

Protective Effects of Anthocyanin Fraction of *Berberis integerrima* Bunge Fruits against H₂O₂ Induced Cytotoxicity in MCF7 and HepG2 Cells

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ABSTRACT

Objective: *Berberis integerrima* Bunge show different pharmacological effects. Its fruits are rich sources of anthocyanin with biological activities. The aim of this study was evaluation of protective effects of isolated anthocyanin fraction of *Berberis integerrima* Bunge fruits (AFBI) against cytotoxicity induced by hydrogen peroxide (H₂O₂) in HepG2 and MCF-7 cell lines. **Method:** Cytotoxicity of ABIF and H₂O₂ was identified by MTT assay. In pretreatment studies, the cells were pre-exposed to nontoxic concentrations of AFBI for 24 h then cytotoxic concentration of H₂O₂ was added. In co-treatment study, the cells were exposed to nontoxic concentrations of AFBI and then cytotoxic concentration of H₂O₂, simultaneously. **Result:** ABIF exhibited significant toxicity at concentration of >800 µg/ml in both cell lines. The IC₅₀ for H₂O₂ were 253 µM and 200 µM for MCF7 and HepG2 cells, respectively. In pre-treatment assay, anthocyanin able to increase viability of MCF7 cells at 200 and 400 µg/ml concentrations compared with the control. In HepG2 cells no significant difference in cell viability was observed compared with the control. In co-treatment study in MCF7 cells, the significant difference in cell viability was observed at all

concentrations of ABIF (25-400 µg/ml), while the significant increase in cell viability were showed at 100, 200 and 400 µg/ml in HepG2 cells. **Conclusion:** This study suggests that AFBI is cytoprotective against H₂O₂-induced oxidative stress in HepG2 and MCF7. This is likely due to its antioxidant activities. This protective effect could be considered as approach for treatment of oxidative stress related diseases.

Key words: Anthocyanin, *Berberis integerrima* Bunge, H₂O₂.

Key message: *Berberis integerrima* fruits might be consider as cytoprotective against H₂O₂-induced oxidative stress

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INTRODUCTION

Reactive oxygen species (ROS) are products of oxygen metabolism in aerobic organisms, comprise: superoxide radical (O₂^{•-}), hydroxyl radical (OH[•]), Hydrogen peroxide (H₂O₂) and singlet oxygen.¹ Several anticancer drugs, chemical carcinogens and radiation are other sources of ROS.² ROS and oxidative stress are related to different cellular dysfunction such as changes in signal transduction pathways, gene expression, mutagenesis and apoptosis.³

Different mechanisms in cancer cells lead to increase levels of hydrogen peroxide and other ROS as well as oxidative stress. This stress plays critical role in both cancer initiation and cancer promotion by redox signaling. It seems that cancer cells are resistance to high level of ROS which are cytotoxic to normal cells. This process, follow by increased cellular antioxidants defense and change of apoptotic pathways which result in increased proliferation and cell survival.⁴

Among various ROS, H₂O₂ is considered due to its role in DNA damages.² Different antioxidant enzymes are protecting cells against H₂O₂ damages such as superoxide dismutase, catalase and glutathione peroxidase as well as the intracellular scavenger: glutathione, histidine-peptides, the iron-binding proteins, dihydrolipoic acid, melatonin, urate and plasma protein thiols.^{1,5-7}

Intracellular concentration of H₂O₂ is firmly regulated by antioxidant system and increased intracellular concentration (more than 1 µM) lead to oxidative stress, resulting in cell cycle arrest and apoptosis.⁸

Phenolic compounds structures comprise aromatic ring(s) with hydroxyl group(s). This structure help them to be antioxidant. They protect plants

against ultraviolet radiation, pathogens and parasites. They know as powerful antioxidants, reduce risk of oxidative stress and associated diseases. This antioxidant activities mediated by different mechanism: scavenging different radical species, inhibition of radicals formation by up regulating antioxidant defense gene.⁹

Anthocyanin belongs to phenolic compounds, known as natural pigments with wide color from blue to orange, Beside its different role in plants, they possess various biological activities.¹⁰ Different evidence reveal the potential biological effects of anthocyanin consumption such as: robust antioxidants, apoptosis induction in tumor cells.¹¹ *Berberis* sp. fruits are rich sources of anthocyanin. *Berberis integerrima* Bunge belongs to Berberidaceae.¹² Because of various secondary metabolite in this plant, several confirmed therapeutic effects reported in previous researches such as anti-inflammatory effects, hypoglycemic, hypolipidemic and antioxidant effects.¹³

According to our previous results anthocyanin fraction of *Berberis integerrima* Bunge fruits are powerful antioxidants so the aim of this study is evaluation of cytoprotective effects of AFBI against H₂O₂ cytotoxicity in HepG2 and MCF7 cell lines.

