IN VITRO ANTITUMOR EFFECTS OF EGG EXTRACT AND PURPLE FLUID FROM MARINE *APLYSIA FASCIATA*

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ABSTRACT

Background: Although anticancer chemotherapy is effective, it is associated with significant side effects, as well as limited anti tumor efficacy, resulting in tumor recurrence. Novel agents with potent anti tumor effects are needed. One potential source could be products from marine animals since they possess agents with potential anticancer effect.

Aims: To test the potential in vitro anti tumor effects of egg extract and purple fluid from the marine mollusk *Aplysia fasciata*.

Methods: Ehrlich ascites carcinoma (EAC) cell line, a breast carcinoma, was cultured *in vitro* with different concentrations of egg extract (50, 100, 150, 200 and 250 µg/mL) and purple fluid (0.5, 1, 1.5, and 2 µg/ml) of *A. fasciata* for 24 hours. Cell cycle and apoptosis of EAC were analyzed by flow cytometry cell survival was analyzed by trypan blue exclusion and MTT assays. Cells incubated with media were used as negative control, while cells treated with anticancer drug cisplatin (CIS) were used as positive control.

Results: Incubation of EAC cells with egg extract and purple fluid resulted in significant decreases in the number and survival of EAC cells associated with increases in the cell apoptosis in a dose dependant effects. Of interest, high doses of egg extract (250 µg/ml) and purple fluid (2 µg/mL) induced 45% and 38% decrease in the EAC cell numbers as compared to control positive while EAC cells treated with cisplatin induced (25%) decrease in EAC cell number as compared to control positive. **Conclusion**: the results of this pilot study indicate that both egg extract and purple fluid of *A. fasciata* possess potential anti tumor effects. Further *in vitro* and *in vivo* studies are required to analyze the anti tumor effect of these agents.

Key words: *Aplysia fasciata*, Apoptosis**,** bioactive materials, Ehrlich Ascites carcinoma, flow cytometry.

INTRODUCTION

Although anti-cancer chemotherapy is effective to kill cancer cells, it associates with significant side effects (Airley, p. 265). Additionally, cancer cells may develop resistance to the chemotherapeutic *drugs* **(Brydøy M. *et***

al.*, 2007*). As such searching for new agents with potential anti-cancer effects is of paramount significance.

Several natural products from microorganisms, plants and animals have been found to be rich in potential anti-tumor effects ***(*Strobel G, Daisy B 2003).*** *Marine organisms, because of their accessibility and their therapeutic* applications for many other diseases, are of interest. With this regard, several studies have identified and isolated some compounds with antitumor activities **(Kisugi *et al.*, 1987)**. Earlier studies have reported that sea hares, Opistobranch mollusks, have been found to contain biologically active compounds (Falkner et al., 1973; Kinnel et al., 1977; Yamamura and Terada, 1977). Similarly, *Aplysia fasciata,* one of the sea hares genus, has multiple chemical defenses to deter predators. The passive chemical defenses of *A. fasciata* are present in the skin, thus producing a distasteful surface to predators, and many of these deterrent compounds have been identified (**Kinnel et al., 1979).** The active chemical defenses are released from *A. fasciata* only upon predatory attack. It includes secretions from two separate glands. The ink gland secretes ink, which is generally a bright purple fluid. The opaline gland secretes opaline, which is a whitish and extremely viscous substance. These two secretions are released into the mantle cavity of the animal and pumped out of the siphon toward the attacker **(Walters and Erickson, 1986)**. Besides the gland secretions, a 250 kDa glycoprotein named aplysianin E was purified from the egg of *A. kurodai* which exhibited anticancer activity against some marine and human tumor cell lines in vitro and in vivo in mice **(Kisugi *et al.*, 1987).** Another 320 kDa glycoprotein was also isolated from the albumen gland of *A. kurodai* with anti-tumor activity **(Takamatsu et al., 1995)**

Given the potential anti-tumor effects of secretions from *A. fasciata*, this study was aimed to assess the antitumor effects of purple fluid and egg extract of *Aplysia fasciata* inhabiting the Egyptian water. Studies showed that these secretions possess direct anti-tumor effects on EAC cells in vitro through induction of cells apoptotic as well as anti proliferating effects.

