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Cytotoxicity assay of *Typhonium flagelliforme* Lodd against breast and cervical cancer cells

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ABSTRACT

BACKGROUND

Cancer is one of the causes of high mortality. Breast and cervical cancers are two of the most frequent cancers affecting women around the world, including Indonesia. Natural materials such as rodent tuber (*Typhonium flagelliforme*) have anticancer potentials. The rodent tuber extract contains ribosome inactivating proteins (RIPs) capable of cutting the DNA or RNA of cancer cells and blocking the growth of cancer cells. The purpose of this study was to evaluate the cytotoxic effects of *Typhonium flagelliforme* Lodd extract on HeLa cervical cancer and Michigan Cancer Foundation-7 (MCF-7) breast cancer cells.

METHODS

Subjects were cultured cell lines of HeLa cells in Rosswell Park Memorial Institute (RPMI) and of MCF-7 cells in Dulbecco's Minimum Essential Medium (DMEM). Rodent tuber ethanolic extract was diluted in dimethyl sulfoxide (DMSO). The cytotoxicity assay used the 3-(4,5-dimethyl thiazol-2-yl,5-diphenyl) tetrazolium bromide (MTT) method. Absorbance was read in an ELISA reader at a wavelength of 595 nm.

RESULTS

Rat tuber extract at all dilutions (500; 250; 125; 62.5; 31.25; 15.625;7.81; 3.9 ì g/mL) showed cytotoxic effects against HeLa and MCF-7 cells. Higher concentrations of the extract gave a higher proliferation inhibition effect. Calculated IC50 values of the extract by probit analysis were 30.19 ìg/mL against HeLa cells and 5.586 ì g/mL against MCF-7 cells.

CONCLUSIONS

Ethanolic extract of *Typhonium flagelliforme* Lodd has cytotoxic effects against HeLa cells and MCF-7 cells. The cytotoxic effects against MCF-7 cells are greater than the cytotoxic effects against HeLa cells.

Key words : *Typhonium flagelliforme*, tetrazolium bromide, cytotoxic, HeLa cells, MCF-7 cells

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Uji sitotoksisitas ekstrak Typhonium flagelliforme Lodd terhadap sel kanker payudara dan rahim

ABSTRAK

LATAR BELAKANG

Kanker menjadi salah satu penyebab kematian yang cukup tinggi. Kanker payudara dan kanker leher rahim menempati dua urutan terbanyak diderita wanita di seluruh dunia termasuk Indonesia. Bahan alam seperti keladi tikus (Typhonium flagelliforme) memiliki potensi sebagai anti kanker. Di dalam ekstrak keladi tikus mengandung ribosom inactivating protein *(RIPs) yang mampu memotong DNA atau RNA sel kanker dan dapat memblokir pertumbuhan sel kanker. Tujuan penelitian adalah untuk menilai efek sitotoksik ekstrak tanaman keladi tikus (Typhonium flagelliforme* L*) terhadap sel kanker rahim Hela dan sel kanker payudara* Michigan Cancer Foundation-7 (MCF-7).

METODE

*Subyek penelitian adalah kultur sel (*cell line*) HeLa dalam medium* Rosewell Park Memorial Institute *(RPMI) dan sel MCF-7 dalam* Dulbecco's Minimum Essential Medium *(DMEM). Ekstrak etanolik tanaman keladi tikus diencerkan dengan* dimethyl sulfoxide *(DMSO). Uji sitotoksisitas menggunakan metode 3-(4,5-dimetil tiazol-2-il,5-difenil* tetrazolium bromide *(MTT). Serapan warna dibaca dengan ELISA* reader *pada panjang gelombang 595 nm.*

HASIL

Dari beberapa pengenceran ekstrak yang digunakan yaitu 500; 250; 125; 62,5; 31,25; 15,625; 7,81; dan 3,9 µg/mL masing-masing menunjukkan adanya efek sitotoksik ekstrak keladi tikus terhadap sel HeLa dan sel MCF-7. Konsentrasi ekstrak yang lebih tinggi memberikan efek hambatan proliferasi yang lebih tinggi. Perhitungan nilai IC50 ekstrak keladi tikus dengan analisis probit diperoleh nilai IC50 30,19 µg/mL terhadap sel HeLa dan 5,59 µg/mL terhadap sel MCF-7.

