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Neuroprotective effects of Myricetin on Epoxiconazole-induced toxicity in F98 cells

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Abstract

Epoxiconazole is one of the most commonly used fungicides in the world. The exposition of humans to pesticides is mainly attributed to its residue in food or occupational exposure in agricultural production. Because of its lipophilic character, Epoxiconazole can accumulate in the brain [1]. Consequently, it is urgent to explore efficient strategies to prevent or treat Epoxiconazole-related brain damages. The use of natural molecules commonly found in our diet represents a promising avenue. Flavonoids belong to a major sub-group compounds possessing powerful antioxidant activities based on their different structural and sterical properties [2]. We choose to evaluate Myricetin, a flavonoid with a wide spectrum of pharmacological effects, for its possible protective functions against Epoxiconazole-induced toxicities. The cytotoxicity induced by this fungicide was evaluated by the cell viability, cell cycle arrest, ROS generation, antioxidant enzyme activities, and Malondialdehyde production, as previously described in Hamdi et al. 2019 [3]. The apoptosis was assessed through the evaluation of the mitochondrial transmembrane potential ($\Delta\Psi_m$), caspases activation, DNA fragmentation, cytoskeleton disruption, nuclear condensation, appearance of sub-G0/G1 peak (fragmentation of the nucleus) and externalization of Phosphatidylserine. This study indicates that pre-treatment of F98 cells with Myricetin during two hours before Epoxiconazole exposure significantly increased the survival of cells, restored DNA synthesis of the S phase, abrogated the ROS generation, regulated the activities of Catalase (CAT) and Superoxide Dismutase (SOD), and reduced the MDA level. The loss of mitochondrial membrane potential, DNA fragmentation, cytoskeleton disruption, chromatin condensation, Phosphatidylserine externalization, and Caspases activation were also reduced by Myricetin. Together, these findings indicate that Myricetin is a powerful natural product able to protect cells from Epoxiconazole-induced cytotoxicity and apoptosis.

Keywords: Epoxiconazole, Myricetin, Oxidative stress, Apoptosis, DNA fragmentation, Cytoskeleton disruption.

List of Abbreviations: EPX: Epoxiconazole; MYR: Myricetin; ROS: Reactive Oxygen Species; $\Delta\Psi_m$: Mitochondrial Transmembrane Potential; IC₅₀: Inhibitory Concentration of 50% of the population; PBS: Phosphate Buffer Saline; DCFH-DA: 2, 7-Dichlorofluoresce Diacetate; FCS: Fetal Calf Serum; MTT: 3-(4, 5-Dimethylthiazol-2-yl), 2, 5-Diphenyltetrazolium Bromide; DMSO: Dimethylsulfoxide; PI: Propidium Iodide; Rh-123: Rhodamine 123; H₂O₂: Hydrogen Peroxide; MDA: Malondialdehyde; DO: Optical Density; GSH: Glutathione; CAT: Catalase; SOD: Superoxide dismutase; V:V: Volume:volume, NAC: N acetylcysteine; H₂O₂: hydrogen peroxide.

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Introduction

Although the large spectrum use of pesticides for pest control, these products represent a risk of poisoning for food and agricultural security [4-5]. In particular, Triazole fungicides have appeared as a category of pollutants widely applied in various sectors, including agricultural, industrial, and pharmaceutical sectors [6-7]. Their mode of action is the inhibition of a specific enzyme of the fungus Cytochrome P450 (CYP: lanosterol-14 α -demethylase), which is important for the synthesis of the fungal membrane [8]. Triazole fungicides are characterized by their chemical stability, their low biodegradability and also by their strong bioaccumulation [9-11]. Various effects of Triazole fungicides have been demonstrated in diverse toxicological studies; these chemicals have been shown to be hepatotoxic, tumorigenic, teratogenic, endocrine disruptors, genotoxic, and neurotoxic. In particular, Triazole fungicides exposure affects the mammalian nervous system, inducing neurobehavioral deficits and neuropathology [12]. One neurotoxic effect of these compounds is the alteration of the dopaminergic neurotransmission, via the inhibition of depolarization-evoked Ca²⁺influx [13].

Epoxiconazole (EPX) is a widely used synthetic fungicide, which aims to stop the biosynthesis of the steroid ergosterol, an essential component of the fungal membrane, by competitively inhibiting the enzyme lanosterol 14 α -demethylase [14]. It belongs to pesticides having “risky” properties: very high bioaccumulation potential with log KOW > 3, strong adsorption in soil with Koc 68 > 500, and long persistency with DT50 > 30 [15]. Combining its properties with increased levels of usage explains the long-term residues of EPX in soils of Europe [16-18]. In addition, it is highly persistent in soil (half-life in soil of over 1500 days) [19-20]. The analysis of soil 7 days after EPX application showed that the residual concentrations of this pesticide were about 0.11-0.35 mg/kg [21]. Following repeated applications, this pollutant enters freshwater ecosystems. Otherwise, EPX has been detected in aquatic environments and wastewaters [22-24], as well as in agricultural topsoils [25]. Previous studies have shown that the potential target of Triazole fungicides is the mitochondrion. Mitochondrial dysfunction would lead to energy depletion, modulation of autophagy, followed by programmed cell death [26]. In line with these findings, we recently showed that EPX is particularly toxic for cells of the nervous system, like F98 cells, where it produces cell cycle arrest, cytoskeleton disruption, and DNA damage. It also alters mitochondrial function and provokes apoptosis via caspases dependent signaling pathway. Finally, EPX induces ROS generation and lipid peroxidation [3]. Such oxidative stress is a

major cause of neurotoxicant-associated cellular damage, and ROS production during oxidative stress has been reported to initiate signaling cascades leading to apoptosis in Parkinson's disease [27].

We used the F98 cells due to their biological resemblance with primary astrocytes and their broad use as an astrocyte cell model and also because of their large size, which allows clear-cut microscopic evaluation of the effects of EPX on organelles, in particular the cytoskeleton and mitochondria. Moreover, because of their strong and well-characterized proliferative properties, it is a convenient model for precise evaluation of the cell cycle and survival. Flavonoids are ubiquitous in nature and abundant in food, providing an essential source for the prevention of chronic diseases, including cancer [28]. In particular, Myricetin (MYR) is a Flavonoid compound enriched in fruits and vegetables, with antioxidants [29], anti-tumor [30-34], free-radical scavenging [35-36], and many other pharmacological activities. Also, it has been reported that MYR can easily pass through the blood-brain barrier [37].

In this study, we investigated the possible antioxidant effects of MYR toward EPX-induced oxidative stress on F98 cells [3]. In particular, we showed its capacity to alleviate most, if not all, the known EPX-induced cytotoxicity and apoptosis on this neural cell model.

2. Materials and methods

2.1. Chemicals

Epoxiconazole, Myricetin, Pyrogallol, N acetylcysteine and Hydrogen peroxide were purchased from Sigma - Aldrich (St. Louis, MO, USA). 3-4,5-Dimethylthiazol-2-yl, 2,5-Diphenyltetrazolium Bromide (MTT), Cell Culture Medium (DMEM), Fetal Calf Serum (FCS), Phosphate Buffer Saline (PBS), Trypsin-EDTA, Penicillin and Streptomycin mixture and L-Glutamine (200 mM) were bought from GIBCO-BCL (UK). 2,7-Dichlorofluoresce Diacetate (DCFH-DA) was supplied by Molecular Probes (CergyPontoise, France). The Mito-tracker Red CMXROS dyes were purchased from Invitrogen. All other chemicals used were of analytical grade.

2.2. Cell culture and treatment

Rat F98 cells were cultured in DMEM, supplemented with 10 % FBS, 1 % L-Glutamine (200 mM), 1 % of mixture Penicillin (100 IU/ml), and Streptomycin (100 µg/ml), at 37 °C with 5 % CO₂ and 95 % O₂ as previously described [3]. The media was replaced by new medium on alternate days and pre-treated with increasing concentrations of MYR (10, 20 and 50 µM) during 2 hours before treatment with different concentrations of EPX dissolved in water and DMSO (1:4, v:v). A negative control containing cells treated with vehicle (DMSO) was also evaluated. The final DMSO concentration in the treated and control cultures does not exceed 0.1%. Positive controls treated with H₂O₂ (hydrogen peroxide at 20 µM) and NAC (N acetylcysteine at 1mM) were added. Positive controls containing cells treated only with increasing concentrations of MYR (10, 20 and 50 µM) was also presented in Fig. 2.

2.3. Cell viability assay (MTT assay)

Cell viability was quantitatively determined by 3-4,5-Dimethylthiazol-2-yl, 2,5-Diphenyltetrazolium Bromide (MTT) assay [38]. F98 cells were grown in 96-well plates and incubated at 37 °C for 24 h with EPX (50 µM, corresponding to the IC₅₀) and with increasing concentrations of MYR (10, 20 and 50 µM) separately or with the combination of them (pre-treatment with MYR two hours before adding EPX). The results were expressed as the percentage of MTT reduction relative to the absorbance measured from negative control cells.

2.4. Cell cycle analysis

The F98 cells were grown in 6-well plates and treated with EPX (50 μ M) alone or with MYR (10, 20, and 50 μ M) at 37 °C for 24 hours. As previously described by Hamdi et al. (2019), F98 cells were harvested, washed with cold PBS, and processed for cell cycle analysis by flow cytometry. The percentages of cell distribution in sub-G0/G1, G0/G1, S, and G2/M phases were evaluated by using WinMDI 2.9 [39].

