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### **Anticancerous Effect of *Typhonium flagelliforme* on Human T4-Lymphoblastoid Cell Line CEM-ss**

<sup>1</sup>S. Mohan, <sup>1,2</sup>A. Bustamam, <sup>1</sup>S. Ibrahim, <sup>1,4</sup>A.S. Al-Zubairi and <sup>3</sup>M. Aspollah

<sup>1</sup>UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience,

<sup>2</sup>Department of Biomedical Sciences, Faculty of Medicine and Health Sciences,

<sup>3</sup>Department of Chemistry, Faculty of Science,

University Putra Malaysia, 43400, UPM Serdang, Malaysia

<sup>4</sup>Department of Clinical Biochemistry, University of Sana'a, Sana'a, Yemen

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**Abstract:** *Typhonium flagelliforme* (Lodd.) Blume, commonly known as rodent tuber in Malaysia, is one of the widely used alternative medicines in cancer therapy by South East Asian population. Intake of this plant is common among patients with malignancies especially Leukaemia, breast and cervical cancer; however no data available regarding the possible direct effect of *T. flagelliforme* in these cancers. The purpose of the present study was to investigate the potential *in vitro* cytotoxic effect of leaves and tubers of *T. flagelliforme* extracts against human T4-lymphoblastoid cell line CEM-ss. Among the 8 extracts Dichloromethane and Ethyl acetate extracts of *T. flagelliforme* demonstrated significant anti proliferative effect with a marked level for both leaves (10.8 and 5.8  $\mu\text{g mL}^{-1}$ ) and tuber (6.5 and 8.2  $\mu\text{g mL}^{-1}$ ), against CEM-ss cells. Considering all the results collectively *T. flagelliforme* appears to be a promising plant demonstrating anti cancer activity, that requires further investigation.

**Key words:** *Typhonium flagelliforme*, cancer, apoptosis, leukaemia, CEM-ss

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#### **INTRODUCTION**

Leukaemia is a malignant disease (cancer) of the bone marrow and blood. It is characterised by the uncontrolled accumulation of blood cells. Lymphoblastic leukaemia or Lymphocytic leukemia is a type of blood cancer. The terms lymphoblastic or lymphocytic indicate that the cancerous change takes place in a type of marrow cell that forms lymphocytes.

Management of cancer will vary according to the individual. Some of the possible treatments are transplantation, chemotherapy and radiation. In recent times focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional system (Umamaheswari and Niveditha, 2007). Various medicinal plants have been studied using modern scientific approaches. The results from these plants have revealed the potential of medicinal plants in the area of pharmacology (Dahanukar *et al.*, 2000).

The genus *Typhonium* had quite some taxonomic and pharmacological attention recently, which is a member of aroid family. The occasionally beautiful and often bizarre combination of spathe and spadix called the inflorescence, sometimes referred to as a flower, is a distinguishing feature of all aroids, which has been used for trapping their pollinators because of their particular morphology and organization of their inflorescences (Jerome *et al.*, 2003). *Typhonium* species are common in Malaysian lowlands, often found in disturbed places (Dassanayake and Fosberg, 1988). The plant

*T. flagelliforme*, commonly known as rodent tuber in Malaysia, is often used as traditional remedy for alternative cancer therapies, including leukaemia by various ethnic populations (Neoh, 1992). This plant is widely distributed in soft, damp and shady habitats in South-East Asia reaching Northern Australia and south India (Choon *et al.*, 2008).

Several fractions of the hexane and dichloromethane extracts of *T. flagelliforme* were found to inhibit the growth of NCI-H23 non-small cell lung carcinoma cell line significantly, however, most of these active fractions were also found to inhibit the growth of non-tumorigenic BALB/c 3T3 mouse fibroblast cell line also (Choon *et al.*, 2008). One of the previous studies had reported that the hexane extract of *Typhonium flagelliforme*, displayed poor cytotoxic activity against *in vitro* P 388 murine leukemia cells (Chee *et al.*, 2001a). A low cytotoxic activity has been exhibited by the polar fraction of this plant, with crude water extract being able to reduce lymphoid cells growth *in vitro* (Chee *et al.*, 2001b).

*Typhonium flagelliforme*, were also used to provide relief in cough and asthma, which was experimentally verified that water, alcohol and ester extract could significantly decrease cough times, prolong asthma incubation period, decrease twisting times, inhibit ear swelling and decrease autonomic action times (Zhong *et al.*, 2001).

