International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 18-07-2021, Accepted: 03-08-2021, Published: 18-08-2021
Volume 6, Issue 4, 2021, Page No. 765-771

Identification of phytochemicals and anticancer activity of ethanolic extract from the tubers of *Typhonium flagelliforme* (Lodd.)

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Abstract
Typhonium flagelliforme, is a natural source of anticancer compounds. The present study was an attempt to obtain bioactive fraction of its tuber for the phytochemical characterization and experimental evaluation for anticancer activity. Anti-proliferative activity of the extract was studied on cancer cell lines of different tissue origin i.e., MCF-7, L6, PA1, MIApaCa2 and A549. The tuber extract was found to bind tubulin with a reduction in fluorescence intensity in concentration dependent pattern. Owing to interference with tubulin, an induction in apoptotic cell death of MCF-7 cancer cell line was observed. Upon the extract treatment, there was a 15 and 30 % of cell early and late apoptosis respectively, was recorded. A consistent result was demonstrated with the appearance of numerous fragmented nuclei by using DAPI staining. The IC_{50} value of the extract was found to be 650.8 µg/ml for L6, 677.1 µg/ml for PA1, 704.0 µg/ml for MIApaCa2, 2098.8 µg/ml for A549 and 150.1 µg/ml for MCF-7 cell lines, respectively which reflects modest anti-proliferative activity. Thus, it was concluded that the tuber extract of *T. flagelliforme* inhibit proliferation of cancer cells of different tissue origin albeit at higher concentration, possessing a great potential as a lead molecule for the management of human cancers.

Keywords: *Typhonium flagelliforme*, tuber, phytochemicals, anticancer activity, tubulin binding

Introduction

The current scenario of cancer in the country is quite disturbing as the number of people living with this deadly disease continues to rise with mortality rate around 0.3 million per year. As per the recent report of ICMR (2020) [6] on prevalence of cancer in nation, tobacco related cancers are estimated to contribute 3.7 lakhs (27.1 %) of the total cancer burden (Mathur, Prashant *et al*, 2020) [6]. According to national cancer registry programme (2020), breast cancers contributed 2.0 lakhs (14.8 %) among women and cervix cancer contributed 0.75 lakhs (5.4 %), whereas gastrointestinal tract cancers contributed 2.7 lakhs (19.7 %) out of the total cancer incidence both for men and women. An overall cancer data compiled by ICMR from 2004 to 2010 the number of males, females and total cancer patients were 3,90,809; 4,28,545 and 8,19,354 respectively. Worldwide, the prevalence of different types of cancer revealed high incidence of lung cancer (1.8 million, 13.0 % of the aggregate), bosom (1.7 million, 11.95 % of the aggregate) and colorectal (1.4 million, 9.7 % of the aggregate) cancer (Mathur, Prashant *et al*, 2020) [6]. Although progress has been made in the detection and treatment of *in situ* (pre-invasive) cancer, there has been relatively little progress made in the treatment of advanced metastatic (invasive) carcinoma. An improved understanding of novel therapeutic agents will provide new treatment modalities to treat this disease.

Herbal drugs were used for many years and are still used as the predominant source of medical treatment in developing countries. The anticancer properties of plants have been recognized for centuries. Vinca alkaloids, one of the oldest classes of therapeutic agents used to treat cancer, are the second most commonly utilized agents in the clinic. These alkaloids have been isolated from *Catharanthus roseus*. Vincristine and Vinblastine are the two main vinca alkaloids and few structural derivatives have been designed such as vinorelbine, vindesine and vinflunine. Taxanes are another important class of anticancer agent. As an example, paclitaxel obtained from *Taxus brevifolia*, is administered to patients with breast, ovarian, lung, head and neck, oesophageal, prostate and bladder cancers (Kuruppu *et al*, 2019) [5]. A semi-synthetic derivative of paclitaxel is docetaxel, which is also used as an effective anticancer agent. The methanolic extract of leaves of *Euphorbia hirta* (Euphorbiaceae) was also investigated to treat cancer. Similarly, curcumin the major chemical constitutent of *Curcuma longa* is used in colon and gastric cancer. For, the therapeutic management of many cancer types, there is a great need for new lead compounds that target microtubules because of the enormous promise already offered by two such leads: vinblastine and taxol. Taxol and the derivatives of vinblastine (e.g., vincristine and vinorelbine) are clinically used in the management of many aggressive cancers (Rowinsky and Donehower, 1991; Kavanagh and Kudelka, 1993) [12, 4]. However, primarily because of the poor solubility of these compounds, the clinical use of these compounds has been somewhat cumbersome and expensive. For example, taxol is dissolved first in 50 % castor oil and 50 % dehydrated alcohol, and then diluted and infused intravenously over long periods of time. In addition, there are several challenges that face the use of this drug: first, the vehicle itself may cause hypersensitivity reaction; second,

