CHAPTER IV

RESULTS AND DISCUSSION

Effect of UV-B irradiation on ARPE-19 cell viability

The MTT assay was firstly used to test cell viability of ARPE-19 cells in this study. Cells were exposed to UV-B at various intensity (20-500 mJ/cm^2) for 12 or 24 h (Figure 3). The results showed that the viability of ARPE-19 cells was decreased depending on the intensity of UV-B. However, UV-B dose response was observed only at low level of UV-B radiation (< 150 mJ/cm^2). Unexpectedly, high dose UV-B (150-500 mJ/cm^2) reduced cell viability to the similar levels (approximately 60 %). The viability of ARPE-19 cells at 12 h after UV-B exposure was not much different from 24 h after the exposure. This result suggests that UV-B irradiation reduced ARPE-19 cell viability in a dose dependent pattern. However, MTT assay was not able to show the dose dependent response of cell viability at high intensity of UV-B. By visual observation under microscope, the numbers of ARPE-19 cells were dramatically decreased after exposed to high intensity UV-B. It is possible that MTT assay may not be a suitable approach to determine the number of viable cells under influences of UV-B irradiation.

Therefore, trypan blue staining was then used to evaluate cell viability after UV-B irradiation in comparison with MTT assay. Numbers of trypan blue staining cells were counted under microscope. In this experiment, cell viability of ARPE-cells was determined 24 h after exposure UV-B (20-150 mJ/cm^2). The results showed that viability of ARPE-19 cells was decreased depending on UV-B intensity by both methods (Figure 4). However the percent reductions of cell viability detected by trypan blue assay were higher than those of MTT assay. Some reported the limitation of MTT assay that cells cytotoxicity was likely to be underestimated as compare to other methods [145, 146]. It was shown that MTT formazan dye can be increased by superoxide reaction [147, 148]. In this study, UV-B was later shown to induce ROS generation within ARPE-19 cells which might then induce the formation of formazan

dye. Therefore, trypan blue assay was chosen to determine cell viability of further experiments.

UV-B radiation can induce cell damage by several mechanisms. UV-B can be absorbed by molecular chromophores, for example DNA leading to DNA break [10, 149]. UV-B is known as a potent inducer of ROS within cells. ROS generated by photo-oxidative reaction can promote lipid peroxidation which causes oxidative change in structure of plasma membrane and damage mitochondria [150, 151]. UV-B also mediates cells apoptosis by activation of membrane bound cell death receptor such as tumor necrosis factor (TNF) receptor, death receptor-3 (CR-3) and CD95 [152, 153, 154].

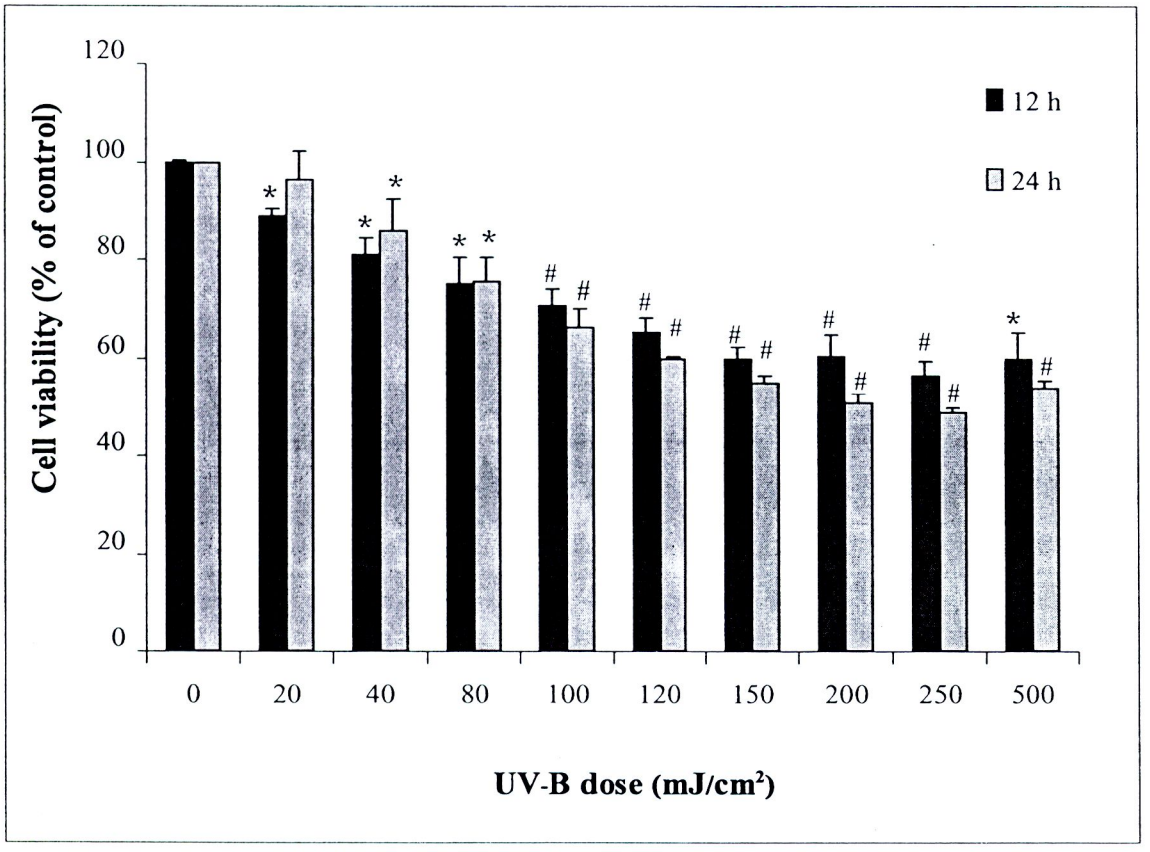


Figure 3 Effect of UV-B irradiation on viability of ARPE-19 cells. Cells were exposed to UV-B (20-500 mJ/cm^2) and cell viability was determined after 12 or 24 h by MTT assay. Cell viability was expressed as a percent of control cells. Value present as mean ± SEM of three independent experiments. (*$p \leq 0.05$, and #$p \leq 0.005$, UV-B vs control)

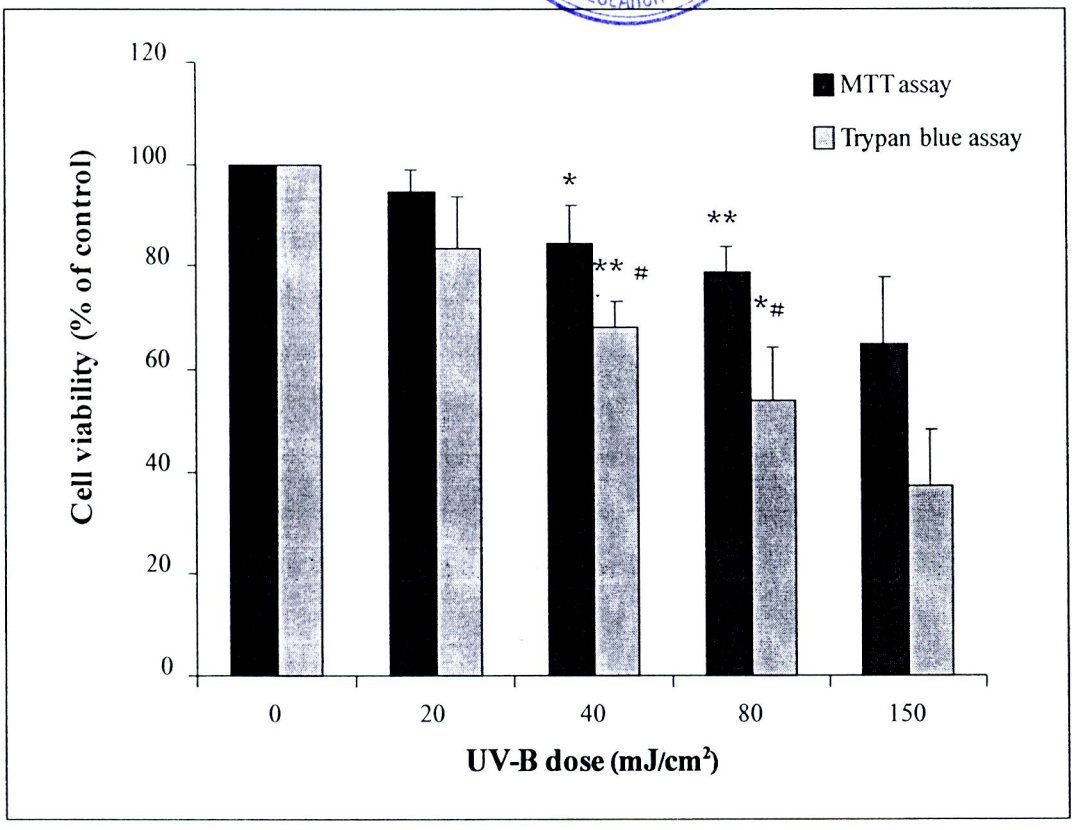
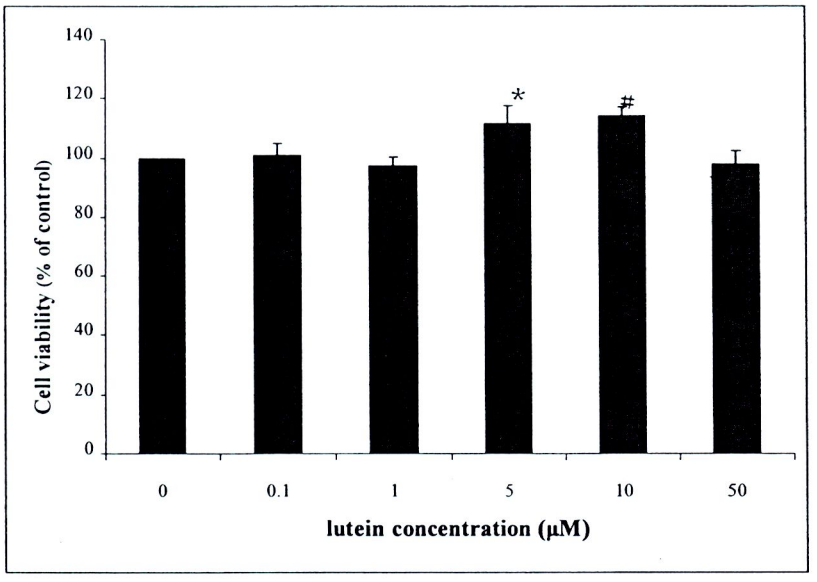


Figure 4 Determination of ARPE-19 cells viability after UV-B exposure by trypanblue exclusion and MTT assays. The viability was present as percent of control (non treated cells). Values present as mean ± SEM of three independent experiments. * $p \leq 0.05$, and ** $p \leq 0.005$; UV-B vs control, and # $p \leq 0.05$; MTT vs trypan blue

Effect of lutein on ARPE-19 cells

Before using lutein for further experiments, the cytotoxic effect of lutein on ARPE-19 cells was examined. In this experiment, the cell viability was determined by MTT assay. Lutein from two sources were tested, lutein extracted from yellow silk cocoon (silk lutein extract) and commercial lutein from marigold flower. The results showed that both luteins at concentrations up to 50 μM did not affect the viability of ARPE-19 cells (Figure 5). Because the solubility of lutein in culture medium was limited, higher concentrations could not be tested. These data indicate that lutein at concentration 0.1-50 μM is relatively safe for ARPE-19 cells. The concentration of lutein and zeaxanthin commonly used to test their effect in retinal pigment epithelial cell and photoreceptor cell culture is 1-20 μM [23, 24, 155, 156, 157], therefore the concentration of luteins between 10-50 μM were chosen to use in the following experiments.

