Possible anticancer effects of rutin and orlistat in Ehrlich-ascites carcinoma in mice.

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Abstract:

Cancer is a broad term used to describe a large number of diseases characterized by uncontrolled cell proliferation that leads to tumor production. Cancer is associated with mutations in genes controlling proliferation, oxidative stress and other mechanisms. Currently, most antineoplastic drugs have severe adverse effects and new effective and safe drugs are needed. This study aims to investigate the possible anticancer activity of rutin and orlistat which are both safely used clinically in humans against an *in vivo* (Ehrlich ascites carcinoma; EAC) model of cancer.

Our results have shown that both rutin and orlistat exerted an *in vivo* anticancer activity as evidenced by the decrease in tumor weight and the exerted antioxidant action (increased catalase content). In conclusion, the anticancer activity of rutin and orlistat makes them promising candidates for cancer treatment alone or in combination with other anticancer drugs specially that they are used clinically with acceptable safety profile.

Key words: Rutin, Orlistat, Ehrlich ascites carcinoma, mice.

Introduction

Cancer is a non-communicable disease that represents the second largest cause of mortality in the world(Islam *et al.* 2014). Breast cancer is considered as the leading cause of cancer-related mortality among women (Youlden *et al.* 2012). Breast cancer is expected to account for 29% all new cancer diagnoses in women. Breast cancer incidence rates are highest in more developed countries with lower incidence in less developed countries (Siegel *et al.* 2016).The mechanisms by which cancer occurs are not completely understood (Posey 2005).

Oxidative stress has been suggested to play a role in carcinogenesis (Uotila *et al.* 1994; Papas 1996). With regard to tumour development, ROS (reactive oxygen species) have been considered as DNA-damaging agents that increase mutation rate and promote oncogenic transformation (Jackson *et al.* 2001). Several factors of our modern life style, e.g. alcohol consumption, tobacco chewing and smoking habits, exposure to toxic chemicals and radiation, all add to the free radical production in the body and increase the risk of cancer (Clayson *et al.* 1994). Evidence from recent studies suggests that cancer cells, compared to normal cells, are under increased oxidative stress associated with oncogenic transformation, alterations in metabolic activity, and increased generation of ROS (Toyokuni *et al.* 1995; Hileman *et al.* 2001; Kang *et al.* 2003; Hileman *et al.* 2004).

Many anticancer drugs have been shown to be teratogenic and carcinogenic in experimental systems (Sorsa *et al.* 1985; Cherry *et al.* 2004) and are associated with many other adverse effects including alopecia, bone marrow suppression, constipation, diarrhea and anemia in humans (Perry, 1969). Therefore, there is a great demand for novel safe and effective anticancer drugs.

Flavonoids, a class of polyphenols found in fruits and vegetables, have been shown to have promising chemopreventive properties against different cancer types (Nair *et al.* 2004; Chen *et al.* 2013). Rutin (5, 7, 3', 4'-OH, 3-rutinose) is the glycosidic form of quercetin, being classified as a flavonol (Ross *et al.* 2002). Rutin is a powerful antioxidant with pharmacological benefits including anticarcinogenic, cytoprotective, antiplatelet, antithrombic, anti-inflammatory, antidiarrhoeal, antimutagenic, vasoprotective and cardioprotective activities (La Casa *et al.* 2000; Janbaz *et al.* 2002; Schwedhelm *et al.* 2003; Sheu *et al.* 2004; Mellou *et al.* 2006; Trumbeckaite *et al.* 2006). Orlistat (tetrahydrolipstatin), an over-the-counter anti-obesity drug, decreases absorption of dietary fats by inhibiting gastric and pancreatic lipases through covalent modification of the enzymes (Dowling *et al.* 2009). It is also a potent irreversible inhibitor of the thioesterase activity of FAS (fatty acid synthase) (Kridel *et al.* 2004). A decrease in DNA synthesis, arrest of cell progression through the G1/S boundary and apoptotic cell death are all consequences of inhibiting FAS in cultured cancer cells treated with orlistat (Pizer *et al.* 1998; Knowles *et al.* 2004).

