



Typhonium flagelliforme inhibits cancer cell growth *in vitro* and induces apoptosis: An evaluation by the bioactivity guided approach

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ABSTRACT

Aim of the Study: *Typhonium flagelliforme* (Lodd.) Blume (Araceae) is a Malaysian plant used locally to combat cancer. In order to evaluate its antiproliferative activity *in vitro* and to possibly identify the active chemical constituents, a bioactivity guided study was conducted on the extracts of this plant.

Materials and Methods: The active extracts of *Typhonium flagelliforme* were fractionated by flash column chromatography and each fraction was evaluated for antiproliferative activity using MTT assay. The apoptotic effect of the active fraction was determined microscopically and by using TUNEL colorimetric assay. GC–MS and NMR were used to determine the chemical constituents of this active fraction.

Results: Several fractions of the hexane and dichloromethane extracts were found to inhibit the growth of NCI-H23 non-small cell lung carcinoma cell line significantly, with $IC_{50} < 15 \mu\text{g/ml}$. However, most of these active fractions were also found to inhibit the growth of non-tumorigenic BALB/c 3T3 mouse fibroblast cell line except for fraction 21 of the dichloromethane extract (D/F21). This particular fraction was not only less cytotoxic to the non-tumorigenic cells, where the IC_{50} was $48.6 \mu\text{g/ml}$ compared to $IC_{50} 7.5 \mu\text{g/ml}$ for NCI-H23, but it was also found to induce apoptosis in the cancer cell line. GC–MS analysis revealed that D/F21 contains hexadecanoic acid, 1-hexadecene, phytol and a derivative of phytol. The presence of non-saturated fatty acids in this fraction was confirmed by nuclear magnetic resonance spectroscopy.

Conclusions: D/F21 was found to be the active and cancer cell line specific fraction of *Typhonium flagelliforme*. Its major chemical constituents had been determined spectroscopically.

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1. Introduction

Typhonium flagelliforme (Lodd.) Blume (Araceae) is a herbal plant which grows up to 30 cm in height. It has an oblong whitish tuber, triangular leaves and a spathe which is dilated and round at the base enclosing the yellowish spadix (Ridley, 1967; Hsuan et al., 1998). This plant grows wild in wasteland and is native to the South East Asian countries and the southern part of India and Sri Lanka (Nicolson and Sivadasan, 1981).

Typhonium flagelliforme, previously mistakenly referred as *Typhonium divaricatum* (Nicolson and Sivadasan, 1981), is commonly known as Rodent Tuber in Malaysia. Being described ethnomedically as toxic, warming and phlegm resolving, this plant is used to soothe swelling, coughing and more predominantly for

the treatment of cancer (Teo and Ch'ng, 1999; Lee and Wong, 2004). As a general practice, the juice of the fresh whole *Typhonium flagelliforme* plant is prepared in honey to be consumed as a drink (Teo and Ch'ng, 1999). There are also other practices where the leaves are wrapped in Longan flesh and taken raw (Lee and Wong, 2004).

Several chemical constituents had been identified from *Typhonium flagelliforme*. The hexane extract was reported to contain saturated hydrocarbons and aliphatic acids (Choo et al., 2001a), 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 antihepatotoxic activity were reported from the root of this plant (Huang et al., 2004).

The polar extracts were investigated *in vivo* and found to be able to ease expectoration, it is also antiasthmatic, anti-inflammatory, analgesic and sedative (Zhong et al., 2001). Pharmacological studies conducted on rats also indicated that the juice extract was able to prevent hepatocarcinogenesis (Karuppiyah et al., 1999).

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The anticancer activity of *Typhonium flagelliforme* however, had only been investigated *in vitro* on two murine cancer cell lines, namely P388 lymphocytic leukemia (Choo et al., 2001b) and a lymphoid cell line for which the cell line designation was not mentioned (Neoh, 1992). Both the studies indicated that the non-polar extracts possessed significant antiproliferative activity. Since no study had been carried out on carcinoma cell lines and the effect of this plant on non-cancerous cell lines was also unknown, we evaluated the antiproliferative effect of *Typhonium flagelliforme* on a human lung carcinoma cell line (NCI-H23) and a non-tumorigenic fibroblast cell line (BALB/c 3T3) using the bioactivity guided approach. We also evaluated the apoptotic activity and determined the major chemical constituents of an active and cell specific fraction.

2. Materials and methods

2.1. Plant material

Fresh *Typhonium flagelliforme* plants were collected from Kampung Baru Cina, Sungai Buloh, Selangor. The taxonomic identity of the plant was authenticated by Mr. Baharuddin Sulaiman, a taxonomist from the School of Biological Sciences, Universiti Sains Malaysia, where a voucher specimen of the plant was deposited (No. 10937).