2.5. ROS determination

The conversion of non-fluorescent 2,7-dichlorofluorescein diacetate (DCFH-DA) to fluorescent DCF was used extensively to monitor oxidation in biological systems detecting and quantifying intracellular products such as Superoxide radicals, Hydroxyl radicals, and Hydrogen peroxide [40-42]. F98 cells were grown in 96-well culture plates (Polylabo, France) and pre-treated with increasing concentrations of MYR (10, 20, and 50 μ M) during 2 hours before treatment with EPX for 24 hours at 37 °C. Then, cells were incubated with 20 μ M of cell-permeable DCFH-DA for 30 min, treated with EPX at 50 μ M and incubated for 24 hours at 37 °C. The DCF fluorescence was measured using a fluorometer (Biotek FLx800) and the amount of ROS produced was proportional to the DCF fluorescence.

2.6. Protein extraction

F98 Cells (10⁶ cells/well) were seeded for 24 h in six-well multi-dishes (Polylabo, France) and incubated at 37 °C for 24 h with EPX alone or combined to MYR (10, 20 and 50 μ M). Cells were rinsed with ice-cold PBS, scraped, collected in a lysis buffer (Hepes 0.5 M containing 0.5 % Nonidet-P40, 1 mM PMSF, 1 μ g/ml Aprotinin, 2 μ g/ml Leupeptin, pH 7.4), and incubated for 20 min in ice before centrifugation. Protein concentration was determined in cell lysates using the Protein Bio-Rad assay [43].

2.7. Measurement of SOD activity

Superoxide Dismutase (SOD) activity was determined according to the method described by Marklund and Marklund [44] by assaying the auto-oxidation and illumination of Pyrogallol at 440 nm for three minutes. One unit of SOD activity was calculated as the amount of protein that caused 50 % Pyrogallol auto-oxidation inhibition. The SOD activity is expressed as units per milligram of protein.

2.8. Measurement of Catalase (CAT)

The CAT activity was measured according to the method described by Aebi (1984) [45] assaying the hydrolysis of H₂O₂ and the resulting decrease of absorbance at 240 nm over a 3-min period at 25 °C. The activity of CAT was calculated using the molar extinction coefficient (0.04/mM/cm). The results were expressed as micromole per minute per milligram of protein.

2.9. Lipid peroxidation

For the detection of lipid peroxidation, Malondialdehyde (MDA) product was measured. F98 cells were seeded and exposed to EPX alone or combined with MYR (10, 20, and 50µM) for 24 h at 37 °C. After treatment, the test was done according to the original procedure of Ohkawa et al. (1979) [46], as described in detail by Hamdi et al. (2019). The concentration of MDA was determined according to a standard curve.

2.10. Immunocytochemistry analysis

In order to view the effect of EPX and MYR on the cytoskeleton network, immunocytochemistry assay was done. F98 cells were grown in 24-well plates containing coverslips and exposed at 37 °C for 24 h to EPX alone or combined to MYR (10, 20, and 50µM). After the treatment period, the assay was performed according to the original procedure of Balzeau et al. (2012) [47], as described in detail elsewhere [3]. Images were taken with an Olympus Confocal Microscope (BX50) after incubation of cells with anti-Alpha-Tubulin antibody or anti-mouse Vimentin antibody and revelation with an anti-mouse Alexa antibody (568 nm).

2.11. DNA damage assessed by the Comet assay

For the detection of DNA damage, F98 cells were seeded in six-well culture plates (Polylabo, France) and exposed to EPX alone or were pre-treated with MYR during 2 hours before treatment with EPX for 24 h at 37 °C. At the end of exposure period, the test was performed according to the original method of Collins et al. (1996) [48], as described in detail elsewhere [3]. One hundred cells were randomly selected and analyzed from each experimental point. The percentage of DNA in the comet tail (ie, tail intensity %) was used as a measure of the extent of DNA damage.

2.12. Annexin V-FITC/PI double staining assay

For the detection of apoptosis, F98 cells were grown in 6-well plates and treated with EPX (50 μ M) and/ or MYR at the indicated concentrations for 24h. After the treatment, F98 cells were harvested and analyzed for apoptosis by flow cytometry analysis after staining with Annexin V FITC and Propidium Iodide (PI) [49].

2.13. Mitochondrial Membrane Potential and Mito-tracker Assay

For the estimation of mitochondrial membrane potential, the cationic fluorescent dye Rhodamine-123 has been used. The F98 cells were grown in 96-well culture plates and treated with EPX alone or combined to MYR for 24 h. At the end of treatment, the assay was done according to the original method of Debbasch et al. (2001) [50], as described in detail by our previous work [3]. The results were expressed as the percentage of absorbed Rhodamine-123 fluorescence relative to the fluorescence measured from negative control cells.

F98 cells were also seeded onto Ibidi 4 well slides and cultured for 24 h. After treatment with EPX (50 μ M) and/or MYR (10, 20, and 50 μ M) for 6 hours, the cells were stained with the Mito-tracker Red CMXRos according to the manufacturer's instructions and observed by confocal microscopy.

2.14. Caspases activation

The evaluation of Caspase-3 and/or caspase-9 activity was performed using a commercially available kit, according to the manufacturer's instructions (Promega, France), and according to Rjiba-Touati et al. (2013) [51]. In the presence of active Caspase-3, cleavage and release of pNA from the substrate occur. Free pNA produces a yellow color detected by a spectrophotometer at 405 nm. A standard curve was realized in order to determine the correspondence between optical density and pNA concentration.

2.15. Statistical analysis

Data are expressed as the mean \pm standard deviation (SD) of the means. The analysis parameters were tested for homogeneity of variance and normality. The data were therefore analyzed using a one-way analysis of variance (ANOVA) with a post hoc Tukey–Kramer test to identify significance between groups and their respective controls. In all cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Measurement of cell viability and proliferation

F98 cells were exposed to increasing concentrations of EPX and MYR separately and to the combination of the two products during 24 hours, and then their viability was determined by MTT assay. The results indicate that EPX induced a dose-dependent decrease of cell viability ($p < 0.05$) (Fig. 1A) and altered the cell morphology (Fig. 1B) as previously published [3]. The IC₅₀ value determined after 24 hours of cell treatment was about 50 μ M. In this study, we used this value to investigate the possible effects of MYR.

While MYR alone exhibited no detectable toxicity towards F98 cells (Fig. 1A), pre-treatment with this Flavonoid at 10, 20, and 50 μ M during 2 hours before treatment with EPX, significantly decreased the EPX-mediated cytotoxicity (Fig. 1C) and restored normal cell morphology (Fig. 1B). Indeed, cell viability was increased from $49 \pm 0.05\%$ in the presence of EPX alone to $57.43 \pm 0.02\%$, $65.5 \pm 2.43\%$ and $75.6 \pm 0.05\%$, when cells were pre-treated with MYR at respectively 10, 20 and 50 μ M, and during two hours before EPX treatment (Fig. 1C).

Then, to examine at which level of the cell cycle MYR protects cells from the cytotoxic effect of EPX, we used flow cytometry to determine the effect of EPX alone (50 μ M) or following the pre-treatment with MYR at these concentrations (10, 20 and 50 μ M). The cell cycle analysis showed that F98 cells accumulated in the G2/M phase with IC₅₀ of EPX (50 μ M), with the inhibition of DNA synthesis at the S phase. Interestingly, the pre-treatment with MYR reduced this effect in a dose-dependent manner (Fig. 1D).

3.2. Myricetin inhibits Epoxiconazole-induced ROS generation

The toxicity of EPX has been linked to the generation of oxidative stress in F98 cells [3]. Therefore, we evaluated whether MYR exerts its proposed antioxidant properties and modulates the level of ROS induced by EPX. The level of intracellular ROS was measured by recording the fluorescence of DCF, which is the result of DCFH oxidation mainly by H₂O₂. As shown in Fig. 2A, the 50 μ M of EPX treatment induced a significant increase of ROS production to about 3.50-fold when compared to untreated cells. Positive control treated with H₂O₂ (20 μ M) also increased the ROS levels to 4.8-fold to untreated cells ($p < 0.001$). Moreover, the pre-treatment with MYR during two hours at different doses (10, 20, or 50 μ M)

before the exposure to EPX significantly reduces the intracellular ROS generated by EPX. On the other hand, in order to examine the role of ROS in the EPX-induced cell death, F98 cells were pre-treated for 2 h with the N-acetylcysteine (NAC) at 1mM before exposure to EPX for 24h. Our results showed that NAC pre-treatment strongly abolishes the ROS generation induced by EPX.

3.3. Effect of Myricetin on Epoxiconazole-induced Lipid peroxidation

As an index of lipid peroxidation, the intracellular concentration of MDA was measured in F98 cells. As shown in Fig. 2B, the EPX treatment (50 μ M) provoked a strong increase in the intracellular concentration of MDA (3.5 ± 0.08 μ mol/mg protein), versus (0.5 ± 0.06 μ mol/mg protein) for untreated cells, which indicates an important oxidative insult to cell lipids. Positive control treated with H₂O₂ (20 μ M) increased the MDA level to 5.1 ± 0.02 μ mol MDA/mg of protein ($p < 0.001$). However, pre-treatment of cells with MYR significantly reduced the level of lipid peroxidation induced by EPX. In fact, the MDA level reached 2.1 ± 0.07 , 1.5 ± 0.08 and 0.9 ± 0.04 μ mol/mg protein following MYR pre-incubation at respectively 10, 20 and 50 μ M. The pretreatment with NAC at 1mM 2 hours before EPX exposure significantly reduced the level of lipid peroxidation induced by EPX.