MATERIALS AND METHODS

Extraction

Anthocyanin fraction was extracted as described previously from fruits of *Berberis integerrima* Bunge collected from Kohmar (Fars Province, Iran).¹⁴

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Cell culture and Treatment

The human cancer cell lines MCF-7 and HepG2 were obtained from Pasteur Institute (Tehran, Iran). They maintained at 37°C incubator under 5% CO₂ and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg of streptomycin/ml. After 80 % confluency, Cells were harvested by trypsin.

Effect of anthocyanin on viability of MCF7 and HepG2 cells

The different concentrations (25- 800 µg/ml) of AFBI were added to the cells and cell viability were analysis by modified MTT assay.³ Briefly, MCF7cells (10⁴ cells per well) were seeded in 96-well cell culture plates. After 24 h incubation, growth media was replaced with growth medium containing different concentrations of AFBI then incubated for 24 and 48h. The medium replace with MTT solutions (5 mg/ml in PBS) was added to the cells and were incubated at 37°C for 4 h, the reached formazan was solubilized with DMSO. The absorption was measured at 570 nm by ELISA reader. The percentage cell viability for each anthocyanin concentration was calculated.

Assay of H₂O₂-induced toxicity

Regarding to determine the cytotoxic effect of H₂O₂, after 24 h cell incubation in plates (10⁴cells/ well), the cells were treated with H₂O₂ (0–800 µM) for 0.5, 1 and 2 h and then cell viability were for both cell lines analysis by the MTT assay as described previously.

Cytoprotective activity assay

In order to analyze the protective effects of AFBI extracts against H₂O₂ cytotoxicity both pre-treatment and co-treatment protocol was used.

Pre-treatment protocol

Cells growth in 96-well culture plates (10⁴ cells /well) were incubated for 24 h in the presence of different concentrations of AFBI (25-400 µg/ml) and quercetin (200 µg/ml) as positive control. After washing the cells with fresh medium, H₂O₂ (400 µM) was added to cells for 2h then cell viability was quantified by MTT.¹⁵

Co-treatment protocol

Before treatment, the cells were seeded in a 96-well plate (10⁴ cells/ well) and incubated for 24 h at 37°C. Then the cells were incubated with different concentrations of AFBI or quercetin and 400 µM H₂O₂ simultaneously, after 2 h incubation, cell viability was determined by MTT assay.

Statistical analysis

All of the experiments were performed in triplicate. One way analysis of variance (ANOVA) test was used to compare difference between groups. All results were presented as mean ± SD where p < 0.5 was taken to indicate Results.

RESULTS

Effect of ABIF on HepG2 and MCF7 cell viability

The cytotoxicity of AFBI was examined on HepG2 and MCF7 which were exposing to different concentrations of anthocyanin for 24 and 48 h. There is significant toxicity at concentration of 800 µg/ml and higher (p < 0.001) for both cell line (Table 1).

Effect of H₂O₂ on MCF7 and HepG2 cell viability

In propose to define the cytotoxic concentrations of H₂O₂ for MCF7 and HepG2 cells, MTT assay was performed. After 24 h incubation, cells were incubated with different H₂O₂ of concentrations (0–800 mM) for 0.5, 1

Table 1: Effect of different concentrations of anthocyanin on MCF7 cells viability after 24 h and 48 h incubation.

Concentration (µg/ml)	24 h	48h	24h	48h
	MCF7 cell line		HepG2 cell line	
6.2	98.58± 0.40	97.13 ± 1.32	91.36 ± 1.0 1	88.94 ± 2.36
12.5	92.28 ± 0.33	96.65 ± 5.81	90.72 ± 0.45	90.15 ± 2.27
25	90.87± 0.15	97.53 ± 1.12	89.20 ± 0. 87	88.56 ± 2.82
50	94.00 ± 0.801	95.15±0.75	87.72 ± 2.83	87.35 ± 0.39
100	86.55± 1.22	87.06±1.18	80.89 ±1 .21	81.76 ± 2.04
200	90.907 ± 0.09	84.10 ± 0.93	72.77 ± 2 .46	76.33 ± 2.31
400	75.42± 0.21	79.05 ± 1.14	66.68 ± 0.95	78.06 ± 8.32
800	28.09± 0.24***	39.00 ± 0.71***	32.8 ± 2.47***	37.55 ± 2.66***

Viability was quantitated by MTT assay.

