

Ethyl acetate extract from marine sponge *Hyattella cribriformis* exhibit potent anticancer activity by promoting tubulin polymerization as evidenced mitotic arrest and induction of apoptosis

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ABSTRACT

Background: Marine sponges are important sources of bioactive compounds. **Objective:** This study investigated the anticancer properties of *Hyattella cribriformis* ethyl acetate (EA) fraction in various cancer and normal cell lines. **Materials and Methods:** anticancer assay was carried out in 15 cell lines to evaluate the anticancer potential of the EA fraction. Impact on cell cycle distribution was determined using flow cytometry. The fraction was investigated for interfering microtubules assembly in both *in vitro* and cellular assay. Further studies were conducted to determine the fraction induced cell death (apoptosis) using calcein/propidium iodide dual staining, activated caspase-3 and phosphorylation of Bcl-2 protein at Ser70. DNA fragmentation assay was performed to confirm the apoptosis. **Results:** EA fraction exhibited potent inhibition of cancer cell growth and resulted in 50% growth inhibition (GI₅₀) of 0.27 µg/mL in A673 cell line. Sarcoma (MG-63, Saos-2) and ovarian (SK-OV-3 and OVCAR-3) cancer cell lines also showed superior anticancer activity GI₅₀ of 1.0 µg/mL. Colon and breast cancer cell lines exhibited moderate GI compare other cancer cell lines and normal human lung fibroblast showed GI₅₀ of 15.6 µg/mL. EA fraction showed potent G2/M phase arrest in A673 cell line and induced apoptosis at 48 h exposure. EA fraction promoted microtubule polymerization in tubulin polymerization assay and increased level of polymerized tubulin in the HeLa cells. Fraction induced the activation of caspase-3 and phosphorylation of Bcl-2 anti-apoptotic protein. Fraction induced DNA fragmentation in HeLa cells as evidence of apoptosis. **Conclusion:** Marine sponge *H. cribriformis* EA fraction exhibited potent anticancer activity through tubulin polymerization and induction of apoptosis.

Key words: Apoptosis and DNA fragmentation, ethyl extract fraction, G2/M arrest, *Hyattella cribriformis*, microtubule

INTRODUCTION

With a long history of clinical efficacy, microtubule-targeting agents (MTAs) remain the most potent antimetabolic drugs used in the treatment of human diseases especially for cancer. This class of compounds disrupts normal microtubule dynamics, leading to abnormal spindle formation, chromosome misalignment and induces apoptosis in cancer cells. MTAs can be further classified by their interaction

mode and mechanism into (i) microtubule-destabilizing agents (MDAs), like vinca alkaloids (vincristine and vinblastine), that prevent microtubule polymerization and (ii) microtubule-stabilizing agents (MSAs), like taxanes (paclitaxel), that inhibits depolymerization of microtubules. Both polymerization and depolymerization processes were important for mitosis phase of the cell cycle.^[1] Many MTAs are derived from natural sources including marine sources and plant sources. In recent years, wide array of structurally diverse MTAs have been isolated from marine organisms (dolastatin, halichondrins, spongistatin, curacins and discodermolide).^[2] Among all marine organism marine sponges considered to be a source of bioactive compounds, sponges have proven to

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be one of the most resourceful groups,^[3] Sponges also represent the largest source of unusual metabolites and bioactive compounds isolated from microorganisms, which can account for almost 40% of the sponge's biomass.^[4,5] In many cases, sponge-derived compounds may be produced by symbiotic microbes rather than by the sponges themselves.^[4,6] The biomedical and pharmaceutical importance of these compounds are attributed to their antibacterial, anticancer, antifungal, antiprotozoal, and antiviral activities.^[7,8]

Peloruside A from the *Mycale hentscheli* had potent anti-mitotic activity, blocking cells in G2/M of the cell cycle. Peloruside A stabilized the polymerized form of tubulin and induced microtubule bundling in interphase cells and multiple asters in mitotic cells^[9] in a similar manner similar to Paclitaxel.^[10] Hemiasterlin A, an antimitotic tripeptide, isolated from the sponge, *Hemiaster linaminor* was shown to interfere with mitotic spindle formation at low concentrations and causes tubulin depolymerization at higher concentrations, with higher efficacy than paclitaxel or vinblastine.^[11] Hemiasterlin was shown to bind to the vincapeptide-binding site of β -tubulin, where it competitively inhibits binding of the marine compound dolastatin 10 and noncompetitively inhibits binding of vinblastine.^[8,12] (+)-Discodermolide isolated from the rare deep-water sponge, *Discodermia dissoluta* functions as an immunosuppressant and induces G2/M phase cell-cycle arrest in lymphoid and nonlymphoid cells at nanomolar concentrations.^[12,13] Besides, it causes aberrant mitosis, altered microtubule dynamics and induction of apoptosis.^[9]

Studies conducted with marine natural products during the last decades have yielded many substances with biomedical potential, which raised the interest of many research groups toward marine ecosystems as a source of new drugs.^[14] There are a number of compounds isolated and identifies from marine sources have been progressed to clinical trial studies of the human disease indications.^[4] Even though, the sea has yielded numerous promising drug candidates, the development of real drugs from this source has been extraordinarily slow mainly due to the lack of ethno-medical history and the pressing supply problem. The natural concentrations of many pharmacologically active compounds from marine organisms are often minute and sometimes account for <6–10% of the respective wet weight.^[14] Marine sponges are considered to be true “chemical factories” producing hundreds of unique chemical compounds, many of which have been isolated and their structure determined, but their biological roles and activities are still largely unknown. However, a few marine-derived compounds obtained Food and Drug Administration (FDA) approval for clinical practice that are including Ziconotide (Prialt®) for

the treatment of pain isolated from cone Snail, Eribulin Mesylate (E7389) (Halaven®) used for the treatment of cancer derived from marine sponge.^[15,16]

In the present study, we have investigated the ethyl acetate (EA) fraction of marine sponge *Hyattella cribriformis* for its anti-cancer properties in panel of cancer cell lines. We have investigated the cell cycle distribution by flow cytometry and found that the fraction is causing mitotic arrest in cancer cells and induces apoptosis. Since the compound exhibited potent G2/M arrest, we have evaluated the tubulin polymerization assay and identified that the fraction is inhibiting the depolymerization process of microtubule. We have also explored the mechanism of apoptosis induced by the fraction.