A. Silk lutein extract



B. Commercial lutein (marigold flower)

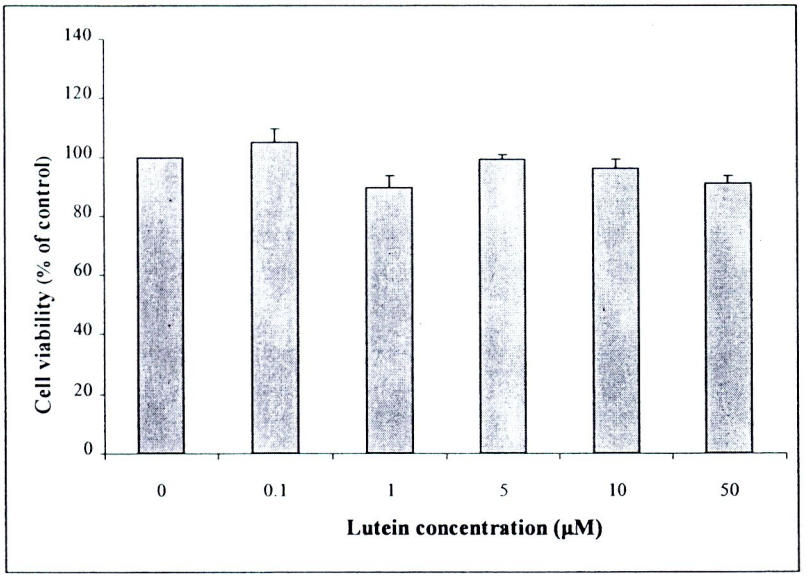


Figure 5 Effect of lutein(s) on ARPE-19 cell viability. Cells were treated with silk lutein extract (A), or commercial lutein from marigold flower (B) for 24, and cell viability were determined by MTT assays. The results were expressed as percent of control cells. Value presents mean ± SEM of three independent experiments. * $p \leq 0.05$, and # $p \leq 0.01$, lutein treatment vs control

Protective effect of lutein against UV-B induced cell death

To determine the protective effect of lutien on UV-B induced ARPE-19 cell death, silk lutein extract, and commercial lutein were tested in this study. Trolox which is a vitamin E analog was used as a control antioxidant. Three doses of UV-B (20, 40, and 80 mJ/ cm^2) and two concentrations of lutein were tested. Viability of ARPE-19 cells was determined by trypan blue assay. Similar to previous results, ARPE-19 cell viability was decreased depending on UV-B intensity. However, these decreases could not be reversed by pre-treatment cells with 10 μM of both lutein(s) and trolox (Figure 6A). At 50 μM concentration of silk lutein extract and trolox, viability of ARPE-19 cells were slightly higher than that of cells exposed to UV-B alone (Figure 6B). Although, these protective effects did not reach statistical significance, these data showed the potential of silk lutein extract for further study. Unexpectedly, commercial lutein from marigold flower at 50 μM induced cell damage by itself and potentiated UV-B induced cell death in this particular experiment (Figure 4B).

It should be noted that cell viability was determined by trypan blue exclusion method in this experiment, whereas cytotoxicity test of lutein was earlier dertermined by MTT assay. Thus, commercial lutein at 50 μM could not be used in the further experiments, since it was toxic to ARPE-19 cells. It was replaced with standard lutein (analytical grade from Sigma) in further experiments.

Commercial lutein from marigold flowers used herein unexpectedly reduces cell viability of ARPE-19 cells. It should be noted that commercial lutein tested in this experiment was not analytical or cell culture grade, it's expected for nutritional or dietary grade. This commercial grade was prepared in high volume for general industrial use. The contaminant levels were expected to be higher to analytical or cell culture grade. In contrast, the analytical grade is associated to products that are suitable for qualitative and semi-quantitative grades that specified impurities are often at 10-1000 ppm levels can used in most analytical application, research and quality control [158]. The reduction could be the result of contaminant substances. Furthermore, cytotoxic effect of commercial lutein was not detected by MTT assay but by trypan blue staining. Thus, to evaluate the cytoxicity of certain compounds, methodology used is important.

Since the highest applicable concentration of lutein (50 μM) showed only slightly protective effect, the effect of silk lutein extract in combination with trolox was tested. As shown in Figure 7, 50 μM of silk lutein extract or trolox slightly prevented ARPE-19 cell death similarly to previous experiment. When cells were pre-incubated with silk lutein extract together with trolox (50 μM each), viability of ARPE-19 cells was not much different from the effect of each compound. It was hypothesized that small protective effect of lutein could be due to of too long incubation period.

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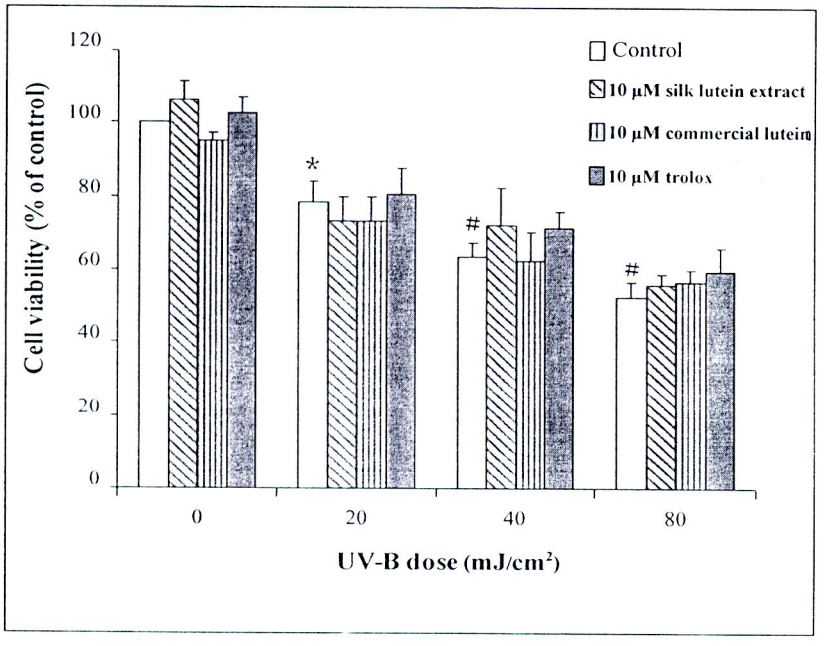

Figure 6 Protective effect of lutein(s) on UV-B induced ARPE-19 cells death. Cells were pretreated with silk lutein extract, commercial lutein, or trolox at 10 μM (A) and 50 μM (B) 24 h before UV-B irradiation. Cell viability was determined 24 h after UV-B exposure by trypan blue exclusion method. Value presents mean±SEM of three independent experiments. * $p \leq 0.05$, and # $p \leq 0.005$; UV-B vs control. ** $p \leq 0.05$ antioxidant+UV-B vs UV-B

B

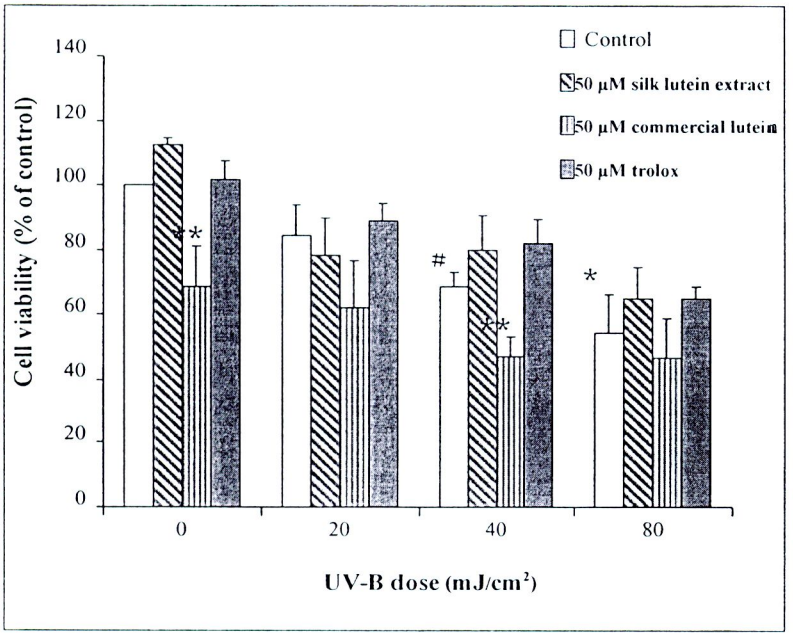

Figure 6 (Cont.)

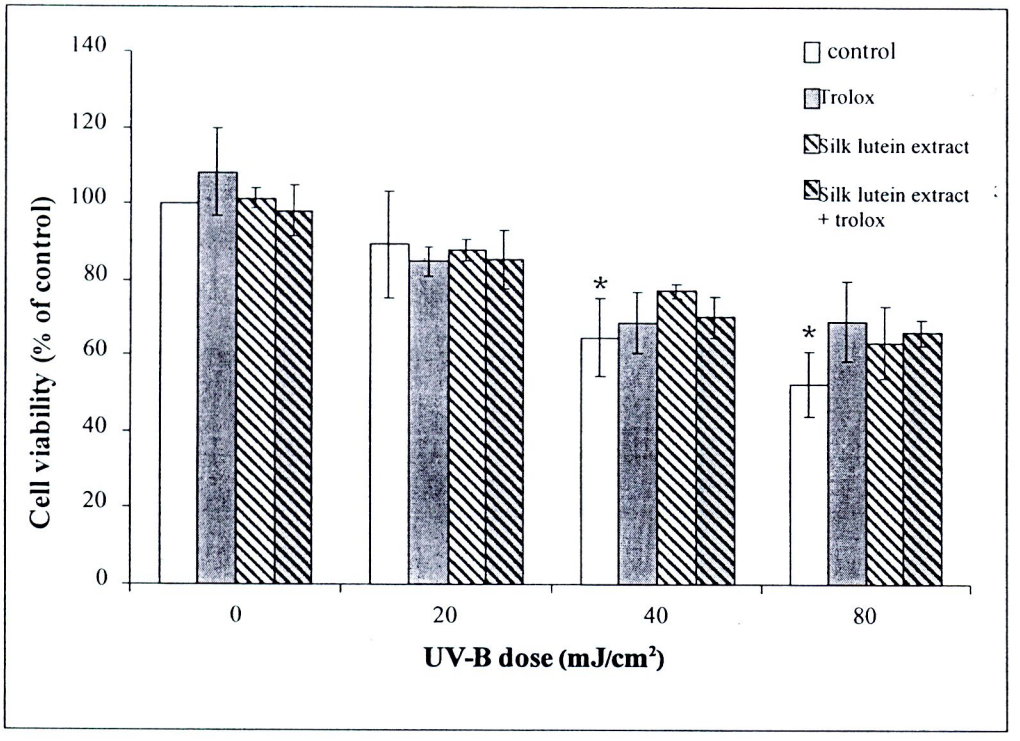

Figure 7 **Protective effect of silk lutein and/or trolox on UV-B induced ARPE-19 cells death. Cells were pretreated with 50 µM silk lutein extract, 50 µM trolox, or combination of both compounds (50 µM each) 24 h before UV-B irradiation. Cell viability was determined 24 h after UV-B exposure by trypan blue exclusion method. Value presents mean ± SEM of three independent experiments. * $p \leq 0.05$; UV-B vs control**

