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RESEARCH ARTICLE

Potential antitumor effects of egg extract and purple fluid from marine *Aplysia fasciata* against experimental Ehrlich ascites carcinoma

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ABSTRACT

Background: The wide biodiversity of marine animals provides a rich source of potential bioactive materials with biomedical applications. **Aim**: This study aimed to test the *in vitro* and *in vivo* anti-tumor effects of the purple fluid and egg extracts from *Aplysia fasciata* inhabiting the Egyptian marine water. **Methods**: The cytotoxicity of both the purple fluid and egg extracts were analysed *in vitro* against Ehrlich ascites carcinoma (EAC) cells by the trypan blue exclusion, MTT reduction assay and flow cytometry. The anti-EAC immunological effects of both extracts were assessed by blood counting and flow cytometry in mice challenged with EAC cells. **Results**: *In vitro* treatment of EAC cells with egg extract and the purple fluid caused significant decreases in the number and viability of EAC cells associated with increases in EAC apoptosis in a dose-dependent manner as compared to untreated EAC cells. *In vivo,* both the purple fluid extract (20µg/ mouse) and egg extract (6.7 µg/mouse) significantly reduced the numbers of EAC cells by 4.3 and 2.3 folds, respectively. However, the anti-tumour effects of the extracts either *in vitro* or *in vivo* were yet lower than that of the anti-tumor reference drug cisplatin. Both extracts induced significant increases in the levels of relative and absolute numbers of lymphocytes, mature macrophages ($CD11b^+$ $Ly6G^-$) while with minimal effects on immature neutrophils ($CD11b^+$ $Ly6G^+$). **Conclusion**: Both egg extract and the purple fluid of *Aplysia fasciata* possess potential anti-tumour effects with less toxicity, opening new avenues for further evaluation of the chemical and biological mechanisms behind these effects.

Keywords: *Aplysia fasciata*, Apoptosis, Bioactive marine extracts, Ehrlich ascites carcinoma, Egg extracts, Purple fluid extract

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INTRODUCTION

Conventional chemotherapeutics seems to be effective against cancer cells. However, they are usually associated with serious side effects such as leucopenia. Moreover, cancer cells consistently develop drug resistance. So, the search for new agents with potential cancer effects became of a paramount significance (Housman *et al.,* 2014). Several natural products from microorganisms, plants and animals are rich in anti-tumor agents *(*Cragg and Newman, 2013). Marine organisms are of interest because they have therapeutic applications for many diseases e.g. Vira-A a potent antiviral drug extracted from the sponge. With this regard, several studies have identified and isolated some compounds with potential antitumor effect from marine organisms (Kisugi *et al*., 1987).

Earlier studies have reported that sea hares contain several biologically active compounds (aplysinine E and aplysinine P) (Falkner *et al*., 1973; Kinnel *et al*., 1977; Yamamura and Terada, 1977). *Aplysia fasciata,* which inhabits the Egyptian water, is one of the sea hares genera which has multiple chemical defences against predators. The passive chemical defences of *Aplysia f.* are found in the skin, producing a distasteful surface to predators (Kinnel *et al*., 1979).

The active chemical defences are released from *Aplysia* only upon predatory attack. It includes secretions from two separate glands. The ink gland secretes ink, which is a bright purple fluid. The other is an opaline gland which secretes opaline; 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, this gland secrets from glycoprotein named aplysianin E was purified from the egg of *Aplysia kurodai* (Takamatsu *et al*., 1995).

Therefore, the present study was designed to detect the ability of purple fluid and egg extracts of *Aplysia fasciata* inhabiting the Egyptian water to induce the anti-cancer effect.

MATERIALS AND METHOD

Sample collection

Aplysia fasciata (8 specimens) were collected from the Mediterranean coast of Alexandria, Egypt in the summer during the spawning season (June and July 2017). They were kept in an aquarium and maintained at 17°C and fed two-three times weekly with *Ulva lactuca* collected along with animals. The snails were then stored frozen at -80°C and then thawed before use.

Preparation of the crude purple fluid extract from *Aplysia fasciata*

The purple fluid was obtained by disturbing the animals with a sudden drop in temperature from 25˚c to -20˚c for 5 minutes. The extracted fluid was then frozen directly at -80°c until use (Yamazaki *et al*. 1989, Zandi *et al.,* 2007).

Preparation of crude egg extract of *Aplysia fasciata*

Eggs were collected from the side of the aquarium 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 min. The homogenate was then centrifuged (Beckman J2-21C centrifuge) at 10,000 rpm for 30 min. The supernatant was collected and centrifuged at 40,000 rpm for 60 min to obtain a clear supernatant for experimental use (Kisugi *et al.,* 1987).

Mice

Adult female Swiss albino mice (CD1 strain; weighting 20±2 g, 10-12 weeks) were purchased from the National Research Centre, Cairo, Egypt. Mice were housed (5 animals per cage) at the animal facility, Zoology Department, Faculty of Science, Tanta University, Egypt in accordance with the ethical guidelines of local Institutional Animals Care and Use Committee at Faculty of Science, Tanta University, Egypt. Mice were divided into seven groups (n= 8/group) including one control group.

Ehrlich Ascites Carcinoma (EAC) cell

Ehrlich Ascites Carcinoma (EAC) cell line was originally obtained from the National Cancer Institute (Cairo University, Egypt). EAC cells were maintained by serial intraperitoneal transplantation of $2.5xl0^6$ cells /0.3 ml saline in CD1 mice. Donor CD1 mice were left for 10 days until ascites is formed. EAC cells were harvested under sterile conditions then purified to be used later for *in vivo* and *in vitro* studies. (Geran *et al.,* 1972)

Assessment of cell viability by trypan blue

Cells were cultured in RPMI 1640 medium with L-glutamine and supplemented with 10% FBS and 1% penicillin-streptomycin at 37^0C, 5% CO_2 (Bioscience, SanDiego, CA, USA) for 48 hours in 6 well-plate at a density of $2.5x10^5$/ml. Then, subconfluent cells were treated with either PBS or different concentrations of egg extract (50, 100, 150, 200, 250 µg/ml) (Kisugi *et al.,* 1987) or purple fluid (0.5, 1, 1.5, 2 µg/ml) for 24 h (Zandi *et al.,* 2007). Also, cisplatin (40 mg/ml) was diluted and used at a final concentration of (40 µg/ml) for in vitro studies. EAC cells were harvested, washed and counted by haemocytometer and then was assessed for viability by trypan blue exclusion assay (Morgan and Darling, 1992)

Assessment of apoptosis by flow cytometry

Treated EAC cells were stained with FITC Annexin V (Apoptosis Detection Kit II; Cat. No 556570 BD Bioscience, U.S.A) according to the manufacturer instructions. Data were obtained and analysed by BD FACSCanto II flow cytometer (BD Becton, Dickinson Company, U.S.A) and BD FACS Diva software (BD Bioscience, U.S.A) respectively.

MTT assay

EAC cells were cultured in a 96-well plate at a density of 2 x 10^5/well. Then, cells were treated with PBS or different concentrations of egg extract or purple fluid for 24 hours as described before. 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 at 540 nm (Van de Loosdrecht *et al*., 1994, Mitry *et al.,* 2005). The viability of cells was calculated according to the following equation (The OD measurements for control wells were considered to correspond 100% growth, their relative OD then calculated the percentage growth in other wells).