KESIMPULAN

Ekstrak etanol keladi tikus (Typhonium flagelliforme Lodd*) memiliki efek sitotoksik terhadap sel HeLa dan sel MCF-7. Efek sitotoksik terhadap sel MCF-7 lebih besar dibandingkan efek sitotoksik terhadap sel Hela.*

Kata kunci: *Typhonium flagelliforme,* tetrazolium bromide*, sitotoksik, sel HeLa, sel MCF-7*

INTRODUCTION

Cancer is a multistep process occurring in stages, consisting of initiation (acquisition of irreversible genetic changes), promotion (increase in the population of initiated cells), and finally development of malignancy. Cancer causes considerable mortality. It has been estimated that each year there are 190.000 new cancer patients, one-fifth of whom will die.[1] Among the various types of cancer, two with the highest ranking are cervical cancer and breast cancer. Modern or conventional (medical) treatments are considered to be extremely expensive, having significant side effects, while the results may not always be satisfactory. To date, tumor or cancer management still has many constraints, such as insensitiveness of the cancer towards antiproliferation signals and their capability of evading the apoptotic program.[2]

In Indonesia various herbals have been applied as alternative drug therapy to aid in the treatment of cancer. Many herbals are known to possess anticancer properties due to their antiproliferative effects against cancer cells, thus inhibiting cancer cell growth. One of the plants

with a potential for development into an anticancer drug is rodent tuber [*Typhonium flagelliforme* (Lodd) Blume]. The extracts of this plant contain antineoplastic or anticancer compounds.[3] Rodent tuber of the species *Typhonium flagelliforme* has long been known to be a potent traditional medication against cancer cells. According to a number of studies conducted in Malaysia, rodent tuber is capable of reducing pain and decreasing the occurrence of metastases or dissemination of cancer cells, including breast, nasopharyngeal, and cervical cancers, and cancers of the prostatic gland, pancreas, and lungs.[4-5] In addition, this plant is found growing in large numbers in Indonesia and Malaysia, and is used to treat hemorrhoids, skin disorders, and to neutralize narcotic drugs. Rodent tuber also has antibacterial and antioxidant activities.[4-6] Several herbal preparations use *Typhonium flagelliforme* for cancer treatment.[7]

There are few data on the chemical composition of rodent tuber. Phytochemical analyses at the Research Station for Medicinal and Aromatic Plants *(Balai Penelitian Tanaman Obat and Aromatik)* demonstrated the presence of alkaloids, saponins, steroids and glycosides.[8] However, the active substances in rodent tuber that specifically play a role in the healing of cancers are still unknown. Rodent tuber saline extracts contain ribosome inactivating proteins (RIPs). These are proteins that are capable of cutting cancer cell DNA or RNA, so as to inactivate cancer cells without damaging the surrounding tissues. In addition, RIPs can also block cancer cell growth,[9] and play a role in triggering apoptosis through cutting DNA and RNA, and through N-glycosidase activity.[10-11]

In a previous study, the dichloromethane fractions of *Typhonium flagelliforme* tuber extract were capable of increasing caspase-3 and p21 expression in the Michigan Cancer Foundation-7 (MCF-7) breast cancer cell line that has a deletion of the caspase 8 gene apoptosis-related cysteine peptidase (CASP-8).[2] However, the abovementioned study did not involve cytotoxicity tests of the rodent tuber extract against the MCF-7 cell line. Another study using chloroform and ethyl acetate extracts of *Typhonium divaricatum* (L) Decne, demonstrated that the extracts had cytotoxic effects on HeLa cells, but with differing IC50 values.[12]

Therefore the purpose of the present study was to test the cytotoxicity of rodent tuber (*Typhonium flagelliforme* Lodd) against cultures of MCF-7 cells as an in vitro model for breast cancer, and of HeLa cells as an in vitro model for cervical cancer.

