IN VITRO ANTICANCER ACTIVITIES OF INK AND INTERNAL SHELL EXTRACTS OF *SEPIA OFFICINALIS* INHABITING EGYPTIAN WATER

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ABSTRACT

The present study was carried out to assess the anticancer activity of extracts of internal shell and ink of *S. officinalis* on cell viability, MTT assay, cell cycle and apoptosis on *Ehrlich Ascites* Carcinoma (EAC) cells. The shell extracts have different anticancer effect on EAC cell in different concentrations (100, 250, 500, 1000 µg/ml) while ink extract has dose dependent effect according to following concentrations (100, 75, 50, 25 µg/ml). The viability of EAC exposed to sepia shell powder ranged from 23% (1000µg/ml) to 76% (100µg/ml) as well as the ink extracts shows cell viability ranged from 18% (100µg/ml) to 83% (25µg/ml). MTT assay showed also dose dependent manner of cell survival according to different concentrations of ink and shell extracts ranged from 61% (100µg/ml) to 100% (25µg/ml) of ink extract and from 61% (1000µg/ml) to 83% (100µg/ml) of sepia shell extract. EAC cells experienced a significant decrease in viability at low concentration with an eventual decline at the highest concentration compared to the reference drug cisplatin effect (40, 20µg/ml). The flow cytometry analysis of cell cycle and apoptosis of EAC cell treated with different concentrations of sepia shell and ink extracts of *S. officinalis* confirmed the previous results of viability and MTT assay sepia shell extracts. The obtained results lead to the conclusion that the cephalopod shell and ink are promising sources to be considered in the drug discovery for cancer therapy.

Keywords: Anticancer, cell cycle, Flow cytometry, marine extracts, MTT, sepia.

INTRODUCTION

Over the past 80 years, the war of cancer was fought with 3 tools: surgery, radiation therapy and chemo therapy over the same period there had been rapid improvements in technology and medical science but unfortunately the

overall cancer incidence increase by 44% since 1950. Traditional cancer chemotherapy causes anticancer immune dysfunction and illness Cancer is one of the leading causes of malignancy- related death worldwide because of tumor heterogeneity and the development of multi drug resistance phenotypes. Till now the availability of treatments for cancer remains unsatisfactory. Hence, the search for active drugs from alternative sources including marine environment, obviously becomes imperative

An immune dysfunction includes hypoactivity (immunodeficiency) due to deficiency in the number and function of immune cells. On the other hand, the current treatments showed different types of resistance.

So, Natural products are very acceptable answer for the traditional anti-cancer drugs problems. There are the trends of natural products used as anticancer agents or immunity enhancers during chemotherapy:

1-medicinal plants

2-marine products (algae, fungi, invertebrate animals)

3- non -pathogenic microbes

In this preclinical study we will use bioactive materials extracted from marine Mollusca as invertebrate animals not only for the successful examples drugs being derived from various types of terrestrial marine Mollusca but also for their very advanced immune system, which have had a profound impact on the history of various types of cancer therapy.

Cancer is one of the leading causes of malignancy-related death worldwide because of tumor heterogeneity and the development of multi-drug resistance phenotypes **(Huang *et al.*, 1992).** Currently, the treatments available for cancer remain unsatisfactory (**Honda *et al.*, 1998)**. Hence, it is essential to search and find biologically active compounds from alternative sources including marine environment. Cephalopods are the largest single group of biotoxic invertebrates, which may be largely useful in the biomedical arena. The biological activities of squid and sepia ink such as antimicrobial (**Patterson and Murugan, 2000, Rajaganapathy *et al.*, 2000, Chacko and Patterson, 2005, Du-Tie-ping *et al.*, 2005, Suba *et al.*, 2008, Gomathi *et al.*, 2010, Smiline Girija *et al.*, 2011, Pasiyappazham *et al.*, 2011, Rajasekharan *et al.*, 2011, Nithya *et al.*, 2011** and **Vennila *et al.*, 2011);** antioxidant (**Liu *et al.*, 2009: Wang et al., 2010** and **Ilamparithi *et al.*, 2011),** antiradiation **(Lei *et al.*, 2007**), anti-tumour (**Takaya *et al.*, 1994, Saskai *et al.*, 1997**, **Russo *et al.*, 2003, Sherief *et al.*, 2004**, **Su Wei-ming *et al.*, 2005, Wang *et al.*, 2008** and **Priya *et al.*, 2013,** immunity promotion (**Huazhong *et al.*, 2011)** and induction of many cytokines (**He *et al.*, 2003)** have been widely studied in recent years.