MATERIALS AND METHOD

Animal:

Aplysia fasciata (commonly named as sea hare; ranging in weight from 200 g to 450 g) was collected from the Mediterranean coast of Alexandria, Egypt in the summer during the spawning season (June and July 2015). Animals were kept in aquarium and maintained at 17°C and fed two or three times weekly with *Ulva lactuca* collected along with animals, stored frozen and then thawed before use.

Reagents and chemicals:

- Isopropanol

- MTT solution, (Sigma, U.S.A.)
- Phosphate buffered saline (PBS)
- trypan blue, (Sigma, U.S.A.)
- Trypsin-EDTA
- FITC Annexin V apoptosis (Apoptosis Detection Kit II; Cat. No 556570; BD Bioscience, U.S.A)
- RNAse, (BD Bioscience, U.S.A)
- 70% ethanol
- RPMI 1640 culture medium with L-glutamine (life technologies)
- Fetal bovine serum (FBS), (life technologies)
- Penicillin-streptomycin solution, (Sigma, U.S.A.)

Harvesting the purple fluid from *A. fasciata*:

The purple fluid was obtained by disturbing the animals by extreme change in temperature from 25˚C to -20C for 5 minutes and was collected in sterile falcons and then frozen directly at -80°C until use. Aplysianin P, which has been reported to induce tumor lysis **(Yamazaki et al., 1989**), is the major active ingredient of this purple fluid of the sea hare *Aplysia kurodai*.

Preparation of egg extract of *Aplysia fasciata*:

Eggs of were collected from fresh *A. fasciata* and stored directly at -80°C until use. Before use, egg masses were thawed at room temperature and homogenized with 2 volumes of 0.9% saline for 10 minutes. The homogenate was then centrifuged at 10,000 rpm for 30 minutes. The supernatant was collected and then re-centrifuged at 40,000 rpm for 60 minutes to obtain a clear supernatant, which was used as starting material for our experimental studies. The egg mass has a moisture content of 91 %, 0.85 % fat 2.85 % protein %, ash of 3.43 % ash and 1.77 % carbohydrates **(Ador R. Pepito et al., 2015)**

Cell lines:

Ehrlich ascites carcinoma (EAC) cell line was originally obtained from the National Cancer Institute (Cairo University, Egypt). EAC cells were collected from donor mouse on the eight day of tumor growth and were suspended in sterile isotonicsaline, the viable EAC cells were counted by using trypan blue method and were adjusted at 1x106 EAC cells before their culture in vitro.

Cell culture:

EAC cells were cultured in RPMI 1640 culture medium with L-glutamine and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. Cultured cell line was incubated for 24 hours at 37ºC in the presence of 5% CO_2 (**Keivan Zandi et al., 2007)**. EAC cells were incubated with different concentrations of egg extract (50, 100, 150, 200and 250 µg/ml) or with purple fluid (0.5, 1.0, 1.5 and 2.0 µg/ml). Total cell

number was counted by haemocytometer and cell viability was assessed by trypan blue exclusion assay (**Morgan and Darling, 1992**).

MTT assay:

Growth of cancer cells was assessed in vitro by MTT assay **(He QJ *et al*.** 2005**).** EAC cells **(**2 x 105/well) were seeded in a 96-well micro-plate. Cells were cultured in 180 µl complete RPMI-1640 media for 24 h. Different concentrations of egg extract and purple fluid as mentioned above were added to the culture. After 24 h, MTT solution (100 µl, 0.5 mg/ml) was added to each well and the cells were incubated for another 4h. Then, 100 µl acidified isopropanol was added to each well and the plate was gently agitated until the color reaction was uniform. The optical density of each well was read by the ELISA reader in 540 nm (**Van de Loosdrecht *et al*., 1994**)

The % of cell viability=Absorb. of treated cells/Absorb. of cont. cells ×100%

Measuring apoptosis by annexin-V assay:

Annexin V assay was used to detect EAC cell according to the manufacturer's protocol. After 24 hours treatment with purple fluid and egg extract of *A. fasciata*, the cells were re-suspended in 1× Annexin-V binding buffer and then incubated with Annexin V-FITC for cellular staining in dark. The cells were then acquired by BD FACSCanto II flow cytometer (BD (Becton, Dickinson Company), U.S.A) and data was analyzed by BD FACS Diva software (BD Bioscience, U.S.A).

