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The Extract of *Merremia mammosa* Tubers Increases the Cytotoxicity of Doxorubicin and Induces Apoptosis in 4T1 Malignant Cancer Cells

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Abstract

Doxorubicin has been incorporated in cancer therapy regimes for wide-range malignancies, but it causes many side effects. These adverse effects can be overcome by combining doxorubicin with other ingredients that possess anticancer potential but are safe on normal cells. The tubers of *Merremia mammosa*, locally known as "Bidara Upas" in Indonesia, have been proven to have anticancer potential with resin glycosides as the active compounds. This research aims to develop the extract of *M. mammosa*'s tubers (MTE) as a co-chemotherapy agent for doxorubicin. A malignant cancer cell line, 4T1, was used as the model. MTE was obtained by maceration in 96% ethanol. The thin layer chromatography confirmed that MTE contains glycoside compounds. Administration of MTE to 4T1 cells showed cytotoxic activity with an IC_{50} value of 61 µg/mL as evaluated by MTT assay. The combination of MTE and doxorubicin exhibited synergistic cytotoxic effects with a combination index of <0.7. Moreover, MTE at around IC_{50} was able to cause DNA fragmentation indicating apoptosis as observed by the agarose gel electrophoresis.These data support our hypothesis that MTE may serve as a potential co-chemotherapeutic agent for doxorubicin; however, the apoptosis-inducing potency of the combination requires further investigation.

Keywords: *4T1 cell, apoptosis, Bidara Upas, co-chemotherapy.*

INTRODUCTION

Cancer malignancy is still an urgent problem in the world of global health, including cases of breast cancer. Doxorubicin is one of the main chemotherapy agents that has been used in a wide-array of malignancies including both solid tumors and leukemia or lymphoma (Rivankar, 2014). However, it causes many adverse effects such as cardiotoxicity, neuropathy, hepatotoxicity, nephrotoxicity, and myelosuppression (Ajaykumar, 2020). These undesirable side effects can be overcome by combining doxorubicin with other ingredients that have anticancer potential but are

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safe on normal cells. This combination can reduce the dose of doxorubicin, thereby reducing side effects but still maintaining or even increasing the effectiveness of therapy (Zulfin, *et al*., 2021).

Natural ingredients can be used as potential companion agents for doxorubicin. One of the natural plants originating from Southeast Asia that has anticancer potency is *Merremia mammosa*, locally known as "Bidara Upas" in Indonesia. This plant has been used for generations as a traditional medicine (Cahyaningsih, *et al*., 2017). The water extract of the tuber displays cytotoxicity on MCF-7 breast cancer cells possibly by inducing cell apoptosis (Widiyastuti, *et al*., 2019). The tuber of this plant contains resin glycoside, flavonoids, tropane alkaloids, polyphenols, and flavonoids (Kitagawa, *et al*., 1996; Purwitasari, 2022). Resin glycosides can destroy target cells by disrupting cell permeability, causing an imbalance in cellular homeostasis (Pereda-Miranda, *et al*., 2009). Thus, resin glycosides can be used as an anticancer agent with a cytotoxic and anti-metastatic mechanism, as has been proved in HT-29 and HCT-116 colon cancer cells with the IC_{50} value of around 6-7 µg/mL (Zhu, *et al*., 2019). Genus *Merremia* show anticancer potencies in several types of cancer cell lines likely due to their resin glycoside contents as reviewed by Olatunji, *et al*. (2021). Taken together, we hypothesize that tubers of *M. mammosa* with high content of resin glycosides would display cytotoxic potency with apoptosis induction *in vitro*.

As one of the highest cancer cases globally, including Indonesia, treatment strategy for breast cancer is in need to be continuously developed (Osborne, *et al*., 2025). One subtype of breast cancer with high malignancy is triple-negative breast cancer (TNBC), that counts up to 25% cases of breast cancer in Indonesia (Rahmawati, *et al*., 2018). This subtype characterized by the diminished or absent expression of estrogen receptor (ER), progesterone receptor (PR), and human epithelial growth factor receptors (HER-2), thus requires an effective approach (Haryanti, *et al*., 2022). The previous study shows the cytotoxic activity and apoptosis induction potencies of *M. mammosa* in luminal breast cancer cell line (Widiyastuti, *et al*., 2019). But, how its anticancer and apoptosis induction properties in TNBC remains unanswered.