METHODS

Study design

This was a laboratory experimental study conducted from April 2013 until November 2013.

Plants materials

Rodent tuber plants were obtained from medicinal herb gardens in the Jagakarsa area, South Jakarta.

Tumor cell lines

The HeLa and MCF-7 cell lines were supplied by the Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. The reagents used for culture media and cell cultures were 0.5% fetal bovine serum (FBS), Rosswell Park Memorial Institute culture medium [(RPMI) (Gibco)], Dulbecco's minimum essential medium (DMEM), 70% ethanol, sodium bicarbonate, hydroxyethyl piperazineethanesulfonic acid (HEPES), sodium dodecyl sulphate (SDS), and dimethyl sulfoxide [(DMSO) (Sigma)].

Preparation of culture media and cell growth media

Culture media for HeLa and MCF-7 cells were prepared by dissolving RPMI 1640 and DMEM powder, respectively, in 800 mL twice-distilled water, with the addition of 2.0 grams of sodium bicarbonate and 2.0 grams of HEPES,

made up to a volume of one liter with twice-distilled water. The solution was mixed with a magnetic stirrer for about 10 minutes until homogenous, than neutralized with 1 N HCl to a pH of 7.2 – 7.4.

Cell growth media were prepared by mixing 19 mL FBS, 2 mL penicillin streptomycin, and 0.5 mL fungizone, and diluting with RPMI 1640 and DMEM culture medium, respectively, to a volume of 100 mL. The solution was then aseptically filtered through a sterile 0.2 µm polyethylene sulfone filter and stored in the refrigerator in stoppered bottles. The cell cultures were observed under the light microscope (Olympus).

Preparation of rodent tuber extract

Rodent tuber whole plant extract was prepared by washing fresh whole plants, cutting them into strips, and air drying. The dried strips were then pulverized in a blender, then the powder was macerated with 80% ethanol in an Erlenmeyer flask, under continuous shaking. The ethanolic extract was decanted into a rotary flask and concentrated in a rotary evaporator. The concentrated rodent tuber extract was serially diluted with DMSO and filtered through an 0.2 µm filter. The prepared serial dilutions were: 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL, 7.81 µg/mL, and 3.9 µg/mL.

3-(4,5-dimethylthiazole-2-il,5-diphenyl tetrazolium bromide) (MTT) assay

HeLa and MCF-7 cells, grown on a 96-well microplate to a density of 1 x 10^4 cells per well, were incubated in RPMI medium (HeLa cells) or DMEM medium (MCF-7 cells) for 48 hours to obtain optimal growth. After a change of medium, rodent tuber extract serial dilutions in DMSO (Sigma) were added to the respective wells, then the microplate was incubated in a 5% CO_2 incubator at 37°C for 48 hours. At the end of the incubation period, the media and extracts were discarded and the cells washed in phosphate buffered saline (PBS, Sigma). To each microplate well, 100 µL of a 5 mg/mL MTT (Sigma) solution was added. The cells were then again incubated for 4-6 hours in a 5% CO_2 incubator at 37°C. The MTT reaction was stopped with 4 N HCl-isopropanol (stopper reagent, 1:100), and gently agitated on a shaker for 10 minutes or incubated overnight at room temperature. Absorbance was read in an ELISA reader (BioRad) at 595 nm wavelength.

Data analysis

Calculation of IC50 values was performed by probit analysis using SPSS version 16.0.

RESULTS

The calculated percentage inhibition values of rodent ulcer extract against growth of HeLa and MCF 7 cancer cells are presented in Tables 1 and 2.