the bundling of microtubules in other non-dividing cells such as neurons caused by taxol results in peripheral neuropathies (Rowinsky and Donehower, 1991; Kavanagh and Kudelka, 1993) [12, 4]. Nevertheless, the success of taxol in the management of aggressive breast and ovarian cancers is an impetus to identify compounds that target microtubules, but are less toxic, bind differently to tubulin, more soluble in aqueous solutions, available orally, yet equally or more effective against cancer.

According to World Health Organization (WHO), *Typhonium flagelliforme* (family Araceae) is a part of the global traditional medicine. However, very limited study has been done regarding its anticancer property. Hence, the plant source was carefully selected with the aim to identify phytochemicals to address anticancer property. To initiate, a large-scale method of *in vitro* micro-propagation was optimized by using plant tissue culture technique by Swain et al., (2020) [15]. The present study aims at identification of bioactive fraction of the tuber followed by chemical characterization and experimental evaluation to understand the anticancer potential of the *T. flagelliforme* and its mechanism of action. We have obtained exciting preliminary results toward this goal that necessitate to investigate more precisely about its anticancer activity by identifying the principal compound.

Materials and Methods

Collection of plant material and Preparations of plant fractions

Mature and dormant corms of *Typhonium flagelliforme* (Lodd.) were collected from the Garden of Odisha University of Agriculture and Technology, Bhubaneswar, India. Corms were washed thoroughly under tap water for removal of root, dirt, etc., followed by washing in distilled water, cut into 1 cm^3 and dried to constant weight in hot air oven at a temperature of 60 ºC and stored for further use. The dried samples were made into powder using a pulverizer and used for hot solvent extraction was done using hydro-ethanol (40:60) in a soxhlet apparatus for 24 h. The extract was dried using rotary evaporator at 60 ºC for 4 h and stored at -20 ºC for use in subsequent experiments. The voucher specimen was deposited in the Science Foundation for Tribal & Rural Resource Development (SFTRRD) herbaria, India.

Phytochemical profiling of crude extract

Phytochemicals were extracted from the sample of dried crude extract by the method described by Roessner *et al.* (2003) [10]. The sample was extracted in 1.4 ml of methanol and ethanol using 100 µl of ribitol (1 mg ml^{-1}) as an internal standard. The extract was mixed properly and incubated for 15 min at 70 ºC with shaking (200 rpm). After incubation, equal amount of water and chloroform (750 µl) were added and mixed vigorously after each addition. The mixture was centrifuged at 22,000 × g at room temperature for 15 min. 200 µl of this supernatant was transferred into another tube and vacuum dried for further derivatization process.

For gas chromatography/mass spectroscopy (GC/MS) analysis, 2 µl of sample was injected in the GC column connected with the GC/MS system (GC/MS-QP2010, Shimadzu, and Kyoto, Japan). The separation of the derivatized compounds was performed on a SH-Rxi-5 ms column (30 m, 0.25 µm DF, Shimadzu, USA) with split injection mode and the injector temperature was maintained at 250 ºC. Helium was used as the carrier gas with a flowrate of 1 ml min^{-1}. The ion source was tuned to 250 ºC, and the transfer line was set at 300 ºC with the rate of 14.5 ºC s^{-1}. The mass spectrawere recorded at a rate of eight scans per second with a scanning range of 70-700 m/z.

