

Antitumor Immunity and Therapeutic Properties of Marine Seaweeds-derived Extracts in The Treatment of Cancer

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Abstract

Marine seaweeds are important sources of drugs with several pharmacological characteristics. The present study aims to evaluate the antitumor and antitumor immunological potentials of the extracts from *Padina pavonia* and *Jania rubens* inhabiting the Egyptian marine coasts. Hep-G2 cell lines used for assessment of the antitumor efficacy of *Padina pavonia* and *Jania rubens* extracts *in vitro*, while Ehrlich ascites carcinoma (EAC) cells were applied to gain more antitumor immunity and antitumor insights of *P. pavonia* and *J. rubens* extracts *in vivo*. *In vitro* antitumor potentials of *P. pavonia* and *J. rubens* extracts were analyzed against human liver cancer Hep-G2 cells by MTT and trypan blue exclusion assays. *In vivo* antitumor immunological potentials of *P. pavonia* and *J. rubens* extracts at low, high, and prophylactic doses were analyzed by blood counting and flow cytometry in mice challenged with Ehrlich ascites carcinoma (EAC) cells. *In vitro* results revealed that *P. pavonia* and *J. rubens* extracts caused significant decreases in the number and viability of Hep-G2 cells in a dose-dependent manner as compared to untreated Hep-G2 cells or Cisplatin®-treated Hep-G2 cells. *In vivo* findings showed that *P. pavonia* and *J. rubens* extracts at low, high, and prophylactic doses significantly reduced the number and viability of EAC tumor cells accompanied by increases in EAC apoptosis compared to naïve EAC mouse. Additionally, *P. pavonia* and *J. rubens* extracts at low and prophylactic doses remarkably increased both the total WBC count and the relative numbers of lymphocytes and decreased the relative numbers of neutrophils and monocytes. Flow cytometric analysis showed that *P. pavonia* and *J. rubens* extracts at the treatment and the prophylactic doses resulted in a significant increase in the phenotypic expressions of CD4⁺ T, CD8⁺ T, and CD335 cells compared to naïve EAC mouse. Overall, both extracts *P. pavonia* and *J. rubens* possess potential antitumor and antitumor immunological effects with less toxicity, opening new approaches for further studies of the chemical and biological mechanisms behind these effects.

Introduction

Cancers are life-threatening diseases that are widely recognized as a major public health issue around the world. Chemotherapies are the most common cancer treatments, and they consist of a variety of drug treatments that spread throughout the body tissue, affecting the mode of action in both malignant and healthy cells, and they cause a variety of side effects such as anemia, appetite loss, hair loss, peripheral neuropathy, and organ damage (Weinberg, 2013). Furthermore, anticancer drug treatment often results in resistance or tolerance, which is one of the most significant challenges in chemotherapy. Antitumor therapy resistance is a key obstacle in cancer chemotherapy, and it is one of the leading causes of failure. As a result, there is a constant need to develop innovative, effective, and economical antitumor medications (Brabec and Kasparkova, 2005; Torigoe et al., 2005). Due to the harmful effects and lack of selectivity of the majority of chemotherapeutics, natural pharmaceuticals are chosen for their acceptance and decreased toxicity (Odeh et al., 2012). Consequently, there is a growing review on the potential and medicinal characteristics of natural pharmaceuticals in improving anticancer drug therapeutic effects and protecting non-tumor tissues from chemotherapy-induced damage (Badary, 2000).

Despite the fact that cancer has been studied for decades, more research is needed to produce anticancer medications that are extremely effective, have low tolerance, and cause fewer adverse effects. Furthermore, with the continued rise in cancer cases and concerns about toxicity, tumor cell resistance, the formation of secondary malignancies, and the unfavorable side effects seen with synthetic medications in recent decades, there has been a raised interest in using natural products to treat cancer (Wamtinga et al., 2015).

Seaweeds are an attractive species because they can be a rich source of bioactive compounds, including toxins with potential therapeutic applications (Khalid et al., 2018; Rai et al., 2019). Furthermore, the brown seaweed sterol hydrocarbon fraction showed dose-dependent anticancer efficacy against a number of cancer cell lines, including the Hep-G2 liver cancer cell line, the MCF-7 breast cancer cell line, the A549 colon cancer cell line, and the A549 ovarian cancer cell line (Ibrahim et al., 2015). *Jania rubens* methanolic extracts demonstrated anticancer activity against the Molt-4 and Jurkat cell lines (IC₅₀ values of 60.25 and 62.5 g/mL, respectively), indicating that it could be a good source of anti-leukemia medicines (Unissa et al., 2017). By blocking vascular endothelial growth factor (VEGF) and natural killer (NK) cell activation, antitumor chemicals from seaweeds can cause cancer cell death via a variety of signaling pathways, including cell cycle arrest, apoptosis, and anti-angiogenesis (Moussavou et al., 2014). These features of natural seaweed products can help to prevent cancer cells from becoming resistant or tolerating them (Ganesan et al., 2019).

Immune regulators produced from seaweed have been reported to stimulate immunological cells and boost the body's immune function (Costa, et al., 2010; Yende et al., 2014). Some seaweed-derived extracts have been studied in biomedical research for biological activities such as anticancer and immunomodulatory properties (Liang et al., 2014). Adjuvants generated from seaweed that were used in cancer immunotherapy found to have good results in terms of targeted immune stimulation (Xue et al., 2019). Seaweed extracts may have an immune-modulatory impact, which could enhance their anticancer activity *in vivo*.

Seaweed extracts have been reported to exhibit a variety of biological actions, including anticoagulant and antiviral properties, as well as roles in immunity enhancement, anti-inflammation, blood cholesterol reduction, antioxidation, improved liver and kidney activity, and gastrointestinal protection (Cumashi et al., 2007; Lee et al., 2004;; Li, et al., 2008; Mak et al., 2013). Studies on the functional and immunomodulating properties of seaweed extracts have recently been published (Maruyama et al., 2003).

The immunomodulatory, antitumor immunological and anticancer activities of natural seaweed products are increasing worldwide because they are cost-effective, safe, and effective. They also contain a large number of phytoconstituents that can be used for the development of new therapeutic entities such as antitumor immunotherapy and antitumor drugs. Therefore, we aimed to study the immunomodulatory, antitumor immunological and antitumor efficacy of methanolic extracts of two seaweeds; *Padina pavonia* and *Jania rubens* against Ehrlich Ascites Carcinoma (EAC) bearing mice.

Materials And Methods

Reagents and antibodies

Extracts of *P. pavonia* and *J. rubens* were dissolved in phosphate-buffered saline (PBS) and stored at -80°C until use. Monoclonal antibodies (mAbs) used in this study: anti-mouse CD4, anti-mouse CD8, and anti-mouse CD335 (NKp46) (eBioscience, San Diego, CA, USA). Roswell Park Memorial Institute medium 1640 (RPMI 1640) supplemented with heat-inactivated fetal bovine serum (FBS) (10% v/v), 2-mM L-glutamine and penicillin/streptomycin mixture (100 IU/ml), 1-mM sodium pyruvate, and non-essential amino acids (Invitrogen, USA). Ammonium-chloride-potassium (ACK) Lysis buffer was purchased from Lonza, BioWhittaker, USA.

Sample collection and maintenance

Padina pavonia and *Jania rubens* were collected freshly from Rocky Bay of Abu Qir, Alexandria, Egypt. Seaweeds rinsed in seawater, then tap water, and delivered in iced condition to the laboratory, Faculty of Science, Tanta University, Tanta, Egypt. The seaweeds were then air-dried at room temperature in the shade and then in the oven at $38 \pm 2^{\circ}\text{C}$. The dried samples were ground to a fine powder and stored for further investigations. The seaweeds were identified according to Jha et al. (2009), and Kanaan and Belous (2016), then using the Algae Base website to confirm their identification and the habitat details (Guiry and Guiry, 2019).

Preparation of seaweed extracts

The extraction was performed according to El-Sheekh et al. (2020). Briefly, one gram of seaweeds was steeped in methanol (1:30 w/v) in a conical flask for two days at room temperature on a rotary shaker at 120 rpm. The filtrate was placed in a 45°C oven to remove the excess solvent. The crude extracts were suspended in the methanol to a final concentration of 5 mg/mL and stored at -20°C for further studies. The extraction yield percentage was calculated according to Maisuthisakul and Pongsawatmanit (2004):

$$\text{Extraction yield\%} = (W_1/W_2) * 10$$

Where W_1 is the weight of dried crude extract, and W_2 is the weight of the sample before extraction.

Cell culture

Human liver cancer Hep-G2 cells lines were provided by the American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured on RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (10% v/v), 1% L-glutamine penicillin (100 units/ml) and streptomycin (100 units/ml). The cells were subcultured two to three times a week in a humid incubator (Sanyo XD-101; Sanyo) with 5% CO_2 at 37°C .

In vitro antitumor activity assay

For antitumor assays, against human liver cancer Hep-G2 cell lines were suspended in PBS buffer and introduced to the medium at a concentration 5×10^4 cell/well in Corning® 96-well tissue culture plates, which were then incubated for 24 h. The tested compounds were subsequently added into 96-well plates at concentration ranging from 10 -1000 µg/mL, along with reference drug Cisplatin® at concentrations ranging from 5 to 100 µg/ml (three replicates). Three untreated controls with media or 0.5% DMSO were run for each 96 well plate as a control. After incubating for 24 and 48 h, the numbers of viable cells were determined by the MTT assay. Briefly, the media was withdrawn from the 96 well plate and replaced with 100 µl of fresh culture RPMI 1640 medium without phenol red then 10 ml of the 12 mM MTT (Sigma) stock solution (5 mg of MTT in 1 mL of PBS) to each well including the untreated controls. The 96 well plates were then incubated at 37°C and 5% CO₂ for 4 h. An 85 µl aliquot of the media was removed from the wells, and 50 µl of DMSO was added to each well and mixed thoroughly with the pipette, and incubated at 37°C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (Bio-Rad microplate reader, Japan) to determine the number of viable cells.

The percentage of viability was calculated as:

$$(OD_t / OD_c) \times 100\%$$

Where OD_t is the mean optical density of wells treated with the tested sample and OD_c is the mean optical density of untreated cells.

The survival curve of the tumor cell line after treatment with seaweed extracts is depicted using the relationship between surviving cells and drug concentration. The Graphpad Prism software (San Diego, CA, USA) was used to calculate the 50% inhibitory concentration (IC₅₀) using graphic plots of the dose-response curve for each conc. (Mosmann, 1983; Elaasser et al., 2011). The IC₅₀ values for **the** HepG-2 cell line after 24 h were estimated at 613 µg/ml and 475.1 µg/ml for *P. pavonia* and *J. rubens*, respectively.

In vivo 50% lethal dose (LD₅₀) values were calculated from the IC₅₀ values according to the formula: $\log(LD_{50}) = 0.372 \times \log(IC_{50}) + 2.024$ (Halle et al., 2003). LD₅₀ values were estimated at 1150.5 µg/kg and 1047.0 µg/kg for *P. pavonia* and *J. rubens*, respectively.

