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## **Regulatory effects of Apatinib in combination with Piperine on MDM-2 Gene Expression, Glutathione peroxidase activity and Nitric oxide level as Mechanisms of Cytotoxicity in Colorectal Cancer Cells**

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**Ph.D in Clinical Biochemistry**

**Running title; Effects of Apatinib in combination with Piperine in colorectal cancer**

# Regulatory effects of Apatinib in combination with Piperine on MDM-2 Gene Expression, Glutathione peroxidase activity and Nitric oxide level in Colorectal Cancer Cells

## Abstract

**Purpose;** Apatinib has been utilized in colon cancer therapies but its efficiency and molecular mechanism are not fully understood. Chemotherapy in combination with non-toxic compounds can be a strategy to reduce the recurrence of cancer. Consequently, this study was carried out to evaluate the effects of Apatinib and Piperine on colorectal cancer (CRC) cell line and their potential anti-cancerous mechanisms *in vitro*.

**Methods:** The effects of Apatinib and Piperine on HCT-116 CRC cells were detected by assessing cell viability using MTT assay. The potential cytotoxic mechanisms of Apatinib and Piperine were investigated by evaluating the apoptosis-related gene (MDM-2) expression ratio using real-time PCR assay. Moreover, the glutathione peroxidase activity (GPX) and nitric oxide (NO) levels were assessed by colorimetric assays.

**Results:** The proliferation rate of CRC cells decreased by increasing the concentrations of Piperine and Apatinib. When HCT-116 cells were treated with different concentrations of Apatinib in combination with Piperine, the synergistic effects were observed (Combination Index<1). In HCT-116 cells treated with Apatinib or Piperine at the concentrations of  $0.5 \times IC_{50}$  and  $0.2 \times IC_{50}$ , the MDM-2 gene expression was downregulated and NO levels increased compared to the untreated control cells and related single treatments. Furthermore, the cytotoxic effects of Apatinib increased when was combined with Piperine. In addition, GPX activity significantly decreased in combination treatment at  $0.5 \times IC_{50}$  concentration of both agents.

**Conclusion:** Apatinib in combination with Piperin could significantly inhibit the viability of CRC cells. These cytotoxic effects were induced by regulation of apoptosis-related gene and inhibition of antioxidant marker.

Keywords; Colorectal cancer, Apoptosis, HCT-116 cells, Apatinib, Piperine

## Introduction

Colorectal cancer (CRC) is the third-largest cancer world wide<sup>1, 2</sup> and is related to a high rate of mortality.<sup>1-5</sup> Recently, clinical studies have been interested in detecting novel integrating targeted treatments and combination chemotherapy regimens.<sup>2</sup> Combination chemotherapy comes into importance in CRC treatment but drug resistance is inevitable.<sup>3</sup> In this regard, evaluating new drug combinations can improve the treatment outcomes.<sup>3, 5</sup> Numerous efforts have been conducted for *increase understanding* of the CRC heterogeneity and propose the most effective-tailored treatment to any affected patient.<sup>6</sup> Since, oncogene mutations and the existence of multi-drug resistance, and the intolerable side effects limit the efficiency of treatment. So, new treatment protocols for CRC treatment are instantly required.<sup>7</sup> Angiogenesis is an important feature of cancer growth and metastasis. Vascular endothelial growth factor (VEGF) binding to vascular endothelial growth factor receptor (VEGFR) induces the vascular endothelial cells proliferation and angiogenesis. Anti-angiogenic drugs show anti-cancer efficiency through blocking the binding of VEGF and VEGFR. Apatinib mesylate as a

micromolecular VEGFR-2 inhibitor that binds to VEGFR-2 and strongly inhibits it, exerts anti-cancer efficacy.<sup>8</sup> Also, Piperine that is a Piperidine alkaloid in black pepper, can inhibit cancer cell growth, but the mechanism of action of Piperine is not fully understood.<sup>9</sup> Piperine has been extensively described to inhibit colon cancer growth by G<sub>1</sub> arrest in the cell cycle, apoptosis induction, and antitumor activities.<sup>9, 10</sup> In the development of chemotherapeutic agents, it is imperative to assess the cancer response to chemotherapy.<sup>11-14</sup> One of the main purposes of cancer therapy is the induction of apoptosis.<sup>15</sup> Apoptosis is identified as programmed cell death in the damaged and normal tissues.<sup>16</sup> The apoptosis induction in cancerous cells is considered as the main objective of cancer treatment. Indeed, apoptosis is an important regulatory mechanism of normal cells. Definitely, apoptosis dysregulation can cause uncontrolled cell multiplication. This process is described by a series of definite morphological changes along with biochemical features that contain intrinsic and extrinsic pathways through a diverse protein which plays a critical role overall.<sup>17</sup>