The % of cell viability = Absorbance of treated cells ×100% / Absorbance of control cells

Cell cycle analysis

EAC cells were cultured in 6-well plate and incubated using different concentrations of purple fluid and egg extracts as mentioned above for 24 hours. Cells were harvested then washed with PBS twice then fixed and incubated with cold 70% ethanol for 2 h. After washing with cold PBS, cells were centrifuged 1800 rpm, 5 min at 4°C then resuspended with staining buffer (1 ml of 0.1 % triton+ 40 µl propodium iodide (PI) + 20 µl RNase), protected from light and incubated overnight at 4°C. Cell cycle analysis was performed and analysed using BD FACSCanto II flow cytometer and BD FACS Diva software respectively. (Darzynkiewicz, *et al.*, 2011)

Tumour challenge and *in vivo* study design

Naïve CD1 mice were injected intraperitoneal with 2.5 x 10^5 EAC cells/mouse. Control group was treated with 200 µL PBS. Two groups of mice were injected with 6.7 and 3.3 µg/mouse of egg extract. Another two groups were treated with 10 and 20 µl /mouse of the crude purple fluid extract. Cisplatin (40mg/mL) was obtained from Mylan Co., France. Then, diluted with saline to be used at a concentration of (2 mg/kg) for each dose. Treatment was administrated by intraperitoneal injection; one day after tumour challenge, 4 times a week for 10 days as in Figure 1. (Kisugi *et al.,* 1987; Melo *et al.,* 1997).

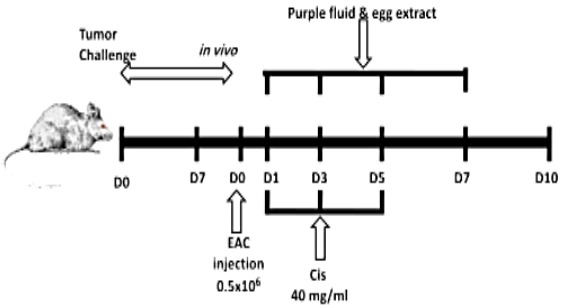

Figure 1. Outline of the experimental design depicted to show the tumor challenge and treatment protocols.

Complete blood count analysis

Blood samples were collected from periorbital sinus under anaesthesia using heparinized microhematocrit tubes. The total and differential leukocytic count was estimated using an automated instrument for complete blood counts (VetScan HM2™ Hematology System, Abaxis, and Union City, CA, USA).

Preparation of spleen cell suspensions

Spleen cell suspensions were prepared according to Salem *et al*. (2007). Briefly, the spleen was homogenized by gently pressing the organ between the rough ends of two glass slides and filtered through nylon mesh filters (100 µm; BD Biosciences, CA, USA). The cells were suspended in PBS (Sigma Chemical Co., St. Louis, USA) and washed twice. Red blood cells were lysed with ammonium chloride_potassium buffer (ACK; Invitrogen, Carlsbad, CA, USA) and the remaining cells were again washed 3 times and counted. Viability was determined by trypan blue exclusion and consistently exceeded 90%.

Analysis of myeloid cells in spleens

Prepared splenocytes were counted and stained with anti-mouse PE-CD11b, FITC-Ly6 antibodies (eBioscience) for 30 min at dark incubation 4°C. The cells were washed twice with PBS and then re-suspended in 0.3 mL of PBS supplemented with 0.5% BSA and 0.02% sodium azide. Cells were then washed and acquired by Partec flow cytometer (Sysmex-Partec Company, Germany). Data were analysed by FlowJo software (Treestar, Ashland, OR, USA).

Assessment of liver enzymes

ALT and AST were assayed by using the commercial kit supplied by Rndox, from Egypt. ALT was estimated according to the method of Retman and Frankel (1957).

Statistical analysis

Numerical data obtained from each experiment were expressed as a mean ± standard error and the statistical differences between the control or diseased with the experimental groups were assessed using one-way analysis of variance (ANOVA) at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons in Graph Pad prism 5.

RESULTS

The effect of the sea hare purple fluid and egg extract on cell count using trypan blue assay

Incubation of EAC with different concentrations of the purple fluid (0.5, 1, 1.5, 2 µg/ml) for 24 h significantly decreased cell viability compared with untreated EAC cells in a dose-dependent manner (Figure 2A). In the same way, Incubation of EAC with different concentrations of eggshell extract (50, 100, 150, 200, 250 µg/ml) also significantly abrogated EAC cell viability compared to untreated cells in a dose-dependent manner. (Figure 2B). The highest doses of both extracts produced nearly the same effect on the viability of EAC compared to the reference drug cisplatin (Supplementary Table 1).

The cytotoxic effect of crude purple fluid and egg extract against EAC cell line using MTT assay

Our results showed that both extracts significantly diminished the proliferative activity of treated cells compared to untreated cells in a dose-dependent manner. However, their anti-proliferative was relatively not as potent as the effect induced by cisplatin (Figure 3A, B; Supplementary Table 1).

Effect of both extracts on EAC cells apoptosis rate using annexin V assay

In the same context, incubated EAC cells with different concentrations of the purple fluid and eggshell extracts showed increased apoptotic rates. Both extracts significantly enhanced apoptosis in treated EAC cells compared to untreated cells. However, cisplatin (40 mg/ml) still has a stronger effect on induction of apoptosis in cisplatin-treated EAC cells (Figure 4; Supplementary Figure 1).

DNA content and cell cycle of EAC cells after treatment with Cisplatin, the purple fluid and egg extracts

EAC cells incubated with different concentrations of the purple fluid (0.5, 1, 1.5, 2 µg/ml) induced a significant decrease in DNA replication (S phase cells) compared to untreated EAC cells relatively even more than cisplatin. Surprisingly, the purple fluid extract was incapable to induce cell cycle arrest or increase the number of G^0 cells (Figure 5A; Supplementary Figure 2A).

On the other hand, treatment with eggshell extract has significantly diminished the number of proliferating cells (S phase) in a dose-dependent manner. Eggshell extracts at a concentration of 200 and 250 µg/ml induced a similar effect as cisplatin the reference drug. However, it was incapable to induce cell cycle arrest (G^0 cells) compare to the untreated EAC cells and cells treated with cisplatin (Figure 5B; supplementary Figure 2B).

Antitumor effects of different doses of egg extract and the purple fluid on EAC growth *in vivo*

High and low doses of the eggshell extract and a high dose of the purple fluid extract produced a significant decrease in the number of EAC harvested cells compared to the control group (Figure 6). Our data reported that treatment with low and high doses of the purple fluid extract significantly decreased the number of harvested EAC cells by 1.09 and 4.3 folds respectively. Meanwhile, treatment with low and high doses of egg extract managed to decrease the total count of EAC cells by 2.3 and 2.8 folds respectively. However, these effects were much lower than the effect of cisplatin that decreased the number of harvested cells by 18 folds (Supplementary Table 2).