3.4. Myricetin modulates Antioxidant Enzyme activities following Epoxiconazole treatment

Changes in the levels of antioxidant enzyme activity represent a sensitive biomarker of the cellular response. SOD catalyzes the dismutation of the highly reactive superoxide anion from oxidative stress to H₂O₂, which can further be decomposed to water and oxygen by CAT or GPx. To characterize the possible protective effects exerted by pre-treatment with MYR, we measured changes in the activities of intracellular antioxidant enzymes (Fig. 2C-D). Treatment of F98 cells with 50 μ M EPX induced significant increases in the enzyme activities. The CAT activity increased from 5 ± 0.1 μ mol/min/mg of protein in untreated cells to 40 ± 0.6 μ mol/min/mg of protein in the presence of 50 μ M EPX (Fig. 2C), and SOD activity raised from 4 ± 0.1 USOD/min/mg of protein in control cells to 23 ± 0.5 USOD/min/mg of protein in the presence 50 μ M EPX (Fig. 2D). The treatment of F98 cells with H₂O₂ (20 μ M) (positive control) strongly increased the antioxidant enzymes activities. However, the two hours pre-treatment with MYR (10, 20 and 50 μ M) before adding EPX

significantly reduced these increases in a dose-dependent manner (Fig. 2C-D). Indeed, the pretreatment with NAC significantly decreased the activity of these antioxidant enzymes.

3.5. Myricetin reduces the Epoxiconazole-induced Cytoskeleton disruption and DNA fragmentation

We next evaluated the ability of MYR to modulate the EPX induced DNA fragmentation and cytoskeleton disruption, two apoptotic hallmarks. DNA damages were analyzed using the alkaline Comet assay. The high sensitivity of the Comet assay allows measurement of DNA fragmentation in individual cells. As shown in Fig. 3A, induced 250 ± 4.2 of total score of DNA damage at concentration of IC₅₀ (50 μ M), as compared to 30 ± 4.48 of the score in control cells (untreated cells). The addition of MYR significantly decreased the DNA fragmentation induced by EPX that reached 90.1 ± 6.2 for 50 μ M of MYR (Fig. 3A).

The effects of the EPX in the presence or absence of MYR on the different cytoskeleton structures in F98 cells are shown in Fig.4. In the control group, the cells exhibited a normal and characteristic network of microtubules (MTs) (Fig. 4A), and intermediate filaments (IFs) (Fig. 4B). On the opposite, in the cells treated with 50 μ M EPX, the cell boundaries were unclear, and the networks of microtubules and intermediate filaments were strongly altered or even lost in multiple cells. Interestingly, the pre-treatment with MYR at different concentrations (10, 20, and 50 μ M) protected the cytoskeleton from a disruption in a dose-dependent manner (Fig. 4A & 4B). Together, these data demonstrated that MYR protects cells from the EPX-induced DNA fragmentation and cytoskeleton disruptions.

3.6. Effect of Myricetin on Epoxiconazole-induced Apoptosis

We have previously reported that EPX induces cell death by activating the intrinsic apoptotic pathway (Hamdi et al., 2019). We thus assessed the possible effect of MYR on the mitochondrial alterations induced by EPX. To this end, cells were stained with Rhodamine 123 (Rh123) to measure the mitochondrial transmembrane potential ($\Delta\Psi$ m).

As shown in Fig. 3F, the EPX treatment reduced the Rh-123 uptake to $49 \pm 1.1\%$, indicating a loss of mitochondrial potential. However, the pre-treatment of cells by MYR restored the percentage of Rh123 uptake in a dose-dependent manner: $62 \pm 2.1\%$, $70 \pm 2.3\%$ and $82 \pm 1.2\%$ for respectively MYR at 10, 20 and 50 μ M. These results indicate that MYR reduces the mitochondrial alterations induced by EPX.

To visualize the structural abnormalities of mitochondria following EPX and/or MYR exposure, we performed a detailed confocal microscopic analysis using a cell-permeable mitochondria selective dye MitoTracker CMXRos Red, which passively diffuses across the plasma cell membrane and accumulates in active mitochondria of F98 cells. Mitochondria from control cells appear as long, thread-like structures that were well linked and randomly distributed throughout the cells. Disruption of the tubular mitochondria network and formation of punctuating staining were observed in EPX-treated F98 cells, indicating fragmentation of mitochondria due to oxidative stress (Fig. 4D).

On the contrary, no fragmentation of mitochondria was observed in EPX+ MYR-treated cells; in fact, MYR-treated cells maintained the normal tubular morphology (Fig. 4D). These results indicate that MYR prevents mitochondria damage induced by oxidative stress in F98 cells.

Besides, the double staining of cells with FITC-labeled-Annexin V and PI allowed to confirm by flow cytometry that EPX induced apoptosis. Compared to the control values, the 50 μ M EPX treatment increased the percentage of early and late apoptotic cells. The rate of apoptosis is the sum of early and late apoptotic cells. We have added in Fig. S2 representative cytograms with quadrant analysis of the time course of EPX treatment to show that late apoptotic cells actually derive from early ones. Nevertheless, when cells were pre-treated with MYR for two hours before treatment with EPX, it significantly reduced the rate of apoptotic cells (Fig. 3E).

When the cells prepare to die with programmed cell death, several morphological changes are produced, like a condensation of chromatin into sharply delineated masses and DNA fragmentation, followed by degradation of nuclei into discrete particles [52-53]. DAPI staining and flow cytometric DNA analysis of sub-G0/G1 peak showed that EPX treatment at 50 μ M for 24 h induced the condensation of chromatin and the fragmentation of nuclei respectively. However, the pre-treatment with MYR at different doses abolished these effects (Fig. 4C, Fig. S1 and Fig. S3).

In order to confirm the possible implication of caspases in the process of apoptotic death, we added the caspase inhibitor Z-vad-fmk (50 μ M) during 2 h before treating F98 cells by EPX. Such a pretreatment with Z-vad-fmk induced a significant inhibition of cell mortality ($P < 0.05$). On the other hand, pretreatment with MYR before adding Z-vad-fmk for 2 hours strongly induced an increase of cell viability.

As illustrated in Fig. 3B, a significant ($p < 0.05$) increase in Caspase-3 activity was observed in F98 cells treated with EPX alone as compared to untreated cells (3 ± 0.1 pmolpNA/h/mg of protein vs. 19 ± 0.5 pmolpNA/h/mg of protein). This activity decreased significantly in the presence of MYR pre-treatments: respectively 14 ± 0.04 ; 10.1 ± 0.01 and 8.3 ± 0.02 pmolpNA/h/mg of protein at 10, 20, and 50 μ M.

Apoptosis can be triggered either by an external death receptor stimulus (extrinsic) or through internal (intrinsic) mitochondria-mediated signaling. In order to confirm the intrinsic apoptotic pathway induced by EPX, specific inhibitor of caspase-9 Z-LEHD-FMK (20 μ M) was added during 2 h before treating F98 cells by EPX. The pretreatment with Z-LEHD-FMK induced a significant inhibition of apoptosis rate ($P < 0.05$). However, pretreatment with MYR before adding Z-LEHD-FMK for 2 hours strongly induced a decrease of apoptosis rate.

As presented in Fig. 3D, a significant ($p < 0.05$) increase in Caspase 9 activity was observed in F98 cells treated with EPX alone as compared to untreated cells (5.6 ± 0.03 pmolpNA/h/mg of protein vs. 0.9 ± 0.05 pmolpNA/h/mg of protein). This activity decreased significantly in the presence of MYR pre-treatments: respectively 4.8 ± 0.03 ; 3.3 ± 0.02 and 2.6 ± 0.05 pmolpNA/h/mg of protein at 10, 20, and 50 μ M.

4. Discussion

Due to their very high use in agriculture, pesticides are widely disseminated in the environment. Increased exposure to pesticide residues poses a serious risk to human health. The great interest of the scientific community is mainly focused on Triazole fungicides, one of the most economical molecules from the fungicide family [54]. Amongst these products, Epoxiconazole is a particularly persistent pollutant with harmful effects on both animals and humans [55]. Increasing evidence indicates that EPX accumulates in the nervous system and induces neurodegeneration [1]. Previous studies showed that the exposure to pesticides induces an imbalance in the redox status within the cells followed by their programmed cell death [3]. As Epoxiconazole is a liposoluble compound, it can easily cross the blood-brain barrier, which makes it frequently exposed to repeated doses of EPX by drinking and ingesting contaminated water and food like fishes [56]. Besides, the acceptable daily intake (ADI) (is an estimate of the amount of a substance in food or drinking water that can be consumed daily over a lifetime without presenting an appreciable risk to health) of EPX was estimated by the EFSA (2008) to 8µg/kg [57]. On the other hand, the determined IC50 (15 µg/ml) on F98 cells seems to be not much higher (1.8 fold) than the daily intake of EPX. Moreover, neurodegenerative disorders are associated with high morbidity and mortality, and few effective options are available for their treatment [58]. Therefore, many studies have been conducted focusing on natural compounds present in food as important molecules against neurodegenerative diseases such as Parkinson's disease [59-62]. Natural polyphenols exert numerous biological activities, like antioxidant, anti-inflammatory, antiviral, antibacterial, antiproliferative, and anticarcinogenic capacities [63], as well as cellular redox state modulation activities through direct action on enzymes, proteins and receptors [64-65]. Myricetin is one of the key constituents of various human foods and beverages including vegetables, teas and fruits, and is recognised mainly for its iron-chelating, anti-oxidant, anti-inflammatory and anticancer properties. It displays several activities that are related to the central nervous system and numerous studies have suggested that these compounds may be beneficial to protect against diseases such as Parkinson's and Alzheimer's [66]. Glial cells represent the first target of oxidative stress in the nervous system, and various natural antioxidants have been described to prevent functional damage of these cells by oxidative stress [67]. Accumulating data proved the strong antioxidant properties of Myricetin (MYR) in various cell models, but it has not yet been evaluated in glial cells. Our study investigates the protective effects and mechanisms of MYR in the prevention of ROS generation and programmed cell death induced by EPX using F98 cell model. As previously shown, we used