Identification of compounds

The phytochemical components of the biologically active fraction, the hydro-ethanolic and methanolic fraction, were identified by comparison of their mass spectra fragmentations and retention indices with those stored in databases, NIST08.LIB (Stein SE National Institute of Standards and Technology, Mass Spectral Database and Software, Version 3.02, USA, 1990) and WILEY8.LIB (McLafferly FW Registry of mass spectral data, ed. 5, Wiley New York, 1989), and also with the published literature.

Cell lines and chemicals

All the chemical reagents and media for cell culture were obtained from Sigma. Cancer cell lines of different tissue origin such as L6 (rat myoblast), PA1 (human ovarian teratocarcinoma), MIApaCa2 (human pancreas adenocarcinoma), A549 (human pulmonary adenocarcinoma) and MCF7 (human breast adenocarcinoma), were obtained from the cell repository of the National Center for Cell Science Pune, Maharashtra, India. The cells were allowed to grow at a temperature of 37 °C in a 5 % CO_2 and 95 % humidity in Dulbecco's Modified Eagle medium (DMEM, Sigma), supplemented with 10 % fetal bovine serum (FBS) and antibiotics (1 % penicillin/streptomycin). Cells with a 70-80 % confluence were sub cultured for bioassays using trypsin-EDTA (0.25 %).

In vitro cell proliferation assays (MTS assay)

The cell proliferation assay was performed using cancer cell lines of different tissue origin such as L6, PA1, MIApaCa2, A549 and MCF7 as reported earlier (Naik *et al.*, 2011) [11]. In brief, cells were cells were grown in culture medium (MEM, DMEM) supplemented with 10 % FBS, 1 % penicillin/streptomycin, 2 mM l-glutamine at 37 °C and 5 % CO_2. In a 96-well plate cells were plated at a density of 5 x 10^3 cells per well and were treated with increasing gradient concentrations of the extract of *Typhonium flageliformae* for 72 hours. The cells were then stained with 0.4 % 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS). The unbound dye was removed by washing with 1 % acetic acid. Inhibition in cell proliferation was measured by MTS assay, using the CellTiter96 AQueous One Solution Reagent (Sigma) as mentioned previously (Santoshi *et al.* (2011). Cells were exposed to MTS for 3 hours and absorbance was measured using a microplate reader (BioRad) at a wavelength of 490 nm. The IC_{50} values that stand for the drug concentration required to achieve a cell kill of 50 % was determined using the online tool Quest GraphTM IC_{50} Calculator (AAT Bioquest, Inc., Sunnyvale, CA, USA, https://www.aatbio.com/tools/ic50-calculator).

Flow cytometry analysis for apoptosis assay

In normal condition, the choline phospholipids (phosphatidylcholine, sphingomyelin) are exposed on the external leaflet, where as aminophospholipids such as

phosphatidylserine, phosphatidylethanolamine are located on the cytoplasmic surface of the lipid bilayer. Such asymmetry is scrambled during apoptosis when the phosphatidylserine (PS) becomes exposed on the outside leaflet of the membrane. The detection of phosphatidylserine (PS) by fluorochrome-tagged 36 KDa anticoagulant protein *i.e.* Annexin V was used for the precise estimation of the apoptotic incidence. This probe reversibly binds to phosphatidylserine residues only in presence of milimolar concentration of the divalent calcium ions.