(A)

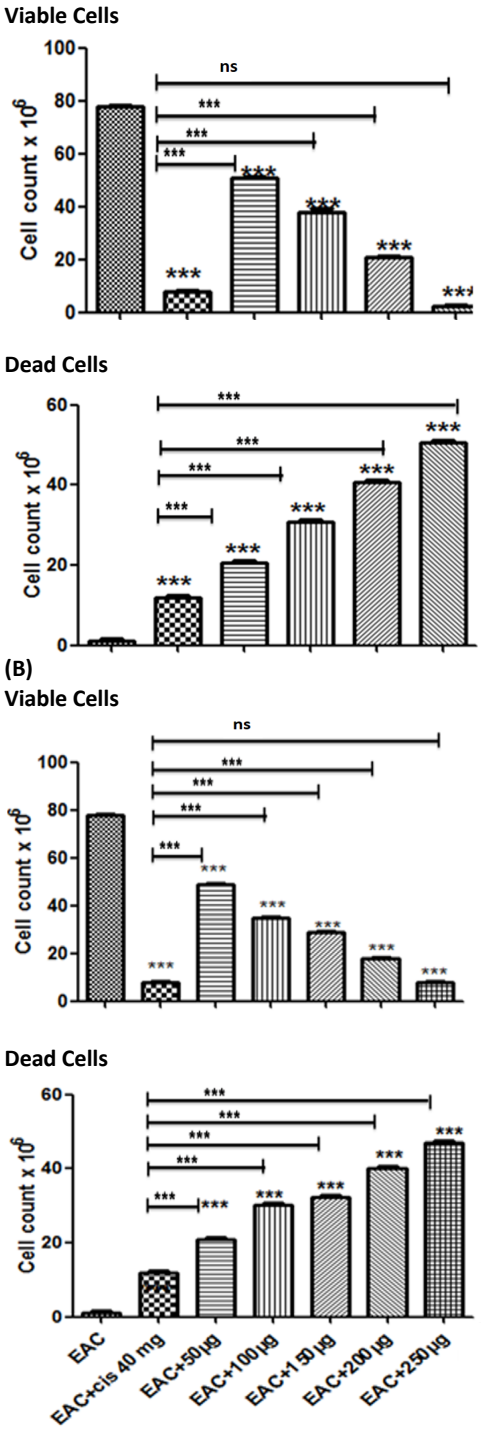

Figure 2. The yield of the viability of EAC Cells counts by trypan blue: (A) Purple fluid extract (B) Eggshell extract. Ehrlich Ascites Carcinoma cells were seeded in RPMI-1640 medium with L-glutamine and supplemented with both 10% FBS and 1% penicillin-streptomycin and treated with CIS (40mg/ml) and different concentrations of crude purple fluid (0.5 - 2.0 µg/ml) for 24hr at the optimum condition of cell culture (37˚C, 5% CO^2). EAC cells were harvested and counted by trypan blue assay. EAC: Ehrlich Ascites carcinoma cell line, CIS: Cisplatin. ANOVA demonstrated a significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

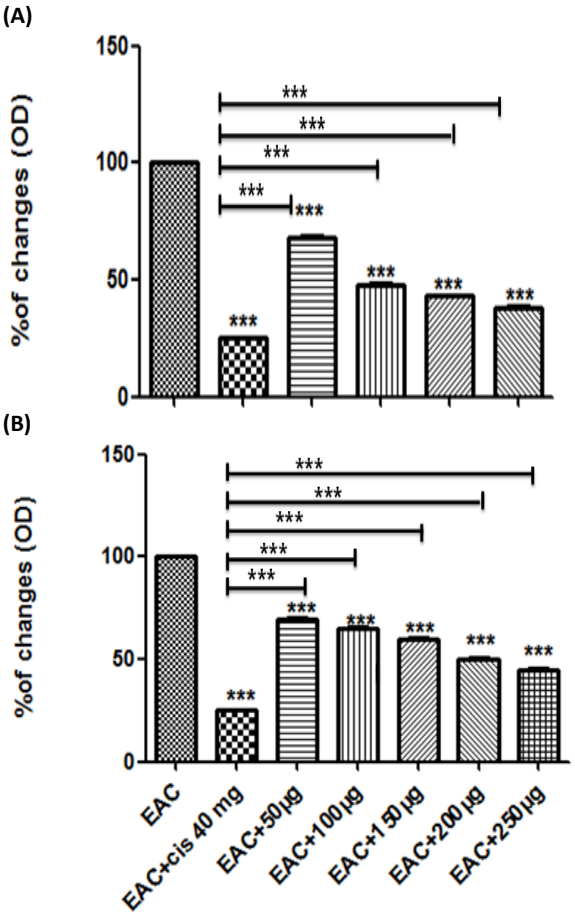

Figure 3. The effect of CIS, egg extract and purple fluid on the proliferative activity of EAC cells: (A) Purple fluid and CIS (B) Eggshell extract and CIS. Ehrlich Ascites Carcinoma cells were seeded in RPMI-1640 medium with L-glutamine and supplemented l with both 10% FBS and 1% penicillin-streptomycin and treated with CIS (40mg/ml) and different concentrations of crude purple fluid (0.5 µg/ml, 1.0 µg/ml, 1.5 µg/ml, 2.0 µg/ml) for 24hr at the optimum condition of cell culture (37˚C, 5% CO^2).EAC cells were harvested and the percentage of O.D of EAC cells was measured after 24 hours using MTT assay. ANOVA demonstrated a significant difference among the groups at $P<0.05$ accompanied by Bonferroni correction for multiple comparisons.

Effect of treatment with Cisplatin, the purple fluid and egg extract on the cellularity of spleen

Treatment of EAC bearing mice with both doses of the crude purple fluid extract significantly decreased the numbers of splenocytes compared to control group. Also, the high and low dose of egg extract induced a significant decrease in splenocytic count as compared to the control group (Figure 7A; Supplementary Table 2).

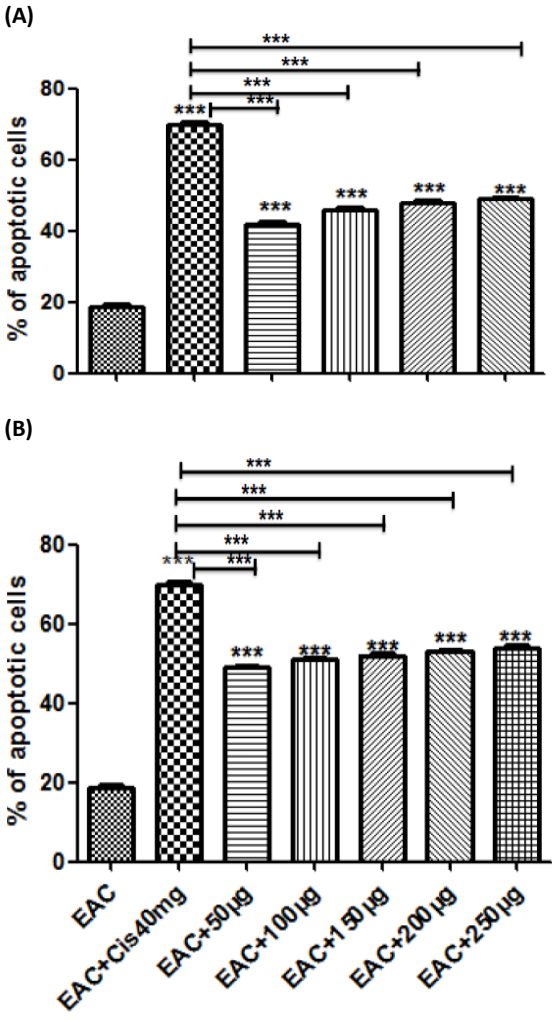

Figure 4. Effect of different concentrations of both extracts on EAC cells apoptosis rate: (A) Purple fluid extract (B) Eggshell extract. EAC cells were harvested from 7 days of tumour-bearing mice, then incubated with different concentrations of purple fluid. After 24hr the cells were harvested and stained with annexin V and were analyzed by flow cytometer. ANOVA demonstrated a significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

The effect of treatment with the purple fluid and egg extract on total white blood cell count and phenotypic characteristics of myeloid cells in the spleen

Inoculation of EAC cells significantly increased the total number of WBCs by 18 folds compared to naïve mice. Meanwhile, treatment with cis-platin induced a significant decrease in the total number of WBCs by 1 fold compared to the EAC control group. Surprisingly, treatment with both extracts exhibited a significant decrease in total WBCs compared to the control group.