Mice

Ninety female Swiss albino mice CD1 (6–8 weeks old, weighing 25 ± 2 g), were purchased from Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. Mice were divided into 9 groups (n = 10) and given a standard pellet diet and tap water *ad libitum* under hygienic conditions in specific pathogen-free conditions in accordance with the National Institutes of Health guide for the care and use of laboratory animals, as well as the recommendations of the proper care and use of laboratory animals, regulated by Faculty of Science, Tanta University, Tanta, Egypt.

Tumor cell line and tumor model preparation

Ehrlich ascites carcinoma EAC cell line, obtained from the Pharmacology and Experimental Oncology Unit, National Cancer Institute, Cairo University, Cairo, Egypt, was kept in ascetic tumor form in naïve female Swiss albino mice CD1 by weekly intraperitoneal (i.p.) inoculation of 2.5×10^6 cells/mouse (Gothoskar and Ranadive, 1971). The ascetic fluid was collected using a sterile syringe and diluted with PBS, and the EAC cells were counted using Trypan Blue dye exclusion method in a Neubauer hemocytometer (cell viability was 95%). EAC cells were resuspended in PBS at a density of 5×10^6 cells/ml PBS and kept at -80°C until needed. To prepare the tumor model, 0.25×10^6 EAC cells were implanted through i.p. injection into naïve female Swiss albino mice.

Tumor challenge and in vivo study design

Eighty female Swiss albino mice were divided into eight groups of 10 mice each. Groups 1–8 i.p. transplanted with EAC cell line (0.5×10^6 EAC cells/mouse). One day post EAC transplantation, group 1 was given i.p. saline (naïve EAC control). Group 2 was i.p. injected with standard drug Cisplatin[®] (10 $\mu\text{g}/\text{mouse}$) (positive EAC control) on days 8 and 10 post EAC transplantation. On days 8, 9, 10, 11, 12, and 13 post EAC transplantation, group 3 and group 4 were i.p. inoculated with *P. pavonia* extract (2.5 $\mu\text{g}/\text{mouse}$, ten times less than LD_{50} and 1.3 $\mu\text{g}/\text{mouse}$, twenty times less than LD_{50} , respectively), and Group 5 and group 6 were i.p. injected with *J. rubens* extract (2.3 $\mu\text{g}/\text{mouse}$, ten times less than LD_{50} and 1.2 $\mu\text{g}/\text{mouse}$, twenty times less than LD_{50} , respectively). Seven days before EAC transplantations, Group 7 and group 8 (prophylactic groups) were i.p. inoculated with *P. pavonia* and *J. rubens* extracts (2.5, $\mu\text{g}/\text{mouse}$ and 2.3 $\mu\text{g}/\text{mouse}$, ten times less than LD_{50} , respectively). On day 14 post EAC transplantation, all groups of mice were sacrificed, immunological and antitumor efficacy were assessed, and sera were collected for hematological and biochemical analysis (Fig. 1).

Hematological analysis

By the end of the experimental study, mice were anaesthetized with mild ether, and blood was drained from their retro-orbital plexus using heparinized microhematocrit tubes. A Nihon Kohden automated haematology analyzer (model MEK-6318K, Japan) was used to assess blood parameters, including leucocytes (WBC) ($10^3/\text{cmm}$) and their differential relative percent (neutrophils, lymphocytes, basophils, and monocytes) in peripheral blood (PB).

Preparation of sera

Blood was drawn from the retro-orbital plexus of experimental mice before they were euthanized by cervical dislocation. Blood samples were gathered in test tubes and left to stand for 3 hours to ensure complete coagulation. After centrifugation at 3000 rpm for 10 minutes, the sera samples were sucked out and stored at -80°C for biochemical analysis.

Tumor cells harvesting and counting

Mice were sacrificed by cervical dislocation and immediately EAC ascetic were individually withdrawn from treated-EAC and nontreated-EAC mice using a syringe containing 5 ml of PBS. The cells were

resuspended 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). EAC cells suspensions were centrifuged for 5 min (3000 rpm) at 4°C, discarding the supernatant. The EAC cell pellets were washed and diluted in supplemented PBS. EAC cells were counted using a Neubauer hemocytometer, and the cell viability was determined using a trypan blue dye exclusion assay. Viability was calculated by trypan blue exclusion assay and consistently exceeded 90%.

Flow cytometry

Fresh single-cell suspensions of tumor EAC cells were collected from the ascites of testing mice. 1×10^6 cells were stained with anti-mouse CD4, anti-mouse CD8, and anti-mouse CD335 (NKp46), which were then incubated for 30 min at room temperature in the dark before being cooled on ice for 1 min. The cells were washed twice and resuspended in 0.3 ml of 0.5% bovine serum albumin and 0.02% sodium azide solution. Flow cytometry was used to detect the CD4 + T, CD8 + T, and CD335 cell subsets using a FACS Calibur system (BD Biosciences, San Jose, CA); all data analysis was done with Cell-Quest software (Becton Dickinson, San Jose, CA). For each sample, a minimum of 50000 events was acquired. In each compartment, the total numbers of different cell populations was calculated each time.

Assessment of apoptosis by flow cytometry

Treated EAC cells from Cisplatin[®]-treated EAC mice, seaweed extracts-treated EAC mice, and nontreated EAC mice were stained with FITC Annexin V (Apoptosis Detection Kit II; Cat. No 556570 BD Bioscience, U. S. A) following the manufacturer's instructions. The data was evaluated using a BD FACSCanto II flow cytometer (BD Becton, Dickinson Company, USA) and analyzed using BD FACS Diva software (BD Becton, Dickinson Company, USA) (BD Bioscience, U.S.A). The apoptosis by Annexin V FITC / PI Assay for EAC tumor cells collected from the peritoneal cavity of tumor-bearing mice after treatment was gated on live cells on FSC v SSC, then single cells were gated using the PI width (W) and area (A) dot plot. Staining by Annexin V is used in association with a vital dye propidium iodide (PI) to recognize early and late apoptotic cells. Viable cells with intact membranes keep PI out, whereas dead and damaged cells' membranes let it in. As a result, cells considered viable when they are both Annexin V and PI negative. Cells in early apoptosis, on the other hand, are Annexin V positive and PI negative. Annexin V and PI are both positive in late apoptosis and dead cells.

Analysis of liver and kidney functions

The levels of serum aspartate aminotransferase (AST) (U/l), alanine aminotransferase (ALT) (U/l), albumin (g/dl), creatinine (mg/dl) were determined colorimetrically using standard ready-to-use kits and methods of Human (Human Gesellschaft Für Biochemica and Diagnostica MBH, Germany) by a fully automated biochemistry analyzer (Vita lab Selectra E, German). Throughout the experiment, the manufacturer's instructions for each biochemical parameter were strictly followed.

Statistical analysis

The numerical data was reported as a mean \pm SD. Statistical analyses was carried out by a one-way analysis of variance (ANOVA). Statistical significance was determined by a *Tukey* and a *post-hoc* test followed by *Dunnett's* multiple comparison tests. Statistical comparisons between groups were done using the paired Student's *t-test*. Statistical. Significant differences were considered at *p*- values < 0.0 5.

Results

In vitro antitumor activity

In the present study, the methanol extract of four algal species: *J. rubens*, *P. pavonia*, *T. atomaria*, and *C. elongate* with different concentrations (10-1000 $\mu\text{g/ml}$) were tested for its growth inhibition activity against human liver cancer HepG2 cell line (24 h incubation) with MTT assay. The human liver cancer HepG2 assayed with methanolic extracts of *J. rubens*, *T. atomaria*, *P. pavonia*, and *C. elongate* resulted in *J. rubens* extract has imparted 10.6 ± 3.7 to $77.0 \pm 0.95\%$ growth inhibition with a decrease in concentration and IC_{50} of 475.1 $\mu\text{g/ml}$, *T. atomaria* extract has imparted 16.1 ± 2.8 to $68.3 \pm 2.3\%$ growth inhibition with a decrease in concentration and IC_{50} of 580 $\mu\text{g/ml}$, *P. pavonia* extract has imparted 19.7 ± 1.3 to $61.6 \pm 9.3\%$ growth inhibition with a decrease in concentration and IC_{50} of 613 $\mu\text{g/ml}$, and *C. elongate* extract has imparted 22.0 ± 5.8 to $56.5 \pm 1.5\%$ growth inhibition with a decrease in concentration and IC_{50} of 630.9 $\mu\text{g/ml}$ (Table 1). It is observed a crude extract of *J. rubens* is influencing the increase in cell growth inhibition in the human liver cancer HepG2 when compared to that of *T. atomaria*, *P. pavonia* and *C. elongate* with IC_{50} values at 475.1 $\mu\text{g/ml}$, 580 $\mu\text{g/ml}$, 613. $\mu\text{g/ml}$, and 630.9 $\mu\text{g/ml}$ for *J. rubens*, *T. atomaria*, *P. pavonia*, and *C. elongate*, respectively (Table 1). Depending on the trypan blue assay, *P. pavonia* and *J. rubens* methanol extracts were selected for advanced studies to determine immunomodulatory and antitumor potentials.

Table 1

In vitro cytotoxic effect of *P. pavonia*, *J. rubens*, *T. atomaria* and *C. elongate* extract by MTT assay at various concentration (10 µg/ml-1000 µg/ml) against human liver cancer Hep-G2 cell line at 24 h-incubation period.

Extract concentration (µg/ml)	% Growth inhibition			
	<i>P. pavonia</i>	<i>J. rubens</i>	<i>T. atomaria</i>	<i>C. elongate</i>
10	19.70 ± 1.30 ^a	10.60 ± 3.70	16.10 ± 2.80a	22.00 ± 5.80a
50	38.00 ± 3.20 ^a	14.30 ± 2.10	21.90 ± 3.90a	27.40 ± 1.80a
100	40.50 ± 5.10 ^a	23.20 ± 3.90a	21.40 ± 7.60a	29.40 ± 3.40a
200	39.30 ± 0.80 ^a	27.40 ± 3.20a	40.50 ± 10.70a	49.90 ± 14.80a
400	42.30 ± 1.10 ^a	66.40 ± 2.00a	46.00 ± 7.20a	48.30 ± 2.40a
600	51.20 ± 11.70 ^a	66.80 ± 2.80a	54.10 ± 1.10a	51.20 ± 6.200 ^a
800	54.10 ± 2.10 ^a	68.50 ± 2.80a	57.90 ± 3.60a	55.10 ± 2.60 ^a
1000	61.60 ± 9.30 ^a	77.00 ± 0.95a	68.30 ± 2.30a	56.50 ± 1.50 ^a
Control	00.00 ± 0.00	0.000 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
IC ₅₀ value (µg/ml)	613.00	475.10	580.00	630.90
Data were represented as mean ± SD. The difference between groups was considered statistically significant at $P < 0.05$. *: Statistically significant vs. control at 24 h-incubation period				

Our result showed that *in vitro* treatment of human liver cancer Hep-G2 with reference therapeutic drug Cisplatin[®] with different concentrations (5-100 µg/ml) resulted in impairing of 20.90 ± 1.50 to 49.50 ± 6.70% growth inhibition with a decrease in concentration at IC₅₀ of 90.40 µg/ml (Table 2).

Table 2

In vitro cytotoxic effect of standard chemotherapeutic drug Cisplatin® by MTT assay at various concentration (5 µg/ml-100 µg/ml) against human liver cancer Hep-G2 cell line at 24 h-incubation period.