2.2. Extraction of the plant material

Based on our initial findings, extraction of the fresh plant of *Typhonium flagelliforme* by maceration produced an extract with highest antiproliferative activity compared with other extraction techniques, such as soxhlet and ultrasonication using fresh or dry plant. As a result, this extraction method was used for the study. Fresh whole plant of *Typhonium flagelliforme* (3.5 kg) was cut with a mill grinder into fine pieces and macerated with hexane (8l) for 1 week at room temperature (ca. 25–27 °C). After removing the hexane extract, the residue was macerated with dichloromethane (DCM) (8l) followed by methanol (MeOH) (8l), each for a week. The extracts were filtered before evaporating to dryness under reduced pressure at 35 °C with a Buchi R110 Rotavapor (Buchi Labor Technik AG, Flawil, Switzerland). The MeOH extract was further lyophilized with a Freezone 121 freeze dryer (Labconco Corp., MO, USA). The % yield of the crude extracts was calculated as: (weight of crude extract/weight of fresh plant) × 100%.

2.3. Bioactivity guided sample purification

All extracts obtained from three different extraction solvents (hexane, DCM and MeOH) were subjected to cell proliferation assay according to the procedure described in Section 2.6. The extracts which showed significant inhibitory effect at 100 µg/ml were further evaluated at six lower concentrations in order to obtain the growth inhibition profile, from which the IC₅₀ values were determined.

From the bioassay results, hexane and DCM extracts were found to be active. As such, purification was done by using normal phase flash column chromatography on these two extracts. The stationary phase was made up of a glass column (50 mm i.d.) packed with silica gel 60, 0.040–0.063 mm (Merck, Darmstadt, Germany) (ca. 1 g extract/100 g silica gel). Compressed oxygen-free nitrogen gas was used to drive the flow of the solvent at a pressure of 1 bar. The mobile phase consisted of combinations of hexane, DCM and MeOH, and the eluting strength of the solvent was increased gradually by increasing the composition of the more polar solvent. For purification of the hexane extract, the initial solvent compo-

sition was hexane (100% v; 2100 ml) and then it was changed to hexane–DCM (4:1 v/v; 500 ml), followed by hexane–DCM (3:2 v/v; 200 ml), hexane–DCM (2:3 v/v; 200 ml), hexane–DCM (1:4 v/v; 400 ml), DCM (100% v; 900 ml), DCM–MeOH (95:5 v/v; 700 ml), DCM–MeOH (9:1 v/v; 1300 ml) and finally to DCM–MeOH (4:1 v/v; 1500 ml). For the purification of the DCM extract, the initial solvent composition was hexane–DCM (9:1 v/v; 1350 ml) and then it was changed to hexane–DCM (4:1 v/v; 400 ml), followed by hexane–DCM (3:2 v/v; 600 ml), hexane–DCM (2:3 v/v; 600 ml), hexane–DCM (1:4 v/v; 600 ml), DCM (100% v; 800 ml), DCM–MeOH (95:5 v/v; 800 ml), DCM–MeOH (9:1 v/v; 800 ml), DCM–MeOH (4:1 v/v; 800 ml) and finally to DCM–MeOH (1:1 v/v; 800 ml).

The eluent was collected in fractions of 40 ml. The chemical composition of each fraction was evaluated by using thin-layer chromatography (TLC) and visualized with UV (254 nm and 365 nm). Since *Typhonium flagelliforme* is known also to contain fatty acids (Chen et al., 1997), long chain unsaturated hydrocarbons (Choo et al., 2001a,b), glycosides and steroids (Huang et al., 2004) which could hardly be detected by UV, anisaldehyde–sulfuric acid reagent was used to identify these compounds. Based on the TLC profiles, those fractions with similar compositions were pooled together and concentrated under reduced pressure. A total of 17 combined fractions were obtained from the hexane extract (designated as H/F1, H/F2, . . . , H/F17) and a total of 21 combined fractions were obtained from the DCM extract (designated as D/F1, D/F2, . . . , D/F21). D/F21 was obtained by removing silica gel from the top of the column to a depth of 4 cm and extracting the residue adsorbed on the silica gel by using two aliquots of 200 ml of DCM. The TLC R_f values of the chemical constituents of each fraction and the weight of the fractions are given in Table 1 (for fractions of hexane extract) and Table 2 (for fractions of DCM extract).