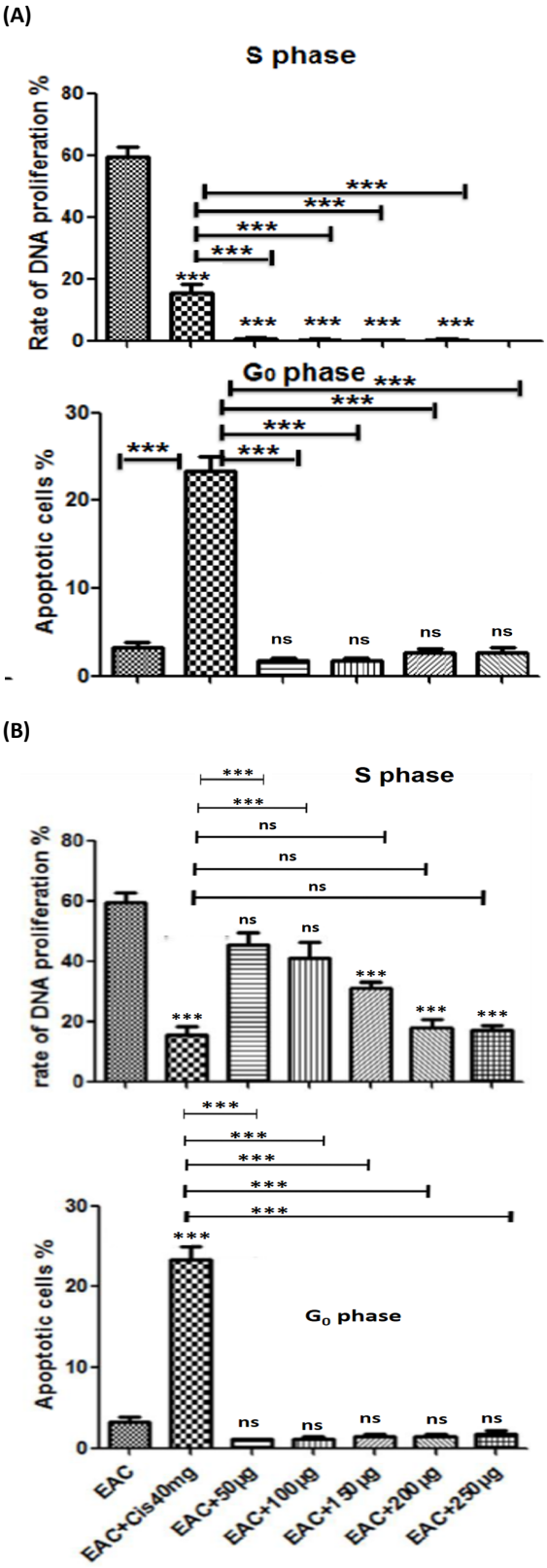

Figure 5. DNA content and cell cycle of tumor cells after treatment: (A) Purple fluid extract (B) Eggshell extract. EAC cells were harvested from 6-well-plate and washed twice. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry. ANOVA demonstrated a significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

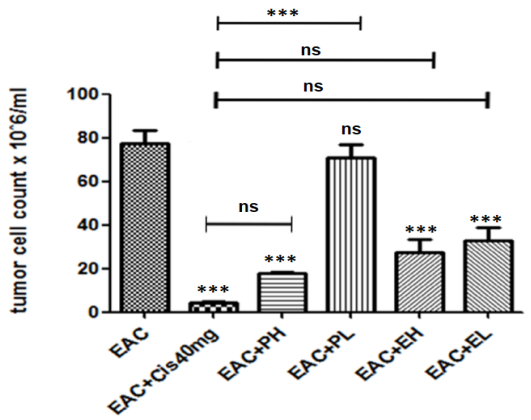

Figure 6. Antitumor effects of CIS, purple fluid and egg extract on tumor cells: mice (n=8) were i.p treated with P.B.S, cisplain, purple fluid or egg extract, mice were sacrificed on day 10 EAC cells were harvested from the peritoneal cavity and counted, PH: high dose of purple fluid, PL: low dose of purple fluid, EH: high dose of egg extract, EL: low dose of egg extract. ANOVA demonstrated a significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

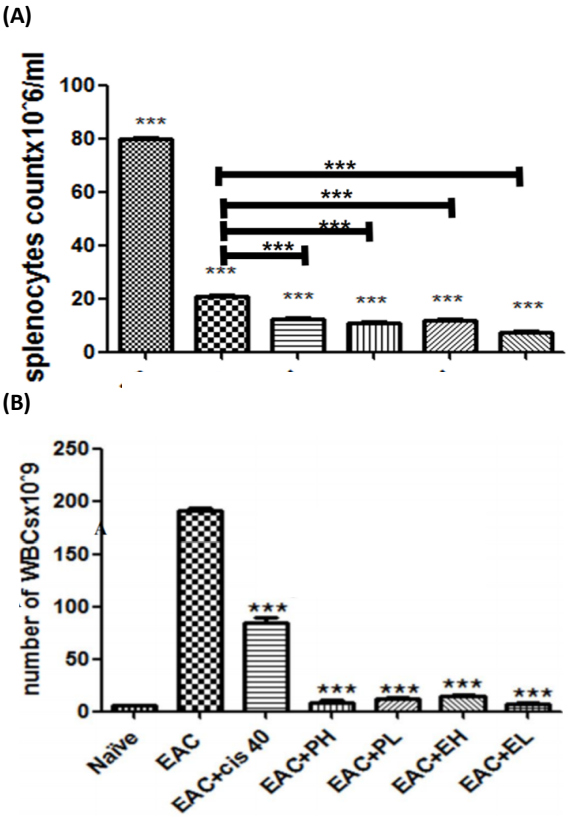

Figure 7. Effect of treatment with Cisplatin, purple fluid and egg extract on: (A) The cellularity of the spleen and (B) Total WBCs count. Mice (n=8) were i.p treated with P.B.S, cisplatin, purple fluid or egg extract, mice were sacrificed on day 10 and spleens were harvested for cell count **(A)** or peripheral blood collection from the tail **(B).** ANOVA demonstrated a significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

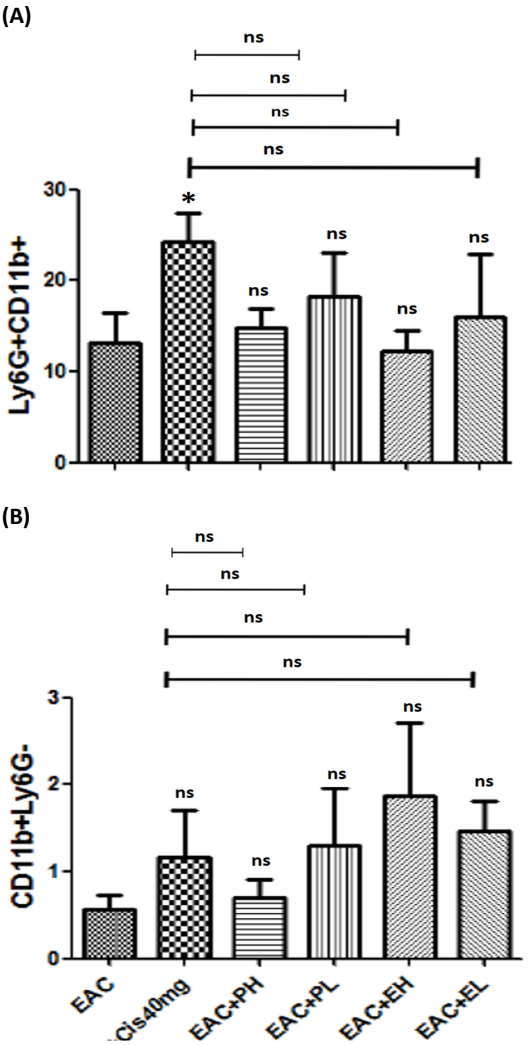

Figure 8. Effect of CIS, purple fluid and egg extract on myeloid cell subsets in the spleen: A) CD11b+ Ly6G+ MDSCs, B) CD11b+ Ly6G- myeloid cells. EAC: Ehrlich ascites carcinoma, CIS: Cisplatin 40mg, PH; purple fluid high dose, PL; purple fluid low dose, EH; egg extract high dose, EL; egg extract low dose. ANOVA demonstrated no significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

The purple fluid (20µg/mouse) and egg extract (6.7µg/mouse) decreased in the total WBCs count by 21.2 and 12.95 fold, respectively compared to untreated EAC-bearing mice (Figure 7B).

