

Antiproliferation activity of water and ethyl acetate red yeast rice fraction against MCM-B2 tumor cells

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ABSTRACT

Red yeast rice (RYR), commonly known as angkak, is a functional food fermented by rice using *Monascus* mushrooms. It has a pigment and flavonoid content that is useful as an antioxidant and plays a role in preventing cancer or tumors. This analysis aims to test the antiproliferation activity of commercial RYR fractions of water and ethyl acetate against Miyazaki Canine Mammary Gland Tumor-Bambang 2 (MCM-B2) breast tumor cells. Research on RYR antiproliferation of cancer-sustaining MCM-B2 cells has not been previously released. In the preliminary study, the antiproliferation bioactivity was measured using the brine shrimp lethality test. The outcome of the brine shrimp lethality test showed that ethyl acetate and water fraction cytotoxicity were more than 1000 ppm and 337.07 ppm, respectively, at a lethal concentration of 50. Antiproliferative activity analyses were performed using direct hemocytometer counting. The antiproliferation activity data collected were analyzed using one-way ANOVA and Duncan continuous testing. The outcome showed that the water and ethyl acetate RYR antiproliferation activity against MCM B2 cancer cells correlated positively with the increasing concentration of each fraction. Ethyl acetate and water fractions at concentrations of 350 ppm may inhibit the growth of MCM-B2 cancer cells *in vitro*, reaching 42.63 percent and 39.84 percent, respectively, not significantly different ($P < 0.05$) with a positive doxorubicin control of 41.24 percent. In conclusion, the ethyl acetate and water fraction of RYR have potent antiproliferation activity against MCM-B2 breast tumor cells.

Keywords: antiproliferation, direct counting, MCM-B2 cell, red yeast rice

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INTRODUCTION

The second leading death rate for women globally is breast cancer (Liu and Chen, 2013; Park et al., 2005). The World Health Organization (WHO) announced that an estimated 627,000 women died of breast cancer, approximately 15% of all cancer deaths among women in 2018 (WHO, 2020). Birth cancer, including radiation, exposure to chemicals with carcinogenic effects, viruses, carcinogenic food intakes, and a lack of consumption of fruits and vegetables, are factors which cause abnormality proliferation of the cell in the body. The creation of abnormal lumps begins with breast cancer, called tumors. One form of breast tumor cell may be isolated from the mammary glands of a 10-year-old female dog (MCM-B2). These tumor cells are formed from atypical cells and stem cells (Priosoeryanto et al., 1995). Natural products, particularly in the field of cancer pharmacology, had long played an essential role in drug discovery. RYR is an anticancer alleged secondary metabolite and pigment of the source.

RYR is a rice-derived product that ferments rice using *Monascus* sp. mushrooms. In Asian society, RYR has become a popular supplement to food, herbal products, and health (Lee et al., 2013). RYR is a commercial market in Asia, including Indonesia. It includes food additives, preservatives, food coloring in fish, and meat (Wei et al., 2003). RYR also acts as a fruit flavor enhancer for yogurt except as a coloring agent. Alternative food coloring and pigments were developed by RYR (Dweck, 2002). As a traditional medicine, RYR also strengthens the digestive system, accelerates blood circulation, reinforces the spleen function and dries the wound into the stomach area (Nijjar et al., 2010; Yang et al., 2014). *Monascus* pigment demonstrates pro-apoptosis effects or the ability to inhibit the growth of various types of tumor cells (Lee et al., 2013). Recent experiments have found Manocalin K, an active metabolite of RYR, as competitive with HMG-CoA Reductase, which can inhibit cholesterol biosynthesis (Zhang et al., 2016). RYR also contains saponins, triterpenoids, terpenoids, coumarin, flobatin and flavonoids. The possible antioxidant of all of these metabolites. Food with antioxidants helps the body process the proliferation of lymphocytic cells, which can lyse cancer cells from natural killers (Agustinisari and Zakaria, 2019).