The shell refers to the internal cartilaginous shell of sepia, squid and octopus. Traditionally shell powder is used as a medicine for some ear ailments, stop bleeding and improve kidney efficiency (**Trivedi and Sarvaiya, 1976** and

Raje and Singh 1992). Antibacterial and antifungal activities of internal shell of cephalopods were studied in different species (**Barwin vino 2003** and **Shanmugam *et al.*, 2008)**. But only a few cephalopods have been tested for anticancer activities, especially in India. Therefore, the aim of the present study was to assess the potential anticancer activity of the methanolic extracts of the internal shell and the ink of cuttle fish using cell viability and toxicity assay (MTT assay) and trypan blue on Ehrlich Ascites Carcinoma cell line.

MATERIALS AND METHODS

2.1 Collection and Preparation of extract

The animals (*S. officinals*) were collected from the Egyptian north and west coast of Alexandria during spring season, brought to the laboratory then cleaned and washed with fresh sea water to remove all impurities.

2.2 Shell Extract:

The internal shells were dissected, washed, air dried and pulverized. 10 gm of pulverized shell powder was mixed with 100 ml of methanol and kept in rotary shaker at 100 rpm overnight then filtered with a filter paper (Whatman No.1). After that, the extract was concentrated to dryness at 40°c, lyophilized and stored at 4°c till further use.

2.3 Ink Extract

The extraction and purification were performed in acidic medium. Fifty gram of commercial sepia ink were added to 100 ml of HCl (molarities ranged from 0, 0.5, 1.0, 2.0 and 3.0 M for each sample) in a dark recipient. The slurry was stirred for 30 min (magnetic or ultrasonic stirring) and then kept for 24 hr at 10^{o} C. Separation of solid from the supernatant fluid was done by centrifugation (10000 rpm at 5^{o} C for 15 min), washed-suspended three times with a 0.5 M HCl solution, water, acetone and finally with water. Following a 24 hr lyophilization to remove all solvent, a very thin black product was obtained at the end of the extraction.

2.4 Anticancer effect on EAC cell lines

The antitumor assay was performed on *Ehrlich Ascites Carcinoma* Cell lines obtained from National Cancer Institute**,** Cairo, Egypt. The cell viability was measured by hemocytomter with trypan blue and MTT assay as described **by Kang *et al*. (2004**).

The cells were grown in a 6-well plate in RPMI Medium obtained from (Al Maadi medical supplements, Cairo) supplemented with 10% fetal bovine serum (Gibco Laboratories) and antibiotics (streptomycin, penicillin-G, kanamycin, amphotericin B). About 1mL cell suspension (10 cells/ml) was seeded in each well and one plate was exposed to various dilutions of methanolic extract of internal shell (1000µg, 500µg, 250µg and100µg) and the other plate was exposed to various dilutions of ink extract (100µg, 75µg, 50µg and25µg). Incubation was at 37^{0} C for 48 hour in 5% CO_2 for the formation of

confluent monolayer. The cell viability was measured by hemocytometer with trypan blue and MTT assay.

2.5 Trypan blue exclusion assay

It relies on the alternation in membrane integrity as determined by the uptake of dye by dead cells and it gives a direct measure of cell viability. Fifty microliter of cell suspension were added to 50 µl of trypan blue (sigma *chemical Co.USA*) (0.4% in distilled water) then mixed well and left for 3 mins. Twenty microliters of this mixture was pippeted into hemocytometer chambers and unstained cell which represent the viable cells were counted.

2.6 MTT assay

With MTT (5 mg/ml) the tetrazolium salt is metabolically reduced by viable cells to yield a blue insoluble formzan product that measured at 570nm by Elisa reader. Controls were maintained throughout the experiment (untreated wells as cell control). The assay was performed in triplicate for both extracts. The mean values of the cell viability were compared to the control to determine the effect of the extract on cells and % of cell viability was plotted against concentration of the extract. The minimum concentration of the extract that was toxic to EAC cells was recorded as the effective drug concentration compared to positive control (CISPLATIN 40 mg/ml).

Cell Viability by Trypan blue was calculated as indicated:

Mean of cell count of hemocytometer chambers* total volume of cell suspension* dilution factor *10000

While MTT calculations were as fellow:

% of viability = Absorbance of the sample / Absorbance of control

% of toxicity = 100 - % of viability

Morphological studies using a normal inverted microscope were carried out to observe the cell death treated with sample EAC cells.

RESULTS

The effect of extract of *S.offinalis* and ink shell on cell viability and cytotoxicity by trypan blue is shown in Table 1, 2 Fig 1&2. The cell viability by MTT assay is shown in Table 3, 4 Fig 3&4.

