

***Original Article***

## **Evaluation of Cytotoxic Effect and Antioxidant Activity of Grape Seed Extract, Crocin, and Phenytoin**

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### **ABSTRACT**

Antioxidant compounds inhibit formation of free radicals, chelate catalytic metals, and scavenge free radicals in biological systems. In addition, antioxidants play a decisive role in prevention of numerous physiological dysfunctions, cancers, and metabolic disorders. This study sought to evaluate the antioxidant capacity and cytotoxic effect of grape seed extract (GSE), crocin (CRO), and phenytoin (PHEN) on a human breast cancer cell line (MCF-7). Methanol extracts of the three mentioned agents were prepared and their antioxidant activity was evaluated by diphenyl-1-picrylhydrazyl method, using Quercetine (QUER) as positive control. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate the cytotoxic effect of the extracts on Michigan Cancer Foundation-7MCF-7 cell line, using doxorubicin hydrochloride (DOX) as the positive control. Given the results, greater scavenging activity was achieved by using GSE in comparison to CRO and PHEN. Further, a significant correlation was found between the antioxidant activity and cytotoxic effects of these agents, and GSE had the highest antioxidant capacity and cytotoxic effect in comparison to CRO and PHEN.

**Keywords:** Antioxidant activity, Crocin, Cytotoxicity, Grape seed extract, Phenytoin

### **Étude comparative de la cytotoxicité et de l'activité antioxydante d'un extrait de pépins de raisin, de la crocine et de la phénytoïne**

**Résumé:** L'objectif de cette étude était d'évaluer et de comparer la capacité antioxydante et la cytotoxicité des extraits de pépins de raisin (GSE), de crocine (CRO) et de phénytoïne (PHEN) sur les cellules cancéreuses du sein. Les extraits de méthanol (MeOH) de ces composés ont été préparés et l'activité antioxydante de ces composés a été mesurée à l'aide de la méthode DPPH en utilisant de la quercétine (QUER) comme témoin positif. Le test a été utilisé pour l'évaluation de la cytotoxicité de ces différents composés sur des lignées cellulaires cancéreuses MCF-7 (cellules cancéreuses du sein). Dans cette expérience la doxorubicine (DOX) a été utilisée comme témoin positif. Les résultats de cette étude ont montré que l'activité anti-oxydante et la cytotoxicité de GSE sont plus élevées que CRO et PHEN, ainsi qu'il existe un lien direct entre l'activité antioxydante et la cytotoxicité de ces composés. Étant donné l'activité antioxydante et la cytotoxicité de GSE contre les cellules cancéreuses, cet extrait pourrait probablement accompagner d'autres composés dans le traitement du cancer. Des études supplémentaires sont nécessaires pour compléter ces résultats.

**Mots-clés:** extrait de pépins de raisin, crocine, phénytoïne, cytotoxicité, activité antioxydante

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## INTRODUCTION

Breast cancer is one of the most common cancers, as well as, the main cause of cancer deaths among women worldwide (World Health Organization, 2014). Postmenopausal women with metabolic syndrome are under higher risk of breast cancer and the likelihood of recurrence (Bruno et al., 2016). Currently, there is a tendency for using natural compounds containing cancer-preventive /antitumor properties in cancer therapy. Medicinal herbs induce their efficacy with various mechanisms including changes in carcinogen metabolism, stimulation of DNA repair systems, immune activation, repression of cell cycle apoptosis and decreasing production of reactive oxygen species (ROS) (Mazzio and Soliman, 2009). The plant kingdom is the most important source of Phenolic antioxidant (PA) and it is considered as one of the most important sources of plant secondary metabolites (Sen and Chakraborty, 2011). PA molecules consisted from at least one aromatic ring with one or more hydroxyl groups and their antioxidant capacity is mainly related to their high activity to chelate metals (Balasundram et al., 2006). Fruits and vegetables are the main source of nutritional phenolic antioxidants and play key roles in health especially prevention from cancers (Sen and Chakraborty, 2011). However, in some cases antitumor activity is not associated with antioxidant properties of phenolic compounds; therefore it has been the subject of researches to prove the presence of antitumor activity in phenolic compounds of various dietary plants. Grape seed extract (GSE) represent the excellent source of natural antioxidants in the form of polyphenols (eg., gallic acid and proanthocyanidins) (Sen and Chakraborty, 2011) and is one of the most widely consumed fruits in the world. Grape seeds are rich in variety of phenolic compounds such as dimers, trimers and other oligomers of flavan-3-ols that are identified as proanthocyanidins generally. Grape seed proanthocyanidins (GSPs) have proved chemopreventive and/or chemotherapeutic properties in *in-vitro* and *in-vivo* studies (Dinicola et al.,

