



Original Research Article

Anti cancer activity of rodent tuber (*Thyphonium flagelliforme* (Lodd.) Blume) on human breast cancer t47d cells

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Abstract

The incidence of breast cancer in developing countries showed an increase from year to year. The efforts of cancer prevention or treatment of the more important given the frequency of occurrence is quite high. Several studies have been focuses on natural products as agents of cancer chemoprevention and as co-chemotherapy agent against cancer. One of medicinal plant that is widely used as anticancer is rodent tuber (*Thyphonium flagelliforme* (Lodd.) Blume). In present study, we investigated the anticancer acitivity of rodent tuber extract (RTE) *in vitro*. Study of anti-proliferative conducted on human breast cancer primary T47D cells. Furthermore, the present study also investigated the molecular mechanisms of cell cycle arrest and induction of apoptosis. The study of rodent tuber as co-chemotherapy is determined by examining the effects on T47D and its combination with tamoxifen (TAM). The results showed the cytotoxic effects of RTE on T47D cells with IC₅₀ value of 632 µg/mL. Low concentration RTE (below 250 ug/mL) are proliferative, while the concentration above 250 µg/mL indicated cytotoxic effect. Based on the calculation of Combination Index (CI), combination of RTE with TAM yielded a value above 10 indicated a strong antagonistic effect. In observation of apoptosis, low concentration of RTE (63 µg/mL) stimulate apoptosis better than high concentration of 250 µg/mL. However, the combination study with 5 nM TAM reduced the induction of apoptosis. Furthermore, the observation cell cycle arrest by flowcytometry, demonstrated that RTE 63 and 250 µg/mL increase the population of sub G1 phase respectively from 14.8% to 53.19 and 32.90%. These results suggest that RTE able to induce cell cycle to apoptosis, but low concentration of RTE more effective than high concentration. Similar to apoptosis observation, the combination of RTE and TAM also demonstrated the antagonistic effect by reducing the population of Sub-G1 RTE (63 µg/mL) and TAM 5 nM, respectively from 53.19% and 44.50% to 35.86%. All finding results of this study provide information that the use of rodent tuber extract (RTE) alone is better than the combination with TAM. In addition, the use of RTE together with TAM reduced the effectiveness of TAM in the treatment of breast cancer.

Keywords: Rodent tuber (*Thyphonium flagelliforme* (Lodd.) Blume), anti cancer, chemoprevention, T47D cells.

Introduction

Breast cancer (BRCA) is the cancer with high prevalence and the second cause mortality women in USA (American Cancer Society, 2005). The incidence of breast cancer in development country also indicated increase every year [1-2]. National Cancer Institute USA in 2005 reported that the percentage of breast cancer patient is the highest rank in the world (24.2%). Due to the frequency of occurrence is quite high, many efforts was done to find the

agent for prevent or treatment breast cancer. Several studies focus to explore the potential of natural products as agents for prevent or treatment breast cancer.

One of the medicinal plant that is widely used as anticancer is rodent tuber (*Thyphonium flagelliforme* (Lodd.) Blume). Rodent tuber rounded average like a nutmeg. The inside and outside is white bulb. Its can use the tubers or seedlings that grow from tubers for breeding. In the dry season, the trunk disappeared while in the

rainy season, these plants appeared again on the surface of the soil tubers are buried in the ground. The crown-shaped flowers are small, white long tail similar to rats. But there are some types that have a red-colored petals. This type of rodent tuber are usually use and grow for hybrid plants. Rodent tuber has been widely used to treat many type of cancer especially breast cancer. Previous study has shown that hexane fraction of rodent tuber are cytotoxic against P388 murine leukemia ($IC_{50} \sim 15 \mu\text{g/mL}$) [3]. Some fraction of hexane and dichloromethane were reported capable to inhibit the growth of NCI-H23 lung cancer significantly, with $IC_{50} < 15 \mu\text{g/mL}$ and found that the fraction D/F21 was an active fraction for the inhibition of cancer growth [4]. Other study found four pheophorbide compounds and fractions D/F19 of rodent tuber that are anti-proliferation against cancer cells [4]. The study on rodent tuber as an anti cancer has not been much done, although its use as an anti cancer already quite extensive, especially in Indonesia and Malaysia as a product of traditional medicine or herbal medicine.