Table 1. Percentage inhibition of rodent ulcer extract against HeLa cells

Dilution of rodent ulcer extract (µg/mL)	Inhibition (%)
500	1.78 ± 0.25
250	0.96 ± 0.39
125	0.58 ± 0.17
62.5	1.98 ± 1.03
31.25	88.08 ± 6.87
15.625	90.32 ± 3.99
7.8125	91.80 ± 4.58
3.90	79.86 ± 3.29

*Values are mean ± SD

Table 2. Percentage inhibition of rodent ulcer extract against MCF-7 cells

Dilution of rodent ulcer extract (µg/mL)	Inhibition (%)
500	67.85 ± 4.66
250	84.69 ± 10.58
125	86.87 ± 0.17
62.5	89.97 ± 1.11
31.25	91.39 ± 4.83
15.625	90.52 ± 4.44
7.8125	94.61 ± 10.21
3.90	82.18 ± 3.37

*Values are mean ± SD

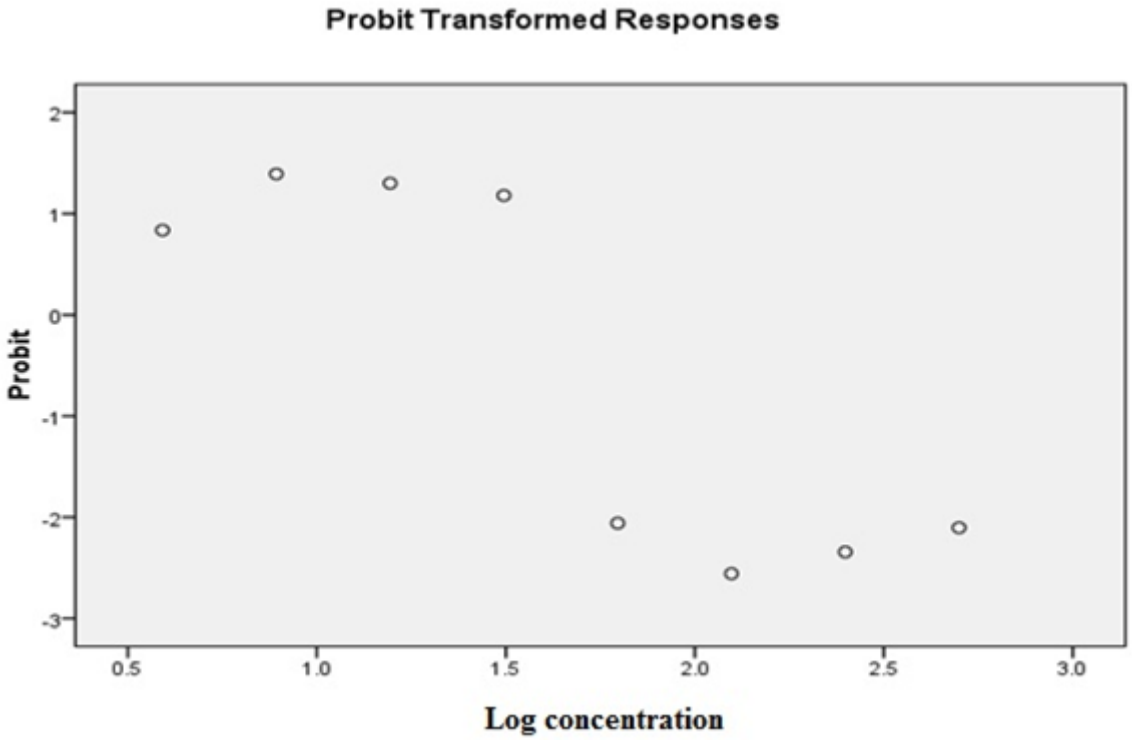

Figure 1. Probit curves for rodent tuber extract against HeLa cells
Note: Higher doses tend to result in greater inhibitory effects

