Evaluation of the antiproliferative and cytotoxic activities of marine invertebrates-derived fungi

Abdulrahman M Alahdal[1], Lamiaa A Shaala[2,3], Ahmad O Noor[1], Mahmoud A Elfaky[4], Sameh S Elhady[4], Ameen Almohammadi[1], Alaa Bagalagel[1], Manar O Lashkar[1], Diena M Almasri[1] and Diaa TA Youssef[4,5]

[1]Department of Clinical Pharmacy, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia
[2]Natural Products Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia
[3]Suez Canal University Hospital, Suez Canal University, Ismailia, Egypt
[4]Department of Natural Products, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia
[5]Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt

Abstract: The present study focuses on the evaluation of the cytotoxicity and antiproliferative activities of the organic extracts of 70 fungal strains associated with twelve Red Sea marine invertebrates. The fungal strains were obtained 10 sponges, one tunicate and one soft coral. Three different media including Sabouraud dextrose agar, malt extract agar and Czapek-Dox agar were used for the purification of the fungal isolates. The purified fungal isolates were cultured in their corresponding media (Sabouraud dextrose broth, Malt extract broth and Czapek-Dox broth) on shaker for 14 days at 26°C. After that, the cultures were lyophilized and the dried cultures were extracted with methanol. The methanolic extracts of these cultures were evaluated for their *in vitro* cytotoxicity and antiproliferative activities against three human cancer cell lines including breast adenocarcinoma (MCF-7), liver hepatocellular carcinoma (HepG2) and colorectal carcinoma (HCT-116). Nine extracts displayed potent and selective activity against MCF-7 with IC_{50} 4.96-8.28µg/mL without any significant effect on the other two cell lines. In addition, six extracts showed strong and selective activity against MCF-7 with IC_{50} 11.37-15.53µg/mL. On the other hand, most of the fungal extracts were inactive or weakly active against HepG2 and HCT-116.

Keywords: Red Sea marine invertebrates, associated-fungi, organic extracts, human cancer cell lines, cytotoxicity and antiproliferative activities.

INTRODUCTION

One of the most important public health problems in Egypt is liver disease related to infectious diseases. Ironically this was exacerbated by attempts to control its primary historical cause, schistosomiasis: intravenous tartar emetic was administered as community-wide therapy in large control campaigns from the 1950s through the 1980s (Strickland, 2006), until the effective oral drug praziquantel became available in the mid-1980s. The use of non-sterile needles in the administration of the tartar emetic has been linked to the Hepatitis C virus (HCV) epidemic in Egypt, and HCV is now the main cause of liver diseases. A major impact of widespread of HCV in Egypt has been the increased incidence of hepatocellular carcinoma (liver cancer) (Hussain *et al*., 2007). Furthermore, there is evidence that HCV increases the risk of other non-hepatic cancers. For example, it seems to have a role in lymphoproliferation and increases the risk of non-Hodgkins lymphoma (Giordano *et al*., 2007). It may also mediate autoimmune thyroiditis leading to thyroid cancer (Montella *et al*., 2003). In Egypt, hepatocellular carcinoma (HCC) is a main cause of cancer-related deaths in men. Moreover, HCC is considered as fifth most common cancer (Alison and Lovell, 2005) and the third leading cause of cancer death world-wide, killing almost all patients who have it within a year. Treatments for HCC rely on surgical resection and ablation, and thus there is a great need for the discovery and development of new, less toxic chemotherapeutic agents for liver cancer. Egypt is also burdened by other cancers which reflect its particular social structure that incorporates both rural and modernized life styles. Bladder cancer, related to schistosomiasis infection predominantly in rural areas, is the most prevalent cancer in Egypt (Vauhkonen *et al*., 2007). On the other hand, cancers usually associated with lifestyles in developed countries, such as breast and gastrointestinal cancers, are a growing health concern in Egypt.

Red Sea was a rich source of diverse biologically active compounds and pharmaceutical probes including latrunculin A, B (actin polymerization inhibitor and microfilament disrupters) from the sponge *Negombata magnifica* and swiholide A (microfilamnet disrupting toxin) from *Theonella swinhoei*. In addition, latrunculins decrease the intraocular pressure in monkeys and increase outflow facility without any corneal effects. It has been patented to Inspire Pharmaceuticals (USA) as possible anti-glaucoma leads (Okka *et al*., 2004; Peterson *et al*.,

**Corresponding author:* e-mail: dyoussef@kau.edu.sa

2000a; Peterson *et al*., 2000b). This diversity represents just a small part of the known bioactive compounds, and the vast majority await discovery.