MATERIALS AND METHODS

Chemicals and reagents

Paclitaxel, vinblastine, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), RNase, propidium iodide, Calcein, bovine serum albumin (BSA), TritonX-100, SIGMAFAST™ 5-bromo-4-chloro-3-indolyl-phosphate (BCIP®)/nitro blue tetrazolium (NBT) and protease inhibitors cocktail were purchased from Sigma-Aldrich (USA). Rabbit Monoclonal antibodies specific for Caspase-3, Bcl-2, Phospho-Bcl-2 (Ser70), α -Tubulin, β -Actin and goat anti-rabbit alkaline phosphate conjugated secondary antibody were purchased from cell signaling technology (USA). EnzChek® Caspase-3 Assay Kit was obtained from Life technologies (USA) and Nitrocellulose membranes were obtained from pall lifesciences (USA).

Cell lines and cell culture

The 14 human cancer cell lines and one normal human cell line were used for *in vitro* anticancer activity evaluation of the active fraction. The cell lines HeLa (Cervical cancer, ATCC# CCL-2) Colorectal adenocarcinoma cell lines, HT-29 (ATCC# HTB-38), HCT-116 (ATCC# CCL-247), HCT-15 (ATCC# CCL-225), Ovarian cancer cell lines, SK-OV-03 (ATCC# HTB-77), OVCAR-3 (ATCC# HTB-161), A-431 (Epidermoid carcinoma ATCC# CRL-1555), ACHN (Renal carcinoma ATCC# CRL-1611), A673 (Rhabdomyosarcoma ATCC# CRL-1598), Osteosarcoma cell lines, MG-63 (ATCC# CRL-1427), Saos-2 (ATCC# HTB-85) cell lines were cultures in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/ml Penicillin, 100 μ g/ml Streptomycin and 2.5 g/l NaHCO₃ at 37°C in an atmosphere of 5% CO₂ and 95% humidity. Breast cancer cell lines, MCF (ATCC# HTB-22), MDA-MB-231 (ATCC# HTB-26),

SK-BR-3 (ATCC# HTB-30) were grown in Leibovitz's L-15 medium (Sigma-Aldrich) supplemented with 10% (v/v) heat inactivated FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin and the culture was maintained at 37°C in incubator without CO₂ environment and 95% humidity was maintained. Normal human lung fibroblast (IMR-90) (ATCC# CCL-186) was grown in Dulbecco's Modified Eagle Medium high glucose medium (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 300 g/l NaHCO₃ at 37°C in an atmosphere of 5% CO₂ and 95% humidity. All cell lines were obtained from the National Centre for Cell Science, Pune (India). The cell lines were cultured in monolayers and cells in log phase growth were used for the experiments.

Sample collection and identification

An eco-friendly collection of sponge as by catch in fishing nets was carried out at Thondi coast (Southeast coast of India). The major species of *H. cribriformis* was collected, identified and taken-up for the isolation of bioactive metabolites. The collected samples were immediately washed with fresh seawater to remove the dirt and symbionts, the excess water was drained off using blotting paper. The sponge sample was brought to the laboratory in the cold container and stored in the -80°C until further process. The morphological and anatomical characteristics of the collected sponges were observed and identified using microscopic and macroscopic analyses.^[17] The typical characteristics taken into account include internal structure, color, size and shape and compared with the existing data and photographs.

Solvent fractionation

For the isolation of bioactive fraction 500 g of sponge sample was minced in a tissue homogenizer (IKA, USA) and extracted with methanol for three times. The combined methanol extract was filtered and concentrated in a rotary vacuum evaporator (Buchi, Switzerland) at room temperature. The completely dried crude extract was defatted with n-Hexane further fractionated with chloroform, EA, n-butanol and methanol in a separating funnel through sequential method using decreasing gradient polar solvents. Each fraction was again concentrated in a rotary vacuum evaporator and stored in -20°C. The fractions were dissolved in dimethyl sulfoxide (DMSO) at concentration to make 20 mg/mL stock and used at appropriate concentration in experiments.

In vitro anticancer assay

Anticancer screening assay was conducted for active fraction (EA fraction) with 15 selected cell lines. The anti-proliferative effect of fraction were tested in five

logarithmic concentrations (100, 10, 1, 0.1 and 0.01 µg) in above mentioned cell lines and the viability of cells was determined by MTT assay, following the method of Carmichael *et al.*^[18] Each concentration of the fraction was used in quadruplicate and cumulative variations were maintained <20% between the data points. Briefly, Cells were seeded in a 96 well cell culture plate at a density of 5000 cells/well and incubated at 37°C in CO₂ incubator. Compounds were added as ×2 concentrations to the cells in 100 µL to maintain the above-mentioned concentration. The plates were further incubated for 48 h in the CO₂ incubator. MTT solution was composed of MTT at 5 mg/ml in phosphate buffered saline (PBS) (1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl; pH 7.4), from this solution 50 µl was pipette out into each well. The plate was further incubated for 2.30 h and the medium was carefully decanted. The formazan crystals were air-dried in dark place and dissolved in 100 µL DMSO and the plates were mildly mixed at room temperature and the absorbance was measured using Synergy H4 microplate reader (BioTek, USA) at 570 nm. At the time of drug addition separate reference plate for cell growth at time 0 h (the time at which drugs were added) was also terminated as described above. From the optical densities the percentage growths were calculated using the following formulae, If T is ≥T₀, percentage growth = 100 × [(T-T₀)/(C-T₀)] and if T is < T₀, percentage growth = 100 × [(T-T₀)/T₀], Where T is optical density of test, C is the optical density of control and T₀ is the optical density at time zero. From the percentage growths, a dose-response curve was generated, and 50% growth inhibition (GI₅₀) values were interpolated from the growth curves.

Flow cytometric analysis for DNA content and apoptosis

A 673 cells were seeded in six-well plates at a density of 3 × 10⁵ cells/well and grown for 24 h. Cells were incubated with active fraction at semi-logarithmic concentrations (0.3 µg, 1 µg, 3 µg and 10 µg) and incubated further for 24 and 48 h. Then cells were collected, fixed in 70% ethanol overnight at 4°C, washed in PBS once, and resuspended in PBS containing 0.5% Triton × -100, 0.1 mg/ml RNase and 40 µg/ml propidium iodide, incubated for 45 min in the dark at temperature. The DNA content and apoptotic cells were analyzed using a flow cytometer (FACS Calibur, Becton Dickinson), equipped with an air-cooled argon laser providing 15 mW at 488 nm (Blue laser) with standard filter setup. 10,000 events were collected, and the percentages of each cell cycle phases were analyzed using Cellquest Pro software (Becton Dickinson, USA). Cell cycle analysis was performed in triplicate for three independent experiments.