Concentration (µg/ml)	% Growth inhibition
5	20.90 ± 1.50 ^a
10	35.20 ± 1.70 ^a
20	39.60 ± 3.90 ^a
40	41.80 ± 5.00 ^a
80	48.30 ± 5.90 ^a
100	49.50 ± 6.70 ^a
Control	00.00 ± 0.00
IC ₅₀ value (µg/ml)	90.40
Data were represented as mean ± SD. The difference between groups was considered statistically significant at $P < 0.05$. *: Statistically significant vs. control at 24 h-incubation period.	

In vivo 50% lethal dose (LD₅₀) values were calculated from the IC₅₀ values according to the formula: $\log(LD_{50}) = 0.372 \times \log(IC_{50}) + 2.024$ (Halle et al., 2003). LD₅₀ values were estimated at 1150.5 µg/kg and 1047.0 µg/kg for *P. pavonia* and *J. rubens*, respectively. Two sublethal doses of *P. pavonia* and *J. rubens* has been chosen for continued immunological and antitumor assays: 2.5 µg/mouse (ten times less than LD₅₀) and 1.3 µg/mouse (twenty times less than LD₅₀) for *P. pavonia* extract and 2.3 µg/mouse (ten times less than LD₅₀) and 1.2 µg/mouse (twenty times less than LD₅₀) for *J. rubens* extract.

Changes in the level of leucocytes profile

Treatment of EAC mice with a low dose of *P. pavonia* extract (1.3 µg/mouse) and a prophylactic dose of *J. rubens* extract (2.3 µg/mouse) significantly increased the total number of WBCs by about 2.2 and 2.5 folds, respectively compared to naive EAC mice and by 1.9 and 2 folds, respectively compared to Cisplatin®-treated EAC mice. Furthermore, treatment with a high dose of *J. rubens* extract (2.3 µg/mouse) induced a significant elevation in WBCs total number by 2 and 1.5 folds compared to naive EAC mice and Cisplatin®-treated EAC mice, respectively. Surprisingly, treatment with a high dose of *P. pavonia* extract (2.5 µg/mouse) and a low dose of *J. rubens* extract (1.2 µg/mouse) exhibited a significant decrease in total WBCs by about 0.5 fold compared to naive (Table 3).

Table 3

Cellular alternation in peripheral blood leucocytes profile of EAC mice treated with Cisplatin®, *p. pavonia* and *J. ruben* extracts

Treatments		WBC count ($\times 10^3$)	Leucocytes differentials relative number (%)		
			Lymphocytes	Neutrophils	Monocytes
<i>P. pavonia</i>	Low dose	11.96 \pm 0.60 ^a	77.00 \pm 7.93	4.66 \pm 0.08	24.33 \pm 3.05
	High dose	2.35 \pm 0.50	76.00 \pm 3.60	2.00 \pm 0.05 ^a	21.33 \pm 2.52
	Prophylactic dose	5.36 \pm 0.66	76.33 \pm 6.42	4.33 \pm 0.09	18.66 \pm 2.23
<i>J. rubens</i>	Low dose	6.46 \pm 0.30	86.00 \pm 5.30	1.66 \pm 0.04 ^a	12.00 \pm 3.29
	High dose	3.63 \pm 0.93	78.00 \pm 6.00	4.00 \pm 0.09 ^a	17.33 \pm 4.32
	Prophylactic dose	9.23 \pm 1.40 ^{ab}	76.66 \pm 8.50	1.66 \pm 0.02 ^a	21.66 \pm 1.02
EAC + PBS		5.20 \pm 0.70	72.66 \pm 9.72	7.66 \pm 0.06	23.66 \pm 3.06
EAC + Cisplatin®		6.46 \pm 0.50	70.00 \pm 7.64	1.33 \pm 0.08	25.33 \pm 2.50
Data were represented as mean \pm SD. The difference between groups was considered statistically significant at $P < 0.05$. ^a : statistically significant vs. EAC mice. ^b : statistically significant vs. EAC mic + Cisplatin®.					

Treatment of EAC mice with a low dose, a high, and prophylactic dose of *P. pavonia* extracts (1.3 $\mu\text{g}/\text{mouse}$, 2.5 $\mu\text{g}/\text{mouse}$, and 2.5 $\mu\text{g}/\text{mouse}$, respectively) and that of *J. rubens* extracts (1.2 $\mu\text{g}/\text{mouse}$, 2.3 $\mu\text{g}/\text{mouse}$ and 2.3 $\mu\text{g}/\text{mouse}$, respectively) resulted in the remarkable decreases in neutrophil relative% compared to naïve EAC mice. Noticeably, treatment of EAC mice at a high dose of *P. pavonia* extract (2.5 $\mu\text{g}/\text{mouse}$) and low dose and prophylactic dose (2.3 $\mu\text{g}/\text{mouse}$) of *J. rubens* extract significantly decreased the neutrophil relative% compared to naïve EAC mice. Remarkably, *P. pavonia* extract at low and prophylactic doses and *J. rubens* at high dose increased the neutrophil relative% compared to Cisplatin®-treated EAC mice (Table 3).

Intraperitoneal inoculation of EAC mice with *P. pavonia* and *J. rubens* extracts at low, high and prophylactic doses resulted in a nonsignificant increase of lymphocytes relative% compared to naïve EAC

and Cisplatin®-treated EAC mice. Furthermore, among all tested doses, a low dose of *J. rubens* recorded the highest lymphocytes percentage compared to Naïve EAC and Cisplatin®-treated EAC mice (Table 3).

Intraperitoneal treatment of EAC mice with *J. rubens* extracts at low, high and prophylactic doses led to a remarkable nonsignificant decrease of monocytes relative% reached the least value at low dose compared to naïve EAC and Cisplatin®-treated EAC mice. Treatment of EAC mice with *P. pavonia* extracts at high and prophylactic doses led to a nonsignificant decrease of monocytes relative% compared to naïve EAC and Cisplatin®-treated EAC mice (Table 3).

Antitumor effects of different doses of seaweeds extract on EAC growth in vivo

Interestingly, high, low, and prophylactic doses of *P. pavonia* extract (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) and *J. rubens* extracts at low, high and prophylactic doses of (1.2 µg/mouse and 2.3 µg/mouse, 2.3 µg/mouse, respectively) produced a significant decrease in the number of viable EAC harvested tumor cells compared to naïve EAC mice (Fig. 2). Importantly our findings revealed that i.p injection of EAC mice with *P. pavonia* extract at low, high and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) and *J. rubens* extracts at low and high doses of (1.2 µg/mouse and 2.3 µg/mouse, respectively) remarkably decreased the total count of EAC tumor cells compared to Cisplatin®-treated EAC mice. However, these effects were higher in EAC mice i.p inoculated with a prophylactic dose of *J. rubens* extract compared to the effect reported in EAC mice i.p treated with Cisplatin®.

(Fig. 2).

Flow cytometric analysis of CD4, CD8, and CD335

The possible proliferative and inhibition efficacy of *P. pavonia* and *J. rubens* extracts on the phenotypic expressions of splenic lymphocytes subsets (helper CD4⁺ T cells, cytotoxic CD4⁺ T cells, and natural killer NK CD335 cells) have been investigated with anti-mouse, anti-mouse CD₄, CD₈, and anti-mouse CD335 staining using flow cytometry (Figures: 3, 4 and 5). Intraperitoneal treatment of EAC mice groups with *P. pavonia* extract at low, high and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) and *J. rubens* extract at high dose (2.3 µg/mouse) increased the percentage of splenic helper CD4⁺ T cells by 6.3, 4.8 and 3.1 fold for *P. pavonia* extract and 3.6, 4.4 fold and 2.5 for *J. rubens* extract, respectively compared to naïve tumor mice (Fig. 3). Interestingly, i.p. inoculation of EAC mice with a low dose of *P. pavonia* extract and high dose of *J. rubens* extracts recorded the highest increase in the percentage of splenic helper CD4⁺ T cells subset population (86.5% and 60.6%, respectively) compared to naïve tumor mice (13.8%). The highest proliferative activity of helper CD4⁺ T cells was evaluated in the EAC mice i.p inoculated with a low dose of *P. pavonia* extract (86.5%) compared to Cisplatin®-treated EAC mice (62.9%) (Fig. 3).

As shown in Fig. 4, phenotypic expressions of cytotoxic T cell subset of spleen harvested from EAC-bearing mice injected with *P. pavonia* and *J. rubens* extracts were detected with anti-mouse CD8 staining using flow cytometry. EAC mice i.p. received *P. pavonia* extract at low, high and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) and *J. rubens* extract at high dose (2.3 µg/mouse) showed a remarkable increase in the percentage of splenic cytotoxic CD8⁺ T cells by 2.9, 2.3 and 1.7 fold for *P. pavonia* extract and 2.3, 2.0 and 1.5 fold for *J. rubens* extract, respectively compared to naïve EAC mice (Fig. 4). Attractively, i.p. administration of EAC mice with a low dose of *P. pavonia* extract and *J. rubens* recorded the highest increase in the percentage of splenic cytotoxic CD8⁺ T cells subset population (71.9% and 56.7%, respectively) compared to naïve tumor mice (24.7%) (Fig. 4).

As shown in Fig. 5, phenotypic expressions of natural killer NK cell subset of spleen harvested from EAC mice injected with *P. pavonia* and *J. rubens* extracts were investigated with anti-mouse CD355(NKP46) staining by flow cytometry. EAC mice i.p. inoculated with *P. pavonia* extract at low doses (1.3 µg/mouse) and *J. rubens* extract at high dose (2.3 µg/mouse) showed a noticeable increase in the percentage of splenic natural killer NK cells by 1.2 fold for *P. pavonia* extract and 1.1 fold for *J. rubens* extract compared with naïve tumor mice (Fig. 5). Remarkably, i.p. treatment of EAC mice with the low dose of *P. pavonia* extract (1.3 µg/mouse) and the high dose (2.3 µg/mouse) expresses the highest increase in the percentage of splenic natural killer NK cell subset population (83.6% and 79.4%, respectively) compared to Cisplatin®-treated EAC mice (74.4%) (Fig. 5).

Assessment of tumor apoptosis by flow cytometry

In the present study, the antitumor efficacy of seaweeds extracts treatment regimen consisting of methanolic extracts of *P. pavonia* and *J. rubens* was assessed. EAC mice were i.p. injected with *P. pavonia* and *J. rubens* at low, high, and prophylactic doses and the apoptotic architecture of ascetic tumor cells were flowcytometrically investigated (Fig. 6). Our data reported that that i.p. treatment of EAC mice with *P. pavonia* extract at low, high and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) and *J. rubens* extract at low, high and prophylactic doses (1.2 µg/mouse, 2.3 µg/mouse and 2.3 µg/mouse, correspondingly) produced remarkable increases in the percentage of apoptosis of Ascetic EAC tumor cells at 53.3%, 71.6%, and 51.8%, respectively for *P. pavonia* extract and 11.0%, 14.9% and 18.4%, respectively for *J. rubens* comparing to naïve tumor mice (2.5%) (Fig. 6). Interestingly, the treatment of EAC mice with *P. pavonia* extract at low, high and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, correspondingly) induced the highest apoptotic effect on EAC tumor cells by 1.3, 1.8 and 1.3 fold compared to a positive control (Cisplatin®-treated EAC mice) (Fig. 6). Meanwhile, treatment with *J. rubens* extract at low, high and prophylactic doses (1.2 µg/mouse, 2.3 µg/mouse, and 2.3 µg/mouse, correspondingly) managed to decrease the total count of EAC cells by 4.4, 5.96 and 7.4 folds, respectively compared to naïve tumor mice. However, these effects were much lower than the effect of cisplatin® decreasing the number of harvested cells by 16.4 folds (Fig. 6).

