CHAPTER III

RESULTS

1. Selection of stable Mn-SOD transfected clones

The stably clones of transfected Mn-SOD RGM1 of clone no. 5, 6 and 8 and RGK1 of clone no. 1,2, 3, 9 and 10 were obtained. The levels of Mn-SOD expression was determined by measuring its ability to protect the reduction of nitro blue tetrazolium (NBT) dye from the superoxide anion ($O_2^{-\bullet}$) free radical using a non-denatured gel assay. This assay depends on the ability of SOD to inhibit the reduction of NBT to blue coloured formazan by $O_2^{-\bullet}$ generated by re-oxidation of photochemically reduced riboflavin. The regions containing active SOD appeared colourless against a background. As can be seen in Figure 4 , all stable clones of transfected Mn-SOD in RGM1 and RGK1 cells highly expressed the Mn-SOD compared with control and pCR3.1-Uni plasmid groups.

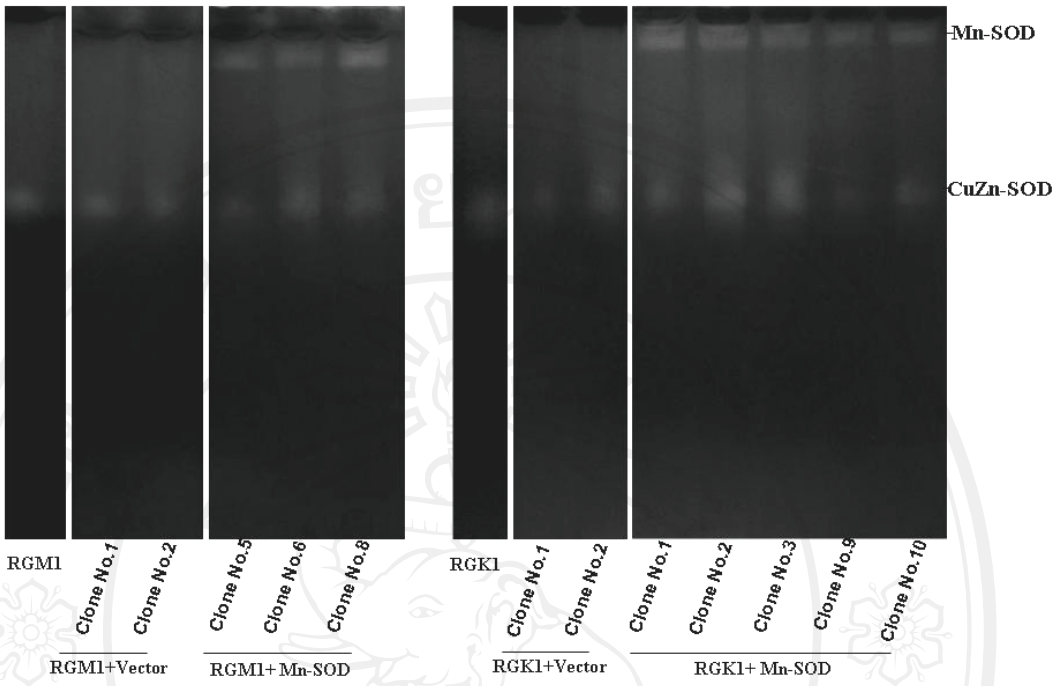

Figure 4. The non-denatureed gel of Mn-SOD activity assay. Cells were lysed and the supernatant containing 20 μg of total gram protein was loaded and separated by electrophoresis through 12% polyacrylamide gel and stained for SOD. The activity of Mn-SOD in parental cells and vector was very low, but the Mn-SOD activity in the Mn-SOD transfected cells (RGM1 clones 5, 6 and 8, and RGK1 clones 1, 2, 3, 9 and 10) were clearly detectable.