Based on the above reasons, it is important to explore and study in deep of rodent tuber as herbal medicine includes mechanisms of action or as co-chemotherapy for cancer treatment. Scientific proven of rodent tuber as anti-cancer drug is needed to ensure public that herbal medicine of rodent tuber is scientifically proven as an anti-cancer. This study also used as a basis for the development of herbal medicine *in vivo* as well as clinic.

Materials and Methods

Materials

Rodent tuber (*Thyphonium flagelliforme* (Lodd.) Blume) was obtained from the Garden of Medicinal Plant Development (CV. Merapi Farma, Yogyakarta, Indonesia). Human breast carcinoma cells used in this study is the T47D cell that was obtained from Prof. Tatsuo Takea (Nara Institute Sciences and Technology, Japan). Tamoxifen was obtained from Sigma Co.. Chem.

T47D cells were grown with RPMI medium containing 10% FBS (Gibco), penicillin-streptomycin 1% (GIBCO), and fungizon 0.5% (GIBCO). Cells culture were growth and incubate in CO_2 incubator. Material for the proliferation assay were [3 - (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide] (MTT) (Sigma Chemical, St Louis, MO), H_2O_2 (Lab Vision Plus), chromogen 3,3-diaminobenzidin (DAB) (Novo Castra) were used in this study. Other materials are pharmaceutical and biochemical grade.

Preparation of rodent tuber extract (RTE)

Rodent tuber sliced into small size and dried in an oven $60^\circ C$, after dried in powder, then sieved to obtain dry powder. A total of 500 grams of powder were macerated with 96% ethanol for 5 days. Ethanol fraction obtained was concentrated by rotary vacuum evaporator to obtain viscous extract. The existence of the remaining traces of water was dried by freeze drying.

Cell proliferation assay

Cell viability was determined by the MTT colorimetric assay (3-[4,5-diethylthiazol-2-yl]-2,5-dipheniltetrazolium bromide (Sigma St. Louis, MO, U.S.A.). After cells reached 80% confluent and good viability, the cells were seeded at a concentration of 5×10^3 cells/well (MCF-7) and 1.0×10^4 cells/well in 96-well plates (Becton Dickinson Co., NJ, USA) and cultured in appropriate medium at $37^\circ C$ in a 5% CO_2 atmosphere. After 48 h of attachment, culture medium was discarded and the cells were treated with various concentrations of rodent tuber extract (RT) (0, 10, 25, 50, 100, 250, 500, 1000 $\mu\text{g/mL}$) and 1, 10, 20, 30, 40, 50, 60 nM (tamoxifen) in 100 μl serum-free and phenol red-free DMEM or RPMI. T47D cells were treated for 48 h. After treatment, cells were incubated with 10 μl of MTT (5 mg/mL) for 6 h at $37^\circ C$. After 6 h, add the stopper 10 % SDS (Sigma Co., St.louis, MO) in 0.01 N HCl (Merck) in incubate for 24 hours in room temperature. After 24 h incubation, measured cells absorbance by

spectrophotometric using ELISA reader (λ 550 nm).

Co-chemotherapy assay

T47D cells were distributed in 96 well plate with the number 5.0×10^3 cells per wells and incubated with test samples (RT extracts and tamoxifen as a single compound) with a series of levels 1/8, 1/4, 1/3; $\frac{1}{2}$ IC₅₀ (RT extract) and 1/10, 1/5, 1/2, 3/5 IC₅₀ (tamoxifen) and also combination of tamoxifen and of RT extracts. Then followed such an experiment like cells proliferation assay above. Synergistic cytotoxicity determined by calculating the interaction index (CI = Combination Index) between the agent chemotherapy with rodent tuber extract (*Thyphonium flagelliforme* (Lodd.) Blume, using the equation:

$$I = (D)_1 / (Dx)_1 + (D)_2 / (Dx)_2$$

Where Dx is the concentration of a single compound required to give effect (in this case is the IC₅₀ against breast cancer cell growth) and (D)₁, (D)₂ is the concentration of both compounds to give the same effect (Notarbartolo et al., 2005). Based on the value of I obtained (synergistic cytotoxicity) can be interpreted: <0.1: very strong synergistic; 0.1-0.3: strong synergistic; 0.3-0.7: moderate synergistic; 0.7-0.9: synergistic; 0.9-1.1: almost additive; 1.1-1.45: moderate antagonist; 1.45-3.3: antagonist; > 3.3: very strong antagonist.