Liver and kidney function

Treatment with *J. rubens* extract at low, high and prophylactic doses (1.2 µg/mouse, 2.3 µg/mouse, and 2.3 µg/mouse, respectively) and *P. pavonia* extract at high and prophylactic doses (2.5 µg/mouse) had a significant decrease in AST level compared to Cisplatin®-treated EAC mice. Meanwhile, i.p inoculation of *P. pavonia* extract at low, high, and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) had a significant raising in AST level compared to naïve EAC mice (Fig. 7). Intraperitoneal injection with *P. pavonia* extract at low, high and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) and *J. rubens* extract at prophylactic doses (2.5 µg/mouse) had a nonsignificant increase in ALT level compared to Cisplatin®-treated EAC mice. Meanwhile, i.p inoculation of *P. pavonia* extract at low and high doses (1.3 µg/mouse and 2.5 µg/mouse respectively) resulted in a significant increase in ALT level compared to naïve EAC mice (Fig. 7). Regarding the inoculation of mice with different doses of extract of *P. pavonia* and *J. rubens*, results show a minor decrease in the albumin concentration in EAC mice post i.p inoculation with *P. pavonia* and *J. rubens* extracts (Fig. 8). Insignificantly decreased the albumin level in serum in all groups, compared to the EAC mice and Cisplatin®-treated EAC mice. Remarkably, a high dose of *P. pavonia* extract significantly diminished the serum albumin level compared to naïve EAC mice (Fig. 8).

Treatment with *P. pavonia* extract at low, high and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) and *J. rubens* extract at high dose (2.3 µg/mouse) had a significant decrease in urea concentration compared to naïve EAC mice. Meanwhile, i.p inoculation with *P. pavonia* extract at prophylactic doses (2.5 µg/mouse) and *J. rubens* extract at a low and prophylactic dose (1.2 µg/mouse and 2.3 µg/mouse, respectively) resulted in a nonsignificant increase in urea concentration compared to Cisplatin®-treated EAC mice (Fig. 9). Interperitoneal inoculation with *P. pavonia* extract at low, high and prophylactic doses (1.3 µg/mouse, 2.5 µg/mouse, and 2.5 µg/mouse, respectively) and *J. rubens* extract at low, high and prophylactic doses (1.2 µg/mouse, 2.3 µg/mouse and 2.3 µg/mouse, respectively) led to a nonsignificant decrease in creatinine concentration compared to naïve EAC mice. (Fig. 9). High and prophylactic doses of *J. rubens* extract i.p. inoculated into EAC mice resulted in a significant decrease in creatinine level compared to Cisplatin®-treated EAC mice (Fig. 9).

Discussion

In vitro antitumor activity

The current research was planned to detect the potential immunomodulation and antitumor efficacy of the extracts of *P. pavonia* and *J. rubens* inhabiting the Egyptian water. Our overall data has succeeded to present the extracts derived from *P. pavonia* and *J. rubens* as novel immune regulators and a promising anticancer agent against Ehrlich ascites breast carcinoma (EAC). Cancer is the world's most serious health concern, and the best treatment is cancer chemotherapy, which is cytotoxic to normal cells as well. Seaweeds are new, inexpensive, healthy, and efficient anticancer agents with minimal side effects on normal cells (Alves et al., 2018; Okolie et al., 2018; Zandi et al., 2010). Seaweed-derived extracts, such as *P. pavonia* and *J. rubens*, have been described as immunomodulatory and anticancer drugs, immunological regulators stimulating immune cells, enhancing immune function, and acting as

adjuvants in cancer immunotherapy (Costa, et al, 2010; Liang et al., 2014; Xue et al., 2012; Yende et al., 2014;).

In a dose-dependent manner, treatment of EAC cells with *P. pavonia* and *J. rubens* extracts resulted in significant reductions in the number and survival of EAC tumor cells in an *in vitro* investigation. Regardless of differences in the chemical composition of the *P. pavonia* and *J. rubens* extracts; both induced direct antitumor effects, indicating that the biological products of genus *pavonia* and *rubens* have potent antitumor effects. Despite the antitumor effects of these two agents were not the same as those of cisplatin® (reference drug), our data had shown the promising antineoplastic effects of *P. pavonia* and *J. rubens* extracts especially at high doses (1000 µg/ml) *in vitro*. It is well known that cancer cells possess a high proliferative rate and low apoptotic rate -opposite to normal cells and the antitumor potential of conventional chemotherapeutic drugs such as Cisplatin® mostly mediated at least in part, by increasing apoptosis, and decreasing DNA proliferation of rapidly dividing cells (Sinicrope et al., 1996; Wang et al., 2005).

We found that *P. pavonia* and *J. rubens* extracts caused an elevation in EAC cell apoptosis Accompanied by a decrease in cell proliferation post i.p. treatment of EAC tumor cells for 24 h *in vitro*. *In vitro* studies also showed that the *J. rubens* extract had a higher apoptotic impact than *T. atomaria*, *P. pavonia*, and *C. elongate* extracts in enhancing the rate of EAC apoptosis, with IC₅₀ values of 475.1 µg/ml, 580 µg/ml, 613 µg/ml, and 630.9 g/ml, respectively. Previously, it was reported that polysaccharides crude extracts of *P. pavonia*, *J. rubes*, and *Corallina officinalis* significantly inhibited the human breast MCF7 and colon cancer CoCa2, liver cancer cell line HePG2 cell lines proliferation *in vitro* when compared to the reference drug doxorubicin (Gheda et al., 2018; Matloub et al., 2015). The biological and antitumor activity detected in the tested seaweed extracts may be associated with the presence of oxygenated sterols and diterpenes (Awad et al., 2008), even though these should be further studied. The differences in the antitumor potentials between *P. pavonia* extracts and *J. rubens* extracts may be due to specific variations, environmental habitats, the used concentrations, or other factors that need further studies.

Antitumor effects of different doses of seaweeds extracts on the host tumor growth in vivo

In vitro investigations of growth suppression of EAC tumor cells are consistent and strongly suggest that *P. pavonia* and *J. rubens* extracts may have anticancer characteristics. Next, we tended to evaluate whether the direct immunomodulation and antitumor efficacy of the *P. pavonia* and *J. rubens* extracts can also be observed *in vivo*. In comparison to naïve EAC mice and Cisplatin®-treated EAC mice, i.p. treatment of EAC-bearing mice with *P. pavonia* extract at low, high, and prophylactic doses (1.3 g/mouse, 2.5 g/mouse, and 2.5 g/mouse, correspondingly) or *J. rubens* extract at low, high, and prophylactic doses (1.2 µg/mouse, 2.3 µg/mouse and 2.3 µg/mouse, respectively) have resulted in a considerable reduction in the number of ascetic EAC tumor cells. Previously, *Ulva rigida* extracts significantly decrease the tumor's developmental size, *J. rubens* extract at 250 mg/kg and 500 mg/kg arrest the cell proliferation

and tumor cell growth, and *Eucheuma cottonii* extract suppressed tumor growth and reduce mammary gland tumour development in rats (Ahmed et al., 2011; Mohini et al., 2014; Shamsabadi et al., 2013).

Changes in the level of leucocytes profile

In addition to the direct antitumor effect exerted by both extracts, we hypothesized that both extracts may exert an immunomodulatory effect that could indirectly potentiate their antitumor efficacy *in vivo*. We investigated the systemic immunomodulatory potentials of tumor inoculation and the ameliorative effect of *P. pavonia* and *J. rubens* extracts. The i.p. inoculation of EAC tumor cells induced significant leukocytosis. Furthermore, an elevated leukocyte count linked to the onset of cancer has been found as one of the predictors of an increased risk of venous thromboembolism (VTE) in cancer patients. Thrombosis is the second greatest cause of cancer-related mortality. (Khorana and colleagues, 2008). Interestingly, we explored that treatment of EAC mice with either Cisplatin® or *P. pavonia* extract or *J. rubens* extract significantly increased the leukocytosis induced by tumor inoculation. Moreover, both *P. pavonia* and *J. rubens* extracts displayed a more powerful efficacy than Cisplatin® in the inhibition of leukocytosis. Accordingly, this revealed that *P. pavonia* and *J. rubens* extracts may be potent tools for not only cancer treatment but also, for inhibition of thrombosis. Hence, they will help better the prognosis for cancer patients.

We hypothesized that i.p. inoculation of EAC mice with *P. pavonia* and *J. rubens* extracts did not increase the total number of WBCs but elevated their flow to the tumor site. The migratory capacity of leukocytes is crucial to their role as defensive cells implicating their role in the progression and spread of tumors. Different leukocyte populations, including neutrophils, dendritic cells, macrophages, eosinophils, mast cells, and lymphocytes, are known to have a role in tumor-associated inflammation (Balkwill and Mantovani, 2001). Our results revealed that i.p. inoculation with *P. pavonia* and *J. rubens* extracts managed to decrease neutrophilia induced by tumor inoculation. Neutrophilia is one of the haematological findings related to poor prognosis in human metastatic melanoma, pancreatic carcinoma, and renal carcinoma (Fogar et al., 2006; Schmidt et al., 2005).

Remarkably, treatment of EAC mice with *P. pavonia* and *J. rubens* extracts at low, high, and prophylactic doses induced a slight increase in the lymphocytes relative number compared to naïve EAC mice and Cisplatin®-treated EAC mice. Unlike neutrophils, the increased lymphocytic count is considered a good prognostic candidate in diverse cancers (Lissoni et al., 2006).

Monocyte subsets are immune cells that have recently been identified as key regulators of cancer growth and development. Phagocytosis, tumouricidal mediator secretion, angiogenesis, extracellular matrix remodeling, lymphocyte recruitment, and tumor-associated macrophages differentiation and dendritic cells are all functions performed by different monocyte subsets that associate to the antitumoral immunity (Olingy et al., 2019). Intraperitoneal treatment of EAC mice with *P. pavonia* at high or prophylactic doses and *J. rubens* extracts at low, high or prophylactic doses has successfully decreased the monocytes relative number compared to naïve EAC mice and Cisplatin®-treated mice. Meanwhile, a low dose of *P. pavonia* extract seemed to increase the relative number of monocytes compared to naïve

EAC mice. Such data could be consistent with the associated neutropenia induced by EAC tumor cells treatment. As, withdrawal of neutrophils numbers is accompanied by their transformation into other myeloid cells including MDSCs (Sagiv et al., 2015).

Flow cytometry analysis

Algal extracts have been widely used in cancer immunotherapy due to their properties as an initiator and modulator of immune responses, and their vaccinations appear to be safe and enhance immunological responses in cancer treatment as well as long-term clinical remissions, but there are some issues that need to be addressed before they can be used clinically. According to flow cytometric analysis of phenotypic expression of immune lymphocyte cells subset surface markers, treatment of mice with *P. pavonia* and *J. rubens* extracts at low, high, and prophylactic doses showed putative immune-modulatory effects on the expression and proliferative response of spleen lymphocyte subpopulations; CD4 + T, CD8 + T, and NK cells.