F98 cells because of their large size necessary to elucidate more precisely the effects of EPX on organelles, in particular the cytoskeleton and mitochondria. Moreover, their high capacity for proliferation allows the careful characterization of their different cellular pathways. The ability to sense oxidative and proteotoxic insults and to coordinate defensive stress response are basic elements for cellular adaptation and survival [68-69]. In this regard, plant polyphenols are produced by plants to defend themselves against pests such as bacteria, fungi, and insects, to which they are toxic. One possible mode of action of natural polyphenols is the hormesis effect [70]. Consistently, hormesis dose response is characterized by low dose stimulation and a high dose inhibition. The biphasic dose-response phenomenon is characterized by a U-shaped or inverse dose response curve, depending on the different measured endpoints [71-73]. The biological processes underlying hormetic dose-response, recently focused attention in the field of neuroprotection, which was mainly elucidated through the exploitation of bioactive polyphenols with the result of activating various cell defense systems aimed at controlling the redox environment, the proteostatic and metabolic homeostasis, organelle turnover, the inflammatory response, and others, thus making cells more resistant to subsequent toxic stimuli, particularly in aged people [74-75]. The polyphenolic compounds such as flavonoids have been commonly used in the human diet. They are known to restore DNA damage and repair, cell cycle, and apoptosis-related multiple cellular signaling pathways [2]. The protective effects of MYR, a potent antioxidant Flavonol, have been studied in several studies against ROS production-induced injuries [76]. Several studies showed the neuroprotective effects of MYR. It has been shown that MYR protects against rotenone-induced cytotoxicity in SH-SY5Y cells [77], MYR also inhibits the formation and extension of β -amyloid protein for the prevention of Alzheimer's disease [78], and the 6-hydroxydopamine which induce neurotoxicity in rats [79]. Moreover, previous findings support that MYR induces an anti-apoptotic activity as described by Shimmyo et al. (2008) [80] who demonstrated that MYR alleviated beta-amyloid (A β)-induced apoptosis in Alzheimer's Disease (AD) brain of the rat by the inhibition of chromatin condensation and caspases-3 activity. To our knowledge, these protective effects of MYR are attributed to its antioxidant properties and the protective effect against mitochondrial failure [81].

In this study, the pre-treatment of MYR increased in a dose-dependent manner the viability and the proliferation of EPX-treated F98 glial cells concomitant with a proportional decline in their reactive oxygen species (ROS) generation. These results are in line with the study of Molina-Jimenez. (2003), demonstrating that MYR suppressed the production of ROS [82]. In

addition, we also found that MYR pre-treatment inhibits EPX-induced lipid peroxidation and DNA damage, and decreases cytoskeleton disruption in F98 cells. Our results are in accordance with the finding of Wang and al. (2010) which proved the efficiency of MYR to prevent H₂O₂-induced DNA damage and lipid peroxidation in Chinese Hamster Lung Fibroblasts (V79-4) cells [83]. During the development of oxidative stress, the organism will trigger enzymatic/nonenzymatic antioxidant mechanisms to protect the cells against oxidative damage. SOD and CAT are the first line of defense against oxidative damage [84]. MYR significantly decreased the activities of these enzymes. Novel data presented the involvement of Nrf2, a vital defender of the anti-oxidative regulation system in MYR-mediated neuroprotection and function recovery [85]. Emerging research has recently focused on biological relevance for cell protection in many degenerative diseases and for neuroprotection in several neurodegenerative disorders, particularly Alzheimer disease and Parkinson disease, of the redox homeostasis elicited by plant polyphenols through the activation of vitagene signaling pathways. The latter involves redox sensitive genes such as the Hsp70, heme-oxygenase-1 (HO-1), thioredoxin/thioredoxin reductase, and sirtuins system. Gene expression of these cytoprotective proteins is coordinately regulated by a common molecular mechanism that involves the Nrf-2 Kelch-like ECH-associated protein 1 (KEAP1)-dependent signal pathways. An essential feature of the stress response initiated by electrophilic species is the nuclear localization of the transcription factor Nrf2 (NF-E2 related factor 2) and the subsequent binding of Nrf2 to a DNA recognition sequence known as antioxidant response element (ARE) or electrophile response element (EpRE), defined phase 2 response. Nrf2 is a key transcription effector for the activation of wide range of cytoprotective genes (>500). The Nrf2 activity induces a mild stress response, providing a healthy physiological steady state and extending lifespan in different cells and animal models. Chemicals that stimulate the nuclear accumulation of Nrf2, thereby enhancing the expression of phase 2 detoxification enzymes. Under basal conditions the pathway operates at low levels due to the repressor function of the cytosolic protein Keap1, which binds to the E3 ubiquitin ligase Cullin3-RING box1 and presents Nrf2 for ubiquitination and subsequent proteosomal degradation. Indeed, there is now compelling experimental evidence that exogenous and endogenous inducers chemically modify specific and highly reactive cysteine residues of Keap1, which, in addition to being a repressor for Nrf2, also functions as the intracellular sensor for inducers. This reaction leads to conformational changes in Keap1 that abrogate its ability to repress Nrf2, ultimately resulting in Nrf2 stabilization, binding to the ARE and recruitment, the basal transcriptional machinery to activate transcription of cytoprotective genes. On the other hand,

a chronic long-term Nrf2 stimulation may lead to pathophysiological events, therefore the Nrf2 signaling can be considered as a hormetic-like pathway. Increasing evidences show that plant polyphenols activate the phase 2 response leading to the expression of various Nrf2-dependent antioxidant vitagenes. These effects represent a powerful instrument supporting redox homeostasis under stressful conditions and support the assumptions that the helpful properties of polyphenols carry out through adaptive stress response vitagenes. Currently, increasing evidence suggests that plant polyphenols may offer beneficial effects acting in a hormetic-like manner by activating adaptive stress-response pathways and making the hormesis concept fully applicable to the field of nutrition. Moreover, it has been considered that plant polyphenols may be protective through hormetic processes that involve the stress-activated “vitagenes” [74], [86-92].

Mitochondria represent the major source and target of oxidative stress. Previous finding proved that the production of ROS had been involved in the disturbance of mitochondrial forms and functions [93]. Our results show that MYR treatment recovered the damaged mitochondria post oxidative stress, as evident by regaining the tubular filamentous mitochondrial morphology in F98 cells. Moreover, apoptosis can be categorized into two pathways, generally mitochondria-dependent intrinsic and death receptor-mediated extrinsic apoptotic signaling pathways. Although previous research has extensively implicated the importance of mitochondria in the pathogenesis of stroke, the excessive ROS generation in EPX-treated F98 cells induces the loss of mitochondrial membrane potential, which is considered to be a characteristic of the onset of the intrinsic apoptotic pathway [94-95]. Pre-treatment of MYR was also effective in inhibiting the mitochondrial-mediated apoptosis induced by EPX in F98 cells. In accordance with our findings, Wu et al., (2016) demonstrated that MYR improves mitochondrial function in the ischemic cerebrum, as indicated by the recovered mitochondrial membrane potential ($\Delta\psi$) [96]. In addition, Zou et al., (2015) revealed that MYR counteracts the mitochondrial dysfunction, which is mainly attributed to its role in the induction of mitochondrial biogenesis by the activation of AMP-activated protein kinase (AMPK) followed by the activation of the peroxisome proliferator-activated receptor gamma coactivator-1-alpha (PGC-1 α), a master regulator of mitochondrial biogenesis during acute hypoxia in rats [97]. When cells are subjected to a variety of oxidative environmental stresses, they typically respond by inducing phase II detoxifying enzymes and by stimulating mitochondrial biogenesis, two of the most important pathways for cells to react against oxidative stress. Nrf2 controls the orchestrated expression of phase II

enzymes and genes involved in oxidative defense. The increase in nuclear Nrf2 level should be the key index of the activation of the phase II detoxifying enzymes including γ -glutamyl-cysteinyl-ligase, NADPH (nicotinamide adenine dinucleotide phosphate)-quinone-oxidoreductase 1, heme-oxygenase-1, superoxide dismutase, peroxiredoxin and thioredoxin as well as other antioxidant enzymes [98-100]. Moreover, Phase II enzymes perform a variety of vital cellular functions important for protecting against oxidative damage. On the other hand, the stimulation of mitochondrial biogenesis is important as an adaptive mechanism in the cellular response to different stressors. Mitochondrial biogenesis also seems to be a promising therapeutic target as it provides a protective mechanism in a broad spectrum of acute and chronic diseases manifested by mitochondrial dysfunction [101]. This may be achieved by activation of the key factor promoting mitochondrial biogenesis, peroxisome proliferator-activated receptor coactivator 1 alpha (PPARGC1 α) [26]. PPARGC1 α is a co-transcriptional regulation factor that regulates the process of mitochondrial biogenesis by interacting with many transcription factors/proteins such as nuclear respiratory factors (NRF-1 and NRF-2), mitochondrial transcription factor A (Tfam), uncoupling proteins (UCP2) [102-104]. NRF-1, NRF-2, and Tfam regulate the transcription of genes encoding respiratory complex subunits and mtDNA synthesis [105].