Several chemical constituents had been identified from *T. flagelliforme*. The hexane extract was reported to contain saturated hydrocarbons and aliphatic acids (Chee *et al.*, 2001b), while the ethyl acetate extract was found to contain aromatic fatty acids (Chen *et al.*, 1997). No biological activities were indicated for these compounds. In addition, phenylpropanoid glycosides, sterols and a cerebroside which has anti hepatotoxic activity were reported from the root of this plant (Huang *et al.*, 2004). Pharmacological studies conducted on rats also indicated that the juice extract was able to prevent hepatocarcinogenesis (Choon *et al.*, 2008).

Regardless of the proclaimed activity, this plant is still used as an ingredient in a herbal remedy, marketed as Typhonium Plus in various parts of South East Asia, as an effective cure for leukaemia, cervical and breast cancer.

The utilization of natural products in all industrial sectors is increasing and studies of the various biotherapeutic properties of *T. flagelliforme* extracts will better equip us to further develop its therapeutic applications and commercialization of this plant. Hence to provide scientific evidence for their reported uses, based on the ethnomedical information, the present study was carried out to evaluate the cytotoxic effect by the *T. flagelliforme* extracts on Human T4-lymphoblastoid cell line CEM-ss.

## MATERIALS AND METHODS

The present research was carried out in the UPM-MAKNA Laboratory for Cancer Research, Institute of Bioscience, University Putra Malaysia.

### Collection of Plant Materials

*Typhonium flagelliforme* (Lodd.) Blume (Araceae) leaves and tubers were collected in July 2007 from the state of Selangor, Malaysia. Authentication was done at the Department of Botany, Faculty of Science, University Putra Malaysia where voucher specimen TF-L100156 was deposited.

### Extraction Procedure

Fresh Plant (10 kg) were harvested and washed thoroughly with running tap water and then distilled water, followed by separated into aerial parts as well as tubers before drying. All the plant materials were air dried and then oven dried at reduced temperature. The fully dried plants were powdered and weighed before cold maceration. The powdered leaves (284 g) and tuber (398 g) were

Table 1: Yield value of different crude extracts of *T. flagelliforme*

Crude extracts	Yield (%)
<b>Leaves</b>	
Hexane (L1)	17.15
Dichloromethane (L2)	9.49
Ethyl acetate (L3)	0.96
Methanol (L4)	50.20
<b>Tuber</b>	
Hexane (T1)	5.49
Dichloromethane (T2)	3.54
Ethyl acetate (T3)	0.86
Methanol (T4)	29.73

extracted with different solvents in the order of increasing polarity. The solvents used were hexane, dichloromethane, ethyl acetate and methanol. The extraction done for 7 days with occasional shaking and the process repeat for three times. The combined extracts were filtered through Whatman® No. 41 filter paper (pore size 20-25 µm) and dried under vacuum using a rotary evaporator and then weighed to calculate the yield of the extracts (Table 1) and kept at 4°C until required.

#### Preparation of Extracts

Dried extracts of the plants were dissolved in 1 mL of DMSO to give a desired stock solution of extract (10 mg mL<sup>-1</sup>). All the extracts were stored in 4°C till the end of experiments. During the experiment, stock solutions were diluted with the complete media to obtain an original concentration.

#### Cell Culture Condition

Human T4-lymphoblastoid cell line CEM-ss were obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: USA and used in this study (Order No. 20081911). The cell lines were grown at 37°C at humidified CO<sub>2</sub> incubator with 5% CO<sub>2</sub> in RPMI-1640 (Sigma, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen Corp., Auckland, N.Z.).

#### Cell Growth Inhibition Assay

The cell suspension (0.1 million cells mL<sup>-1</sup>) was plated out into 96-well microtitre plates. Plant extracts were dissolved with dimethylsulfoxide (DMSO) and the final concentration of DMSO was 0.1% (v/v). Different concentrations of the sample were prepared with serial dilution. Dimethylsulfoxide (0.1%) was used as a control.

The toxicity profiles of the extracts were assessed using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) microculture tetrazolium viability assay as described by (Mosmann, 1983). Thereafter, the various concentrations of plant samples were plated out in triplicate. Each plate included untreated cell controls and a blank cell-free control. After 68 h of incubation, MTT (5 µg mL<sup>-1</sup>) was added to each well and the plates incubated for a further 4 h and the media removed. DMSO was later added into each well to solubilize the formazan crystals. The absorbance was read at wavelength of 595 nm using a microtitre plate reader (Labsystems iEMS Reader MF). The percentage cellular viability was calculated with the appropriate controls taken into account. The concentration which inhibited 50% of cellular growth (IC<sub>50</sub> value) was determined. All experiments for each extract were carried out in triplicate.

