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# CYTOTOXICITY EFFECTS OF TYPHONIUM FLAGELLIFORME AND CLINACANTHUS NUTANS ON BREAST CANCER CELLS

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## Graphical abstract

*Typhonium flagelliforme*

*Clinacanthus nutans*

## Abstract

Cancers are diseases that can cause death and breast cancer is one of the prevalence cancers. Conventional treatments have been used to treat cancer. However, these treatments produced inefficient effects and low survival rate. Therefore, most cancer patients began to consume complementary and utilized alternative treatments to alleviate their pain. This study is to identify the cytotoxicity effect of methanolic leaves extract *Typhonium flagelliforme* and *Clinacanthus nutans* on breast cancer cells (MDA-MB-231). MTT assay has been used to determine the cytotoxicity effect of both plants on MDA-MB-231 cells and CHO cells (non-cancerous cell), which acted as the positive control cells. Result revealed that *T. flagelliforme* extract has shown higher cytotoxic effect on MDA-MB-231cells ($IC_{50}$: 0.11 mg/mL) compared to *C. nutans* extract ($IC_{50}$: 0.17 mg/mL). Then, the $IC_{50}$ values of *T. flagelliforme* and *C. nutans* extract on CHO cells were 0.10 mg/mL and 0.24 mg/mL respectively. Based on these results, *T. flagelliforme* represented higher toxicity effect on CHO compared to the MDA-MB-231 cells. Thus, in future CHO cell (as a positive control) can be replaced with a normal breast cell line such as HCC1395 (epithelial mammary duct of normal breast cells) to understand clearly the toxicity effect of *T. flagelliforme* extract towards the normal breast cell. Moreover, identification of potential compounds that can inhibit MDA-MB-231 cells growth is also important for future research.

*Keywords*: Breast cancer cell (MDA-MB-231), *Typhonium flagelliforme* and *Clinacanthus nutans*

## Abstrak

Kanser adalah penyakit yang boleh menyebabkan kematian dan salah satu darinya adalah kanser payudara. Rawatan konvensional telah digunakan untuk merawat kanser, walaubagaimanapun, rawatan ini telah menunjukkan kesan ketidakcekapan dan kadar kemandirian yang rendah. Oleh itu, kebanyakan pesakit kanser mula mencari rawatan pelengkap dan alternatif untuk merawat penyakit ini. Oleh itu, kajian ini adalah penting untuk mengenalpasti kesan ketoksikan ekstrak metanol bagi daun *Typhonium flagelliforme* dan *Clinacanthus nutans* pada sel kanser payudara (MDA-MB-231). MTT assai telah digunakan untuk mengukur kesan ketoksikan kedua-dua pokok ini pada sel MDA-MB-231 dan CHO (sel normal) adalah sebagai sel kawalan positif. Keputusan menunjukkan bahawa ekstrak *T. flagelliforme* telah menunjukkan kesan sitotoksik yang tinggi kepada sel MDA-MB-231 ($IC_{50}$: 0.11 mg/mL) berbanding dengan ekstrak *C. nutans* ($IC_{50}$: 0.17 mg/mL). Kemudian, nilai $IC_{50}$ bagi ekstrak *T. flagelliforme* dan *C.nutans* kepada sel CHO telah menunjukkan nilai masing-masing adalah 0.10 mg/mL dan 0.24 mg/mL. Berdasarkan nilai $IC_{50}$, *T. flagelliformee* telah menunjukkan kesan sitotoksik yang tinggi kepada sel CHO berbanding sel MDA-MB-231. Oleh itu, di masa akan datang sel CHO (sebagai kawalan positif) boleh diganti dengan sel payudara yang normal seperti HCC1395 (duktus susu epitelium bagi sel payudara normal) untuk memahami dengan jelas kesan toksik ekstrak *T. flagelliforme* terhadap sel normal payudara. Selain itu, pengenalpastiansebatian yang memberikan perencatan terhadap sel MDA-MB-231 juga penting untuk penyelidikan di

masa akan datang.