Furthermore, Caspase-3 is considered the key effector of Caspases, involved in both intrinsic and extrinsic apoptotic pathways that will be activated in response to cytotoxic drugs [106]. The exposure to EPX induced a significant increase in the activity of Caspase-3 of F98 cells. Nevertheless, pre-treatment with MYR alleviates the Caspase-3 induction. The protective action of MYR may be due to its direct inhibition of the active site of Caspase-3 and thus reducing directly Caspase-3 activity [80]. Indeed, Caspase-9 is a key player in the intrinsic or mitochondrial pathway. The EPX treatment significantly increased the activity of Caspase-9 of F98 cells. However, pre-treatment with MYR strongly reduced this increase.

Our results suggest that the underlying mechanism of the protective action of MYR against EPX-induced toxicity probably operates through the free radical scavenging activity of MYR. The ability of MYR to boost up the cellular antioxidant defense system can be explained by the following mechanisms: one possible underlying mechanism may involve its catechol-like structure since it is known that catechol-containing polyphenols are potent radical scavengers and chelators of ferric ion [107-108]. In addition, the antioxidant activity of MYR could also operate through oxidation of the three hydroxyl groups (3',4',5'-position) attached in its B-ring [109].

In conclusion, our findings showed for the first time that MYR, the common antioxidant food component, protects F98 cells from EPX by inhibiting oxidative stress upstream event, which then leads to DNA damage, cytoskeleton disruption, and apoptotic cell death. These results indicate that the use of MYR as a dietary supplement might be helpful to prevent EPX-related neurotoxicity.

562 **Conflict of interest**

563 The authors declare that there is no conflict of interest.

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568 5. References

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- 900

6. Figure legends

Fig.1. Cytotoxic effect of Epoxiconazole on F98 cells and the protective effect of the two hours Myricetin pre-treatment.

(A) Cells were treated with EPX and MYR separately at the indicated concentrations during 24 h. Cell viability was determined using the MTT assay and expressed as percentages of viability. Values are significantly different ($p < 0.05$) from control ($n = 5$).

(B) Cells were pretreated for 2 h with MYR before adding EPX (50 μ M). Cell morphology was visualized microscopically and then photographed using IncuCyte® Live-Cell Analysis.

(C) Cells were treated with EPX (50 μ M) and MYR (10, 20, 50 μ M). Data are expressed as the mean \pm SD of three separate experiments. $^{##}P < 0.01$ vs. control $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. EPX alone.

(D) Effects of MYR on EPX-induced cell cycle arrest. F98 cells were pre-treated during 2 hours with MYR (10, 20 and 50 μ M) before the EPX treatment for 24 h (50 μ M). The cell cycle arrest was analyzed by flow cytometric analysis. Percentages of cells in different phases of the Cell Cycle were measured and expressed as the Mean \pm SD of three separate experiments. $^{##}P < 0.01$ and $^{###}P < 0.001$ vs. control, $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. EPX alone. The triangle indicates the increasing concentrations of MYR (10, 20 and 50 μ M).

Fig.2. Protective effects of Myricetin on the Epoxiconazole-induced oxidative stress.

(A) Cells were pre-treated with MYR (10, 20 and 50 μ M) for 2 h before EPX treatment for 24 h (50 μ M). The relative intracellular ROS production was evaluated by recording the fluorescence of DCF, the product of DCFH oxidation mainly by H_2O_2 . H_2O_2 , at 20 μ M and NAC at 1mM, were used as positive controls. Data are expressed as the mean \pm SD of three separate experiments. $^{##}P < 0.01$, $^{###}P < 0.001$ vs. control, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. EPX alone.

(B) Effects of MYR on EPX-induced lipid peroxidation. Cells were pre-treated with MYR (10, 20 and 50 μ M) for 2 h before EPX treatment for 24h (50 μ M). The peroxidation of lipids was recorded by measuring the accumulation of MDA. H_2O_2 , at 20 μ M and NAC at 1mM, were used as positive controls. Data are expressed as the mean \pm SD of three separate experiments. $^{###}P < 0.001$ vs. control, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. EPX alone.

(C and D) Effects of MYR on catalase and superoxide dismutase activities. Cells were pre-treated with MYR (10, 20 and 50 μ M) for 2 h before EPX treatment for 24 h (50 μ M). H_2O_2 , at 20 μ M and NAC at 1mM, were used as positive controls. Data are expressed as the mean \pm SD of three separate experiments. $^{###}P < 0.001$ vs. control, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. EPX alone.

Fig.3. Effects of Myricetin on the Epoxiconazole-induced DNA damage, caspases activation, mitochondrial trans-membrane potential ($\Delta\Psi_m$) and externalization of Phosphatidylserine.

(A) Cells were pre-treated with MYR (10, 20 and 50 μ M) for 2 h before EPX treatment for 24 h (50 μ M). Data are expressed as the mean \pm SD of three separate experiments. $^{###}P < 0.01$ vs. control, $^{**}P < 0.01$ vs. EPX alone.

(B) Effects of MYR on EPX-induced caspase-3 activation. Data are expressed as the mean \pm SD of three separate experiments. $^{###}P < 0.001$ vs. control, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. EPX alone.

(C) The effect of Z-VAD-FMK pretreatment (2 h, 50 μ M) on EPX-induced cytotoxicity. Data are expressed as the mean \pm SD of three independent experiments. ($^{*}P < 0.05$) values are significantly different from Z-vad-fmk pretreated cells.

(D) Effects of MYR on EPX-induced caspase-9 activation. Data are expressed as the Mean \pm SD of three separate experiments. $^{###}P < 0.001$ vs. control, $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. EPX alone.

(E) Different subsets of cells were measured by AnnexinV/PI staining after treatment with EPX (50 μ M) and/or MYR (10, 20 and 50 μ M). The rate of apoptosis is the sum of the early and late apoptosis. Data are expressed as the mean \pm SD of three separate experiments. $^{###}P < 0.001$ vs. control, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. EPX alone. ($^{***}P < 0.01$) values are significantly different from Z-LEHD-FMK pretreated cells.

(F) Effects of MYR on EPX-induced loss of mitochondrial trans-membrane potential ($\Delta\Psi_m$). F98 cells were pre-treated with MYR (10, 20 and 50 μ M) for 2 h before EPX treatment for 24 h (50 μ M). The mitochondrial potential was assessed by measuring the uptake of Rhodamine-123. Data are expressed as the mean \pm SD of three separate experiments. $^{##}P < 0.01$ vs. control, $^{*}P < 0.05$ vs. EPX alone.

Fig.4. Effects of Epoxiconazole and Myricetin on the cytoskeleton, chromatin and mitochondria of rat F98 cells.

Microtubules (A) and intermediate filaments (B) were detected using respectively an anti-Tubulin and an anti-Vimentin antibody. Cells were examined with a confocal microscope. Cells treated with 50 μ M EPX lack a normal cytoskeleton network and typically adopt a spherical shape, while the pre-treatment with MYR strongly reduced this effect.

Fig.5. Graphical abstract

A proposed mechanism of pretreatment with Myricetin in alleviating Epoxiconazole-induced neurotoxicity. The protective effect is mainly attributed to its ability to reduce intracellular

969 reactive oxygen levels, modulating the antioxidant enzymes activity, reducing
970 malondialdehyde level, stabilizing the cytoskeleton organization and mitochondrial membrane
971 potential Finally, Myricetin pretreatment prevented the increase of DNA
972 fragmentation,caspases-3 and 9 activities and DNA condensation.

