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Cytotoxic activities of hexane, ethyl acetate and butanol extracts of marine sponges from Mauritian Waters on human cancer cell lines

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ABSTRACT

The ocean is an exceptional source of natural products with many of them exhibiting novel structural features and bioactivity. As one of the most interesting phylum with respect to pharmacological active marine compounds, Poriferas have been investigated widely in the last few decades. A total of 60 organic extracts (hexane, ethyl acetate and butanol) from 20 species of marine sponges from Mauritius were screened at 50 μg/ml in an *in vitro* screening assay against 9 human cancer cell lines. From these tested extracts, many exhibited pronounced cytotoxic effect at least in one of the cell lines and cell type cytotoxic specificity was observed. 27% of ethyl acetate, 11% of hexane and 2% of butanol extracts were found to possess a cytotoxicity ≥75% on 9 different cancer cell lines with the sponges *Petrosia* sp. 1, *Petrosia* sp. 2, *Pericharax heteroraphis* and *Jaspis* sp. being the most active. Overall, the HL-60 cells were much more sensitive to most of the extracts than the other cell lines. We further evaluated the properties of the ethyl acetate (JDE) and hexane extract (JDH) of one sponge, *Jaspis* sp. on KB cells. JDE displayed a smaller IC_{50} than JDH. Clonogenic assay confirmed the antiproliferative effect of both extracts while mitochondrial membrane potential change and microscopic analysis demonstrated extracts-induced apoptosis. Treatment with 100 ng/ml of JDE led to a significant increase of cells (24 h: 4.02%; 48 h: 26.23%) in sub-G1 phase. The cytotoxic properties of the tested extracts from these sponges suggest the presence of compounds with pharmacological potential and are currently undergoing fractionation to isolate the active constituents.

Abbreviations: NCI, National Cancer Institute; FDA, Food and Drug Administration; EMEA, European Medecines Agency; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); WSTs, Water soluble tetrazolium salts; XTT, (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide); DMEM, Dulbecco's minimum essential medium; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; EDTA, ethylenediaminetetra acetic acid ;DMSO,dimethylsulfoxide; Hela, cervical adenocarcinoma; HepG2, hepatocellular carcinoma; MCF7, breast adenocarcinoma; ATCC, American type culture collection; HL-60, acute promyelocytic leukemia; A549, lung carcinoma; HCT 116, colorectal carcinoma; Mia Paca-2, pancreatic carcinoma; HCT-15, colorectal adenocarcinoma; KB, epidermoid carcinoma; MRC-5, lung fibroblast; ANOVA, one way analysis of variance; HCT-8, colon cancer cells; B16, murine melanoma cancer cells; BE(2)-M17, neuroblastoma; NCG, National Coast guard; PBS, Phosphate buffered saline; JC-1, 5,5′ 6,6′-tetrachloro-1 1′ 3,3′ tetraethylbenzimidazolcarbocyanineiodide.

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1. Introduction

Marine organisms have shown to be potential sources of bioactive compounds with pharmaceutical interest (Munro et al., 1999; Faulkner, 2000) with sponges, bryozoans and tunicates being the most promising marine organisms as sources of new active compounds for drug development (Faulkner, 2001). Because of their prevalence, ease of collection and ability to biosynthesize an array of structurally diverse natural products, marine sponges have been the primary source of biologically active marine natural products (Belarbi et al., 2003; Blunt et al., 2007). The initial work of Bergmann on the sponge nucleosides, spongothymidine and spongouridine in the marine sponge *Cryptotethia crypta* (Ireland et al., 1993) played a major role in promoting the search for marine bioactive molecules. In the recent years, marine natural products bioprospecting has yielded a considerable number of drug candidates, most still being in preclinical or early clinical development, with only a limited number already in the market (Haefner, 2003). Among these, several anticancer agents derived from marine sources especially sponges have entered preclinical and clinical trials (Mayer et al., 2010; Andavan and Lemmens-Gruber, 2010; Miller et al., 2010) and have shown cytotoxic activity against various tumor types (da Rocha et al., 2001). Cancer, as one of the most important diseases in humans, has always attracted the scientific and commercial communities with an effort of continuously discovering new anticancer agents from natural products sources (Kinghorn et al., 2003). Even the US National Cancer Institute (NCI) has embarked on this avenue since the 50 s and has subsequently made significant contributions to the discovery of new naturally occurring anticancer agents (Cragg and Newman, 2005). The recent example is of eribulin mesylate, a derivative of halichondrin B isolated from the marine sponge *Halichondria okadai* that has achieved success in phase III clinical trials (Twelves et al., 2010) and has been recently approved by US Food and Drug Administration (FDA) and the European Medecines Agency (EMEA).

Several *in vitro* assay methodologies have been developed to study the effects of stimulatory or inhibitory agents in cancer research (Cook and Mitchell, 1989). A battery of several different cancer and normal cell lines can assess and determine the selectivity of a test substance. The need for rapid tests has led to the development of a variety of assays notably tetrazolium salts. MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by the cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. This formazan product is impermeable to cell membranes and thus accumulates in healthy cells. The validity of MTT assay has been tested on various cell lines (Mosmann, 1983) and over the years several modifications have been proposed to improve the repeatability and sensitivity of this assay (Denizot and Lang, 1986; Hansen et al., 1989). More recently, newer tetrazolium salts *e.g.* MTS, WSTs, XTT have been introduced that can pass back out the cell after reduction.

This study provides data on the cytotoxic potential of 60 organic extracts derived from 20 species of marine sponges using a combination of MTT/MTS assays on nine human cancer cell lines. Toxicity to a normal lung fibroblast (MRC-5) was studied as a control. Additionally, to validate our observation, we investigated the detailed cytotoxic properties of the ethyl acetate and hexane extract from a sponge, *Jaspis* sp. on KB cells. This study is a project of the Mauritius Oceanography Institute, Mauritius and has as aim the screening of a variety of biological activities of marine sponges with an attempt to improve the knowledge about the marine fauna and in finding new substances with potential pharmaceutical applications.

2. Materials and methods

2.1. Materials

Hexane, ethyl acetate and butanol were from SDFC Limited. DMEM, RPMI-1640, fetal bovine serum, amphotericin B, Trypsin-EDTA and L-glutamine were purchased from PAA Cell Culture. Ethoposide, campthothecin, dimethylsulfoxide (DMSO), sodium pyruvate, JC-1, MTT and gentamycin were from Sigma. Paclitaxel was from Enzo Biologicals. CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) was from Promega.

2.2. Species collection

Twenty species of sponges involved in this study were collected through SCUBA diving at depth varying from 5 to 40 m between 2004 and 2011. Samples were photographed *in situ* for better species characterization and identification. Voucher samples of each species were identified by Prof. Rob W.M. van Soest and deposited at the Zoological Museum of University of Amsterdam, The Netherlands .The species investigated in this study are detailed in Table 1.