Apoptosis in cancer cells was detected by Annexin-V-FITC apoptosis detection method as reported previously, Ye *et al.* (1998) [16]; Zhou *et al.* (2003) [19] using Apoptosis detection kit (Sigma-Aldrich, USA). For experimental purpose, $3x10^4$ MCF-7 cells per well were seeded on 12 well culture plate and incubated for 24 hrs with complete medium. The cells were treated with IC_{50} concentration of the extract and were harvested at 72 hrs. Cells were typsinized and stained with surface marker antibodies (biotin-conjugated Annexin V, FITC-conjugated streptavidin) and propiumdium iodide. Cells were allowed to suspend in 1X binding buffer and incubated with Annexin V FITC conjugate for 20 min in dark at room temperature. Flow cytometer data for propiumdium iodide (PI) with excitation at 488 nm and emission at 530 nm were collected. The viable cells (Annexin V^- / PI^-), early apoptotic cells (Annexin V^+ / PI^-), late apoptotic/necrotic cells (Annexin V^+ / PI^+) and late necrotic cells (Annexin V^-/PI^+) were identified and their parentage was determined.

DAPI staining

The MCF7 cells were grown on poly-L-lysine coated coverslips in 6-well plates and were treated with the IC_{50} concentration of the extract for 72 hrs. After incubation, the cover slips were fixed in cold methanol and washed with phosphate buffered saline (PBS), stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted on slides. The images were captured using a fluorescent microscope (Nikon Eclipse Ts2R-FL). Apoptotic cells were identified by alterations of morphological features (e.g. nuclear condensation, formation of membrane blebs and apoptotic bodies).

Tubulin purification

Tubulin devoid of microtubule-associated proteins (MAPs) was purified from bovine brain in presence of 1 M glutamate and 10% v/v dimethyl sulfoxide (DMSO) by two cycles of temperature-dependent polymerization and depolymerization followed by phosphocellulose chromatography (Hamel and Lin, 1981) [3]. Tubulin was then purified using phosphocellulose chromatography (Hamel and Lin, 1981; Panda *et al.* 2000) [3, 9]. Tubulin concentration was determined by the method of Bradford (Bradford, 1976) [1]. The purified tubulin was quickly frozen in liquid nitrogen and stored at -80 ºC until further use.

Tryptophan quenching assay

Due to the presence of many tryptophan residues, tubulin displays an intrinsic fluorescence characteristic (excitation at 295 nm and emission at 332 nm). The tubulin binding agents change the dichroic spectrum of tubulin and quench its fluorescence intensity in a concentration dependent and saturable fashion (Ye *et al*, 1998) [16]. This has allowed an easy method for measuring the tubulin-drug interactions. The IC_{50} concentration of the extract was incubated with 2 μM tubulin in 25 mM piperazine-N, N′-bis (2-ethanesulfonic acid (PIPES, pH 6.8), 3 mM MgSO4, and 1 mM EGTA for 30 min at 25 ºC. The fluorescence intensities of tubulin in the absence and presence of different concentrations of the extracts were monitored in a JASCO FP-6500 spectrofluoremeter (JASCO, Tokyo, Japan) by exciting the samples at 295 nm and measuring the emission in the range of 310-380 nm. The inner filter effects were corrected using a formula: $F_{corrected} = F_{observed}\ x\ antilog\ [(A_{ex} + A_{em})/2]$, where A_{ex} and A_{em} are the absorbances at the excitation and emission wavelengths. The dissociation constant (K_d) was estimated using the following equation: $\Delta F = \Delta F_{max}\ L/(K_d + L)$; where, ΔF is change in the fluorescence intensity of the tubulin upon binding to extract, ΔF_{max} is the maximum change in the fluorescence intensity of the protein when it is fully bound with extract, and L is the concentration of the extract. ΔF was calculated by subtracting the fluorescence intensity of tubulin in the absence of extract from the fluorescence intensity of tubulin in the presence of different concentrations of extract.

