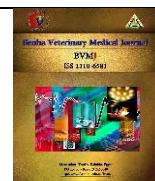




Official Journal Issued by
Faculty of
Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: <https://bvmj.journals.ekb.eg/>



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Original Paper

The combination of atorvastatin with Proanthocyanidin enhances antioxidant and reactive oxidative stress on HepG2 hepatocellular Carcinoma Cells.

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ARTICLE INFO

Keywords

Hepatocellular carcinoma

HpG2

Taxol

Atorvastatin

Proanthocyanidin

Received 13/07/2023

Accepted 17/08/2023

Available On-Line

01/10/2023

ABSTRACT

The purpose of this study was to measure the antioxidant state and reactive oxidative stress in the HPG2 cell line in order to explore the effects of Taxol, Atorvastatin, and proanthocyanidin. In this study, the effects of Atorvastatin, a novel 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, and Proanthocyanidin, the most prevalent polyphenol class in the human diet, on a range of metabolic diseases and chemotherapeutic effects were investigated. Taxol, a chemotherapeutic drug is substance derived from the bark and trunk of the Yew Pacific tree, was shown to assist induce and enhance tubulin polymerization and assembly while also preventing its depolymerization effects on oxidative stress and antioxidant enzymes. Taxol, Atorvastatin, and Proanthocyanidin inhibited the viability of HepG2 cells in a time- and dose-dependent manner, Superoxide dismutase (SOD) and catalase antioxidant enzyme activities were dramatically reduced as a result of the treatment, but glutathione reductase activity, malondialdehyde (MDA) concentrations, and malondialdehyde reductase activity were all significantly higher in treated groups than in untreated cells.

1. INTRODUCTION

As the fifth most frequent cancer globally, hepatocellular carcinoma (HCC) has risen to become the third largest cause of cancer-related fatalities. The incidence of HCC is expected to rise further through 2020 (Siegel et al., 2016). Recently, there has been a surge in public interest in the use of dietary botanical supplements for cancer prevention and adjuvant therapy (Martinez et al., 2012). Compounds with high antioxidant activity have gained popularity among dietary supplements. Free radicals are known to cause oxidative stress and DNA damage. Persistent oxidative stress causes chronic inflammation, which can contribute to cancer development (Wang et al., 2013). Several studies have found a strong positive association between the anti-proliferation action of chemicals isolated from diverse plants and their free radical-scavenging activity (You et al., 2011). Over 60% of current anticancer drugs are sourced from natural sources such as plants, fungus, and microorganisms. Large-scale anticancer natural product screening programs, such as those launched in the 1950s by an Italian research company or in the 1960s by the National Cancer Institute (NCI), enabled the identification of bacteria-produced doxorubicin and taxol (paclitaxel) derived from yew tree bark. Both of these compounds are often employed in cancer treatment regimens. Despite the fact that their mechanisms of action differ in that doxorubicin intercalates into DNA and prevents replication, (D'Arpa and Liu, 1989) Taxol slows microtubule depolymerization during mitosis (Weaver, 2014), and they both cause significant oxidative stress, albeit

in distinct ways (Yadav., et al 2015). Total cellular antioxidant capability has been identified as a predictor of cancer susceptibility to these medications (Chen PM., et al 2015). Chemotherapeutic-induced oxidative stress is critical for their effectiveness, but it also contributes to the cumulative and permanent cardiotoxicity observed clinically. (Adão R., et al 2013).

Statins are lipid-lowering medications that prevent the enzyme HMG-CoA reductase from working in the pathway that produces cholesterol (Feingold, 2020). They have been routinely used for approximately 40 years to treat and prevent coronary artery disease (CAD) in hypercholesterolemic individuals, despite the development of newer medications (Fernandez et al., 2018; Nurmohamed et al., 2021). Statins, in addition to their lipid-lowering function, can alter several metabolic pathways in numerous tissues and organs, allowing them to have good therapeutic effects on a wide range of illnesses (Almuti et al., 2006). Statins such as pravastatin (PRA), pitavastatin (PTV), rosuvastatin (RSV), lovastatin (LOV), simvastatin (SIM), atorvastatin (ATV), and fluvastatin (FLV) have pleiotropic benefits in addition to lowering LDL-C, including anti-inflammatory, antioxidant, anti-apoptotic, and immunomodulatory effects. (Gorabi et al., 2020; Vahedian-Azimi., et al. 2021). Oxidative stress has an important role in the release of nitric oxide (NO), the development of foam cells, the advancement of atherosclerotic lesions, and the instability of atherosclerotic plaque, all of which are implicated in the process of atherogenesis (Ikezaki et al., 2021). They have been associated to cancer because of their

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effects on antioxidant enzyme systems such as lipoxygenases, myeloperoxidase, nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase, catalase, GPX, and superoxide dismutase (SOD). Fattman et al. (2003).