2014). CRO is well known as a unique source of water soluble carotenoids which are found in the stigmas of *Crocus sativus* Linne and in the fruits of *Gardenia jasminoides* Ellis (eg. gallic acid and proanthocyanidins), which could be used in the pharmaceutical, cosmetic and food industries. It has been shown *in- vivo* and *in- vitro* that crocin and crocetin, the main carotenoids derivatives of saffron, present significant antitumor property in breast, lung, pancreatic and leukemic cells. Numerous previous studies reported that crocin contain antitumor properties and inhibit growth of several tumor cells (Alavizadeh and Hosseinzadeh, 2014). It has shown that long-term administration of CRO in colorectal cancer exerts a potent cytotoxic effect on human and animal adenocarcinoma cells *in vitro*; further confirmation came from three colorectal (HCT-116, SW-480 and HT-29) and two breast cancer cell lines, without affecting normal cells (Alavizadeh and Hosseinzadeh, 2014). Moreover, encapsulation of CRO in liposomal form also induced cell toxicity in the HeLa and MCF-7 cells. Altogether, these evidences obviously show that CRO can be valuable for treatment of cancers (Mousavi et al., 2011). One study reported that higher blood levels of some carotenoids may be decreased the risk of breast cancer, therefore the use of colored plant in diet may show preventing effect on breast cancer (Aune et al., 2012) and cardioprotective effect of CRO is reported (Razmaraii et al., 2016a). PHEN or dilantin, is a hydantoin derivatives and one of the oldest anticonvulsive agents that classified as a subdivision of class-1B-anti-arrhythmic drugs. This compound is used in treatment of epilepsy and shortens the potential duration (Rizzon et al., 1987). In addition, PHEN recently introduced as a wound healing agent (Pereira and Alchorne Ade, 2010). The aim of this study was exploration of antioxidant activity and cytotoxic effects of GSE, CRO and PHEN extract on MCF7 breast cancer cell lines. The antioxidant activity of extracts were examined by DPPH

methods and the cytotoxicity of compounds were conducted by MTT assay.

## MATERIALS AND METHODS

**Materials.** Doxorubicin hydrochloride was purchased from Exir Nano Sina Company, (Iran), phenytoin was kindly provided by Caspian Tamin Pharmaceutical Company, (Iran). Human breast adenocarcinoma MCF7 cell were acquired from National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide) and DPPH (1, 1-diphenyl-2-picrylhydrazyl) were from Sigma; methanol Merck, Germany; RPMI-1640, fetal calf serum (FCS), DMSO (dimethyl sulfoxide), penicillin, streptomycin, L-glutamine and sodium pyruvate were from Gibco.

**Free radical scavenging activity assay.** The capacity of the GSE, CRO and PHEN to scavenge radicals were determined by the method based on the reduction of DPPH (molecular formula C<sub>18</sub>H<sub>12</sub>N<sub>5</sub>O<sub>6</sub>) in the presence of a hydrogen donating antioxidant (Duan et al., 2006). The electron donation abilities of the corresponding pure compounds were measured from the bleaching of the purple-colored MeOH solution of 2, 20-diphenylpicrylhydrazyl (DPPH). Briefly, 8 mg DPPH was dissolved in 100 ml MeOH (solvent) to obtain a concentration of 80 µg/ml and GSE, CRO and PHEN separately were dissolved in solvent to prepare a concentration of 1 mg/ml. Dilutions were made to obtain different concentrations of agents and 5 ml of each solution were mixed with 5ml DPPH. Then solutions incubated for 30 minutes at room temperature and the absorbance was measured at 517 nm with a Shimadzu UV/Visible Spectrophotometer. The reduction percentage was assessed in order to analyze RC50 values which are the extract concentration providing 50% loss of DPPH activity. QUER was used as positive control and all experiments were performed in duplicate.