This research investigates the potency of the extract of *M. mammosa* tuber as a co-chemotherapy agent for doxorubicin and whether it stimulates apoptosis in malignant cancer cells. The 4T1 TNBC cell line was used as the malignant cancer cell model. After successfully obtaining the extract containing resin glycosides, the cytotoxicity assay was carried out by MTT assay both in the single and in combination, followed by the DNA fragmentation assay to show the apoptosis incidence in 4T1 cells.

MATERIALS AND METHODS

Extract Preparation

Tubers of *M. mammosa* (Figure 1A) were obtained from Tawangmangu, Central Java. The samples were correctly identified as *Merremia mammosa* Hallier f. based on the determination that was carried out by the Department of Pharmacognosy, Faculty of Pharmacy, Universitas Gadjah Mada (UGM) (reference letter no. 44.25.9/UN1/FFA.2/BF/PT/2023). The tubers were dried and powdered through a 40-mesh sieve, weighed as much as 2.7 grams in a conical tube and 20 mL of 96% ethanol was added as the solvent (Nurrosyidah, *et al*., 2022). The extraction was carried out by constant stirring in a rotary shaker for 24 h at room temperature, followed by filtration. The macerate was then placed in a porcelain cup and evaporated in a fume cupboard until a thick extract remains and was weighed periodically to obtain a fixed weight. The obtained extract is abbreviated as MTE from here after.

Phytochemical Identification by Thin Layer Chromatography (TLC)

A silica gel plate (60 F254 nm) was used as the stationary phase and a mixture of butanol: ethyl acid: water (4:6:1 v/v) was used as the mobile

phase (Putri & Sakinah, 2018). MTE was dissolved in 96% ethanol and spotted on the stationary phase. After elution, the plate was observed under visible light following reaction with alpha naphthol to identify the glycoside compounds.

Cytotoxicity Test with MTT Assay

Available from the collection of Cancer Chemoprevention Research Center, Faculty of Pharmacy, UGM, the 4T1 cells were grown and cultured in a complete media as described previously (Ramadani, *et al*., 2021; Zulfin, *et al*., 2021). Cells that were 80% confluent were harvested and then seeded into a 96-well plate (5×10^3 cells/well). After 24 h, cells were treated with MTE or doxorubicin in a final volume of 100 µL for each well. Five milligrams of MTE were dissolved in 50 µL of dimethyl sulfoxide as the stock solution and the serial concentration 1, 10, 25, 50, and 100 µg/mL in complete media was prepared from it. Doxorubicin (Sigma) solution was prepared according to the previous method (Putri, *et al*., 2021; Zulfin, *et al*., 2021). After the 24-h treatment, 100 µL of MTT reagent (0.5 mg/mL) was added into each well. The formazan formed after 2-4 h was dissolved by the stopper reagent (10% SDS in 0.01 N HCl) and the absorbance was read in a microplate reader at λ 595 nm. The percentage of live cells wes calculated based on the curve between the percentage of cell viability and the concentration of the tested compound after treatment to calculate the concentration that can inhibit 50% of cell growth (inhibitory concentration 50, IC_{50}) using non-linear regression data analysis tools available *i.e*. Microsoft Excel (Ikawati, *et al*., 2020).

As for the cytotoxic combination assay, the same method as the single cytotoxic assay was used. The IC_{50} values of each MTE and doxorubicin were used to determine the concentration for the combination assay. The combination index (CI) was calculated following the established method (Ikawati, *et al*., 2023).

DNA Fragmentation Assay

As many as 1×10^5 cells/mL of 4T1 cells were cultured on a 10-cm dish and incubated for 24 h. The cells were treated with single MTE at around and doxorubicin as a positive control at the concentration of around IC_{50} and ¼ IC_{50}, respectively. After 24-h of treatment the cells were washed and harvested, followed by the DNA isolated. The cells used for DNA isolation were at the same passage and under identical culture conditions to ensure consistency in the experimental setup. The cell pellets were mixed with 500 µL of DNA extraction buffer (containing 0.1 M Tris-HCl pH 7.5, 0.05 M EDTA pH 8.0, 1.25% SDS, and 2 µg/mL RNase) and centrifuged at 5,000 rpm for 10 min. After discarding the supernatant, 100 µL of SE buffer, 100 µL of 4 M guanidine isothiocyanate, 700 µL of chloroform, and 400 µL of NaCl were added and homogeneously mixed. After centrifugation at 10,000 rpm for 10 min, the upper layer was transferred into a new tube. The DNA was precipitated by the cold absolute ethanol and dissolved in the TE buffer. The extracted DNA was quantified with a spectrophotometer. DNA samples and the DNA ladder marker (DM2400, AccuBand 100, Smobio) were electrophoresed on a 1.5% agarose gel containing 1 µL/100 mL of DNA gel staining (S33102, SYBR Safe DNA Gel Stain, Invitrogen). The gel was observed and photographed with an ultraviolet gel documentation system (Saadat, *et al*., 2015).