Fractions number H/F1 and H/F13 were further purified with silica gel 60 preparative-TLC plates of 1 mm thickness (Merck, Darmstadt, Germany) to yield purer subfractions. H/F1 was developed with hexane and resulted in 10 bands which were visualized with UV 254 nm (R_f =0.09, 0.20, 0.25, 0.32, 0.36, 0.42, 0.46, 0.56, 0.70, 0.93). These bands were scraped from the prep-TLC plate and the compounds contained in these bands were extracted with hexane to yield 10 subfractions (designated as H/F1/01, H/F1/02, . . . , H/F1/10). Fraction 13 was developed with hexane–chloroform–ethyl acetate, 4:5:1 v/v/v and resulted in four bands which could not be visualized under UV. However, after spraying a small section of the TLC plate with anisaldehyde–sulfuric acid reagent, the sprayed section of the bands became visible (R_f =0.08, 0.35, 0.68, 0.88). The unsprayed sections of the bands were then scraped from the plate and the compounds were extracted with DCM to yield four subfractions (designated as H/F13/01, . . . , H/F13/04).

The fractions and subfractions obtained through flash column chromatography were subjected to antiproliferation assay on NCI-H23 and following that, 10 most active samples were selected for evaluation of their cell line specificity. Subsequently, the fraction which showed specific action towards NCI-H23 was evaluated for its apoptotic effect towards the cell line.

2.4. Cell culture

NCI-H23 (human non-small cell lung carcinoma cell line, ATCC No.: CRL-5800) was contributed by Dr. Tengku Sifzizul Tengku Muhammad (School of Biological Sciences, Universiti Sains Malaysia). This cell line was cultured in RPMI-1640 (Sigma, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen Corp., Auckland, N.Z.). The non-tumorigenic cell line, BALB/c 3T3 (mouse fibroblast cell line, ATCC No.: CCL-163) was purchased from the American Type Culture Collection (ATCC) and was cultured in

Table 1
TLC profile and weight of the hexane fractions

Fraction	TLC mobile phase	TLC R_f values	Weight (mg)
H/F1	Hexane-CHCl ₃ , 9:1 v/v	0.06, 0.46, 0.60, 0.78, 0.96	30.49
H/F2	Hexane-CHCl ₃ , 9:1 v/v	0.06, 0.38, 0.46, 0.60	10.39
H/F3	Hexane-CHCl ₃ , 9:1 v/v	0.13	21.63
H/F4	Hexane-CHCl ₃ , 2:3 v/v	0.75, 0.78	36.55
H/F5	Hexane-CHCl ₃ , 2:3 v/v	0.60, 0.70, 0.75, 0.78	22.28
H/F6	Hexane-CHCl ₃ , 2:3 v/v	0.45, 0.50, 0.60, 0.70, 0.75, 0.78	23.31
H/F7	Hexane-CHCl ₃ , 2:3 v/v	0.45, 0.50, 0.60	18.52
H/F8	Hexane-CHCl ₃ , 2:3 v/v	0.08, 0.13, 0.21, 0.30, 0.45	73.13
H/F9	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.35, 0.48, 0.55, 0.73, 0.88	11.26
H/F10	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.08, 0.18, 0.35, 0.48, 0.55, 0.73, 0.88	15.25
H/F11	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.08, 0.18, 0.35, 0.65, 0.73, 0.88	57.13
H/F12	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.08, 0.18, 0.35, 0.68, 0.88	50.25
H/F13	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.08, 0.35, 0.68, 0.88	18.14
H/F14	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.03, 0.10, 0.20, 0.35	775.72
H/F15	CHCl ₃ -MeOH, 95:5 v/v	0.28, 0.33, 0.40, 0.53	45.95
H/F16	CHCl ₃ -MeOH, 95:5 v/v	0.28, 0.33, 0.40	105.63
H/F17	CHCl ₃ -MeOH, 95:5 v/v	0.28, 0.33 with tailing effect	398.18