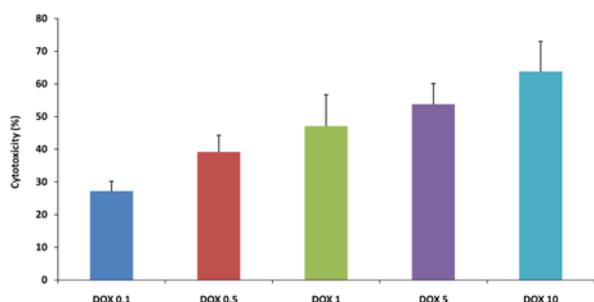
**Antitumor activity.** MTT assay was used to determine the effect of CRO, GSE and PHEN on growth and proliferation of the MCF-7 malignant cell

line, (Razmarai et al., 2016b). Briefly, the cells were cultured (5000 cells/well) in a 96-well plates in RPMI-1640 medium supplemented with 10% fetal-calf serum (FCS) and antibiotics (Penicillin G 50,000 units/l, Streptomycin 38,850 units/l and Nystatin 9078 units/l), and incubated in at 37°C with 10% CO<sub>2</sub> for 24 h. The cells were then exposed to a serial concentrations of GSE (250 and 500 µg/ml), CRO and PHEN (25, 50 and 100 µg/ml), and Doxorubicin (0.1, 0.5, 1, 5, 10 µg/ml as positive control) and incubated for 24 h. At the end of incubation time, MTT (20 µl with the concentration of 5 mg/mL) was added to each well and the plates incubated for further 6 h. Then, the culture medium was removed, 200 µl of DMSO was added to each well and the plates were shaken for 15 min. Finally, the optical density was measured at 570 nm using a microplate reader (AD 340; Beckmann Coulter). All the experiments were performed in triplicate.

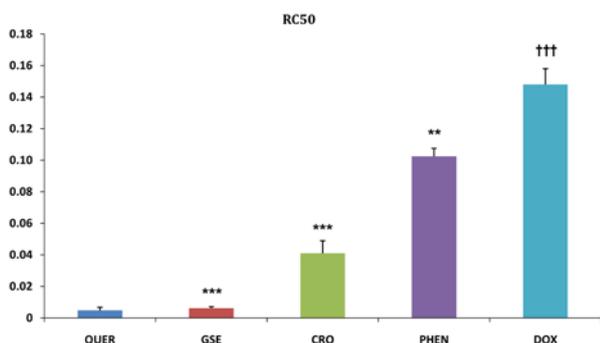
**Statistical Analysis.** All experiments were conducted in duplicate or triplicate measurements and expressed as the mean ± SEM. The groups were compared using student t-test or one-way analysis of variance (ANOVA) followed by Tukey's HSD test for pairwise comparison as appropriate. The data was analyzed using SPSS (v. 13.0) for Windows. All data are presented as the mean ± standard error of the mean (SEM). A *P*-value less than 0.05 were considered statistically significant.

## RESULTS

**Antioxidant activity.** The results of antioxidant activity of samples are illustrated in Figure 1. RC50 values of the DPPH radical scavenging activity of QUER, GSE, CRO, PHEN and DOX were 0.0048±0.002 (µg/mL), 0.0062±0.001 (µg/mL), 0.0410± 0.005 (µg/mL), 0.1025± 0.008 (µg/mL) and 0.1480± 0.01 (µg/mL), respectively. QUER was used as positive control. Experiment was performed in duplicate and values expressed as mean ± SD. †††: *p*<0.001 vs. Quercetin, \*\*: *p*<0.01, \*\*\*: *p*<0.001 vs. DOX.



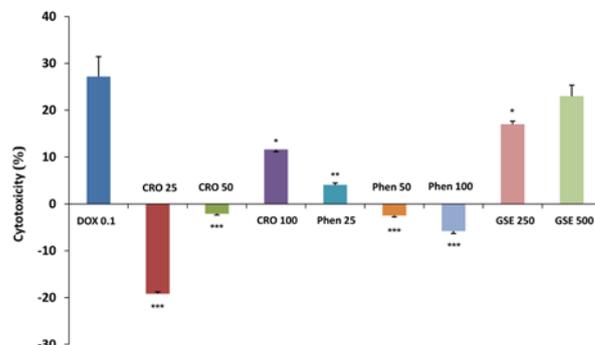
**Figure 1.** Comparison of Antioxidant activity of grape seed extract (GSE), crocin (CRO) and Phenytoin (PHEN) of MeOH extract. Doxorubicin (DOX) and Quercetine (QUER) were used as control. GSE: grape seed extract, DOX: doxorubicin, CRO: crocin, PHEN: phenytoin, QUER: Quercetine; the values are expressed as mean  $\pm$  SEM. †††:  $p < 0.001$  vs. Quercetine, \*\*:  $p < 0.01$  and \*\*\*:  $p < 0.001$  vs. DOX.