2. Effects of *Mn-SOD* gene transfect on growth rate

The typical results of the effects of an overexpression of Mn-SOD on proliferation of RGM1 and RGK1 patterns were shown in Figure 5. By using the same initial concentration of cells (5×10^4 cells), both RGM1 and RGK1 has the same lag phase time about 72 hours, but RGK1 can increase the cell numbers faster than its parental RGM1 cells. Under these Mn-SOD over-expression conditions clearly

affected the growth pattern of both RGM1 and RGK1 cells. Figure 5 shows that the overexpression of Mn-SOD caused a decrease in cellular growth or arrest of RGM1 while increased in the specific growth rate of transfected RGK1 cells. The specific growth rate (γ) was determined as indicated in Table 2.

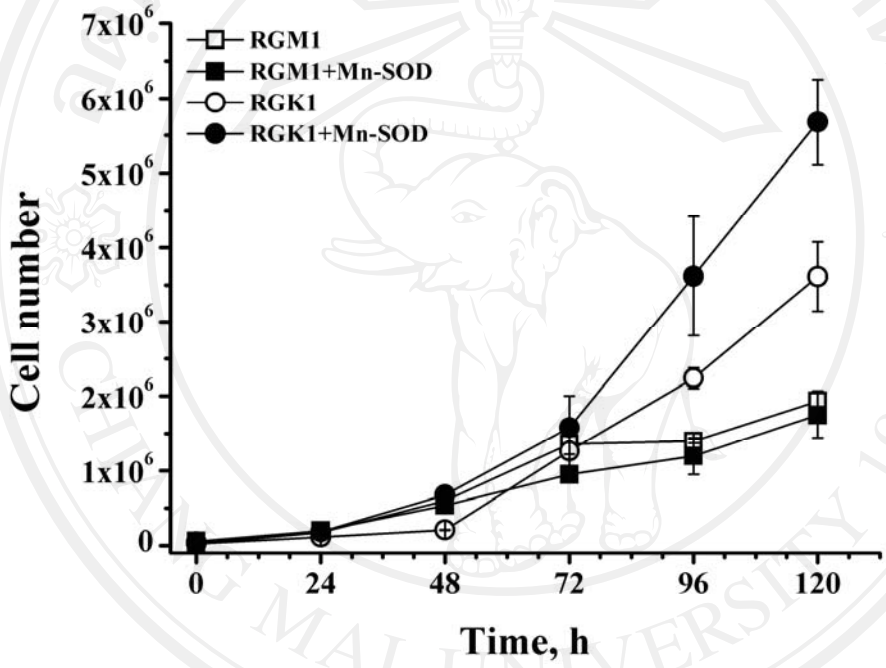

Figure 5. Growth curves of RGM1, Mn-SOD transfected RGM1 clone no. 6 and Mn-SOD transfected RGK1 clone no. 10 cells.

Table 2 . The specific growth rate of RGM1, RGK1 and Mn-SOD transfected cells.

Normal cell	Clone No.	Specific growth rate (γ)	Cancer cell	Clone No.	Specific growth rate (γ)
RGM		0.0495 ± 0.0017	RGK1		0.0417 ± 0.0006
RGM1 + Vector	1	0.0584 ± 0.0003	RGK1 + Vector	1	0.0501 ± 0.0011
	2	0.0582 ± 0.0004		2	0.0603 ± 0.0022
RGM1 + Mn-SOD	5	0.0233 ± 0.0001	RGK1 + Mn-SOD	1	0.0574 ± 0.0028
	6	0.0293 ± 0.0005		2	0.0633 ± 0.0012
	8	0.0393 ± 0.0032		3	0.0582 ± 0.0013
				9	0.0515 ± 0.0019
				10	0.0567 ± 0.0022

3. Intracellular reactive oxygen species (ROS_i)

The amount of intracellular reactive oxygen species (ROS_i) was determined using a fluorescence probe, HPF, which allows the selective detection of hydroxyl radicals under a confocal microscope. The HPF staining of the cells can not only reflect the ROS_i levels but also provide the evidence of vacuole formation in the cytoplasm. Figure 6 demonstrates that the HPF fluorescence of RGM1cells without transfection was found distributed throughout the cytoplasm. These typical of fluorescence micrographs are still the same when the similar conditions of experiments were performed but this time, additional of 100 and 200 μM quercetin. In comparison with RGM1, micrographs of RGK1 show the degree of HPF fluorescence intensity but similar pattern of these RGM1 cells. It should be noted that in a dose dependent manner of quercetin, some vacuoles excluding HPF appeared in the cytosol of RGK1 cells.