Cell viability with trypan blue:

The cell viability of shell extract ranged from 23% (1000µg/ml) to 76% (100µg/ml) and the toxicity was 76% for 1000µg/ml and 23% for 100µg/ml. The minimum effective concentration that was toxic to EAC cell was recorded at a concentration greater than 500 µg.

The cell viability of ink extract ranged from 16% (100µg/ml) to 81% (25µg/ml) and the toxicity was 83% for 100µg/ml and 18% for 25µg/ml. The minimum effective concentration that was toxic to EAC cell was recorded at a concentration greater than 75 µg.

The viability by MTT assay:

The viability of methanolic extract of *S. officinalis* shell on EAC cell line was found to be 61% for 1000µg/ml and 83% for 100µg/ml and the IC 50 at a concentration of >1000µg. The viability of extract of ink extract of *S. officinalis* on EAC cell line was found to be 61% for 100µg/ml and mor than 100% for 25µg/ml and the IC 50 at a concentration of >100µg.

Table 1: Percentage of viability and toxicity - extract *S. officinalis* shell powder

Conc.	CIS 40	CIS 20	1000 µg	500 µg	250 µg	100 µg	Control
Viability (%)	16.6	33.3	23.1	52.6	61.1	76.5	100
Cytotoxicity (%)	83.4	66.6	76.9	47.36	38.8	23.5	0

Table 2: Percentage of viability and toxicity-extract *S. officinalis* ink powder

Concentrations	CIS 40	CIS 20	100 µg	75 µg	50 µg	25 µg	Control
Viability (%)	392	73.4	16.4	40.4	63.5	81.6	100
Cytotoxicity (%)	60.8	26.6	83.5	59.6	36.4	18.4	0

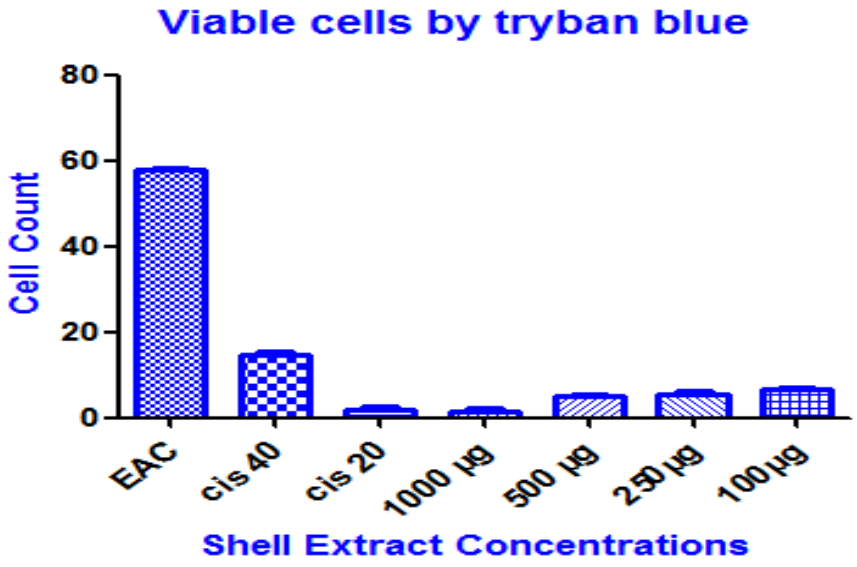

Fig. 1: Analysis of cell viability - methanol extract from *S. officinalis* shell powder.

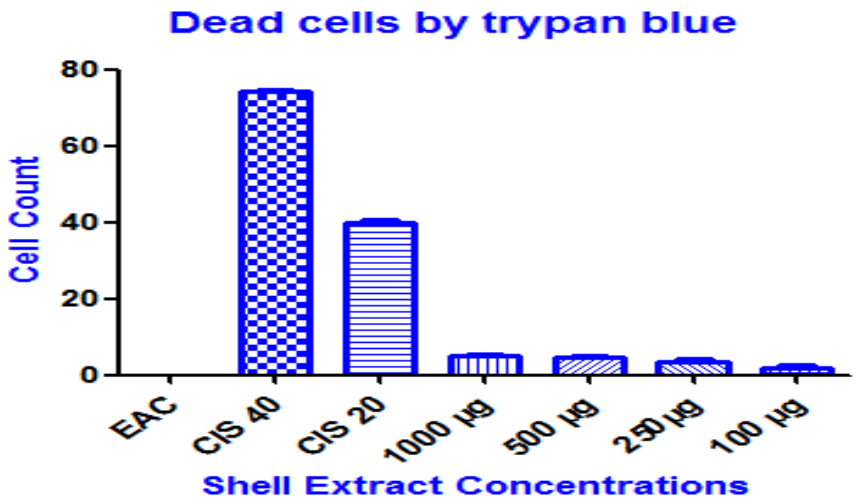

Fig. 2: Analysis of dead cell - methanol extract from *S. officinalis* shell powder.