The objective of the current investigation is to study the anticancer activity of rutin and orlistat against an *in vivo* model of cancer in mice (solid ehrlich carcinoma model; SEC).

2-Materials and Methods
2.1.Drugs used:

Doxorubicin HCl (doxorubicin vial®, 50mg/25ml, Ebewe, Australia) was administered by intraperitoneal injection in a dose of 2mg/kg (Sugiyama *et al.* 1998). Rutin (RTN), (Acros Organics, Belgium), suspended in saline using 0.5% w/v carboxymethyl cellulose, was administered by intraperitoneal injection in a dose of 20mg/ kg (Alonso-Castro *et al.* 2013).

Orlistat (ORL, Xenical®120mg/capsule, Roche Pharmaceuticals) was administered by intraperitoneal injection in a dose of 240mg/kg (Kridel *et al.* 2004). The content of each capsule (120 mg) of orlistat was solubilized in 33% ethanol during 30 min and vortexed every 10 min. After centrifugation for 10 min at 14,000 r.p.m, supernatants were retrieved and stored at -80 ^{0}C (Kridel *et al.* 2004; Carvalho *et al.* 2008).

2.2.Solid Ehrlich carcinoma (SEC) tumor model:

A model of SEC was used, where 2.5 x 10^6 of the Ehrlich carcinoma cells (ECC) obtained from the Pharmacology and Experimental Oncology Unit of the National Cancer Institute, Cairo University, Egypt. Tumor cells implanted subcutaneously into the right thigh of the lower limb of Swiss albino mice (Osman *et al.* 1993; Fahim *et al.* 1997).

2.3. Animals:

Adult female Swiss albino mice weighing (20–30 g) were purchased from Theodor Bilharz Research Institute, Cairo, Egypt. The mice were kept under standard environmental and nutritional conditions throughout the investigation. All experimental procedures were approved by the Ethical Committee for Animal Handling at Zagazig University (ECAHZU), approval no. (P3-3-2014). Mice were randomly distributed into 5 groups (n=20) as follows:
Group (1): Control. Group (2): Ehrlich tumor cells implanted subcutaneously into the right thigh of the lower limb of mice (Osman *et al.* 1993; Fahim *et al.* 1997). Group (3): Rutin given by intraperitoneal injection daily for 21 days (Lin *et al.* 2009) after subcutaneous implantation of Ehrlich tumor cells. Group (4): Orlistat given by intraperitoneal injection daily for 21 days (Kridel *et al.* 2004; Chuang *et al.* 2011) after subcutaneous implantation of Ehrlich tumor cells. Group (5): Doxorubicin (the standard anticancer drug) given by intraperitoneal injection, on days

14, 16, 18 and 20 after subcutaneous implantation of Ehrlich tumor cells (Sugiyama *et al.* 1998).

2.4. Assessment of relative tumor weight and animal weight:

At the end of the study, mice were weighed, sacrificed, tumor excised and weighed. The relative tumor weight was calculated as follows:

Relative organ weight = $\frac{\text{tumor weight}}{\text{body weight}} \times 100$

2.5. Determination of relative liver and heart weight:

Additionally, after sacrifice, the liver and the heart were quickly removed and weighed individually. The relative organ weight was calculated as follows:

Relative organ weight = $\frac{\text{organ weight}}{\text{body weight}} \times 100$

2.6. Assessment of the biochemical parameters:

At the end of the study, blood samples were collected, clear sera were obtained and stored at -20 ^{0}C until further analysis. Alpha-Fetoprotein (AFP) in serum was determined by Enzyme-Linked Immunosorbent Assay (ELISA) using kit obtained from Chemux BioScience,Inc (USA) as described by (Hirai *et al.* 1973; Hirai 1982).