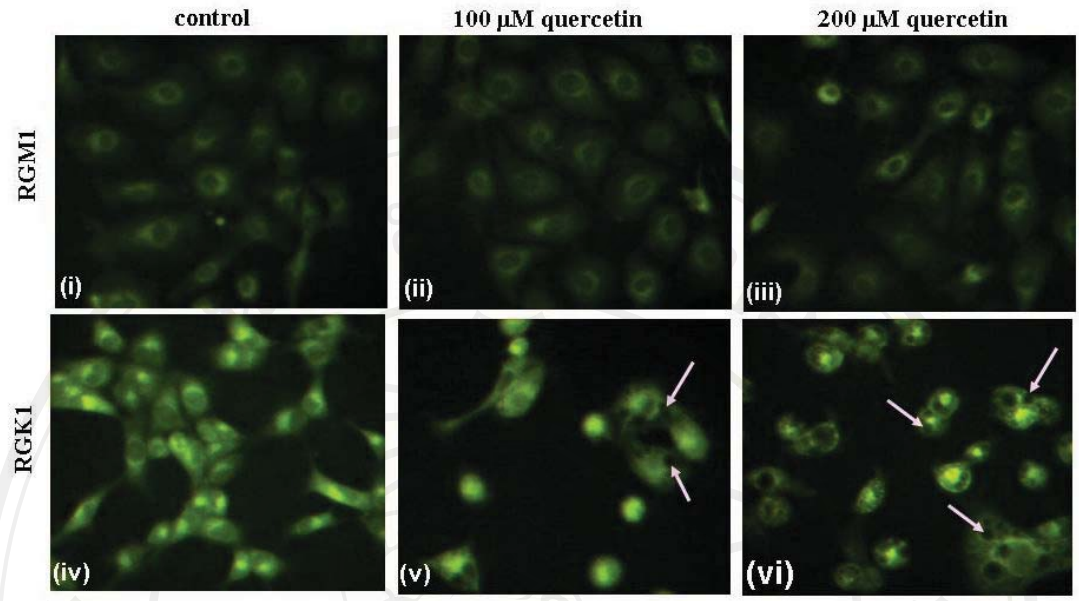

Figure 6. Confocal micrographs of untreated RGM1 (i) and RGK1 (iv) and treated with 100 µM (ii, v) and 200 µM (iii, vi) quercetin, respectively. Cells were stained with HPF at 24 hours after treatments according to materials and methods.