The Red Sea tunicate, *Didemnum* sp.

The Red Sea sponge, *Hyrtios erectus*

Fig. 1: Underwater photographs of Red Sea marine invertebrates with highest fungal diversity.

Remarkably, most marine invertebrates (sponge, soft coral and tunicate species) harbor commensal microorganisms, which exist as intra- and extra-cellular symbionts (Dunlap *et al*., 2007). In some sponges, microbes may constitute as much as 40% of the organism volume, and there is now abundant evidence to suggest that a significant portion of the biologically active metabolites isolated from marine invertebrates are produced or synthesized by associated microbes. Many anticancer compounds produced by sponges such as discodermolide, halichondrin B and bryostatin 1, are believed to be products of the microbiotic associations.

Microbial symbiosis in invertebrates gained significance as an important source of biologically active compounds (Gordon and Leggat, 2010). A recent review describing the bioactive compounds produced by symbiotic microbes isolated from marine sponges showed that the majority of the associated microbes included bacteria and fungi (Thomas *et al*., 2010). Compounds produced by fungal symbionts represented by 65.71% of the total compounds produced by sponge-microbial associations, while 34.28% of the total compounds were bacterial products (Thomas *et al*., 2010). Among the fungal phyla, Ascomycota was the producer of 73% of the total fungal compounds, suggesting the importance of this phylum and the fungi in general as a future source for the process of drug discovery (Thomas *et al*., 2010). The investigation of microbial communities present within marine invertebrates has significant effects for the production of symbiont-derived secondary metabolites and for the application of marine invertebrates as future source material for microbes in screening efforts for discovery of anticancer drug leads (Evidente *et al*., 2015 ; Gomes *et al*., 2015, ; Jin *et al*., 2016 ; Imhoff, 2016). In the course of our ongoing search to identify marine microbial drug leads from associated microbes with Red Sea invertebrates (Shaala *et al*., 2016; Murshid *et al*., 2016; Asiry *et al*., 2015; Shaala and Youssef, 2015; Mourshid *et al*., 2016), the associated fungal community with twelve Red Sea marine invertebrates was examined. The organic extracts of the culture of 70 fungi were evaluated for their cytotoxic and antiproliferative activities against HCT-116, HepG2 and MCF-7.

MATERIALS AND METHODS

Collection of Red Sea marine invertebrates

Marine invertebrates (sponges, tunicates and soft corals) were collected from the Red Sea by hands using SCUBA at the Egyptian Red Sea coast off Hurghada at 10-30m depth during May 2013. The collected invertebrates include 10 sponges, one tunicate and one soft coral (table 1 and fig. 1). The sponges were identified by Prof. Dr. van Soest. The tunicates were identified by Dr. Francoise Monniot at Muséum national d'histoire naturelle (MNHN), Paris. Voucher specimens for the sponges were kept in the collections of the Naturalis Biodiversity Center at Leiden, The Netherlands. Additional voucher specimens were placed in the Red Sea Invertebrates Collection at the Department of Pharmacognosy, Faculty of Pharmacy at Suez Canal University.

Preparation of the fungal isolates from the marine invertebrates

The preparation of the marine invertebrates for the isolation and purification of fungal isolates was performed as previously described (Shaala and Youssef, 2015).

Culture of fungal strains and preparation of organic extracts

Colonies of each fungal isolate were directly inoculated into 250-mL Erlenmeyer flasks, each containing 50-mL of the corresponding media: The media used in this study include: Sabouraud dextrose broth (Sabouraud dextrose broth 30.0g, NaCl 20.0g, dissolved in distilled water 1,000mL), Malt extract broth (malt extract 30.0g, peptone 3.0g, NaCl 20.0, dissolved in distilled water 1,000mL) and Czapek-Dox broth ($NaNO_3$ 3.0g, KCl 0.5g, K_2HPO_4

Table 1: Source of fungal isolates and culture media used in this study.