In vitro tubulin turbidity assay

Tubulin polymerization assay was performed according to the manufacturer's protocols (tubulin polymerization assay kit; Cytoskeleton Inc., Denver, CO). In brief, tubulin proteins (>99% purity, included in this kit) were suspended in G-PEM buffer containing 80 mM PIPES, 2 mM MgCl₂, 0.5 mM Ethylenediaminetetraacetic acid, and 1.0 mM GTP (pH 6.9) and 5% glycerol with or without paclitaxel (1.0 µg/ml), vincristine (0.3 µg/ml), and EA extract (1 and 5 µg/ml) in a 96-well plate, and the absorbance was measured at 340 nm from 0 to 30 min (Synergy H4 multimode microplate reader BioTek, USA). *In vitro* tubulin, turbidity assay was performed in three independent experiments.^[19]

In vivo microtubule assembly assay

We used a well-established method to measure soluble (depolymerized) and assembled (polymerized) tubulin.^[20] HeLa cells (5 × 10⁶/flask) were seeded into the T-75 flask. Cells were exposed to paclitaxel (1 µg), vincristine (1 µg), and active fraction (1 and 5 µg) for 12 h. After treatment, cells were collected and washed twice with ice-cold PBS then lysed at 37°C for 5 min with 50 µl of hypotonic buffer (1 mM MgCl₂, 2 mM EGTA, 0.5% NP-40, 5 mM amino caproic acid, 1 mM benzamidine, 20 mM Tris-HCl containing protease inhibitors cocktail, pH 6.8). The cell lysates were centrifuged at 13,000 rpm for 10 min at 25°C. The supernatants containing soluble (cytosolic) tubulin were separated from the pellets containing polymerized (cytoskeletal) tubulin. The pellets were resuspended in 100 µl of hypotonic buffer, sonicated on ice, mixed with ×5 sample buffer, and heated for 5 min at 100°C. Equal amounts of the two fractions were partitioned by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Immunoblots were probed with anti-α-tubulin monoclonal antibody and secondary alkaline phosphate-conjugated antibody. Using BCIP/NBT substrate developed the blots.

Assessment of live/dead (calcein/propidium iodide) in confocal microscopy

HeLa cells were plated on coverslips in each well of a six-well plate at density of 3 × 10⁵ cells/well. Cells were treated with 0.3, 1.0 and 3 µg concentrations of EA fraction for 48 h. At the end of the incubation period, cells were trypsinized and washed with ice-cold PBS and resuspended in 200 µl of PBS and 100 µl Calcein (1 µM) was added and incubated for 20 min in dark place at room temperature. Cells were washed with 5 ml of PBS and resuspended in 200 µl PBS and 100 µl propidium iodide (1 µM) solution and incubated for 1 min. Cells were washed with 5 ml PBS and resuspended in 100 µl PBS and 50 µl of cell suspension was placed on a microscope coverslip (22 × 40 mm) and it was covered with another microscope coverslip (22 × 22 mm)

cell images were observed under a LSM Meta 510 Confocal Scanning Microscope (Carl Zeiss, Germany).

Immunoblotting

HeLa cells were seeded in T-25 flask at density of 2 × 10⁶ cells and the cells were grown for 24 h in CO₂ incubator. Cells were incubated with active fraction at semi-logarithmic concentrations (0.3 µg, 1 µg, 3 µg) incubated further for 48 h. Cells were lysed in 1% Triton× -100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM proteinase inhibitor cocktail (Sigma-Aldrich), 1 mM sodium orthovanadate and 25 mM NaF for 30 min on ice and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant was collected and samples containing equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to Biotrace™ Polyvinylidene difluoride membranes (Pall lifesciences, USA), the membranes were blocked in tris buffered saline with tween (TBS-T) (50 mM Tris-base; 150 mM NaCl; 0.2% Tween-20) with 5% BSA for 1 h at room temperature. The primary antibodies included those to, caspase-3, Bcl-2, phospho-Bcl-2 (Ser70), α-Tubulin and β-Actin (Cell Signaling, USA). The primary antibodies were washed with 0.1% Tween-20/TBS and then incubated with alkaline phosphate-conjugated secondary antibody. The bound antibodies were visualized using SIGMA FAST™ BCIP®/NBT (Sigma-Aldrich, USA). The percentages of increase or decrease of protein were estimated by comparison to vehicle control.

DNA ladder assay

HeLa cells were seeded in T-25 flask at density of about 0.75 × 10⁶ cells. After 24 h incubation, cells were treated with 0.3, 1.0 and 3 µg concentrations of EA fraction for 48 h. After incubation, cells were trypsinized and resuspended in 1 ml of Hank's balanced salt solution. Cell suspension was transferred into 10 ml of ice-cold 70% ethanol and stored in -20°C for 34 h. Fixed cells were centrifuged at 800 ×g for 5 min and cell pellet was resuspended into 40 µl phosphate-citrate buffer (192 parts of 0.2 M Na₂HPO₄ and eight parts of 0.1 M citric acid (pH 7.8) and incubated at room temperature for 30 min. Cell suspension was centrifuged at 1000 ×g for 5 min and supernatant was gently decanted without disturbing the pellet. To lyse the cells 3 µl of 0.25% Nonidet NP-40, 3 µl of RNase (1 mg/ml in water) added to the suspension and incubated for 30 min at 37°C. Further 3 µl of proteinase K (1 mg/ml and incubated at 56°C for 30 min. After incubation, 12 µl of gel loading buffer (0.25% bromophenol blue, 30% glycerol) was added. Finally, Samples were loaded in to 1.5% agarose gel and electrophoresis was carried out at 4 V/cm for 4 h and DNA was analyzed under ultraviolet transilluminator.

Statistical analysis

The data were subjected to one-way analysis of variance, followed by Student-Newman-Keul's test using a computer based software, Statistical Package for Social Sciences Graphed Prism and $P < 0.05$ was considered to be statistically significant.