Values are expressed as a percentage of negative control values. Data are mean ± SEM (n=3) and

***p<0.001, significantly different from controls.

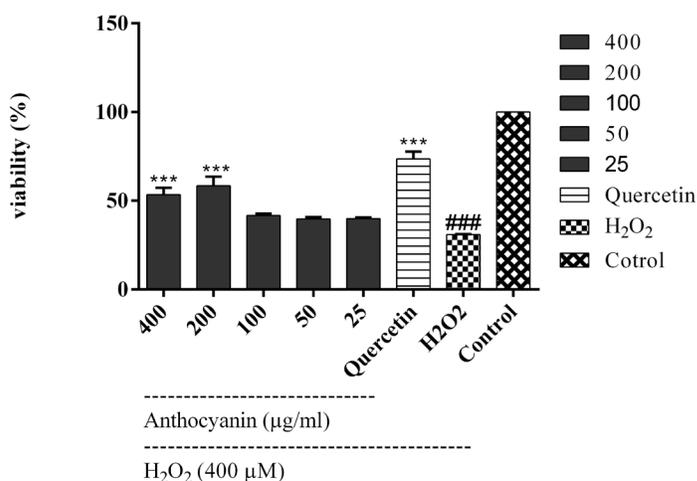


Figure 1: MCF7 cell line treated by different concentration of anthocyanin (µg/ml) and quercetin (200 µg/ml) for 24h then add H₂O₂ (400 µM) viability measure after 2h by MTT assay **p<0.001 versus H₂O₂ stimulated cells, ### p < 0.001 versus control.

and 2 h. There were no significant toxicity show in 0.5 and 1h incubation time in both cell lines. The IC₅₀ for H₂O₂ with 2 h incubation were 253 µM and 200 µM for MCF7 and HepG2 cells, respectively. The significant cytotoxicity (>50%) was detected at concentration of 400 µM and higher with 2h incubation time compared to untreated control (p<0.001). So, proper concentration of H₂O₂ to induce toxicity to assay the effects of the AFBI is 400 µM (for 2 h) in both cells.

Effect of ABIF on viability of MCF7 and HepG2 cells against H₂O₂-induced cytotoxicity

In pre-treatment assay, AFBI increase viability of MCF7 cells from 30 to about 60% at 200 and 400 µg/ml (p < 0.001) concentrations compared with the control (H₂O₂ alone).

In HepG2 cells no significant difference in cell viability was observed at different concentrations compared with the control (H₂O₂ alone) (Figure 1, 2).

When co-treatment protocol were used, there are significant difference in cell viability was observed at all concentrations of AFBI (25-400 µg/ml) in MCF7 cells (p < 0001) compared with the control (H₂O₂ alone), while

the significant increase in cell viability in HepG2 were showed at 100, 200 and 400 µg/ml concentration ($p < 0.001$, $p < 0.05$, $p < 0.01$ respectively) (Figure 3, 4)

DISCUSSION

This study was aimed to investigate the protective effects of AFBI on two cancer cell lines, MCF7 and HepG2, against H₂O₂ toxicity.

According to previous study sensitivity and reproducibility of MCF-7 cells make them as proper *in vitro* model for study chemically induced oxidative stress.¹⁵ Results were shown that H₂O₂ remarkably reduced viability of both cell lines in a dose and time dependent manner. In this study, 2h incubation time and 400 µM concentration were chosen for assay of protective effect of anthocyanin on adverse effects of H₂O₂ on these cell lines which is consist with results of previous study intermediate concentrations of H₂O₂ (250 to 400 µM) known as toxic concentration. Although incubation time and cell concentration cause the difference in reported cytotoxic concentrations of H₂O₂ in cell cultures.⁸