Table 2
TLC profile and weight of the dichloromethane fractions

Fraction	Solvent for TLC	R_f values	Weight (mg)
D/F1	Hexane-CHCl ₃ , 9:1 v/v	0.45, 0.47, 0.65	11.80
D/F2	Hexane-CHCl ₃ , 9:1 v/v	0.45, 0.47	15.77
D/F3	Hexane-CHCl ₃ , 9:1 v/v	0.45	15.81
D/F4	Hexane-CHCl ₃ , 2:3 v/v	0.28, 0.45	8.88
D/F5	Hexane-CHCl ₃ , 2:3 v/v	0.03, 0.19, 0.28, 0.45	0.31
D/F6	Hexane-CHCl ₃ , 2:3 v/v	0.83, 0.85	19.88
D/F7	Hexane-CHCl ₃ , 2:3 v/v	0.60, 0.65, 0.83, 0.85	17.85
D/F8	Hexane-CHCl ₃ , 2:3 v/v	0.33, 0.38, 0.60, 0.65	15.21
D/F9	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.50, 0.55, 0.70, 0.83, 0.90	6.99
D/F10	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.20, 0.30, 0.43, 0.50, 0.63, 0.70, 0.83, 0.90	3.89
D/F11	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.35, 0.55, 0.70, 0.78, 0.83, 0.90	4.13
D/F12	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.35, 0.55, 0.68, 0.78, 0.88	14.49
D/F13	Hexane-CHCl ₃ -EtOAc, 4:5:1 v/v/v	0.15, 0.20, 0.28, 0.35, 0.48, 0.55, 0.68, 0.88	45.52
D/F14	CHCl ₃ -MeOH, 95:5 v/v	0.15, 0.20, 0.28, 0.35, 0.48, 0.55, 0.68, 0.80	4.81
D/F15	CHCl ₃ -MeOH, 95:5 v/v	0.05, 0.10, 0.18, 0.23, 0.43, 0.60, 0.70, 0.83, 0.90, 0.93	1061.46
D/F16	CHCl ₃ -MeOH, 95:5 v/v	0.05, 0.08, 0.13, 0.23, 0.90	44.34
D/F17	CHCl ₃ -MeOH, 9:1 v/v	0.43, 0.48, 0.50, 0.58, 0.63, 0.70, 0.95	94.37
D/F18	CHCl ₃ -MeOH, 9:1 v/v	0.23, 0.43, 0.48, 0.50, 0.58, 0.63, 0.70, 0.95	32.83
D/F19	CHCl ₃ -MeOH, 9:1 v/v	0.43, 0.48, 0.50, 0.58	130.77
D/F20	CHCl ₃ -MeOH, 9:1 v/v	0.18, 0.25, 0.33	262.00
D/F21	CHCl ₃ -EtOAc, 4:1 v/v	0.03, 0.10, 0.20, 0.38, 0.50, 0.68, 0.75, 0.88	42.93

DMEM (Sigma, MO, USA) supplemented with 10% calf serum (Sigma, MO, USA). The cell cultures were maintained in a humidified CO₂ incubator with 5% CO₂ at 37 °C.

2.5. Sample preparation for cell proliferation assay

Stock solutions of the test samples were prepared at concentrations of 20 mg/ml in either ethanol (EtOH) or dimethyl sulfoxide (DMSO) depending on their solubility and these were kept at -20 °C. Immediately before the assay, dilution was made from the stock solutions in complete culture medium to produce test solutions with a final concentration of 100 µg/ml for initial screening. When a test solution was found to inhibit the growth of NCI-H23 almost completely (>85% inhibition) at this concentration, it was further evaluated for its antiproliferative effect at six lower concentrations ranging from 0.78–100 µg/ml. Solutions of 0.5% EtOH and 0.5% DMSO in complete culture medium were used as the vehicle control.

2.6. MTT cell proliferation assay

Based on some experiences in our laboratory, NCI-H23 was found to respond most strongly towards *Typhonium flagelliforme*

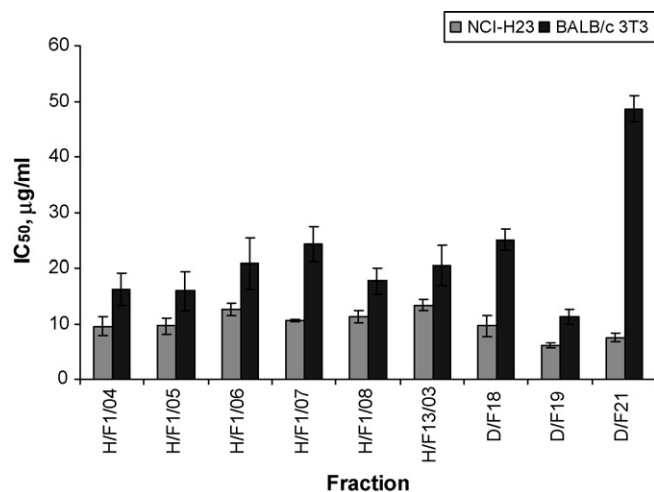


Fig. 1. Specificity of the antiproliferative action of the active fractions/subfractions towards NCI-H23, in comparison to BALB/c 3T3. The IC₅₀ values are the average of three independent triplicate assays and the error bars indicate S.E.M. Subfraction H/F13/04 was not analyzed due to insufficient sample amount for the assay.