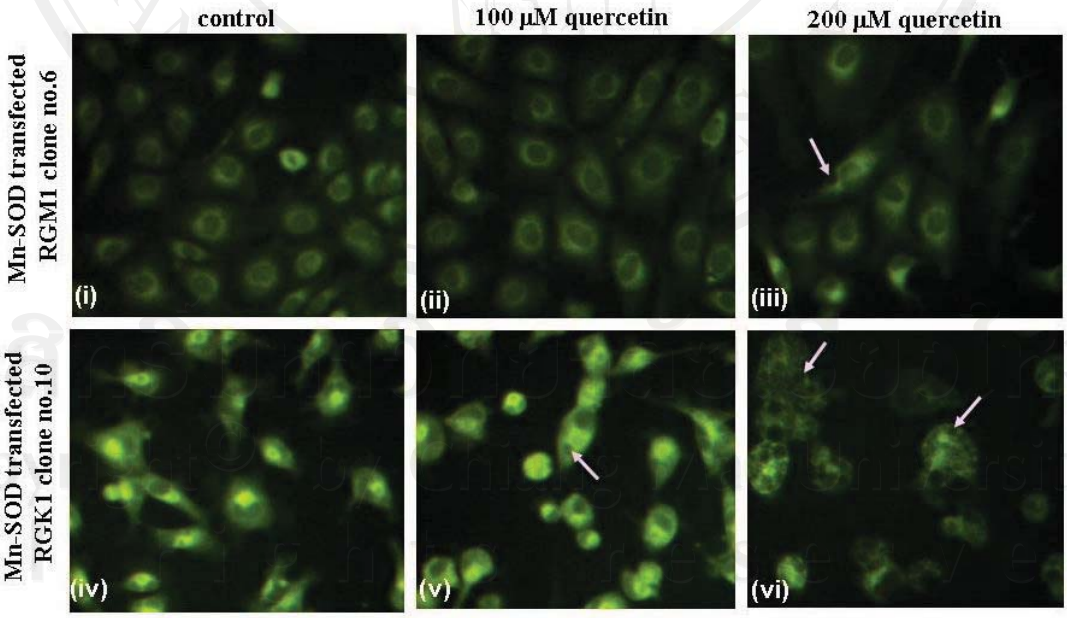

Figure 7. Confocal micrographs of untreated Mn-SOD transfected RGM1 clone no. 6 (i) and Mn-SOD transfected RGK1 clone no. 10 (iv) and treated with 100 µM (ii, v)

and 200 μM (iii, vi) quercetin, respectively. Cells were stained with HPF at 24 hours after treatments according to materials and methods.

The typical fluorescence micrographs of the Mn-SOD transfected RGM1 clone no. 6 without and with treatment using 100 μM or 200 μM quercetin were indicated in the Figure 7. These fluorescence micrographs still have the similar pattern with those of normal RGM1 cells. However, some vacuoles were observed when the cells were treated by 200 μM quercetin. It should be noted that the micrographs of Mn-SOD transfected RGK1 clone no. 10 cells show higher degree of HPF fluorescence intensity but similar pattern of the RGM1 cells. It should be also noted that as dose dependent manner of quercetin, some vacuoles excluding HPF appeared in the cytosol of Mn-SOD transfected RGK1 clone no. 10 cells.

The HPF fluorescence intensity reflects the ROS_i contents were measured by IPLab Spectrum version 3.0 software as shown in Figure 8. The results showed that the amount of ROS_i of cancer cells was almost 2 fold higher than in normal cells and an additional Mn-SOD of transfected RGM1 clone no. 6 did not alter the ROS_i contents but decrease in transfected RGK1 clone no. 10 cells. Contrary to an additional exogenous antioxidant such as quercetin, gradually decreased in ROS_i of the RGK1 and Mn-SOD transfected RGK1 clone no. 10 cells was clearly determined as dose-dependent manner but not for the normal cells (Figure 8 b and c).