**Figure 2.** Cytotoxicity of DOX (doxorubicin; 0.1, 0.5, 1, 5 and 10  $\mu\text{g/ml}$ ) against MCF-7 cells, the results are mean values  $\pm$  SEM of five independent experiments performed in triplicate.

**Cytotoxicity/Antitumor activity.** The results of cytotoxicity obtained for different concentrations of DOX is illustrated in Figure 2 and cytotoxicity effect of GSE, CRO, PHEN and DOX 0.1 in MCF-7 cells are shown in Figure 3. MCF-7 was used as tumor cells in MTT assay to evaluate the antitumor activities of various agents. As illustrated in Figure 2, the data analyses showed that DOX induced dose-dependent cell toxicity. In addition the results indicated that GSE concentrations of 250  $\mu\text{g/ml}$  ( $p < 0.05$ ), CRO concentrations of 25, 50  $\mu\text{g/ml}$  ( $p < 0.001$ ) and 100  $\mu\text{g/ml}$  ( $p < 0.05$ ) revealed significant differences in cell toxicity as compared to the DOX group. Hence PHEN concentrations of 25  $\mu\text{g/ml}$  ( $p < 0.05$ ), 50 and 100

$\mu\text{g/ml}$  ( $p < 0.001$ ) are significantly different in cytotoxicity as compared to the DOX.



**Figure 3.** Cytotoxicity of DOX (0.1  $\mu\text{g/ml}$ ) and GSE (grape seed extract; 250 and 500  $\mu\text{g/ml}$ ), CRO (crocin; 25, 50 and 100  $\mu\text{g/ml}$ ), PHEN (Phenytoin; 25, 50 and 100  $\mu\text{g/ml}$ ). The results are mean values  $\pm$  SEM of five independent experiments performed in triplicate, \*:  $p < 0.05$ , \*\*:  $p < 0.01$  and \*\*\*:  $p < 0.001$  vs. DOX.

## DISCUSSION

The number of worldwide new cases of cancer in 2008 was estimated about 12.7 million (6.6 million men and 5.6 million women) and it is predicted to increase to 21.3 million in 2030. The most common malignant neoplasm among the women is breast, colorectal, cervical, lung and stomach (Ferlay et al., 2010). Breast cancer, the most frequently occurring cancer in females in the developed and less developed countries, is a major public health problem and it is estimated that over 508,000 women were died in 2011 due to breast cancer (World Health Organization, 2014). Despite breast cancer is believed to be a disease of the developed countries, approximately 50% of breast cancer cases and 58% of deaths are reported in less developed countries. Incidence of breast cancer rates differ greatly worldwide from 19.3 per 100,000 women in Eastern Africa to 89.7 per 100,000 women in Western Europe. In most of the developing world the incidence rates are below 40 per 100,000. The lowest incidence rates are reported in most African countries but here breast cancer occurrence rates are also growing (Anderson et al., 2008). Consume of plant derived compounds probably exerts a preventive effect