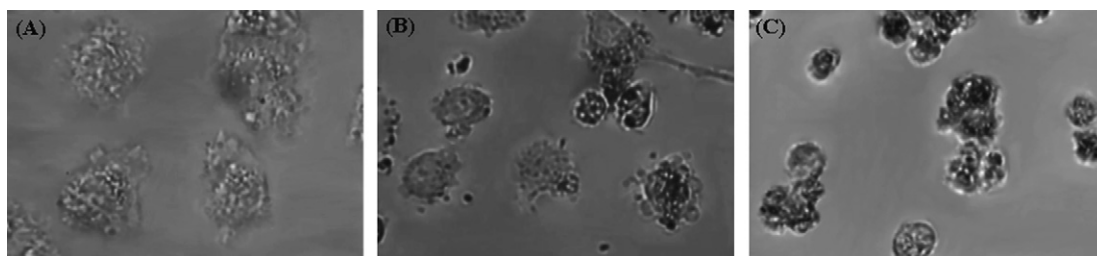


Fig. 2. Microscopic observation of NCI-H23 cells undergoing apoptosis. (A) Control cells; (B) cells treated with 50 µg/ml of D/F21 for 24 h; (C) cells treated with 50 µg/ml of D/F21 for 48 h (original magnification 320×).

extracts compared to other cell lines. Thus, this cell line was used throughout the study to screen for antiproliferative components.

In order to evaluate whether the antiproliferative action was specific to tumor cells, the active fractions were also tested on a non-tumorigenic cell line, BALB/c 3T3. Due to the difference in growth rates for both NCI-H23 and BALB/c 3T3 cell lines, the initial seeding densities for both the cell lines were optimized to ensure that the final absorbance reading falls between the linearity range of the detector. 6×10^3 cells/well of NCI-H23 and 1.5×10^3 cells/well of BALB/c 3T3 were seeded in 96-well microtiter plates. The plates were incubated for 24 h for cell recovery and attachment before test samples and the vehicle control were added to the cells. This was followed by 72 h of incubation. 20 µl of 5 mg/ml MTT (Acros Organics, Belgium) was then added to the cells followed by further 3 h of incubation. The medium was then aspirated and the formazan product generated from the viable cells was dissolved in DMSO before being measured at 570 nm against the reference wavelength of 650 nm. The growth inhibitory effect of the test samples was determined by comparing the optical density of the treated sample against the optical density of the control. The inhibition concentration, IC_{50} of the test samples was calculated using Probit Analysis (SPSS Version 12.0.1, Chicago, IL, USA). All the experiments were carried out in triplicates and three independent experiments were performed for each sample.

2.7. Assessment of apoptosis in cell culture

2.7.1. Cell morphological examination

NCI-H23 cells treated with the selected fraction were observed under the phase contrast microscope at 320× magnifications. Cytoplasmic shrinkage, membrane blebbing and the ultimate formation of smaller membrane bound vesicles were observed as signs of apoptosis.

2.7.2. DNA nick-end labelling

For further confirmation of the occurrence of apoptosis, terminal deoxynucleotidyl transferase (TdT) mediated biotin-16-deoxyuridine triphosphate (dUTP) Nick-End Labelling (TUNEL) assay was performed using DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA). Cells cultured on a chamber slide were treated with the selected fraction for 24 h at concentrations close to the IC_{50} . The assay was then conducted according to the manufacturer's instructions. Briefly, treated cells were fixed with 4% paraformaldehyde solution in phosphate buffer saline (PBS). After washing with fresh PBS, the cells were permeabilized with 0.2% Triton X-100 solution. The fragmented 3'-OH DNA ends were then labeled by incorporating biotinylated dUTP in rTdT reaction mixture at 37 °C for 1 h in the incubator, followed by addition of 0.3% hydrogen peroxide for blocking endogenous peroxidase. Streptavidin HRP (1:500 in PBS) was then added to the slide. After incubating at room temperature (ca. 25 °C) for 30 min, cell nuclei with fragmented DNAs were stained with

hydrogen peroxide and chromagen diaminobenzidine for visualization.

2.8. Identification of the selected fraction with GC–MS

Hyphenated gas chromatography and mass spectrometry (GC–MS) analysis of the selected fraction was carried out on an Agilent 6890 N Network GC system coupled to an Agilent 5973i Mass Selective Detector. Separation was obtained on a HP-5 MS column, 30 m × 0.25 mm, 0.25 µm, with helium as the carrier at a flow rate of 1.0 ml/min. The injection volume was 1 µl with a split ratio of 10:1. The initial column temperature was held at 100 °C for 3 min and then increased to 280 °C at the rate of 10 °C/min. The column temperature was then maintained at 280 °C for 20 min. The temperature of the injector was 280 °C and the temperature of the detector was 230 °C. Mass acquisition was performed in the range of 40–550 a.m.u. using electron impact ionization at 70 eV. The major components in this sample were identified by performing a spectral database matching against the library of the National Institute of Standards and Technology (NIST02).