Isolate No.	Source organism	Organism class	Culture Media	Isolate No.	Source organism	Organism class	Culture media
1	*Callyspongia* sp.	S	SDB	36	*Ircinia echinata*	S	CDB
2	*Callyspongia* sp.	S	SDB	37	*Dragmacidon coccinea*	S	SDB
3	*Callyspongia* sp.	S	SDB	38	*Dragmacidon coccinea*	S	SDB
4	*Hyrtios erectus*	S	SDB	39	*Dragmacidon coccinea*	S	SDB
5	*Hyrtios erectus*	S	SDB	40	*Callyspongia siphonella*	S	SDB
6	*Hyrtios erectus*	S	SDB	41	*Callyspongia siphonella*	S	SDB
7	*Hyrtios erectus*	S	SDB	42	*Callyspongia siphonella*	S	MEB
8	*Hyrtios erectus*	S	SDB	43	*Theonella swinhoei*	S	SDB
9	*Hyrtios erectus*	S	SDB	44	*Theonella swinhoei*	S	SDB
10	*Hyrtios erectus*	S	SDB	45	*Theonella swinhoei*	S	SDB
11	*Hyrtios erectus*	S	SDB	46	*Theonella swinhoei*	S	SDB
12	*Hyrtios erectus*	S	SDB	47	*Suberea mollis*	S	SDB
13	*Hyrtios erectus*	S	SDB	48	*Suberea mollis*	S	MEB
14	*Hyrtios erectus*	S	SDB	49	*Suberea mollis*	S	MEB
15	*Hyrtios erectus*	S	SDB	50	*Didemnum* sp.	T	SDB
16	*Hyrtios erectus*	S	SDB	51	*Didemnum* sp.	T	SDB
17	*Hyrtios erectus*	S	SDB	52	*Didemnum* sp.	T	SDB
18	*Hyrtios erectus*	S	SDB	53	*Didemnum* sp.	T	SDB
19	*Hyrtios erectus*	S	MEB	54	*Didemnum* sp.	T	SDB
20	*Hyrtios erectus*	S	CDB	55	*Didemnum* sp.	T	SDB
21	*Hyrtios erectus*	S	CDB	56	*Didemnum* sp.	T	SDB
22	*Amphimedon chloros*	S	SDB	57	*Didemnum* sp.	T	SDB
23	*Amphimedon chloros*	S	SDB	58	*Didemnum* sp.	T	SDB
24	*Amphimedon chloros*	S	MEB	59	*Didemnum* sp.	T	SDB
25	*Mycale euplectellioides*	S	SDB	60	*Didemnum* sp.	T	SDB
26	*Mycale euplectellioides*	S	SDB	61	*Didemnum* sp.	T	SDB
27	*Mycale euplectellioides*	S	SDB	62	*Didemnum* sp.	T	SDB
28	*Mycale euplectellioides*	S	SDB	63	*Didemnum* sp.	T	SDB
29	*Mycale euplectellioides*	S	CDB	64	*Didemnum* sp.	T	SDB
30	*Mycale euplectellioides*	S	CDB	65	*Didemnum* sp.	T	SDB
31	*Cliona vastifica*	S	MEB	66	*Didemnum* sp.	T	ME
32	*Cliona vastifica*	S	MEB	67	*Didemnum* sp.	T	CDB
33	*Cliona vastifica*	S	CDB	68	*Didemnum* sp.	T	CDB
34	*Ircinia echinata*	S	SDB	69	*Nephthea* sp.	SC	CDB
35	*Ircinia echinata*	S	CDB	70	*Nephthea* sp.	SC	CDB

S) Sponge; T) Tunicate; SC) Soft coral; SDB) Sabouraud dextrose broth; ME) Malt extract broth; CDB) Czapek-Dox broth.

0.1g, $MgSO_4 \cdot 7H_2O$ 0.5g, $FeSO_4$ 0.01g, sucrose 30.0g, NaCl 20.0g, dissolved in distilled water 1,000mL, pH 6.7). Cultures of the fungal isolates were incubated on a shaking bed at 120 rpm at 26°C for 14 days. After that, all cultures were freeze-dried followed by extraction with methanol (3 x 150mL). The combined extracts of each culture was dried under vacuum and the resulting extracts were used for evaluation of the cytotoxic and antiproliferative activities.