The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{OD control} - \text{OD treated}}{\text{OD control}} \times 100$$

The cytotoxicity of sample on cancer cells was expressed as IC<sub>50</sub> values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

### Morphological Studies of Cell Lines Using Normal Inverted Microscope

In order to determine apoptosis may play an important role in mediating the cell death of CEM-ss cells elicited by the dichloromethane and ethyl acetate extracts, where only these two extracts showed  $IC_{50}$  below  $30 \mu\text{g mL}^{-1}$  for both the plant parts of *T. flagelliforme*, morphological studies by using the normal inverted microscope was carried out. The concentration of the  $IC_{50}$  value of the respective crude extracts of both plant parts were used for the morphological studies. The Human T4-lymphoblastoid cell line CEM-ss were treated with crude extracts for 72 h. The untreated cells were served as the negative control. The morphological changes of the cells were observed under the normal inverted microscope after 72 h post-treatment (Jun *et al.*, 2007).

### Morphological Characterization by Fluorescence Microscopy

A cell-permeable DNA-binding dye, i.e., Acridine Orange (AO), was used in combination with plasma membrane-impermeable, DNA-binding dye propidium iodide (PI). AO and PI excite a green and orange fluorescence, respectively, when they are intercalated into DNA. But only AO is able to cross the plasma membrane and will stain all the cells, while PI is excluded by cells with intact membrane (viable and early apoptotic cells). Late apoptotic and necrotic cells take up the two stains and fluoresce orange for the predominance of PI fluorescence. After treatment, the cells were stained with a 1:1 mixture of AO  $100 \mu\text{g mL}^{-1}$  and PI  $100 \mu\text{g mL}^{-1}$  for 2 min. Ten milliliter of the suspension was placed onto a glass slide and examined with a fluorescence microscope (Gorman *et al.*, 1996).

## RESULTS

In the search for new anti cancer drugs, the most common screening method employs cytotoxicity test against panel of cell lines. These are high tough point screening assays, revealing compounds with the highest cytotoxic activity.

### Cell Growth Inhibition Assay

The MTT is a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular growth. The American National Cancer Institute guidelines set the limit of activity for crude extracts at a 50% inhibition ( $IC_{50}$ ) of proliferation of less than  $30 \mu\text{g mL}^{-1}$  after an exposure time of 72 h (Suffness and Pezzuto, 1990). Among the 8 extracts dichloromethane and ethyl acetate extracts of *T. flagelliforme* showed excellent activity for both leaves ( $10.8$  and  $5.8 \mu\text{g mL}^{-1}$ ) and tuber ( $6.5$  and  $8.2 \mu\text{g mL}^{-1}$ ), against CEM-ss cells. On the same time hexane and methanol extracts of both plant parts showed  $IC_{50}$  above  $50 \mu\text{g mL}^{-1}$ . As a result, only the dichloromethane and ethyl acetate extracts were selected for further studies (Fig. 1, 2).

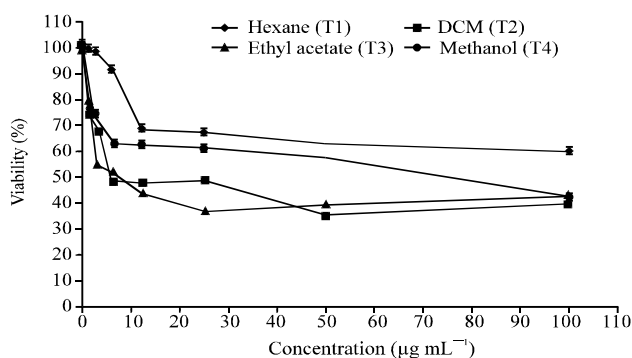


Fig. 1: Effect of different concentration of crude extracts of *T. flagelliforme* tuber on the viability of human leukemic cells, CEM-ss for 72 h. Each value represents Means $\pm$ SD