2.9. Identification of the selected fraction with ¹H NMR

¹H NMR spectrum for the selected fraction was acquired using Bruker Avance 400 spectrometer equipped with a 5 mm bore gradient-pulse inverse probe head. The sample (5 mg) in the form of a viscous liquid was dissolved in 1 ml CDCl₃ in a 5 mm NMR tube. Analysis was carried out at 298 K where the spectrum was accumulated with 64 k data points, 16 scans, 30° flip angle, 8.3 kHz sweep width and 1 s pulse repetition time. The spectrum was processed using Gaussian window function. The chemical shifts were recorded in δ (ppm) relative to that of TMS (δ = 0.00 ppm).

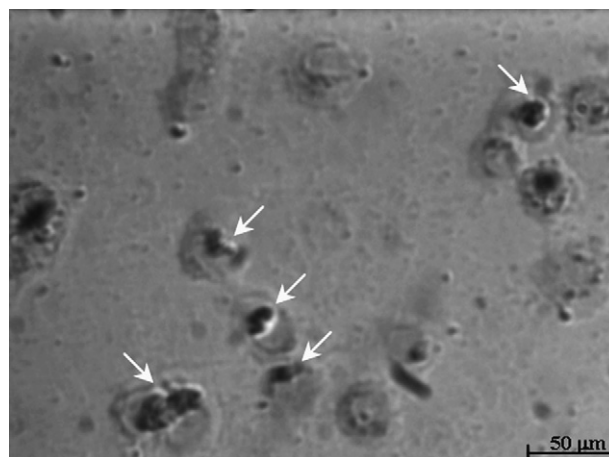


Fig. 3. NCI-H23 cells assayed by DeadEnd™ Colorimetric TUNEL System after treatment with 12.5 µg/ml of D/F21 for 24 h. Arrows show dark stained cell nuclei that indicate DNA fragmentation.

Table 3
The yields and the growth inhibitory activity of crude extracts

Extract	% yield (w/w)	IC ₅₀ (μg/ml, mean ± S.E.M.)
Hexane	0.07	53.89 ± 1.86
Dichloromethane	0.10	15.43 ± 2.70
Methanol	0.99	>100.00

The IC₅₀ are average values of three independent triplicate assays.

3. Results

3.1. Yields of crude extracts and the growth inhibitory effects

The extraction of *Typhonium flagelliforme* with both hexane and DCM provided <0.1% yields compared to the extraction with methanol which gave approximately 1% yield. The hexane and DCM extracts of *Typhonium flagelliforme* inhibited NCI-H23 cell growth with IC₅₀ = 53.9 μg/ml and 15.4 μg/ml, respectively, while the MeOH extract was found to be inactive even up to the concentration of 100 μg/ml (Table 3). As a result, only the hexane and DCM extracts were selected for purification by flash column chromatography.

3.2. The growth inhibitory effects of hexane and DCM fractions

Six fractions of the hexane extract and almost all the subfractions of H/F1 and H/F13 were found to cause >85% growth inhibition at 100 μg/ml. Further investigation of these active samples revealed

that H/F1/04–08, H/F13/03 and H/F13/04 had IC₅₀ < 15 μg/ml. Twelve fractions of the DCM extract showed significant inhibition at 100 μg/ml. Further investigation of these active fractions revealed that D/F18, D/F19 and D/F21 had IC₅₀ < 15 μg/ml. The growth inhibition concentrations of the active fractions are given in Table 4.

All the active samples which showed IC₅₀ < 15 μg/ml were further evaluated for their specificity towards NCI-H23 tumor cells compared to BALB/c 3T3 non-tumorigenic cells. Results of this study demonstrated that most of these test samples were not significantly specific for the tumor cells except for D/F21. As shown in Fig. 1, D/F21 inhibited the growth of NCI-H23 seven times more strongly than it did to BALB/c 3T3 as reflected by the IC₅₀ of 7.5 ± 0.8 μg/ml of the former to 48.6 ± 2.4 μg/ml of the latter.

3.3. Assessment of apoptosis in cell culture

Fraction D/F21 was investigated for its apoptotic inducing effect. Morphological changes of NCI-H23 were observed under the phase contrast microscope after treatment with D/F21 at 24, 48 and 72 h. After 24 h, clear signs of apoptosis such as cytoplasmic shrinkage and membrane blebbing were observed at the treatment concentrations of 50 μg/ml (Fig. 2B). By the 48 h, the cells had already broken into small apoptotic bodies (Fig. 2C). This observation of apoptosis was confirmed by the results from the DeadEnd™ Colorimetric TUNEL assay. Fig. 3 shows the stained nuclei of NCI-H23 after treatment with D/F21, which indicates

Table 4
Growth inhibition concentrations of the active fractions of *Typhonium flagelliforme* extracts