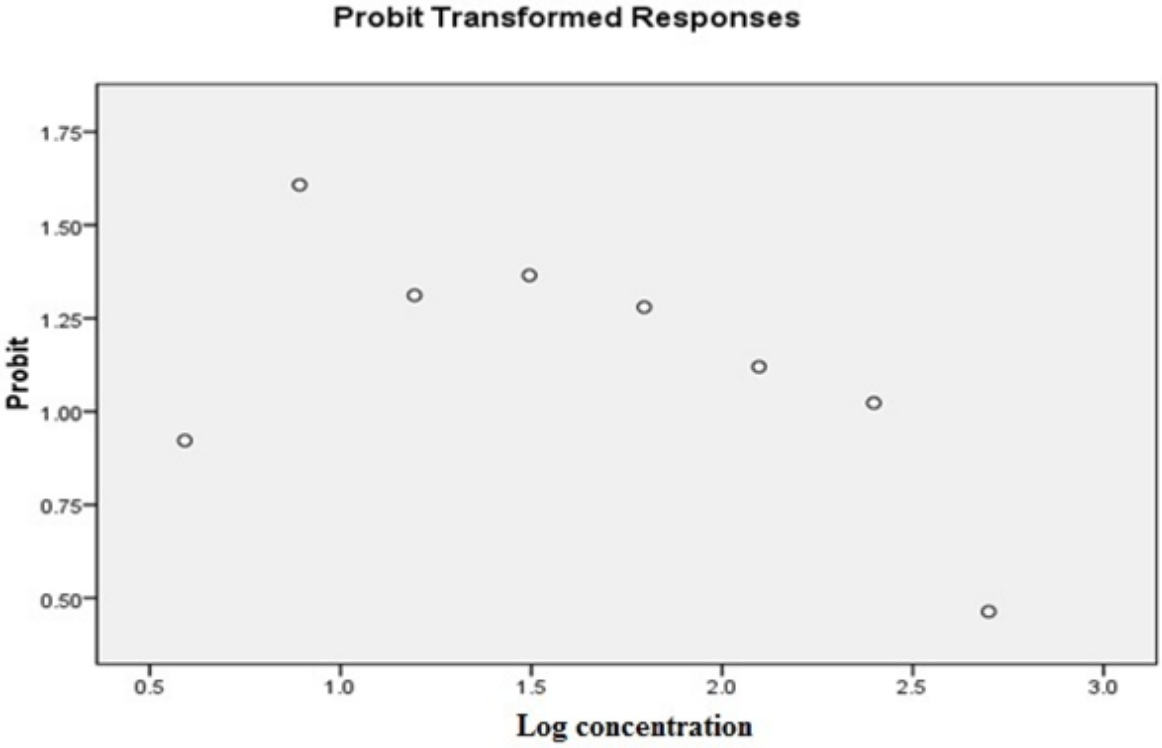

Figure 2. Probit curves for rodent tuber extract against MCF-7 cells
Note: The highest dose (500 µg/mL) showed lower inhibition than the dose of 250 µg/mL. For the other doses, lower inhibition was associated with lower doses

Inhibition of HeLa and MCF-7 cells tended to increase with higher dilutions of rodent tuber extract. Maximum inhibition of HeLa cells was 91.8 ± 4.58% at a dilution of 7.1825 µg/mL (Table 1), while for MCF-7 cells, maximum inhibition was also at a dilution of 7.1825 µg/mL, the percent inhibition being 94.61 ± 10.21% (Table 2).

Calculation of IC50 values by probit analysis, from a probability of 0.01 up to 0.99, gave IC50 values of 30.19 µg/mL for HeLa cells and 5.59 µg/mL for MCF-7 cells. The probit curves for rodent tuber extract are presented in Figures 1 and 2.