Proanthocyanidins have been demonstrated to inhibit tumor growth in a variety of human cancer, including squamous cell carcinoma of head and neck, non-small cell cancer of the lungs, colorectal cancer, HCC, and pancreatic cancer. (Prasad et al., 2012), according to studies that are currently available (Nandakumar et al., 2008). Proanthocyanidins have been shown to be safe in vitro and in vivo at specific dose ranges by studies (Yamakoshi et al., 2002). Many foods and beverages are rich in proanthocyanidins, and the most abundant source is grape seeds (Santos-Buelga et al., 2000). Grape seed proanthocyanidins (GSPs) are a polymerization of catechins and/or epicatechins that results in a variety of dimers, trimers, tetramers, and oligomers/polymers (Lan et al., 2015). Numerous studies have found that the generation of drug-induced ROS is a vital role in the apoptosis of many malignancies. Grape seed extract, for example, kills Caco-2 human colon cancer cells (Dinicola et al., 2013). Proanthocyanins, like many drugs, have the capacity to induce cancer cells to die via releasing ROS. Apoptosis is induced by proanthocyanins in human colorectal cancer cells (Chung et al., 2009). Procyanidins derived from *Vitis vinifera* seeds cause apoptosis in squamous cell carcinoma cells (Hah et al., 2017). This study was designed to investigate the effects of Taxol, Atorvastatin, and Proanthocyanidin on the antioxidant status and reactive oxidative stress in the HPG2 cell line.

2. MATERIAL AND METHODS

2.1. Cell culture:

Taxol, Atorvastatin, and Proanthocyanidin were tested for their anticancer effects individually and in combination using the HepG2 hepatocellular carcinoma cell line. HepG2 cells (Sigma-Aldrich, Inc., St. Louis, MO 68178, United States) were cultured in monolayers in Roswell Park Memorial Institute (RPMI) culture medium with glucose and L-glutamine (LSP, UK); WI-38 cells were cultured in Dulbecco's modified Eagle medium with glucose and L-glutamine (Lonza, USA), supplemented with 10% fetal bovine serum (Sigma, Germany), 20 µg/mL penicillin, and 100 µg/mL streptomycin (Biowest, USA) in a humidified 5% CO₂ atmosphere at 37 °C. Trypan blue was used to determine the vitality of the cells (Strober, 2015). HepG2 cells were treated with various concentrations of Taxol, Atorvastatin and Proanthocyanidin, then incubated for 24 hours. Wells containing HepG2 cells without drugs were considered as the control wells.

2.2. Cytotoxicity assay:

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega, USA) was used to determine the cytotoxicity. Briefly, triplicates of 1 × 10⁴ HepG2 cells were treated with 200 L/well of the Taxol, Atorvastatin, and Proanthocyanidin working solution prepared in RPMI. Taxol was added to the medium at concentrations of 0, 0.006, 0.06, 0.6, 6 and 60 µg/ml, Atorvastatin at concentrations of 0, 5, 10, 20, 40, 60 µg/ml and Proanthocyanidin at concentrations 0, 20, 40, 60, 80, 100 µg/ml. For 24 or 48 hours, all plates were incubated at 37 °C in a humidified 5% CO₂ incubator. The Mosmann method was used to perform the MTT assay, with a few

changes (Mosmann, 1998). Using a microplate reader (LMR-9602, U.S.A.), the cell growth in each plate after 24h was measured at 570 nm, and the IC₅₀ concentrations were determined.

2.3. Determination of oxidative stress markers and antioxidant enzymes activity:

Oxidative stress markers and antioxidant enzymes activity were estimated by using commercial kits (Biodiagnostics, Egypt). MDA was estimated according to the method of (Ohkawa et al., 1979). SOD activity according to the method of (Nishikimi et al., 1972), Catalase activity was determined according to the method described by of catalase in the original sample (Aebi, 1984). The decrease in absorbance of Glutathione reductase (GR) at 340 nm is measured (Goldberg and Spooner, 1983).

2.4. Statistical analyses:

All experiments were done in triplicates; the data are presented as means ± standard deviation. The statistical analysis and plotting of data were done using Excel. *P* < 0.05 was considered statistically significant.

3. RESULTS

The Cellular Toxicity and Viability Data by MTT Assay:

Toxicity of HepG2 Cells Treated with Taxol for 24hrs:

Table 1 represent the cell viability percentages of HepG2 cells after 24 hours of incubation with serially increasing Taxol concentrations. According to the findings, the percentages of surviving cells decrease as Taxol concentrations increase in a dose-dependent way. After 24 hours, the inhibitory concentration (IC₅₀) estimated from the treatments that caused toxicity in HepG2 cells was 3.320 µg/mL.