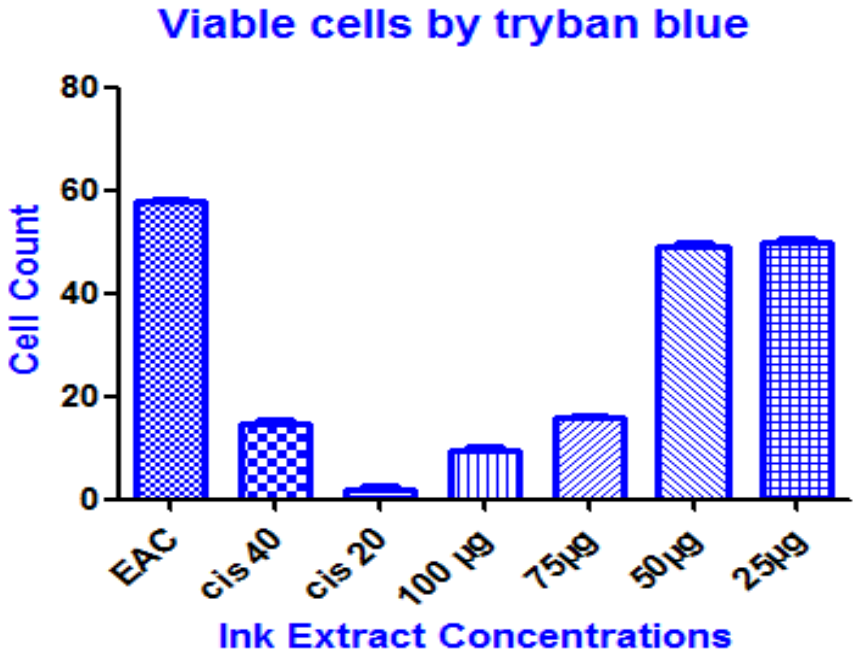

Fig. 3: Analysis of cell viability – extract from *S. officinalis* ink powder.

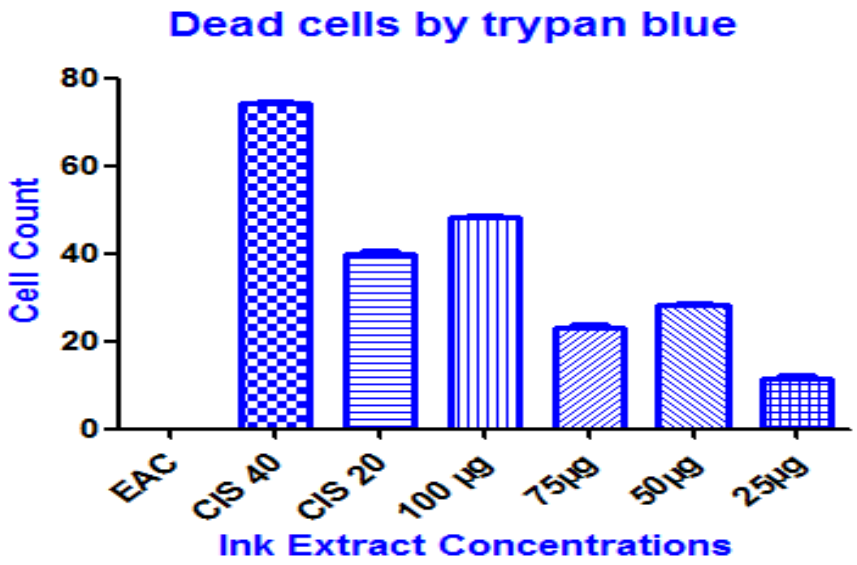

Fig. 4: Analysis of dead cell – extract from *s.officinalis* ink powder.