2.3. Preparation of extracts

Freshly collected marine sponges were set free of any debris, cut into small pieces, weighted and freeze dried. Depending on the availability of the specimen, the dried sponge (49–400 g) was macerated with methanol and dichloromethane (1:1) for 24–48 h. After maceration, the solution was filtered and evaporated to dryness on a rotatory vacuum evaporator set at a maximum temperature of 40 °C. This constituted the crude extract, which was dissolved in distilled water to be partitioned subsequently with *n*-hexane, ethyl acetate and *n*-butanol to afford non-polar, semi-polar and polar fractions respectively. The extracts were weighed and stored at −20 °C until used.

2.4. Preparation of test materials and reference drug

Extracts were dissolved in DMSO (10 mg/ml) and further diluted in medium yielding a final testing concentration of 50 μg/ml with a final DMSO concentration of 0.5%. This concentration of DMSO did not affect cell viability. Positive control ethoposide (42 μg/ml), paclitaxel (50 μg/ml) and camptothecin (62.5 μg/ml) were prepared in DMSO.

Table 1 – Sponge species examined in this study.

Species	Family	Physical appearance
Petrosia sp. 1	Petrosiidae	Large, compact structure without defined shape, visible oscules.
Dragmacidon coceieum	Axinellidae	Thin sheet-like coating of the substrate with rough surface
Dragmacidon durrissima	Axinellidae	Thick sheet-like coating of the substrate with rough surface
Jaspis sp.	Coppatiidae	Very soft texture with prominent oscules
Plakortis nigra	Plakinidae	Very soft texture with small occules on the smooth surface
Petrosia sp. 2	Petrosiidae	Distinct tubes with oscules at the apex with rough surface
Dysidea aff. *cinerea*	Dysideidae	Soft and flexible texture with many flattened lobes projected from base
Iotrochota purpurea	Mycalidae	Thin, string like sponge with spiny surface
Rhabdastrella globostellata	Geodiidae	Massive, bitter gourd shape with prominent oscules
Biemna tuberosa	Desmacellidae	Large, compact specimen without a definite shape with smooth surface.
Acanthella pulcherrima	Axinellidae	Soft texture with large oscules, thickly encrusting
Axinella donnani	Axinellidae	Hard texture firmly attached to substrate with very small oscules
Acanthella cavernosa	Axinellidae	Massive and spherical with soft texture
Pericharax heteroraphis	Leucettidae	Solid free-standing sponge with a big opening at one end
Acanthostylotella sp.	Raspailiidae	Soft, thickly encrusting with randomly distributed oscules
Liosina paradoxa	Dictyonellidae	Body is fleshy, resilient and tough
Haliclona sp.	Chalinidae	Multiple clumps of hollow cylinders; smooth and delicate.
Dactylospongia sp.	Thorectidae	Thick, fleshly encrusting sponge
Spheciospongia sp.	Spirastrellidae	Medium size smooth sponge
Stylissa sp.	Dictyonellidae	Soft thickly encrusting with prominent oscules raise on short stalks

2.5. *Cell cultures*

Nine human cancer and one human normal cell lines were used for cytotoxicity assays: cervical adenocarcinoma (HeLa), hepatocellular carcinoma (HepG2) and breast adenocarcinoma (MCF7) cell lines were purchased from the American type culture collection (ATCC, Rockville, MD); acute promyelocytic leukemia (HL-60), lung carcinoma (A549), colorectal carcinoma (HCT 116), pancreatic carcinoma (Mia Paca-2), colorectal adenocarcinoma (HCT-15), epidermoid carcinoma (KB) and lung fibroblast (MRC-5) cells were a generous gift from Dr.Thierry Cresteil.HeLa, HL-60, A549, HCT 116, HCT-15 and MCF-7 were cultured in RPMI-1640 while MRC-5, KB, HepG2 and Mia Paca-2 cells were grown in D-MEM, both medium being supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, amphotericin B (10 ml/l) and 0.1% gentamycin with the medium for MCF-7 having an additional supplementation of 1 mM sodium pyruvate. Cells were grown in a humidified incubator with 5% CO_2 at 37 °C and upon reaching 80% confluence were passaged with a solution of 0.25% trypsin-EDTA.

2.6. *Cytotoxicity screening*

Cytotoxicity was determined as previously described with modifications (Mosmann, 1983). Adherent cells (HeLa, A549, HCT 116, HCT-15, MRC-5, MCF-7, KB, Mia Paca-2 and HepG2) were plated at 1×10^4 cells/well and allowed to incubate for 24 h at 37 °C. After this incubation period, cells were treated with extracts (hexane, ethyl acetate and butanol) and incubated for 48 h. Control groups received DMSO. After treatment, the old medium was replaced with 200 μl of fresh medium, containing MTT (5 mg/ml) and plates were further incubated for 4 h at 37 °C. The formazan was dissolved in 100 μl DMSO and the absorbance was measured using a microplate reader at 560 nm and subtracted at 670 nm. Cytotoxicity was determined using [{1 − (OD treated/OD control)} × 100]. For MTS assay, 1×10^4 HL-60 cells/well were plated in 96 well plates. After 24 h, 100 μl medium containing extracts was added to the wells (final volume = 200 μl) and further incubated for 72 h. Control groups received DMSO. At the end of incubation period, 20 μl of MTS was added and kept for incubation for 4 h at 37 °C. Absorbance was read at 490 nm, subtracted at 670 nm and cytotoxicity was determined taking the absorbance of the blank (no cells) into consideration. Cytotoxicity of etoposide, paclitaxel and camptothecin were determined as positive control using either MTT or MTS depending on the cell line.

2.7. *IC_{50} determination*

KB cells were plated in 96-well tissue culture microplates at a density of 650 cells/well in 200 μl medium and treated 24 h later with extracts (JDH and JDE) dissolved in DMSO using a Biomek 3000 automation workstation (Beckman-Coulter). Controls received the same volume of DMSO (1% final volume). After 72 h exposure MTS reagent (Celltiter 96 Aqueous One solution, Promega) was added and incubated for 3 h at 37 °C. Absorbance was monitored at 490 nm, subtracted at 670 nm and results expressed as the inhibition of cell proliferation calculated as the ratio [(1 − (OD490 treated/OD490 control) × 100]. For IC50 determinations (50% inhibition of cell proliferation) experiments were performed with compound concentrations ranging from 0.001 μg/ml to 10 μg/ml in duplicate.

2.8. *Clonogenic survival determination*

The cells were seeded in a 12-well plate (300/well) and incubated overnight. Cells were then exposed for 24 h to different concentrations of JDE and JDH (0.05–50 μg/ml) after which medium was aspirated and cells were rinsed with PBS.Fresh medium was added to each well and plate was incubated at 37 °C. After 7 days of incubation, the cells were stained with Giemsa stain and colonies with >50 cells were counted under a dissection microscope. Experiments were conducted in triplicate. The plating efficiency of the cells was taken into consideration when calculating the surviving fraction.