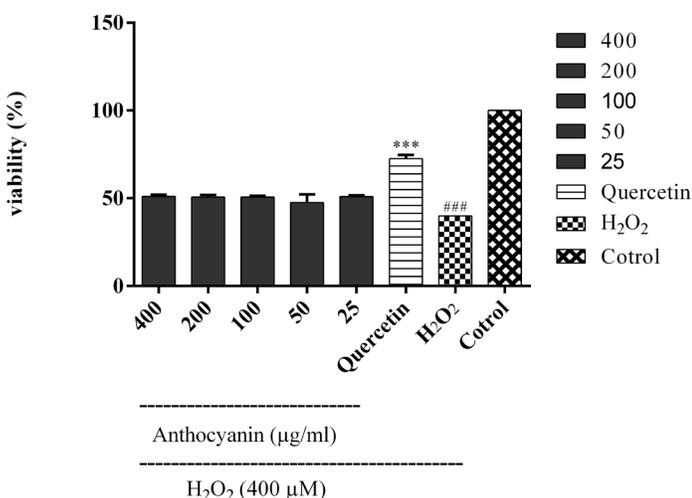


Figure 2: HepG2 cell line treated by different concentration of anthocyanin (µg/ml) and quercetin (200 µg/ml) for 24h then add H₂O₂ (400 µM) viability measure after 2h by MTT assay *** $p < 0.001$ versus H₂O₂ stimulated cells, ### $p < 0.001$ versus control.

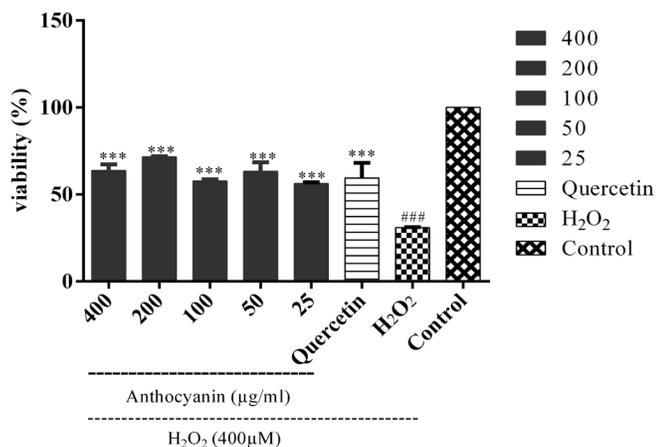


Figure 3: MCF7 cell line treated by different concentration of anthocyanin (µg/ml) and quercetin (200 µg/ml) and H₂O₂ (400 µM) for 2h simultaneously, then viability measure by MTT assay *** $p < 0.001$ versus H₂O₂ stimulated cells, ### $p < 0.001$ versus control.

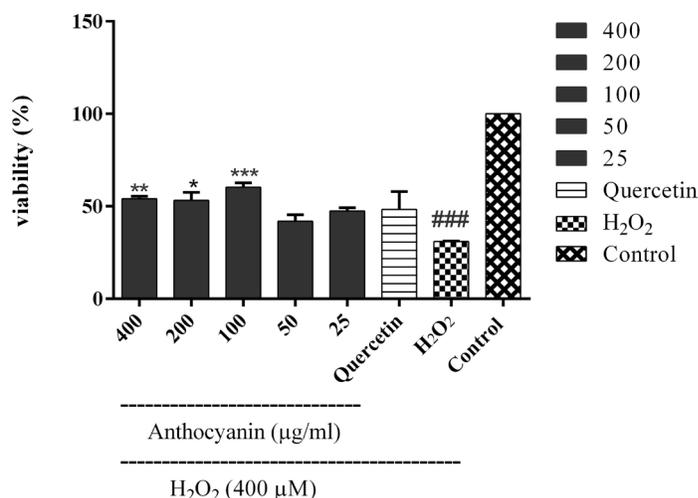


Figure 4: HepG2 cell line treated by different concentration of anthocyanin (µg/ml) and quercetin (200 µg/ml) and M H₂O₂ (400 µ) for 2h simultaneously, then viability was measured by MTT assay, ** $p < 0.01$ and *** $p < 0.001$ versus H₂O₂ stimulated cells, ### $p < 0.001$ versus control.