Both doses of the purple fluid extract in addition to the low dose of egg extract increased the percentage of MDSCs (CD11b+, Ly6G+) by 0.09, 0.39, and 0.22 fold respectively compared to untreated EAC. On the other hand, treatment with cisplatin was of more potent effect as it increased the percentage of the MDSCs by 1.23 fold compared to the control group (Figure 8A; Supplementary Table 2).

(A)

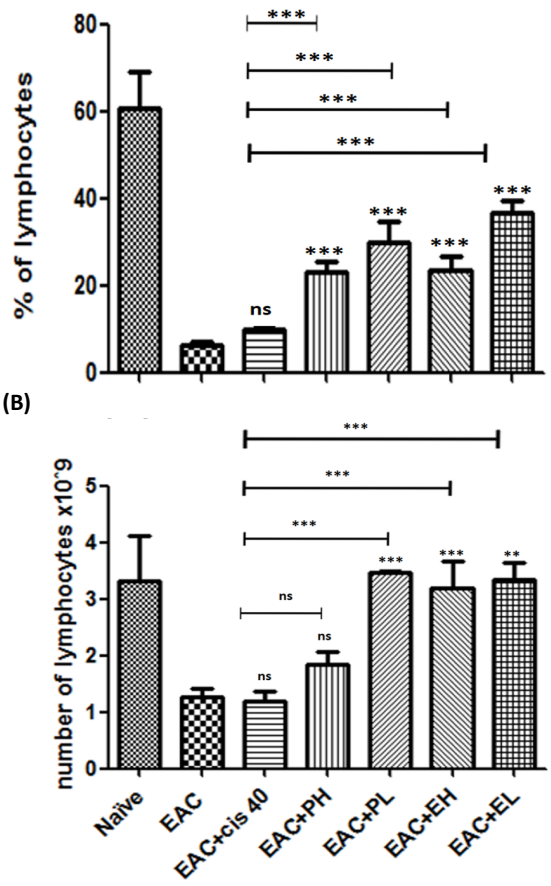

Figure 9. Effects of different concentrations of egg extract and purple fluid on the relative and absolute number of Lymphocytes: (A) Relative number (B) Absolute number. mice (n=8) were i.p treated with P.B.S, CIS, purple fluid or egg extract, mice were sacrificed on day 10 and bled for differential cell numbers of peripheral blood mononuclear cells. ANOVA demonstrated a significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

Interestingly, treatment of EAC bearing mice groups with cisplatin, the low and high dose of the purple fluid extracts and low and high doses of egg extract increased the number of myeloid cells (CD11b+, Ly6G-) by 1.07, 1.3, 0.24, 1.6 and 2.3 folds respectively compared to control group (Figure 8B; Supplementary Table 2).

Effect of the purple fluid and egg extract on the total lymphocytes, monocytes and neutrophils

Treatment with eggshell extract and the low dose of the purple fluid had minimal effect on the absolute and relative numbers of total lymphocytes compared to naïve group, untreated control EAC group and cisplatin-treated EAC group (Figure 9A, B; Supplementary Table 2).

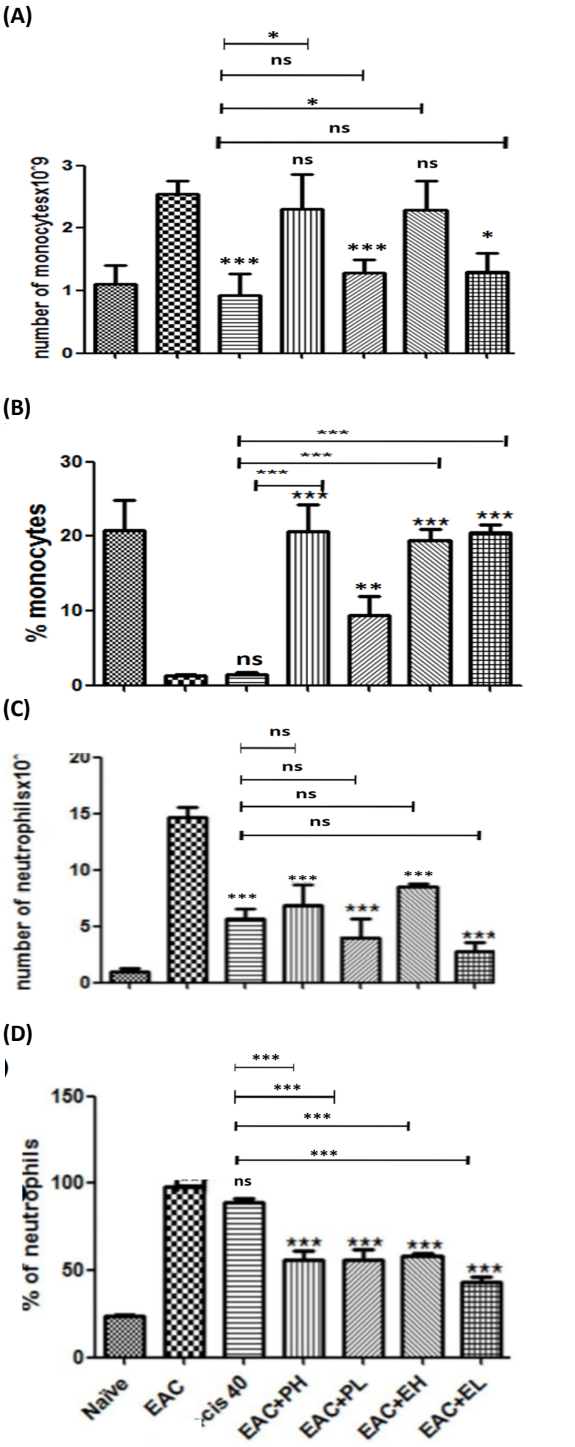

Figure 10. Effects of different concentrations of egg extract and purple fluid on the relative and absolute number of monocytes and neutrophils: (A) Absolute number (B) Relative number of monocytes (C) Absolute number (D) Relative number of neutrophils. Mice (n=8) were i.p treated with P.B.S, CIS (40mg/mouse), purple fluid (20, 10µg/mouse) or egg extract (6.7, 3.3 µg/mouse), mice were sacrificed on day 10 and bled for differential cell numbers of peripheral blood mononuclear cells. ANOVA demonstrated a significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