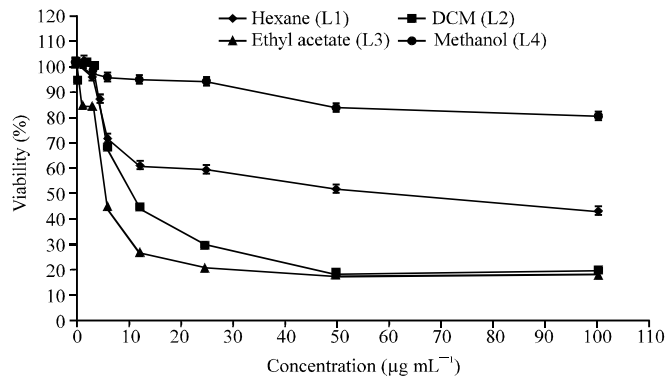


Fig. 2: Effect of different concentration of crude extracts of *T. flagelliforme* leaves on the viability of human leukemic cells, CEM-ss for 72 h. Each value represents Means±SD

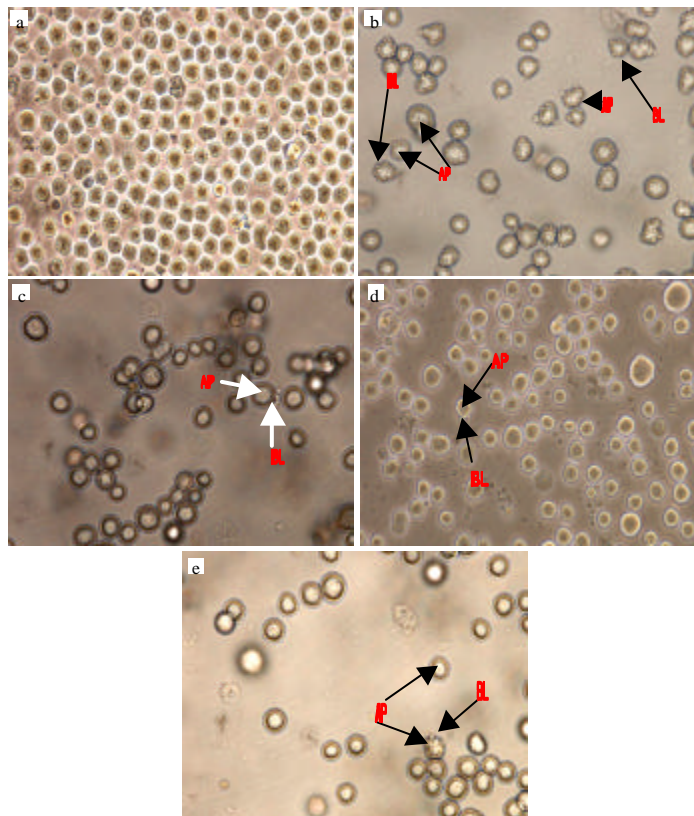


Fig. 3: Morphological changes of CEM-ss cells after 72 h treatment at IC<sub>50</sub> concentration with Dichloromethane extract of tuber (b), Ethyl acetate extract of tuber (c), Dichloromethane extract of leaves (d), Ethyl acetate extract of leaves (e) compared with untreated cancer cell (a) (a-e: 400 X magnification). AP: Apoptotic Cell; BL: Blebbing of the cell membrane