DISCUSSION

Rodent tuber ethanolic extract demonstrated inhibitory effects on the growth of the test cancer cells. The cytotoxicity assay of rodent tuber extract against HeLa and MCF-7 cells used DMSO at a concentration of 0.1% as the solvent. A previous study on the cytotoxicity of another rodent tuber species, *Typhonium divaricatum* L, against HeLa cells reported that the use of DMSO as a solvent at concentrations of up to 0.1% or below 3%, had no effects on cancer cells.[12] The results obtained from our cytotoxicity assays showed a tendency of the rodent tuber ethanolic extract to have a cytotoxic potential against both HeLa and MCF-7 cancer cells. Essentially similar results were obtained in an in vivo study, showing that *Typhonium flagelliforme* ethanolic extract was capable of decreasing tumor cell proliferation in mice, as shown by the expression levels of Ki67 as a proliferation marker.[13]

The cytotoxic effect of rodent tuber extract in the present study, i.e. inhibition of HeLa and MCF-7 cell proliferation, tended to increase with increasing rodent tuber concentration. This is in agreement with the abovementioned study on *Typhonium divaricatum* L cytotoxicity against HeLa cells, using chloroform and ethyl acetate extracts of the plant, where the highest dose used was 250 µg/mL and the lowest dose 5 µg/mL.[12]

From phytochemical analyses by previous investigators, it is known that rodent tuber contains alkaloids, saponins, steroids, phenols, and glycosides, but the specific active principle of rodent tuber that plays a role in the healing of cancer is still unknown.[8] These studies showed that phenolic compounds in rodent tuber are responsible for its anticancer potential. In addition, it has been reported that phenolic compounds in herbal extracts can play a role as chemopreventive agents or may promote inhibition of the cell cycle and trigger apoptosis of the cells.[4,14] Other studies demonstrated that phenolic compounds of the flavonoid group are able to inhibit cancer cell growth. Flavonoids containing phenolic groups are capable of inhibiting the growth of rat H4IIE cells at low doses and also promote apoptosis at these low doses.[12]

Furthermore, it is known that rodent tuber saline extract contains ribosome inactivating proteins (RIPs), which are capable of cutting cancer cell DNA or RNA, thus inhibiting cancer cell growth without damage to the surrounding tissues; in addition, RIPs are also able to block cancer cell growth. Ribosome inactivating proteins are in general of plant origin and are cytotoxic against mammalian cells.[9]

Rodent tuber extract IC50 against HeLa cells and that against MCF-7 cells are different in value, the IC50 against MCF-7 cells being lower than that against HeLa cells. This shows that rodent tuber extract is more toxic against MCF-7 cells than against HeLa cells. As is well known, HeLa cells are commonly used for in vitro assays of cervical cancers, whereas MCF-7 cells are commonly used for assays of breast cancer cells, in addition to T47D cells. Thus rodent tuber extract has a greater anticancer potency against breast cancer cells than against cervical cancer cells. Rodent tuber is reportedly capable of inhibiting the growth of breast cancer cells and enhance apoptosis.[15,16] Previous investigators have reported that chloroform and

hexane extracts of rodent tuber roots and tubers had IC50 values of 15.0 µg/mL and 6.0 µg/mL, respectivley, against P288 leukemia cells, while chloroform and hexane extracts of rodent tuber stalks and leaves had IC50 values of 8.0 µg/mL and 65.0 µg/mL, respectively.[17] Another study has demonstrated that the dichloromethane extract fraction number 7 (DCM/F7) of *Typhonium flagelliforme* inhibited leukemia cancer-cell proliferation selectively, via the induction of apoptosis.[18] Dichloromethane and ethyl acetate extracts of *Typhonium flagelliforme* inhibit the in vitro proliferation of the CEM-ss human T4-lymphoblastoid cell line.[19] These study results indicate that *Typhonium flagelliforme* possesses anticancer potential, for which further studies are necessary.

The present study is still in its early stages, therefore more advanced studies are required in connection with the potential of rodent tuber plants as anticancer agents, e.g. through an antitelomerase mechanism, in view of the fact that cancer cells possess high telomerase activity.

CONCLUSIONS

The study results indicate that the DMSO extract of rodent tuber plants (*Typhonium flagelliforme* Lodd) have cytotoxic potential against HeLa and MCF-7 cells. The cytotoxic effect against MCF-7 cells is greater than the cytotoxic effect against HeLa cells. Further investigations are necessary on the effects of rodent tuber extract against telomerase activity or expression in MCF-7 and HeLa cancer cells.

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