*Kata kunci*: Sel kanser payudara (MDA-MB-231), pokok *Typhonium flagelliforme* dan *Clinacanthus nutans*



## 1.0 INTRODUCTION

Cancer is defined as an abnormal cell (malignant cell) that grows without control in the body[5]. Based on previous researches, one million cancer cases were reported with 400, 000 deaths within a year[22,38]. The World Health Organization (WHO) claimed that the cancer death rate will rise to the number of twelve million deaths per year in 2030 if there is no prevention on cancer disease[14,17] .

There are more than 100 types of cancer existed which include breast, skin, lung, colon, prostate cancer and lymphoma[3,36,37]. Recently, breast cancer has emerged as the most common female malignancy in majority Asian countries such as Thailand, Indonesia and Malaysia[2]. In Malaysia, one out of twenty women will suffer from breast cancer during their lifetime and worst most of the cases was identified at the critical or late stage of cancer.[14,39].

The formation of breast cancer is caused by several factors including external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors will act in order to initiate or promote the breast cancer to be revealed[12,20,34]. Therefore, conventional treatment have been developed to treat breast cancer in order to reduce mortality and increase the survival rate of the cancer patient[20,24,28,31].

In addition, common advanced treatments that have been used to treat breast cancer such as radiation therapy, surgery treatment, and chemotherapeutic agents were found to be less effective with low survival rate of breast cancer patients and have potential of long-term negative side effects[25,28,31]. Due to this shortcomings, patients turn to alternative therapies for their primary health care and one of it is by using natural product derived from plants[31]. The secondary metabolites of plant were found to contain bioactive components that can cure and treat many diseases including cancer[11]. Therefore, plant herbs are widely used as an alternative remedy and as a main choice for scientists to find out new discovery of cancer disease remedy.

*Typhonium flagelliforme* is commonly known as rodent tuber or Keladi tikus in Malaysia and this medicinal herb belongs to the Araceae family[21]. It is characterized by its oblong, whitish tuber, triangular leaves and a spathe which is dilated and rounded at the base enclosing the yellowish spadix[21,32]. *T. flagelliforme* has been categorized as toxic, warming, and phlegm resolving plant and have potential to soothe swelling, coughing and more predominantly for the treatment of cancer[35]. Previous study reported that *T. flagelliforme* extraction inhibit the proliferation of *in vitro* cancer such as P388 murine leukaemia, human lung carcinoma and breast carcinoma cell lines[6,9,21].

Other than that, *Clinacanthus nutans,* which belongs to acanthaceae family is grown widely in tropical Asia and also known as Sabah Snake Grass or Belalai gajah[7]. This plant can be identified by its characteristic consisting of cylindric-shaped stems and peculiar leaves which are opposite, simple and slightly serrated[40]. This herb is not only accepted as remedy in neutralizing venomous insect and snake bites but it also has potential to treat Herpes Simplex Virus infection, minimize inflammations and reducing in vitro carcinogenic effects[36,40].

Hence, the present study was undertaken to evaluate the cytotoxicity effect of *T. flagelliforme* and *C. nutans* on MDA 231 breast cancer cell lines *in vitro*.

## 2.0 EXPERIMENTAL

### 2.1 Materials

Isopropanol, 37% hydrochloric acid and methanol were purchased from Sigma Chemical Co. (Subang Jaya, Selangor, Malaysia) while dimethyl sulfoxide (DMSO) was purchased from Gibco, Life Technologies (Petaling Jaya, Selangor, Malaysia). Phosphate Buffer Saline (PBS) tablets were acquired from Bio Basic Canada Inc. (Medigene Sdn Bhd, Puchong, Selangor, Malaysia). The Rosewell memorial Institute (RPMI 1640) medium and Dulbecco's Modified Eagle's Medium (DMEM) medium were purchased from Biowest Company (Puchong, Selangor, Malaysia). Trypsin, Fetal Bovine Serum (FBS) and Penicillin-Streptomycin (Pen-Strep) were acquired from Gibco Company (Bio-Diagnostic Sdn. Bhd., Petaling Jaya, Selangor, Malaysia). Tetrazolium/formazan reagent was

purchased from Sigma Chemical Co. (IChem Solution, Johor, Malaysia). Reagents that were used in preparing phosphate buffer saline (PBS) such as sodium chloride (NaCl), potassium chloride (KCl), and phosphate dibasic ($Na_2HPO_4$) were obtained from Sigma Chemical Co. (Subang Jaya, Selangor, Malaysia).