The tumor was homogenized in cold potassium phosphate buffer (50mM, pH 7.5, 5 ml/g tissue), centrifuged at 4000 r.p.m for 15 minutes then the supernatant was used for the determination of tissue catalase using Kits obtained from Biodiagnostic Co. (Aebi 1984)

2.7. Blood sampling, Assesment of Hematological Parameters:

Aliquot of blood was collected from each mouse into ethylenediamine tetra-acetic acid- (EDTA-) coated tubes for the analysis of hematological parameters using an automated analyzer Swelab Alfa (BouleMedical AB, Sweden).

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's post Hoc test using Graph pad Prism$^®$ software$^®$ version 5. For all analysis, the level of statistical significance was set at P< 0.05.

3-Results

1. **Effect of cancer and treatment with rutin, orlistat and doxorubicin on relative tumor weight and body weight:**

Doxorubicin, rutin and orlistat induced a significant decrease in relative tumor weight by 52, 45 and 41% compared with cancer group (9.9, 11.3 and 12 vs. 20.4% respectively) and they did not induce any significant change in animal weight (fig.1).

2-Effect of cancer and treatment with rutin, orlistat and doxorubicin on relative liver and heart weight:

Figure (2) represents the relative liver and heart weight of mice under different treatment conditions. Subcutaneous implantation of Ehrlich tumor cells into the right thigh of the lower limb of mice and treatment with doxorubicin, rutin and orlistat did not induce any significant change in relative liver and heart weight.
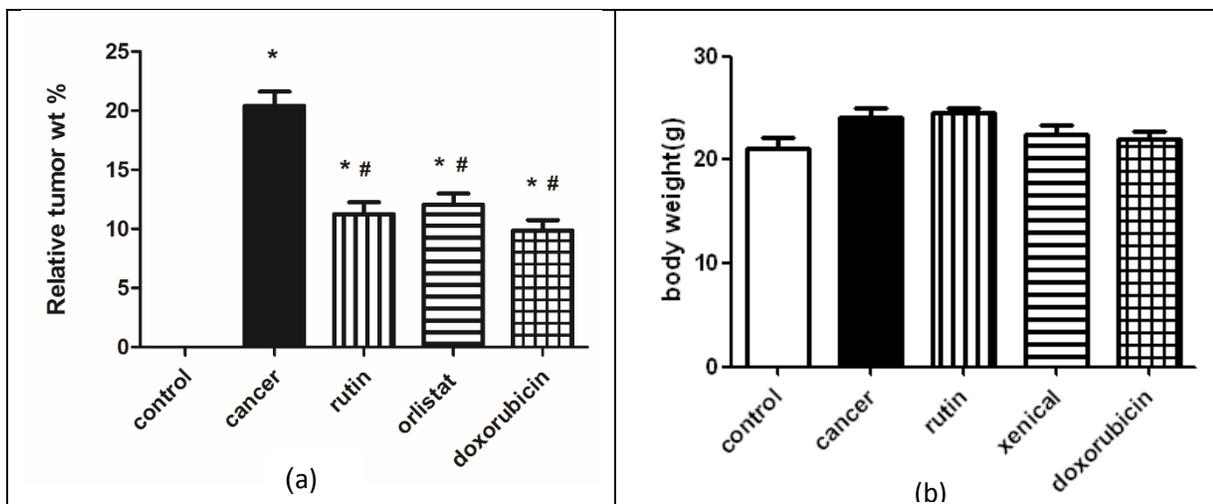
Fig. (1): Effect of cancer and treatment with rutin, orlistat and doxorubicin on a) relative tumor weight and b) body weight. Data are expressed as mean ± S.E.M. n=20.
* Significantly different from the control group at P<0.05. # Significantly different from cancer group at P<0.05 using ANOVA followed by Tukey's posthoc test.
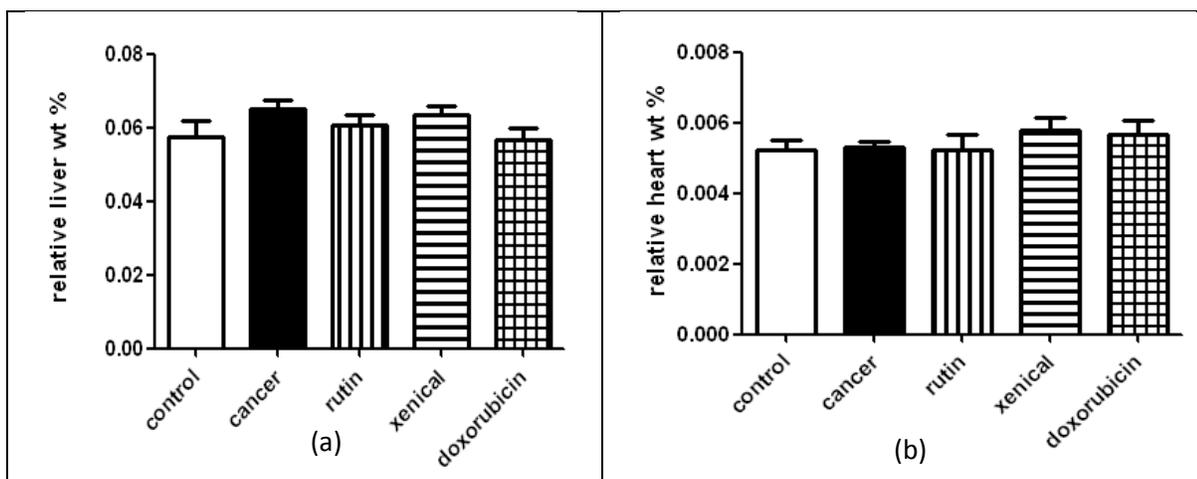