The MDM2 protein encoded through the mouse double minute 2 (*MDM2*) gene is considered as the negative regulatory factor of the p53 protein and can preserve the p53 signaling pathway stability. MDM2 amplification has been assessed in numerous human cancers, comprising colon cancer.<sup>18</sup> MDM2 over-expression can induce transformation in cultured cells.<sup>19</sup>

In this regard, Nitric oxide (NO) has been revealed to induce apoptosis by post-translational alterations and has an anti-cancer role.<sup>20</sup> On the other hand, antioxidant enzymes have a critical function in protecting cells against oxidative stress, dysregulation of antioxidant enzymes activity, such as glutathione-peroxidase (GPX), are related to cancer.<sup>21</sup> So, in this study, we evaluated the effects of co-treatments of Apatinib and Piperine with evaluating the related molecular mechanism in CRC cells.

## Materials and Methods

### Cell culture

HCT-116 cell line was obtained from the Pasteur Institute in Iran. The culture medium contained 10% fetal bovine serum, 1% penicillin and streptomycin, and Dulbecco's modified Eagle medium (DMEM). CRC cells were seeded in 96 well cell culture plate and maintained at 37 °C in a 5% carbon dioxide in the incubator.

### Cell proliferation assay

HCT-116 cells were cultured in 96-well plates at a density of  $1 \times 10^4$ /well. Following incubation at 37 °C and 5% CO<sub>2</sub> for 24 h, CRC cells were exposed to increasing concentrations of Apatinib (0, 5, 10, 15, 20, 25, 50, 75, 100 μM) and Piperine (10, 20, 40, 80, 100, 150, 200, 250 μM). After 48 h of treatment time, a cell viability kit (Kia zist, Iran) was used to detect cell proliferation. In this regards, 10 μl MTT reagent was added to each well, and the cells were incubated for 3 h at 37 °C and 5% CO<sub>2</sub>, the supernatants were discarded and a solubilizer was added to each well. The absorption rate was measured by ELISA Reader at 550 nm. The rate of viable cells was determined by measuring the absorbance. Each test in all treatments was repeated at least three times. The IC<sub>50</sub> values of each agent were determined based on their corresponding dose-response curve and with Compusyn software (ComboSyn, Inc., Paramus, NJ 07652, USA).

In all treatments, the concentrations of  $0.50 \times \text{IC}_{50}$ ,  $0.2 \times \text{IC}_{50}$ , and  $0.1 \times \text{IC}_{50}$  were utilized for cell viability assay and all other experimental analysis including glutathione peroxidase (GPX) activity, MDM-2 gene expression, and NO level assays. The interaction between two therapeutic agents based on Chou<sup>22</sup> method and compusyn software (Combosyn, Inc.,

Paramus, US) were evaluated, and the combination index (CI) determined. In this regard, the  $CI < 1$ ,  $CI > 1$ , and  $CI = 1$  display synergism, antagonism, and additive effect, respectively.

In addition, the Fraction affected (fa) amount (indicating the cell fraction that affected by the combination treatment) and DRI (Dose reduction index which shows the dose reduction level in combination treatment compared to single treatments) were evaluated.

### **Real-time polymerase chain reaction (PCR)**

Total RNA was extracted from HCT-116 cells (untreated and treated with Apatinib and Piperine at different concentrations as mentioned above) using an RNA extraction kit based on the kit protocol (GeneAll, South Korea). The First-strand cDNAs were synthesized by SuperScript III™ First-Strand synthesis kit (GeneAll, South Korea) and utilized as templates to perform real-time PCR on a real-time PCR System, following the manufacturer's instruction. The MDM2 gene expression was measured by real-time PCR using an AccuPower®2× Green StarqPCR master mix (Ampliqon, Denmark). Real time-PCR via cDNAs and specific primers were carried out in annealing temperature at 57°C for 30 seconds. Relative MDM2 expression was normalized to b-actin housekeeping gene and calculated by  $2^{-\Delta\Delta Ct}$ . Error bars in control and treated groups show the Standard deviation.