2.9. Cell observation using an inverted microscope

1×10^4 KB cells were grown in 6-well plates and treated with both sponge extracts. The cells were then washed with 1X PBS. Morphological changes in cells, in both the treated group (10 μg/ml of extract treated cells) and control group (1.0% DMSO treated-cells) were observed using an inverted microscope (Leica DM IL).

2.10. Measurement of mitochondrial transmembrane potential

We used the mitochondrial-specific cationic dye (JC-1) that undergoes potential-dependent accumulation in the mitochondria. 1×10^4 cells were seeded in a 96-well plate and treated with 1, 10 and 50 μg/ml of JDH and JDE for various time points. After treatment, cells were washed and stained with 100 μl JC-1 for 30 min at 37 °C. Fluorescence was monitored with the fluorescence plate reader (Synergy HT, BioTek USA) at wavelengths of 485 nm (excitation)/528 nm (emission) and 540 nm (excitation)/590 nm (emission) pairs. Changes in the ratio between the measurements at test wavelengths of 590 nm (red) and 528 nm (green) fluorescence intensities are indicative of changes in the mitochondrial membrane potential. Experiment was performed in triplicate.

2.11. Cell cycle analysis

KB cells (25,000 cells/well in 96-well microplates) were exposed for 24 h and 48 h at 37 °C under 5% CO_2 to extracts (JDH and JDE) in 100 μl complete RPMI-1640 medium. Controls received the same volume of DMSO (1% final volume). Culture media were carefully collected and gently centrifuged to collect floating cells, adherent cells harvested after addition of trypsin, mixed with the pellet of floating cells, washed with PBS and fixed in ice-cold absolute ethanol. After 2 h at 4 °C, cells were spun down by centrifugation, washed with 2% FBS in PBS and stained with 50 μg/ml propidium iodide in hypotonic buffer in the presence of RNase A (50 μg/ml), incubated at dark for 30 min before being analyzed by flow cytometry with a Guava Easycyte cytometer (Millipore). Cell populations were quantified using Modfit LT (Verity Software House).

2.12. Statistical analysis

Results were expressed as means ± SD of replicates ($n = 3$). Comparison between data sets was performed using one way analysis of variance (ANOVA) followed by Tukey test. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Differences were accepted as statistically significant at $p < 0.05$.

3. Results

3.1. Cytotoxicity screening

Screening of crude extracts is the preliminary step in the study of cytotoxicity of marine sponges since it reveals which species has the ability of producing and accumulating bioactive molecules. Tables 2–4 shows the results of the *in vitro* testing of the extracts against each cell line. This is the first step in our anticancer drug-screening project and is designed to identify potent extracts with a cytotoxicity ≥75% at 50 μg/ml. MTT/MTS assay revealed considerable variability with 27% (45/180) of ethyl acetate, 11% (19/180) of hexane and 2% (3/180) of butanol extracts having a cytotoxicity ≥75% on 9 different cancer cell lines. Ethyl acetate extracts from 14 sponge species (*Petrosia* sp.1, *Petrosia* sp. 2, *Dragmacidon coceieum*, *Dragmacidon durrissima*, *Jaspis* sp., *Dysidea* aff. *cinerea*, *Rhabdastrella globostellata*, *Biemna tuberosa*, *Acantella pulcherrima*, *Acantella cavernosa*, *Pericharax heteroraphis*, *Haliclona* sp., *Dactylospongia* sp., *Spheciospongia* sp.) exhibited a cytotoxicity ≥75% in atleast one cancerous cell line with the sponges *Petrosia* sp. 2 and *Pericharax heteroraphis* showing broad activity on 7 out of 9 cancer cell lines (Table 2). In the case of hexane extracts, only 9 sponges (*Petrosia* sp. 1, *Petrosia* sp. 2, *Jaspis* sp., *Plakortis nigra*, *Dysidea* aff. *cinerea*, *Acanthella pulcherrima*, *Acanthella cavernosa*, *Pericharax heteroraphis*, *Liosina paradoxa*) demonstrated a potent cytotoxicity in at least one cancer cell line (Table 3). For butanol extracts, only sponges *Biemna tuberosa* and *Axinella donnani* demonstrated potent activity. Overall, the HL-60 cells were much more sensitive to the cytotoxic effects of the organic extracts than the other cell lines. It is of note that, at a testing concentration of 42–62.5 μg/ml and exposure time of 48–72 h, the positive controls (paclitaxel, etoposide and camptothecin) showed a cytotoxicity 75% in most of the cell lines, with a few exceptions (Table 5).

3.2. Inhibitory effect of extracts on proliferation and morphology of KB cells

IC_{50} was determined using different concentrations (0.001–10 μg/ml) of JDE and JDH extracts. A 72 h exposure to both extracts dramatically decreased the proliferation of KB cells in a concentration dependent manner. The concentration require to inhibit growth by 50% (IC_{50}) was 0.0018 μg/ml for JDE and 1.46 μg/ml for JDH. The results of colony formation inhibition of JDE and JDH are presented in Fig. 1. Control cells showed a surviving fraction of 80 ± 15% whereas with increasing JDE and JDH concentration the survival capacity of KB cells decreased. Thus a dose-dependent colony-forming inhibition effect was observed. To visualize the morphological changes in KB cells, cells treated with 10 μg/ml extracts. Rapid morphological changes were observed within 6 h of addition of extracts (Fig. 2). At this concentration cells exhibited the characteristic morphological changes of apoptosis including membrane blebbing and formation of apoptotic bodies. The control cells were normal with round and homogeneous nuclei.

3.3. Effect of JDE on the mitochondrial apoptotic pathway in KB cells

The collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) is an indication of an irreversible checkpoint in apoptosis. To investigate the mitochondrial apoptotic events involved in JDE and JDH induced apoptosis, we analyzed the mitochondrial membrane potential using JC-1. As shown in Fig. 3, the $\Delta\Psi_m$ of

Table 2 – Percentage cytotoxicity of cancer and normal cell lines treated with ethyl acetate extract from a total of 20 sponge species at the concentration of 50 μg/ml.