RESULTS

Solvent fractionation

We have obtained total 6.3 g of sponge crude extract by initial three methanol extraction. The entire 6.3 g of crude extract was defatted using n-Hexane for three times and yielded 3.7 g. Subsequent solvent fractionation has yielded different dry weight of the fractions chloroform fraction was accounted 2.0 g, EA fraction showed weight of 0.8 g, n-Butanol and methanol yielded 0.6 g and 0.3 g respectively [Table 1].

In vitro anticancer assay

Among four solvent fractions EA fraction exhibited superior anticancer activity in the preliminary screening, and other fraction does not exhibit much cell GI (data not shown). Thus, EA fraction was selected for further investigations.

In the extended cancer panel screening, the EA fraction of sponge *H. cribriformis* exhibited anticancer activity against most of the tested cell lines and the activity was dose-dependent and tissue-specific activity was obtained [Table 2]. Among the 15 cell lines tested, most potent anticancer activity exhibited against A673 cell line at concentration GI_{50} of $0.27 \pm 0.2 \mu\text{g/ml}$ and less activity was observed in the HT-29 cell line it accounted 23.3 ± 1.9 . The GI_{50} for 48 h treatment of EA fraction in SK-OV-3, MG-63, Saos-2, OVCAR-3, HeLa, MCF-7, ACHN, A431, HCT-116, SK-BR-3, HCT-15, IMR-90 and MAD-MB-231 cell line were 0.46 ± 0.2 , 0.72 ± 0.12 , 0.93 ± 0.21 , 1.4 ± 0.31 , 1.5 ± 0.6 , 4.1 ± 0.4 , 5.0 ± 1.0 , 5.6 ± 1.3 , 8.6 ± 0.8 , 11.0 ± 3.1 , 13.41 ± 1.6 , 15.61 ± 3.1 , $17.4 \pm 2.0 \mu\text{g/ml}$ concentrations [Table 1 and Figure 1]. The MTT assay results found that EA fraction has an anticancer effect against all the tested cell lines. The EA fraction also showed less cell GI against normal lung fibroblast cell line IMR-90 in GI_{50} value at $15.61 \pm 3 \mu\text{g/ml}$ concentrations compared with other tested cancer line. Similarly, breast cancer cell line MAD-MB-231 exhibited less anticancer activity compare to normal lung fibroblast cell line (IMR-90).

Flow cytometry analysis for DNA content and Apoptosis

Cell cycle progression in A673 cells, exposed to EA fraction at four different concentrations for 24 and 48 h is depicted in Tables 3 and 4 and Figures 2 and 3. The

Table 1: Fractionation of marine sponge *Hyattella cribriformis* extract using different solvent and fraction weight (g)

Solvent fraction	Dry weight (g)
1 st methanol extract (crude)	6.3
After n-hexane defatting	3.7
Chloroform fraction	2.0
EAF	0.8
n-butanol fraction	0.6
Methanol fraction	0.3

EAF: Ethyl acetate fraction

Table 2: Anticancer activity of ethyl extract from *Hyattella cribriformis* against panel of cancer and normal cell lines cell viability was assessed after 48 h and exposure using MTT assay

Cell line	$GI_{50} \mu\text{g/ml}$ (EAF)
HeLa	1.5±0.6
HT-29	23.3±1.9
HCT-116	8.6±0.8
HCT-15	13.41±1.6
SK-OV-3	0.46±0.2
OVCAR-3	1.4±0.31
A-431	5.6±1.3
ACHN	5.0±1.0
A673	0.27±0.2
MG-63	0.72±0.12
Saos-2	0.93±0.21
MCF-7	4.1±0.4
MDA-MB-231	17.4±2.0
SK-BR-3	11.0±3.1
IMR-90*	15.61±3.1

Data represent percentage mean±SD, n=3. *IMR-90: Normal human lung fibroblast. GI_{50} : 50% growth inhibition; EAF: Ethyl acetate fraction; SD: Standard deviation; MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

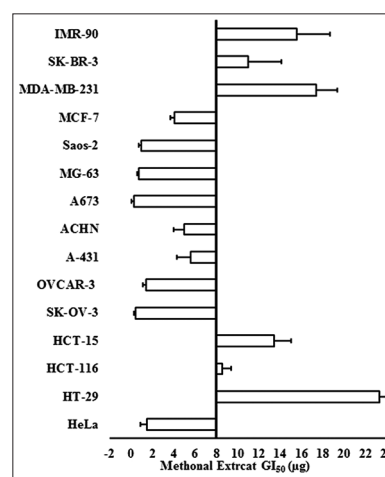


Figure 1: Comparative analysis of growth inhibition (GI_{50}) values of different cell lines treated with ethyl acetate fraction for 48 h. Left side bars signifies the potency and right side bars signify the less anticancer activity. Data represent percentage mean of GI_{50} ± standard deviation, n = 3

Table 3: Cell cycle distribution (%) in A673 cell line after exposure of EAF for 24 h

Phase/% population	Control	EAF				Paclitaxel 1 µg/ml
		0.3 µg/ml	1.0 µg/ml	3.0 µg/ml	10 µg/ml	
Sub G0-G1 phase	0.66±0.06	1.59±0.17	2.37±0.05	2.46±0.47	3.07±0.3	2.77±0.7
G0-G1 phase	57.5±1.7	51.6±2.95	49.5±7.45	35.4±1.21*	26.26±2.5*	20.8±4.3*
S phase	14.9±1.57	13.6±0.21	12.6±1.43	14.0±0.90	14.0±1.5	11.8±3.1
G2-M phase	26.5±4.03	34.2±3.62*	34.8±0.76*	48.4±3.95*	53.53.2*	64.9±17.3*

*P<0.05; Significantly different compared with control by one-way ANOVA. Data represent percentage mean±SD, n=3. Data marked with stars are significantly different at P<0.05, in comparison with untreated cells. SD: Standard deviation; ANOVA: Analysis of variance; EAF: Ethyl acetate fraction

Table 4: Cell cycle distribution (%) in A673 cell line after exposure of EAF for 48 h

Phase/% population	Control	EAF				Paclitaxel 1 µg/ml
		0.3 µg/ml	1.0 µg/ml	3.0 µg/ml	10 µg/ml	
Sub G0-G1 phase	1.04±0.2	1.6±0.1	3.35±1.0	15.57±1.6*	25.6±2.3*	24.7±6.2*
G0-G1 phase	57.2±2.4	52.0±1.3	51.4±2.4	36.6±5.8*	26.6±0.7*	15.4±1.3*
S phase	18.7±0.5	15.2±2.4	18.5±3.8	16.6±2.1	20.8±2.0	15.1±3.6
G2-M phase	23.6±2.7	31.09±1.9	28.5±3.9	31.5±1.3	27.3±1.5	45.6±4.2*

*P<0.05; Significantly different compared with control by one-way ANOVA. Data represent percentage mean±SD, n=3. Data marked with stars are significantly different at P<0.05, in comparison with untreated cells. SD: Standard deviation; ANOVA: Analysis of variance; EAF: Ethyl acetate fraction

effects of EA fraction at four different semi-logarithmic concentrations (0.3, 1, 3 and 10 µg) for 24 and 48 h were studied to further characterize the growth inhibitory effects on cell cycle analysis. As EA extract exerted maximum GI in A673 cells with minimum GI₅₀ value (0.27 µg), this cell line was selected for further investigations.