### **Nitric oxide assay**

In order to assay the NO level in untreated and treated cells with various concentrations of Apatinib and Piperine in single and combined treatments, the cell supernatants were collected carefully. Assessments of NO level in various treatments were performed based on the kit protocol [ZellBio GmbH (Germany)]. The absorbance of each sample was read at 550nm and NO levels were detected based on a standard curve.

### **Glutathione peroxidase assay**

To evaluate the possible cytotoxic mechanism of Apatinib and Piperine, the GPX enzyme activity was assessed with the colorimetric method. In this regard, after various treatments as mentioned, cell culture supernatants of each sample were collected carefully and then centrifuged and the GPX activity was determined according to the kit protocol [ZellBio GmbH (Germany)]. In the next step, the absorbance at 412 nm was measured and GPX activity was calculated by this formula:

$$\text{GPX activity (U/ml)} = (\text{OD control} - \text{OD sample}) / (\text{OD standard} - \text{OD blank}) \times 6000$$

## **Results and Discussion**

The cytotoxic effects of Apatinib and Piperine in HCT-116 CRC cells were detected by MTT assay. The potential mechanisms were investigated by assessing the apoptosis-related gene (MDM-2) expression ratio in vitro using the real-time PCR assay. Moreover, the glutathione peroxidase activity (GPX) and nitric oxide (NO) levels were evaluated by colorimetric assays. In the first step, we evaluated the cytotoxic effects of monotherapies at different concentrations of Apatinib or Piperine after 48 h treatment and dose-response curves were plotted. Results of cell viability assays in single therapies were presented in **Figure1**. Both agents (Apatinib and Piperine) decreased the cellular viability in a concentration-dependent pattern.

Treatment of HCT-116 cells with increasing concentrations of Apatinib or Piperine exhibited a reduction in the cellular viability in dose-dependent patterns. The  $IC_{50}$  values for Apatinib and Piperine

drugs were equal to 26 and 94  $\mu\text{M}$ , respectively. All combined *treatments* lead to the *synergistic* interaction in *comparison* to their respective *single treatments*.

Results of DRI, CI, and fa for combined treatments at different concentrations were presented in **Table 1**.

As presented in **Figure 2**, after combined treatments with Apatinib and Piperine, the cell viability in terms of  $0.5 \times \text{IC}_{50}$ ,  $0.2 \times \text{IC}_{50}$ , and  $0.1 \times \text{IC}_{50}$  concentrations were decreased significantly compared with untreated control cells ( $p < 0.05$ ). In all combination treatments, the cell viability was lower than that of corresponding monotherapies including Apatinib and piperine in  $\text{IC}_{50}$  concentrations ( $p < 0.05$ ).

In order to study the molecular mechanism of Apatinib and Piperine in CRC cells treated with single and combined drugs, the MDM2 gene expression ratio was evaluated by Real-time PCR assay and normalized to  $\beta$ -Actin as a house-keeping gene. The mean fold changes for each treatment and control cells were calculated by  $2^{-\text{R}\Delta\Delta\text{Ct}}$ . The results of gene expression levels (**Figure 3**) showed that the mean gene expression ratio was downregulated in single and combined treatments ( $p < 0.05$ ). The MDM2 gene expression ratio in combined treatment groups (at concentrations of  $0.5 \times \text{IC}_{50}$  and  $0.2 \times \text{IC}_{50}$ ) decreased versus single treatments ( $p < 0.05$ ).

In this presented study, the GPX activity was evaluated by the colorimetric assay. The results (**Figure 4**) showed that there was a significant decrease in GPX activity in the combined treatment group at  $0.5 \times \text{IC}_{50}$  concentration of both drugs in comparison with the control group and related single treatments ( $p < 0.05$ ).

The results of the NO assay (**Figure 4**) showed that the level of NO in the combination groups at the concentrations of  $0.5 \times \text{IC}_{50}$  and  $0.2 \times \text{IC}_{50}$  were more than that of the untreated control group ( $p < 0.05$ ). In addition, there was an elevation in NO levels in combined groups ( $0.5 \times \text{IC}_{50}$  and  $0.2 \times \text{IC}_{50}$  concentrations) compared to monotherapies (Apatinib and Piperine in  $\text{IC}_{50}$  concentration).