Pigments and other compounds in RYR have contributed to antiproliferative effects (Hong et al., 2011). Recent studies have shown that RYR pigment inhibits skin cancer (Hsu and Pan, 2012), tumor growth in mice; c67 BL pulmonary carcinoma is inhibited (Ho and Tzu-Ming, 2009). The use of RYR metabolites as a new approach to cancer prevention and treatment may be expected, but further verification remains important in preclinical and clinical studies. A naturally occurring compound can be named anticancer as anticancer, antiproliferation with different types of cancer cells has been reported in several studies. Flavonoid has a secondary anticancer potential in addition to the pigment present in RYR (Lee et al., 2013). RYR antiproliferation activity has not been identified in Miyazaki Canine Mammary Gland Tumor-Bambang 2 (MCM-B2) breast tumor cells. Since each cell varies in its sensitivity and mechanism from the natural product, MCM-B2 can be generated from atypical stem cells or cells (Priosoeryanto et al., 1995). Observation in vitro of the MCMB2 cell line shows that these cells exhibit homogeneous morphological and immunohistochemistry characteristics and also display less differentiation in cell culture, with this characteristic of the MCM-B2 cell being clearly capable of inhibiting the spread, but with a low potential of the natural product (Elsyana et al., 2016), as expected in commercial RYR used in this study.

The purpose of this analysis is to test the antiproliferation activity of commercial RYR fractions of water and ethyl acetate against MCM-B2 breast tumor cells. The use of water and ethyl acetate as fractional solvents has polar and semipolar solvent characteristics that can be optimally used as anticancer properties for the extraction of pigmentation components and secondary compounds.

MATERIALS AND METHOD

Materials

In this study, Mean material RYR was collected from the traditional market in Bogor, West Java. Besides, MCM B-2 tumor cells were collected from the tissue culture collection of Faculty Veterinary Medicine, IPB University.

Methods

Preparation of water and ethyl acetate fraction of red mold rice

Sample preparation, extraction, and fractionation were carried out based on the previous study with modification (Hasim et al., 2017). Samples of commercial RYR were dried for 6 hours in an oven at 50°C. In addition, the blender has been mashed to 40 mesh scale. The moisture content of the sample to be used is < 10 percent. Maceration and removal with 96 percent ethanol up to 800 mL with 110 rpm shaker is used to extract 40 grams of RYR. The filter was evaporated with a 50°C rotating evaporator. Fractionation is done with water and ethyl acetate as a solvent using the liquid-liquid partition process. A rotary evaporator at a temperature of 50°C was used for a fraction of ethyl acetate and water.

Brine Shrimp Lethality Test (BSLT)

The BSLT test was performed to determine the range of concentrations that would then be used to evaluate the antiproliferation test. BSLT tests were carried out using the (Rampe and Tombuku, 2015) method with modification. A steady supply of oxygen has been in the artemia salina shrimping larvae for 48 hours. Dissolving the ethyl acetate and water fraction of RYR with seawater in order to produce test stock solutions with a concentration of 2000 µg / mL was performed. The stock solution has been diluted in a plate using sea waters to measure levels of 0, 100, 200, 400, 600, 800, and 1000 µg/mL. After 24-hour incubation, dead shrimp larvae have been counted and LC50 values have been determined. The test was conducted with a triplicate.

Antiproliferation activity

Tumor cell inhibitory activity test was performed using Priosoeryanto et al. (Priosoeryanto, 2009), Starting by planting MCM-B2 cells using DMEM media on a 24-well tissue plate. The planting media contained 850 µL of growth media, 10 µL of fungizone, 10 µL of gentamicin and 30 µL of serum. Water fraction and ethyl acetate fraction were weighed at 0.02 g to be liquefied in 10 mL of sterile distilled water. The extract doses used were 87.5, 175, 350, 700 and 1400 µg / mL (as defined by BSLT LC50). In addition, first thawing treatment is given to the cell suspension. MCM-B2 Sustainable Cell Suspension Plate up to 50 µL per hole. Wells that have not been applied to the fraction as a negative control (0 ppm) and wells that have been applied to 100 µg/mL of doxorubicin as a positive control. MCM-B2 sustainable cells were grown by incubation at 37 ° C (5% CO₂) in the incubator for 3 days.

The tumor cell inhibitory activity test is conducted by cell harvesting and counting. Sustainable cell harvesting is carried out when the negative control has expanded optimally to cover about 70% of the entire surface after 3 days of planting. The cell suspension is then homogenized by up and down pipetting using a micropipette. Next, 80 µL cells were injected into a 20 µl trypan blue dye microplate. The homogeneous cell suspension was dropped on the hemacytometer of Neubauer. The number of cells was counted with a light microscope. The cells that are counted are living and dead cells in the center of the measured space. This test was performed with a triplicate.