Table 3: Percentage of viability by mtt assay - extract from *S. officinalis* shell powder

Concentrations	**CIS 40**	**CIS 20**	**1000 µg**	**500 µg**	**250 µg**	**100 µg**	**Control**
Viability (%)	53%	31%	61%	74%	77%	83%	100%

Table 4: Percentage of viability by mtt assay - extract from *S. officinalis* ink

Concentrations	**CIS 40**	**CIS 20**	**100 µg**	**75 µg**	**50 µg**	**25 µg**	**Control**
Viability (%)	31%	53%	108%	97%	66%	61%	%100

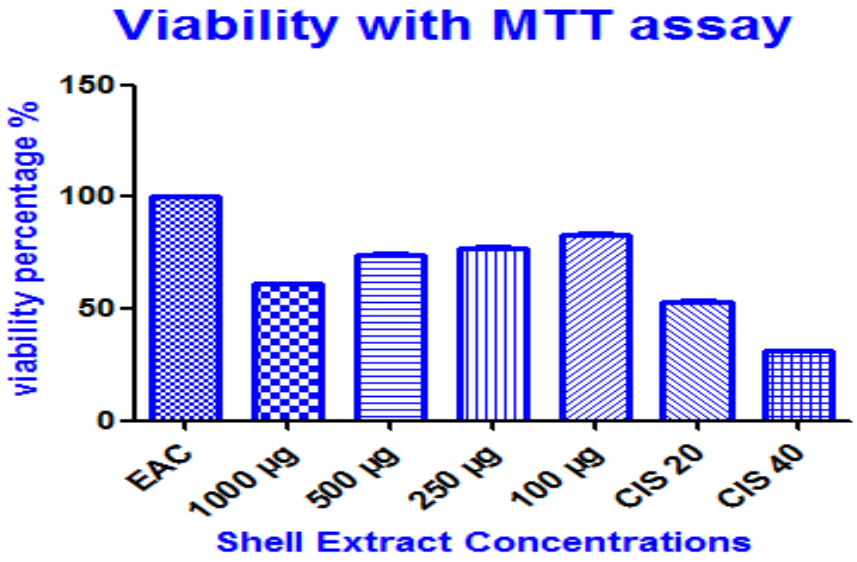

Fig. 5: Analysis of cell viability by mttassay-methanol extract from *S. officinalis* shell powder

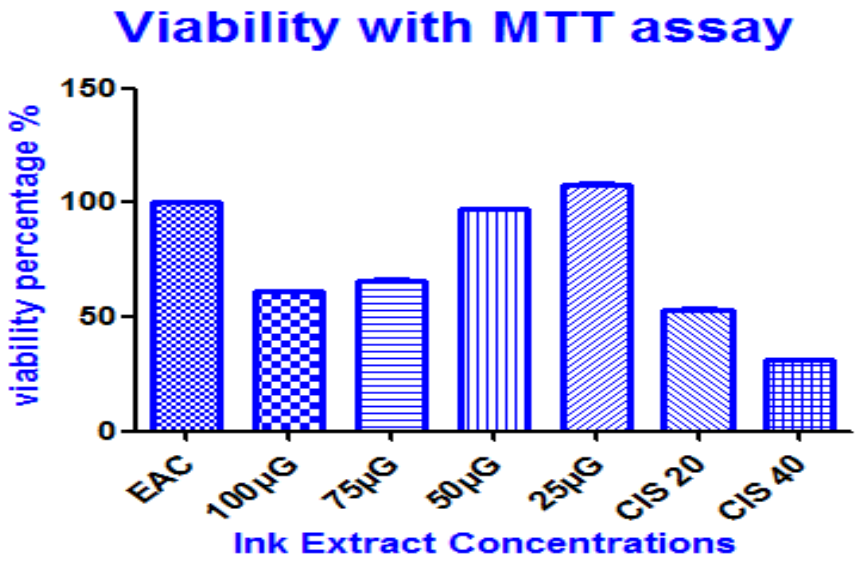

Fig. 6: Snalysis of cell viability by mttassay-methanol extract from *S. officinalis* ink