### 2.2 Plant Materials

Fresh leaves of *C. nutans* were collected from the Institut Pertanian Air Hitam, Johor while *T. flagelliforme leaves* were collected from Taman Botani Perak. The matured leaves were harvested and subsequently washed with distilled water. Then, the leaves were left dried at shady room temperature for 2 weeks[28,33].

### 2.3 Plant Extraction

The dried plant (50 g) was grounded into powder and then extracted with methanol solvent at room temperature for 72 hours[16,22,28]. Then, the plant extract was filtered through sterile cotton and filtered again using Whatman No. 1 paper which was purchased from Sigma-Aldrich (Petaling Jaya, Selangor, Malaysia). The sample was evaporated under reduce pressureuntil dryness using rotary evaporator (ELEYA N-100, EYELA, Tokyo, Japan). The yield of the extract was weighed and stored in freezer at 4°C prior to use[27,33].

### 2.4 Cell Lines

Breast cancer (MDA-MB-231) and non-cancerous Chinese Hamster Ovary (CHO) cell lines were purchased from American Type Culture Collection (ATCC) and were a generous gift from Dr Salehhuddin Hamdan (Animal Tissue Culture Laboratory, Faculty of Biosciences and Medical Engineering, UTM). MDA-MB-231 cells were cultured in DMEM while CHO cells were grown in RPMI medium supplemented with 10% v/v fetal calf serum, 1% v/v of penicillin/streptomycin as a complete growth medium. Cells were maintained in tissue culture flask at 37°C with 5 % $CO_2$ [4].

### 2.5 Cytotoxic Activity

The 80 - 90% of confluence cells were harvested by adding 2-5 mL of trypsin to detach the cells from the flask's surface[1,15]. The harvested cells were seeded in 96-well plate with the seeding density of 5 × $10^4$ cells/mL and incubated overnight in the $CO_2$ incubator at 37 °C with 5% $CO_2$. After 24 hours, the cells were exposed with the serial dilutions of plant extracts (100 µl/well) with a range of (0.00781-1.0 mg/mL) for 3 days[15,26].

### 2.6 MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a colorimetric assay developed by Mosmann[29]. The assay was used to determine the viability of the cell after the drug treatment. After 72 hours of incubation, 20 µl of 5 mg/mL MTT was added to each well and then the plates were incubated for 4 hours at 37°C in the incubator. After incubation, the media in each well was discarded and 225 µl of 100mM acidified isopropanol was added to each well to dissolve formazan crystal. Then, it was mixed homogeneously and the absorbance was measured directly using the spectrophotometer from the Thermo Scientific Company (Shah Alam, Selangor Darul Ehsan, Malaysia) with OD at 570 nm[8]. The viability of the cell (in percentage) can be calculated by using formula below:

$$\% \text{ cell viability} = \frac{\text{sample (mean)} \times 100\ \%}{\text{control (mean)}}$$

### 2.7 Statistical Analysis

All experiments were conducted with 3 replicates and the results were expressed as mean ± standard deviation. Differences between the control and the treated groups were analyzed by using the SPSS (version 16.0) software. The normality of the data in this study has been expressed by using the Shapiro-Wilk test which had showed normal and abnormal data. The normal data was analyzed by using the independent *t*-test while, the abnormal data were tested using Man-Whitney test [15,25]. The differences were considered to be significant if the probability $p <$ (0.05-0.001)[26].

## 3.0 RESULTS AND DISCUSSION

In this study, cytotoxicity activities of methanolic *T. flagelliforme* and *C. nutans* leave extracts were evaluated against breast cancer cells (MDA-MB-231) and Chinese Hamster Ovary (CHO) cells (positive control) by using MTT assay. The plant extract with the concentration range within 0.00781-1.0 mg/ml have been used to observe its toxicity response on MDA-MB-231 and CHO cells as shown in Figure 1, 2 and 3.

Figure 1 shows the morphology of MDA-MB-231 and CHO cells after being treated with methanolic leaves extract of *T. flagelliforme* for 72 hours. The images were generated using the inverted microscope (Nikon, Tokyo, Japan). Then, the treated MDA-MB-231cells (Figure 1D) and CHO (Figure 1B) revealed morphological changes as compared to untreated cells in Figure 1A and Figure 1C. The treated MDA-MB-231 cells became rounded-shape and shrunk after 3 days of treatment with the plant extract. In contrast, the untreated cells remained confluence and retained its morphology throughout the 72 hours incubation period.