Fig. (2): Effect of cancer and treatment with rutin, orlistat and doxorubicin on relative a) liver weight and b) heart weight. Data are expressed as mean ± S.E.M. n=20 using ANOVA followed by Tukey's posthoc test.

3. Effect of cancer and treatment with rutin, orlistat and doxorubicin on catalase activity:

Subcutaneous implantation of Ehrlich tumor cells into the right thigh of the lower limb of mice resulted in a significant decrease in tissue catalase by 77% compared to the control group (2.9 vs. 12.4u/g).

On the other hand, doxorubicin induced a significant increase in tissue catalase by 108% compared with cancer group (6 vs. 2.9u/g). Rutin induced a significant increase in tissue catalase by 90% compared with cancer group (5.6 vs. 2.9u/g). Orlistat

induced a significant increase in tissue catalase by 71% compared with cancer group
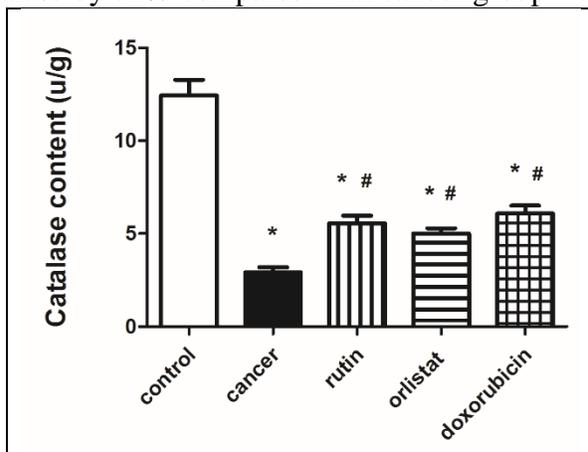
(5 vs. 2.9u/g) (fig. 3).

Fig. (3). Effect of cancer and treatment with rutin, orlistat and doxorubicin on tissue catalase. Data are expressed as mean ± S.E.M. n=20.
* Significantly different from the control group at P<0.05. # Significantly different from cancergroup at P<0.05 using ANOVA followed by Tukey's posthoc test.

4. Effect of cancer and treatment with rutin, orlistat and doxorubicin on Alpha-Fetoprotein (AFP):

Figure (4) recorded AFP levels in tumor tissue of Ehrlich carcinoma bearing mice under different treatment conditions.