In these experiments, ARPE-19 cells were pre-treated with silk lutein extract for 24 h before UV-B exposure. It is possible that lutein was degraded in the significant amount when it presented in culture medium for 24 h. It was reported that the concentration of lutein was significantly reduced after exposure of light [159]. Since degradation of lutein after long time incubation was thought to be a reason of low level of cell protection, various incubation periods were then tested. ARPE-19 cells were pre-treated with silk lutein extract at 2, 4, 12, and 24 h before 40 mJ/cm^2 of UV-B exposure. The result showed that 2 and 4 h incubation provided higher cell protection than longer incubation periods (12 and 24) (Figure 8). Due to high variation at 2 h pretreatment, 4 h time period were then chosen for further experiments.

Several studies show that lutein and zeaxanthin protect ARPE-19 cell death induced by light and oxidative stress [24, 155, 160]. The lutein at concentration 6 and 8 μM was reported to increase approximately 10 % in viability of ARPE-19 cells after exposure to blue light (430 nm) [160]. Protective effect of lutein at 2 μM was rather small in ARPE-19 cells exposed to visible light [24]. In cellular oxidative stress induced by H_2O_2, lutein could increase viability of ARPE-19 cells for 20 % [155]. According to these studies, lutein showed potential to prevent ARPE-19 cells from various types of insults, but the efficacy might be depending on lutein concentration and degree of cell injury of individual study. In the present study, the protective effect of lutein was not observed at 10 μM but slightly prevention was found at 50 μM. UV-B light was likely to cause high degree of cellular damage, thus high concentration of lutein is required to prevent cells when compare to other insults such as H_2O_2.

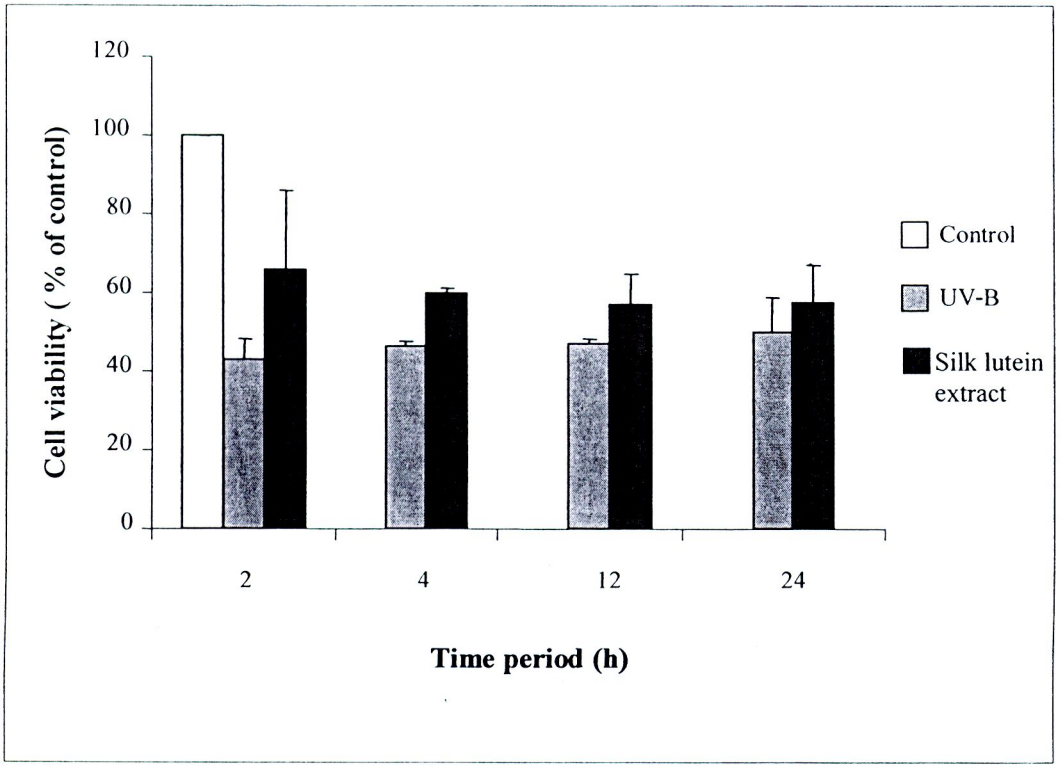

Figure 8 Effect of lutein incubation period on UV-B induced ARPE-19 cells death. ARPE-19 cells were pre-treated with 50 µM lutein for 2, 4, 12 and 24 h before 40 mJ/cm^2 UV-B exposure. Cell viability was determined using the trypan blue assay. The data present in mean ± SD of two experiments which each experiment was performed in duplicate

Lutein is a lipid soluble and an unstable molecule that contains the conjugated double bonds, which can be readily isomerized, oxidized, and degraded to metabolites with lower activity and antioxidant capacity [161]. Lutein and zeaxanthin can be bleached rapidly *in vitro* by photolysis resulting in the loss absorption as the function to defends free radicals [162]. The mechanisms of silk lutein extract for protecting ARPE-19 cells from UV-B radiation may be similar to protection from other types of insults in other studies. Lutein and zeaxanthin function as light screening pigments by absorbing damaging blue light, but they may also contribute to the antioxidant defense of retinal structures [24, 116]. It is well known that lutein is an efficient lipophilic antioxidants, acting as singlet oxygen quenchers and as scavengers of reactive oxygen intermediates [163].

Protective effect of lutein and combination with trolox on UV-B induced ARPE-19 cell death.

In this experiment, ARPE-19 cells were pre-treated with silk lutein extract, standard (std.) lutein, or trolox for 4 h before UV-B irradiation (40 mJ/cm^2). The results in figure 12A showed that only 50 μM but not 25 μM of tested compounds could protect cell from UV-B induced cell death. The viability of ARPE-19 cells was approximately 55 % after UV-B exposure, and it was significant increased to approximate 72 % after treating cells with 50 μM of either antioxidant (Figure 9A). In addition, the viability of control cells was slightly increased by these antioxidants at high concentration (50 μM). To test the enhancing effect, cells were treated with 25 μM of both luteins in the present of 25 μM trolox. The results showed significant increases in cell viability of these combinations in the similar degree as individual compound at 50 μM (Figure 9B).

The present results suggest the protective effect of silk lutein extract in ARPE-19 cells against oxidative stress by UV-B radiation. This protection was similar to standard lutein and vitamin E. These data also suggest that the enhancing effect of lutein combined with vitamin E that could be the results of efficient antioxidative capacity of the combination. Previous report demonstrated the augmentation effect of combine caroteiniods and α-tocophorol for protection ARPE-19 cells due to better antioxidant efficiency [24, 164].

A

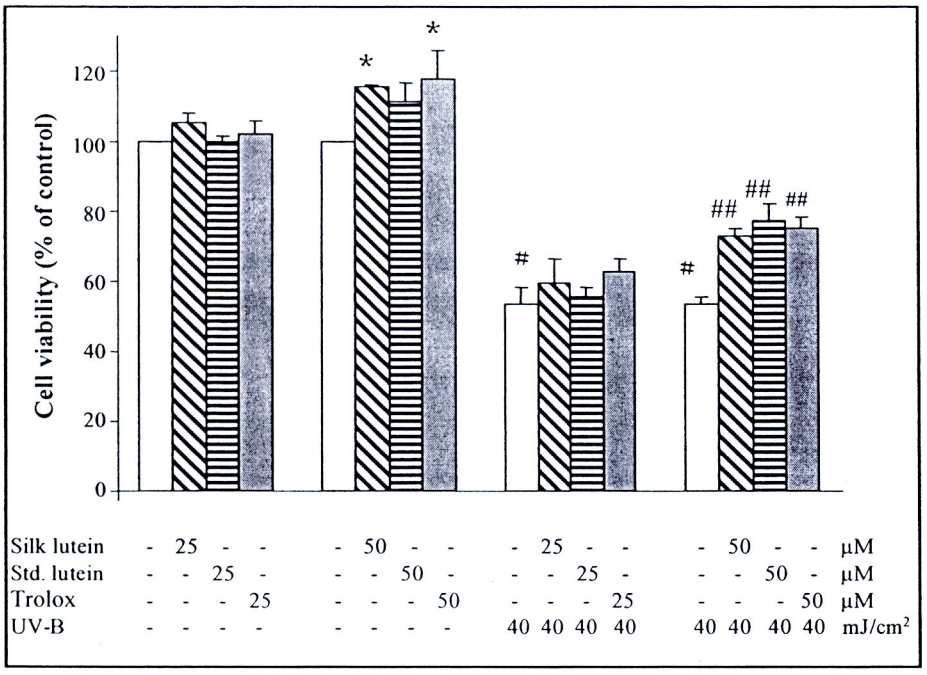

Figure 9 Protective effect of luteins and/or trolox on UV-B induced ARPE-19 cells death. Cells were pretreated with silk lutein extract, std. lutein, or trolox (A) and combination of both compounds (B) 4 h before UV-B irradiation. Cell viability was determined 24 h after UV-B exposure by trypan blue exclusion method. Value presents mean ± SEM of three independent experiments. # $p \leq 0.05$, UV-B vs control, * $p \leq 0.05$; antioxidant vs control, and ** $p \leq 0.05$, ## $p \leq 0.005$, antioxidant+UV-B vs UV-B

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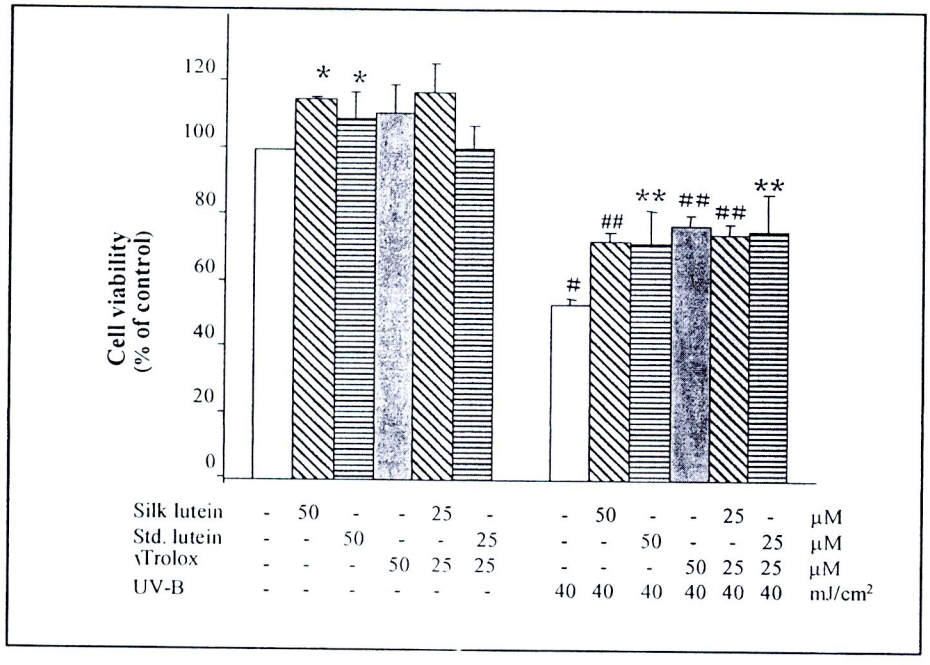


Figure 9 (Cont.)