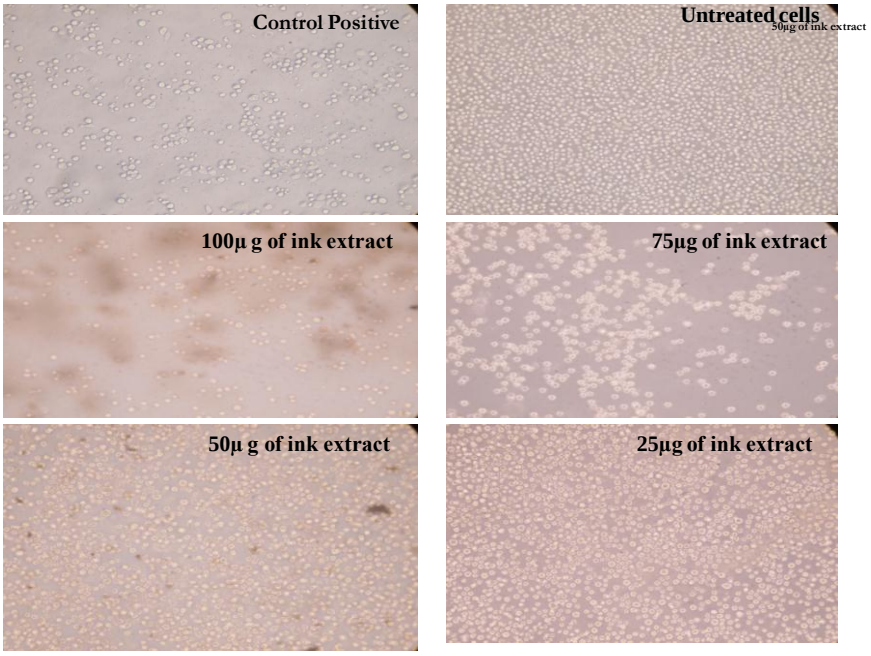

Fig. 7 Microscopic pictures of EAC cells after 24 hours of seeding in 6-well plats with different concentrations of *S. officinalis* ink

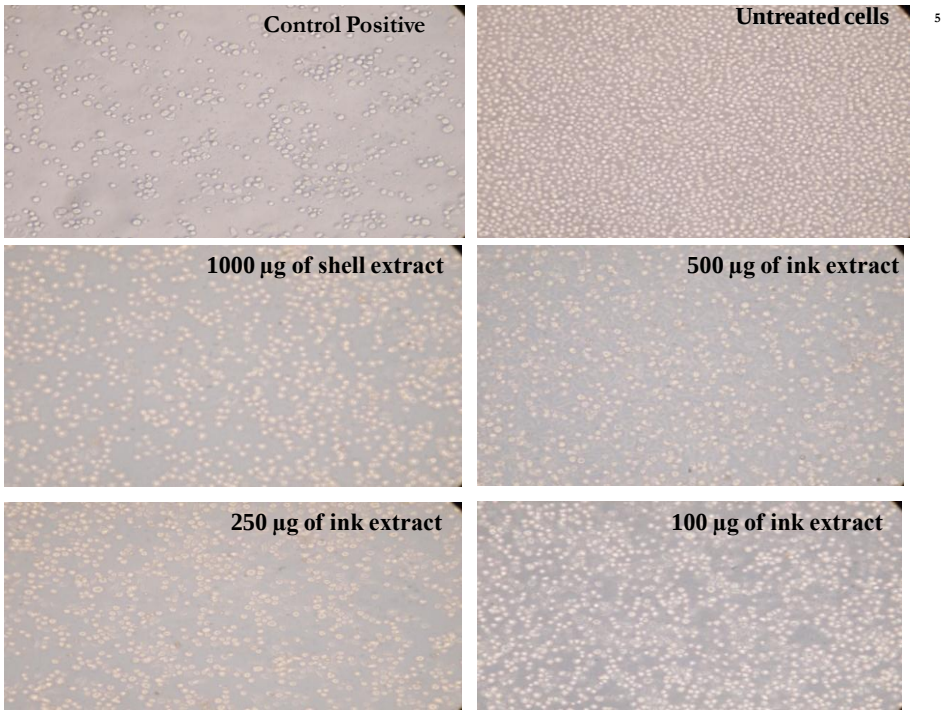

Fig. 8 Microscopic pictures of EAC cells after 24 hours of seeding in 6-well plats with different concentrations of *S. officinalis* shell.

Cell cycle analysis:

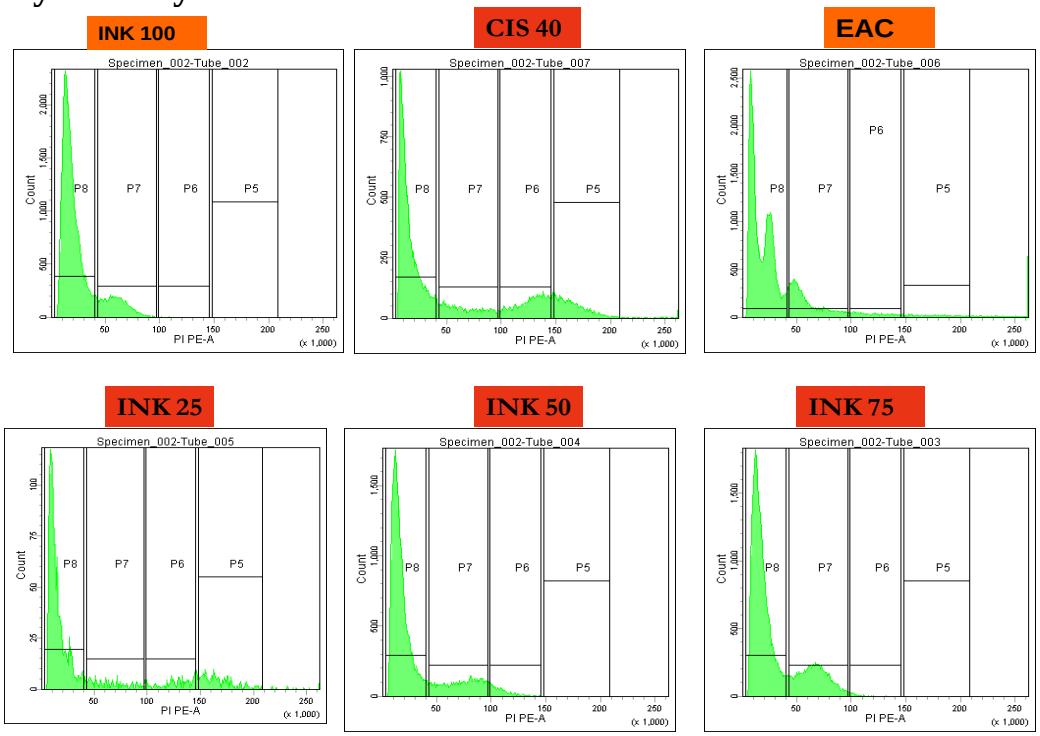

Fig.9 cell cycle analysis by flow cytometry of EAC cell treated with different concentrations of sepia ink

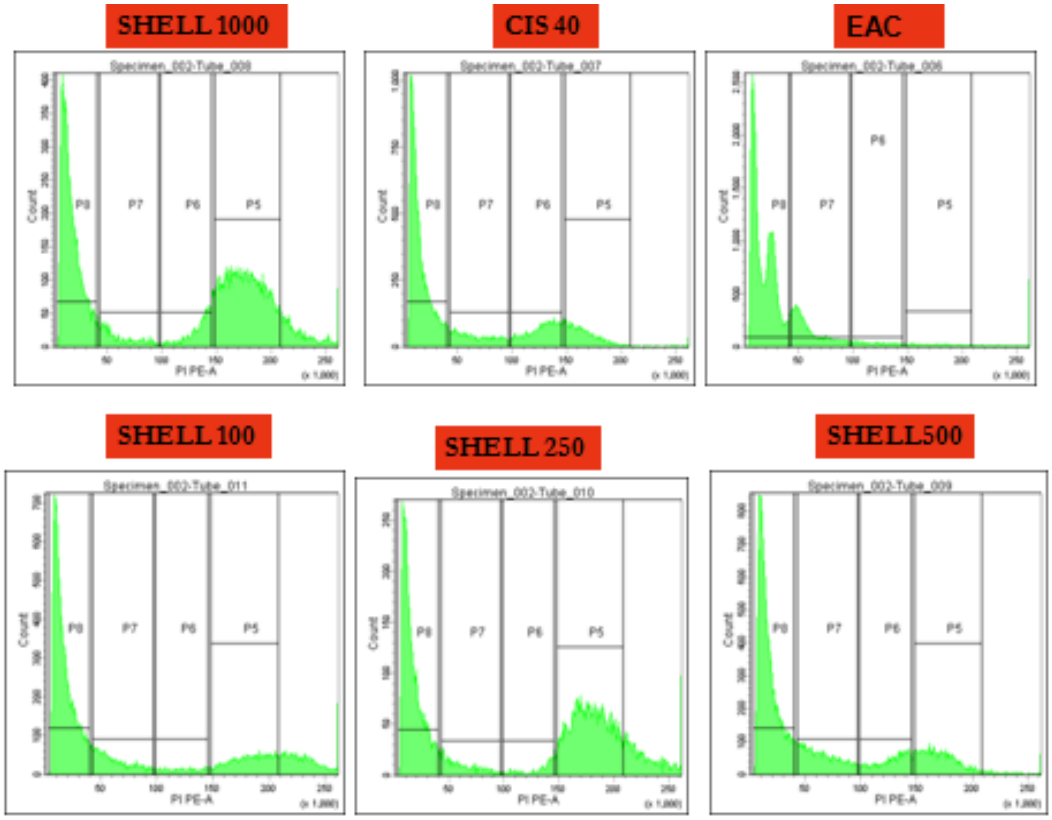

Fig.10 cell cycle analysis by flow cytometry of EAC cell treated with different concentrations of sepia ink