P. pavonia and *J. rubens* administration increased the percentage of lymphocyte subpopulations; CD4⁺ T cells, CD8⁺ T cells, and CD335 cells and lymphocyte proliferative responses that were stimulatory at all tested doses, and there was a dose-dependent increase in the proliferative activity of CD4 + T-cells, CD8 + T cells, and NK cells in EAC-bearing mice comparing to naïve EAC mice and Cisplatin®-treated EAC mice.

More specifically, i.p. inoculation of EAC mice with *P. pavonia* and *J. rubens* extracts induced an immune response favoring tumor cell death, tumor antigen cross-presentation *in vivo*, and the production of cytokines favoring homeostatic proliferation and/or ablation of immunosuppression mechanisms (Baxevanis et al., 2009). The change in the ratio of CD4⁺T-cell, CD8⁺T-cell, may be attributed to the migration of immune cells populations to the developed tumor, leading to a significant reduction of the same population in the spleen.

Natural killer (NK) cells are an innate immune system subpopulation of lymphocytes that play a key role in the host defense against tumor growth and infectious pathogens (Shen et al., 2008). NK cells can detect and kill a broad variety of abnormal cells, like tumor cells, without damaging healthy cells (Caligiuri, 2008). The same findings as polysaccharide from *Laminaria digitate* activated immune systems, B and helper T lymphocytes were evaluated in the literature (Hoffman et al., 1995). Later, the fucoidan isolated from *Sargassum* sp and *Fucus vesiculosus* stimulates natural killer cell activity *in vivo* in noncancerous mice (Ale et al., 2011). Data from *in vivo* studies observed inhibitory activity of cancer, which could be attributed in part to increased innate and specific immunity (Lowenthal and Fitton, 2015). Fucoidan from *Undaria pinnatifida* sporophyll increased survival in P-388 tumor-bearing mice, which was linked to the enhancement of natural killer (NK) lymphocyte activity and increased interferon-gamma production by T cells (Maruyama et al., 2003). *Gracilaria lemaneiformis* Sulfated polysaccharides prevented tumor growth; increased CD8⁺ T cells, splenocyte proliferation, macrophage phagocytosis (Fan et al. 2012; Gesheva et al., 2014).

Our results show that one of the antitumor mechanisms of the extract of *P. pavonia* and *J. rubens* may be to mediate an increase in T-lymphocytes subsets of helper (CD4⁺ T-cell), cytotoxic (CD8⁺ T-cell), and NK cells in the spleen that resulted in the production of immunostimulation of the immune system triggering the antitumor response development.

Assessment of apoptosis by flow cytometry

In the current study, we aimed to analyze the potentiation of both extracts on the apoptosis rate and proliferative rate of EAC tumor cells by Annexin V-FITC Apoptosis assay using flow cytometry, which targets the loss of integrity of the plasma membrane (Vermes et al., 1995; Wlodkowic et al., 2009). It is well known that cancerous cells have a high proliferative degree and low apoptotic rate -contradictory to normal cells. The antitumor efficacy of conventional chemotherapeutic agent such as Cisplatin® is generally mediated at least in part, by elevating apoptosis rate, and reducing DNA proliferation of rapidly dividing cells (Sinicrope et al., 1996; Wang et al., 2005).

Interestingly, *P. pavonia* and *J. rubens* extracts at all tested doses increased the percentages of apoptosis in a dose-dependent manner as high doses increase the percentages of apoptosis compared to low ones. Besides, analysis of the cell cycle revealed that the *P. pavonia* extract was of a more potent effect than *J. rubens* extract in increasing apoptosis of EAC cells *in vivo*. As it successfully increased EAC apoptosis even more than the therapeutic dose of Cisplatin®. It is recommended a strongly related activity of *P. pavonia* and *J. rubens* extracts to develop an antitumor response in a tumor-bearing host.

Our data revealed that i.p. inoculation of EAC mice with *P. pavonia* and *J. rubens* extracts at low, high, and prophylactic doses resulted in an elevation in EAC cell apoptosis accompanied by a decrease in cell proliferation. Additionally, *in vivo* data indicated that the *P. pavonia* extract was of a more apoptotic effect than *J. rubens* extracts in increasing the rate of EAC apoptosis. The apoptosis mode of action may vary among different anticancer bioactive compounds, and the main anticancer mechanism of the *P. pavonia* and *J. rubens* extracts is the apoptosis induction (Jose, 2015). Several other anticancer studies of sulfated polysaccharides from *P. pavonia* and *Jania rubens* (Gheda et al., 2018; Jose et al., 2018) were published, with identical findings for both qualitative and quantitative apoptosis evaluation.

Liver and kidney functions

Finally, serum liver transaminases (ALT and AST) and albumin are tested to rule out hepatocellular injury and hepatotoxicity as a side effect of therapy. Their increases in serum are a cellular outflow marker and a loss of functional integrity of hepatocyte cell membranes (Ghoury et al., 2010; Rajesh and Latha, 2004). We also investigate the serum kidney creatinine and urea, the most widely used biomarkers for determining the amount of nephrocellular damage, to rule out nephrocellular damage and nephrotoxicity caused by chemical stressors (Orinya et al., 2016; Singh et al., 2011).

Our data revealed that i.p. inoculation of *J. rubens* extracts at low, high or prophylactic doses, but not *P. pavonia* extract doses, has successfully decreased the level of serum AST, ALT, and albumin and showed a significant effect in restoring their levels to be very close to the naïve mice comparing to naïve EAC mice

and Cisplatin®-treated EAC mice, indicating restoring liver function and integrity and promoting the antioxidant hepatoprotective role of *J. rubens* extracts (Bodansky, 1973). A previous study reported that treatment of experimental animals with extract of *J. rubens*, *Sargassum subrepandum*, and *Ulva lactuca* maintained liver enzymes in the range of the normal levels compared to the naïve tumor group. Therefore, methanol extract of *J. rubens* may be promising hepatoprotective agents that could synergistically inhibit the process of hepatocellular injury initiation and progress in combination with its antioxidant activity (El Gamal, 2010).

Hepatotoxicity may be induced by a high dose of Cisplatin® (Cüre et al., 2016). One of the most significant pathway implicated in Cisplatin® toxicity is oxidative stress and may be the primary source of Cisplatin®-induced toxicity attributable to decreased GSH glutathione depletion (Bentli et al., 2013). Transaminases are the most important biomarkers specifically involved in causing cell damage and toxicity, as they are released into circulation after cell damage.

Importantly, our results revealed that i.p. inoculation of *J. rubens* extracts at low, high or prophylactic doses, but not *P. pavonia* extract doses, has successfully decreased the concentrations of serum creatinine and urea and reported a significant effect in restoring their concentrations to be close to the naïve mice comparing to naïve EAC mice and Cisplatin®-treated EAC mice. As a result, methanol extract of *J. rubens* may be promising nephroprotective agents that could synergistically inhibit the initiation and progression of nephrocellular injury in combination with its antioxidant activity. This effect could be due to its antioxidant activities and active antioxidant constituents.

Nephrotoxicity is Cisplatin's main dose-limiting side effect (Sastry and Kellie, 2005). Cisplatin®, as well as direct cellular toxicity, triggers an inflammatory cascade where neutrophils may induce kidney injury via different pathways such as the development of proinflammatory mediators, reactive species, proteases, and other products that may provoke tubular damage resulting in acute kidney injury (Tadagavadi and Reeves, 2017).

Conclusion

Further studies are needed to isolate the therapeutic bioactive components of *P. pavonia* and *J. rubens*, identify the underlying mechanisms of their antitumor immunity and antitumor cytotoxic effects, investigate their immunomodulatory effect that could indirectly potentiate their antitumor efficacy, and their ameliorative effects on tumor inoculation. Overall, both extracts of *P. pavonia* and *J. rubens* possess potential antitumor and antitumor immunological effects with less toxicity, opening new approaches for further studies of the chemical and biological mechanisms behind these effects.

Abbreviations

Hep-G2, human hepatoma G2 cells; MTT, (3-(4,5-Dimethylthiazol-2-yl)); EAC, Ehrlich ascites carcinoma; WBC, white blood cells; MCF-7, acronym of Michigan Cancer Foundation-7; IC₅₀, 50% inhibition

concentration; VEGF, vascular endothelial growth factor; NK, natural killer; FBS, fetal bovine serum; RPMI-1640, Roswell Park Memorial Institute Medium-1640; ACK, Ammonium-chloride-potassium; DMSO, Dimethyl sulfoxide; LD₅₀, 50% lethal dose; TBRI, Theodor Bilharz Research Institute; PB, peripheral blood; ANOVA, a one-way analysis of variance; SD, standard deviation

Declarations

Acknowledgments: Not applicable.