Cell cycle analysis:

Cell cycle analysis was performed to evaluate the effect of purple fluid and egg extract of *A. fasciata* on the distribution of tumor cells in G1, S and G2/M phases of the cell cycle. This test was performed by flow cytometry after DNA staining to reveal the total amount of DNA. Approximately, $1x10^6$ cells/well of EAC tumor cells were cultured in 6-well plate in the presence of the tested products used at their different concentrations. After 24 h of incubation, cells were collected, washed with PBS, fixed with cold 70% ethanol and kept at -20°C overnight. After washing cells twice by adding 2 ml cold PBS (1800 rpm, 5 min), the supernatant was discarded, cells were stained with a solution containing 300 µg/mL of PI/ triton X 100 staining solution (1000µl of 0.1 % triton+ 40µl PI + 20µl RNAse)**.** The samples were analyzed using BD FACSCanto II flowcytometer(BD (Becton, Dickinson Company), U.S.A) and data was analyzed by BD FACS Diva software **(**BD (Becton, Dickinson Company), U.S.A)**.**

RESULTS AND DISCUSSION

Effects of egg extract and purple fluid on EACcell count and viability:

Changes in count and cell viability of EAC cells after incubation with different concentrations of purple fluid and egg extract were measured by counting cells. Overall, incubation of EAC cells with purple fluid (Fig. 1) and

egg extract (Fig. 2) induced decreases in number of EAC cells as well as their viability as compared to EAC cells incubated with medium alone. With regard to purple fluid, its at concentrations of 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml induced decreases in cell number from 796 x 10ˆ6 to 71.6x10^6, 69.86x10ˆ6, 61.46x10ˆ6, 53.76x10ˆ6, respectively, as compared to control positive EAC cells (treated with CIS) (20x10ˆ6). With regard to egg extract at concentrations of 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml induced decreases in cell numbers from 796 x 10ˆ6 to 69.6 x10ˆ6, 65.4 x10ˆ6, 61.4 x10ˆ6, 58.2 x10ˆ6, 55.1 x10ˆ6, respectively, as compared to control positive EAC cells treated with CIS (20x10ˆ6). You have to write the effects of CIS as compared to negative control.

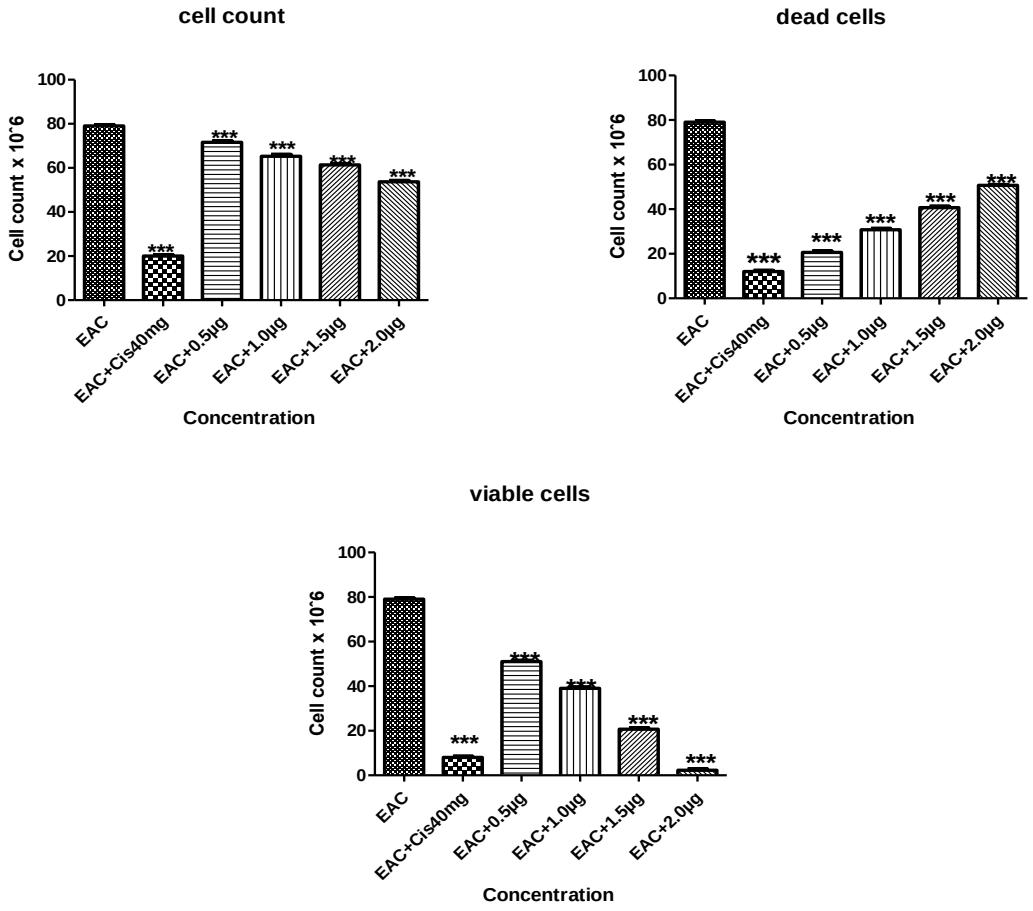
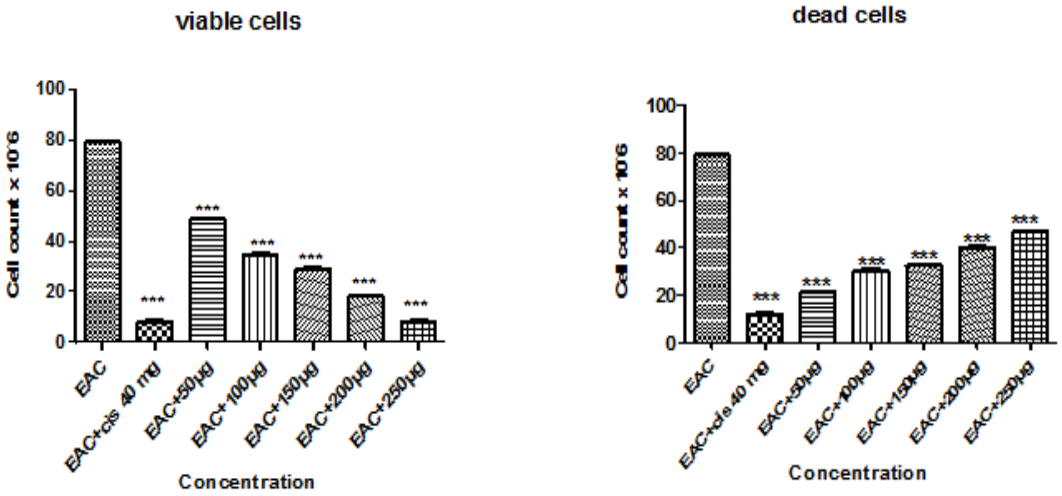