Determination of the antiproliferative and cytotoxic activities of the extracts

Three human cell lines were used in this study including HepG2 (liver hepatocellular carcinoma, HB-8065, ATCC, Manassas, VA, USA), HCT-116 (colorectal carcinoma, CCL-247, ATCC, Manassas, VA, USA) and MCF-7 (breast adenocarcinoma, HTB-22, ATCC, Manassas, VA, USA). These cells were used for the evaluation of the cytotoxic and antiproliferative activities of the fungal organic extracts. The effects of 70 fungal extracts on cell proliferation and cytotoxicity were evaluated using sulforhodamine B (SRB) assay as described previously (Vichai and Kirtikara, 2006). Doxorubicin was used as positive control drug.

RESULTS

The present study investigates the fungal community of twelve marine invertebrates including 10 sponges, one tunicate and one soft coral and the evaluation of the fungal extracts for their cytotoxic and antiproliferative activities against three human cancer cell lines.

Table 2: *In vitro* antiproliferative and cytotoxic activities of fungal extracts against human cancer cell lines.

Extract No.	IC_{50} (µg/mL)			Extract No.	IC_{50} (µg/mL)		
	MCF-7	HepG2	HCT-116		MCF-7	HepG2	HCT-116
1	25.52	>50	>50	36	48.19	>50	>50
2	34.87	>50	>50	37	>50	48.65	>50
3	41.29	35.96	37.52	38	7.40	30.75	42.6
4	8.28	39.65	>50	39	26.61	40.55	>50
5	6.83	>50	>50	40	37.78	>50	>50
6	23.14	47.33	>50	41	39.48	>50	>50
7	6.86	>50	>50	42	36.24	>50	>50
8	21.10	>50	>50	43	6.26	>50	>50
9	15.27	>50	>50	44	26.26	>50	>50
10	>50	>50	>50	45	>50	>50	>50
11	>50	>50	>50	46	>50	>50	>50
12	15.53	31.88	39.00	47	35.02	>50	>50
13	23.97	>50	>50	48	39.21	>50	>50
14	27.75	>50	>50	49	23.56	>50	>50
15	>50	>50	>50	50	5.93	>50	>50
16	17.93	>50	>50	51	23.84	34.90	>50
17	8.22	41.00	38.40	52	21.10	40.75	>50
18	>50	>50	>50	53	48.07	45.50	>50
19	6.65	>50	>50	54	49.75	>50	>50
20	41.51	>50	>50	55	>50	>50	>50
21	>50	>50	>50	56	38.01	>50	>50
22	31.46	>50	>50	57	41.42	>50	>50
23	>50	>50	>50	58	30.05	>50	>50
24	40.01	>50	>50	59	11.37	>50	>50
25	>50	>50	>50	60	21.87	>50	>50
26	39.00	>50	>50	61	26.39	>50	>50
27	49.68	>50	>50	62	>50	>50	>50
28	>50	42.90	>50	63	>50	>50	>50
29	28.2	>50	>50	64	20.47	41.93	45.12
30	>50	>50	>50	65	23.04	>50	>50
31	>50	>50	>50	66	>50	>50	>50
32	27.12	>50	>50	67	12.30	>50	>50
33	>50	>50	>50	68	30.85	>50	>50
34	>50	>50	>50	69	4.96	>50	>50
35	27.65	48.97	>50	70	12.77	>50	>50
Doxorubicin*	0.41	0.85	0.11	Doxorubicin*	0.41	0.85	0.11

*) Positive control drug.

Representative examples of the marine invertebrates are shown in fig. 1. A total of 70 fungal strains were isolated from these invertebrates (table 1). The maximum fungal diversity was obtained from the sponge *Hyrtios erectus* (fig. 1) and the tunicate *Didemnum* sp. (fig. 1) on Sabouraud dextrose agar, while it was minimal on the other two media (malt extract agar and Czapek-Dox agar) (table 1). The *in vitro* antiproliferative and cytotoxic activities of 70 fungal extracts against three human cancer cell lines was presented in tables 2 and 3.