Sponge	HL 60	Hela	A549	MRC5	HCT 116	HCT 15	KB	MCF 7	Mia Paca	HepG2
Petrosia sp. 1	99 ± 14	83 ± 0	67 ± 1	95 ± 1	81 ± 1	83 ± 2	82 ± 2	−6 ± 12	87 ± 1	70 ± 2
Dragmacidon coceieum	100 ± 19	83 ± 1	50 ± 1	91 ± 1	64 ± 3	68 ± 3	72 ± 3	−5 ± 6	83 ± 1	63 ± 2
Dragmacidon durrissima	72 ± 7	81 ± 1	36 ± 1	55 ± 8	52 ± 1	66 ± 4	67 ± 8	9 ± 11	72 ± 1	53 ± 4
Jaspis sp.	100 ± 7	81 ± 1	63 ± 1	70 ± 4	66 ± 8	60 ± 5	78 ± 3	37 ± 2	81 ± 4	61 ± 3
Plakortis nigra	3 ± 8	54 ± 4	35 ± 8	6 ± 6	20 ± 8	24 ± 24	37 ± 5	−4 ± 10	11 ± 19	3 ± 9
Petrosia sp 2	100 ± 12	84 ± 1	33 ± 9	89 ± 2	77 ± 3	80 ± 3	80 ± 2	27 ± 2	87 ± 1	79 ± 3
Dysidea aff. *cinerea*	100 ± 12	87 ± 3	26 ± 7	43 ± 7	58 ± 5	68 ± 3	36 ± 13	10 ± 11	64 ± 2	25 ± 3
Iotrochota purpurea	32 ± 4	25 ± 4	16 ± 25	25 ± 7	27 ± 4	23 ± 13	−1 ± 24	0 ± 5	40 ± 18	19 ± 7
Rhabdastrella globostellata	100 ± 15	85 ± 3	17 ± 26	85 ± 3	74 ± 7	78 ± 2	66 ± 3	49 ± 2	87 ± 1	75 ± 4
Biemna tuberosa	98 ± 11	88 ± 1	10 ± 10	73 ± 6	4 ± 12	51 ± 4	72 ± 3	24 ± 17	61 ± 4	49 ± 12
Acanthella pulcherrima	100 ± 6	84 ± 2	53 ± 4	87 ± 3	35 ± 14	56 ± 2	72 ± 6	14 ± 5	74 ± 1	56 ± 1
Axinella donnani	61 ± 9	72 ± 2	38 ± 14	53 ± 5	40 ± 3	43 ± 8	52 ± 1	24 ± 7	64 ± 1	43 ± 3
Acanthella cavernosa	100 ± 10	91 ± 1	43 ± 3	84 ± 1	60 ± 2	40 ± 5	72 ± 3	30 ± 4	81 ± 1	36 ± 8
Pericharax heteroraphis	98 ± 10	86 ± 1	82 ± 1	93 ± 3	77 ± 1	77 ± 1	95 ± 1	50 ± 5	84 ± 2	66 ± 3
Acanthostylotella sp.	−41 ± 8	−20 ± 7	−7 ± 21	6 ± 15	17 ± 3	16 ± 15	−16 ± 30	9 ± 3	51 ± 3	44 ± 6
Liosina paradoxa	71 ± 9	51 ± 10	42 ± 3	27 ± 2	46 ± 6	52 ± 14	55 ± 5	34 ± 7	65 ± 6	67 ± 4
Haliclona sp.	52 ± 25	68 ± 3	51 ± 8	87 ± 13	65 ± 3	77 ± 3	85 ± 1	29 ± 1	74 ± 4	49 ± 10
Dactylospongia sp.	88 ± 6	43 ± 8	17 ± 9	18 ± 13	15 ± 30	46 ± 15	46 ± 9	−2 ± 7	69 ± 5	40 ± 1
Spheciospongia sp.	83 ± 9	13 ± 3	44 ± 13	51 ± 2	30 ± 16	24 ± 3	17 ± 9	23 ± 24	68 ± 14	63 ± 2
Stylissa sp.	63 ± 2	−32 ± 16	34 ± 8	51 ± 11	48 ± 15	49 ± 8	0 ± 15	22 ± 5	66 ± 3	55 ± 8

Table 3 – Percentage cytotoxicity of cancer and normal cell lines treated with hexane extract from a total of 20 sponge species at the concentration of 50 μg/ml.

Sponge	HL 60	Hela	A549	MRC5	HCT 116	HCT 15	KB	MCF 7	Mia Paca	HepG2
Petrosia sp. 1	22 ± 2	80 ± 3	31 ± 20	74 ± 5	33 ± 4	8 ± 13	76 ± 2	−6 ± 6	63 ± 5	21 ± 5
Dragmacidon coceieum	−18 ± 11	21 ± 7	3 ± 3	15 ± 7	40 ± 10	33 ± 16	22 ± 15	−2 ± 18	16 ± 17	−27 ± 9
Dragmacidon durrissima	0 ± 14	18 ± 10	19 ± 14	52 ± 13	31 ± 9	37 ± 5	21 ± 16	−14 ± 3	16 ± 4	−25 ± 31
Jaspis sp.	100 ± 1	83 ± 0	56 ± 3	90 ± 1	66 ± 3	54 ± 7	81 ± 4	42 ± 2	81 ± 5	52 ± 6
Plakortis nigra	94 ± 2	69 ± 9	35 ± 16	33 ± 3	38 ± 10	63 ± 8	68 ± 8	30 ± 4	54 ± 6	54 ± 9
Petrosia sp. 2	−18 ± 45	76 ± 2	31 ± 10	27 ± 16	31 ± 12	11 ± 15	52 ± 5	−10 ± 14	43 ± 4	9 ± 19
Dysidea aff. *cinerea*	28 ± 13	92 ± 2	28 ± 7	49 ± 11	52 ± 9	6 ± 22	79 ± 2	1 ± 18	58 ± 6	31 ± 8
Iotrochota purpurea	−36 ± 36	−6 ± 5	21 ± 24	31 ± 49	40 ± 10	23 ± 15	21 ± 9	−14 ± 26	34 ± 17	1 ± 17
Rhabdastrella globostellata	38 ± 41	8 ± 13	−14 ± 18	−71 ± 35	44 ± 1	36 ± 8	19 ± 5	17 ± 13	65 ± 3	63 ± 8
Biemna tuberosa	71 ± 2	−21 ± 8	10 ± 11	3 ± 21	9 ± 19	21 ± 16	38 ± 7	5 ± 4	53 ± 12	16 ± 4
Acanthella pulcherrima	74 ± 64	6 ± 13	30 ± 5	74 ± 7	28 ± 8	75 ± 1	82 ± 3	1 ± 1	62 ± 9	33 ± 8
Axinella donnani	55 ± 21	41 ± 6	17 ± 8	39 ± 5	26 ± 14	54 ± 2	29 ± 18	24 ± 11	69 ± 5	51 ± 6
Acanthella cavernosa	85 ± 6	92 ± 1	22 ± 12	44 ± 19	57 ± 4	81 ± 1	68 ± 2	34 ± 2	81 ± 3	44 ± 8
Pericharax heteroraphis	100 ± 2	29 ± 3	57 ± 9	33 ± 3	38 ± 10	74 ± 1	6 ± 20	33 ± 3	52 ± 1	50 ± 15
Acanthostylotella sp.	−44 ± 68	19 ± 11	3 ± 7	− 31 ± 3	17 ± 6	−16 ± 11	−3 ± 26	15 ± 13	38 ± 7	29 ± 14
Liosina paradoxa	80 ± 2	61 ± 12	17 ± 2	19 ± 7	32 ± 6	48 ± 1	46 ± 10	24 ± 9	74 ± 8	64 ± 1
Haliclona sp.	58 ± 8	65 ± 4	44 ± 8	79 ± 3	68 ± 2	29 ± 21	58 ± 6	6 ± 14	72 ± 2	67 ± 3
Dactylospongia sp.	36 ± 24	39 ± 13	22 ± 9	56 ± 4	44 ± 13	34 ± 16	44 ± 10	−3 ± 14	73 ± 3	21 ± 8
Spheciospongia sp.	62 ± 9	1 ± 14	38 ± 2	51 ± 10	13 ± 16	7 ± 6	13 ± 6	3 ± 24	54 ± 12	56 ± 5
Stylissa sp.	56 ± 8	−10 ± 1	10 ± 6	16 ± 9	−2 ± 13	31 ± 8	−55 ± 31	3 ± 17	57 ± 6	20 ± 6

Table 4 – Percentage cytotoxicity of cancer and normal cell lines treated with butanol extract from a total of 20 sponge species at the concentration of 50 μg/ml.