Results and Discussion

Chemical profiling of aqueous ethanolic extract of tuber

The hydro-ethanolic extract of mature and dormant corms of *T. flagelliformae* was used for chemical profiling using GC-MS technique. Since the hydro-ethanolic extracts of mature and dormant corms of *T. flagelliformae* does not contain any volatile components, we have derivatized with silylating agents (vide infra) and then analysed with GC-MS (Fig. 1). Broadly, three groups of compounds found in the extract such as organic acids, monosaccharides and polyols (Table 1). Ten organic acids were found with a total composition of 50.54 % of the extract. There existed one aromatic acid (0.88 %), one amino acid (0.15 %), four long chain alkanoic acids (46.59 %) and two dicarboxyalic acids (2.23 %). It was also found to contain myo-inositol (1.29 %) and four monosaccharides (26.28 %). Four esters of long chain alkanoic acids (13.26 %) and one amide of 9-Octadecenoic acid were also detected in the extract. In addition to these, glycerol (5.55 %) and monoglyceryl ester of palmitic acid (0.63 %) were also present as detected by their mass spectrum. The major group of compounds of the extract are the organic acids. Among them, four long chain alkanoic acids constituted 46.59 % of the extract. Major component of the extract is palmitic acid (29.14 %) and oleic acid (13.84 %). The biological activities of the aqueous ethanol extract are probably due to the presence of these four organic acids (Bordoloi *et al*., 2017). Lactic acid and benzoic acid also have synergistic effect with other organic acids contributing towards these activities. Glycidyl ester of oleic acid may also contribute towards antibacterial activity of the extract as it contains an epoxide moiety (Nicolau *et al*, 2010). Epoxides are active components of organic compounds usually due to its strained three membered rings. Further, the monosaccharide, D-Psicofuranose was also found to occur in 18.82% of the extract.

Table 1: Putative identification of the phytochemicals of the aqueous ethanol extract from rhizome of *Typhonium flagelliformae*

Peak#	Compounds present*	Retention Time of Silyl derivatives (min)	Molecular Weight (MW)	Yield (%)
5	Lactic acid	11.786	90	0.47
6	L-valine	12.22	117	0.15
8	Benzoic acid	14.94	122	0.88
9	Glycerol	15.48	92	5.55
10	Butanedioic acid	16.00	118	0.28
11	Beta-hydroxypyruvic acid	17.18	104	0.22
12	Malic acid	18.57	134	1.95
14	D-Psicofuranose	22.27	180	18.82
15	α-Methyl galactoside	22.79	194	0.88
16	β-Methyl galactoside	23.14	194	0.72
17	1,3,4,5,6-pentahydroxy-D-Fructose	23.38	180	4.76
18	Methyl glucoside	23.64	194	1.10
19	Palmitic acid	24.95	256	29.14
20	9-Octadecenoic acid, methyl ester	25.48	282	4.57
21	Myo-Inositol	25.66	180	1.29
22	Oleic acid	26.71	282	13.84
23	Stearic acid	26.88	284	2.04
24	Hexadecanoic acid	27.36	568	2.36
25	9-Octadecenamide	28.01	281	0.72
26	13-Docosenoic acid	28.38	338	1.57
27	Glycidyloleate	28.96	338	7.09
28	Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester	29.13	625	0.97
29	1-Monopalmitin	29.85	330	0.63

*Bistrimethyl silyl trifluroacetamide octamethyltrisiloxane N, N'-methane tetraylbis [1,1,1 trimethyl-silanamine], Bis (trimethylsilyl) monomethyl phosphoric acid, Ribitol penta trimethyl silyl derivatives are also detected in the gas chromatogram; but they are not listed in the table as they are added as silylating agents and ribitol is added as internal standard (vide experimental).

We have also obtained another bioactive fraction of *Typhonium flagelliformae* using aqueous methanol. The GC-MS analysis of the methanolic extracts leads to identification of number of compounds. These compounds were identified through mass spectroscopy attached with gas chromatography. The lists of compounds identified are included in Table 2.

Table 2: Compounds identified in methanolic extracts of *Typhonium flagelliformae* using GC-MS analysis.