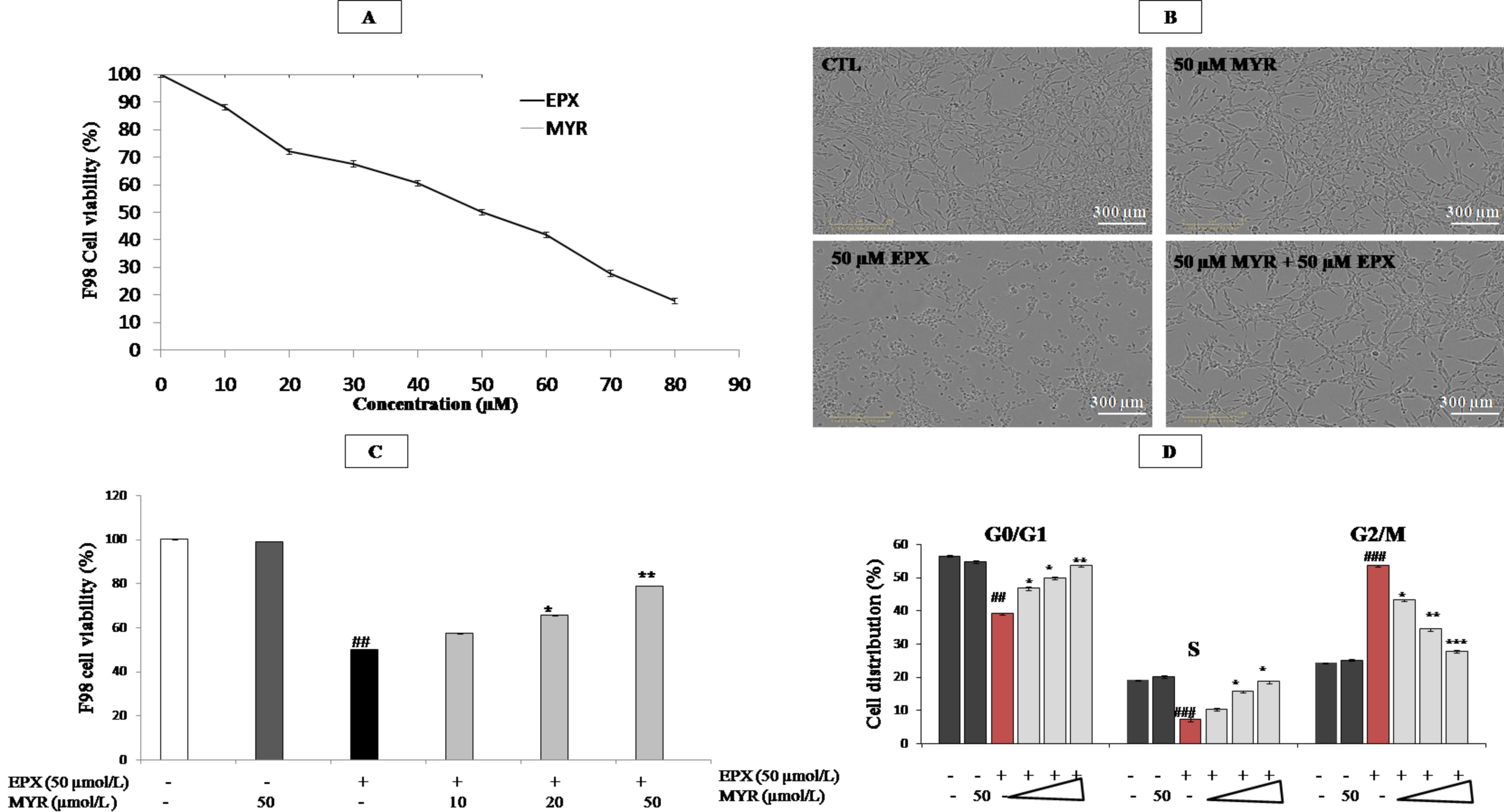


Fig. 1

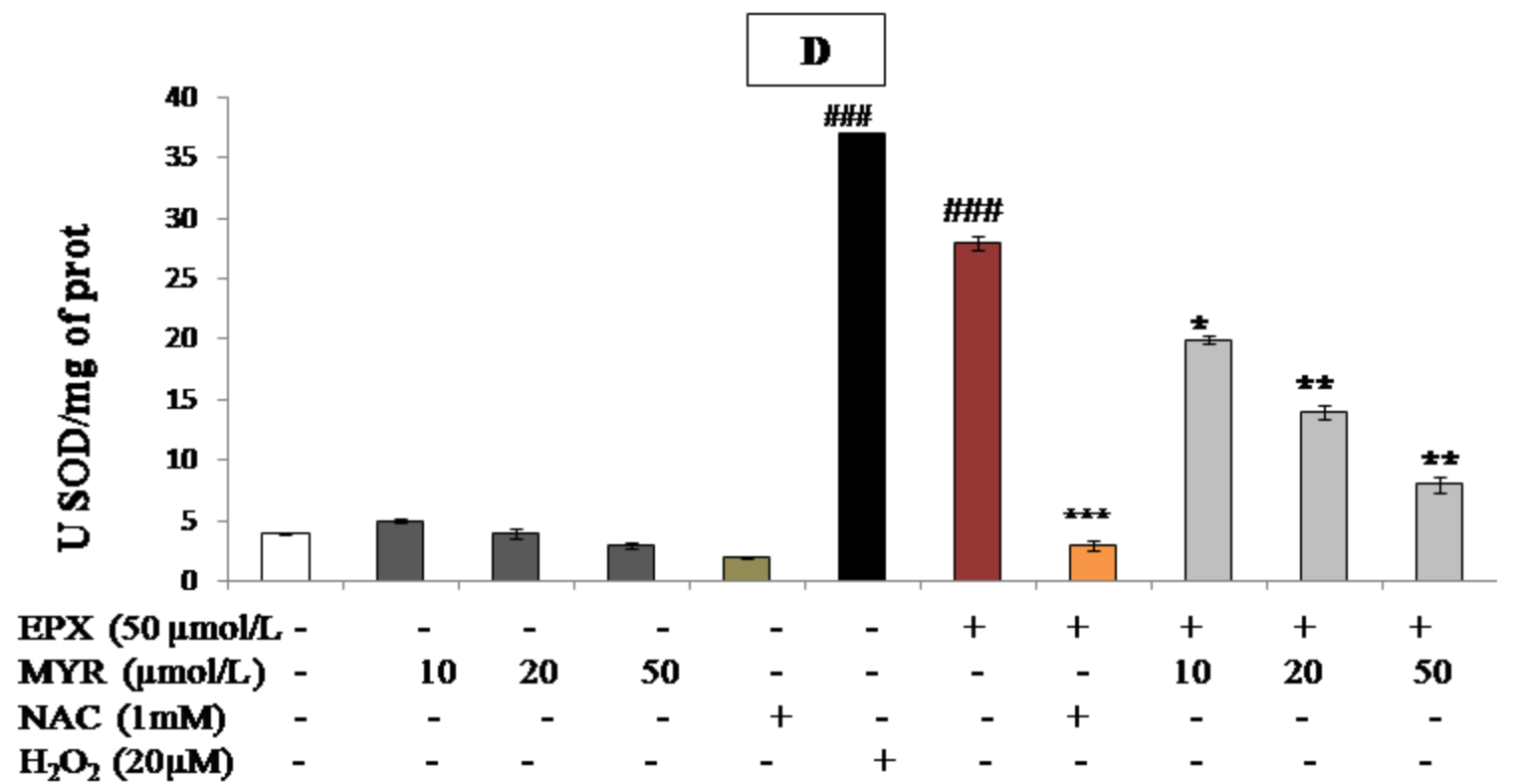