Apoptosis observation

T47D cells were grown on coverslips which placed in the microplate 24 wells in order to obtain the density of 3×10^4 cells /wells and incubated until 50-60% confluent. After that it was incubated with RT extracts for 48 hours. Medium was removed, then washed with PBS. Coverslips containing the cells is removed, put on the object glass and add 10 mL 1X Working Solution acridin orange-ethidium bromide and then allowed to stand for 5 minutes. Cells immediately observed under the microscope fluorescence (Zeiss MC80). Green fluorescence

indicated the life cells (with acridin orange) and orange fluorescence indicated the apoptosis cells with ethidium bromide.

Cell cycle analysis

T47D cells were seeded in complete medium (5×10^5 per 60-mm plate). After 24 h, cells were incubated in an incubator overnight to synchronize them in G2/M phase. Then samples were washed and placed in complete medium with DMSO (control), RT extract (63 and 250 μ g/mL) or tamoxifen (TAM) (5 nM) for 24 and 48 h to assess the effects on cell cycle progression. Both floating and adherent cells were collected, washed twice with cold PBS and centrifuged. The pellet was fixed in 70% ethanol in PBS at 4°C for 1 h, washed twice with cold PBS and then resuspended in PBS containing 40 μ g/ml PI and 100 μ g/ml RNase, at 37°C for 30 min. Samples were then analyzed on the FACScan flowcytometer. After activation of the 'doublet discrimination module' and exclusion of cell debris defining a gate in the side and forward scatter dot-plot, at least 10 000 events per sample were acquired in linear mode. Percentage of cells in subG1, G1, S and G2/M phases were calculated using the CellQuest software (Becton Dickinson).

Statistical Analysis

Data from the *in vitro* experiments were expressed as the mean \pm S.E.M. The statistical significance of differences between the groups were assessed with a one-way ANOVA, followed by Bonferroni or LSD post-hoc test analysis using rel 13.0 software SPSS (Chicago, IL, USA). *p* values of less than 0.05 were considered to indicate significant differences.

Results and Discussion

Cell proliferation assay

In this study, rodent tuber extracted using ethanol that can extract both polar and semipolar compounds in the rodent tuber. The extractive value of ethanol from dried powders of rodent tuber (*Thyphonium flagelliforme* (Lodd.) Blume was calculated as % w/w yield and was found to

be 10.71%. Cell proliferation assay of rodent tuber (RT) extract performed on T47D cells as a model of primary human breast cells. The concentrations of extract used were 10, 25, 50, 100, 250, 500, and 1000 $\mu\text{g/mL}$. The results demonstrated that the extract in the range concentration of 10-250 $\mu\text{g/mL}$ showed a tendency to T47D cell proliferation, whereas increasing concentrations of extract more than 250 $\mu\text{g/mL}$ showed inhibitory or cytotoxic effect on T47D cells. Biphasic effect of these study showed that the extract induced cells proliferation at levels less than 250 $\mu\text{g/mL}$ and inhibition of cells proliferation or cytotoxic for concentrations above 250 $\mu\text{g/mL}$. It is likely there are compounds in the extracts that are estrogenic such like phytoestrogens.

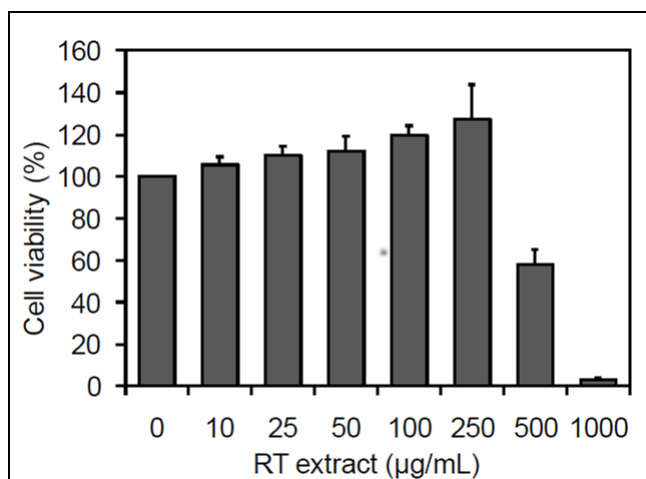


Figure 1. Effect of rodent tuber extract (RTE) on T47D cells viability. Experiments were conducted with an incubation time of 48 hours. Data represent mean \pm SD of triplicate.