Hexane fraction	IC ₅₀ (μg/ml, mean ± S.E.M.)	Dichloromethane fraction	IC ₅₀ (μg/ml, mean ± S.E.M.)
H/F1/02	21.65 ± 1.68	D/F1	24.49 ± 2.05
H/F1/03	22.10 ± 4.15	D/F2	26.55 ± 0.69
H/F1/04	9.51 ± 1.68	D/F3	30.17 ± 1.94
H/F1/05	9.60 ± 1.45	D/F4	29.80 ± 4.93
H/F1/06	12.59 ± 1.21	D/F9	22.92 ± 2.46
H/F1/07	10.63 ± 0.25	D/F15	43.55 ± 6.75
H/F1/08	11.16 ± 1.10	D/F16	16.10 ± 3.40
H/F1/09	18.50 ± 2.00	D/F17	18.92 ± 3.64
H/F8	46.90 ± 4.44	D/F18	9.56 ± 1.89
H/F9	18.45 ± 2.35	D/F19	5.99 ± 0.43
H/F10	24.20 ± 2.16	D/F21	7.49 ± 0.77
H/F13/02	31.06 ± 4.52		
H/F13/03	13.37 ± 1.05		
H/F13/04	12.85 ± 2.12		
H/F14	37.71 ± 4.99		
H/F15	27.17 ± 2.50		
H/F16	17.46 ± 1.49		

The IC₅₀s are average values of three independent triplicate assays. Samples given in bold characters were selected for further evaluation on the specificity of antiproliferative action.

Table 5
Chemical constituents of D/F21 as determined by GC–MS

Retention time (min)	Mass spectral data ^a , <i>m/z</i> (relative intensity)	Compound	Relative composition ^b (%)
14.93	Obtained: 270(M ⁺ , 5), 74(100), 87(71), 43(46), 55(35), 143(23), 97(11), 129(9), 185(6), 239(5), 227(4), NIST Database: 270(M ⁺ , 8), 74(100), 87(58), 43(37), 55(25), 143(10), 227(6), 97(5), 129(5), 185(3), 239(3)	Hexadecanoic acid	9.52
16.45	Obtained: 55(100), 43(94), 97(81), 83(75), 41(71), 69(69), 111(41), 125(20), NIST Database: 224(M ⁺ , 3), 41(100), 55(92), 43(86), 83(60), 69(59), 97(51), 111(25), 125(10)	1-Hexadecene	16.45
16.75	Obtained: 71(100), 43(47), 57(44), 123(30), 81(30), 95(21), 111(14), 137(4), NIST Database: 71(100), 43(38), 57(33), 81(22), 123(18), 95(11), 111(7), 137(2)	Phytol	25.33
17.58	Obtained: 43(100), 71(81), 57(80), 81(30), 95(27), 111(20), 123(16), 115(15) ^c , 207(7) ^c , 137(7), NIST Database for phytol: 71(100), 43(38), 57(33), 81(22), 123(18), 95(11), 111(7), 137(2)	Phytol derivative	12.83

^a Major and representative *m/z* values only.

^b Composition calculated based on the % peak area of a total of 11 peaks.

^c Peaks not found in the spectral database of phytol.

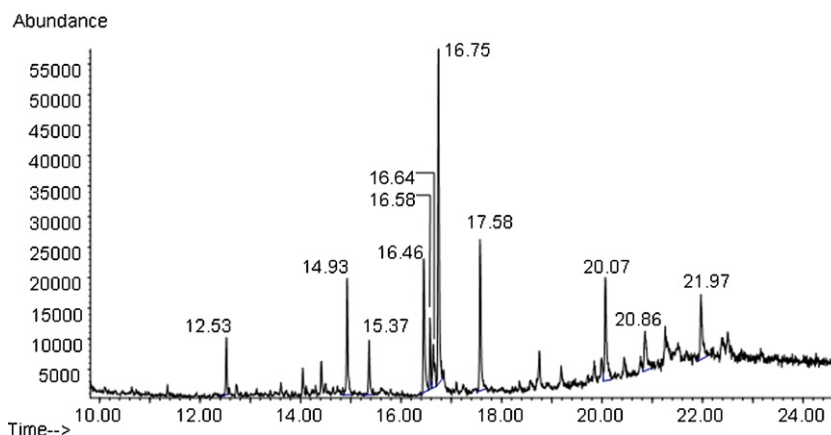


Fig. 4. Total ionic chromatogram (GC–MS) of D/F21 obtained with 70 eV using a HP-5MS column (30 m × 0.25 mm) with He gas as the carrier at a flow rate of 1.0 ml/min.

the occurrence of DNA fragmentation—a clear sign of apoptosis.