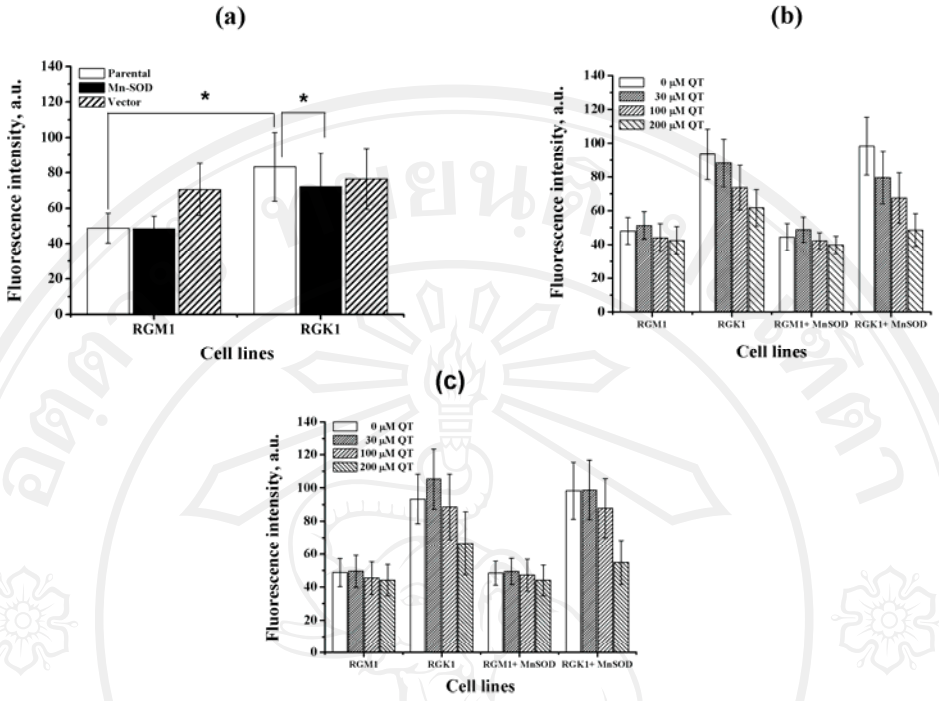

Figure 8. Effects of Mn-SOD and exogenous quercetin (QT) on ROS_i; for Mn-SOD in RGM1 and RGK1 cells (a) and for indicated concentration of quercetin demined at 2 hours (b) and 24 hours (c). * Represents $P<0.05$.

4. Determination of 4-HNE Protein adducts

Figure 9 shows the typical distribution of 4-HNE protein adducts in the cells after treated using 0.1% DMSO. The confocal micrographs demonstrated the perinuclear accumulation of 4-HNE protein adducts probably the endoplasmic reticulum. As can be seen, at 2 h after addition of DMSO, the cell morphology was found in round shape homogeneously distributed throughout the microscopic field and 24 h later the morphology of the cells was changed in the spindle form. There was an increase in number cells counted at 24 hr. These signified that DMSO even at very low concentration caused an increase in lipid peroxidation in Mn-SOD transfected

RGM1 cells, probably the majority of 4-HNE protein adducts was found in the acidic organelle such as lysosome and endoplasmic reticulum thus caused a cellular morphological changes. It seems that the cell can adapt themselves as can be seen that the different morphologies and an increase in numbers of cells at 24 hours later.

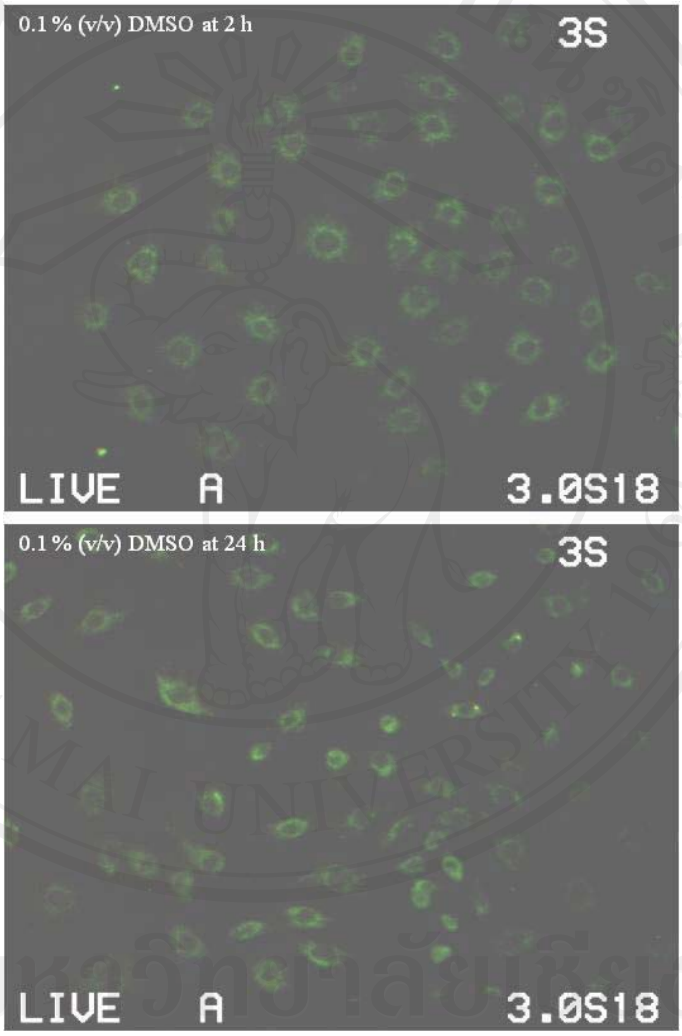

Figure 9. Confocal micrographs of Mn-SOD transfected RGM1 clone no. 6 in the presence of 0.1% (v/v) DMSO for 2 and 24 hours.

