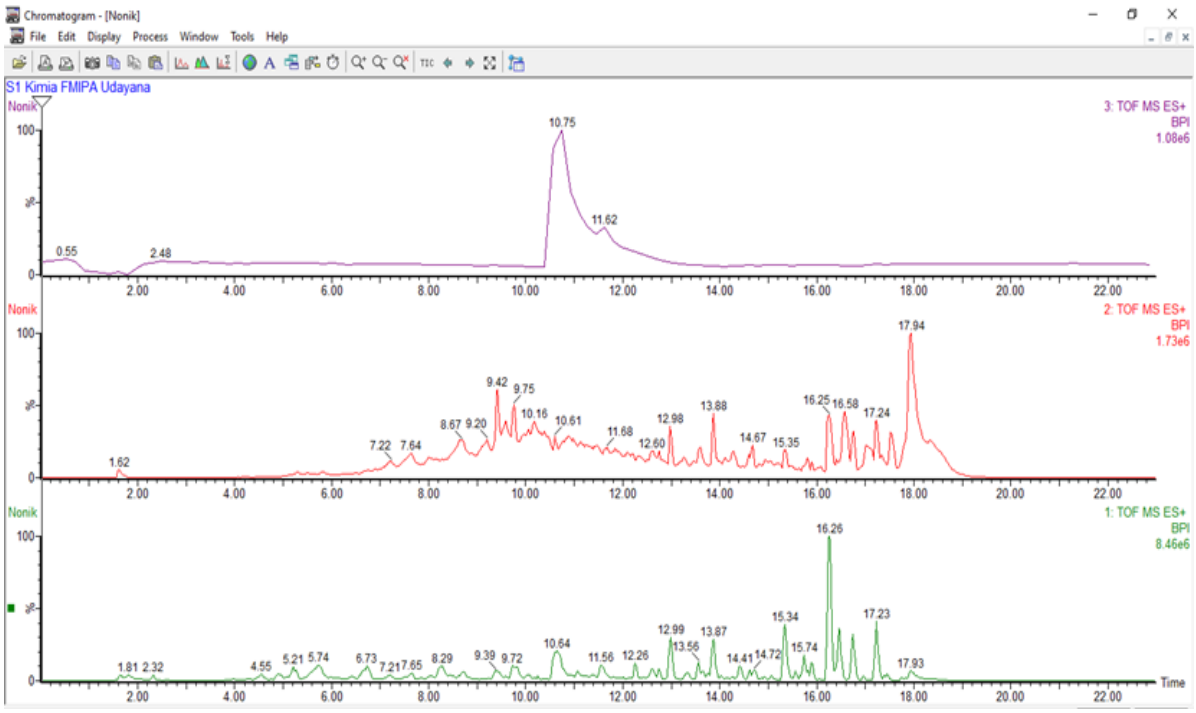


Figure 1. Chromatogram of Ethanol Fraction of Red Galanga Rhizomes

Table 3. Results of compound identifikasi of red galanga rhizomes with LCMS/MS.

Retention time	Ion Result $[M+H]^+$ (m/z) Precusor Ion and Prouduct ion	Molecular formula	Suspected compound
12,60	318 **(Precusor Ion)**, 300, 282, 270	$C_{18}H_{39}NO_3$	Phytosphingosine
12,75	519 **(Precusor Ion)**, 502, 472, 398	$C_{26}H_{38}N_{12}$	N-[2-(Dimethylamino)ethyl]-N′-[4-(4-methyl-1-piperazinyl)phenyl]-6-[4-(2-pyrimidinyl)-1-piperazinyl]-1,3,5-triazine-2,4-diamine
12,99	519 **(Precusor Ion)**, 502, 472, 398	$C_{26}H_{38}N_{12}$	N-[2-(Dimethylamino)ethyl]-N′-[4-(4-methyl-1-piperazinyl)phenyl]-6-[4-(2-pyrimidinyl)-1-piperazinyl]-1,3,5-triazine-2,4-diamine
13,87	-	-	-
15,34	-	-	-
16,26	-	-	-
16,75	278 **(Precusor Ion)**, 236, 222, 194, 96	$C_{19}H_{36}N$	Perhexiline
17,23	-	-	-

This dihydrodiol epoxide compound will covalently bind the purine ecocyclic amino group on DNA with the deoxyadenosine (dA) or deoxyguanosine (dG) exocyclic amino group on DNA to form a stable adduct. Adducts formed on nucleotide bases result in a mutation process caused by errors in DNA repair or replication in lesions. Meanwhile, the formation of radical cations is influenced by the removal of one electron due to oxidation in the π electron system of the molecule. Cell cycle abnormalities due to induction of this compound will result in uncontrolled cell division and result in apoptosis of lymphoma cells. So, based on this mechanism,cancer cells will form in mouse breasts (Wuyung, 2016).