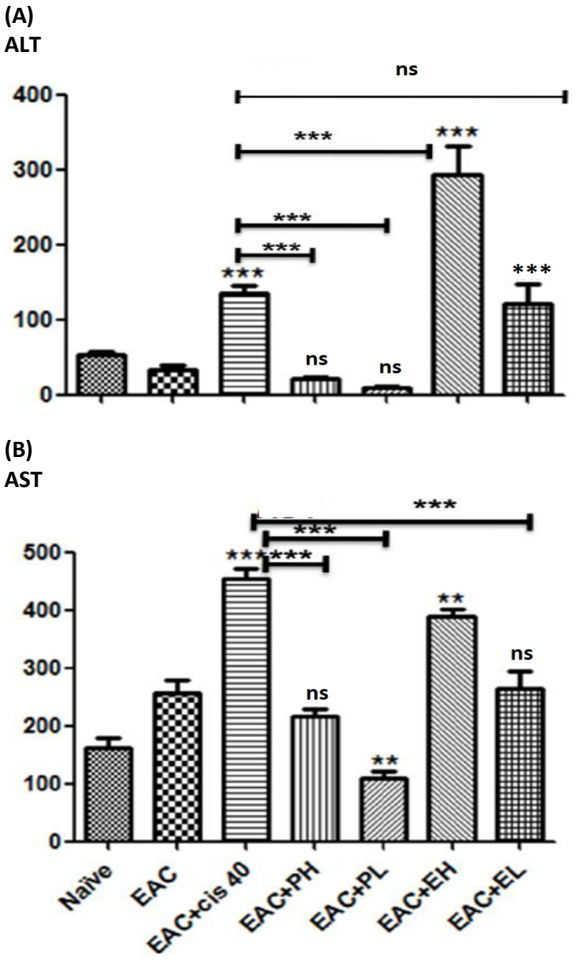

Figure 13. The effect of purple fluid and egg extract on liver function tests: mice (n=8) were i.p treated with P.B.S, CIS (40mg/mouse), purple fluid (20, 10µg/mouse) or egg extract (6.7, 3.3 µg/mouse), mice were sacrificed on day 10 and bled for measurement of liver enzymes ALT (A) and AST (B). ANOVA demonstrated a significant difference among the groups at $P < 0.05$ accompanied by Bonferroni correction for multiple comparisons.

Treatment with cisplatin and a low dose of the purple fluid extract significantly decreased the absolute number of monocytes compared to untreated EAC group. While other treated mice groups exhibited an insignificant difference. However, treatment with both extracts significantly increased the relative number of monocytes compared to cisplatin and untreated control group (Figure 10A, B; Supplementary Table 2).

Interestingly, all treated mice groups showed a significant decrease in the absolute number of neutrophils compared to the untreated control group (Figure 10C). In the same way, all treated groups also showed a significant decrease in the relative number of neutrophils except for cisplatin-treated group (Figure 10D).

The effect of the purple fluid and egg extract on liver function tests

The eggshell extract significantly increased the serum transaminases AST and especially ALT even higher than cisplatin EAC group. In contrast, the purple fluid extract did not affect ALT or AST (Figure 11A, B; Supplementary Table 3).

DISCUSSION

The present study was designed to detect the potential anti-tumor effect of the egg and purple fluid extracts of *Aplysia fasciata* inhabiting the Egyptian water. Our overall data has succeeded to present the purple fluid and eggshell extracts derived from *A. fasciata* as a novel anti-cancer agent against Ehrlich ascites breast carcinoma (EAC). *In vitro* study has shown that incubation of EAC cells with egg extract and the purple fluid have resulted in significant decreases in the number and survival of EAC cells in a dose-dependent manner. Despite differences in the chemical composition of the purple fluid and egg extract; both induced direct antitumor effect, indicating that the biological products of Genus *Aplysia* produce chemicals with potent antitumor effect regardless to the active ingredient. Although the antitumor effects of these two agents were not the same as those of cisplatin (reference drug), our results have indicated the promising anti-neoplastic effects of these marine products of interest especially high doses of egg extract (250 µg/ml) and the purple fluid (2 µg/ml) *in vitro*. It is well known that cancer cells possess a high proliferation rate but low apoptosis rate - Opposite to normal cells (Sinicrope *et al.,* 1999). The antitumor effect of conventional chemotherapeutic drugs as (Cisplatin) mostly mediated at least in part, by increasing apoptosis, and decreasing DNA proliferation of rapidly dividing cells (Wang *et al.,* 2005). In the present study, we tended to investigate the effect of both extracts on the rate of apoptosis and proliferation of EAC cells using MTT assay and annexin- V (Mosmann and Tim, ???? and Vermes *et al.,* 1995). We found that both extracts caused an increase in EAC cell apoptosis coincided with a decrease in cell proliferation after treatment of EAC cells for 24 hours *in vitro*.

Besides, analysis of the cell cycle revealed that the purple fluid extract was of a more potent effect than eggshell extract in decreasing the number of proliferating cells (S phase) by 0.98-fold. As it successfully diminished cell proliferation even more than the therapeutic dose of cisplatin. However, both extracts were incapable of induction of complete cell cycle arrest as cisplatin.

Previously, it was reported that the crude purple fluid derived *from A. dactylomela*, another Genus from south-west Iran, induced a decrease in the number of proliferating cells by 85% against HL60 human cell line (Zandi *et al.,* 2007). The differences in the anti-tumor effects between the purple fluid extracted from *A. fasciata* and *A. dactylomela* may be caused by specific variations, environmental habitats, the used concentrations or the cancer cell type or other factors.

However, both studies are consistent and strongly recommend the potential anti-tumor properties of the purple fluid of Genus Aplysia. Next, we tended to investigate whether the direct anti-tumor effects of the purple fluid and egg extract can also be observed *in vivo*. The present study showed that treatment with the purple fluid (20 or 10 µg/mouse) or with egg extract (6.7 or 3.3 µg/mouse) have induced significant decreases in EAC harvested tumor cell count compared to untreated control and cisplatin-treated mice groups. Interestingly, a high dose of the purple fluid induced less increase in tumor cell count than a high dose of egg extract. Previously, A 250 kDa glycoprotein named Aplysianin E was purified from the egg of *Aplysia kurodai*. Aplysianin E exhibited anticancer activity against some human tumor cell lines as well as on experimental mice models (Kisugi *et al.,* 1987).

In addition to the direct antitumor effect exerted by both extracts, we hypothesized that both extracts may exert an immune-modulatory effect that could indirectly potentiate their anti-tumour effect *in vivo*. At first, we tested the effect of both extracts on the number of splenocytes. The treatment of EAC-bearing mice with both extracts induced a significant decrease in the splenocytes number compared to untreated control EAC-bearing mice. In contrast, cis-platin-treated mice exhibited an increase in the number of splenocytes. This could be caused by the migration of cells from to the tumour sites which is a fundamental event associated with cancer initiation. As, tumour releases chemical chemokines that induce local changes in the tumour microenvironment and also, control the cell migration (Jarnicki *et al.,* 2010, Coussens and Werb, 2002)

Secondly, we explored the systemic immunomodulatory effect of tumour inoculation and the ameliorative effect of our extracts of interest. The inoculation of EAC cells induced significant leucocytosis. Moreover, an elevated leukocyte count associated with cancer initiation was recently identified as one of the predictive factors of increased risk for venous thromboembolism (VTE) in cancer. Thrombosis is the second leading cause of death in cancer (Khorana *et al.,* 2008). Importantly, we found that treatment of EAC cells with either cisplatin or *Aplysia* extract significantly alleviated the leucocytosis induced by tumour inoculation. Moreover, both *Aplysia* extracts exhibited a more powerful effect than cisplatin in inhibition of leucocytosis. Accordingly, this indicates that Aplysia extracts could be powerful tools for not only cancer treatment but also, for inhibition of thrombosis. Hence, they will help better prognosis for cancer patients.