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Figures

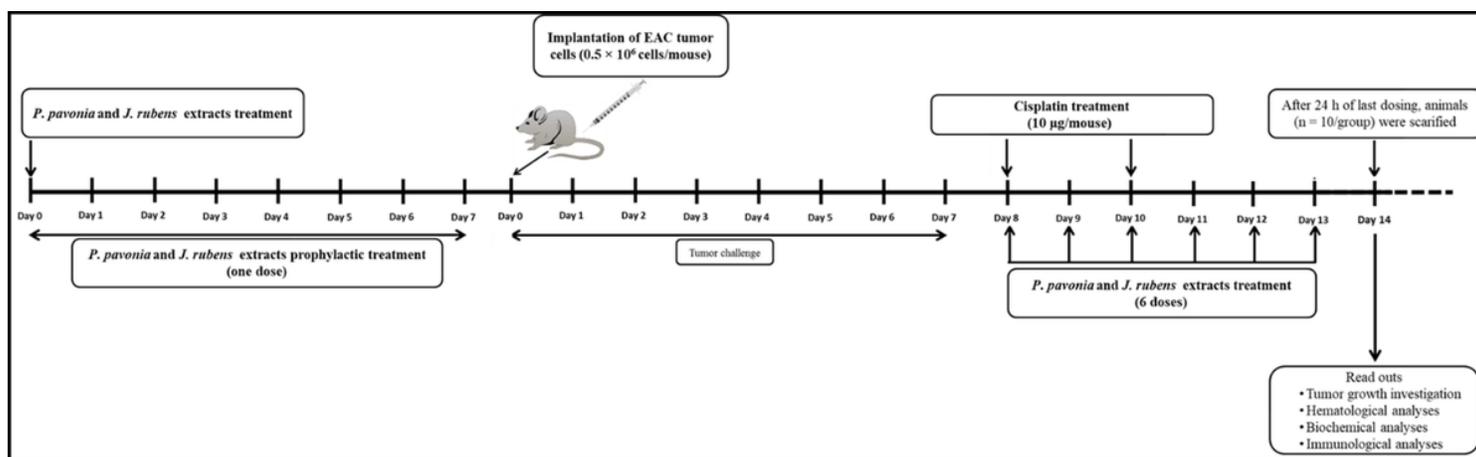


Figure 1

In vivo experiment dosage plan

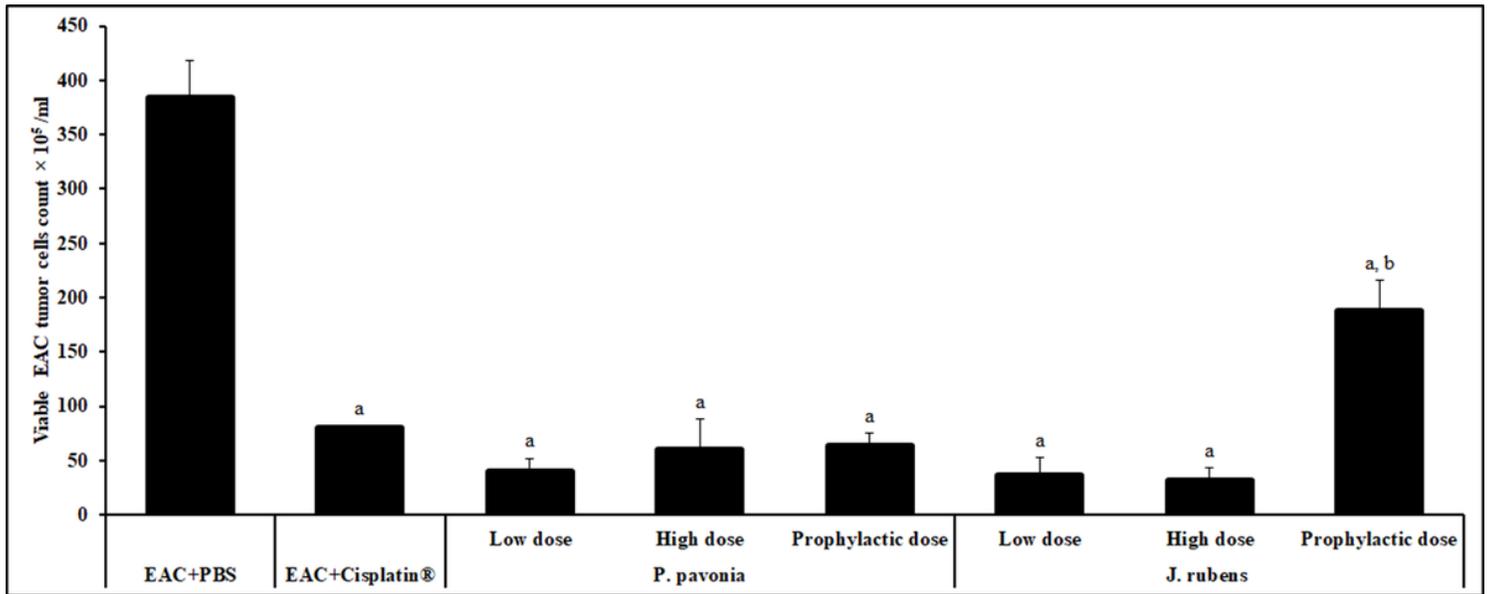


Figure 2

Antitumor effects of *p. pavonia* and *J. ruben* extracts on EAC tumor growth *in vivo*: Mice (n=10) were i.p inoculated with *p. pavonia* at low dose (1.3 µg/mouse), high dose (2.5 µg/mouse) or prophylactic dose (2.5 µg/mouse), *J. rubens* at low dose (1.2 µg/mouse), high dose (2.3 µg/mouse) or prophylactic dose (2.3 µg/mouse), Cisplatin® (10 µg/mouse) or phosphate buffer saline (PBS). Data were represented as mean ± SD. The difference between groups was considered statistically significant at $P < 0.05$. ^a: statistically significant vs. EAC mice. ^b: statistically significant vs. EAC mic + Cisplatin®

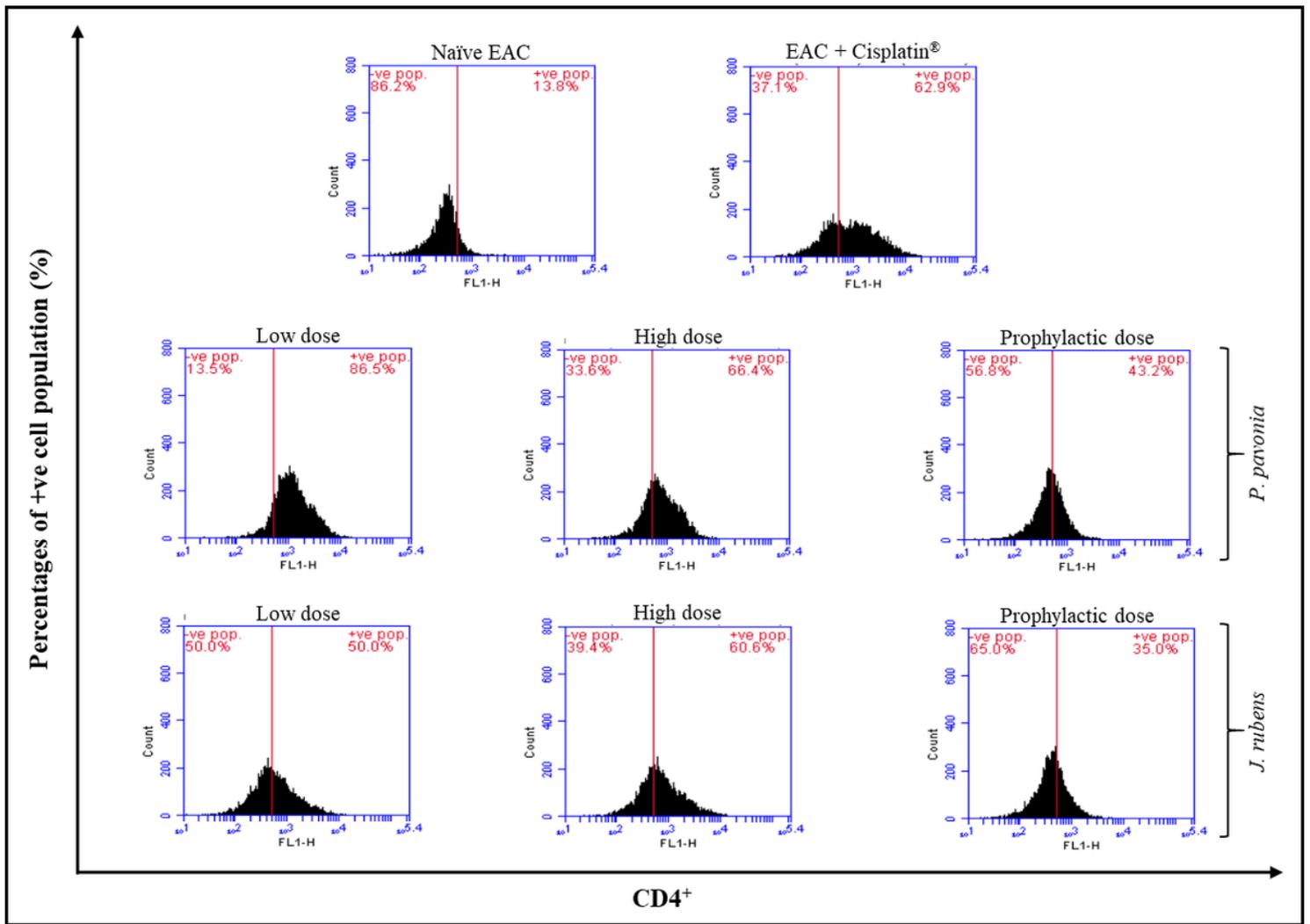


Figure 3

Flow cytometric analysis of Phenotypic modifications helper CD4⁺ T cells induced in EAC mice by treatment with *p. pannonia* and *J. rubens* extracts : Mice (n=10) were i.p inoculated with *p. pannonia* at low dose (1.3 µg/mouse), high dose (2.5 µg/mouse) or prophylactic dose (2.5 µg/mouse), *J. rubens* at low dose (1.2 µg/mouse), high dose (2.3 µg/mouse) or prophylactic dose (2.3 µg/mouse), Cisplatin® (10 µg/mouse) or phosphate buffer saline (PBS).

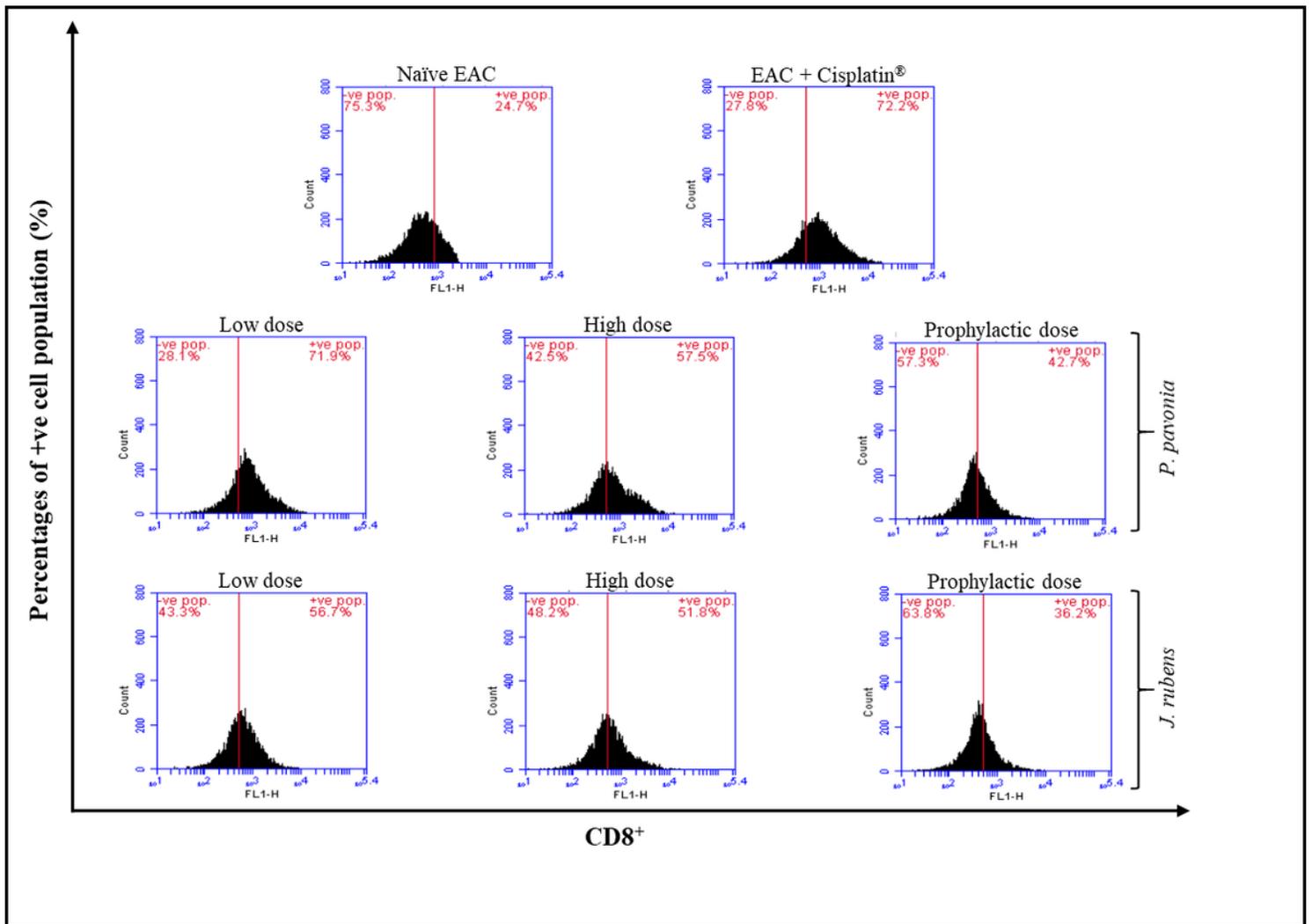


Figure 4

Flow cytometric analysis of phenotypic modifications cytotoxic CD8⁺ T cells induced in EAC mice by treatment with *p. pavonia* and *J. rubens* extracts : Mice (n=10) were i.p inoculated with *p. pavonia* at low dose (1.3 µg/mouse), high dose (2.5 µg/mouse) or prophylactic dose (2.5 µg/mouse), *J. rubens* at low dose (1.2 µg/mouse), high dose (2.3 µg/mouse) or prophylactic dose (2.3 µg/mouse), Cisplatin® (10 µg/mouse) or phosphate buffer saline (PBS).