The results are consistent with previous studies that phytoestrogens such as genistein could inhibit the growth of cancer cells *in vitro* include hormone-dependent breast cancer or not with the IC_{50} ranged between 10-50 $\mu\text{mol/L}$ [5-8]. While at concentrations <10 $\mu\text{mol/L}$, the growth of MCF-7 (estrogen receptor-positive cell line) stimulated by genistein [7,9,10]. The calculation result using semilog linear regression of concentration versus cell viability (%) obtained the equation $y = 121.40x - 0.113$. The calculation

result obtained IC_{50} value of RT extract 632 $\mu\text{g/mL}$ (Figure 1). Mechanism of cytotoxic or cell proliferation possibility could be through inhibition of one or more cellular molecules that control cell signaling, cell growth and apoptosis. The IC_{50} of RT extract on cell inhibition of T47D human carcinoma cells showed a low value that is equal to 632 $\mu\text{g/mL}$. This result indicated that the active compound in rodent tuber extract small and needed purified to get greater anti cancer activity.

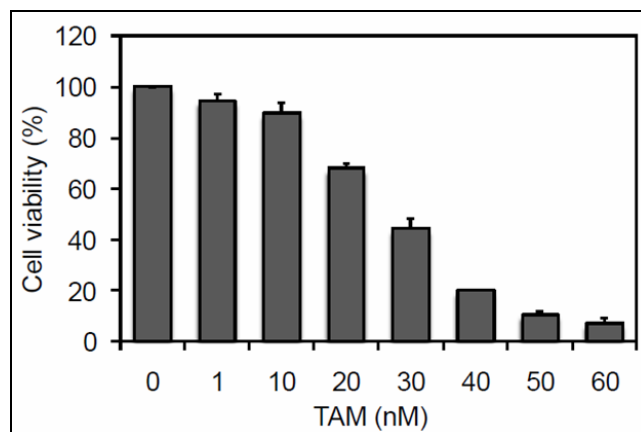


Figure 2. Effect of TAM on T47D cells viability. Experiments were conducted with an incubation time of 48 hours. Data represent mean \pm SD of triplicate.

Furthermore, treatment of tamoxifen (TAM) as one of SERMs showed very effective against cancer cells T47D (Figure 2). Tamoxifen is a drug that is often used for the treatment of breast cancer as in research International Breast Cancer Intervention Study-1 [11], Royal Marsden Hospital [12], and Italian [13], that women who receive tamoxifen women randomly shows 36% reduction in the incidence of ductal carcinoma-in-situ (DCIS) and 46% reduction on the incidence of invasive breast cancer [14]. In this study, TAM administration very effectively inhibits the growth of T47D cells with very low concentrations in the range 1-60 nM. Results of linear regression calculations indicate the value of IC_{50} was 27.48 nM. T47D cells are ER-positive breast cancer as evidenced by the response to increased proliferation as a result of exposure to 17-estradiol [15]. Tamoxifen (TAM) is one of the

estrogen antagonist which inhibits the activity of estrogen in most tissues that are sensitive to estrogen. TAM has become the standard treatment for all stages of breast cancer in both pre-menopausal women and post-menopausal [16]. The potential benefits of the use of TAM as a precaution has been widely studied, in addition reported that TAM can reduce the risk of breast cancer approximately 50% of most women who have a high risk of breast cancer [17]. These results of TAM on cell proliferation assay were supported by previous studies.