SI. No.	Retention Time	Compound Name	Formula	Molecular Weight (MW)
1	9.389	Chloro (Diethyl) Phosphine	C4H10ClP	124
2	11.295	Methyl 3-[(Trifluoroacetoxy) Methylthio] Prop-2-Enoate	C7H7O4F3S	244
3	12.61	Boronic Acid, Ethyl-, Bis (2-mercaptoethyl ester)	C6H15O2S2B	194
4	13.03	Methyl 6-Thia-Dodecanoate	C12H24O2S	232
5	13.526	N-(1-Methoxycarbonyl-1-Methylethyl)-4-Methyl-2-Aza	C9H17O4N	203
6	14.256	4-Methylthiane, S-Oxide	C6H12OS	132
7	15.801	Hexadecane, 1,16-Dichloro-	C16H32Cl2	294
8	16.217	Trans-2-Methyl-4-N-Butylthiane, S, S-Dioxide	C10H20O2S	204
9	16.722	Methyl 9,10-Methylene-Hexadecanoate	C18H34O2	284
10	17.777	Phenol, 2,6-Bis(1,1-Dimethylethyl)-	C14H22O	206
11	18.167	Dl-Proline, 5-Oxo-, Methyl Ester	C6H9NO3	143
12	18.893	Tetradecane, 1-Chloro-	C14H29Cl	232
13	19.373	3-N-Hexylthiane, S, S-Dioxide	C11H22O2S	218
14	19.948	Methyl 12-Methyl-Tridecanoate	C15H30O2	242
15	20.944	Methyl 13-Methyltetradecanoate	C16H32O2	256
16	21.149	Methyl 14-Methylhexadecanoate	C18H36O2	284
17	21.674	Pentadecanoic Acid, Methyl Ester	C16H32O2	256
18	22.439	Methyl-d-Mannopyranoside	C7H14O6	194
19	22.864	D-Glucopyranoside, Methyl	C7H14O6	194
20	23.545	Methyl 11-Methyl-Dodecanoate	C14H28O2	228
21	23.69	Methyl 11-Hexadecenoate	C17H32O2	268
22	25.04	l-(+)-Ascorbic Acid 2,6-Dihexadecanoate	C38H68O8	652
23	25.095	Methyl 15-Methylhexadecanoate	C18H36O2	284
24	25.27	11-Bromoundecanoic Acid	C11H21O2Br	264
25	25.965	3-Pyrrolidin-2-Yl-Propionic Acid	C7H13O2N	143
26	26.991	Methyl 11,14-Octadecadienoate	C19H34O2	294
27	27.301	Methyl 9-Cis,11-Trans-Octadecadienoate	C19H34O2	294
28	27.491	Methyl 11,14,17-Eicosatrienoate	C21H36O2	320
29	27.851	Ethyl 9. Cis.,11.Trans.-Octadecadienoate	C20H36O2	308
30	28.251	Cyclopropanebutanoic Acid, 2- [[2- [[2- [(2-Pentylcyclop	C25H42O2	374
31	28.367	Cis-13,16-Docasadienoic Acid	C22H40O2	338

32	28.452	Ethyl 9,12-Hexadecadienoate	$C_{18}H_{32}O_2$	280
33	28.712	4-Methyl-2,7-Dioxa-Tricyclo [4.4.0.0(3,8)] Decane	$C_9H_{14}O_2$	154
34	28.952	1,10-Hexadecanediol	$C_{16}H_{34}O_2$	258
35	29.722	Methyl 8-Methyl-Nonanoate	$C_{11}H_{22}O_2$	186
36	29.922	Methyl 9,12-Hexadecadienoate	$C_{17}H_{30}O_2$	266
37	30.187	Butyl 9,12,15-Octadecatrienoate	$C_{22}H_{38}O_2$	334
38	30.242	Methyl 6-Cis,9-Cis,11-Trans-Octadecatrienoate	$C_{19}H_{32}O_2$	292
39	30.367	2-Nonyl-1-Ol, Diethyl Acetal	$C_{13}H_{24}O_2$	212
40	30.507	Butyl 6,9,12-Hexadecatrienoate	$C_{20}H_{34}O_2$	306
41	31.108	Methyl 11-Cyclopentylundecanoate	$C_{17}H_{32}O_2$	268
42	31.703	Cis-13,16-Docasadienoic Acid	$C_{22}H_{40}O_2$	336
43	32.458	Methyl 20-Methyl-Heneicosanoate	$C_{23}H_{46}O_2$	354
44	32.683	Methyl 12,15-Octadecadienoate	$C_{19}H_{34}O_2$	294
45	33.303	Methyl 2-Octylcyclopropene-1-Octanoate	$C_{20}H_{36}O_2$	308
46	34.554	2-Cyclohexylpiperidine	$C_{11}H_{21}N$	167
47	35.524	i-Propyl 7,10,13,16-Docosatetraenoate	$C_{25}H_{42}O_2$	374
48	36.33	Cyclohexanecarboxylic Acid, 4-Propyl-, 4-Cyanoph	$C_{17}H_{21}O_2N$	271
49	36.835	Geranylgeraniol	$C_{20}H_{34}O$	290