Data Analysis

The data were analyzed qualitatively. The classification of cytotoxicity based on the IC_{50} value was as follows: <2 µg/mL as strong cytotoxic, 2-89 µg/mL as moderate cytotoxic, and >90 µg/mL as not cytotoxic (World Health Organization, 2017 *cit* Haryanti, *et al*., 2018). The classification of combination potency based on the CI value was as follows: 0.1-0.7 as synergist, 0.7-0.9 as moderately synergist, 0.9-1.1 as addictive, while >1.1 as antagonist (Haagensen, *et al*., 2012). The DNA

fragmentation pattern in 4T1 cancer cells under MTE treatment was compared qualitatively with the doxorubicin-treated cell as the positive control (Saadat, *et al*., 2015).

All of experiments in this study were approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, UGM) (reference no. KE/FK/1003/EC/2023).

RESULTS

Extraction and Phytochemical Profiles of MTE

The obtained MTE extract weighed as many as 78.6 mg with a yield of 2.91% w/w (Figure 1B). MTE was analyzed for its phytochemical content using the TLC as described in Methods. To identify the content of the resin glycoside groups, MTE was dyed with alpha naphthol and was observed under visible light. Alpha naphthol reacts with the aldehyde group of glycoside compounds, which have been dehydrated with sulfuric acid to produce a purple color (Rathod, *et al*., 2022). The positive-resin glycoside spots appeared as purple spots in visible light that were located around 2-3 cm of the chromatogram (Figure 1C). In agreement to the previous reports, the MTE consisted of resin glycosides (Kitagawa, *et al*., 1996).

The Cytotoxicity Effect of MTE

A single cytotoxic test was carried out to determine the toxicity of MTE on 4T1 cells with the IC_{50} value as an evaluation parameter. MTE treatment caused changes in the cell morphology and decreases in cell density in a dose-dependent manner. The control cells did not experience changes in cell morphology, while MTE-treated cells became irregularly round, smaller, and not attached to the bottom of the well (Figure 2A). After treatment at a serial concentration ranging from 1 to 100 µg/mL for 24 h, MTE resulted an IC_{50} value of 61 µg/mL in 4T1 cells (Figure 2B). Thus, MTE was categorized as moderately cytotoxic in 4T1 cells.

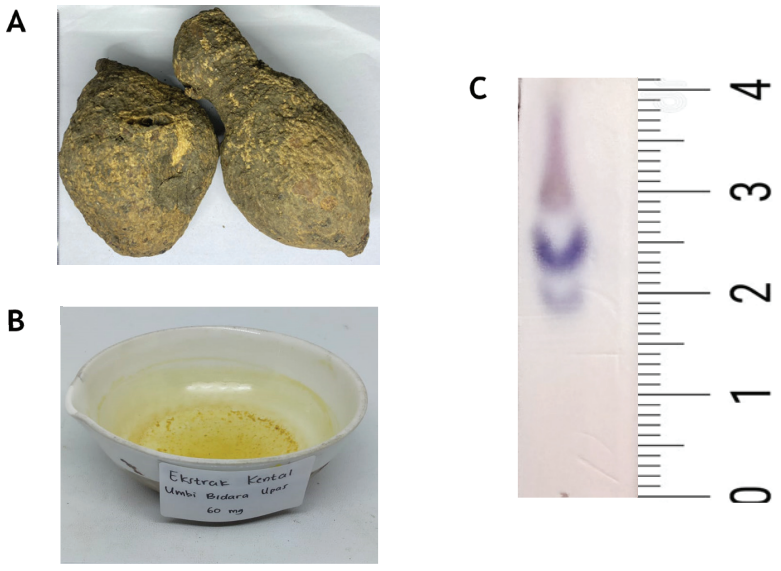

Figure 1. Preparation and phytochemical of MTE. (A) Tubers of *Merremia mammosa*. (B) The ethanolic extract of *M. mammosa* tuber after thickening. (C) TLC profile of MTE under visualization of visible light after dyeing with alpha naphthol reagent. The purple spots indicate resin glycosides.