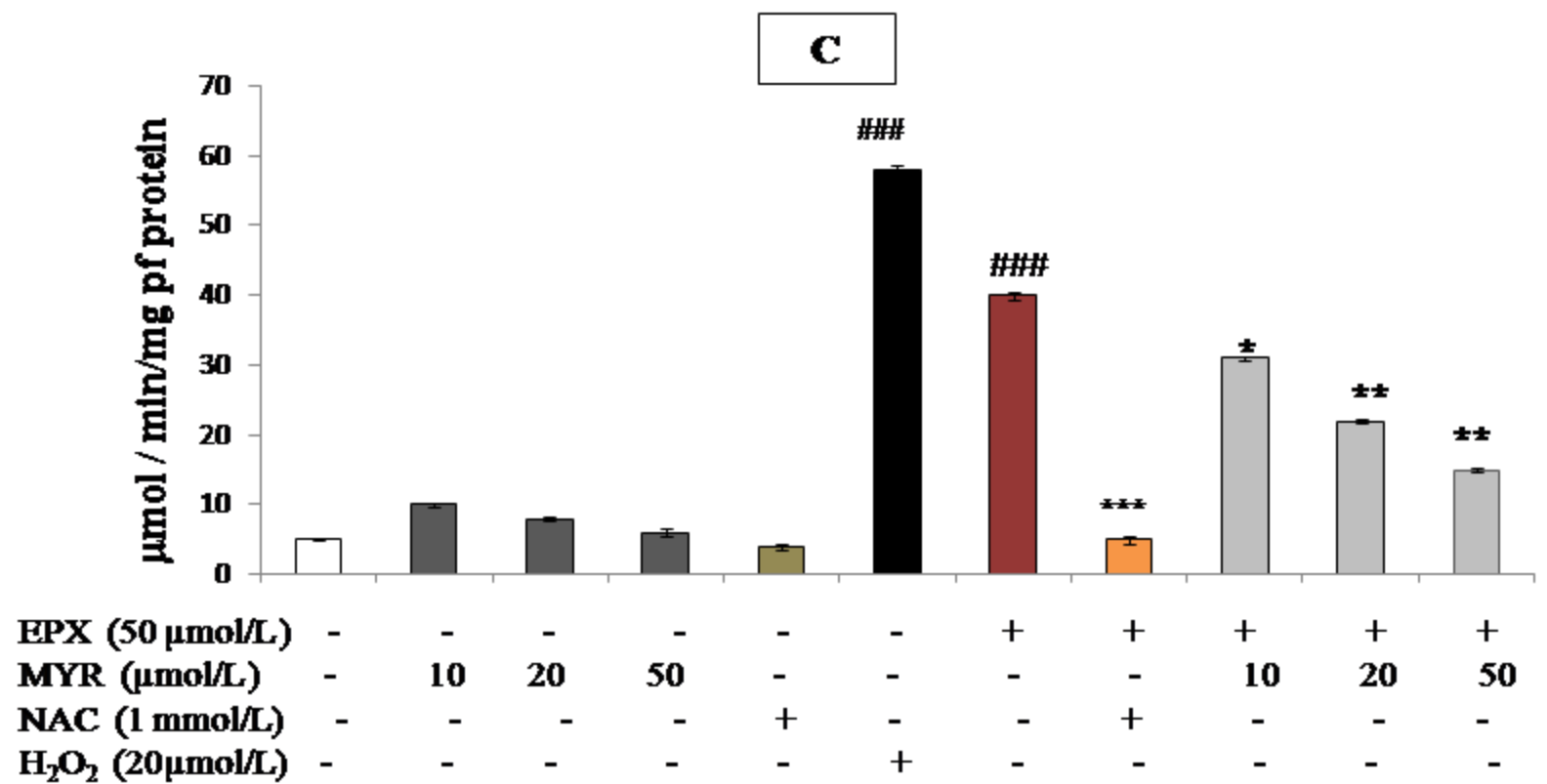
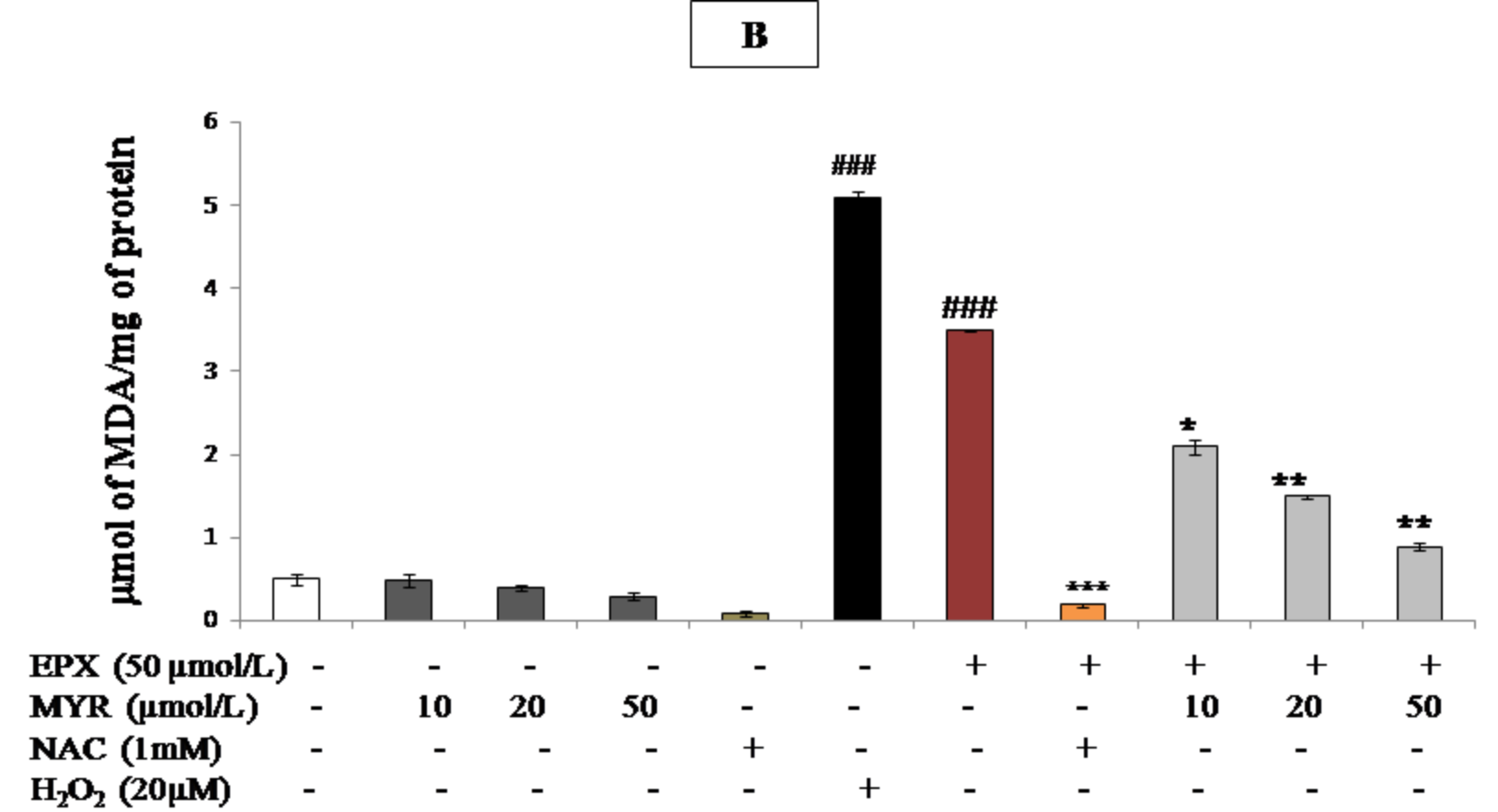
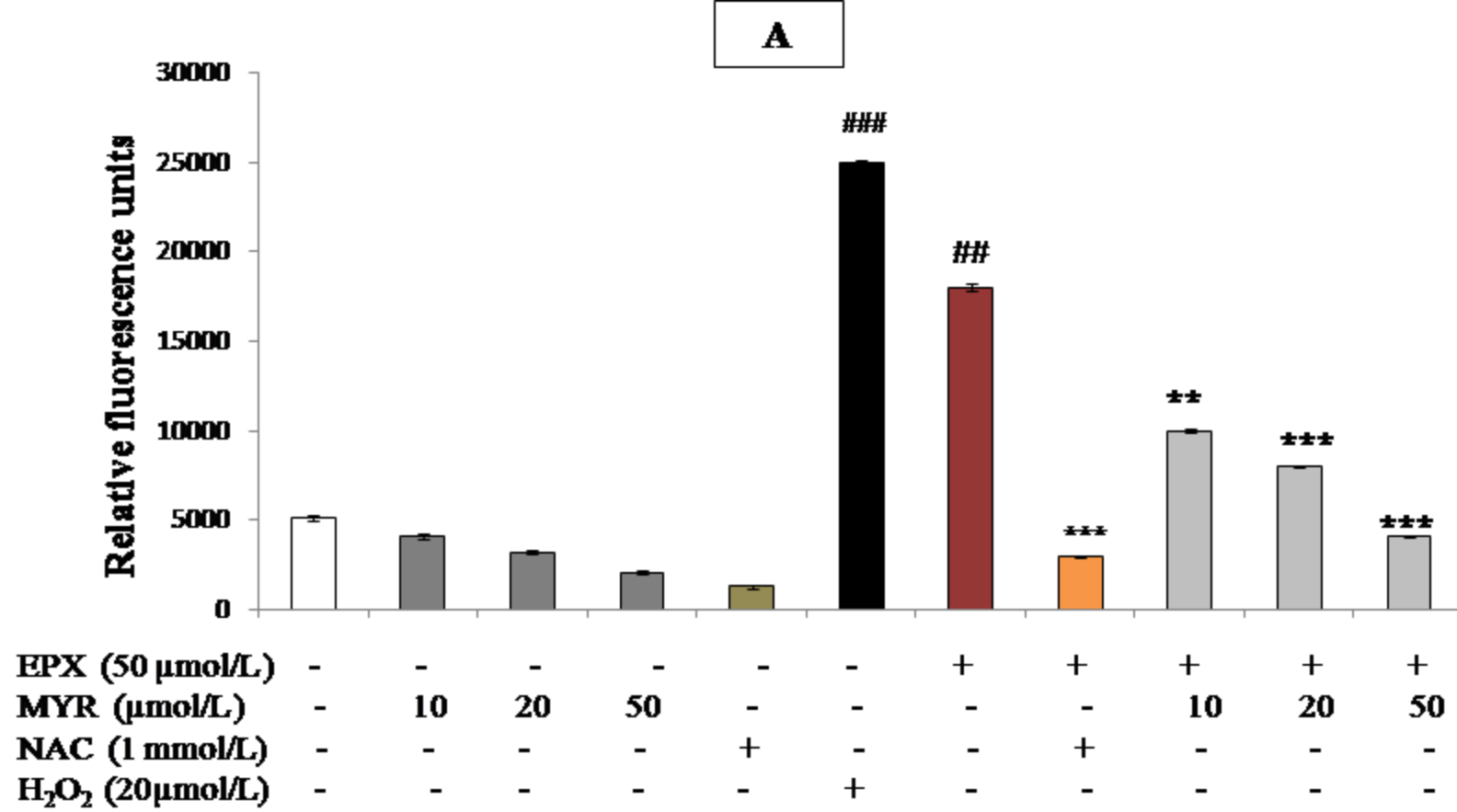