We also hypothesized that treatment with both extracts did not increase the total number of WBCs but increased their trafficking to tumour site. The migratory capacity of leukocytes is critical to their role as defence cells implicating their role in the progression and spread of tumours. As we know, tumour associated inflammation is carried out by different leukocyte populations including neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes (Balkwill and Mantovani 2001). Our data showed that treatment with both Aplysia extracts managed to decrease neutrophilia induced by tumour inoculation. Neutrophilia is one of haematological findings related to poor prognosis in human metastatic melanoma, pancreatic carcinoma, and renal carcinoma (Schmidt *et al.,* 2005, Fogar *et al.,* 2006 and Donskov and von der Maase 2006).

Interestingly, the low dose of the purple fluid, low dose and high dose of egg extract induced a significant increase in the absolute number of lymphocytes compared to untreated EAC-bearing mice by 1.89, 1.78 and 1.8 fold respectively. Unlike neutrophils, the increased lymphocytic count is considered a good prognostic factor in diverse cancers (Lissoni *et al.,* 2006).

Monocytes are innate immune cells that lately; have been considered as important regulators of cancer development and progression. Different monocyte subsets perform functions that contribute to both pro- and antitumoral immunity, including phagocytosis, secretion of tumouricidal mediators, promotion of angiogenesis, remodelling of the extracellular matrix, recruitment of lymphocytes, and differentiation into tumour-associated macrophages and dendritic cells (Olingy *et al.,* 2019).

Our present study showed that treatment of EAC cells with either cisplatin or low doses of both extracts has successfully reduced the total number of monocytes. Meanwhile, all doses of Aplysia extract managed to increase the relative number of monocytes, unlike cisplatin. Considering controversial role of monocytes; we tried to investigate the different subsets of monocyte populations. We found that both Aplysia extracts exhibited an insignificant effect on either MDSCs or macrophages compared to the untreated cells. Such data might be consistent with the associated neutropenia induced by EAC cells treatment. As, withdrawal of neutrophils numbers is accompanied by their transformation into other myeloid cells including MDSCs as postulated by Sagiv *et al.,* 2015.

Finally, we test the serum liver transaminases (ALT and AST) to exclude hepatotoxicity that might be produced as a side effect to therapy. (Ghouri, 2010). Interestingly, the purple fluid extract did not affect the serum transaminases, unlike the eggshell extract. At last, our results have demonstrated the differential anti-tumor effect between the purple fluid and eggshell extracts that might be caused by different active ingredients. Our overall data were consistent with other studies of other extracts of similar components. Yang and co-workers have isolated a 60-kDa antibacterial protein from the defensive secretions of the sea hare *Aplysia californica* (Yang CA *et al.,* 2011). This protein, escapin, has been characterized as an l-amino acid oxidase with bacteriostatic and bactericidal activities (Barsby, 2006). Antitumor effect of purple fluid is suggested to be due to l-amino acid oxidase which has been reported in another study on extracts derived from snake venom (Ahn *et al.,* 1997; Costa *et al.*, 2014) which was capable of promoting cytotoxicity in different cell lines (Imlay, 2003, Guo *et al.,* 2012; Okubo *et al.,* 2012). However, further studies are needed to isolate the active bioactive components of both extracts and also to identify the underlying mechanisms of their anti-tumor cytotoxic effect.

Conflicts of interest

All authors have approved this article and declare no conflicts of interest.

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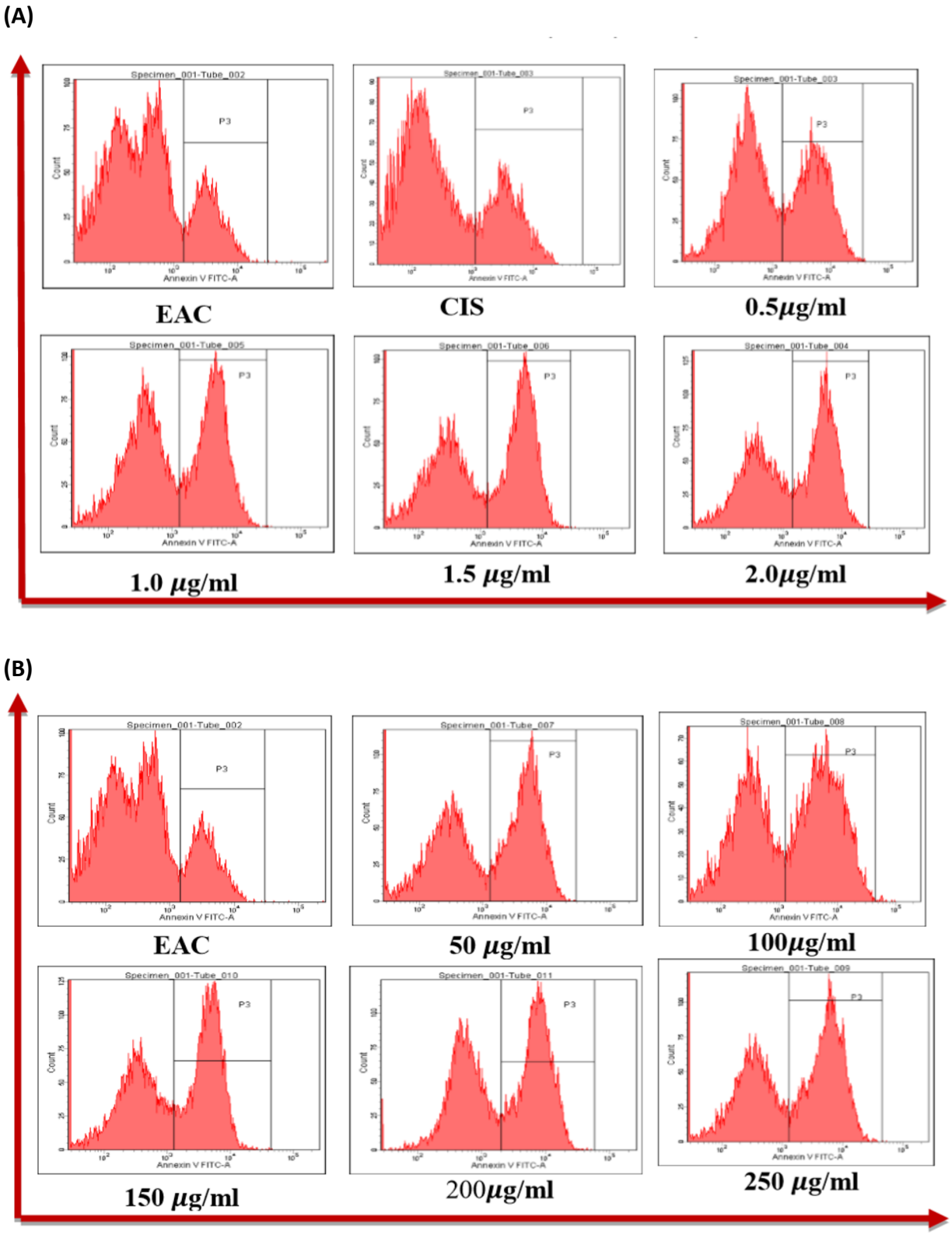
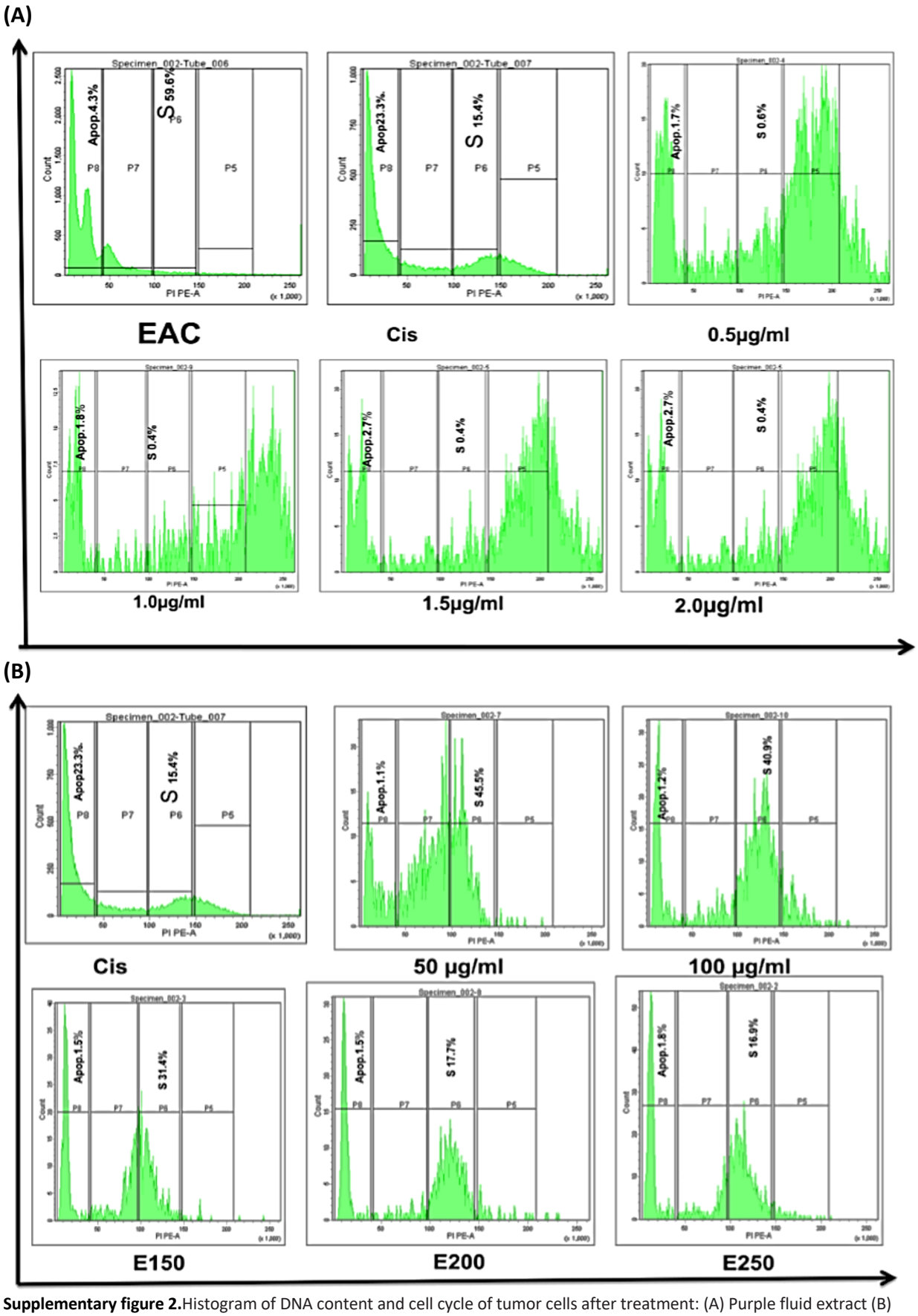