Tuber extract inhibits proliferation of cancer cells of different tissue origin

We want to determine whether the rhizome extracts of *Typhonium flagelliformae* affect proliferation of cancer cells of different tissue origin. The extract was analyzed for its anti-proliferative activity using cancer cell lines *viz*. L6, PA1, MIApaCa2, A549 and MCF7. The extract exhibited improved cytotoxic activity in increasing concentration (Figure 2) using all the cell lines. The IC_{50} value of the extract was found to be 650.8 µg/ml for L6, 677.1 µg/ml for PA1, 704.0 µg/ml for MIApaCa2, 2098.8 µg/ml for A549 and 150.1 µg/ml for MCF7 cell lines, respectively, which reflects modest anti-proliferative activity.

Tubulin binding activity of tuber extract

We found that the tuber extract of *T. flagelliformae* reduced the intrinsic fluorescence of tubulin in a concentration dependent manner (Fig. 3a). Incubation of the extract with tubulin showed a concentration-dependent quenching of the intrinsic tryptophan fluorescence, indicating the binding of the extract to tubulin in a concentration-dependent fashion. The double reciprocal plots yielded a dissociation constant (Kd) of 78 ± 6 µg/ml (Fig. 3b). Although significant correlation of the sensitivity of cancer cells to the extract cannot be established at this stage, but the study indicated that tubulin represents a potential target for the extract.

Extract of *T. flagelliformae* induced apoptosis to cancer cells

The induction of apoptosis with the treatment of extract of *T. flagelliforme* was determined using MCF7 cells. The apoptotic process is characterized by the alterations in lipid composition of cell membrane *i.e.* phosphatidylserine, which is normally on the inner leaflet of cell membrane translocates to outer leaflet, which can be measured using Annexin V by fluorescent binding. In contrast, the cell impairment DNA-binding fluorescent dye *i.e.* propodium iodide can only enter the cells at the stage of late apoptosis when the membrane permeability is compromised. The percentage of early and late apoptotic cells using MCF7 cell lines for treatment of IC_{50} concentration of the plant extract (150.1 µg/ml) for 72 hrs is collated in Table 1. The representative figure of flow cytometry analysis treated with the extract was represented in Figure 4. After 72 hrs of culture, the control untreated cell culture represented only few early apoptotic (2.5%) and late apoptotic cells (1.0%), which were considered as the background cell death due to regular trauma during cell culture (Table 5.3). In contrast, the percentage of early apoptotic cells of 15% and late apoptotic cells of 30% treated with the extract were found to be relatively higher compared to the controlled untreated cells (Ji *et al.,* 2017; Sivakumaran *et al.,* 2018; Fan *et al.,* 2018) [17, 14, 2]. (Table 3).

Table 3: Percentage of early apoptotic (Q1), late apoptotic (Q2), viable (Q3) and necrotic (Q4) cell measured by flow cytometry.

Viability/Apoptotic	Control	Extract
Q1	2.5%	15%
Q2	1%	30%
Q3	94%	50%
Q4	0.5%	1%

Besides, the morphological examination using DAPI staining revealed apoptotic cell death of MCF7 breast cancer cells characterized by chromatin condensation along with the appearance of numerous fragmented nuclei (Figure 5).