UV-B induced intracellular ROS production in ARPE-19 cells

This experiment aimed to investigate the effect of UV-B on intracellular ROS production in ARPE-19 cells. DCFH-DA_2 was used as an indicator of intracellular levels of ROS. ARPE-19 cells were exposed to UV-B (20–80 mJ/cm^2), and fluorescence intensity of oxidized $DCFH_2$ was determined at 0.5, 1, and 24 h afterthat. Non-fluorescent DCFH-DA dye freely penetrates into the cells, gets hydrolyzed by intracellular esterase to DCFH, and traped inside the cells [165]. The increased DCF dye indicated the production of intracellular ROS within ARPE-19 cells. The results showed that UV-B induced the production of intracellular ROS of ARPE-19 cells in a dose-dependent pattern at 0.5 and 1 h after UV-B exposure (Figure 10). The level of intracellular ROS was highest at 0.5 h after UV-B irradiation. The ROS level was expectedly lowest and not depending on UV-B intensity at 24 h after UV-B exposure. This result demonstrates that UV-B markedly increased the production of intracellular ROS in ARPE-19 cells and this increment is occurred shortly after UV-B exposure. This result corresponds to previous study showing that the intracellular ROS could be observed within 30 min after UV irradiation [166].

It is well known that UV-B radiation has higher photon energy, that rapidly produces the photochemical reaction which elevates the oxygen molecule from its ground triplet state to the singlet state by direct energy transfer [167]. This reactive oxygen species is itself a potent oxidant [168], including singlet oxygen, the superoxide radical, hydroxyl radicals, and hydrogen peroxide radical [169]. From our observation, ROS was dramatically generated by 30 min after UV-B exposure and the level of ROS was significantly reduced after that. It is possible that ROS can interact with oxidation sensitive biomolecules inside the cells, especially lipids which then leading to the reduction of ROS level. Therefore when the fluorescence was measured at 24 h after UV-B exposure, low level of fluorescence was determined. This result could not directly indicate that cells had low level of cellular oxidative stress because number of viable cells was also reduced at this time points. Furthermore, it was thought that fluorescent DCF may not be stable and degraded quickly. Thus, most studies using this dye for ROS measurement would determine the fluorescence no longer than 4 h after cell treatment [170].

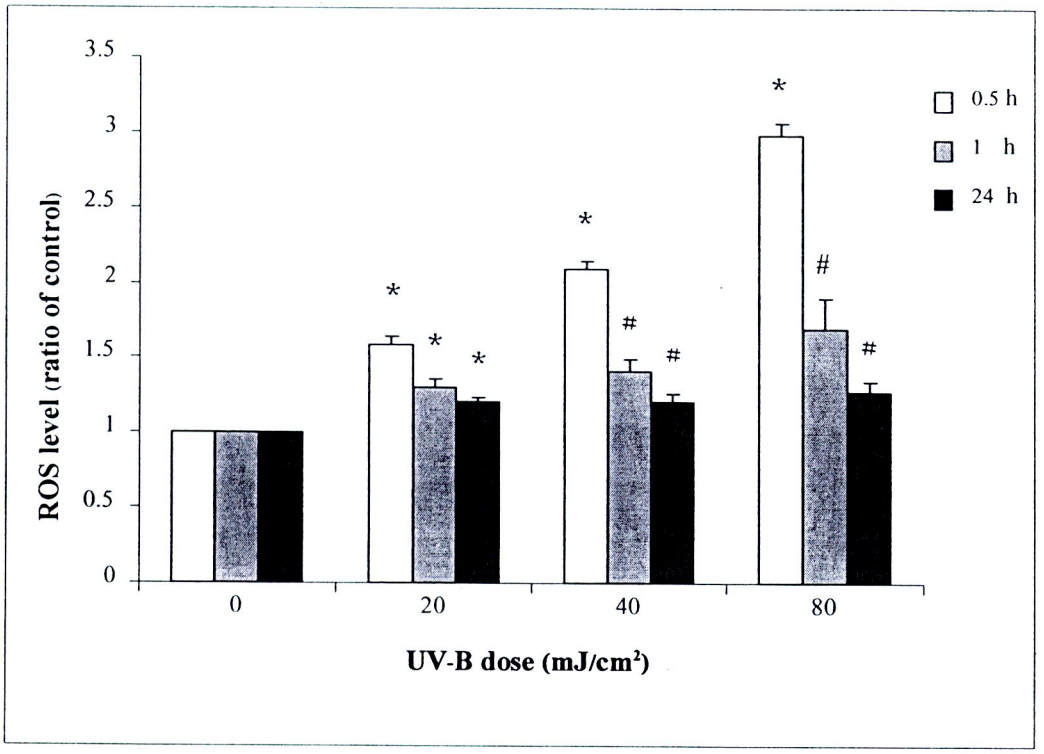


Figure 10 Effect of UV-B on intracellular ROS generation in ARPE-19 cells. ROS formation was measured at 0.5, 1 and 24 h after cells exposed to UV-B (20-40 mJ/cm^2). Results are presented as ratio of ROS levels to control or untreated cell. Values represent means ± SEM of three independent experiments. # $p \leq 0.05$ and * $p \leq 0.005$; UV-B vs control

Lutein inhibits intracellular ROS production in ARPE-19 cells

One mechanism of UV-B induced ARPE-19 cell death could be due to the elevation of ROS production. Therefore, this experiment determined the inhibitory effect of lutein and their combination with trolox on UV-B-induced ROS production. The results showed that 40 mJ/cm^2 UV-B induced the increase in intracellular ROS production approximately 2 folds (Figure 11). Pretreatment with silk lutein extract, std. lutein, or trolox at 50 μM significantly inhibited ROS generation. Luteins combined with trolox (25 μM each) also reduced ROS production. Standard lutein showed the highest degree of inhibition in this experiment. This result also demonstrated that all tested compounds (individual and combinations) suppress the formation of intracellular ROS in control cells that were not exposed to UV-B. These data suggest that lutein and/or trolox inhibit ROS generation in ARPE-19 cells in both normal (endogenous) and stress (UV-B induced) conditions.

Based on this finding, it is possible that lutein protect ARPE-19 cells from UV-B via its ability to eliminate intracellular ROS levels. The ability to reduce intra cellular ROS may indicate that lutein can enter ARPE-19 cells and interact with ROS. Lutein is belong to the carotenoids that composed of a high number of conjugated double bond that can directly to quench ROS [171]. Lutein plays a role for cell protection because it possess of singlet oxygen and triplet excite state of photosensitizing that facilitated it ability to scavenge free radicals that involved the oxidation of lipid [163, 172]

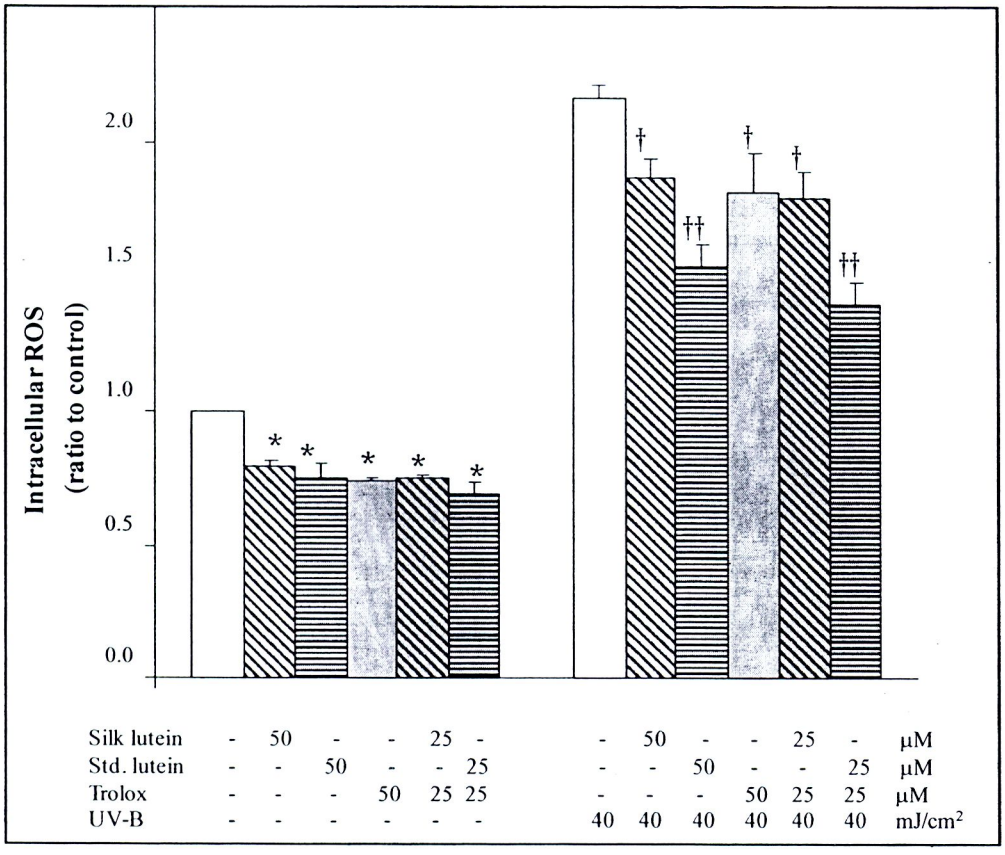


Figure 11 Protective effect of lutein(s) and/or trolox on UV-B induced intracellular ROS production in ARPE-19 cells. Cells were pre-treated silk lutein extract, std. lutein, or trolox, and combination for 4 h before UV-B irradiation. Intracellular ROS was determined 30 min after UV-B exposure. Value presents mean ± SEM of three independent experiments. * $p \leq 0.05$; antioxidant vs control, # $p \leq 0.05$; UV-B vs control, and † $p \leq 0.05$, †† $p \leq 0.005$; antioxidant+UV-B vs UV-B

UV-B induced lipid peroxidation in ARPE-19 cells

In this experiment, lipid peroxidation was determined at 24 h after UV-B exposure by TBARs assay which evaluates the level of MDA, a lipid peroxidation product. The result showed that UV-B induced lipid peroxidation in ARPE-19 cells in dose dependent pattern (Figure 10). All tested intensity of UV-B significantly increased the level of MDA suggesting that UV-B irradiation can induce the oxidative damage of lipid compartment of ARPE-19 cells which may consequently lead to cell injury or death.