DISCUSSION

Cephalopods play an important role in marine ecosystem and are valuable to man as food, in biomedical research and proven to be a very rich source of extremely potent compounds that have significant anticancer activity (**Sherief *et al*., 2007, Guo-Fang *et al*., 2011** and **Jie-Ping *et al*., 2009**). The cephalopod ink extract have protective effect towards hemopoietic injuries from chemotherapeutics **(Okutani, 1976**). In the present study extract from squid ink and cuttle shell powder was tested for anticancer activity against EAC cells and the cells experienced a significant decrease in viability as the concentration increases. Theextract obtained from the internal shell of squid contains polysaccharides showed an antitumor activity against mouse sarcoma -180 was reported **(Okutani, 1976**). There are previous report on the cytotoxic activity of the methanolic extract obtained from flesh of *Sepia brevimana* and *Sepiella inermis* using Dalton's ascites and the cytotoxic activity was found to be *S.brevimana >S. innermis* **(Ilamparithi *et al*., 2011**) . The results of this study revealed the presence of bioactive compounds in extract of shell and ink of *S.officinalis* so it can be used as a source of drug in the treatment of cancer.

CONCLUSION

The results of this study shows that **a**mong the two extracts tested here methanol fraction from shell of *S.fficinalis* has less toxicity towards EAC cell line than the ink extract of *S. officinalis* and hence sepia are the best targets for cancer therapy. If their nature, structure and mechanism of action are explored they would be better drugs for site-specific chemotherapy

Both extract shows anticancer effect by:

- Decrease proliferation of cancer cells
- Decreasing number of viable cells
- Arresting cell cycle

Ink extraction show stronger anticancer effect than shell extracts in all used techniques.

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التأثير المعملى المضاد للسرطان لمستخلصات الحبر والصدفة الداخلية ل سيبيا اوفيسينياليس التي تقطن المياه المصرية

محمد لبيب سالم و محمد حسن منا و مني الجمل و ميرفت منصور و انجي غنيم

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أجريت هذه الدراسة لتقييم نشاط السرطان مستخلصات الحبر والصدفة الداخلية ل سيبيا اوفيسينياليس *S.officinalis* على حيوية الخلية بواسطة تقنية MTT، دورة حياه الخلية و الموت المبرمج في خلايا سرطان استسقاء ايرليش (EAC). تم دراسة أثر مستخلص الصدفة الداخلية ل سيبيا اوفيسينياليس علي خلايا السرطان باستخدام تركيزات مختلفة (١٠٠، ٢٥٠، ٥٠٠، ١٠٠٠ ميكروغرام/مل) بينما تأثير مستخلص الحبر للتركيزات التالية (١٠٠، ٧٥، ٥٠، ٢٥ ميكروغرام/مل). حيوية الخلايا ل مستخلص الصدفة الداخلية تراوحت بين ٢٣% (١٠٠٠ميكروغرام/مل) إلى ٧٦% (١٠٠ميكروغرام/مل)، فضلا عن حيوية خلية لمستخلص الحبر تراوحت بين ١٨ في المائة (١٠٠ميكروغرام/مل) إلى ٨٣ في المائة (٢٥ميكروغرام/مل). فحص MTT أظهرت أيضا ان حيوية الخلية تتغير وفقا لتركيزات مختلفة من مستخلصات الحبر والصدفة الداخلية بنسب تراوحت بين ٦١ في المائة (١٠٠ميكروغرام/مل) إلى ١٠٠% (٢٥ميكروغرام/مل) للحبر استخراج ومن ٦١ في المائة (١٠٠٠ميكروغرام/مل) إلى ٨٣ في المائة (١٠٠ميكروغرام/مل) للصدفة الداخلية. في خلايا سرطان استسقاء ايرليش (EAC) شهدت انخفاضا طفيفا في الحيوية مع التركيزات المنخفضه بينما حدث انخفاضا ملحوظا مع التركيزات الأعلى مقارنة بتأثيرالعقار المختار لعلاج هذا النوع من الاورام السرطانية المسمي سيسبلاتين بتركيزي (٤٠، ٢٠ميكروغرام/مل). كل من تقنيتي تحليل التدفق الخلوي لدورة حياة الخلية و الموت المبرمج للخلية لخلايا سرطان استسقاء ايرليش (EAC) مع التركيزات المختلفة لمستخلصات الحبر والصدفة الداخلية من س.اوفيسينياليس أكدت النتائج السابقة لتقنية MTT لدراسة حيويه الخلايا. النتائج التي تم الحصول عليها تؤدي إلى الاستنتاج لمستخلصات الحبر والصدفة الداخلية من س.اوفيسينياليس يمكن اعتبارها من المصادر الواعدة التي سينظر فيها في اكتشاف الأدوية لعلاج السرطان.