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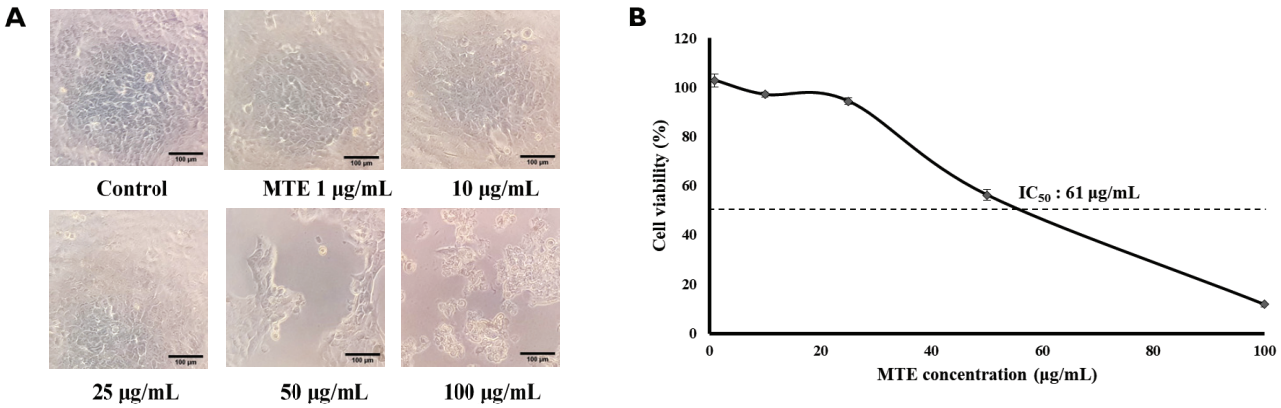
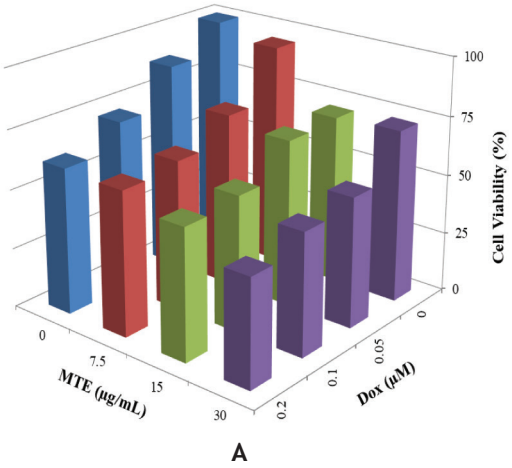


Figure 2. Cytotoxicity of MTE on 4T1 cells. (A) Morphology of 4T1 cells after treatment for 24 h. (B) The cell viability was performed by MTT method and is presented in the graph as MTE concentration against percent cell viability, data are shown as the mean ±SD. The dashed line indicates 50% cell viability. The cytotoxic assays were performed in a three separate experiments in triplicate for each experiment. The representative results are shown.

Cytotoxicity Effects of the Combination of MTE and Doxorubicin

A combined cytotoxic assay was performed to evaluate the effect of MTE in increasing the cytotoxicity of doxorubicin on 4T1 cells. The IC_{50} values of MTE and doxorubicin were used to determine the combination concentration, *i.e.* at around 1/8, ¼, and ½ of $IC5_0$. The IC_{50} of doxorubicin was around 0.5 µM (data not shown), in agreement with the previous finding (Zulfin, *et al*., 2021). The combination of MTE and doxorubicin was able to inhibit cell growth to a greater extent compared to the single treatment of MTE or doxorubicin at the same concentration (Figure 3A). All the tested combination concentration resulted in CI values of 0.32-0.69, indicating synergistic combination effect (CI<0.7) (Figure 3B) based on the classification by Haagensen, *et al.* (2012). These findings validated our hypothesis that MTE is a potential co-chemotherapy candidate for doxorubicin.