Data analysis

Probit analysis was performed using MINITAB 17 as BSLT data to assess the 50 percent mortality concentration (LC50) at 95 percent confidence intervals. Data on antiproliferation behavior were analyzed using variance analysis (ANOVA) by Duncan's continued $P \leq 0.05$ test to identify the significance of differences between treatment and control groups.

RESULT AND DISCUSSION

In this analysis, the extraction of RYR was performed using maceration and remaceration dynamically, with 96% ethanol. The advantages of using 96% ethanol as a solvent is nontoxic, noncorrosive, has a strong absorption rate, require only a little heat in the evaporation process and is readily available (Lestari, 2015). Ethanol is a polar solvent and has a lower polarity than water, so it can attract polar and semipolar compounds in RYR simplicia.

Recent studies have recorded that ethanol extract and ethyl acetate fraction of RYR contain flavonoid: 8.84 and 22.09 mg QE/g RYR simplicia, respectively (Hasim et al., 2017). Another research reported that pigments in RYR are yellow pigment (monascin, ankaflavin), pink pigment (monascurbin, ribropunctamine) (Pattanagul et al., 2007). The pigment contains flavonoid anthocyanin, which makes RYR a potentially anticancer. Increasing epidemiological studies indicate that high intake of flavonoids could be associated with a decreased risk of cancer (Batra and Sharma, 2013). In addition, active anticancer compounds such as manocalin K would increase with an increase in the ethanol concentration used for extraction (Singgih et al., 2014).

Meanwhile, fractionation was conducted using two separate polarity solvents. Pigment-containing anthocyanin has a polar structure that makes them dissolve easily in polar and semipolar solvents. This research, therefore, uses ethyl acetate as a semipolar solvent and water as a polar solvent. The difference in the degree of polarity and the specific gravity of each solvent is the concept of separation of the compounds in the sample used (Pratiwi et al., 2016). The fractionation outcomes showed that water has a higher yield compared to ethyl acetate fractions, as shown in Figure 1. These data indicate that the fraction of water has a higher yield than the fraction of ethyl acetate. Water and ethyl acetate fractions yield 18.80 ± 0.29 and $11.58 \pm 0.64\%$, respectively. It can be indicated that the RYR used in this analysis has more polar metabolites.

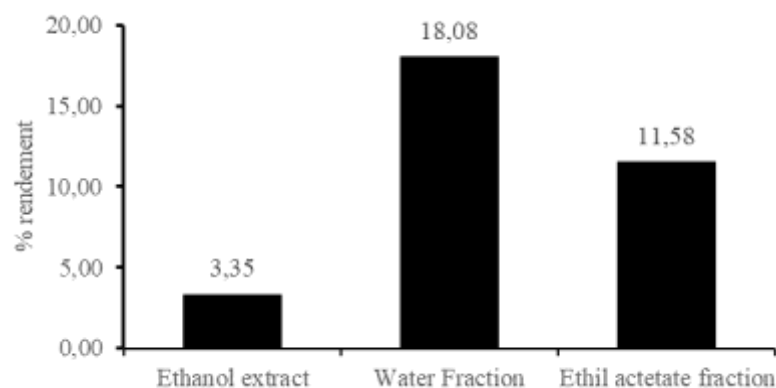


Figure 1. % Yield extraction of red yeast rice

The BSLT was used as an initial measure of RYR antiproliferation activity. The parameter calculated was the mortality rate of *Artemia salina* shrimp larvae in the nauplii process after a red yeast rice fraction sample. Death of *Artemia salina* larvae is correlated with the potential of bioactive compounds in the sample with the reduced anti-inflammatory activity of larvae (Vitalia et al., 2016). Flavonoid compounds can cause the death of *Artemia salina* larvae through the gastric poisoning mechanism of larval death by inhibition of food inhibition (antifedant) (Vitalia et al., 2016). Based on Meyer's toxicity index, a sample was reported to have a potential for bioactivity if it had an LC50 value (50% mortality rate for *Artemia salina* shrimp larvae) of < 1000 µg / mL (R. Hamidi et al., 2014). Based on this categorization, the RYR water fraction in this study included anticancer activity as a bioactivity potential. While the fraction of ethyl acetate has a value of LC50 > 1000 µg/mL. The BSLT test results show that the more polar solvent (water), the more toxic the effect. The results of the BSLT test are shown in Tables 1 and 2. These data indicate that ethyl acetate and water fractions have values of LC50 > 1000 ppm and 337.07 ppm, respectively. The LC50 BSLT value of the 337.07 ppm rounding water fraction to 350 ppm was invented as a mean value to assess the concentration range in the antiproliferation analysis. Thus, the LC50 values obtained from the cytotoxic pre-screening test of *Artemia salina* larvae provide a picture of RYR extract with potential bioactivity, including an anticancer compound, for further testing of MCM-B2 breast cancer cells in vitro.