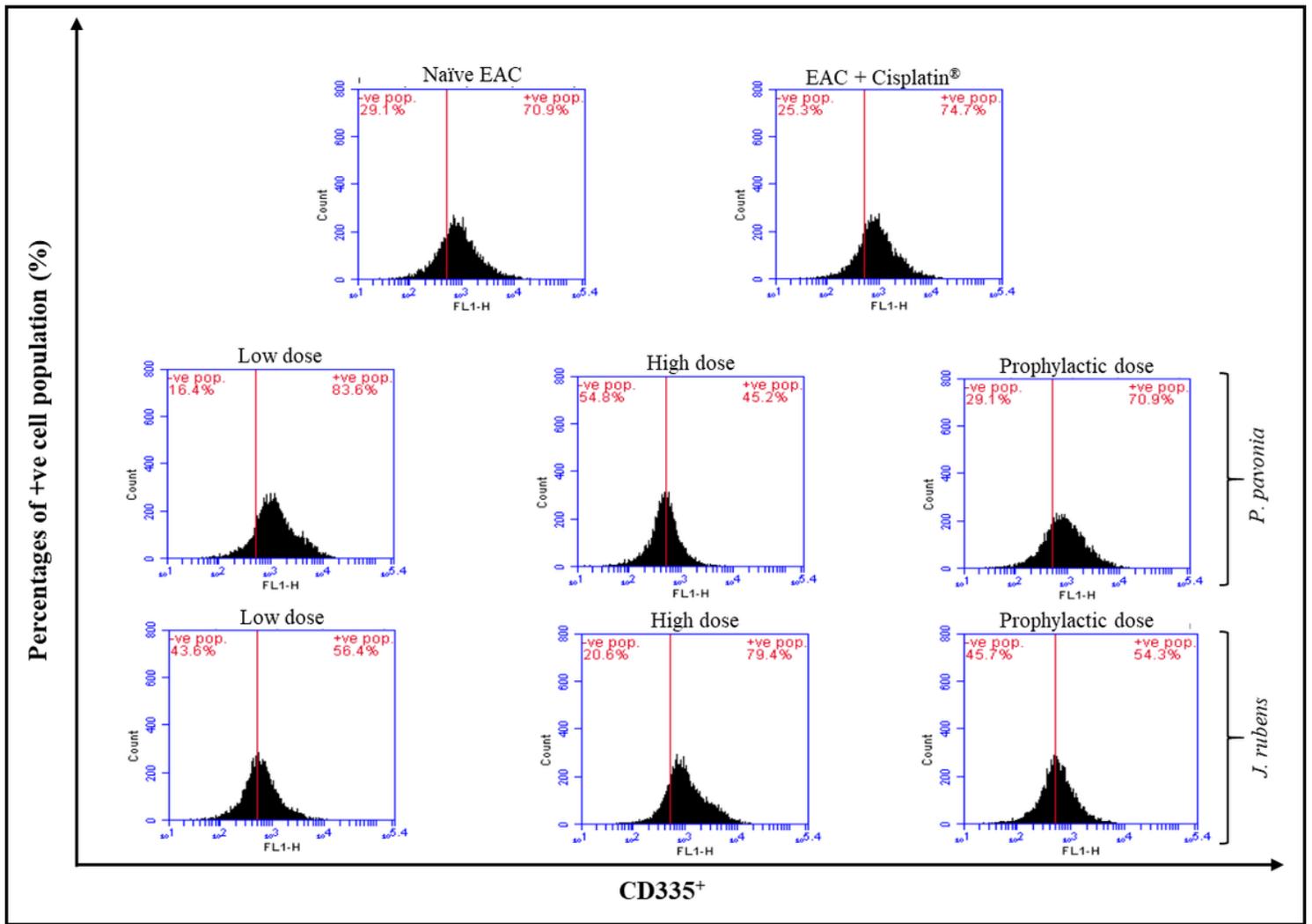


Figure 5

Flow cytometric analysis of Phenotypic modifications natural killer (NK) CD335⁺ cells induced in EAC mice by treatment with *p. pavonia* and *J. rubens* extracts : Mice (n=10) were i.p inoculated with *p. pavonia* at low dose (1.3 µg/mouse), high dose (2.5 µg/mouse) or prophylactic dose (2.5 µg/mouse), *J. rubens* at low dose (1.2 µg/mouse), high dose (2.3 µg/mouse) or prophylactic dose (2.3 µg/mouse), Cisplatin® (10 µg/mouse) or phosphate buffer saline (PBS).

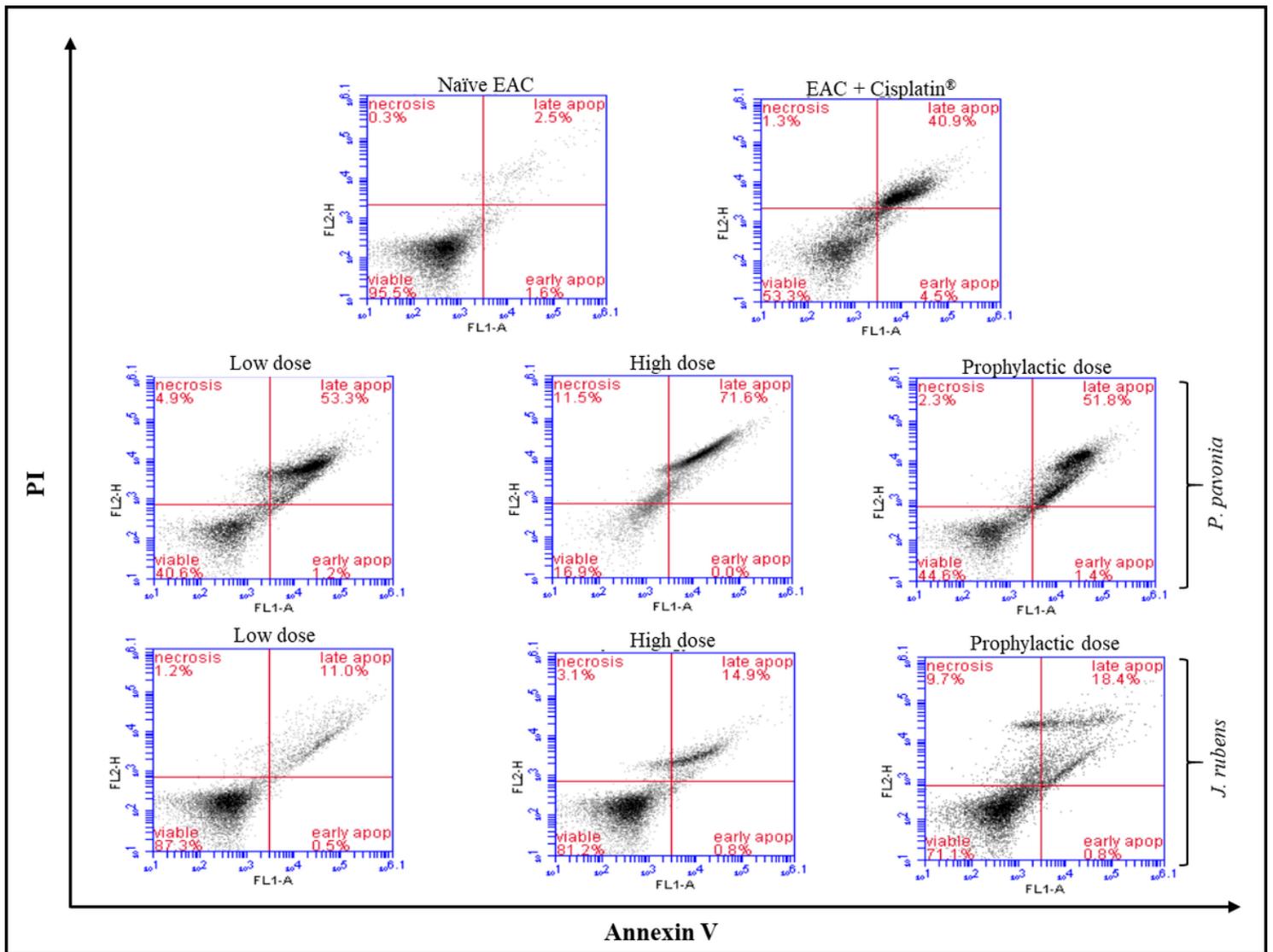


Figure 6

Flow cytometric analysis by Annexin-V/propidium iodide (PI) staining method of EAC tumor cells harvested from EAC mice treated with *p. pavonia* and *J. rubens* extracts : Mice (n=10) were i.p inoculated with *p. pavonia* at low dose (1.3 µg/mouse), high dose (2.5 µg/mouse) or prophylactic dose (2.5 µg/mouse), *J. rubens* at low dose (1.2 µg/mouse), high dose (2.3 µg/mouse) or prophylactic dose (2.3 µg/mouse), Cisplatin® (10 µg/mouse) or phosphate buffer saline (PBS).