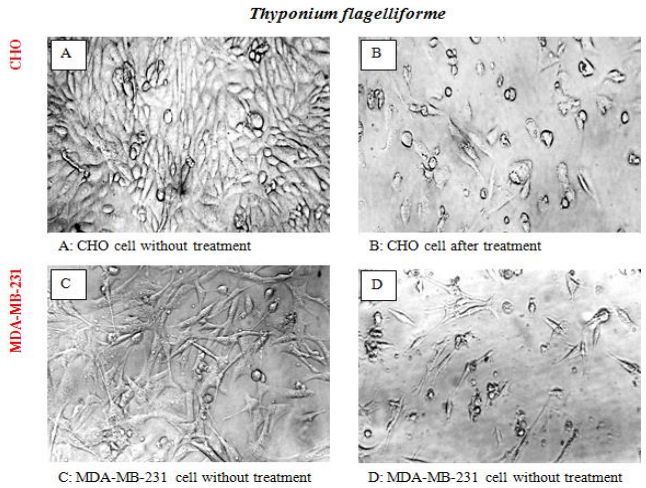


**Figure 1** The morphology of MDA-MB-231 and CHO cells when treated with the *T. flagelliforme* methanolic leaves extract (20× magnification)

Figure 2 shows the morphological changes of the MDA-MB-231 and CHO cells after being treated with the methanolic leaves extract of *C. nutans* for 72 hours. The result of untreated cells of CHO and MDA-MB-231 are shown in Figure 2E and 2G, respectively. While for the treated CHO and MDA-MB-231 cells, the results were shown in Figure 2F and 2H. The treated cells displayed significant growth inhibition, rounded-shape and shrinkage of the cells. In contrast, the untreated cells remained confluence throughout the experimental period.

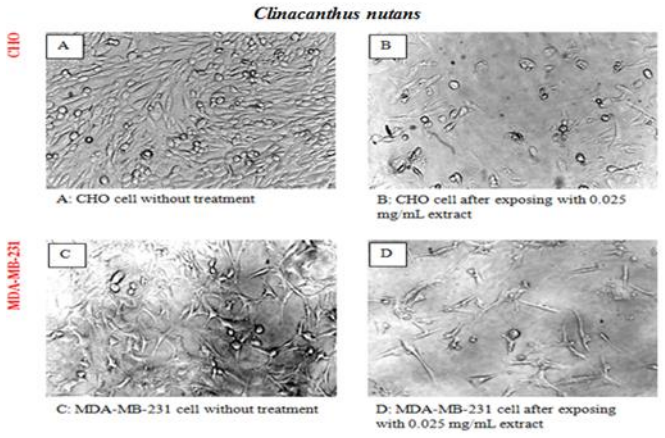


**Figure 2** The morphology of MDA-MB-231 and CHO cells when treated with *C. nutans* methanolic leaves extract (20× magnification)

Table 1 demonstrated the cytotoxic effect of *T. flagelliforme* and *C. nutans* methanolic leaves extract against MDA-MB-231 and CHO cells. From the results, the inhibition of the cells growth was being impaired by the concentration of the plant extracts. The $IC_{50}$ values of *T. flagelliforme* and *C. nutans* extracts towards MDA-MB-231 and CHO cells exhibited a significant value ($p<0.001$) when compared to the control (untreated cell).

Figure 3 shows the $IC_{50}$ values of methanolic leaves extract of *T. flagelliforme* and *C. nutans* towards MDA-MB-231 and CHO cells. *T. flagelliforme* extract was identified to have higher cytotoxic effect on MDA-MB-231 cell with $IC_{50}$ value of 0.11 mg/mL as compared to *C. nutans* extract which has $IC_{50}$ value of 0.17 mg/mL. On the other hand, the $IC_{50}$ values of *T. flagelliforme* and *C. nutans* extracts against CHO cell were 0.10 mg/mL and 0.24 mg/mL, respectively. However, *T. flagelliforme* extract demonstrated higher toxicity effect on CHO rather than MDA-MB-231 cells. This result is considerable due to the presence of hormone-independent character in CHO cell which is similar to MDA-MB-231 cell[17,18]. Thus, *T. flagelliforme* extract was not only toxic on MDA-MB-231 cells but also onto the CHO cells.