The results were along with those of cytotoxicity assay by which the concentration of DMSO used (0.1% (v/v) did not inhibit cell growth. It should be noted even at non-cytotoxic concentration determined at 72 hours, DMSO provoked an increase in 4-HNE protein adducts reflects stimulate lipid peroxidation.

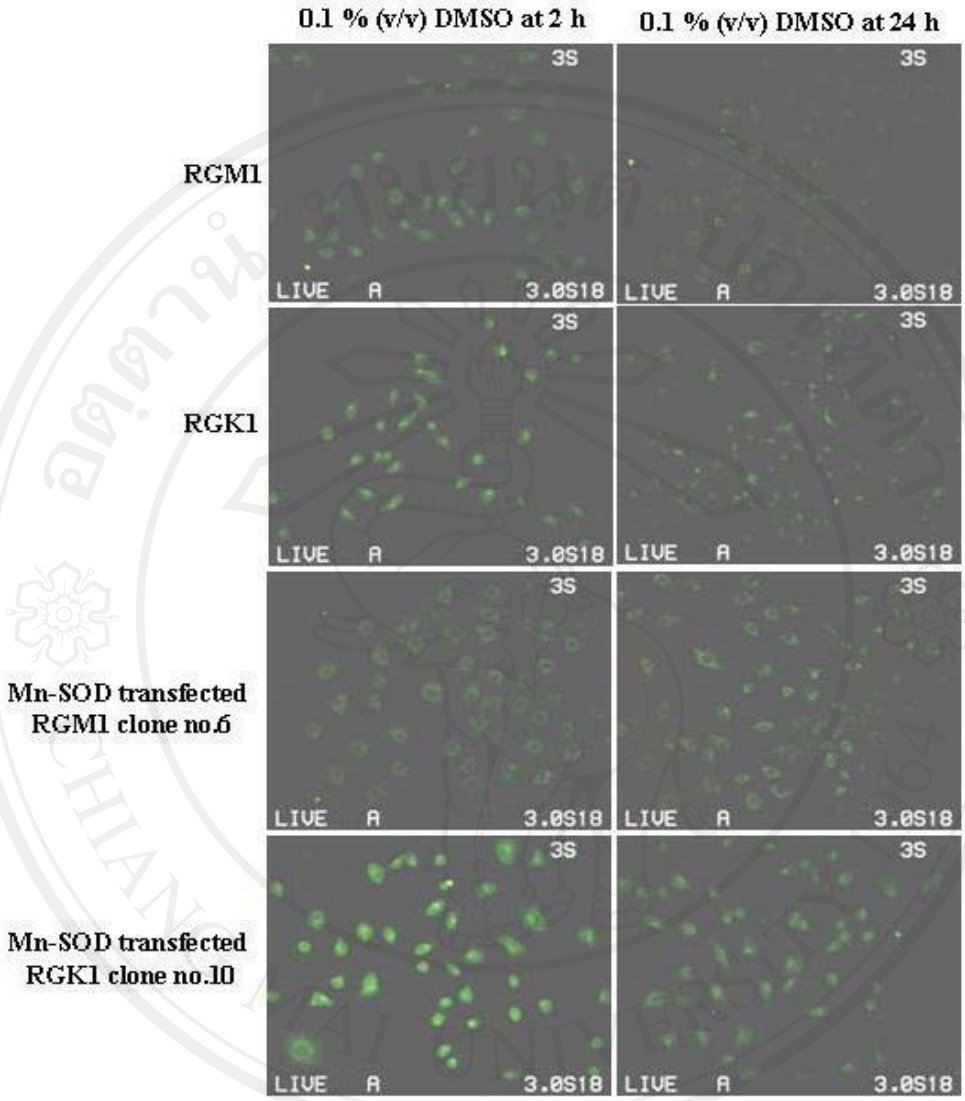

Figure 10. Confocal micrographs of RGM1, RGK1, Mn-SOD transfected RGM1 clone no. 6 and RGK1 clone no. 10 cells in the presence of 0.1% (v/v) DMSO for 2 and 24 hours.