Table 1. The percentages of cellular toxicity after treatment of HepG2 cells with different concentrations of Taxol for 24 hrs.

Treatment Compound	Conc. (µg/ml)	Average Absorbance Reading ± S.D.	Cyto-toxicity (%) *	IC50 Conc.
Taxol	0	0.60 ± 0.00	00.00	3.32 µg/mL
	0.006	0.6 ± 0.00	00.00	
	0.06	0.57 ± 0.01	5.56	
	0.6	0.49 ± 0.02	18.89	
	6	0.21 ± 0.01	65.00	
	60	0.09 ± 0.01	84.56	

*: Mean percentage of cytotoxicity (absorbance of experimental wells/absorbance of control wells) x 100; Taxol.

The Cellular Toxicity (IC₅₀) of HepG2 Cells After Treatment with Atorvastatin for 24 hrs.:

Table 2 show the percentage of dead cells after 24 hrs. of incubation of HepG2 cells with different concentrations of Atorvastatin. The results show that the percentage of survival cells is decreased with increased Atorvastatin concentrations in a dose-dependent manner. the IC₅₀ of Atorvastatin-treated cells was calculated to be 88.56 µg/ml after 24 hrs.

Table 2. The percentages of cellular toxicity after treatment of HepG2 cells with different concentrations of Atorvastatin for 24 hrs.

Treatment Compound	Conc. (µg/ml)	Average Absorbance Reading ± S.D.	Cyto-toxicity (%) *	IC50 Conc.
Atorvastatin	0	0.6 ± 0.00	0.00	88.56 µg/mL
	5	0.58 ± 0.02	2.22	
	10	0.51 ± 0.01	14.44	
	20	0.47 ± 0.01	22.22	
	40	0.41 ± 0.01	32.22	
	60	0.35 ± 0.01	42.22	

*: Mean percentage of cytotoxicity (absorbance of experimental wells/absorbance of control wells) x 100; Atorvastatin.

The Cellular Toxicity (IC50) of HPG2 Cells After Treatment with Proanthocyanidin for 24 hrs.:

Table 3 and Figure 1 show the percentage of dead cells after 24 hrs. of incubation of HepG2 cells with different concentrations of Proanthocyanidin. The results show that the percentage of survival cells is decreased with increased Proanthocyanidin concentrations in a dose-dependent manner. the IC50 of Proanthocyanidin-treated cells was calculated to be 32.99µg/ml after 24hrs.

Table 3. The percentages of cellular toxicity after treatment of HepG2 cells with different concentrations of Proanthocyanidin for 24 hrs.

Treatment Compound	Conc. (µg/ml)	Average Absorbance Reading ± S.D.	Cytotoxicity (%) *	IC50 Conc.
Proanthocyanidin	0	0.6 ± 0.00	0.00	32.99 µg/ml
	20	0.48 ± 0.02	20.55	
	40	0.12 ± 0.01	80.38	
	60	0.08 ± 0.01	86.22	
	80	0.07 ± 0.00	88.33	
	100	0.045 ± 0.003	92.38	

*: Mean percentage of cytotoxicity (absorbance of experimental wells/absorbance of control wells) x 100; Proanthocyanidin.

Oxidative stress markers and antioxidant enzymes activities. Treatment of HepG2 cells with Taxol, Atorvastatin and Proanthocyanidin, at doses corresponding to their respective IC₅₀ concentrations, induced a significant increase in GR activity and MDA concentration in all treated groups compared to an untreated control group. SOD activity in all treated groups was significantly decreased compared with untreated cells. in the same manner catalase activity in all treated groups was significantly decreased comparing with untreated cell. (Table 4 and Figure 1).

Table 4. Antioxidant enzymes activities and oxidative stress markers levels in A-431 in different experimental groups.

Group	MDA (nmol/ml)	SOD (U/ml)	CAT (µmol/g tissue. Min-1)	GR (U/L)
Untreated cells	1.55 ± 0.09	286.6 ± 0.96	1.5 ± 0.01	80.3 ± 0.35
Taxol	3.46 ± 0.07 *	195.2 ± 0.6 *	0.5 ± 0.04 *	92.4 ± 0.09 *
Atorvastatin	3.98 ± 0.15 *	170.45 ± 0.7 *	0.56 ± 0.08 *	72.36 ± 0.17 *
Proanthocyanidin	2.37 ± 0.07 *	184.2 ± 1.2 *	0.07 ± 0.01 *	140.6 ± 0.06 *

Data is represented by means ± SD, *: Significant at P<0.05 vs. untreated cells, MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase; GR: Glutathione reductase.