During peroxidation of membrane lipids, the free radicals attack the molecule of lipids such as plasma membrane, and lipid radicals propagate the peroxidation process which leads to the accumulation of MDA byproduct [173]. Cell membranes contain numerous amount of polyunsaturated fatty acids which are susceptible to ROS and oxidation of lipids can break down membranous structures [44]. Our earlier experiment showed the UV-B radiation elicited a large increase of intracellular ROS production of ARPE-19 cells. Therefore, the overproduction of ROS induced by UV-B was likely to cause the oxidation of lipids leading to RPE cell damage.

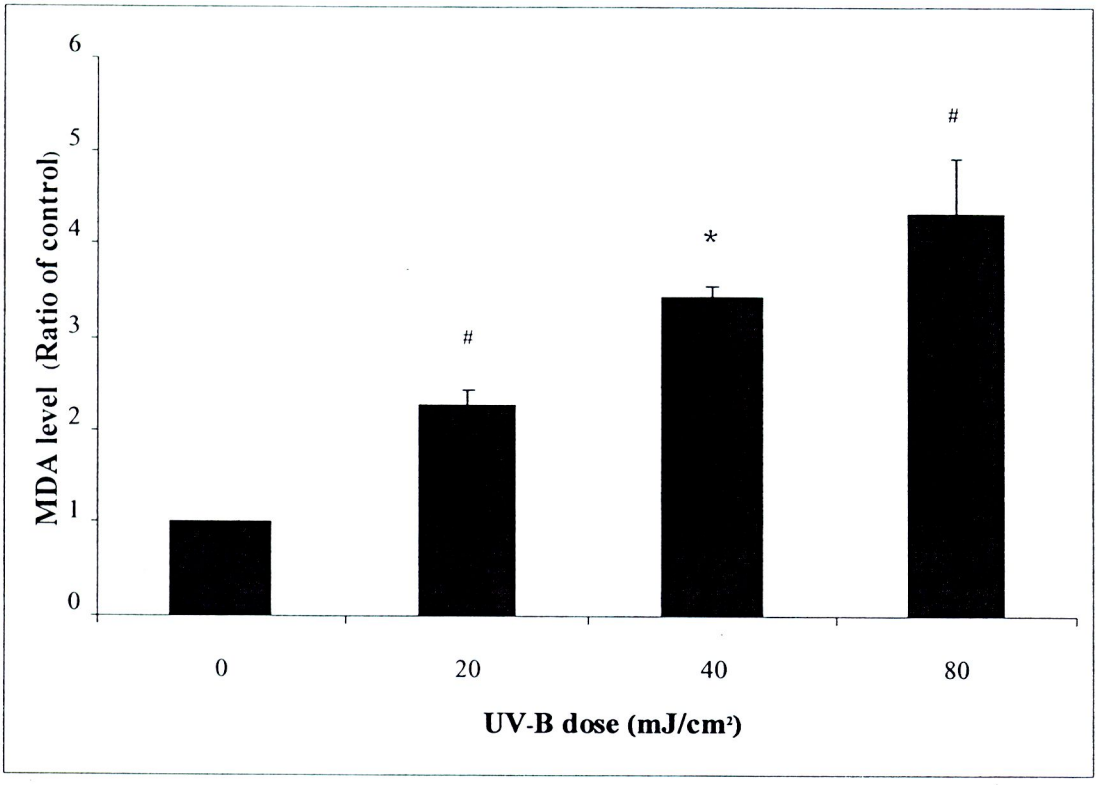


Figure 12 **UV-B induced lipid peroxidation in ARPE-19 cells. The measurement was determined using TBARs assay after 24 h of UV-B exposure at 20-80 mJ/cm^2. Results are presented as ratio of control or untreated cell. Values represent means±SEM of three independent experiments. # $p \leq 0.05$, and * $p \leq 0.01$; UV-B vs control**

Lutein inhibits lipid peroxidation in ARPE-19 cells

The lipid peroxidations in ARPE-19 cells were affected by UV-B generated the ROS induced cells damage. Therefore, this study investigated the inhibitory effect of lutein on UV-B induced lipid peroxidation in ARPE-19 cells was investigated by TBARs assay. The results showed that 40 mJ/cm^2 UV-B increased lipid peroxidation about 1.8 fold from control at 24 h after UV-B irradiation (Figure 13). Silk lutein extract, std. lutein, or trolox at 50 μM as well as their combinations significantly reduced lipid peroxidation induced by UV-B. Similar to the effect on ROS production, std. lutein and its combination showed the highest inhibitory effect on lipid peroxidation. Compared to ROS reduction, lipid peroxidations were reduced more effectively by these tested compounds. In addition, ARPE-19 cells treated with these compounds had slightly lower level of MDA than control cells. These data suggest that both lutein(s) and trolox can reduce endogenous lipid peroxidation and effectively prevent UV-B-mediated lipid peroxidation in ARPE-19 cells.

The ability of lutein to inhibit lipid peroxidantion was demonstrated in several studies. The study in animal model reported that lutein could prevent diabetes-induced increase in retinal damage and decrease the level of malondialdehyde and oxidative induced DNA damage [174]. Some studies suggest that lutein and zeaxanthin reduce lipid peroxidation by quenching the singlet oxygen and scavenging free radicals [24, 111]. Lutein is a dipolar carotenoid presenting hydroxyl group in their ionone rings, and by this property it can accumulate in bilayer surface of cellular membrane. In addition, lutein can penetrate in cells membrane, strengthen the membrane and act to reduce the oxygen radical penetration into lipid bilayer membrane [112]. Therefore, lutein could effectively protect lipid molecule from peroxidation as also observed in the present study. Trolox can effectively interact with acoxyl and peroxyl radicals [175]. Trolox is very effective to protect lutein since trolox can regenerate lutein from the corresponding caroteniod radicals [176]. By these properties of trolox, it can enhance the anti-oxidative effect of lutein. In addition, trolox shows coactive effect with lutein by reducing lutein degradation [24].

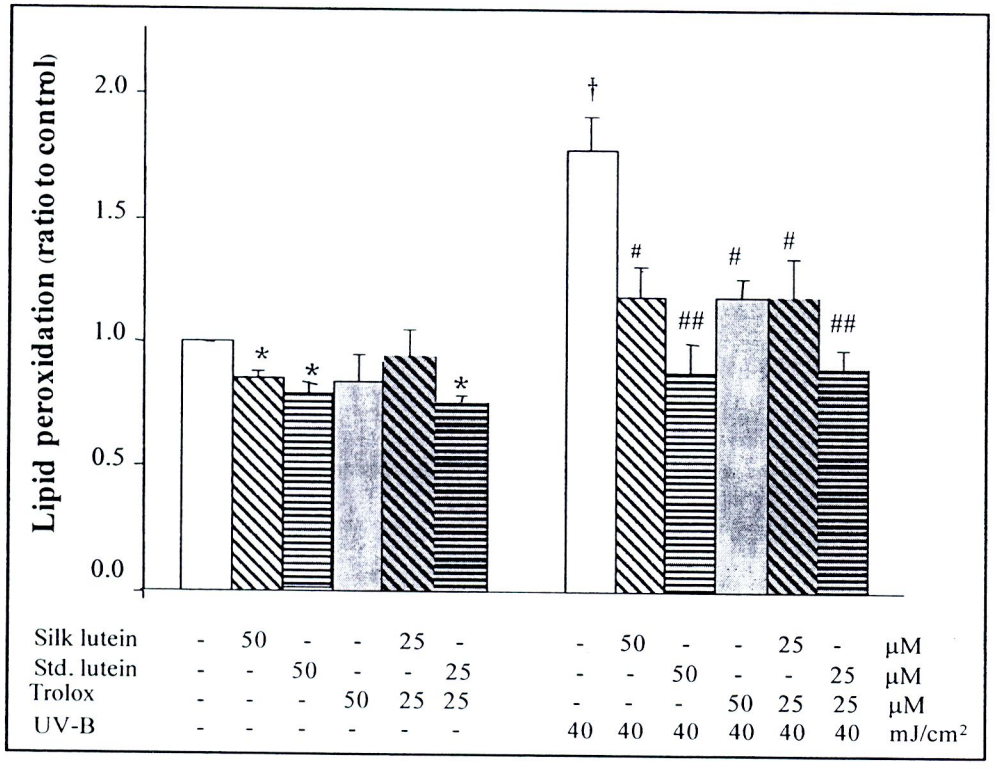


Figure 13 Protective effect of luteins and/or trolox on UV-B induced lipid peroxidation in ARPE-19 cells. Cells were pretreated silk lutein extract, std lutein, or trolox, and combination for 4 h before UV-B irradiation. Lipid peroxidation was determined 24 h after UV-B exposure by TBARS assay. Value presents mean ± SEM of three independent experiments. * $p \leq 0.05$; antioxidant vs control, † $p \leq 0.005$; UV-B vs control, and # $p \leq 0.05$, and ## $p \leq 0.005$; antioxidant+UV-B vs UV-B

Correlation of cell viability, intracellular ROS, and lipid-peroxidation induced by UV-B

UV-B significantly decreased ARPE-19 cell viability in a dose dependent pattern that was correlated to the increase in intracellular ROS production and lipid peroxidation (Figure 14 A, B, and C). UV-B intensity showed linear correlation to ROS level suggesting its direct effect on free radical generation. In addition, ROS were generated shortly after UV-B irradiation. Cell viability was however correlated to lipid peroxidation more than intracellular ROS. Compared to 40 mJ/cm^2 UV-B, 80 mJ/cm^2 UV-B produced almost double ROS level but similar in lipid peroxidation. Retinal pigment epithelial cells contain an abundance of photosentizers which can potentiate the generation of intracellular ROS [44, 45]. Cells damage caused by ROS was mostly mediated through contribution of lipid peroxidation. Endogenous antioxidative defense mechanism probably plays crucial role to eliminate the excess amount of ROS under extreme cellular stress.

Lutein was earlier showed to inhibit the effect of oxidative stress in UV-B treated ARPE-19 cells by suppressing intracellular ROS and lipid peroxidation. Lutein partially reduced ROS production but completely suppressed lipid peroxidation. Thus there may be other mechanisms that lutein inhibit the oxidation of lipid. Therefore in further experiments were conduct to test the effect of lutein on anti-oxidant enzymes in UV-B treated ARPE-19 cells.