On the other hands, there is probability the present of compounds in *T. flagelliforme* extract were more toxic on CHO cell than the MDA-MB-231 when compared to *C. nutans* extract. However, the specific compound in these plants which had shown the side effect on CHO cell mechanism has yet to be identified.

**Table 1** Comparison cytotoxic effect *T. flagelliforme* with *C. nutans* methanolic leaves extract against MDA-MB-231 and CHO cells. Control = 0.00 mg/m

| Methanolic leaves extract of plants | Concentration of drug treatment (mg/mL) | Control | 0.0078 | 0.0156 | 0.0313 | 0.0625 | 0.125 | 0.25 | 0.50 | 1.0 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Typhonium flaelliforme* | Viability of MDA-MB-231 cell (%) | 100.00 ±0.004 | *** 86.956 ±0.007 | *** 83.939 ±0.007 | *** 75.512 ±0.002 | *** 60.338 ±0.003 | *** 48.074 ±0.012 | *** 30.350 ±0.012 | *** 19.338 ±0.003 | * 5.944 ±0.001 |
| | Viability of CHO cell (%) | 100.00 ±0.007 | * 84.602 ±0.018 | ** 81.556 ±0.014 | *** 76.169 ±0.040 | - 68.721 ±0.048 | *** 41.965 ±0.019 | *** 11.023 ±0.005 | *** 4.266 ±0.003 | *** 6.003 ±0.03 |
| *Clinacanthus nutans* | Viability of MDA-MB-231 cell (%) | 100.00 ±0.006 | * 93.274 ±0.002 | *** 84.047 ±0.007 | * 79.810 ±0.003 | * 69.279 ±0.001 | *** 57.737 ±0.019 | *** 40.122 ±0.004 | *** 33.116 ±0.007 | * 5.834 ±0.0 |
| | Viability of CHO cell (%) | 100.00 ±0.013 | *** 80.594 ±0.001 | *** 73.972 ±0.005 | *** 69.707 ±0.016 | *** 66.556 ±0.020 | *** 61.502 ±0.004 | *** 49.218 ±0.032 | *** 25.016 ±0.024 | *** 3.349 ±0.014 |

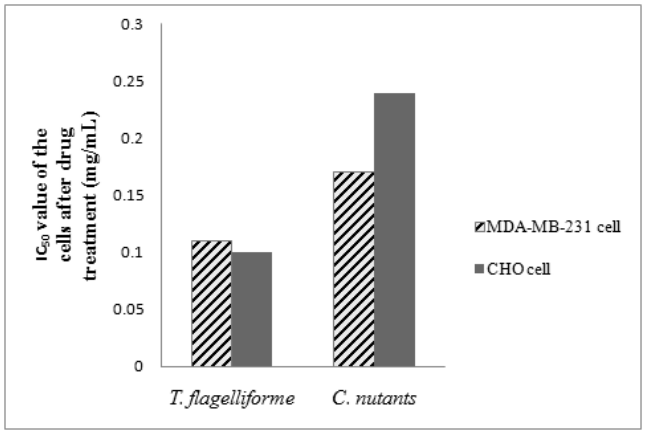


**Figure 3** The graph shows the $IC_{50}$ value of MDA-MB-231 and CHO cells after being treated with *T. flagelliforme* and *C. nutans* methanolic leaves extracts. Value are mean ±STDEV

## 4.0 CONCLUSION

In this study, methanolic leaves extract from both plants showed higher potential to inhibit the proliferation of MDA-MB-231 cells. However, *T. flagelliforme* resulted in more toxic (negative result) on CHO rather than to MDA-MB-231 cells. For further investigation, CHO cell as a positive control can be replace with the normal breast cell such as HCC1395 (epithelial mammary duct of normal breast cells) to investigate if there is any toxicity effect on the normal breast cells. Apparently a good anticancer drug should inhibit or kill the cancer cells but remain harmless to the normal breast cells. Furthermore, study on the identification of compounds that showed inhibition towards breast cancer cells were also crucial for future research.

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