Subcutaneous implantation of Ehrlich tumor cells into the right thigh of the lower limb of mice and treatment with doxorubicin, rutin and orlistat failed to cause any significant change in AFP.
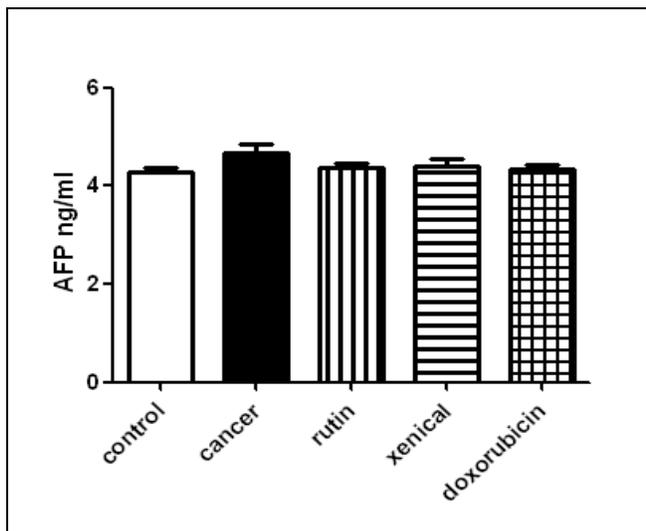

Fig.(4): Effect of cancer and treatment with rutin, orlistat and doxorubicin on AFP. Data are expressed as mean ± S.E.M. n=20 using ANOVA followed by Tukey's posthoc test.

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5. Effect on hematological parameters:

Table (1) shows that different treatment regimens used in the present study resulted in non significant alterations of hematological

parameters compared to untreated tumor-bearing mice

Table (1): Effect of tumor inoculation and intra-peritoneal injection of doxorubicin, rutin and orlistat on hematological parameters:

Items	control	cancer	rutin	xenical	doxorubicin
RBC (10^{12}/l)	8.٢+0.٤	8.335+0.22	7.6+0.٤	7.1+0.1	7.2+0.٣
WBC (10^9/l)	7.1+0.٥	7.9+0.٧	8+0.6	6.2+0.3	9.١+0.٧
HCT (%)	48.3 +2.1	50.7+1.١	50.4 +2.٧	44.2 +1.٥	42.٧+2.1
LYM (10^9/l)	5.٤+0.3	5.٧+0.5	5.9+0.4	5.٨+0.٤	5.٤+0.4
GRAN (10^9/l)	0.4	0.٤	0.3	0.٤	0.٤
MCHC (g/dl)	25.١+0.8	23.9+0.٧	24+0.٥	23.1+0.3	23.7+0.5
HGB (g/dl)	12 +0.3	12.٢+0.5	11.8+0.٧	10.2+0.٤	10.٢+0.5
MCV (fl)	59.٢+1.4	6١+1.١	61.٨+1.١	62.6+2.1	58+1.٦
MPV (fl)	6.5+0.1	6.9+0.1	6.٧+0.٢	6.٧+0.1	6.7+0.1
MCH (pg)	14.٨+0.5	14.٦+0.٣	14.٨+0.٣	14.٥+0.6	13.7+0.٤
RDW (%)	21.٦+1.١	1٨+1.٥	19.6+1	23.1+1.٧	22.٥+1.٦
platelet 10^9/l	524.3+48.2	687+54.6	855.7+81.6[a]	1059+65[ab]	1138+78.6[ab]

Data are expressed as mean $\pm$ SEM, n=20
[a]Significantly different from the control group
[b] Significantly different from the cancer group at $p < 0.05$ using ANOVA followed by Tukey's post hoc test.

4- Discussion

Cancer is the second leading cause of death by disease in the world (Xia *et al.* 2014). Breast cancer is the most common cancer among women (Ponzone *et al.* 2006). Chemotherapy is one of three major therapeutic methods for cancer treatment (Xia *et al.* 2014). Patients receiving chemotherapeutic agents suffer several adverse effects which might be transient (e.g. alopecia) or irreversible (e.g. cardiac or pulmonary toxicity), so the search for new drugs with suitable anticancer activity and acceptable adverse effect profile is rational approach in cancer therapy. Ehrlich carcinoma has been chosen to be a model for breast cancer in the present study.