Flow cytometric profiles of nuclear DNA content revealed that EA fraction at all concentrations provoked a strong G2/M arrest in a dose-dependent manner (34%, 34%, 48% and 53% vs. 27%). Consequently, the fraction of cells in G0-G1 phase was significantly reduced (51%, 49%, 35% and 26% vs. 57% control). S phase population of vehicle-treated cells was found to range between 14% and 15%. The proportion of cells with S phase DNA content remained approximately the same in cells exposed to EA fraction at all doses for 24 h. Cell cycle analysis further revealed that the EA at all concentrations had no effect on the sub G1 phase in cells exposed for 24 h, indicating no effect on apoptotic induction.

As observed in 24 h, cells exposed for 48 h were also primarily arrested in G2-M phase (31%, 28%, 31% and 28% vs. 23%) with a concomitant decrease in G0-G1 population (52%, 50%, 36% and 27% vs. 58% control). The percentage of cells in S phase remained stable at all doses. Cells exposed to 10 and 3 µg concentration of EA for 48 h alone exhibited an increase in sub G0-G1 population, indicating an induction of apoptosis [Figure 4].

In vitro tubulin turbidity assay

Since EA fraction exhibited potent G2/M arrest, we have investigated whether EA fraction affects the microtubule

assembly. As shown in the Figure 5, in the *in vitro* microtubule assembly assay EA fraction inhibited the tubulin depolymerization in a dose dependent manner. Concomitantly, the results also indicated that paclitaxel promoted tubulin polymerization significantly, and Vincristine inhibited tubulin polymerization. The EA extract promoted the tubulin polymerization similar to paclitaxel as a tubulin stabilizing agent.

Cell based microtubule assembly assay

We have investigated the EA fraction for promoting the microtubule polymerization by measuring the density of cellular microtubules. Exposure with EA fraction (1 and 5 µg/ml), paclitaxel (1 µg/ml) and vinblastine (1 µg/ml) resulted in the increase of microtubule polymerization and decrease in short microtubules in the cytoplasm [Figure 6]. Our results indicate that EA fraction induces mitotic arrest through stabilizing the microtubules in HeLa cells.

Assessment of live/dead (Calcein/propidium iodide) in confocal microscopy

HeLa Cells exposed with 0.3, 1.0 and 3.0 µg/ml concentration of EA extract for 48 h depicted clear signs of increase in apoptosis through increased number of propidium iodide stained cells and the cell death was observed in a dose dependent manner. This dual staining method enables differentiating the live/dead cells, dead cells nucleus stained with propidium iodide in red color and live cells were appeared in green color due to the calcein uptake in the live cells and enzyme conversion [Figure 7]. Compared to unexposed control cells 3.0 µg/ml exposed well showed a significant number of propidium iodide stained cells.

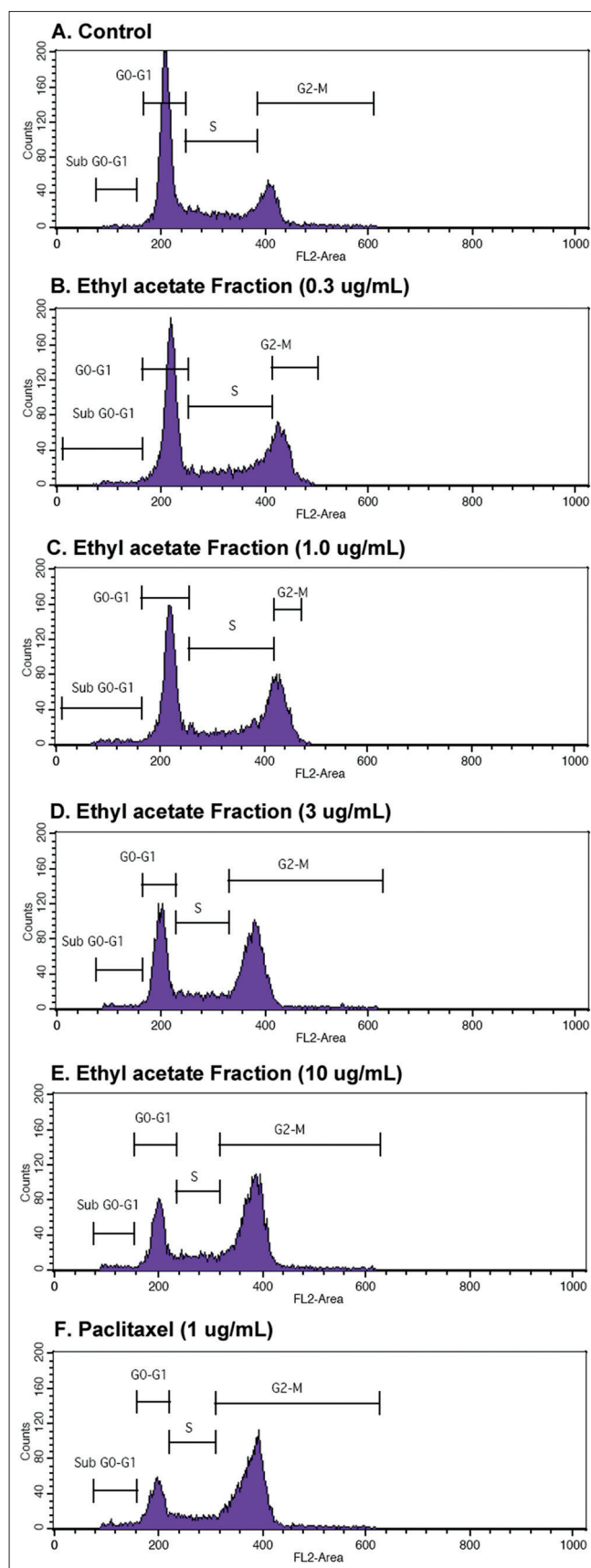


Figure 2: Analysis of cell cycle distribution after 24 h exposure of different concentration of ethyl acetate fraction in A673 cell line