Combination treatments involve the administration of conventional chemotherapy agents together with one or more natural bioactive (typically from a plant). This anticancer drug combination may be applied to cancer cell cultures.<sup>23</sup> Indeed, there are limitations to the effectiveness of several cancer therapies because of the systemic toxicity. Therefore, Chemotherapy combined with non-toxic compounds can be a strategy for decreasing cancer incidence. Numerous natural agents have revealed chemotherapeutic potential in a diversity of bioassays.<sup>12</sup> In this regard; we evaluated the efficacy of Apatinib as a chemotherapy drug combined with Piperine as a natural agent in the CRC cell line. Our results demonstrated that synergistic effects were observed in the combination treatment g of Apatinib and Piperine at concentrations lower than the  $\text{IC}_{50}$  values of each agent. Also, these combination treatments regulated the MDM2 gene expression levels and increased NO levels in cell culture, which these effects are related to apoptosis induction. Moreover, combined therapy at the concentration of  $0.5 \times \text{IC}_{50}$  decreased the GPX enzyme activity that indicates the efficacy of this combination treatment and induction of cytotoxicity.

The p53 is the main transcription factor regulating cellular pathways including apoptosis and cell cycle. It acts as a central defense mechanism toward cancer progression and is controlled by interaction with the MDM2( oncoprotein). The inhibition of MDM2-p53 interaction is considered as a striking treatment approach for cancer therapy.<sup>24</sup>

At the molecular level, Piperine can affect numerous effector proteins involved in the apoptosis pathway and can stimulate extrinsic and intrinsic apoptosis process. Piperine repressed the cancer development and metastasis in a cancer model.<sup>25</sup> In our study, Piperine could downregulate the MDM-2 gene expression as an oncogenic mediator.

In a similar study, Piperine elevated the anti-proliferative and cytotoxic effects of doxorubicin and paclitaxel in cell lines,<sup>26</sup> which was parallel to our results.

Piperine has elevated the cytotoxicity of paclitaxel and doxorubicin in cell line. Moreover, Piperine in combination with doxorubicin and paclitaxel induced P21 expression. These researchers recommended that the molecular mechanism has to be further assessed to recognize



the definite function of Piperine.<sup>26</sup> Likewise in another study, the isobologram and the CI of the combination of Paclitaxel and Piperine showed synergistic effects which were in accordance with our results.<sup>27</sup>

Correspondingly, in another study similar to our research Apatinib in combinatorial cases showed the anti-cancer effect. Furthermore, Apatinib exhibited synergistic interactions with Paclitaxel plus 5-fluorouracil chemotherapeutic agents in-vivo.<sup>28</sup> Also, a related study confirmed that Apatinib displayed potentially inhibitory impacts in pancreatic cancerous cells and Astragalus polysaccharide increased the anti-cancer effects of Apatinib by decreasing phosphorylation of AKT, ERK expression, and MMP-9.<sup>29</sup> In addition, a recent study indicated that in AGS cells Astragalus polysaccharide improved the antitumor efficacy of Apatinib by inhibition of AKT signaling pathway.<sup>30</sup>

Our study found that both Piperine and Apatinib induced cytotoxicity effects in a dose-dependent manner. Nevertheless, when Piperine was combined with Apatinib, the expression of the anti-apoptotic gene was decreased significantly compared with the Apatinib and Piperine alone treated groups. Therefore, we speculated that apoptosis was associated with the synergetic effects of Piperine and Apatinib in the present study. Our current study is strong evidence that pharmacological regulation of the MDM2 gene expression improves cancer treatment strategy. Piperine in combination with Apatinib was showed more cytotoxic effects in compared to Apatinib monotherapy by reducing its concentration in combination treatment and may increased the apoptotic induction potency. Further studies including clinical trials should be carried out for these new cancer therapies because the combination of Apatinib and Piperine as *MDM2* inhibitors with the which not only reduce drug concentrations but also promoted its efficiency in inducing apoptosis.

## Conclusion

Overall, in this investigation, Piperine as a natural anti-cancer agent is proving efficacious in combination with Apatinib at lower concentrations, which could be accounting for possible anti-cancer effects of this combination in CRC cells. Based on our results elevating cytotoxic activity of both agents in the combined treatment group might be related to the release of NO as a nitrosative stress marker. Nevertheless, the effects of this combination in cell cycle regulation as well as the decreased expression level of *MDM-2* might be examined by further studies.

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**Ethical issues;** This article does not contain any studies with human subjects or animals performed by authors.