A

MTE (µg/mL)	Doxorubicin (µM)		
	1/8 IC_{50} (0.05)	¼ IC_{50} (0.1)	½ IC_{50} (0.2)
1/8 IC_{50} (7.5)	0.32	0.34	0.49
¼ IC_{50} (15)	0.48	0.44	0.57
½ IC_{50} (30)	0.61	0.64	0.69

B

Figure 3. The effect of the combination of MTE and doxorubicin on 4T1 cells. The cells were treated with the combination of MTE and doxorubicin (Dox) at the particular concentration as indicated for 24 h, assayed with MTT method to obtain the percentage of cell viability (A), and the combination index was calculated (B).

The Effect of MTE on 4T1 Cell Apoptosis

Apoptotic cells exhibit internucleosomal DNA fragmentation producing fragments of approximately 180–200 bp and their multiples (*e.g.*, 350 and 540 bp, and so on so forth), resulting in a ladder pattern on agarose gel electrophoresis (Kari, *et al*., 2022). After treating 4T1 cells with MTE for 24 h, the isolated DNA were resolved in the agarose gel. The anticancer molecular mechanism of doxorubicin is complex, but it has been established that doxorubicin induces cell apoptosis (Rivankar, 2014). Therefore, doxorubicin at around ¼ IC_{50} was used as a positive control. Doxorubicin was indeed caused cell apoptosis as indicated by thick fragment DNA bands at around 2,000-3,000 bp and 1,000-1,200 bp as mutiples of the smaller fragments that did not appear in control cells without any treatment (Figure 4, 1st vs 4th lane). On the other hand, both MTE at around and above IC_{50} (60 and 100 µg/mL, respectively) showed the DNA fragmentation profile comparable to the doxorubicin (Figure 4, 2nd and 3rd lane). In contrast, necrotic cell death results in random DNA degradation, producing a smear rather than a ladder pattern. Therefore, the DNA laddering observed in Figure 4, corresponding to fragments of the multiples of ~180–200 bp consistent with the theoretical hallmark of apoptosis, indicates that MTE induces apoptosis in 4T1 cells. These results suggest that MTE inhibits the cancer cell growth possibly by apoptosis induction.

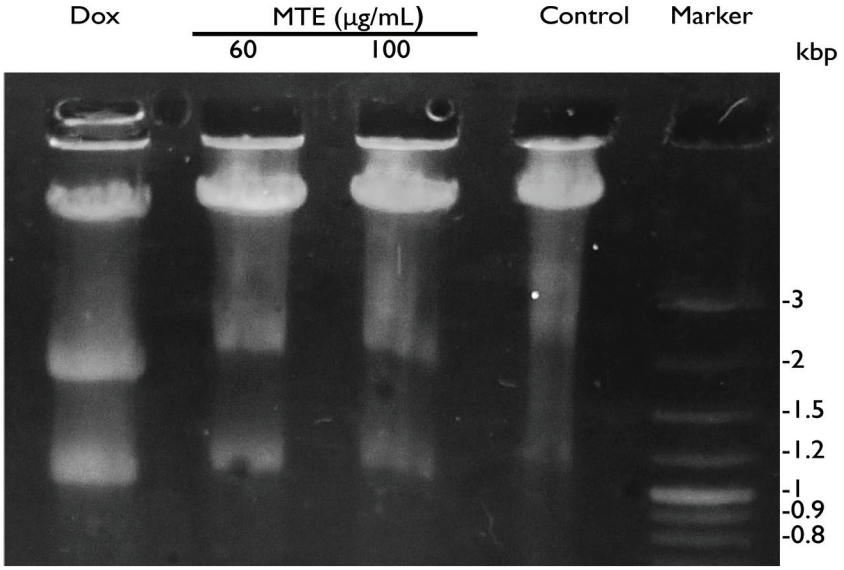


Figure 4. DNA fragmentation in 4T1 cells analyzed by the agarose gel electrophoresis. The cells were treated as indicated for 24 h prior to DNA isolation. Doxorubicin 100 nM (Dox) was used as the positive control, while cells without any treatment served as control. The size of DNA ladder marker is presented in kilobase pair (kbp).

DISCUSSION

Originated from southeast Asia, *Merremia mammosa* is easily to be found in Indonesia. As part of the family of Convolvulaceae that contains relatively high alkaloids and resin glycosides (Nagano, *et al*., 2009), we predicted that the ethanolic extract of *M. mammosa* tubers (MTE) is also rich in those compounds. Positively reacted with alpha naphthol, glycosides are confirmed to be present in MTE (Figure 1C). There were several positive spots in the chromatogram, indicating that several glycosides exist instead of single compound. Previously, resin glycoside

mammosides A, B, H1, and H2 have successfully isolated from the chloroform-soluble portion of the *M. mammosa* tuber methanol extract (Kitagawa, *et al*., 1997) in addition to merremosides a, b, c, d, and e (Kitagawa, *et al*., 1996). To further identify the compound, TLC by using standard compounds, *i.e*. mammoside B, can be implemented.