Sponge	HL 60	Hela	A549	MRC5	HCT 116	HCT 15	KB	MCF 7	Mia Paca	HepG2
Petrosia sp 1	−51 ± 6	46 ± 6	14 ± 6	28 ± 12	48 ± 3	16 ± 20	17 ± 11	−6 ± 15	61 ± 5	0 ± 29
Dragmacidon coceieum	−2 ± 20	20 ± 17	−18 ± 42	−17 ± 13	16 ± 13	4 ± 6	6 ± 27	18 ± 8	28 ± 5	29 ± 5
Dragmacidon durrissima	−44 ± 40	29 ± 16	45 ± 3	31 ± 6	34 ± 8	−7 ± 14	24 ± 9	−1 ± 11	11 ± 12	3 ± 15
Jaspis sp.	−2 ± 20	−20 ± 13	−2 ± 7	1 ± 10	6 ± 6	34 ± 9	65 ± 4	11 ± 20	30 ± 3	17 ± 13
Plakortis nigra	−24 ± 21	20 ± 16	28 ± 3	− 27 ± 42	10 ± 11	23 ± 4	−3 ± 12	15 ± 5	0 ± 22	54 ± 12
Petrosia sp. 2	−51 ± 18	12 ± 7	43 ± 9	27 ± 13	41 ± 4	−21 ± 16	20 ± 10	6 ± 5	−2 ± 22	10 ± 9
Dysidea aff. *cinerea*	−59 ± 45	−33 ± 2	−1 ± 3	−18 ± 11	0 ± 3	3 ± 11	8 ± 16	19 ± 3	43 ± 12	20 ± 10
Iotrochota purpurea	12 ± 6	18 ± 11	16 ± 15	44 ± 14	26 ± 9	−2 ± 26	25 ± 5	−4 ± 12	37 ± 7	−11 ± 54
Rhabdastrella globostellata	5 ± 11	−13 ± 14	−20 ± 32	−3 ± 18	40 ± 5	38 ± 6	30 ± 7	8 ± 9	33 ± 5	40 ± 7
Biemna tuberosa	100 ± 23	6 ± 11	6 ± 10	31 ± 10	26 ± 8	10 ± 3	32 ± 7	−20 ± 18	15 ± 9	7 ± 3
Acanthella pulcherrima	51 ± 16	21 ± 13	0 ± 3	3 ± 20	15 ± 12	41 ± 8	14 ± 12	15 ± 5	43 ± 16	14 ± 13
Axinella donnani	78 ± 8	84 ± 9	26 ± 8	70 ± 1	46 ± 10	8 ± 18	67 ± 1	11 ± 8	71 ± 3	46 ± 7
Acanthella cavernosa	−1 ± 31	13 ± 1	0 ± 15	−9 ± 7	10 ± 42	3 ± 22	5 ± 10	22 ± 4	13 ± 11	24 ± 7
Pericharax heteroraphis	−99 ± 62	43 ± 12	4 ± 12	−49 ± 15	3 ± 3	21 ± 29	−7 ± 31	2 ± 4	26 ± 5	30 ± 17
Acanthostyllotela sp.	−25 ± 11	22 ± 13	49 ± 16	47 ± 5	44 ± 12	3 ± 26	−10 ± 2	−34 ± 16	53 ± 3	14 ± 8
Liosina paradoxa	−35 ± 31	24 ± 22	−17 ± 34	−48 ± 25	13 ± 19	2 ± 21	29 ± 4	13 ± 1	−4 ± 36	36 ± 10
Haliclona sp.	−29 ± 52	33 ± 5	6 ± 16	11 ± 11	21 ± 14	21 ± 6	35 ± 9	3 ± 3	34 ± 5	48 ± 14
Dactylospongia sp.	61 ± 6	44 ± 4	2 ± 16	5 ± 19	51 ± 16	52 ± 2	27 ± 3	0 ± 30	44 ± 6	51 ± 7
Spheciospongia sp.	−26 ± 5	−54 ± 22	25 ± 22	−44 ± 18	12 ± 11	12 ± 16	30 ± 3	9 ± 10	30 ± 23	38 ± 18
Stylissa sp.	59 ± 4	2 ± 12	15 ± 16	16 ± 15	12 ± 21	40 ± 2	43 ± 7	5 ± 31	31 ± 18	53 ± 4

Table 5 – Percentage cytotoxicity of paclitaxel, etoposide and camptothecin.

	μg/ml	HL 60	HeLa	A549	MRC5	HCT 116	HCT 15	KB	MCF 7	Mia Paca	HepG2
Paclitaxel	50	18 ± 5	75 ± 3	18 ± 6	51 ± 11	46 ± 1	51 ± 11	65 ± 13	4 ± 7	ND	63 ± 1
Etoposide	42	98 ± 1	58 ± 5	24 ± 1	30 ± 17	54 ± 0	30 ± 17	65 ± 13	23 ± 15	ND	42 ± 1
Camptothecin	62.5	100 ± 3	74 ± 1	12 ± 6	65 ± 5	55 ± 1	78 ± 5	58 ± 1	30 ± 1	ND	61 ± 6

ND: Not determined.

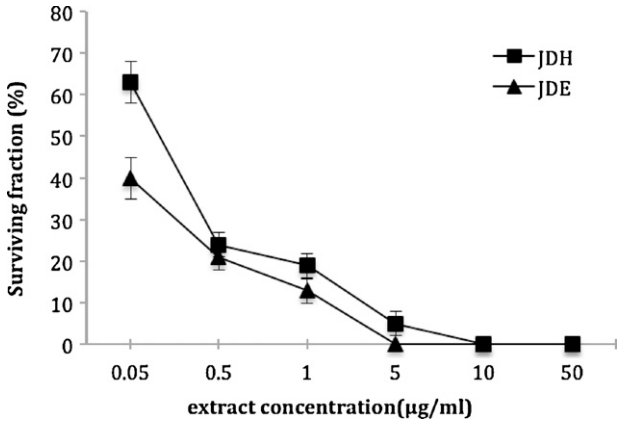

Fig. 1 – Clonogenic survival determination. Cells were exposed to extracts, washed with PBS and allowed to grow for 7 days. Colonies with >50 cells were counted. The plating efficiency of the cells was taken into consideration when calculation the surviving fraction.