Doxorubicin is a versatile anthracycline anti-tumour agent, which is effective against wide range of cancers including solid tumours, leukemias and lymphomas (SKCarter 1975). However, the clinical use of doxorubicin is restricted by its severe adverse reactions such as cardiotoxicity (Sazuka *et al.* 1989).

Rutin, a poly-phenolic bioflavonoid has shown wide range of pharmacological applications due to its significant antioxidant properties. Conventionally, it is used as antimicrobial, antifungal, and anti-allergic agent (Chen *et al.* 2013). However, current

Zagazig J. Pharm. Sci. Dec, 2017
Vol. 26, Issue 2, pp, 55-66

lSSN 2356_9786
ISSN (on-line) 2356_9786

research has shown its multispectral pharmacological benefits for the treatment of various chronic diseases such as cancer, diabetes, hypertension, and hypercholesterolemia (Hunyadi *et al.* 2012). Its use is advantageous over other flavonoids as it is a nontoxic and nonoxidizable molecule (Sharma *et al.* 2013).

Moreover, orlistat is an irreversible inhibitor of pancreatic and gastric lipases which is clinically used because of its anti-obesity properties (Dowling *et al.* 2009). Orlistat blocks the activity of the thioesterase domain of fatty acid synthase (FAS) (Kridel *et al.* 2004). In fact, FAS inhibition by orlistat reduces proliferation and promotes apoptosis in prostate, breast and stomach cancer cell lines (Knowles *et al.* 2004; Kridel *et al.* 2004; Menendez *et al.* 2005; Menendez *et al.* 2005) and has shown antitumor activity by inhibiting the growth of prostate cancer xenografts (Kridel *et al.* 2004).

Implantation of ECC in the thigh of female Swiss albino mice induced the formation of solid tumor which showed marked increase in its volume continuously with time (Osman *et al.* 1993; Fahim *et al.* 1997). The results revealed a significant reduction in the level of catalase (fig.3).

It was previously shown that the presence of tumor caused disequlibria of the antioxidant defense system including both complex enzyme systems e.g. catalase and non-enzymatic antioxidant, low molecular weight free radical scavenger, e.g. glutathione (Kumaraguruparan *et al.* 2002).

Tumor inoculation led to a non significant difference in tumor marker AFP (fig.4), which may be due to the time factor of experiment (Ahmed *et al.* 2011) showed that the post-treatment of the Ehrlich ascites carcinoma EAC-bearing mice for two months with their tested antitumour and antioxidant agent produced a significant decrease in the serum level of the tumor markers CEA and AFP in comparison with the untreated EAC-bearing mice so, we expected that the duration of the experiment may affect change in the tumor markers concentrations.

Doxorubicin showed marked regression in tumor growth that was evidenced by the reduction of tumor weight (fig.1a). This finding resembles that of the previous study on tumor growth (Ahmed *et al.* 2013). In addition, it induced a significant improvement in tissue antioxidant status that was evidenced by a significant increase in tissue catalase (fig.3). These results were in accordance with El-Dayem *et al.* (2013) and Adwas *et al.*(2016).

Different mechanisms have been proposed for doxorubicin antitumor effects: free-radical generation, DNA intercalation/binding, activation of signaling pathways, inhibition of topoisomerase II and apoptosis (Mordente *et al.* 2001). Takahashi, (2011) reported that doxorubicin has a protective effect against tissue damage induced by oxidative stress which was in the same line with the results of our study.

Rutin showed marked regression in tumor growth by the reduction of tumor weight (fig.1a). This finding was in accordance with that of (Alonso-Castro *et al.* 2013) who stated that rutin exerts antitumor effects on nude mice bearing SW480 tumor. In addition, it induced a significant improvement in antioxidant activity that was evidenced by the significant increase in tissue catalase (fig.3). These results were in accordance with Gautam *et al.* who stated that rutin has antioxidant effects (Gautam *et al.* 2016).