**Competing interests;** The authors declared no conflicts of interest.

**Study highlights;**

**What is current knowledge?**

Although clinical studies are active in detecting new targeted treatments for CRC, unsuccessful treatment and drug resistance are inevitable.

**What is new here?**

Apatinib and Piperine could significantly inhibit the CRC cells.

Combined treatment with Apatinib and Piperin showed anti-cancer effects by regulating apoptosis-related genes.

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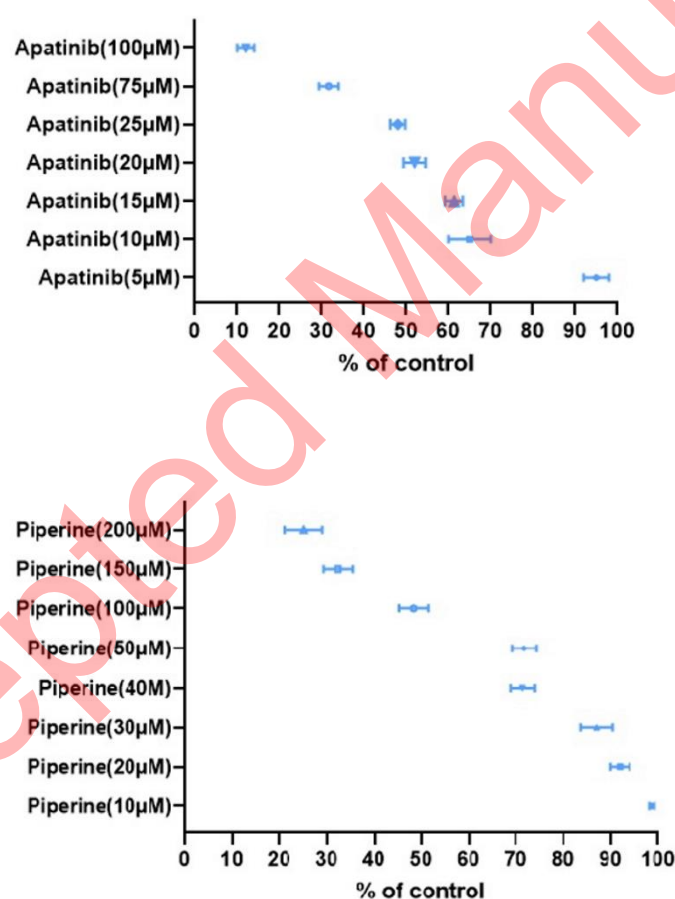
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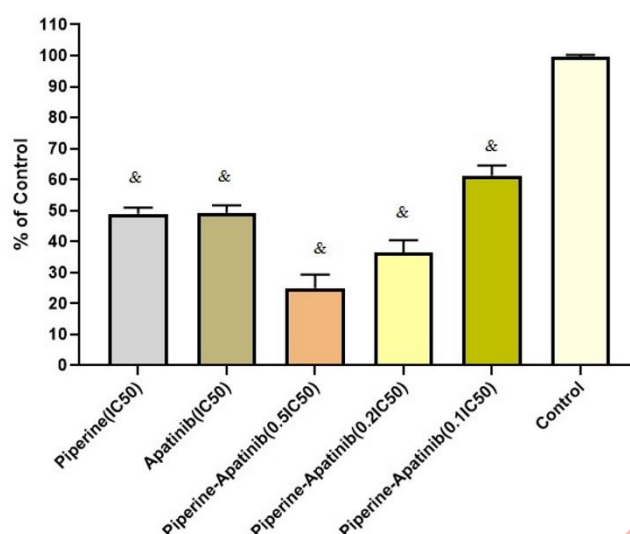
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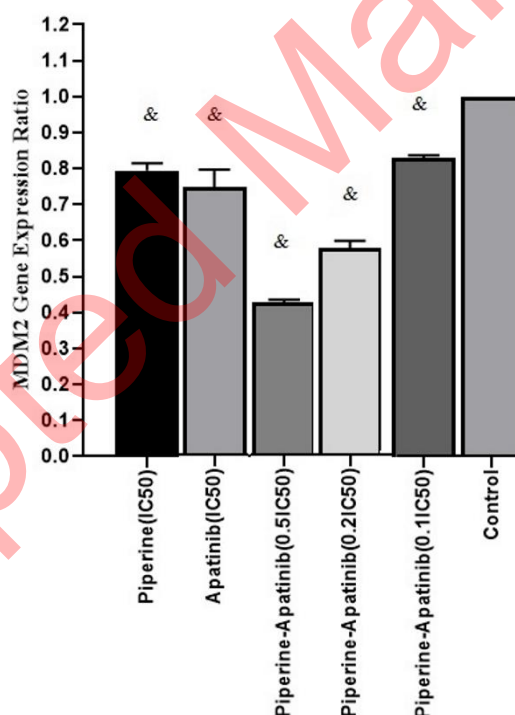
**Table 1.** Results of Combination Index (CI), Fa, and DRI in drug reaction at the various concentration in Apatinib and Piperine combined treatments which were measured by Compusyn software.