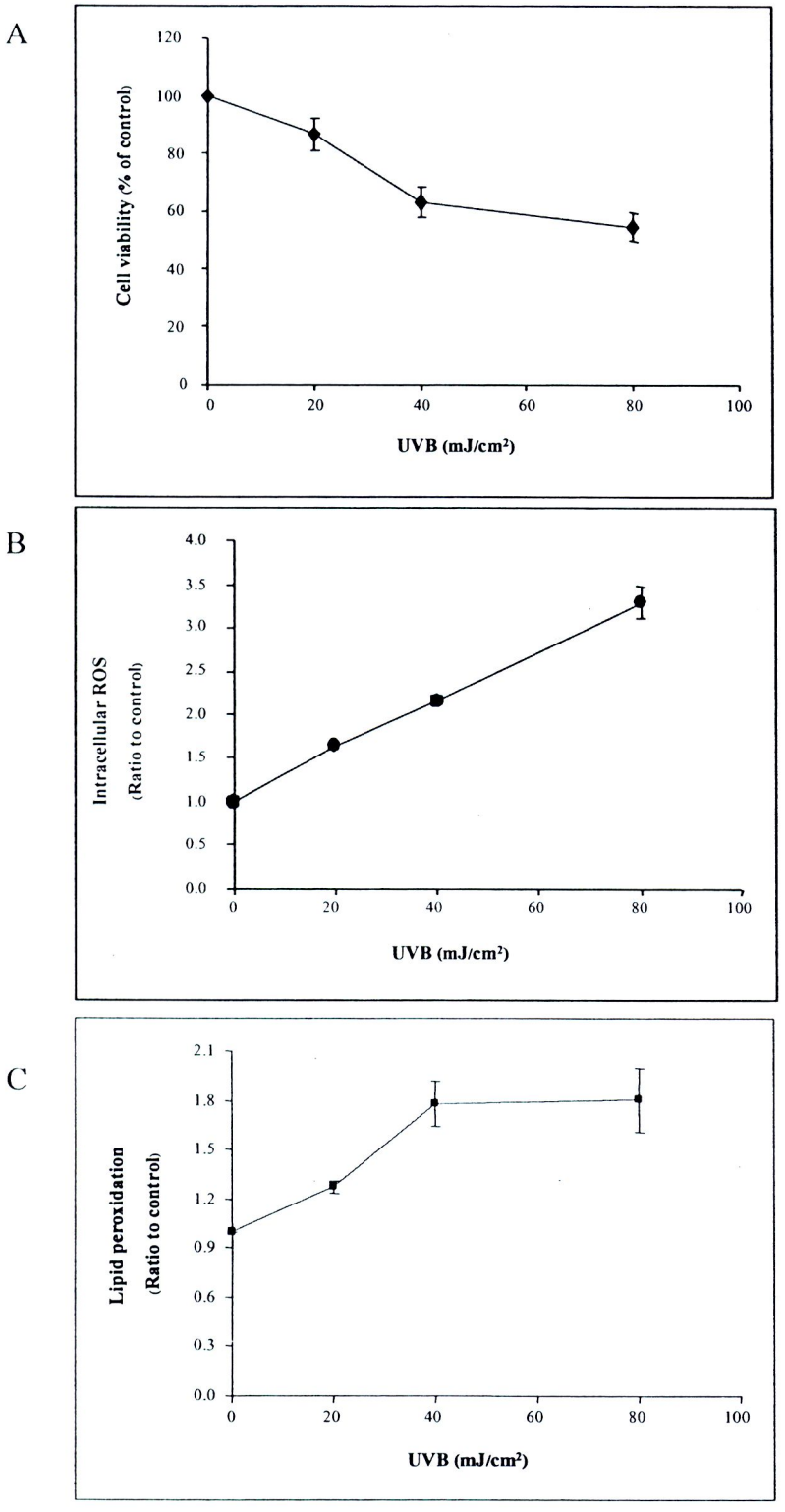


Figure 14 Dose-dependent effect of UV-B on ARPE-19 cells death (A), intracellular ROS production (B) and lipid peroxidation. The cells were treated with UV-B intensity at 20, 40 and 80 mJ/cm^2. Values represent means ± SEM of three independent experiments

Effect of lutein on superoxide diamutase (SOD) in ARPE-19 cells

This experiment demonstrated the effect of lutein on SOD activity in UV-B exposed ARPE-19 cells. The results showed that UV-B irradiated ARPE-19 cells had approximately 20% reduction of SOD activity compared with non exposed cells (Figure 15). Silk lutein extract, std. lutein, trolox, and combinations slightly but not significantly increased SOD activity in UV-B treated cells. Under circumstances of this experiment, UV-B radiation only slightly affected SOD activity, therefore insignificant effect from all tested compounds was observed. These data however suggest that reduction of SOD activity might be one of action of UV-B to induce cellular oxidation stress of ARPE-19 cells.

It is well known that the action of SOD enzyme is important in the cellular antioxidative defense to catalyse superoxide radical which is the first step of the enzymatic antioxidant protection. H_2O_2, which is produced by SOD, should be converted simultaneously to water and oxygen by glutathione peroxidase (GPx) and catalase enzymes. UV-B may increase degradation of SOD protein resulting in loss of SOD activity. However this alteration is small which is consistent with other study showing that SOD level was not markedly changed in response to any of the treatments in ARPE-19 cells [177]. Therefore, the activity of SOD may reflect only portions of the antioxidant defend in retinal pigment epithelial cells.

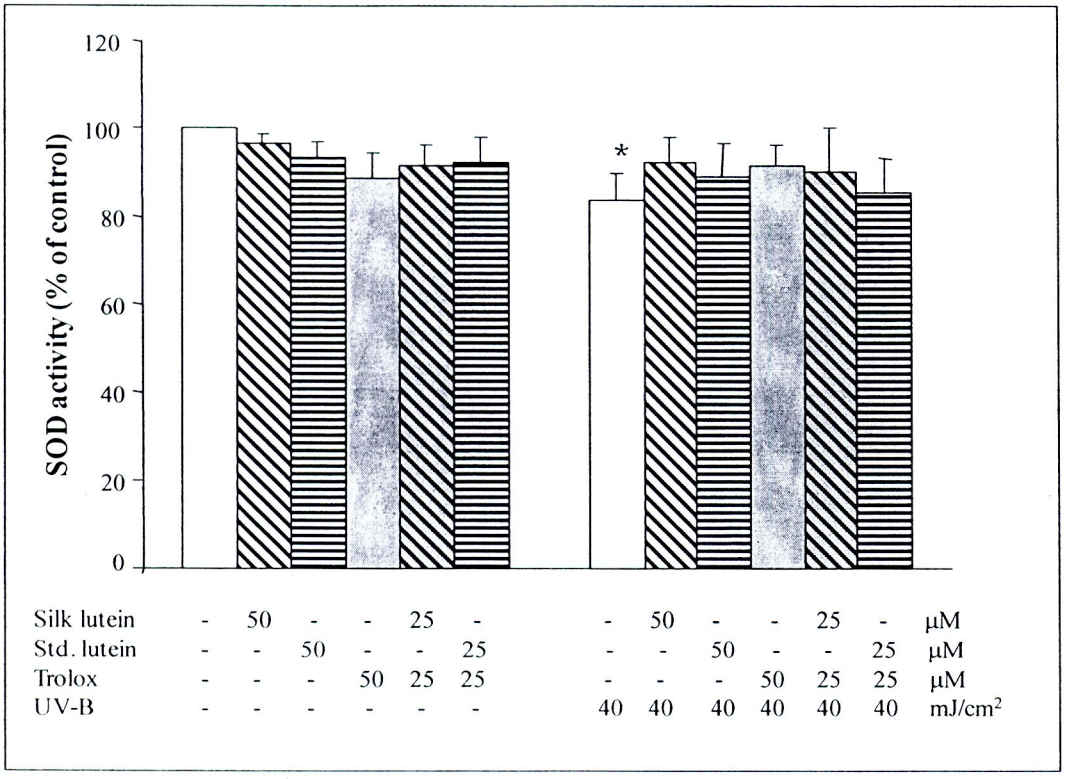


Figure 15 Effect of lutein(s) and/or trolox on SOD activity in UV-B exposed ARPE-19 cells. Cells were pretreated with silk lutein extract, std. lutein, or trolox, or its combination for 4 h before UV-B irradiation. SOD activity was determined 24 h after UV-B exposure by enzymatic assay. Values present mean±SEM of three independent experiments. * $p \leq 0.05$; UV-B vs control

Effect of UV-B irradiation on catalase (CAT) activity in ARPE-19 cells.

This experiment demonstrated the effect of UV-B (40 mJ/cm^2) on CAT activity. The data showed that CAT activity of control (non-exposed) ARPE-19 cells at 1, 4, and 24 h were 30, 34, and 42 unit/mg proteins, respectively (Figure 16). At the same time periods, cells exposed to UV-B showed CAT activity at 40, 37, and 28 unit/mg proteins, respectively. From these results, CAT activity was higher when cells were older (1 h vs 24 h). Interestingly, CAT activity in UV-B exposed cells was varied depending on time of cell collection. The increase in CAT activity was observed at 1 h, whereas CAT activity was decreased at 24 h after UV-B exposure. This data might indicate that CAT activity was rapidly induced by UV-B probably due to the increased level of intracellular ROS. As shown earlier, the intracellular ROS was dramatically increased within 30 min after UV-B exposure.

The catalase enzymes are the key antioxidants cleaving hydrogen peroxide to water and oxygen. The upregulation of CAT enzyme is a part of defense mechanism to combat oxidative stress. The activation of CAT was found in apelin-induced oxidative stress in rat primary cultures of cardiac myocytes which the increase in mRNA level and CAT activity was observed at 80 min after exposure [178]. Many types of oxidants such as acrolein [179], H_2O_2 [155, 180], iron [180], and paraquat [180] could induce oxidative stress in RPE-19 cells in which the decrease in the activity and expression of catalase were also observed. In those studies, CAT activity and/or expression were determined 24 h after oxidative insults. In addition, UV-B was shown to reduce CAT activity in mice skin culture at 24 h after the exposure [181]. Based on all of these informations, CAT enzyme seems to be activated shortly after exposure to the insult and it might be exhausted in cells undergoing long period of oxidative stress.