Table 1. The BSLT test result of Red yeast rice

Fraction	Concentration (ppm)	% mortality of cell
Water	100	36.7
	200	50
	400	66.7
	800	80
	1000	96.7
	2000	10
Ethyl acetate	100	0
	200	0
	400	0
	800	0
	1000	0
	2000	40

Table 2. LC₅₀ of water and ethyl acetate fraction of red yeast rice using BSLT methods

Sample (fractions)	LC ₅₀ (ppm)
Ethyl acetate	>1000
Water	337.07

The antiproliferation study of both water and ethyl acetate fractions showed that MCM-B2 cancer cell proliferation decreased with increasing concentration. The variation of fraction concentration of the antiproliferation test was based on the outcome of the LC50 of BSLT test. A negative control containing only culture media and MCM-B2 cells was considered to have a proliferation activity of 100 percent and an antiproliferation activity of 0 percent. A positive control containing 100 ppm cancer drug (doxorubicin), DMEM rising media and MCM-B2 cells are considered to have an antiproliferation effect of 41.24%. Doxorubicin has been used as a proactive

Antiproliferation Activity of (Hasim et al.,)

antiproliferation regulation in MCM-B2 cells (Djamiludin et al., 2019). Doxorubicin is used as an anthracycline synthesis drug in cancer patients for chemotherapy (Yun et al., 2019). Two proposed mechanisms of doxorubicin act in cancer cells, i.e. DNA intercalation and topoisomerase-II-mediated DNA repair and free radical generation and damage to cell membranes, DNA, and proteins (Thorn et al., 2011). However, doxorubicin can cause many side effects such as hair loss, nausea, dry lips, vomiting, and heart problems (Atiqoh et al., 2011; Hasim et al., 2020).

Average inhibition of MCM-B2 ethyl acetate cancer cells and the water fraction of RYR is expressed as a percentage of antiproliferation. Based on the graphs shown in Figures 2 and 3. These findings indicate that the distribution of MCM-B2 cancer cells decreases with increasing concentration. The antiproliferation test results show that concentration of 350 µg/ml, the antiproliferation activity of the water fraction and the ethyl acetate fraction did not have a significant difference with doxorubicin as a positive control. In general, the fraction of ethyl acetate will reduce MCM-B2 cells proliferation.

The fraction of 350 ppm ethyl acetate has an antiproliferation activity of 42.63% greater than 100 ppm doxorubicin and has no significant difference through the Duncant test. The higher the concentration of ethyl acetate fraction used, the higher the antiproliferation activity.

The water fraction antiproliferation activity is shown in Figure 3. Water fractions, in general, can reduce the proliferation of MCM-B2 cells. Water fraction 350 ppm has an antiproliferation activity 39.84% lower than the antiproliferation activity of 100 ppm doxorubicin 41.24% but has no substantial difference from the Duncant test. The higher the concentration of the water fraction used, the greater the antiproliferation activity.

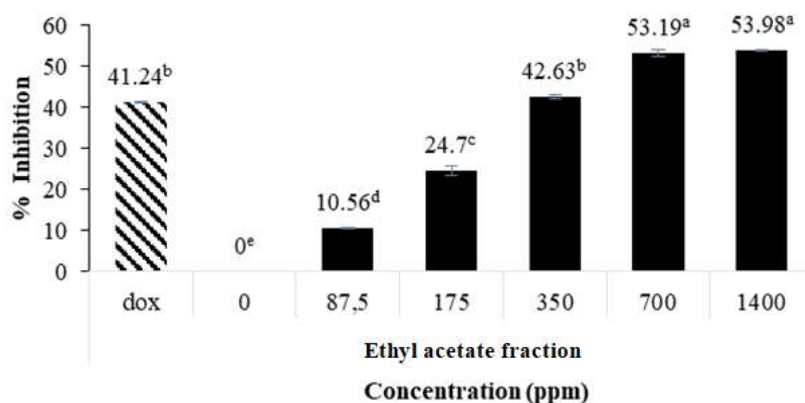


Figure 2. Antiproliferation activity of ethyl acetate fraction against MCM-B2 cells