Immunoblotting analysis

We have evaluated the important apoptosis regulators proteins through immunoblotting to study the mechanism underlying in the EA fraction induced apoptosis. We have primarily investigated the effector caspase-3 activation, our results revealed that EA fraction induces the caspase-3 activity by increasing cleaved form of caspase-3 (Active enzyme) in dose-dependent manner. Then we have evaluated the anti-apoptotic protein Bcl-2 phosphorylation levels at Ser70 it correlates with the apoptosis induction. EA extract treated HeLa cells showed pattern of increased phosphorylation at Ser70 in the Bcl-2 protein in a dose-dependent manner [Figure 8]. This indicates that anti-apoptotic protein Bcl-2 in activity was reduced as anti-apoptotic activity it does not inhibit the apoptosis process.

DNA ladder assay

From the above experiments, it was prudent that the EA fraction is inducing the apoptosis through tubulin polymerization. In order to confirm the apoptosis, a hallmark assay DNA ladder assay has been performed. DNA fragmentation is the final process of apoptosis, and this explains the DNA fragmentation between the nucleosomes and forms 140–180 base pairs fragments. The EA fraction treated HeLa cell's nucleus has showed laddering pattern compared to untreated control. This fragmentation was significant in the 3 µg/ml concentration treated cells, but untreated control cells did not show any sign of DNA fragmentation [Figure 9]. Hence, this data gives the confirmation of apoptosis induction in HeLa cells that were treated by EA fraction.

DISCUSSION

Disturbing the microtubule polymerization and depolymerization process in the mitosis is a popular approach for cancer therapy.^[21] The ability of the chemical to kill rapidly dividing cells is the hallmark of chemotherapy. Anticancer drugs often act by killing the rapidly dividing cells of a tumor by inhibiting the synthesis of DNA directly (DNA interacting agents) or suppress microtubule dynamics, leading to disruption of the mitotic spindle in dividing cells, cell cycle arrest at M phase, and late apoptosis (Microtubule-damaging agents).^[1] Thus discovering novel and less toxic MTAs are the current requirements. In the present study, EA fraction exerted concentration-dependent *in vitro* anti-cancer activity in a panel of cancer cell lines viz., HT-29, A673, SK-OV-3, MG-63, Saos-2, OVCAR-3, HeLa, MCF-7, ACHN, A431, HCT-116, SK-BR-3, HCT-15, IMR-90 and MDA-MB-231. Nevertheless, the degree of GI varies with dose, extract and cell line. Of the 15 cell lines tested, A673 cells showed

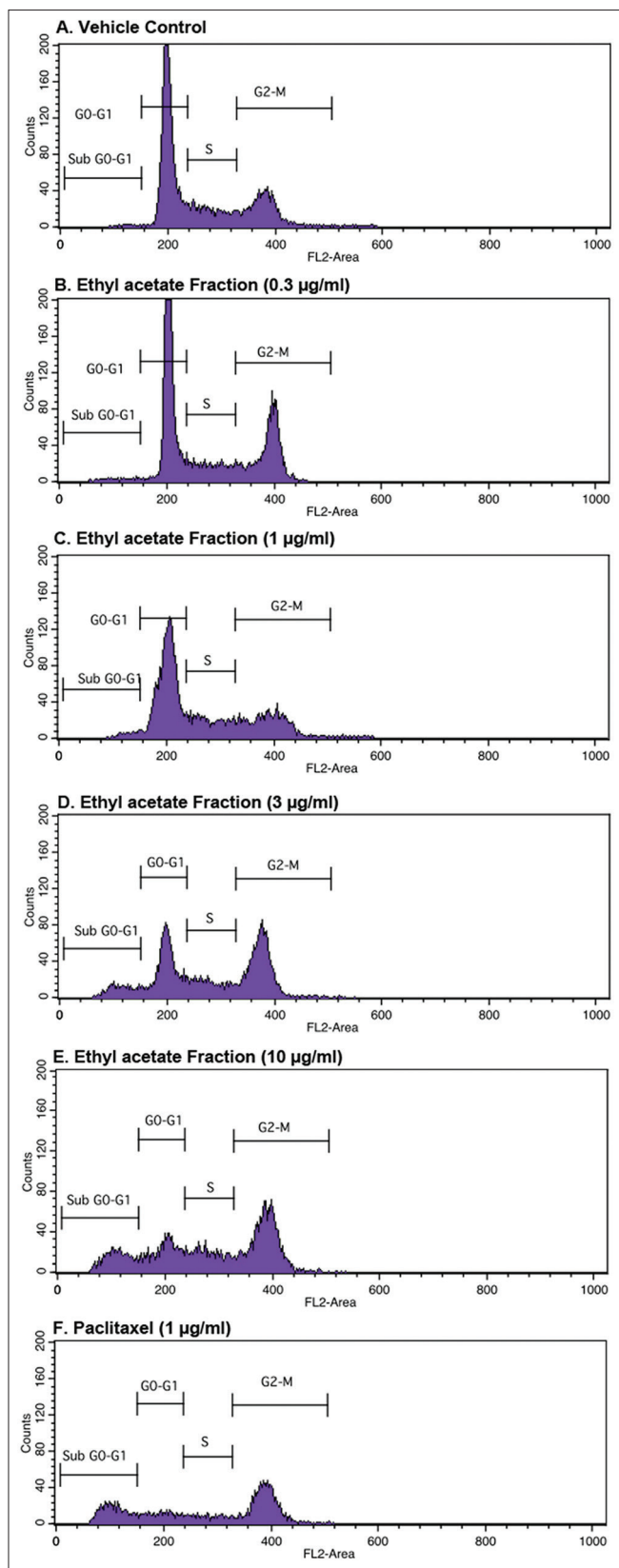


Figure 3: Analysis of cell cycle distribution after 48 h exposure of different concentration of ethyl acetate fraction in A673 cell line

the greatest sensitivity to EA fraction with a GI_{50} value of $0.27 \mu\text{g/ml}$ we also observe a trend of potency toward