According to research by Liangan *et al*., (2015), induction using benzopyrene, which is a compound in the same group as DMBA, namely polycyclic aromatic hydrocarbons (PAH), was carried out for 14 days in groups of mice B (treatment I: induction and breast excision) and C (treatment II : induction and galangal extract 4.5 mg/head/day) causes cuboidal epithelial hyperplasia in the lactiferous ducts so that the walls become thicker (>4 layers epithelial cells). Induction with DMBA resulted in death in 3 mice. This is caused by the DMBA sonde process being inappropriate and resulting in inappropriate incoming compounds in the pathway. After 2 weeks, mice in the treatment group were given thick extract of red galangal rhizomes for 2 weeks according to the dose for each treatment for anticancer treatment. Based on the results of compound identification analysis using LC-MS/MS, the ethanol extract of red galangal rhizomes contains alkaloid compounds (Perhexiline) and polyphenols (N-[2-(Dimethylamino)ethyl]-N′-[4-(4-methyl-1 - piperazinyl)phenyl]-6-[4-(2-pyrimidinyl)-1-piperazinyl]-1,3,5-triazine-2,4-diamine) which has the potential as anticancer.

The mechanism of this phenolic compound as an anticancer is shown by the inhibitory effect of this compound on Nrf_2 (Nuclear factor erythroid 2-related factor 2). Inhibition of Nrf_2 by phenolic compounds leads to increased sensitivity of cancer cells to conventional anticancer therapy, reducing tumor growth and cancer cell death. Nrf_2 has an important role in cellular defense against oxidative stress and exogenous toxicants. So Nrf_2 has emerged as a therapeutic target for

cancer prevention and therapy. However, because Nrf_2 has a paradoxical role in cancer biology, it is necessary to understand the molecular pathways leading to the tumor suppression or oncogenic effects of Nrf_2 for the development of drugs with highly specific and limited side effects. Natural products, including phenolic compounds, mediate the Nrf_2/ARE pathway and can act as chemopreventive or chemotherapeutic agents (Sharifi-Rad *et al*., 2023).

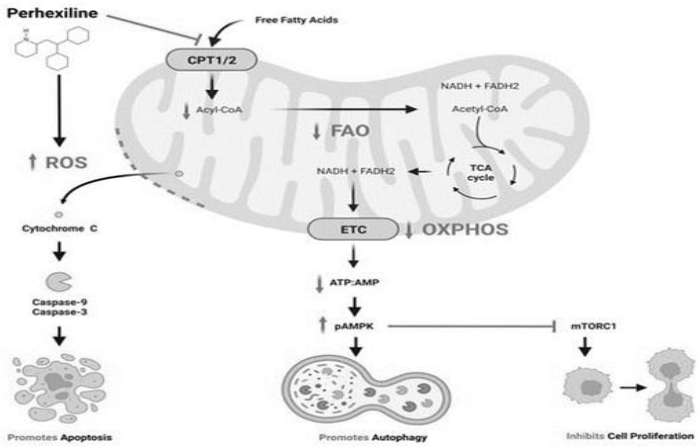


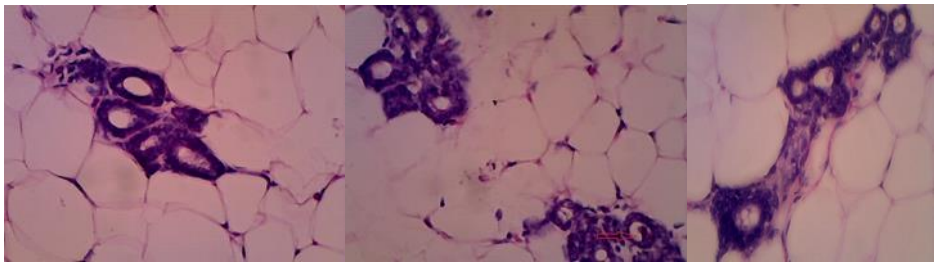
Figure 2. Anticancer mechanism of perhexiline