Supplementary Figure 1. Histogram of apoptosis % of EAC cells: (A) Purple fluid extract (B) Eggshell extract. EAC cells were harvested from 7 days of tumor-bearing mice and then incubated with different concentrations of egg extract. After 24hr the cells were harvested and stained with annexin V and were analyzed by flow cytometer.



Supplementary figure 2. Histogram of DNA content and cell cycle of tumor cells after treatment: (A) Purple fluid extract (B) Eggshell extract. EAC cells were harvested from 6-well-plate and washed twice. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry.

Supplementary table 1: Mean and standard deviation for in vitro experiments

	Untreated EAC	Cisplatin (40 mg/ml)	Crude purple fluid extract				Crude egg shell extract				
			0.5 µg/ml	1 µg/ml	1.5 µg/ml	2 µg/ml	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Total EAC cell count (n x 10^6)	79 ± 7.098	20 ± 6.234	71.6 ±3.983	65 ±8.319	61.4 ±3.985	53.7 ±4.583	69.6 ± 2.93	65.4 ± 3.98	61.4 ± 5.32	58.2 ± 5.671	55.1 ± 6.241
MTT assay (O.D %)	100 ± 5.02	25 ± 7.987	68.2 ± 8.32	48.5 ± 4.953	43.3 ± 6.915	38.738 ± 2.203	69.5 ± 3.641	65 ± 4.726	60 ± 6.92	50 ± 4.972	45 ± 6.34

Supplementary table 2: Mean and standard deviation for in vivo antitumor and immunological experiments

	Untreated EAC (Control)	Cisplatin (2 mg/kg)	Crude purple fluid extract		Crude egg shell extract	
			Low dose	High dose	Low dose	High dose
Total EAC cell count (n x 10^6)	79.37 ± 6.95	4.4 ± 2.451	71 ± 3	17.9 ± 5.9	32.6 ± 6.455	27.5 ± 5.5
***P* value Vs control**		< 0.01	0.4	<0.01	< 0.01	< 0.01
Total splenocytic count (n x 10^6)	80 ± 3	21 ± 7	11.2 ± 4.01	12.5 ±3.9	7.5 ± 3	12 ± 5
***P* value Vs control**		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
CD11b+Ly6G+ %	13.16 ± 5.67	30.5 ± 2.0432	18.3 ± 8.265	14.83 ± 3.7	16.033 ± 11.78	12.3 ± 3.78
***P* value Vs control**		> 0.01	> 0.01	> 0.01	> 0.01	> 0.01
CD11b+Ly6G-%	0.566 ± 0.289	1.17 ± 0.929	1.3 ± 1.136	0.7 ± 0.36	1.467 ± 0.586	1.87 ± 1.457
***P* value Vs control**		> 0.01	> 0.01	> 0.01	> 0.01	> 0.01
Absolute number of lymphocytes x 10^9	1.2 ± 0.265	1.1667 ± 0.379	3.47 ± 0.017	1.83 ± 0.39	3.335 ± 0.43	3.377 ± 1.078
***P* value Vs control**		> 0.01	< 0.01	> 0.01	< 0.01	< 0.01
Relative number of lymphocytes x 10^9	6 ±1.73	10 ± 2	31.5 ± 5.138	22.367 ± 5.835	36.83 ± 7.67	23.57 ± 5.03
***P* value Vs control**		> 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Supplementary table (3): Effect of Cisplatin, purple fluid extract and egg extract on liver enzymes

	Naïve	Untreated EAC (Control)	Cisplatin (2 mg/kg)	Crude purple fluid extract		Crude egg shell extract	
				Low dose	High dose	Low dose	High dose
ALT	53 ± 7	32.67 ± 10.5	135.67 ± 15.5	9.6 ± 3.055	22 ± 3.606	121.3 ± 11.015	292.6 ± 8.08
***P* value Vs Naive**		> 0.01	< 0.01	< 0.01	> 0.01	< 0.01	< 0.01
AST	162 ± 29.445	256 ± 14	454.33 ± 15.04	109.667 ± 19.5	216 ± 10	261 ± 10.15	388.33 ± 12.5
***P* value Vs control**		> 0.01	> 0.01	> 0.01	> 0.01	> 0.01	> 0.01

Egyptian Association for Cancer Research (EACR)
http://eacr.tanta.edu.eg/

EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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