Figure 1: Change in cell count according to trypan blue assay including viable and dead cells after treatment with purple fluid

Crude purple fluid and egg extract against EAC cell line using MTT assay:

Anti-proliferative activity of different concentrations of purple fluid and egg extract against EAC cells was assessed by measuring by MTT assay. Incubation of EAC cells with purple fluid (Fig. 3) and egg extract (Fig. 4) induced inhibitory effect on number of cells of EAC cells as compared to

EAC cells incubated with medium alone. With regard to purple fluid at concentrations of 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml, it induced decreases in cell proliferation by 68% , 48% , 43% , 38%, respectively, as compared to (98%) for control (CIS-treated EAC cells) positive condition. , with regard to egg extract at concentrations at 50 µg/ml , 100 µg/ml , 150 µg/ml , 200 µg/ml , 250 µg/ml, it induced decreases in the cell proliferation by (69.5% , 65% , 60% , 50% , 45%, respectively, in as compared to (98%) for control positive



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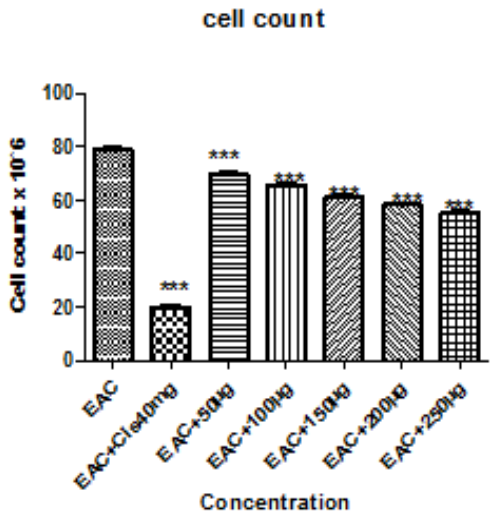


Figure 2: Change in cell count according to trypan blue assay including viable and dead cells after treatment with egg extract