Perhexiline's inhibition of carnitine palmitoyltransferase 1 (CPT1) and CPT2 limits the entry of free fatty acids into the mitochondrial matrix, thereby inhibiting fatty acid oxidation (FAO). This can limit the production of the electron transport chain (ETC) coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), which will inhibit oxidative phosphorylation (OXPHOS) and the formation of adenosine triphosphate (ATP). A decrease in the ratio of ATP to adenosine monophosphate (AMP) activates AMP-activated protein kinase (AMPK) through phosphorylation (pAMPK). pAMPK triggers autophagy, and inhibits cell proliferation by inhibiting mammalian target of rapamycin complex 1 (mTORC1). Additionally, perhexiline increases reactive oxygen species (ROS) levels, which compromise the integrity of mitochondrial membranes, leading to the release of cytochrome C and activation of caspases that initiate apoptosis (Dhakal *et al*., 2023).

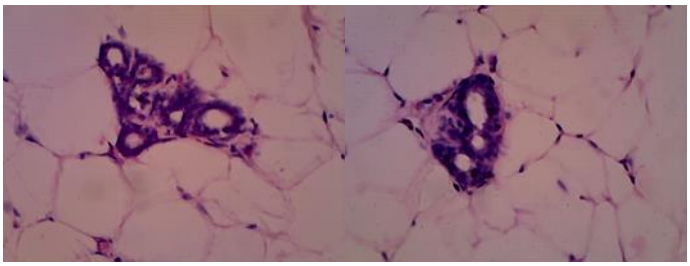
Image of histopathological changes in breast cancer

The examination uses the Haematoxylin-Eosin staining method. Tissue staining is based on the interaction of the chemicals used with cell and tissue components. The dyes used are able to interact and repel specific components which are influenced by their chemical properties. Haematoxylin is a chemical compound that is basic (basophilic), this compound can interact or bind to cells and tissues (such as DNA and RNA) which are acidic and produce a blue color change. Meanwhile, Eosin is a chemical compound that is acidic (eosinophilic), this compound is able to interact or bind to cells and tissues (such as cytoplasmic proteins) which are alkaline and produce a color change to red or pink (Digambiro and Parwanto, 2024).

Therefore, based on these results, it can be concluded that DMBA induction is able to cause cell proliferation which is an early sign of the formation of cancer cells. Meanwhile, administration of red galangal rhizome extract to Wistar rats after DMBA induction at a dose of 70 mg/kgBW/day (Figure IV) and 90 mg/kgBW/day (Figure V) resulted in suppression of cuboidal epithelial cell hyperplasia so that cancer cells did not form. However, the dose of 50 mg/kgBW/day (Figure III) in treatment was not optimal in preventing cell proliferation caused by the DMBA compound. Cancer is excessive and uncontrolled cell proliferation, causing gene mutations which are a factor in the occurrence of cancer. Cells that undergo abnormal division will accumulate and form tumors. Tumor cells are divided into benign and malignant tumor cells. These malignant tumor cells are cancer cells.



(I) (II) (III)



(IV) (V)

Figure 3. Histopathological examination of breast gland tissue uses five.

According to the mechanism, if the DNA repair mechanism fails, the cell mutations that occur will accumulate in genes that regulate the cell cycle, such as proto-oncogenes becoming oncogenes, causing uncontrolled cell growth. This mutation is also able to interfere with cell apoptosis, which functions to destroy cells that are badly damaged so that the cells will continue to grow. When this cell proliferation occurs simultaneously with uncontrolled genetic changes, the cells will undergo transformation into cancer cells. This process is referred to as carcinogenesis which consists of stages of initiation or initial mutation, cell proliferation and progression or invasive cancer growth. Proliferation in the long term will increase the opportunity for additional mutations to occur that encourage the development of malignant tumors.

CONCLUSIONS

Ethanol extract of red galangal rhizome (*Alpinia purpurata* K. Schum) has an effect on the histopathology of Wistar rat breast cancer cells at doses of 70 mg/kgBW/day and 90 mg/kgBW/day by preventing the proliferation of cuboidal epithelial cells so that red galanga rhizome has potential as a anticancer. The secondary metabolite compound contained in the rhizome of red galangal (*Alpinia purpurata* K. Schum) which has the potential to act as an anticancer is Perhexiline (cinnamaldehyde) which is a polyphenol compound.

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