In other studies, rutin has suppressed cell proliferation by inducing G2/M cell cycle arrest and promoting apoptosis in human neuroblastoma cells LAN-5 (Chen *et al.* 2013).

On the other hand, orlistat is a potent inhibitor of FAS (Kridel *et al.* 2004), which showed a marked regression in tumor growth that was observed by the reduction of tumor weight (fig.1a), this is in agreement with Kridel *et al*. (2004). Also, it affected

oxidative stress parameters by causing a significant increase in tissue catalase (fig.3). These results were in accordance with that shown by (Bougoulia *et al.* 2006) and (Vincent *et al.* 2007). Orlistat was reported to induce a decrease in DNA synthesis, arrest of cell progression through the G1/S boundary and apoptotic cell death in cultured cancer cells due to inhibition of FAS (Pizer *et al.* 1998; Knowles *et al.* 2004). Previously, it was shown that orlistat could suppress the tumor growth of melanoma, prostate and grastric cancers *in vivo* (Martinez-Villaluenga *et al.* 2010; Kridel *et al.* 2004; Carvalho *et al.* 2008; Dowling *et al.* 2009).

In the present study, there is no toxic effects of rutin and orlistat on mice during the period of treatment as evidenced by non significant change (P<0.05) in the relative organ weight of liver and heart (fig.2a&2b). In addition, there was no hematotoxicity as evidenced by non significant alterations in complete blood count (CBC) parameters (table.1). It was previously shown that orlistat treatment exhibited no outward signs of toxicity on animals, experienced no loss of weight nor were there any effects of orlistat (240 mg/kglday) on hematocrit or WBC levels (Kridel *et al.* 2004). In keeping with this line, (Alonso-Castro *et al.* 2013) reported that rutin at all concentrations (1-20 mg/kg) did not affect the relative organ weight and total body weight.

In conclusion, this study showed that rutin and orlistat exert *in vivo* antitumor activity against solid Ehrlich tumour in mice as evidenced by the significant reduction in tumor weight and improved antioxidant status.

Rutin and orlistat may be a good option for cancer treatment, especially that they are clinically used with acceptable side effects, following further experiments and clinical trials to confirm their anticancer activity on humans which might allow their use in cancer chemotherapy alone or in combination with other anticancer drugs.

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التأثيرات المحتملة المضادة للسرطان من روتين وأورليستات

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السرطان هو مصطلح واسع يستخدم لوصف عدد كبير من الأمراض التي تتميز بتكاثر الخلايا الغير المنضبط الذي يؤدي إلى إنتاج الورم، ويرتبط السرطان مع الطفرات في الجينات المسيطرة على التكاثر وموت الخلايا المبرمج، والإجهاد التأكسدي، وتكوين الانزيم المسئول عن تصنيع الاحماض الدهنية وغيرها من الآليات. حاليا، فإن معظم الأدوية المضادة للسرطان لها آثار ضارة شديدة وهناك حاجة إلى أدوية فعالة وآمنة جديدة. تهدف هذه الدراسة إلى التحقيق في النشاط المحتمل المضاد للسرطان من الروتين والأورليستات والمستخدمان سريريا بأمان في البشر على حد سواء في الجسم الحي (فئران التجارب الحاملة لخلايا ايرليخ السرطانية) كنموذج للسرطان وقد أظهرت نتائجنا أن كلا من الروتين والأورليستات تمارس في الجسم الحي نشاط مضاد للسرطان كما يتضح من انخفاض في وزن الورم وممارسة عمل مضاد للأكسدة (زيادة محتوى انزيم الكاتاليز) ويمكننا استنتاج ان النشاط المضاد للسرطان من الروتين والأورليستات يجعلهم مرشحين واعدين لعلاج السرطان وحدهما أو بالاشتراك مع أدوية أخرى مضادة للسرطان خصيصا لأنهما يستخدمان سريريا مع سلامة مقبولة.