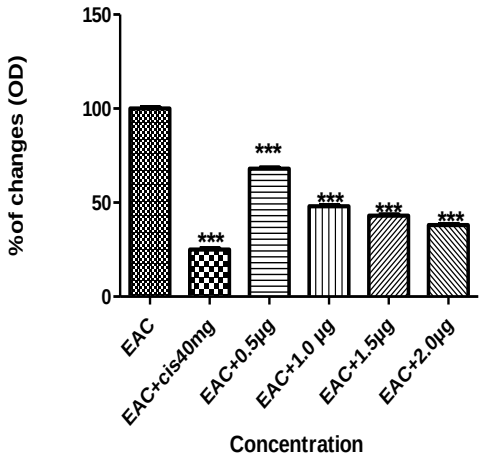


Figure3: Anti-proliferative activity of of purple fluid of *Aplysia fasciata* against EAC cells

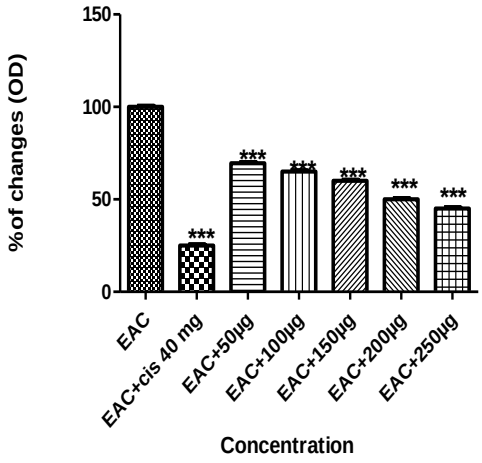
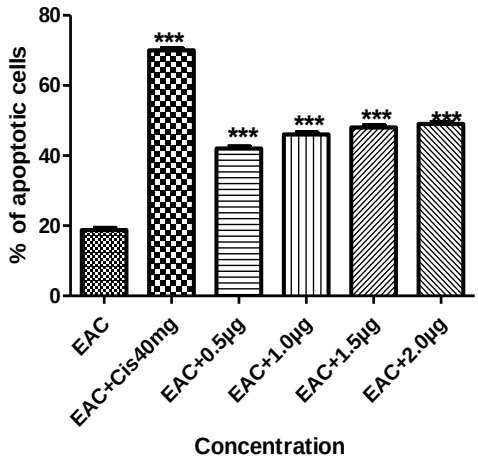


Figure 4: Anti-proliferative activity of egg extract of *Aplysia fasciata* against EAC cells

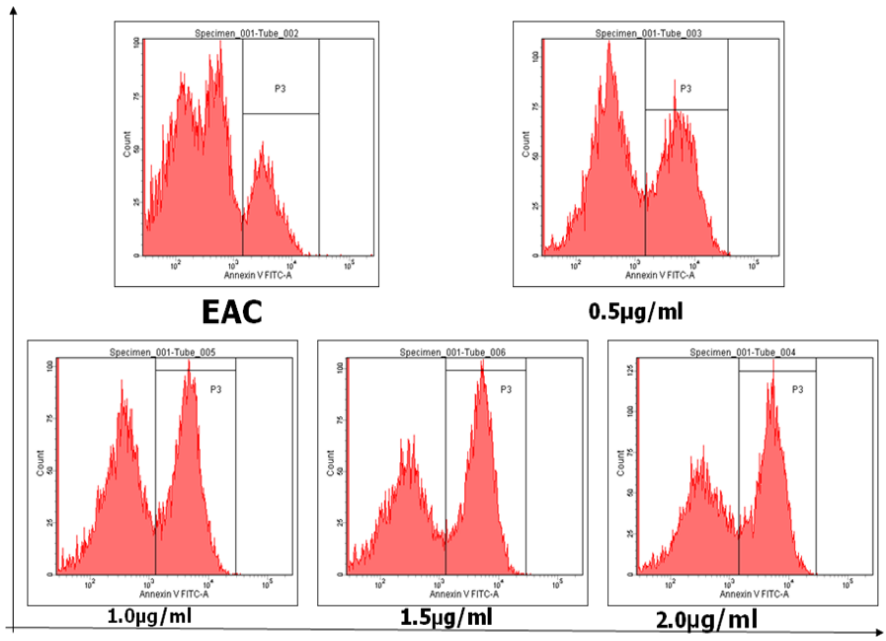
(3) Effect of purple fluid and egg extract on EAC cell apoptosis measured by annexin-V assay:

Incubation of EAC cells with 40ug/ml CIS induced70% increases in EAC cell apoptosis as compared to PBS-treated EAC cells (Figure 5 and 6). Incubation of EAC cells with either purple fluid or egg extract at different concentrations induced significant apoptosis of EAC cells in a dose-dependent manner. With regard to purple fluid at concentrations of 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml, it induced increases in cell apoptosis by 42%, 46%, 48%, 49%, respectively as compared to CIS-TREAD cells (Fig. 5A and B). With regard to egg extract at concentrations of 50 µg/ml, 100 µg/ml, 150

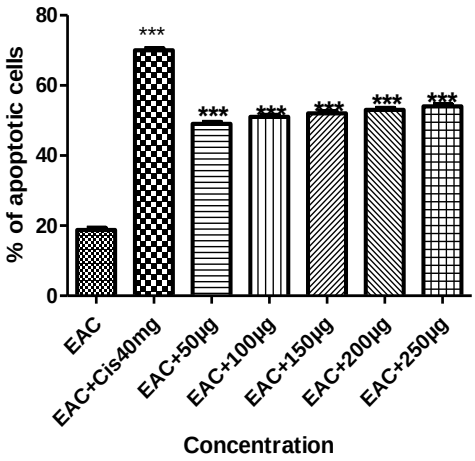
µg/ml, 200 µg/ml, 250 µg/ml, it induced increases in cell apoptosis by 49%, 51%, 52%, 52.7%, 54%, respectively, as compared to CIS-treated EAC cells (Fig. 6 A and B).



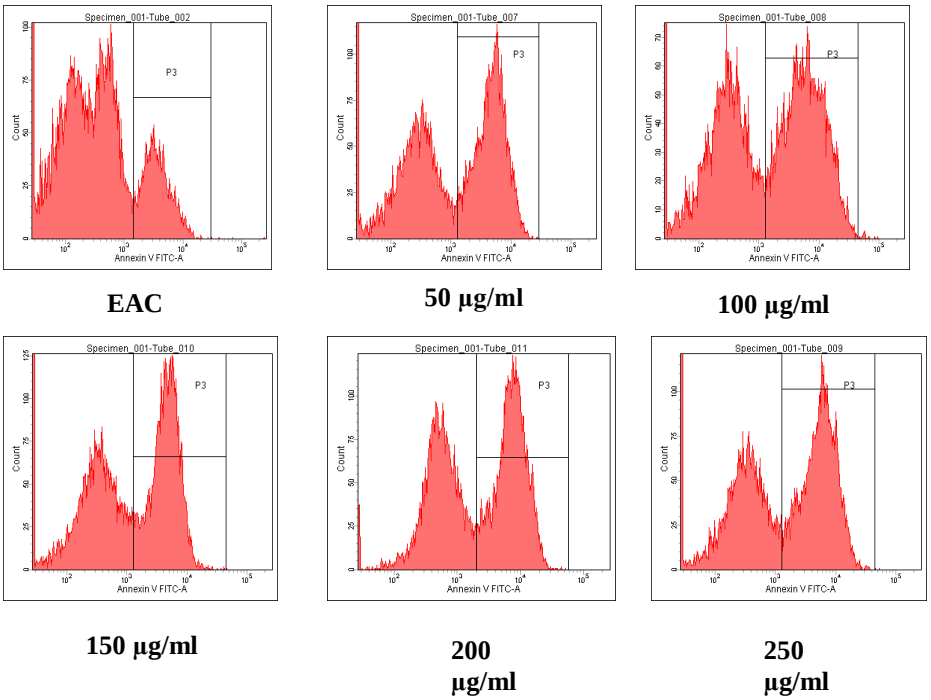
(Figure 5, a): Effect of purple fluid on EAC cell apoptosis rate