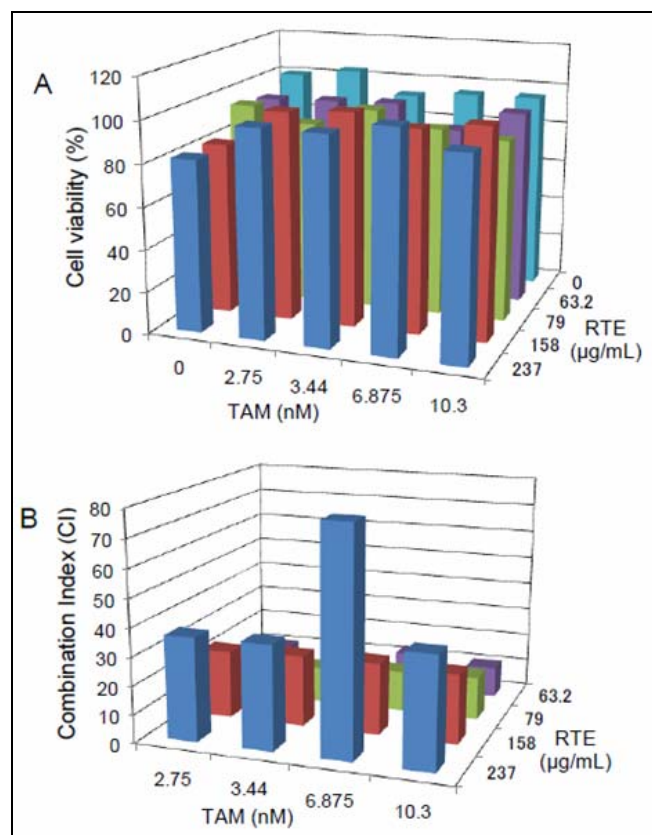


Figure 3. The combination study of tamoxifen (TAM) and rodent tuber extract (RT) on the percentage of cells viability of T47D and combination index (CI) using MTT method. Graph indicated relationship the combination of RTE and TAM on the T47D cells viability (A), and the combination index (B). Data are mean \pm SD of 3 replications. Experiments conducted with an incubation time of 48 hours.

Co-chemotherapy assay

Further study carried out co-chemotherapy to know the efficacy of tamoxifen as the standard treatment of breast cancer. Combination study

treatment made by combining tamoxifen (TAM) with RTE. The concentrations of TAM and RTE used in this study were 2.75, 3.44, 6.875, 10.3 (TAM) and 63.2; 79; 158, 237 (RTE). Figure 3 described the effect of combination of extracts RTE-TAM. The potential combination of tamoxifen with RTE is determined by the combination index (CI). Almost all treatment combinations of tamoxifen with RTE is a combination that gives a very strong antagonistic effect ($CI > 3.3$), indicated a decreased effect exceeds the sum of the effect produced when a single administration. From the results of RTE-TAM combination can be inform that administration of combination would reduce the effectiveness of extract or TAM as anti cancer. The use of RTE as co-chemotherapy is not effective to increase the efficacy of tamoxifen for treatment in patients with breast cancer.

Apoptosis observation

In this study, apoptosis observation carried out by double staining method using ethidium bromide-acrydine orange. Observations showed that in control cells visible only green fluorescence due to just absorb acrydine orange. Ethidium bromide can not penetrate the cell because it controls cell integrity is still good. In cells treated with RTE (63 $\mu\text{g}/\text{mL}$) largely cells seen as green fluorescence and some orange. This orange fluorescence indicated that RT extract capable to induce apoptosis. It is can be understand that treatment of RTE (63 $\mu\text{g}/\text{mL}$) shown only a small fraction of cells indicated orange fluorescence because the concentration are still below IC_{50} . Increased concentration of RTE to 250 $\mu\text{g}/\text{mL}$, further increase induction of apoptosis which is marked by the increasing number of cells seen as orange fluorescence. This shows that the treatment of RTE can induce apoptosis (Figure 4). In contrast, RTE and TAM combination were reduce the number of cells undergoing apoptosis (Fig. 4B-E, and CF). All of the observation of apoptosis showed that the combination of RT with TAM is less effective in promoting apoptosis than the single administration of RTE. In addition, the use of RT

and TAM will simultaneously reduce the effectiveness of tamoxifen in promoting cancer cell induce apoptosis.