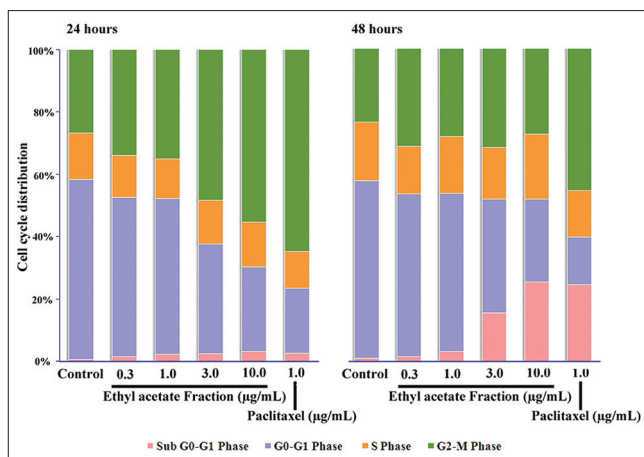


Figure 4: Flow cytometric analysis of cell cycle distribution on the A673 cells treated 24 and 48 h with different concentration of ethyl acetate fraction from *Hyattella cribriformis* in comparison with untreated control

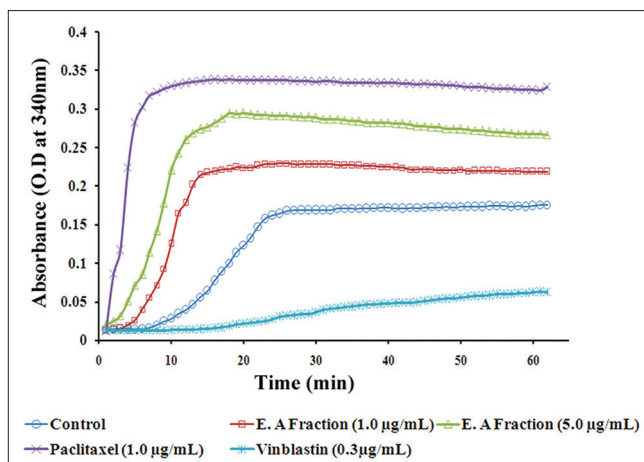


Figure 5: *In vitro* tubulin polymerization analysis ethyl acetate fraction altered the microtubule assembly by promoting the polymerization process in comparison to the control

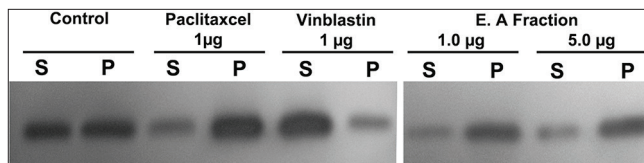


Figure 6: Analysis of cytosolic (S, soluble) and cytoskeletal tubulin (P, Polymerized tubulin) with ethyl acetate fraction exposed HeLa cells for 24 h using α -tubulin antibody in immunoblot

sarcoma cell lines including MG-63 ($0.72 \pm 0.12 \mu\text{g/ml}$), Saos-2 ($0.93 \pm 0.21 \mu\text{g/ml}$). Colon cancer cell lines including HT-29, HCT-116 and HCT-15 these cell line are accounted less potent compare to sarcoma and ovarian panel, similarly, EA fraction exhibited moderate inhibition profile in breast cancer cell lines MDA-MB-231, SK-BR-3 and MCF-7. This indicates that the EA fraction exhibited tissue specific anticancer activity and selectivity. According to American National Cancer Institute, the GI_{50} value to consider a crude extract promising for the development of

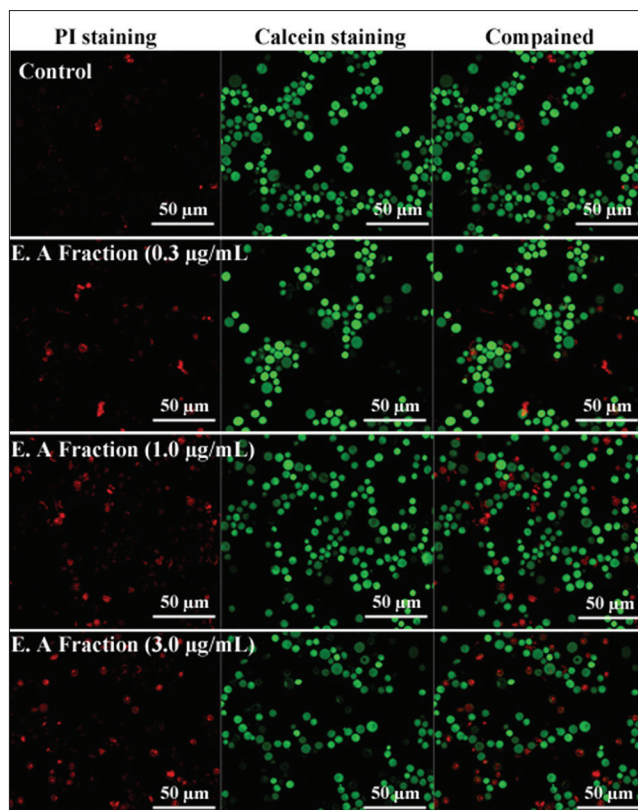


Figure 7: Live/Dead® cell staining of HeLa cells grown as coverslips culture with different concentration of ethyl acetate fraction for 48 h; Red staining indicates dead cells stained with propidium iodide; green indicates live cell stained with Calcein

anti-cancer drug (s) is lower than a limit threshold of 30 µg/ml.^[8] If one considers the values of GI_{50} , in the present study, it is found that the EA fraction of *H. cribriformis* has a strong anticancer effect on the most of the cell lines, except breast and colon cancer, which alone exhibited average GI_{50} values ~15 µg/ml in both cell line panel. Interestingly, EA fraction exhibited less GI against IMR-90 it proves that the fraction has a moderate effect on normal cells. To the best of author's knowledge, this is the first report on the *in vitro* evaluation of anticancer of EA fraction of *H. cribriformis*.

Cell cycle analysis revealed that the EA fraction exerted cell cycle arrest and apoptotic induction. EA fraction at all concentrations, irrespective of the duration, induced a significant growth arrest in G2/M phase, indicated by the massive increase in G2/M population. Earlier studies have shown that most of the mitotic arrest (G2/M phase) are due to microtubule-interfering mechanism and arrest at G2/M transition.^[22,23] We have further investigated towards analyzing the underlying mechanism of G2/M arrest and found that EA fraction is promoting the polymerization process of microtubule in a dose-dependent manner less potent compared to paclitaxel. It was also evidenced that small fragmented tubulin (Soluble form) levels are decreased in and polymerized forms are increased.