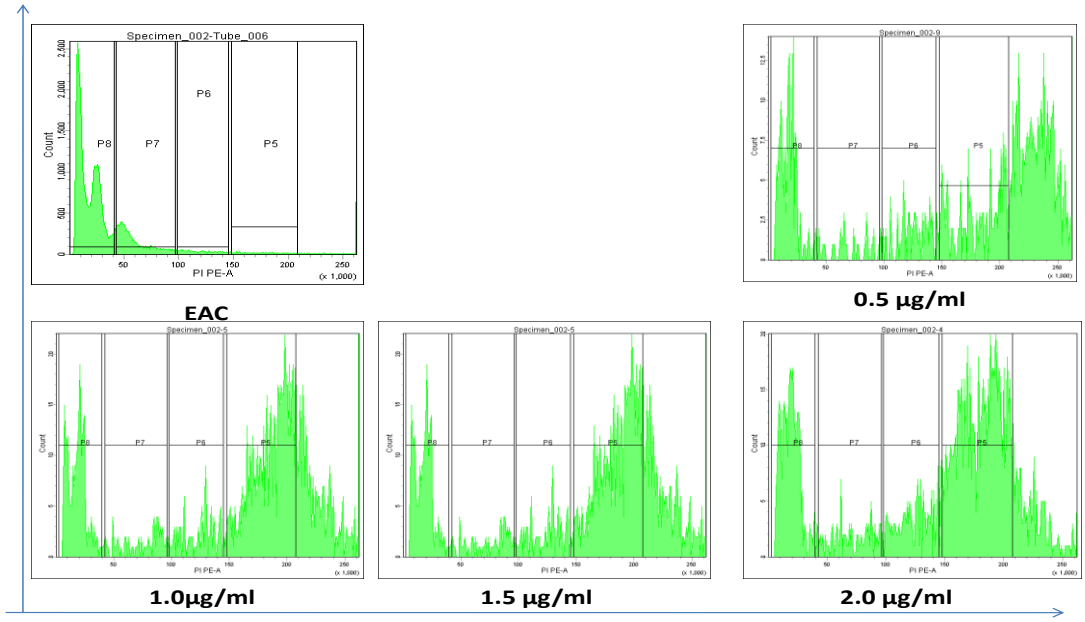
(Figure 5, b)



(Figure 6, a): Effect of egg extract on EAC cell apoptosis rate



(Figure 6, b)



(Figure 7) Cell cycle analysis

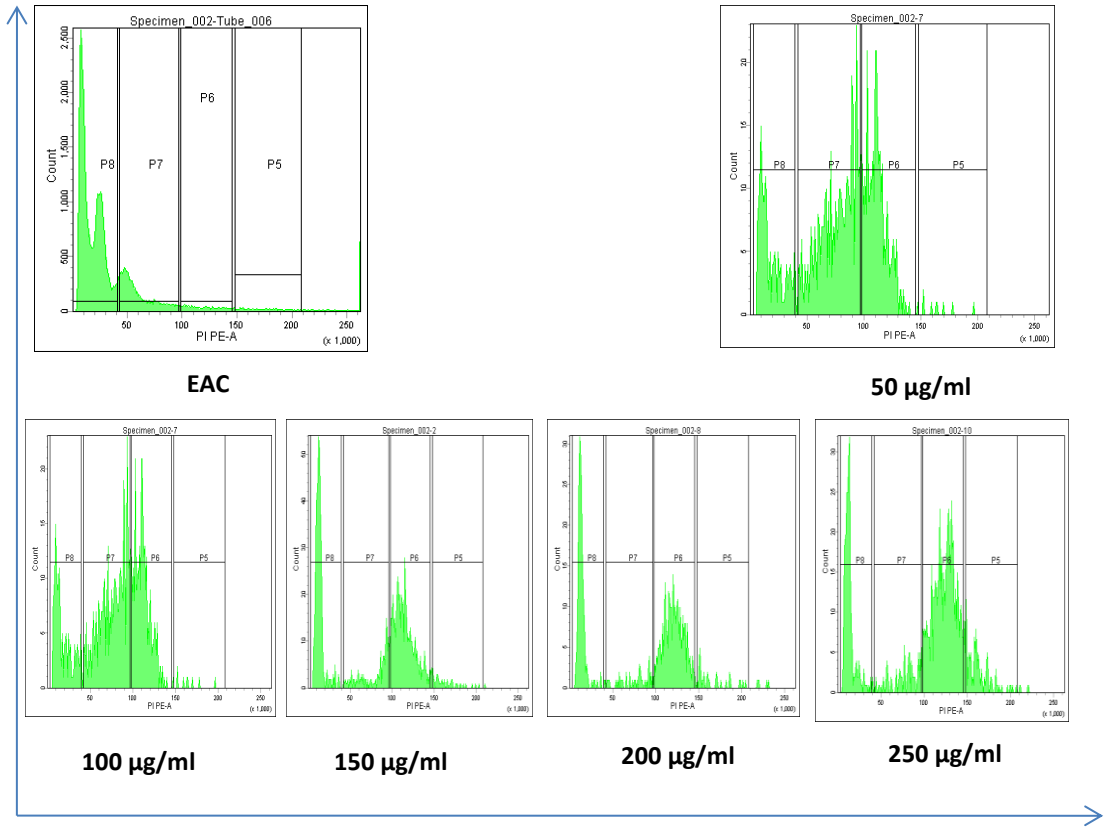


Figure 8

CONCLUSION

In this study *A.fasciata* was searched for in the Mediterranean coast of Alexandria. In a study on *A. fasciata*, two kinds of natural products were identified which had cytotoxic effects on some cancer cell lines (Ehrlich Ascites carcinoma).