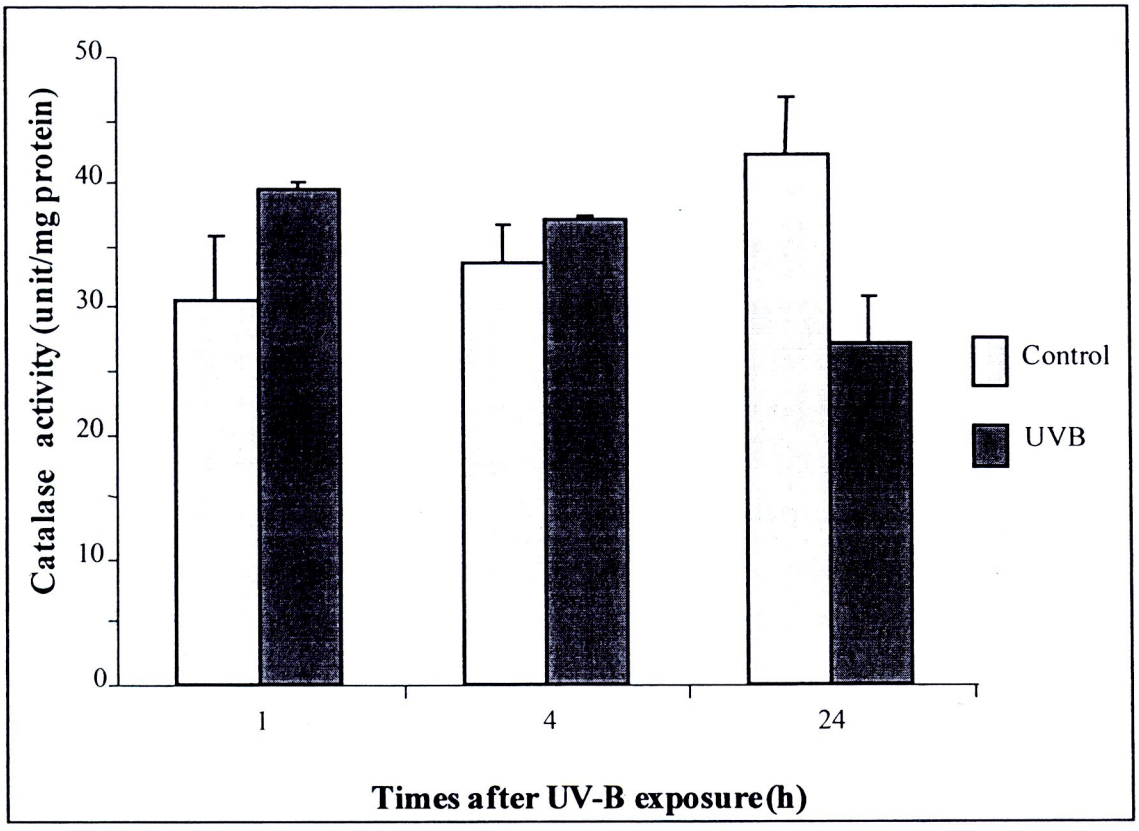


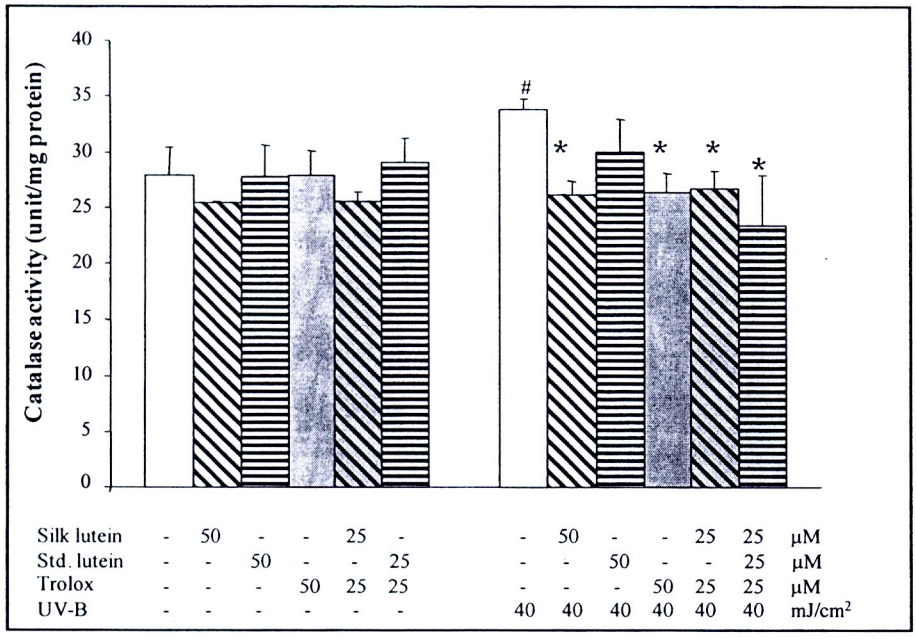
Figure 16 **Effect of UV-B on catalase (CAT) activity in ARPE-19 cells. Cells were collected and lysed with lysis buffer at 1, 4 and 24 h after UV-B irradiation (40 mJ/cm^2). The CAT activities were analyzed by enzymatic method. Data are expressed as means ± SEM from three separate experiments. * $p \leq 0.05$; UV-B vs control**

Effect of lutein on catalase (CAT) activity in UV-B exposed ARPE-19 cells.

The present experiment investigated the effect of lutein on CAT activity in ARPE-19 cells exposed to UV-B. The results showed that silk lutein extract, std. lutein, or trolox at 50 μM and combinations (25 μM each) abolished the influence of UV-B-mediated changes in CAT activity (Figure 17). The ability of these tested compounds could also reduce the increased in CAT activity as the immediate effect (1 h) of UV-B might be the results of their ability to suppress the formation of intracellular ROS in ARPE-19 cells. Long after UV-B exposure (24 h), these antioxidants could also inhibit UV-B induced reduction of CAT activity. Under this circumstance, ARPE-19 cells treated with these compounds underwent low level of intracellular oxidative stress, thus less exhaustion of CAT was observed. These data suggest that both lutein(s) and trolox effectively inhibit the effect of UV-B on CAT activity in ARPE-19 cells.

In the present study, UV-B exhibits the opposite effect on CAT activity depending on the time of measurements, short or long after the exposure. However, lutein(s) and its combination with trolox can inhibit both effects of UV-B. Grape seed proantrocyanin was demonstrated to inhibit UV-B induced depletion of CAT activity when it was measure at 24 h [166]. UV-A light can decrease CAT and SOD activity in rat kidney cells, and lutein can protect this cells by increasing CAT and SOD activity [182]. However, in colon cancer HT 29 cells lutein showed different results from other studies by significantly decreasing activities of CAT on deoxynivalenol induced oxidative stress for 24 h [183]. Taken all data together, CAT activity seems to be altered, decreased or increased probably due to type of oxidative stress, and time of treatment and these alterations can be corrected by antioxidants such as lutein.

A



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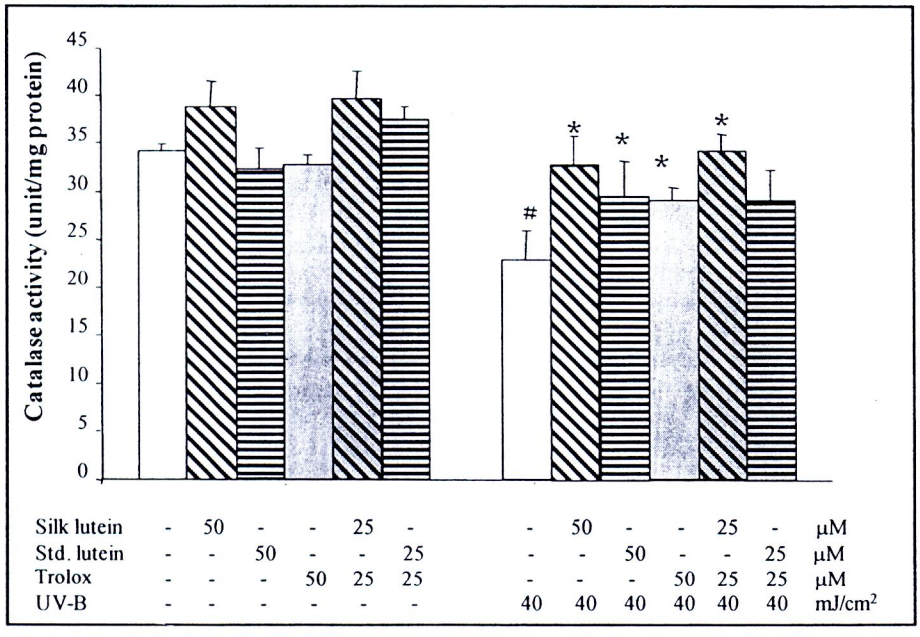

Figure 17 Effect of lutein(s) and/or trolox on CAT activity in ARPE-19 cells. Cells were pretreated with silk lutein extract, std. lutein, or trolox, and combinations for 4 h before UV-B irradiation. CAT activity was determined at 1 h (A) and 24 h (B) after UV-B exposure. Values present mean ± SEM of three independent experiments. # $p \leq 0.05$; UV-B vs control, and * $p \leq 0.05$; antioxidant+UV-B vs UV-B

Effect of lutein on glutathione peroxidase (GPx) activity in UV-B exposed ARPE-19 cells

This experiment investigated the effect of lutein on GPx acivity in ARPE-19 cells exposed to UV-B irradiation. The GPX activity was determined by the reaction in the presence of GSH with hydroperoxide as a substrate and a decrease in the absorbance of NADPH at 340 nm was measured. The result showed that UV-B irradiated ARPE-19 cells had a much higher enzyme activity than those control cells (Figure 18). This increase in GPx activity was effectively inhibited by silk lutein extract, std. lutein, trolox and combinations. These data indicated that UV-B considerably enhanced GPx activity and this enhancement was blocked by both lutein(s) and its combination with trolox.

Glutathione peroxidase is considered to be the most important antioxidant defense system in eukaryotic cells. It is well known that GPx is a selenoprotein that can convert hydrogen peroxide into water and molecular oxygen using GSH as unique hydrogen donor [184]. Consistent with the present study, GPx activity was shown to increase in human skin fibroblast cells exposed to UV-A [185], and keratinocytes irradiated by UV-B [186]. The overexpression of GPx enzyme appears to particularly induce cell apoptosis because this enzyme can catalyze the reduction of peroxides by glutathione (GSH) which protects tissue and DNA against cell damage [187]. The amount of cellular GSH has been found to play an important role in the defense mechanism and its depletion leads to cell apoptosis [188]. It is possible that ROS formation and lipid peroxidation in ARPE-19 cells exposed to UV-B undergo cell apoptosis because of cellular depletion of GHS levels and possibly compensatory increase in GPx. Increased GPx enzyme activity in response to cellular oxidative stress is extensively documented as a key role in combating oxidative stress. In the presence of antioxidant such as lutein, cellular oxidative stress is low, which then preserves normal level of GPx activity. Lutein protects HT-29 cells from deoxynivalenol-induced oxidative stress and its effect to decrease GPx activity might be associated with its protective effect [183].