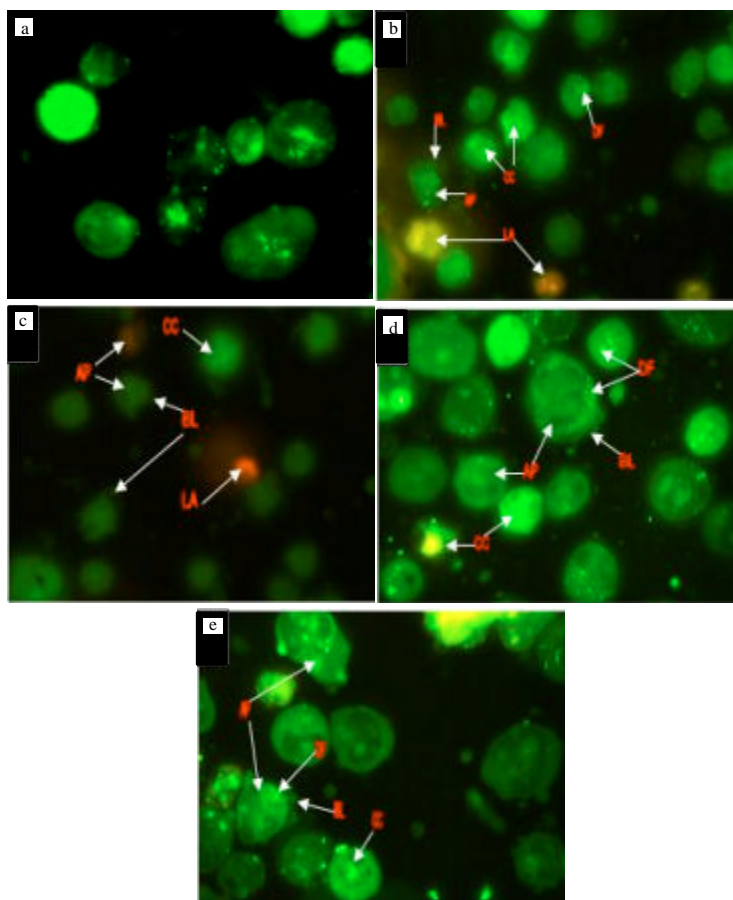


Fig. 4: Fluorescent microscopic pictures of CEM-ss treated for 72 h with Dichloromethane extract of tuber, (b) Ethyl acetate extract of tuber, (c) Dichloromethane extract of leaves, (d) Ethyl acetate extract of leaves, (e) compared with untreated cell and (a) at  $IC_{50}$  concentration. Characteristics of apoptosis like cell shrinkage, blebbing of the cell membrane, chromatin condensation and DNA fragmentations were observed (1000 X magnification); CC: Chromatin Condensation; DF: DNA fragmentation. AP: Apoptotic cell; BL: Blebbing; LA: Late Apoptosis

#### **Morphological Studies of Cell Lines Using Normal Inverted Microscope**

Normal inverted microscope was carried out to observe the morphological changes occur in the cells treated with extracts, which was well compared with untreated cells. The treated cells showed obvious changes as compared to non-treated cells. Figure 3b-e showed several morphological changes of the treated cells compared to untreated cells as shown in Fig. 3a at 72 h post-treatment under 400 x magnification. Treated CEM-ss cells showed the blebbing of the cell membrane, a more prominent growth inhibition and shrinkage of the cells. On the contrary, untreated cells remained confluent throughout the incubation period.

### **Morphological Characterization by Fluorescence Microscopy**

When the cells treated with different crude extracts for 72 h were stained with acridine orange and propidium iodide (AO/PI) and examined under the fluorescent microscope, the characteristics of the apoptotic features such as cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentations were seen. Figure 4 (b-e) shows the fluorescent image of treated CEM-ss cells. These features were however not seen in untreated CEM-ss cells (negative control) as shown in Fig. 4a. The untreated CEM-ss cells demonstrated intact nucleus and cellular membrane and were clearly observed as bright green fluorescent.

### **DISCUSSION**

The polar extracts of *T. flagelliforme* found to be more in yield, which is shown in Table 1. It is interesting to note however that the dichloromethane and ethyl acetate extracts yield only less; the same time the cytotoxicity towards the selected cell line were excellent. The low cytotoxicity of the polar extracts is in agreement with the findings reported previously by Chee *et al.* (2001b).

The objectives of this *in vitro* study were to establish the cytotoxicity of *T. flagelliforme* in Human T4-lymphoblastoid cell line CEM-ss and we tried to establish the possible mechanism of action. The induction of apoptosis in neoplastic cells is an essential step for the treatment of cancer. Most of the available chemotherapeutic agents exert their anticancer property by inducing apoptosis in cancer cells (Kamesaki, 1998). Morphological changes found in this study done by normal inverted microscope shows the morphological signs of apoptosis, which was very clear, especially the cellular blebbing and loss of normal shape of cytoplasm.

By fluorescent microscopy the above said parameters were studied and which shows the characteristics of the apoptotic features such as cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentations. Apart from the leaves, both the extracts of tubers showed the signs of late apoptosis. However this plant has shown anti cancer effect on NCI-H23 cancer cell line by inducing apoptosis (Choon *et al.*, 2008), our findings demonstrate the inhibitory effect of this plant on CEMss leukaemic cell lines for the first time.

In conclusion, the dichloromethane and ethyl acetate extracts of the plant *T. flagelliforme* inhibit the cell proliferation *in vitro* on CEM-ss Human T4-lymphoblastoid cell line. In addition to this, the possible mechanism of action of this plant also were tried to establish. Further work is required in order to fractionate the active crude extracts and to evaluate this plant further for the identification of new anti cancer compounds.

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