Conclusion

According to World Health Organization's International Agency for Research on Cancer, the incidence of cancer cases in the world is increasing day by day. Previous studies indicated that microtubule-targeted drugs are particularly effective for the treatment of many cancer types. However, these drugs are known to cause severe dose-limiting toxicities in patients. Therefore, it is imperative to discover other novel anti-mitotic agents that have fewer side effects and that can be easily administrated. We have identified a novel source of obtaining such compounds from the medicinal herb *T. flagelliforme*. We found that one of its ethanolic extract interfere with the microtubule and inhibit the proliferation of a panel of cancer cell lines of different tissue origin.

The catastrophe effects on interfering with the microtubule lead to induction of apoptosis to cancer cells. However, at this stage it is difficult to say which single compound of the extract is having the anticancer property.

Conflict of Interest

All authors declare no conflict of interest.

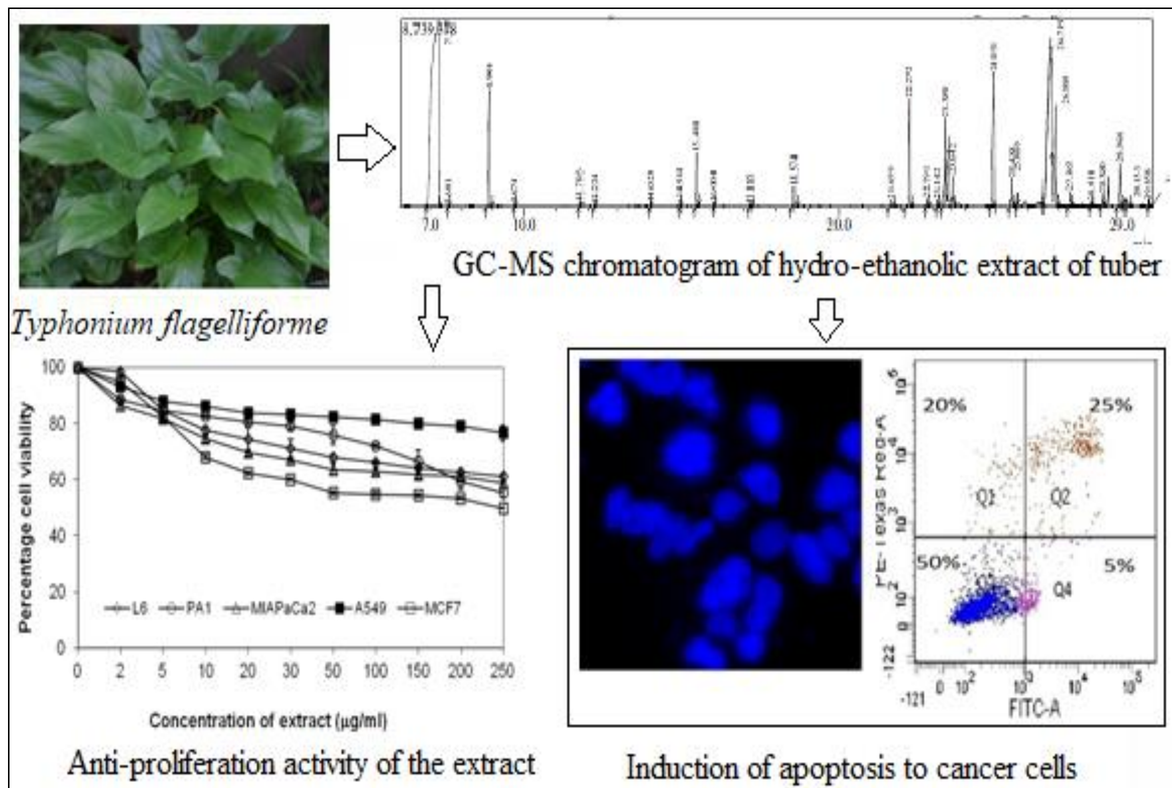

Fig 1

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