Each data represent the mean of triplicate inhibition percentage. Values followed with different superscripts (a, b, c, d and e) represent significant differences at $p < 0.05$. a showed the highest inhibition potential, while e showed the lowest inhibition

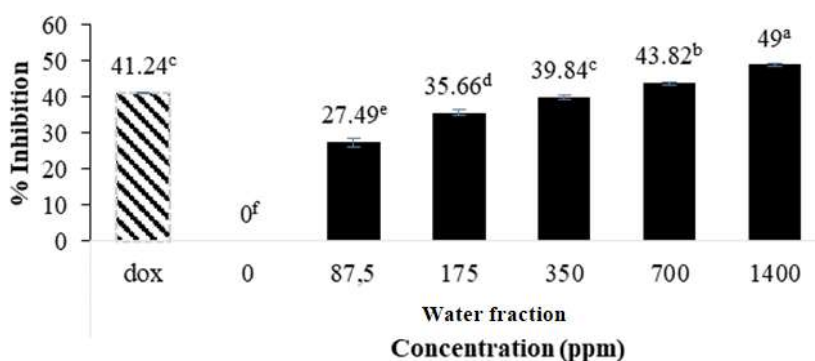


Figure 3. Antiproliferation activity of water fraction of red yeast rice against MCM-B2 cells Each data represents the mean of triplicate inhibition percentage. Values followed with different superscripts (a, b, c, d, e, and f) represent significant differences at $p < 0.0$. a showed the highest inhibition, while f showed the lowest inhibition

The antiproliferation activity of ethyl acetate fraction increased more with an increase in concentration than the water fraction. This is confirmed by the findings of previous studies that the RYR fraction of ethyl acetate contains more phenolic and flavonoid compounds than the water extract (Hasim et al., 2017). Further research on the identification of flavonoid compounds acting as anticancer in this RYR is required. The difference in the antiproliferative activity of the above fraction may be due to variations in the response or sensitivity of the cancer cell receptor MCM-B2 to each of the active compounds in each fraction (Talib and Mahasneh, 2010). Since the LC50 value, the fraction of ethyl acetate is classified as nontoxic to *Artemia salina*, but the results of the MCM-B2 cell tests can inhibit cell growth. The explanation for this is that the MCM-B2 cells used are cells that have not been differentiated. They are, after all, stem cell origin (Priosoeryanto, 2009). Therefore, cell growth is interrupted when given a sample of the ethyl-acetate fraction. Although the test object used in BSLT is an organism that has been differentiated (limbs are complete) and the digestive tract is perfect (Muaja, 2013). As the consequence, concentrations of less than or equal to 1,000 ppm can not kill half of the total population of shrimp larvae.

Previous in vitro experimental analysis of RYR aqueous and ethanol extract demonstrated inhibitory activity in pre-adipocyte proliferation (Bule et al., 2018). RYR anticancer plays a role as antiproliferation by programmatic induction of cell death (apoptosis), based on preclinical in vivo studies, it is established that one of the red pigments (rubropunctatin) of RYR contains anthocyanin flavonoid, induce apoptosis (Zheng et al., 2010). RYR contains monacalin K, phenolic and flavonoids which are useful for regulating the growth of cancer cells. RYR consists of rubropunctatin and manocaline, which has a very powerful anticancer activity against cancer cells (Xu et al., 2017). In addition, flavonoids function by three mechanisms to stimulate the apoptotic pathway in cancer cells by acting as an oxidant, the second inhibit protein kinase activity that induces signal transduction inhibition, and the third inhibits tyrosine kinase receptor activity that increases and plays a role in the growth of cancer cell malignancy (Mardany et al., 2016). In addition, the mechanism of flavonoids as anticancer is the inactivation of carcinogens, antiproliferation, suppression of the cell cycle, induction of apoptosis, differentiation, and inhibition of angiogenesis (Ismaryani et al., 2018). Further analysis of flavonoid structure as secondary metabolites with anticancer properties in both the water and ethyl acetate fractions of this RYR is required.

CONCLUSION

Water and ethyl acetate fraction of ethanol extract of red yeast rice have been shown to have antiproliferation activity against MCM-B2 cells. The fraction of ethyl acetate has greater antiproliferation activity compared to the fraction of water.

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