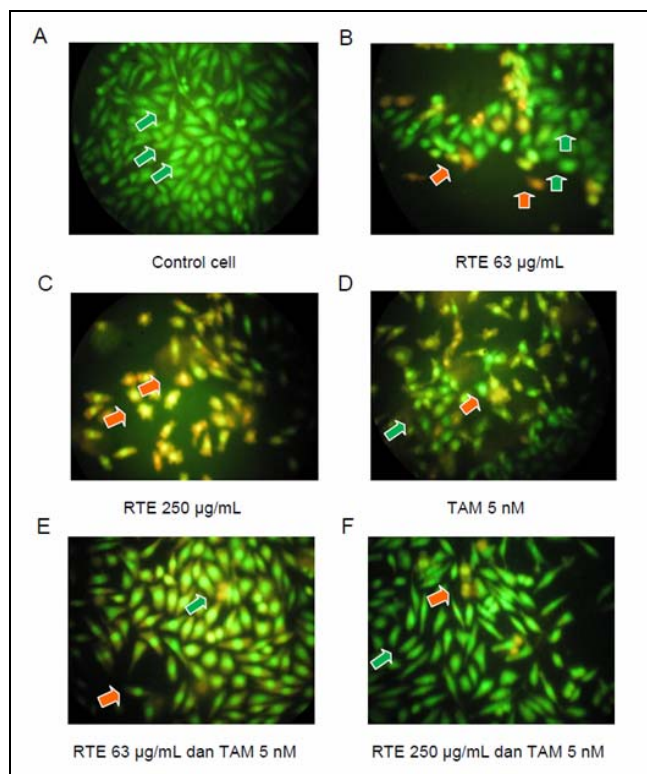


Figure 4. The effect of RTE and its combination with TAM on apoptosis induction in T47D cells after 48 hours of administration. Staining was done by grown T47D cells (5×10^4 cells/well on coverslips in the plate 24, carried acrydine staining using ethidium bromide-orange and viewed with a microscope fluorosence. (A) control cells, (B) RTE 63 $\mu\text{g}/\text{mL}$, (C) RTE 250 $\mu\text{g}/\text{mL}$, (D) TAM 5 nM, (E) RTE 63 $\mu\text{g}/\text{mL}$ and TAM 5 nM and (F) RTE 250 $\mu\text{g}/\text{mL}$ and TAM 5 nM. 100x magnification.

➡ : apoptosis cells; ➡ : life cells

Cell cycle arrest

Furthermore, we also observe and evaluate the effects of RTE and their combinations on cell cycle profiles using flow cytometry as shown in Figure 5. As shown in Figure 5, administration of RTE (63 and 250 $\mu\text{g}/\text{mL}$) increased the population of sub G1 phase respectively from 8.14 to 53.19 and 32.90%. These results show that rodent tuber extract (RTE) was able to induce cell cycle to apoptosis, but low concentration of RTE induced apoptosis more

effective than high concentration. The combination of RTE and TAM demonstrated the antagonistic effect to reduce the population of Sub-G1 RTE (63 $\mu\text{g}/\text{mL}$) and TAM (5 nM) of 53.19% and 44.50%, respectively to 35.86% (Figure 5). The results are consistent with previous observations of both apoptosis and the combination index calculations showed that the combination of RTE and TAM is antagonistic effect. The results that not dose-dependent likely indicate a proliferative compounds that are at such low concentration of phytoestrogens. On the other hand, the possibility of high concentration of RTE, there are any DNA repair mechanisms that can pass through G0 phase. These results provide information that the use of RTE as a single administration is better than the combination. In addition, the use of extracts of RTE together with TAM actually reduced the effectiveness of TAM in the treatment of breast cancer. All the observation of cell cycle arrest, RTE capable to induce apoptosis on T47D cells, but the molecular signaling study is needed on specific protein cyclin, such as CDK4 and CDK6 involved in G1 progression, and a variety of CDK inhibitors that may occur for the regulation of the cell.

In conclusions, this study demonstrated that RTE capable to inhibit cell proliferation of T47D cells. The combination of RTE with tamoxifen was resulted an antagonistic effect. In the observation of apoptosis and cell cycle arrest can be seen that administration of single rodent tuber extract low concentration induced apoptosis effectively than high concentration or its combination with TAM.