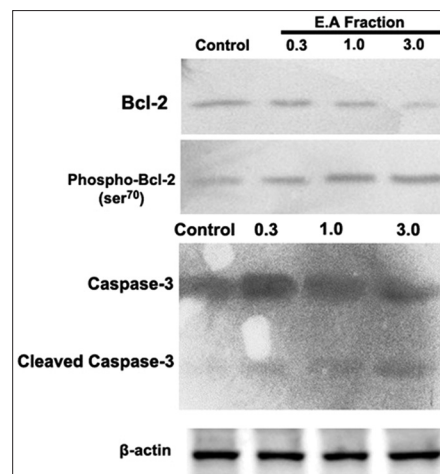


Figure 8: Immunoblot analysis of HeLa cells exposed to different concentration of ethyl acetate fraction exposed for 24 h for activation of caspase-3 and phosphorylation of Bcl-2 Ser70

Microtubule consists of polymers of α - and β -tubulin dimers, and there are drugs that either promote tubulin polymerization MSAs or promote depolymerization MDAs. Paclitaxel was the first MSA discovered and translated into the clinic as an anti-cancer drug, being approved by the U.S. FDA for the treatment of ovarian cancer in 1992.^[24] Marine sponges, however, remain the most prolific source of MTAs^[24-26] and include the MDAs jaspolid, dolastatin, halichondrin, spongistatin, and milnamide and the MSAs hemiasterlin, dictyostatin, discodermolide, laulimalide, peloruside A, and zampanolide.^[24] Since cancer is the result of uncontrolled cell division, it has long been recognized in the field of medical research that microtubule-interfering agents could prove valuable for cancer chemotherapy. In the present study, it is at present clear that EA fraction evidenced its effects through interference with microtubule depolymerization.

One of the goals of anticancer potential of any drug/extract is the induction of apoptosis in cancer cells. Apoptosis or programmed cell death is one of the most important targets for cancer treatment comprising chemotherapy as well as chemoprevention. Membrane blebbing, cytoplasmic condensation, formation of apoptotic bodies, DNA fragmentation, alteration in membrane symmetry, activation of the cascade of caspases, and loss of mitochondrial membrane potential characterize it.^[27] In the present study, an increase in hypo diploid sub-Go population at 48 h indicates the induction of apoptosis, as sub-Go peak is reported to be a quantitative indicator of apoptosis. The observation of apoptosis by flow cytometry was confirmed further investigations of Live/dead cell viability test. A two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell

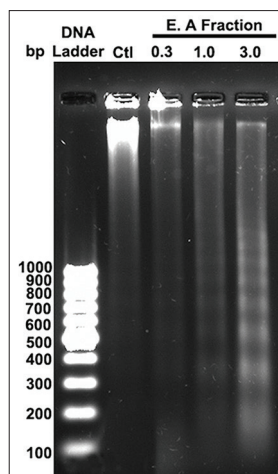


Figure 9: DNA fragmentation assay performed with different concentration of ethyl acetate fraction exposed HeLa cells and isolated DNA samples were analyzed using (2%) agarose gel electrophoresis

viability. The Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein. PI, a nucleic acid stain intercalates into double-stranded nucleic acids.^[28] We have observed a dose dependent increase in apoptotic cells with EA fraction exposed cells an clear evidence of anti-cancer effect.

Similarly, we have investigated the mechanism underlying the apoptosis through immunoblotting of protein markers. The caspase-3 protein is a member of the cysteine-aspartic acid protease family.^[29] Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Tubulin depolymerization induced cell death through activation of caspases causing the apoptosis through caspase-3 activation was recorded earlier.^[30] We have further evaluated the Bcl-2 phosphorylation levels and found that increased Ser70 phosphorylation with EA fraction treated cell lysate in dose-dependent manner. This hyperphosphorylation was observed in taxane-induced Bcl2 hyper-phosphorylation involves both serine and threonine residues at multiple sites majorly it was observed with Ser70.^[31,32] Bcl2 function to prolong cell survival was shown experimentally and demonstrated to be a potent suppressor of the process of programmed cell death, It has been discovered that phosphorylation of Bcl2 may regulate its function furthermore it is negative regulation of it is function that is induction of apoptosis.^[33] Literatures recorded that the anti-apoptotic function of Bcl-2 is inactivated by phosphorylation.^[34,35]

In this study, finally we have also evaluated the DNA fragmentation pattern of EA fraction exposed cells. We have observed a DNA fragmentation in agarose gel electrophoresis in the treated cells. DNA laddering can

be used as final state read-out method of apoptosis it one of the hallmark assay method to distinguish the apoptosis from necrosis.^[36] This DNA fragmentation pattern was observed with cells that exposed to microtubule targeted drugs.^[37]

CONCLUSION

The present study highlights the anticancer potential and anticancer mechanism of EA fraction of *H. cribriformis*. The overall findings revealed that EA fraction exerted a potent anti-proliferative action and GI in tested 14 cell lines. Sarcoma, ovarian and cervical cancer cell lines exhibited potent inhibition. Colon, breast, renal and epidermoid carcinoma cell line exhibited moderated anticancer activity. Interestingly, IMR-90 showed a moderate degree of inhibition compared to cancer cell lines. Flow cytometric analysis revealed that the 24 h and exposure of EA fraction to A673 cells resulted in G2-M phase arrest in cell cycle and it induces apoptosis at 48 h in a dose and time-dependent fashion. Potent G2/M arrest of EA fraction revealed a mechanism of promoting tubulin polymerization (Inhibition of depolymerization at Mitosis) in both *in vitro* and *in vivo* assay. Further investigation of G2/M arrest induced anticancer property resulted in induction of apoptosis in the tested cell lines. It was evidenced with dual fluorescence staining of EA exposed cells that culminated into dose-dependent cell death. Subsequently, we have analyzed the apoptosis markers including activation of caspase-3 and phosphorylation of Bcl-2 protein. Results revealed that induction of apoptosis through Bcl-2 inactivation and activation of caspase-3. Finally, we also have analyzed DNA fragmentation of EA fraction exposed cells. It accounted in DNA laddering as evidence of apoptosis. Further studies are required to isolate, characterize the active compound/compounds responsible for the anticancer effect.

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