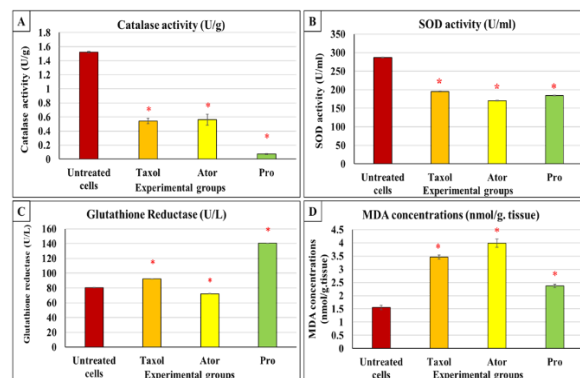


Figure 1. Antioxidants enzyme activities and oxidative stress markers in HepG2 cells.

4. DISCUSSION

During chemotherapy and radiation therapy, free radical generation increases. During antioxidant therapy, catalase levels in most cancer cells increased. It breaks down hydrogen peroxide to produce oxygen and water (Drisko et

al., 2001). Adriana and colleagues (2006) studied Grape extract for its impact on breast cancer. GSE was studied in mice for antitumor effectiveness against Ehrlich ascites carcinoma. GSE has no anticancer activity and does not interfere with doxorubicin's anticancer effects in mice, but it does reduce lipid peroxidation produced by doxorubicin treatment. An experiment was conducted to test the chemo preventive efficacy of blue grape marc powder against colorectal cancer in HT-29 cells. Treatment significantly reduced oxidative DNA damage in cells, with all fractions demonstrating comparable genoprotective effects. These findings show that grape marc powder has chemo preventive potential in colorectal cancer. (Pino-Garcia et al., 2017). Shimura et al. (2021) presented new data in favor of the combined use of Andrographis and Oligomeric Proanthocyanidins (OPCs) as a viable therapeutic alternative in colorectal cancer patients, maybe as an adjuvant treatment to conventional medications. In addition to its antioxidant and anti-inflammatory qualities, a study demonstrates that OPC-B2 is a new allosteric AKT inhibitor with substantial anti-tumor effectiveness (Liu et al., 2020).

The cytotoxicity effects (IC50 dosages) of Taxol, Atorvastatin, and Proanthocyanidin were examined in this study when given to HepG2 cell lines for 24 hours. When the concentrations of the treatment chemicals increased in a dose-dependent way, the percentages of surviving cells decreased. The IC50 doses computed following Taxol administration to HepG2 cells were 3.32 g/mL after 24 hrs., whereas the IC50 doses for Atorvastatin-treated cells were 88.56 g/ml after 24 hours and 32.99 g/ml after 24 hrs. Several studies have evaluated the cytotoxicity values of Taxol at different concentrations on HepG2 cells and various other cell lines. Previously, Taxol, Atorvastatin, or Proanthocyanidin, alone or in combination with -D-glucosidase, enhanced the progression of apoptosis in HepG2 cells. All samples altered HepG2 cellular proliferation in dosage and time response behaviors, according to the MTT viability test. The findings are consistent with those of Mamdouh et al., (2021), who investigated both in vitro and in vivo models of HCC utilizing HepG2 and Huh-7 cell lines as established in vitro models. They tested the cytotoxicity and IC50 of Amygdalin and Doxorubicin on these cells for 24, 48, and 72 hours and discovered a dose-dependent influence on cell growth.

In this study there was depletion in SOD and catalase activities while there was an elevation of MDA and GR in all treated groups compared with untreated cells. This effect may be due to the antioxidant effect of Proanthocyanidin by variance the antioxidant enzymes activity and increasing MDA production in the cells.

These results are consistent with (Alyami et al., 2022) that suggested that Proanthocyanidin can ameliorate oxidative stress, neuroinflammation, and neuronal apoptosis by activating the Nrf2 signaling pathway in PTZ induced seizures in mice.

Superoxide radicals and hydrogen peroxide are directly converted to less harmful species by the enzyme protection molecule SOD. HO-1, GSH, and SOD also modulate the redox state of GSH via the regulation of glutathione reductase (GSR), which regulates the expression of all measured protective molecules; it is likely that the antioxidant activity of paclitaxel is manifested through activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Matusovits et al., 2023). Oral administration of Paclitaxel was reported that it can maintain the expression of important antioxidants, anti-inflammatory molecules, HO-1, SOD and GSH, and suppress the

production of TNF- α , MPO and NF- κ B, which are involved in myocardial damage (Matusovits et al., 2023).

5. CONCLUSION

In conclusion, the current work demonstrated that treatment with atorvastatin or paclitaxel or Proanthocyanadin can effectively maintain the expression of the important antioxidant on HepG2 cell lines. Future studies should also analyze their roles in various conditions.

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