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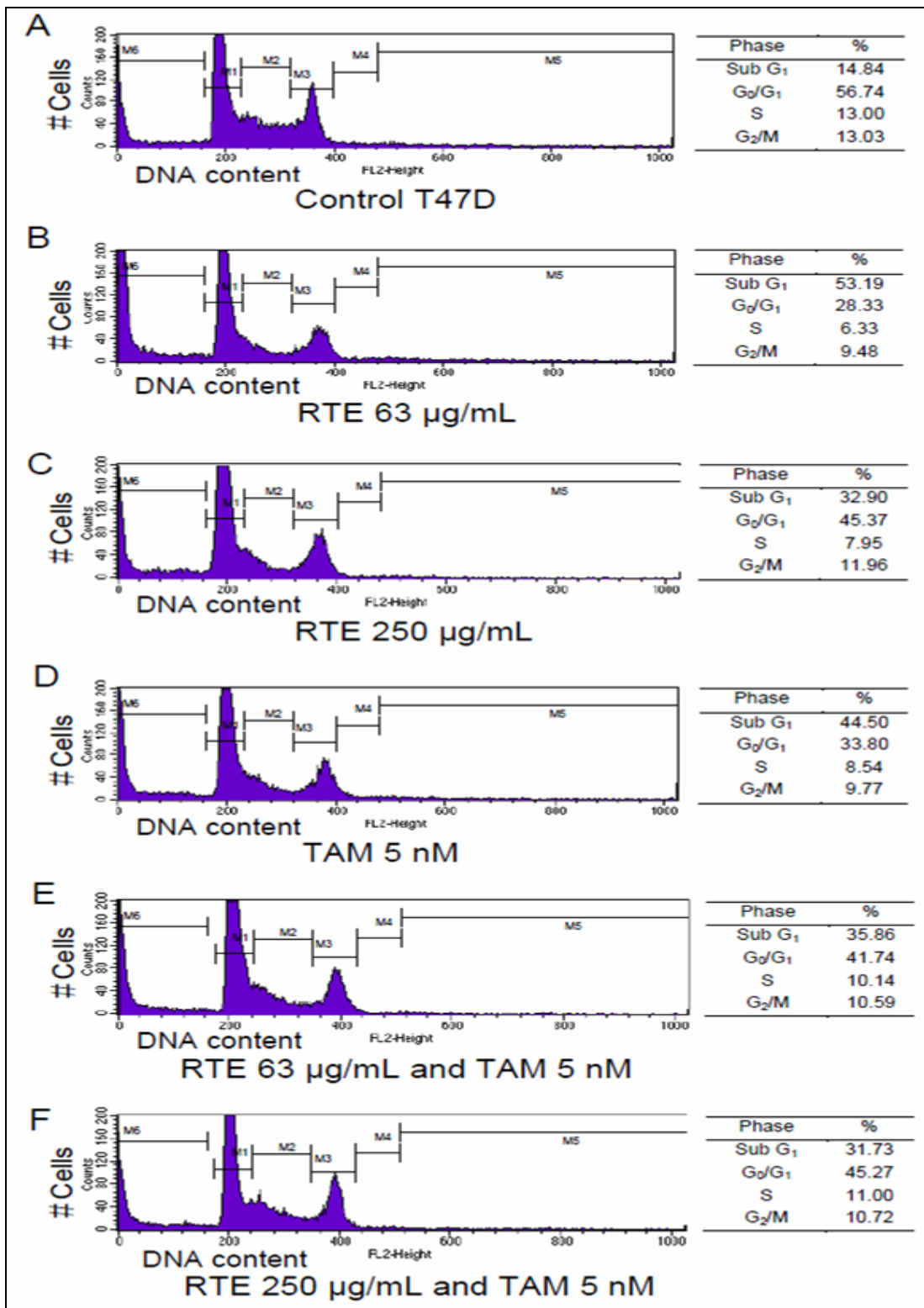


Figure 5. Distribution of Cell Cycle T47D cells using flow cytometry after staining with propidium iodide (PI). (A) Control cells; (B) treatment of RTE 63µg/mL; (C) treatment of 250 µg/mL; (D) TAM 5 nM; (E) combination of RTE 63 µg/mL and TAM 5 nM; (F) combination of RTE 250 µg/mL and TAM 5 nM. I: Sub-G₁; II: G₀/G₁; III: S; IV: G₂/M. All treatment is a representation of 20,000 cells and the percentage of cells in cell cycle phase is indicated beside each cell cycle distribution.

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