H₂O₂ induces oxidative damages by producing free radicals and lipid peroxidation and intercellular mechanism including: perturbing intracellular calcium homeostasis,¹⁶ diminishing intracellular ATP,¹⁷ induction of DNA damage and apoptosis.¹⁸ Different natural products have been reported as powerful antioxidant. This activity is related to present of phenolic compound as second metabolite. Recently, natural antioxidant is more consider by means of cancer chemopreventive agents. Antioxidants able to postponement or inhibit the oxidative damages produced by free radicals.¹⁹

In this study, cytotoxicity of AFBI (800-3µg/ml) on MCF7 and HepG2 cell line was measured after 24h and 48h incubation. According to the results concentration > 800 µg/ml showed significant toxicity. In previous studies, aglycone anthocyanins (delphinidin) at 200 µM concentration reduced cell viability to 50% in HeLa cells where aglycone anthocyanins (cyanidin) not toxic at all the concentrations (50-200 µM) in the three cell lines (HeLa, NHF and CaCo2).²⁰

Also 200 mM delphinidin stimulate apoptosis in Hela and CaCo-2 cells. This study suggested that this anthocyanin able to inhibit cell cycle progression in normal and tumor cells. Apoptotic mitochondrial pathway was recommended mechanism for these cellular events.²⁰

Polyphenols able to induce large amounts of intracellular H₂O₂ in tumor cells so they could be cytotoxic in tumor and normal cells and H₂O₂ is a well-known mediator of apoptosis in cancer cells.

To evaluate whether AFBI able to protect MCF7 and HepG2 cells against H₂O₂ induced toxicity, cells were pre-treated with various concentrations of anthocyanin (25-200 µg/ml) for 24 h, followed by H₂O₂ treatment (400 µM). In these assay, cell viability decreased to about 30% after hydrogen peroxide treatment in comparison to control cells. Viability of cells which were pre-treated by AFBI at 200 and 400 µg/ml concentrations increased to 50% but it doesn't see any significant increase in other concentration.

To evaluate whether AFBI could inhibit H₂O₂-induced toxicity, in co-treatment study, cell expose to anthocyanin (25-200 µg/ml) and hydrogen peroxide (400µM) for 2 h. In this assay, able to increase viability of cells from 30 to 60 (both cell line) in all concentration in MCF7 and 100-400 µg/ml in HepG2. In further study cyanidin was effective to protect PC12 cells against H₂O₂ against at 100µM concentration when cells expose to 400 µM H₂O₂ for 2h.²¹ Previous study showed that hydrogen peroxide increase lipid peroxidation in cells.²² Our former report showed

the powerful antioxidant activity of this fraction so these results point to that anthocyanin play radical scavenging role by reducing lipid peroxidation and cellular damages induced by H₂O₂.¹⁴ Also, these results consist with results of previous investigation which shown anthocyanin increase resistance of endothelial cells against ROS damages.²³

Additionally, *in vivo* study exhibit protection effects of cyanidin-3-glucoside by bilitranslocase-mediated entrance of this anthocyanin to cell and reduce free radical formation and diminish lipid peroxidation induced by Cu.²⁴

Anthocyanin belongs to flavonoids as a subclass of phenolic compounds. The anthocyanin structure variance are refer to the number of hydroxyl groups, the number and type of sugars and the position of attachments in anthocyanin structure.²¹ These compounds engage different mechanism as antioxidant such as hydrogen donating and modulate cell signaling pathways²⁵ These roles confirmed in various *in vitro* and *in vivo* studies.²⁶⁻²⁷

Anthocyanins (Bilberon-25) from bilberry extract show potential in the treatment of chronic pruritus in a mouse model of chronic allergic contact dermatitis.²⁷

These potential effects of anthocyanin is more valuable in preventive medicine against oxidative related disease such as coronary heart disease and cancer.²¹ Epidemiological study revealed consumption of anthocyanin reduce mortality rate in cardiovascular disease (CVD).²⁸ In other study, consumption of flavonoids, including anthocyanin, flavonones, and flavonoid-rich foods in postmenopausal women related with reduced mortality of CVD and coronary heart disease after 16 years of follow up.²⁹

CONCLUSION

Present study determined the protective effect of *Berberis integerrima* Bunge against hydrogen peroxide damage on MCF7 and HepG2 cells.

While more studies are necessitated to clarify the exact mechanism of these protection, but it is consider as potential natural antioxidants, with the cytoprotective capacity from oxidative stress. It may be effective in oxidative related disease such as inflammation and cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AFBI: Anthocyanin fraction of *Berberis integerrima* Bunge fruits; **ROS:** Reactive oxygen species; **DNA:** Deoxyribonucleic acid; **IC₅₀:** The half maximal inhibitory concentration.

SUMMARY

In this study, AFBI show potent cytoprotective effects on H₂O₂-induced oxidative stress in HepG2 and MCF7 cells. The cytoprotective capacity and antioxidant activity of this fruits could be considered as chemopreventive approach in oxidative stress related diseases.

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