DISCUSSION

Most of the fungal extracts showed variable activity against MCF-7. From these, nine extracts (No. 4, 5, 7, 17, 19, 38, 43, 50 and 69) were selective and potent against MCF-7 with IC_{50} of 4.96-8.28µg/mL without any significant effect on the other two cell lines (tables 2 and 3). In addition, six extracts (No. 9, 12, 16, 59, 67 and 70) displayed strong and selective activity against MCF-7 cell line with IC_{50} of 11.37-15.53µg/mL (tables 2 and 3). The rest of the extracts were either moderately active (IC_{50} 20-

Table 3: Fungal extracts with potent and selective activities against MCF-7 cell line.

Extract No.	IC_{50} (µg/mL)			Significance
	MCF-7	HepG2	HCT-116	
4	8.28	39.65	>50	Potent/selective
5	6.83	>50	>50	Potent/selective
7	6.86	>50	>50	Potent/selective
9	15.27	>50	>50	Strong/selective
12	15.53	31.88	39.00	Strong/selective
16	17.93	>50	>50	Strong/selective
17	8.22	41.00	38.40	Potent/selective
19	6.65	>50	>50	Potent/selective
38	7.40	30.75	42.6	Potent/selective
43	6.26	>50	>50	Potent/selective
50	5.93	>50	>50	Potent/selective
59	11.37	>50	>50	Strong/selective
69	4.96	>50	>50	Potent/selective
67	12.30	>50	>50	Strong/selective
70	12.77	>50	>50	Strong/selective

40 µg/mL), weakly active or completely inactive (table 2). Conversely, most of the fungal extracts were inactive (IC_{50} >50µg/mL) against human liver hepatocellular carcinoma cell line (HepG2). Only 14 extracts (No. 3, 4, 6, 12, 17, 28, 35, 37, 38, 39, 51, 52, 53 and 64) were weakly active against HepG2 with IC_{50} of 23.04-49.75µg/mL (table 2). Similarly, most of the fungal extracts were inactive (IC_{50} >50µg/mL) against human colorectal carcinoma cell line (HCT-116). Finally, five extracts (No. 2, 12, 17, 38 and 46) showed weak activity (IC_{50} 37.52-45.12 µg/mL) against HCT-116 (table 2).

Present study revealed the strong and selective antiproliferative and cytotoxic potential of marine fungal extracts (about 13%, 9 out of 70 extracts) (table 3) against MCF-7 and hence strong potential to produce antiproliferative compounds. Marine fungi and their purified extracts have shown to be good producer of cytotoxic and antiproliferatve compounds. This activity could be attributed to different classes of secondary metabolites as shown previously by our group and by other groups (Evidente *et al*., 2015; Gomes *et al*., 2015; Jin *et al*., 2016; Imhoff, 2016; Shaala *et al*., 2016; Murshid *et al*., 2016; Asiry *et al*., 2015; Shaala and Youssef, 2015).

CONCLUSION

It could be concluded from this study that marine invertebrates represent a massive source of fungal diversity. The fungal strains associated with marine invertebrates represent a potential reservoir for production of novel anticancer drugs. The fungal extracts # 4, 5, 7, 17, 19, 38, 43, 50 and 69 showed potent and selective activity against MCF-7 with IC_{50} of <10 µg/mL. These extracts are categorized as "first priority" and are expected to possess lead compounds for future isolation and identification of selective antiproliferative drugs against human breast adenocarcinoma. Currently, efforts are in progress to identify the fungal strains of interest (table 3) and to isolate the active cytotoxic and antiproliferative compounds in their corresponding extracts using bioassay-directed fractionation.

REFERENCES

Alison M and Lovell M (2005). Liver cancer: the role of stem cells. *Cell proliferation*, **38**(6): 407-421.

Asiry IA, Badr JM and Youssef DT (2015). Penicillivinacine, antimigratory diketopiperazine alkaloid from the marine-derived fungus *Penicillium vinaceum*. *Phytochemistry Lett*., **13**: 53-58.

Dunlap WC, Battershill CN, Liptrot CH, Cobb RE, Bourne DG, Jaspars M, Long PF and Newman DJ (2007). Biomedicinals from the phytosymbionts of marine invertebrates: A molecular approach. *Methods*, **42**(4): 358-376.