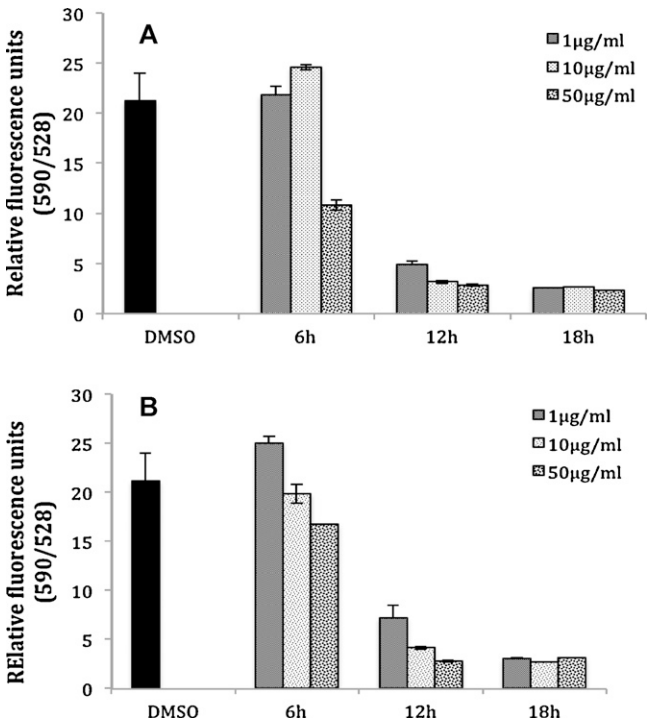

Fig. 3 – Mitochondrial membrane potential change demonstrated by extracts of *Jaspis* sp ((A) JDE; (B) JDH). KB cells were incubation for 6, 12 and 18 h with 11,050 μg/ml extracts. Data presented as means ± SD ($n = 3$). Vertical bars represent standard error.

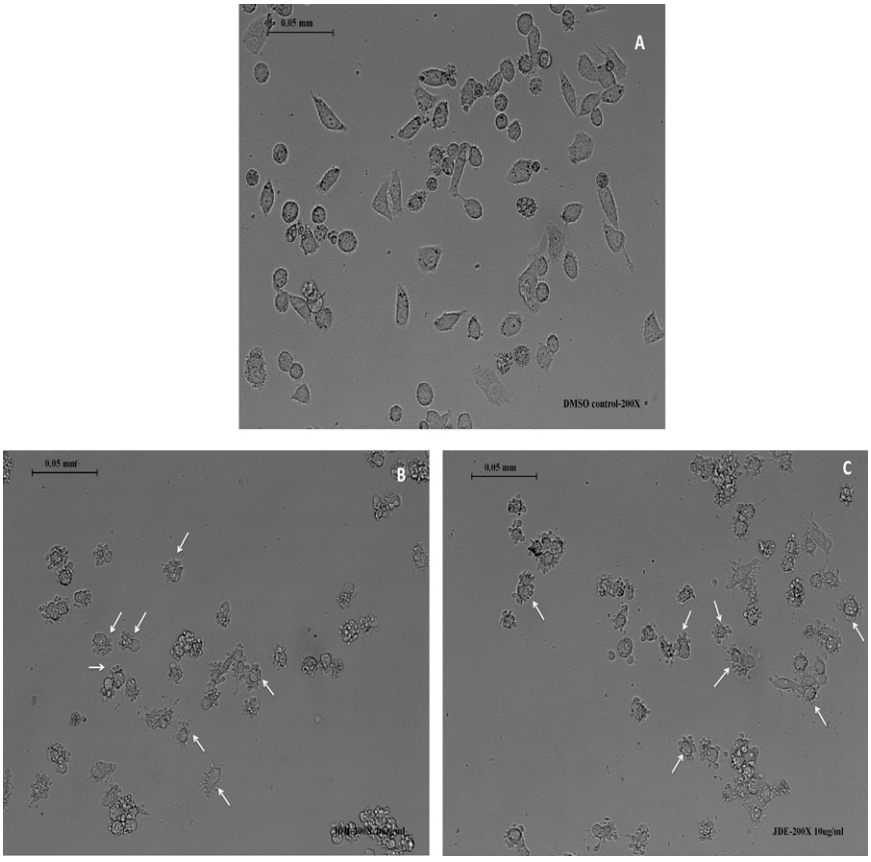

Fig. 2 – Morphological studies by inverted microscope at actual magnification 200×. Photographs of KB cells incubated with 10 μg/ml of JDE and JDH for 6 h. (A) control culture (DMSO); (B) JDH; (C) JDE. Apoptotic bodies indicated by arrows.

JDE and JDH treated KB cells showed a progressive decrease up to 18 h. 50 μg/ml JDE caused a sudden decrease in $\Delta\Psi_m$ within the first 6 h compared to JDH. Control cells indicate $\Delta\Psi_m$ after 6 h of exposure to DMSO.

3.4. Cell cycle analysis

To examine the mechanism of inhibitory effect of JDE on KB cell proliferation, cell cycle distribution was evaluated using flow cytometry as demonstrated in Fig. 4. Table 6 details the percentage of cells in each phase in control (DMSO) and treated cells at 24 h and 48 h. The sub-G1 population indicated apoptotic-associated chromatin degradation. As compared to control, we noticed a significant increase in the sub-G1 group after cells were treated with 100 ng/ml of JDE (24 h: 4.02%; 48 h: 26.23%) whereas treatment with 10 μg/ml JDH resulted in a moderate increase JDE (24 h: 5.03%; 48 h: 16.54). These results suggest that JDE can induce more apoptosis in KB cells at a much lower concentration compared to JDH during the same incubation period.