Fig. 2

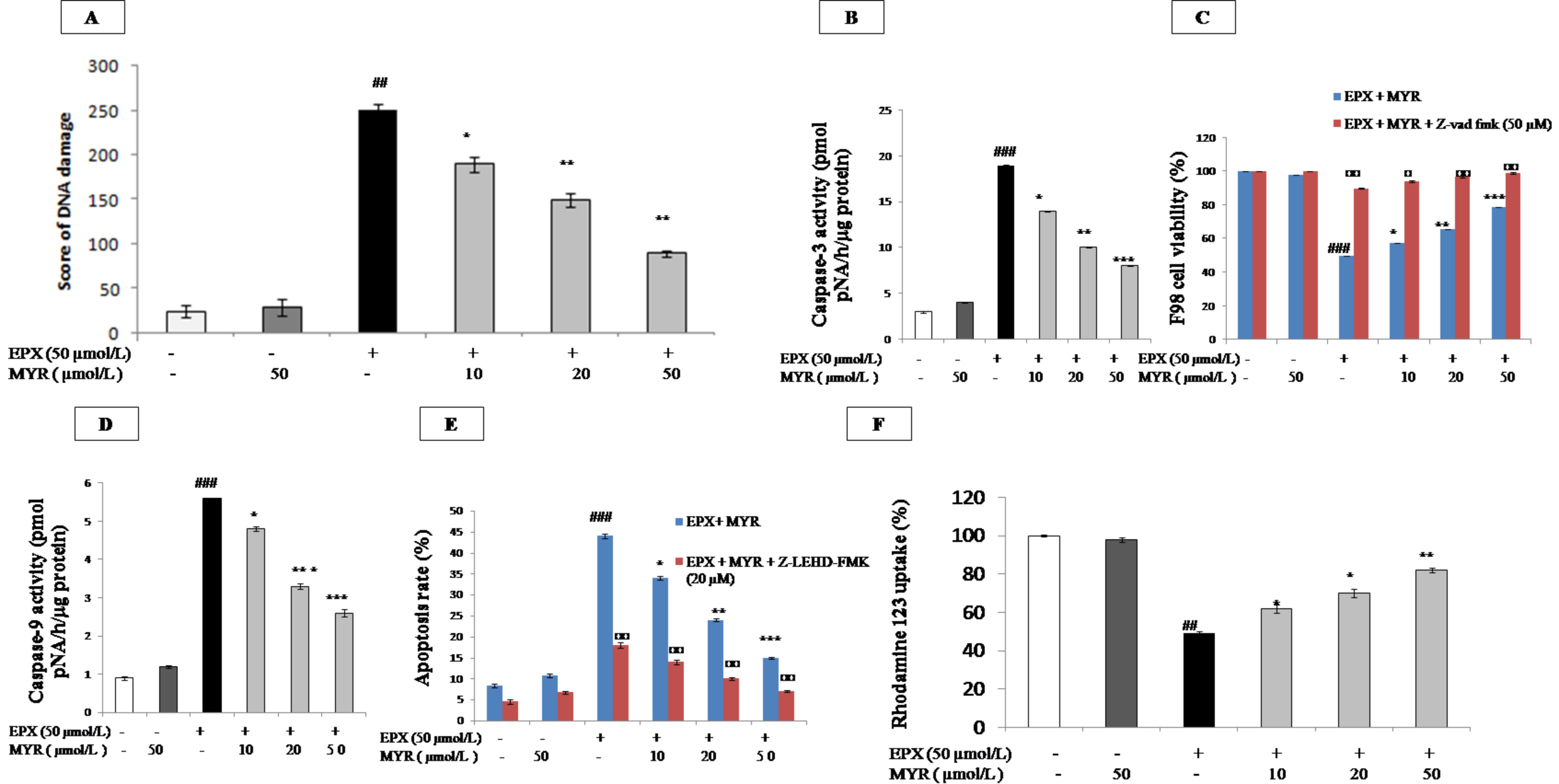
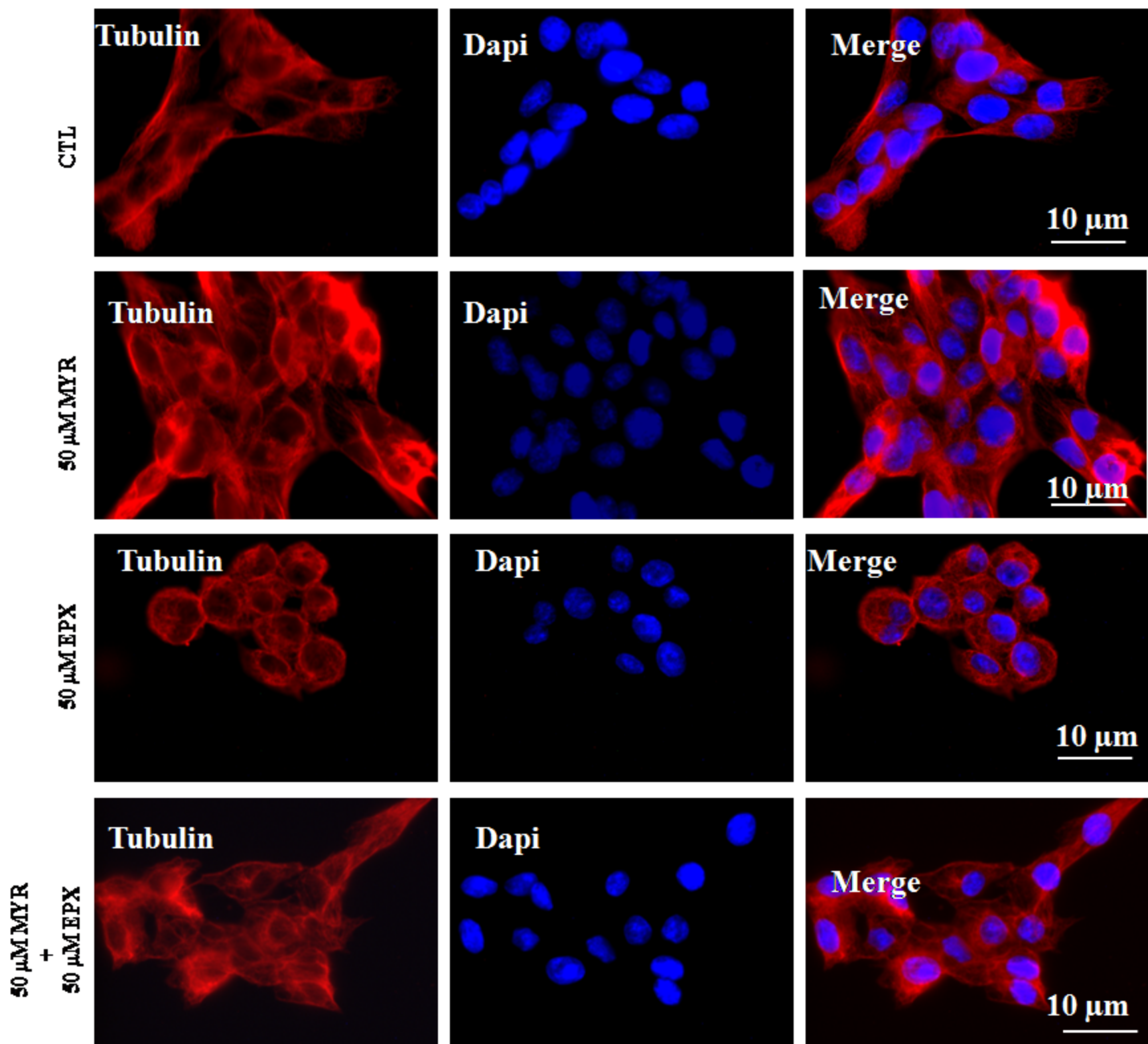
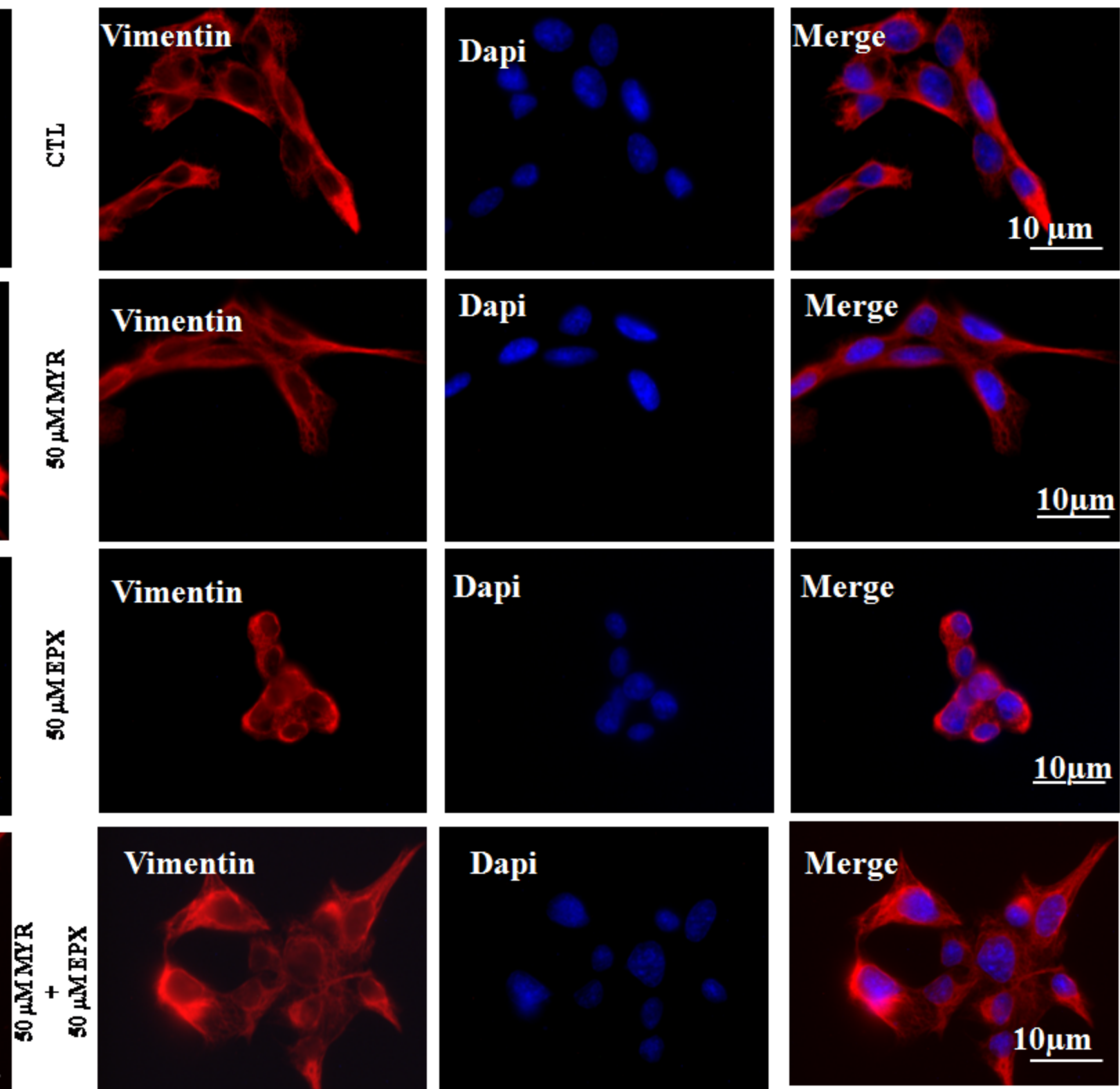


Fig. 3

A**B****Fig. 4**