We validated the MTE-containing resin glycosides display cytotoxicity activity moderately in malignant 4T1 cells (Figure 2). In contrast to our finding, the ethanolic extract of *M. mammosa* tuber does not show cytotoxicity in T47D cells with an IC_{50} of 165 µg/mL, but fractionation with ethyl acetated increasing its activity into moderate cytotoxicity with IC_{50} of 32 µg/mL (A'yun, 2016). Interestingly, the ethyl acetate fraction is practically not toxic in non-cancerous cell line with an IC_{50} value of more than 700 µg/mL (A'yun, 2016), indicating the selectivity the ethyl acetate fraction of *M. mammosa* tubers. Together with the current data, this further rationale the utilization of MTE as co-chemotherapy agent candidate for doxorubicin to minimize its adverse effect on normal tissues, in which MTE has cytotoxic effect toward cancer cell but not to normal cells.

The verification of the potency of MTE as a co-chemotherapy agent for doxorubicin was executed by the combination assay. As displayed in Figure 3, MTE synergistically improve cytotoxicity of doxorubicin in 4T1 cells. All of the combinations at different concentrations produced synergism, meaning less doxorubicin is needed to obtain the same anticancer effect. A synergistic combination can increase the efficacy of doxorubicin while reducing the treatment dose to obtain the same cytotoxic effect (Muna & Jenie, 2018). Doxorubicin exerts its anticancer effects mainly through DNA intercalation, inhibition of topoisomerase II, and induction of oxidative stress–mediated cell death (Tacar, *et al*., 2013). Previous reports have shown that plant-derived extracts contain bioactive constituents with antiproliferative properties that may modulate oxidative stress, disrupt cell cycle progression, and enhance chemosensitivity (Newman & Craff, 2020). Thus, MTE may potentiate doxorubicin activity by sensitizing 4T1 cells to DNA damage, interfering with survival pathways, or improving intracellular drug accumulation, resulting in enhanced cytotoxicity when used in combination. Further studies evaluating apoptosis markers and related signaling pathways will be necessary to elucidate the precise molecular mechanisms underlying the observed synergistic effect.

Although the exact mechanism of MTE in increasing the cytotoxicity of doxorubicin in 4T1 cells remains unclear, we proved that MTE is able to induce apoptosis as indicated by the DNA laddering (Figure 4). This phenomenon can be interpreted as preliminary evidence of its intrinsic anticancer activity, rather than as a mechanistic explanation for the observed synergistic effect. We acknowledge as a limitation of this study that apoptosis assays were not performed for the combined treatment of MTE and doxorubicin. Although combination-induced DNA fragmentation data would strengthen the mechanistic interpretation, such data are not available within the scope of the present study. For further development, it is necessary to measure the apoptosis induction activity under the combination of MTE and doxorubicin, either qualitatively or quantitatively, *i.e*. by Annexin V-propidium iodide flow cytometry (Ramadani, *et al*., 2021; Zulfin, *et al*., 2021). Additionally, the molecular mechanism of the apoptosis induction will become a valuable information. Based on a study by Widiyastuti, *et al*. (2019), the water extract of *M. mammosa* tuber in a combination formula with other Indonesian plants stimulates apoptosis in MCF-7 breast cancer cell by suppressing the expression of the anti-apoptotic protein Bcl-2. The similar investigation on the expression of anti and pro-apoptotic proteins, for example by immunostaining or immunoblotting, can be imposed to predict the molecular mechanism of MTE in inducing apoptosis in 4T1 cells.

CONCLUSION

The tuber ethanolic extract of *Merremia mammosa* (MTE) containing glycosides is moderately cytotoxic to 4T1 malignant cancer cells. Interestingly, the combination of the extract with doxorubicin works synergistically as demonstrated by combination index analysis. This synergism indicates the potential of MTE as a co-chemotherapeutic agent to enhance the anticancer efficacy of doxorubicin at lower doses. However, the underlying molecular mechanisms of this interaction remain to be fully elucidated.

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