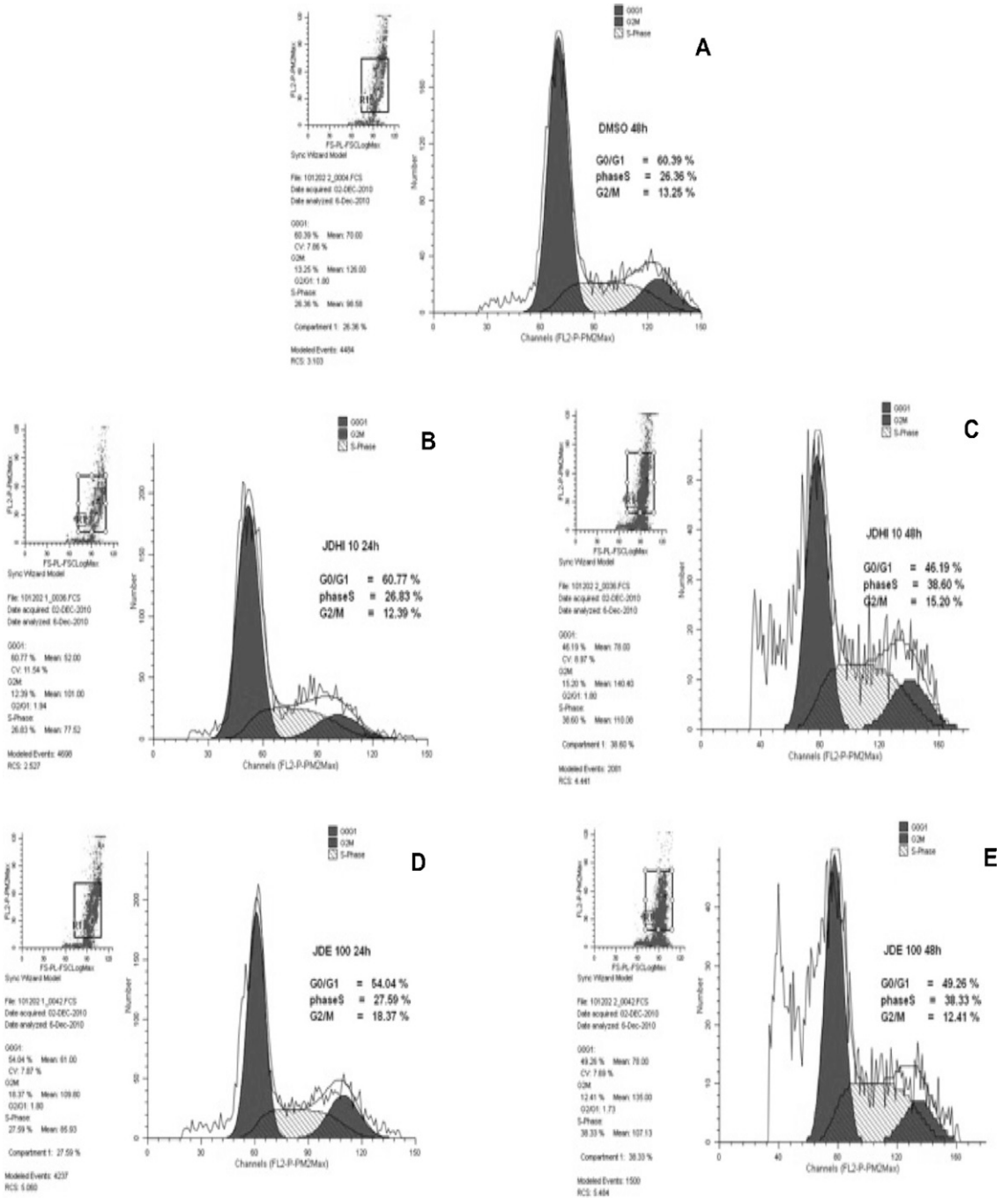

Fig. 4 – Cell cycle analysis of KB cells. Cells were cultured with 1% DMSO (A, control); 10 μg/ml of JDH for 24 h (B); for 48 h (C) and 100 ng/ml of JDE for 24 h (D); for 48 h (E). The percentage of non-apoptotic cells within each cell cycle was determined by flow cytometry. Compared to the control (A), JDE could induce apoptosis (increasing sub-G1 population).

Table 6 – Measurement of Annexin-V-PE/7-AAD staining. KB cells were stained with Annexin V-7-AAD and subsequently analyzed by flow cytometry.

		DMSO	JDH			JDE		
			1 μg/ml	5 μg/ml	10 μg/ml	5 ng/ml	10 ng/ml	100 ng/ml
24 h	G0/G1	57.87	53.52	55.85	58.46	51.50	54.17	53.96
	S Phase	27.90	30.37	26.37	24.52	32.20	26.90	24.62
	G2/M	10.50	11.91	13.73	11.98	13.12	15.11	17.41
	SubG1	3.74	4.20	4.06	5.03	3.18	3.82	4.02
48 h	G0/G1	56.24	53.75	54.57	40.42	53.10	57.61	36.12
	S Phase	26.23	27.29	25.10	31.20	24.37	23.53	28.72
	G2/M	12.60	13.10	13.45	11.83	17.10	13.21	8.93
	SubG1	4.93	5.86	6.89	16.54	5.44	5.64	26.23

4. Discussion

There are increasing evidences that suggest that the marine environment contain different classes of biologically active compounds with strong anticancer properties (Mayer and Gustafson, 2006), in particular marine sponges from which several potent cytotoxic compounds, including alkaloids, steroids, terpenes, peptides, macrolides and polyketides have been isolated. Although the sponges possess relatively few defenses, yet most of them demonstrate an ability to deter predators and inhibit formation of biofilms. The impetus for initiating our own bioprospecting project came from the initial work of Pettit et al. (1997) when a series of cytotoxic peptides including dolastatin 10 were isolated from the shell less mollusc *Dolabella auricularia*, collected from the eastern coast of Mauritius. Dolastatin 10 demonstrated interesting inhibitory activities against various human cancer cell lines and progressed till Phase II clinical trials (Molinski et al., 2009). Wah et al. (2006) described for the first time the anticholinesterase and antibacterial activity of thirty-seven samples of soft corals and sponges collected from Mauritian Waters and justified the need for marine natural product research. Several screening investigations have demonstrated that crude extracts from marine sponges display potent cytotoxic (Rangel et al., 2001; Monks et al., 2002; Becerro et al., 2003; Prado et al., 2004; Xue et al., 2004; Ferreira et al., 2007; Pedpradab et al., 2010).

However, only a limited number of sponge samples and extracts were screened in these studies. Therefore, it is of interest to screen a large number of sponge extracts, in order to verify the percent of active extracts and the relatedness with the taxonomy of the sponges, which yielded the most active crude extracts. One such study (Seleghim et al., 2007) screened 215 Brazilian sponge methanolic extracts, out which, 11% displayed cytotoxic activity against breast cancer cells (MCF-7), 18% against colon cancer cells (HCT-8) and 8% against murine melanoma cancer cells (B16). Recently, Ferreira et al. (2011) described the cytotoxic activities of extracts of six species of marine sponges from Spain whereby several apoptotic markers were evaluated on neuroblastoma BE (2)-M17 cell line. The present study evaluated the cytotoxic effect of 60 extracts derived from 20 sponge species on a panel of nine human cancer cell lines (KB, MCF-7, HeLa, A549, HCT15, HCT116, Mia Paca-2, HepG2, HL-60). Different cell lines exhibit different sensitivities towards cytotoxic compounds, therefore the use of more than one cell line is considered necessary in the detection of cytotoxic compounds, justifying the use of 9 cell lines panel of 8 different histological origins in the present study. Several cell-based assays that quantify cell death associated phenomena are routinely used in drug development and it is also important for such tests to reveal the role of biochemical cascades along with identification of the molecular targets.