3.4. Chemical analysis of D/F21 with GC–MS

Results of the GC–MS analysis showed that at least 11 compounds were present in D/F21 of which 4 were identified through mass spectrometry (Fig. 4). The mass spectra of these compounds were matched with those found in the NIST spectral database and the data are given in Table 5. The fragmentation pattern of the fourth compound (retention time: 17.58 min) was found to be rather similar to that of phytol, except for a few m/z values. As such, this compound is tentatively assigned as a derivative of phytol.

3.5. Chemical analysis of D/F21 with ^1H NMR

In the ^1H NMR spectrum, resonances were observed in the region of δ 0.65–6.0 ppm. No resonance was observed in the region of δ 6.5–8.0 ppm, thus indicating the absence of aromatic moieties. The saturated protons (*ca.* δ 0.5–4.3 ppm) occurred in higher abundance compared to the non-saturated protons (*ca.* δ 4.8–6.0 ppm) as indicated by the peak integration ratio of 27:1 of the former to the latter. This result shows that D/F21 contained only non-saturated aliphatic compounds, which is in keeping with the GC–MS data.

4. Discussion and conclusion

Typhonium flagelliforme consisted of mainly polar compounds as reflected by the yields of the extracts shown in column two of Table 3. The polar constituents made up 1% of the plant while the non-polar constituents (hexane and DCM soluble) made up <0.2% in total. It is interesting to note however that the non-polar extracts, particularly the DCM extract, were more potent compared to the polar extract, which is in agreement with the findings reported previously by Choo et al. (2001b) and Neoh (1992).

Flash column chromatography of the hexane and DCM extracts yielded a total of 38 fractions. Ten subfractions were obtained from fraction 1 and 4 subfractions were obtained from fraction 13 of the hexane extract. *In vitro* screening of all the fractions and subfractions at 100 $\mu\text{g}/\text{ml}$ led to our selection of 29 test samples for evaluation of their cell growth inhibition profiles for determination of the IC_{50} . Ten of these samples exhibited $\text{IC}_{50} < 15 \mu\text{g}/\text{ml}$ and the order of the strength of inhibition, starting from the highest to the lowest is: D/F19 > D/F21 > H/F1/04 > H/F1/05 > D/F18 > H/F1/07 > H/F1/08 > H/F1/06 > H/F13/04 > H/F13/03.

In order to identify the least cytotoxic fractions towards non-tumorigenic cells, the inhibitory effect of the 10 fractions

mentioned above was evaluated on BALB/c 3T3 cells. This cell line is recommended by US National Institute of Environmental Health Sciences (NIEHS), Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to access basal cytotoxicity (NIEHS, 2001). It is important for an anticancer agent to exhibit cytotoxicity but such activity should be specific for cancer cells only. Results from our studies showed that although D/F19 was most active towards NCI-H23, its antiproliferative action was however non-specific. Instead, the second most active fraction, D/F21, was found to have specific antiproliferative action against NCI-H23.

In addition to cancer cell line specificity, the mode of cell death induced by D/F21 was investigated. The induction of apoptosis in neoplastic cells is an essential step for the treatment of cancer. Many chemotherapeutic agents in the market today exert their anticancer property by inducing apoptosis in cancer cells (Kamesaki, 1998). In this present study, microscopic observation of the cells treated with fraction D/F21 showed clear morphological signs of cells undergoing apoptosis. The apoptotic effect of this fraction was confirmed by the TUNEL biochemical assay.

Results from both GC–MS and ^1H NMR analyses revealed that D/F21 contained unsaturated aliphatic compounds with phytol being the main component (*ca.* 25%). This compound was previously found to have non-specific antiproliferative effect against HeLa (human cervix carcinoma), HL-60 (human promyelocytic leukemia) and WI-38 (non-cancer human lung fibroblast) with IC_{50} between 13.8 and 16.4 $\mu\text{g}/\text{ml}$ (Block et al., 2004). Furthermore, phytol was reported to induce apoptotic cell death (Komiya et al., 1999). However, it is interesting to find in our experimental results that the activity of fraction D/F21, which consisted of a combination of phytol and other aliphatic fatty acids, to be cell specific contrasting to the results obtained by Block et al. (2004) where phytol was reported to be non-cell specific. As such, further investigation needs to be carried out to determine whether cell specificity could be increased when phytol is combined with fatty acids.

In conclusion, the non-polar fractions of *Typhonium flagelliforme* inhibit cell proliferation *in vitro* on NCI-H23 human lung cancer cell line. The bioassay guided process led to the identification of a considerably potent fraction D/F21. This fraction was able to induce apoptosis on cancer cell line and its antiproliferative activity was found to be cell specific. Four chemical constituents in D/F21 had been identified, namely, hexadecanoic acid, 1-hexadecene, phytol and a derivative of phytol.

Further work is required in order to establish the identity of the other chemical constituents in D/F21 and to determine the possible correlation of each constituent in the mixture to the activities mentioned.

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