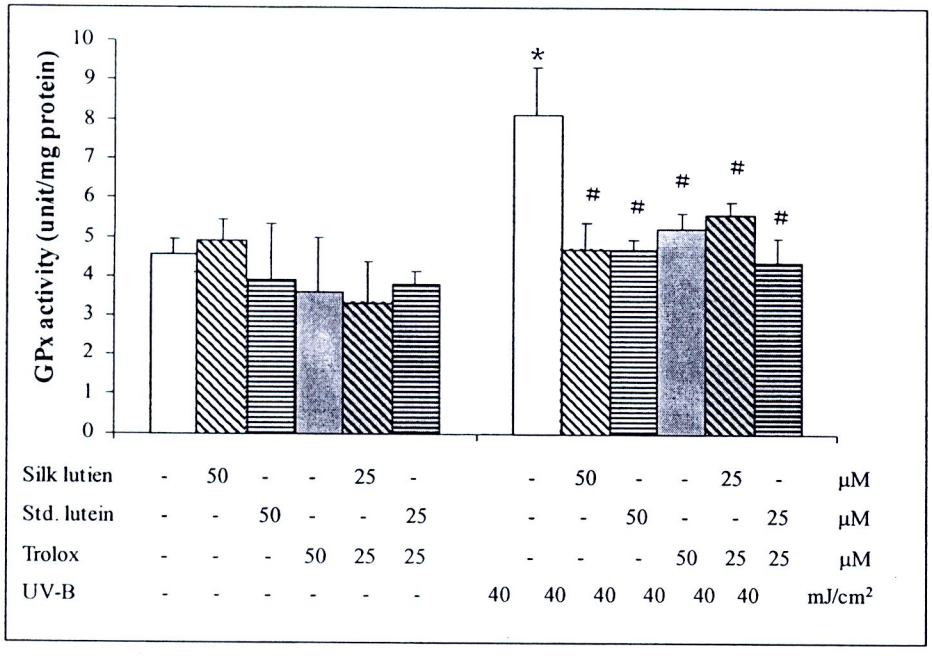

Figure 18 Effect of lutein(s) and/or trolox on GPx activity in UV-B exposed ARPE-19 cells. Cells were pretreated silk lutein, std. lutein extract, or trolox, and combinations for 4 h before UV-B irradiation. GPx activity was determined after 24 h after UV-B exposure. Values present mean ± SEM of three independent experiments. * $p \leq 0.05$; UV-B vs control, and # $p \leq 0.05$; antioxidant+UV-B vs UV-B

Luteins inhibit UV-B induced apoptosis in ARPE-19 cells

To determine whether UV-B induced cell apoptosis, caspase 3 activity was measured. From this assay, the activity of caspase 3 was represented as fluorescent value. The results showed that UV-B caused the elevation of caspase 3 activity in ARPE-19 cells aproximatlty 2 folds at 48 h after the exposure (Figure 19). Cells pre-incubated with silk lutein extract, std. lutein, trolox or its combinations before UV-B irradiation exhibited lower level of caspase 3 activity than UV-B exposed cells. These data suggest that UV-B induce cells apoptosis can be inhibited by both lutein(s) and their combinations with trolox.

Oxidative stress has been implicated in different types of cell death. High levels of ROS can disturb the redox balance and directly cause oxidative damage of nucleic acids, proteins, and lipids which can then lead to cells apoptosis. Oxidative damage can stimulate caspase-3 activity after mitochondrial membrane depolarization and cytochrome C release [189]. Several studies demonstrated that UV-A and UV-B could induce apoptosis of the RPE cells [190, 191, 192, 193]. In the *in vivo* model, xanthophyll has been shown to protect quail photoreceptors by reducing light-induced photoreceptor apoptosis [194]. The anti-apoptotic effect of lutein on UV-B induced RPE cell damage might be result of its antioxidant activity to reduce cellular oxidative stress and activate signaling pathways to enhance expression of opsin genes in photoreceptor cells [23].

Regarding the possible mechanisms by which lutein inhibits UV-B induced ARPE-19 cell death, our results showed that it may act through its anti-oxidative and subsequently anti-apoptosis effect. The biologic effects of UV-B are primarily mediated by the generation of ROS resulting in redox balancing of antioxidant enzyme and stimulating of cells apoptosis. Lutein can accumulate in cells membrane and modulate antioxidant enzymes, especially catalase and glutathione peroxidase that protected cell membrane from peroxide radical, which then effectively suppress apoptotic process.

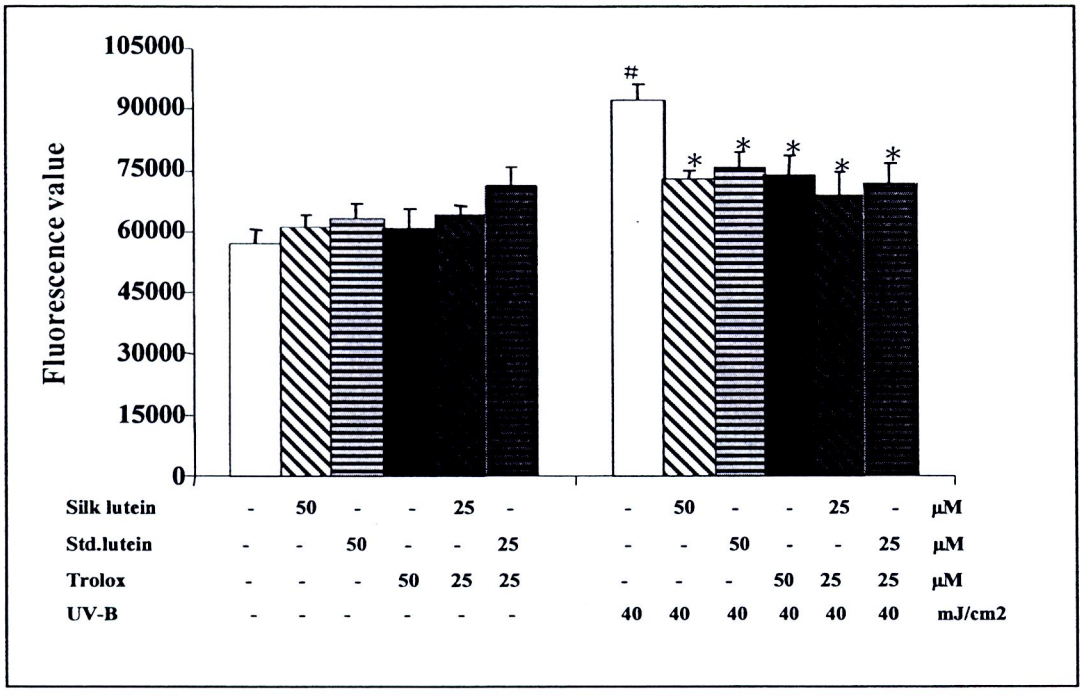

Figure 19 Effect of lutein(s) and/or trolox on caspase 3 activity in UV-B exposed ARPE-19 cells. Cells were pretreated silk lutein extract, std. lutein, or trolox, and combinations for 4 h before UV-B irradiation. Caspase 3 activity was determined 48 h after UV-B exposure. Values present mean±SEM of three independent experiments. # $p \leq 0.05$; UV-B vs control, and * $p \leq 0.05$; antioxidant+UV-B vs UV-B

Trolox reduces lutein degradation

According to the results shown in the previous section, lutein combined with trolox exhibited an effective antioxidant against UV-B irradiation. The effectiveness of this type of combination was previously reported as a role of tocopherol to prevent lutein degradation [24]. Therefore this experiment aimed to measure the level of lutein(s) when it was incubated with or without trolox in serum free medium for 4 h. HPLC was used to quantify the concentration of lutein remained in the medium. The results showed that the level of silk lutein and std. lutein were reduced approximately 25 % when they were suspended in the medium without trolox (Figure 20). When trolox was present in culture medium together with lutein(s), lutein was remained in the same level as in the beginning. This result suggests that trolox may prevent lutein(s) from degradation in the culture medium.

This finding is corresponding with previous studies showing that, trolox can stabilize lutein degradation. Some studies reported that lutein and zeaxanthin combined with tocopherol or ascorbic acid exhibited enhancing effect probably by synergistic mechanism [24, 195, 196]. The possible mechanisms of this enhance protection could be different targets as antioxidants. Trolox acts to scavenge the peroxyl radical by hydrogen atom donation, whereas lutein can prevent lipid peroxidation via singlet oxygen quenching or via scavenging peroxyl radicals [197].

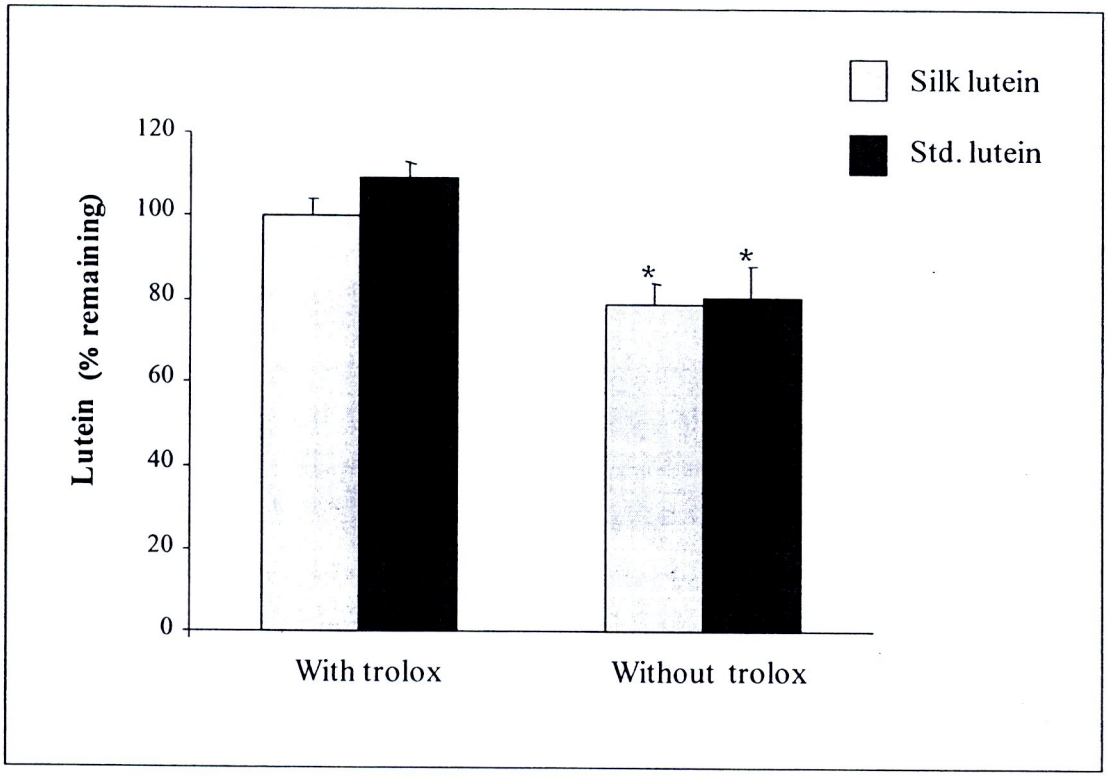

Figure 20 Level of silk lutein or std. lutein in the presence and absence of trolox. Lutein (s) was incubated with trolox at the same concentration (25 μM) in serum free culture medium for 4 h. The remaining of lutein was then analyzed by HPLC. Values present mean ± SEM of three independent experiments. * $p \leq 0.05$; with trolox vs without trolox