As seen from Tables 2–4, cell type cytotoxic specificity is observed. This specificity is likely due to the presence of different classes of compounds in the fractions (Cragg et al., 1994). While the US National Cancer Institute (NCI) 60 human tumour cell line anticancer drug screening program (NCI60) considers an extract to have *in vitro* cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48 and 72 h, is less than 20 μg/ml (Boik, 2001), we chose to screen the sponge extracts for ≥75% at 50 μg/ml, as IC_{50} determination would require investing a higher amount of sponge fractions and this can be a limiting factor when the sponge extract is available in small quantity. The most potent sponges were found to be *Rhabdastrella globostellata*, *Jaspis* sp., *Plakortis nigra*, *Dragmacidon coceieum*, *Dragmacidon durrissima*, *Acanthella pulcherrima*, *Acanthella cavernosa*, *Pericharax heteroraphis*, *Petrosia* sp. 1, *Petrosia* sp. 2 and *Dysidea* aff. *cinerea*.

The sponge *Rhabdastrella globostellata* is known to possess several molecules like Rhabdastrellic acid-A, stellettin B and E which can inhibit growth of cancer cell line HCT-116 (Rao et al., 1997; Tasdemir et al., 2002). New cytotoxic isomalabaricane-type sesterterpenes have also been isolated from the same sponge in New Caledonia (Bourguet-Kondracki et al., 2001). Marine sponges of the genus *Plakortis* (family Plakinidae) have been known to be rich sources of structurally unique and cytotoxic metabolites namely peroxides (Davidson, 1991; Rudi and Kashman, 1993), β-carbolines (Sandler et al., 2002) and polyketides (Sata et al., 2005; Barber et al., 2011). There are relatively few reports of the presence of cytotoxic molecules from sponges of the genus *Dragmacidon* namely bis (indole) piperazine alkaloids derivatives (Kohmoto et al., 1988; Mancini et al., 1997) and β-carboline alkaloids (Pedpradab et al., 2010). The genus *Axinella* is distributed worldwide and is known to possess a variety of metabolites such as bromo compounds, cyclopeptides, polyethers, sterols and terpenes. Several cytotoxic peptides, Axinastatin 1–5 have been reported from Axinella specimen of Palau and Republic of Comoros (Pettit et al., 1991, 1993, 1994a,b). The sponge *Pericharax heterorhaphis* has been known to possess antimicrobial activity

(Wah et al., 2006). However no report was found of cytotoxicity activity being exhibited by this genus. The marine sponge *Petrosia* sp. has been known to yield polyacetylenes, which delivered significant selective cytotoxicity against several human tumour cell lines (Lim et al., 1999, 2001; Kim et al., 2002). Recently Okamoto et al. (2007) reported the presence of two new cytotoxic polyacetylene carboxylic acids, petroformynic acids A and B from a *Petrosia* sp. collected off Katsuo-jima Island, Japan while Hitora et al. (2011) described (-)-Duryne and its homologues B-F as natural products for the first time. *Dysidea* sponges have long been known as a prolific source of bioactive natural products (Sharma and Vig, 1972) with several classes of terpenes, modified peptides and diketopiperazines with uncommon amino acids, bromodiphenyl ethers, and, less frequently, alkaloids.

From our initial screening, we further tested the ethyl acetate (JDE) and hexane (JDH) extracts of the sponge *Jaspis* sp. and found their ability to induce apoptosis on KB cells. The cells exposed to JDE and JDH exhibit the morphological and biochemical changes that characterize apoptosis as shown by survival incapacity (Fig. 1), loss of cell viability (Fig. 2), collapse of the mitochondrial membrane potential (Fig. 3) and sub-G1 phase accumulation (Fig. 4, Table 5). Apoptosis plays an important role in cellular homeostasis (Thompson, 1995; Green and Reed, 2000; Hengartner, 2000; Kaufmann and Hengartner, 2001) and induction of apoptosis in cancer cells is one of the useful strategies for anticancer drug development (Hu and Kavanagh, 2003; Camero, 2002).

Once the inhibitory effects of the extracts, JDE and JDH, on cell growth inhibitions (using MTS) were established, their effects on cell viability were assessed using the clonogenic assay at various concentration range (Fransis et al., 1996). The clonogenic formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony defined as at least 50 cells. It detects all cells retaining the capacity to produce a large number of cells after treatment that can cause cell death as a result of damage to the chromosomes (Brown and Attardi, 2005). Apoptosis in the treated KB cells was conclusive from the characteristic changes in morphology, particularly rounding up of cells, membrane blebbing and formation of apoptotic bodies. Collapse of mitochondrial membrane potential is an indicator of apoptosis in mammalian cells and can happen *via* two different pathways: intrinsic and extrinsic. The intrinsic pathway is involved with the release of cytochrome c and other proteins from mitochondrial intermembrane space, which leads to activation of caspases. In the extrinsic pathway, an increase in the mitochondrial membrane potential is an intermediate link to apoptosis (Desagher & Martinou, 2000). Highest decreases of mitochondrial membrane potential in KB cells were observed in cultures incubated with 50 μg/ml JDE although cultures exposed to 50 μg/ml JDH also underwent moderate and gradual decrease in membrane potential.

Cell cycle is a critical process in the development, differentiation and proliferation of mammalian cells (Schwartz and Shah, 2005). Deregulation of its machinery has been associated with cancer initiation and progression (Wang et al., 2009) and thus suppression of the cell cycle with cytotoxic agents has proven to be an interesting target for management and treatment of tumor cell. The increase in sub G1 population in our results indicates the induction of apoptosis, as sub G1 peak is reported to be a quantitative indicator of apoptosis (Del Bino et al., 1999).

Sponges of the genus *Jaspis* have been a rich source of biologically active and novel molecules. This genus has received much attention since the discovery of the cyclodepsipeptide Jaspamide in the sponge *Jaspis cf. johnstoni* with pronounced antifungal, anthelmintic, insecticidal and cytotoxic activities (Zabriskie et al., 1986; Crews et al., 1986; Scott et al., 1988; Inman et al., 1989). Several other members of this genus have also yielded cytotoxic molecules namely macrolides from the Okinawan *Jaspis* sp. (Kobayashi et al., 1993), bengamides (Groweiss et al., 1999) and bromotyrosine derivatives (Shinde et al., 2008). Here, we demonstrated that constituents of the ethyl acetate and hexane extract of the sponge *Jaspis* sp. can inhibited cell growth and induce apoptosis of KB cells.

5. Conclusion

Marine organisms collected from waters of Mauritius have been known to possess a number of biological activities and our study show that Mauritian sponges are rich sources of organic fractions with significant cytotoxic activity. To the best of our knowledge, this is the first report demonstrating the anticancer activity of Mauritian sponges taken up in this study over such a panel of cancer cell lines. The species with the strongest activities found in their fractions were: *Petrosia* sp. 1, *Petrosia* sp. 2, *Jaspis* sp. and *Pericharax heteroraphis*. From our results, these species prove to be more suitable candidates for further detailed chemical and pharmacological studies. The use of MARINLIT at the beginning of the literature review reveals to be very valuable as it provide clues on the type of work, if any, which has already been undertaken on the specimen, hence avoid us duplicating research work.

Conflict of interest

The authors declare that there are no conflicts of interest.

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