against common cancers. Hence, the association of dietary intake of fruit and vegetables antioxidant with risk of breast cancer have been suggested (Kris-Etherton et al., 2002). Inverse association between blood levels of antioxidant and carotenoids with breast cancer risk have also been confirmed by previous studies (Aune et al., 2012). Different natural sources, especially plants are the main source to discover new medicines. According to the World Health Organization recently reports, about 80% of the world's population trust on traditional medicines (World Health Organization, 2002). Currently, more than 121 types of medicines in USA are originated from natural products among them 91 types of medicines derived from botanicals and over twenty five chemotherapy medicines have been originated from derivatives of natural product (Newman and Cragg, 2007). Antioxidant activity of the GSE, CRO and PHEN determined by DPPH method is based on the ability of antioxidants to accept electron or hydrogen to become a stable diamagnetic molecule (Diphenylpicrylhydrazyl). It was found that these materials reduced DPPH radicals in a concentration-dependent manner. The lower RC50 values shows a stronger capacity of the antioxidant agents to scavenge the DPPH radicals whereas the higher RC50 values reveals a lower scavenging activity of the scavengers as more scavengers were required to achieve 50% scavenging reaction. Various antioxidant and anti-proliferative properties of grape seeds from different cultivars have been reported. The differences of antioxidant activity could be related to cultivated varieties, freshness and storage duration and methods of measurement (Sung and Lee, 2010). Natural phenolic antioxidants of grape seeds and seed fractions could be used as dietary source are flavones, anthocyanins and simple phenolic acids which are the most important GSE ingredients. In addition, the genotype, growing conditions and post-harvest treatments can change the quantity and quality of antioxidants capacity of GSE. This may be an influential factor in developing grape seeds-based nutraceutical ingredients for human consumption. The

results of our study indicated that GSE, CRO and PHEN exhibit moderate to strong radical scavenging activities (RC50 of 0.0062, 0.0410 and 0.1025 mg/ml, respectively) in comparison with QUER (RC50 0.0048 mg/ml) and DOX (RC50 0.1480 mg/ml), but the free radical scavenging of GSE (RC50 0.0062 mg/ml) was superior to the other two extracts. The GSE might contain phenolic compounds, e.g. flavonoids, coumarins or phenylpropanoids, which may contribute in free radical scavenging activity of this extract. These findings were consistent with previous reports about antioxidant capacities of GSE (Ahn et al., 2002). In contrast to current study, Sharma and colleges reported higher cytotoxicity of GSE on MCF-7 cells. This difference might be related to several factors such as cultivated varieties, freshness and storage duration and purity of GSE. Results of cytotoxicity assay showed a growth stimulating effect of very low doses of CRO, whereas higher doses of CRO remarkably inhibited the growth of tumoral cells (Fernández, 2006). These findings are in accordance with some reports and might be related to the antioxidant properties of polyunsaturated terpenoids of CRO and its metabolites (Alavizadeh and Hosseinzadeh, 2014). GSE showed significant antitumor activity on MCF7 cells. Furthermore, it was reported that procyanidin, an antioxidant flavonoid of the GSE, exhibited cytotoxicity effect on MCF7 cells. In this regard, other studies have shown that procyanidins induce cytotoxicity on several cancer cells (Nassiri-Asl and Hosseinzadeh, 2009). PHEN at concentration of 100 µg/ml stimulated proliferation of MCF-7 cells which were in line with some other studies (Correa et al., 2011). In this study, the inhibition of cancer cell proliferation by GSE, CRO and PHEN could be related to polyphenol or flavonoid compounds, but other phytochemicals agents may play a major role in the antiproliferative activity. Further investigations about combination of GSE and CRO for their antioxidant and antiproliferative activities would be valuable. Also, treatment of the MCF-7 cells by GSE and CRO provides cell toxicity. GSE are rich in phenolic

compounds and different activities of the GSE in comparison to CRO can be attributed to their different antioxidant agents. Main potential mechanisms of action of flavonoids could be listed as follows: prevention of ROS formation by suppression of enzymes and chelating trace elements involved in free radical production; scavenging ROS; up regulation of antioxidants defenses against p53 protein down regulation, cell cycle arrest, tyrosine kinase inhibition, prevention of heat shock proteins, estrogen receptor binding capacity and prevention of expression of Ras proteins (Kumar and Pandey, 2013).

In conclusion, results of our study demonstrated that GSE harbor a high antioxidant activity, which plays an important role in combating with reactive oxygen species and also in anticancer activities of GSE on MCF-7 tumoral cell line. CRO showed mild antioxidant and anti-cancer properties only at high doses and PHEN showed lesser antioxidant activity than other agents. Major biological activities of flavonoids are related to their structure. Antibacterial, hepatoprotective, anti-inflammatory, antitumor and antiviral efficacy of flavonoids is well known. These elements are more commonly utilized in the developing world. Medicinal use of new compounds must be confirmed using specific biochemical examinations. Further researches will provide safe, new and strong flavonoid for the treatment of various diseases specially cancers.

### Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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