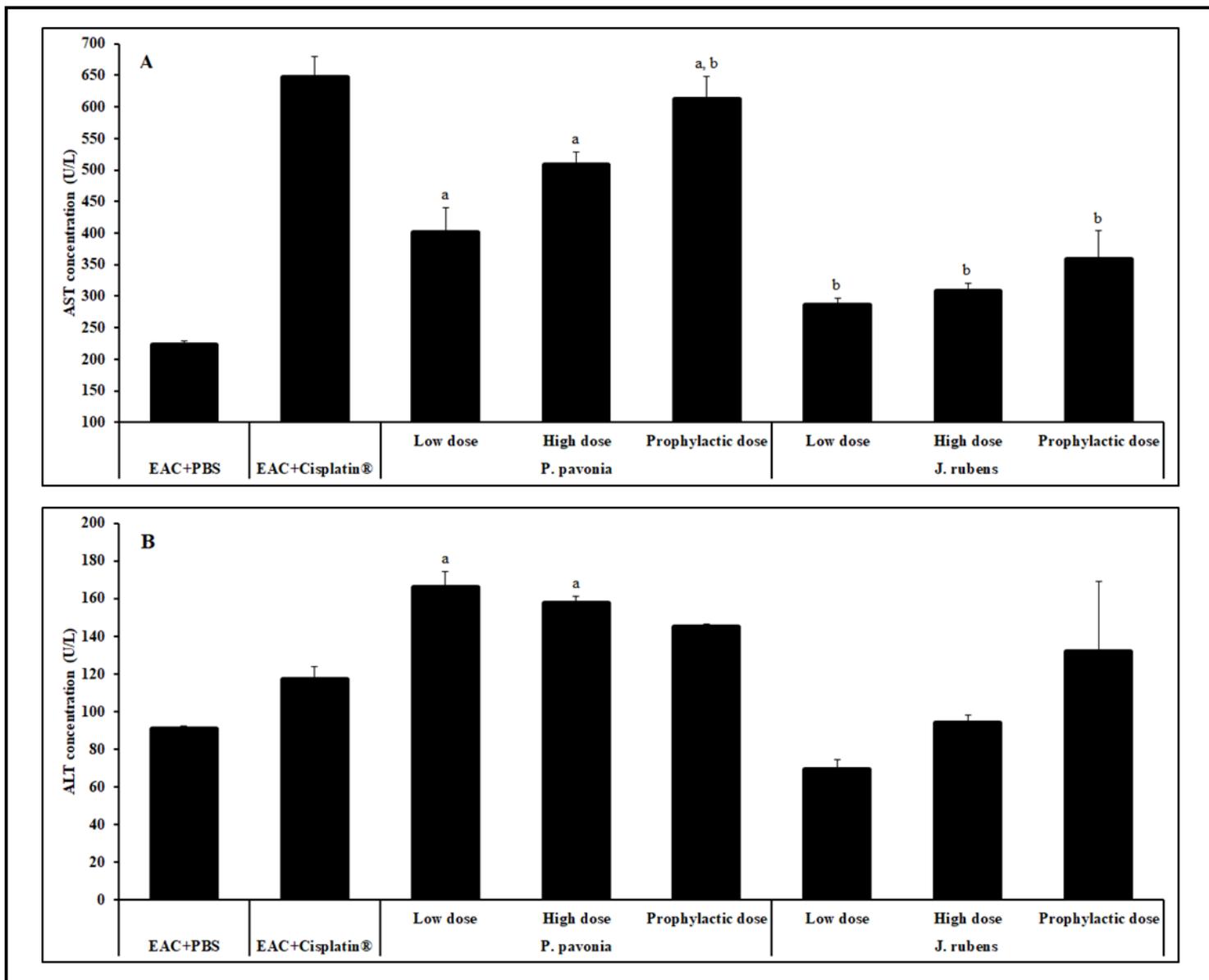


Figure 7

The effects *p. pavonia* and *J. rubens* extracts on liver function in EAC-bearing mice: Mice (n=10) were i.p inoculated with *p. pavonia* at low dose (1.3 µg/mouse), high dose (2.5 µg/mouse) or prophylactic dose (2.5 µg/mouse), *J. rubens* at low dose (1.2 µg/mouse), high dose (2.3 µg/mouse) or prophylactic dose (2.3 µg/mouse), Cisplatin® (10 µg/mouse) or phosphate buffer saline (PBS). Data were represented as mean ± SD. The difference between groups was considered statistically significant at $P < 0.05$. ^a: statistically significant vs. EAC mice. ^b: statistically significant vs. EAC mic + Cisplatin®.

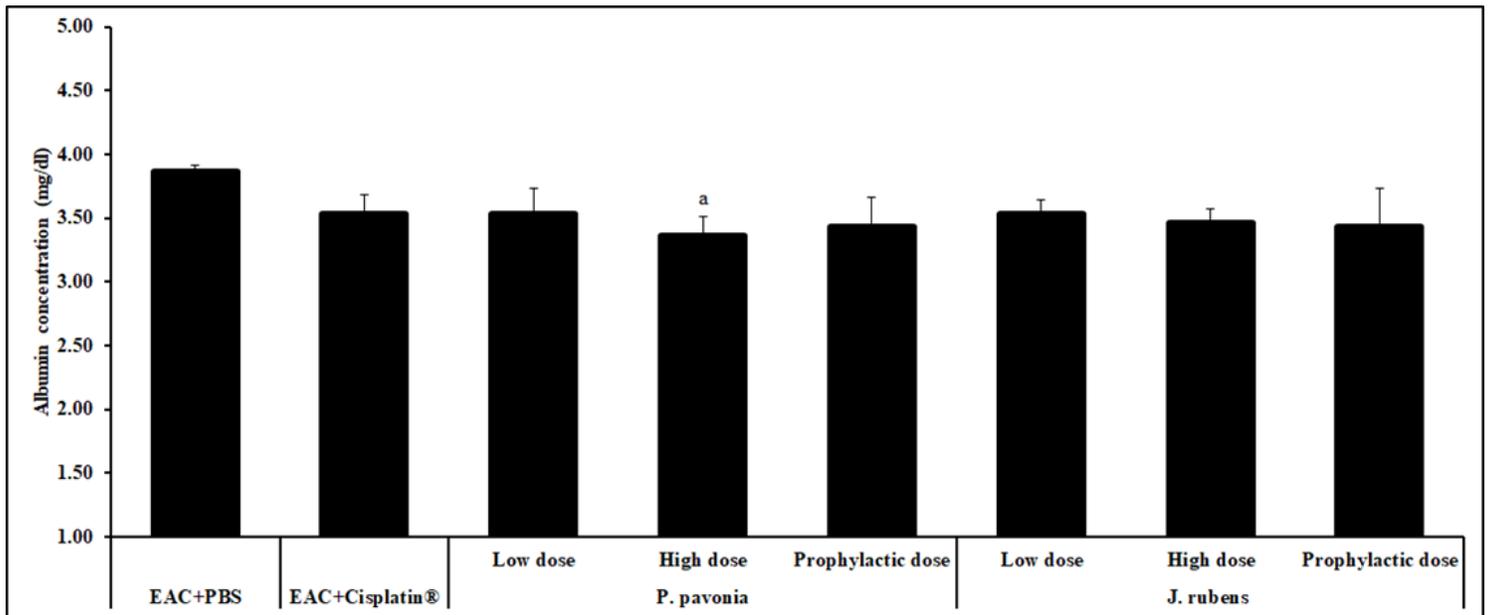


Figure 8

The effects *p. pavonia* and *J. rubens* extracts on Albumin concentration in EAC-bearing mice: Mice (n=10) were i.p inoculated with *p. pavonia* at low dose (1.3 µg/mouse), high dose (2.5 µg/mouse) or prophylactic dose (2.5 µg/mouse), *J. rubens* at low dose (1.2 µg/mouse), high dose (2.3 µg/mouse) or prophylactic dose (2.3 µg/mouse), Cisplatin® (10 µg/mouse) or phosphate buffer saline (PBS). Data were represented as mean ± SD. The difference between groups was considered statistically significant at $P < 0.05$. ^a: statistically significant vs. EAC mice.

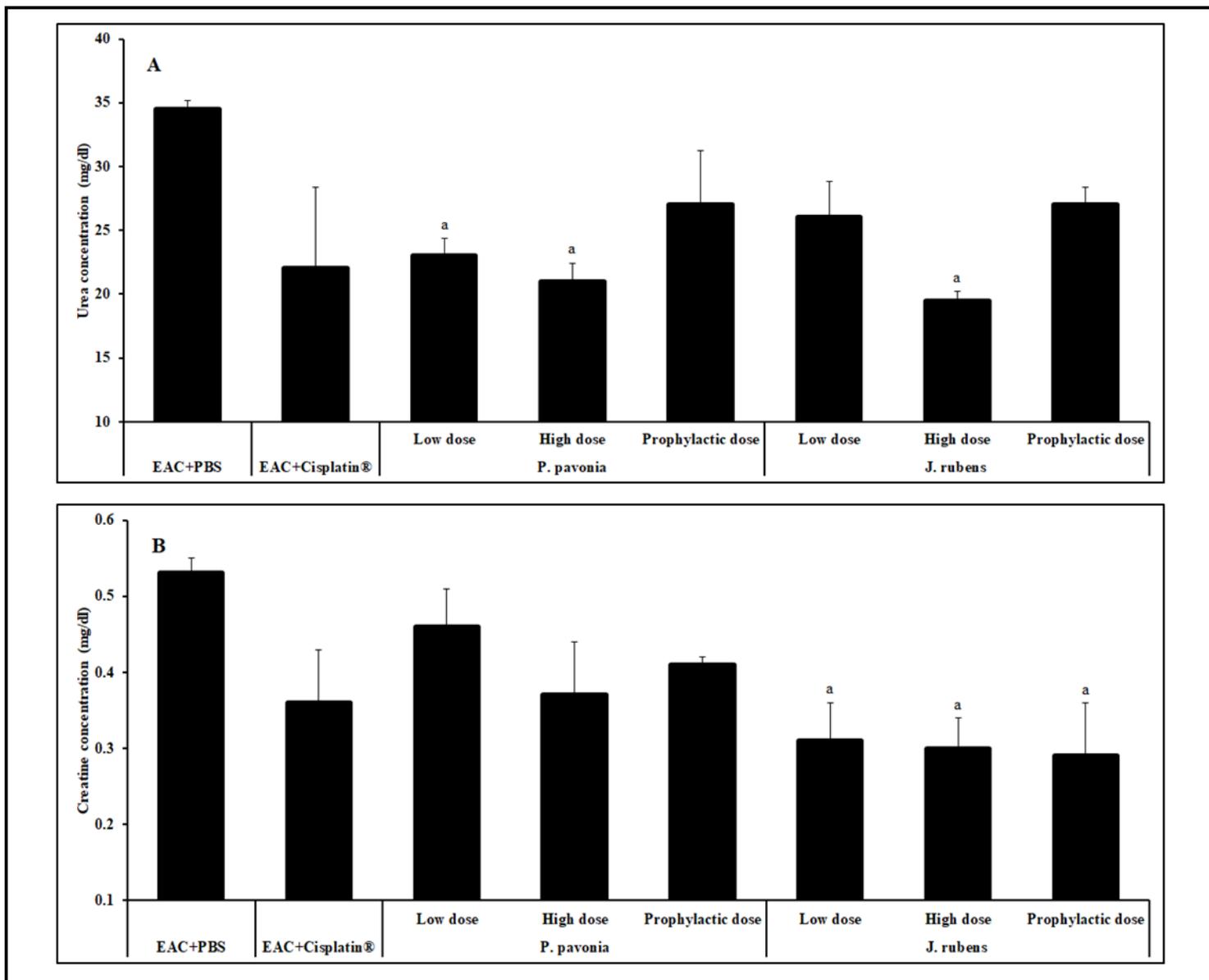


Figure 9

The effects *p. pavonia* and *J. rubens* extracts on kidney function in EAC-bearing mice: Mice (n=10) were i.p inoculated with *p. pavonia* at low dose (1.3 µg/mouse), high dose (2.5 µg/mouse) or prophylactic dose (2.5 µg/mouse), *J. rubens* at low dose (1.2 µg/mouse), high dose (2.3 µg/mouse) or prophylactic dose (2.3 µg/mouse), Cisplatin® (10 µg/mouse) or phosphate buffer saline (PBS). Data were represented as mean ± SD. The difference between groups was considered statistically significant at $P < 0.05$. ^a: statistically significant vs. EAC mice.

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