Based on the results of this study both purple fluid and egg extract of *A.fasciata* exhibited the growth of tumor cells.As shown in figure (1), (2) the increase in concentration of crude purple fluid and egg extract of *A.fasciata* respectively showed increased in the inhibitory effect of EAC cell line according to MTT assay.

And also increasing in cytotoxic effect depending on increased dose of crude purple fluid and egg extract of A.fasciata as shown in figure (3),(4) respectively according to trypan blue assay.

And for confirmation of the results apoptosis by annexin V assay was performed by flowcytometry at center of excellence for cancer research(CECR), Tanta university and the test confirmed the above results as by increasing the concentration of crude purple fluid and egg extract of *A.fasciata* showed increasing in apoptosis as shown in figure (5), (6) respectively. In conclusion, purple fluid and egg extract of *A. fasciata* have inhibitory effect on EAC cell line.

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التأثير المناعي و المضاد للسرطان لمستخلص من ارنب البحر القاطن بـالمياه المصرية باستخدم نموذج فئران تجريبي

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خلفية: على الرغم من أن العلاج الكيميائي المضاد للسرطان فعال الا انه يرتبط مع آثار جانبية كبيرة، فضلا عن محدودية فعاليتة المضادة الورم، مما قد يؤدى إلى عودة الورم.لذلك فاننا بحاجه الى عوامل جديده ذات تأثيرات مضاده للاورام و اكثر فاعليه ، و احد هذه المصادر يمكن أن تكون المنتجات من الحيوانات البحرية لأنها تمتلك مواد ذات تأثير مضاد للسرطان محتمل.

الأهداف:لاختبار التأثير المعملى المضاد للأورام من مستخلص البويضات و السائل الأرجوانى الذى تم الحصول عليهم من أرنب البحر *Aplysia fasciata* .

الطريقه: (EAC) هو الخلايا السرطانيه المستخدمه فى هذه التجربه، تم زراعه تركيزات مختلفه من مستخلص البويضات (٥٠، ١٠٠، ١٥٠، ٢٠٠ و ٢٥٠ ميكروغرام / مل) والسائل الأرجواني (٠,٥، ١، ١,٥، و: طرق ٢ ميكروغرام / مل) من *A. fasciata* لمدة ٢٤ ساعة. وقد تم تحليل دورة الخلية وموت الخلايا المبرمج للEAC التدفق الخلوي تم تحليل بقاء الخلية بواسطه trypan blue and MTT assay. استخدمت الخلايا المحتضنة مع الوسط المستخدم للزراعه ك control negative، بينما تم استخدام الخلايا المعالجه بسيسبلاتين المضادة للسرطان (CIS)، ك control positive.

النتائج: أسفرت الحضانة من الخلايا مع مستخلص البيضات والسائل الأرجواني في انخفاض كبير في عدد وبقاء خلايا EAC يرتبط مع زيادة في موت الخلايا المبرمج في جرعة والآثار التابعة. من الفائدة، والجرعات العالية من استخراج البيض (٢٥٠ ميكروغرام/مل)، والسائل الأرجواني (٢ ميكروغرام/مل) التي يسببها ٤٥٪ و ٣٨٪ انخفاضا في أعداد الخلايا EAC مقارنة ب control positive في حين أن الخلايا التى تم معالجتها بسيسبلاتين الناجم عن (٢٥٪) انخفاض في عدد الخلايا EAC بالمقارنة مع control positive. الخلاصة: نتائج هذه الدراسة التجريبية تشير إلى أن كلا من استخراج البيض والسائل الأرجواني من A. fasciata تمتلك اثار مضاده للاورام. وعلاوة على ذلك في التجارب المختبرية والدراسات المجراة هناك حاجة لتحليل تأثير مضاد الورم من هذه العوامل.