Combinations	Fa (fraction affected)	DRI (dose reduction index) for Apatinib	DRI (dose reduction index) for Piperine	Combination Index(CI)
Apatinib-Piperine (0.5×IC <sub>50</sub> )	0.75±0.044	1.24±0.19	13.32±1.5	0.83±0.1 (synergistic effect)
Apatinib-Piperine (0.2×IC <sub>50</sub> )	0.63±0.04	2.1±0.27	24.7±2.3	0.52±0.06 (synergistic effect)
Apatinib-Piperine (0.1×IC <sub>50</sub> )	0.38±0.032	1.99±0.19	28.1±2.0	0.53±0.05 (synergistic effect)

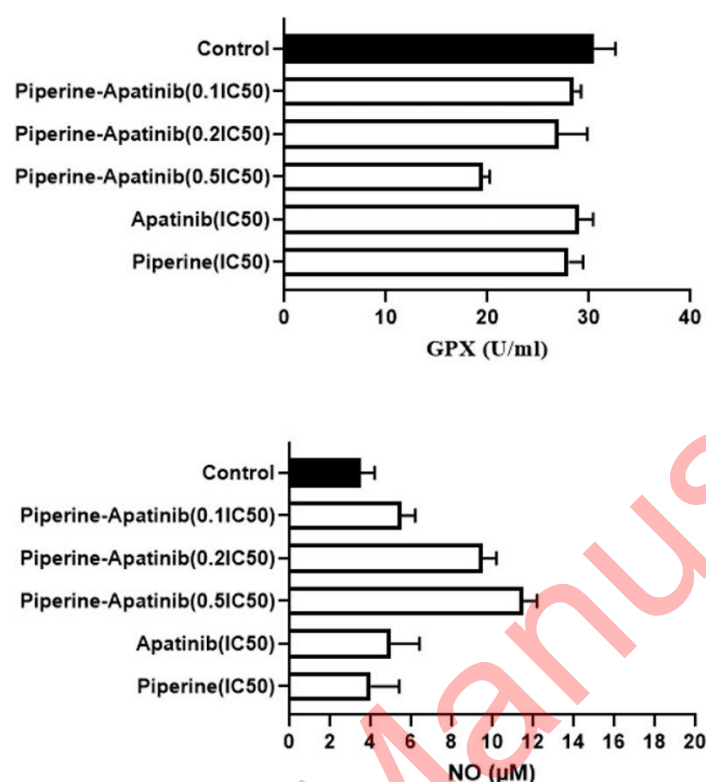
**Figure 1.** The results of cell viability using MTT assay for Apatinib and Piperine at various concentrations after 48 h treatment. Each value shows the mean ± standard deviation.



**Figure 2.** Cellular inhibitory effects of combined therapy with apatinib and piperine at different concentrations after 48h treatment in HCT-116 CRC cells. Each value shows mean  $\pm$  standard deviation). &; Significant differences compared to untreated control cells ( $p < 0.05$ ).



**Figure 3.** The MDM2 gene expression ratio which was assessed by the real-time PCR method in treated and untreated cells with Apatinib and Piperine after 48 h. each column shows the mean fold change  $\pm$  standard deviation which was normalized to a housekeeping gene ( $\beta$ -actin) and calculated by  $2^{-R\Delta\Delta C_t}$ . &; Significant difference compared to untreated control cells ( $p < 0.05$ ).



**Figure 4.** The levels of NO and GPX activity in HCT-116 cells treated with various concentrations of Apatinib and Piperine in single and combination treatments. Data were expressed as mean±standard deviation. \*; Significant difference compared to untreated control cells ( $p < 0.05$ ).