Evidente A, Kornienko A, Lefranc F, Cimmino A, Dasari R, Evidente M, Mathieu V and Kiss R (2015). Sesterterpenoids with Anticancer Activity. *Curr. Med. Chem.*, **22**(30): 3502-3522.

Giordano TP, Henderson L, Landgren O, Chiao EY, Kramer JR, El-Serag H and Engels EA (2007). Risk of non-Hodgkin lymphoma and lymphoproliferative precursor diseases in US veterans with hepatitis C virus. *J. Am. Med. Assoc.*, **297**(18): 2010-2017.

Gomes N, Lefranc F, Kijjoa A and Kiss R (2015). Can Some Marine-Derived Fungal Metabolites Become Actual Anticancer Agents? *Mar. Drugs*, **13**(6): 3950-3991.

Gordon BR and Leggat W (2010). Symbiodinium-invertebrate symbioses and the role of metabolomics.

Mar. Drugs, **8**(10): 2546-2568.

Hussain S, Schwank J, Staib F, Wang X and Harris C (2007). TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene*, **26**(15): 2166-2176.

Imhoff JF (2016). Natural products from marine fungi-Still an underrepresented resource. *Mar. Drugs,* **14**(19): 1-19.

Jin L, Quan C, Hou X and Fan S (2016). Potential Pharmacological Resources: Natural Bioactive Compounds from Marine-Derived Fungi. *Mar. Drugs*, **14**(76): 1-25.

Montella M, Pezzullo L, Crispo A, Izzo F, Amore A, Marone U, Tamburini M, Ronga D, Chiofalo MG, Chiappetta G and Mozzillo N (2003). Risk of thyroid cancer and high prevalence of hepatitis C virus. *Oncol. Rep.*, **10**(1): 133-136.

Mourshid SS, Badr JM, Risinger AL, Mooberry SL and Youssef DT (2016). Penicilloitins A and B, new antimicrobial fatty acid esters from a marine endophytic *Penicillium species*. *Z Naturforsch, C: J. Biosci.*, **71**(11): 387-392.

Murshid SS, Badr JM and Youssef DT (2016). Penicillosides A and B: new cerebrosides from the marine-derived fungus *Penicillium* species. *Rev. Bras. Farmacogn.*, **26**(1): 29-33.

Okka M, Tian B and Kaufman PL (2004). Effects of latrunculin B on outflow facility, intraocular pressure, corneal thickness, and miotic and accommodative responses to pilocarpine in monkeys. *Trans. Am. Ophthalmol. Soc.*, **102**(12): 251-257.

Peterson JA, Tian B, Geiger B and Kaufman PL (2000a). Effect of latrunculin-B on outflow facility in monkeys. *Exp. Eye Res.*, **70**(3): 307-313.

Peterson JA, Tian B, McLaren JW, Hubbard WC, Geiger B and Kaufman PL (2000b). Latrunculins' effects on intraocular pressure, aqueous humor flow, and corneal endothelium. *Invest. Ophthalmol. Visual. Sci.*, **41**(7): 1749-1758.

Shaala LA and Youssef DT (2015). Identification and bioactivity of compounds from the fungus Penicillium sp. CYE-87 isolated from a marine tunicate. *Mar. Drugs*, **13**(4):1698-1709.

Shaala LA, Youssef DT, Badr JM and Harakeh SM (2016). Bioactive 2(1H)-pyrazinones and diketopiperazine alkaloids from a tunicate-derived actinomycete *Streptomyces* sp. *Molecules*, **21**(1116): 1-9.

Strickland GT (2006). Liver disease in Egypt: Hepatitis C superseded schistosomiasis as a result of iatrogenic and biological factors. *Hepatology*, **43**(5): 915-922.

Thomas TRA, Kavlekar DP and LokaBharathi PA (2010). Marine drugs from sponge-microbe association-A review. *Mar. Drugs*, **8**(4): 1417-1468.

Vauhkonen H, Böhling T, Eissa S, Shoman S and Knuutila S (2007). Can bladder adenocarcinomas be distinguished from schistosomiasis-associated bladder cancers by using array comparative genomic hybridization analysis? *Cancer Genet. Cytogenet.*, **177**(2): 153-157.

Vichai V and Kirtikara K (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.*, **1**(3): 1112-1116.