The similar results were obtained when these similar conditions of experiments were performed for RGM1, RGK1 and Mn-SOD transfected RGK1 cells. Figure 10 shows that DMSO caused an increase in lipid peroxidation particularly higher degree in Mn-SOD transfected RGK1 compared with its corresponding parental cells.

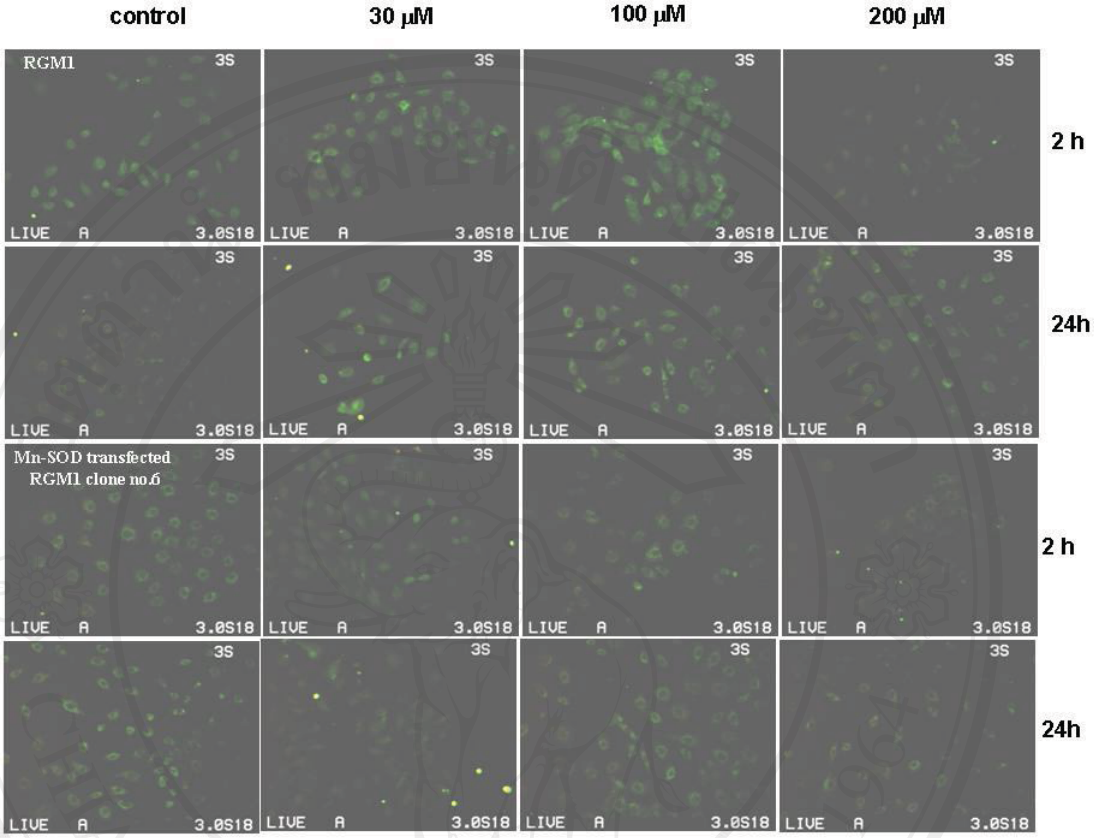

Figure 11. Confocal micrographs of RGM1 and Mn-SOD transfected RGM1 clone no. 6 cells in the presence of indicated concentration of quercetin for 2 and 24 hours.

As previously mentioned, DMSO has pro-oxidant activity at low concentration resulted an increase in 4-HNE protein adducts thus cellular adaptation. It is of important to demonstrate that whether an antioxidant molecule such as quercetin also caused an increase in peri-nuclear accumulation of 4-HNE protein adducts in similar pattern to those of DMSO. The 4-HNE protein adducts of treated cells with varied concentration of quercetin at immediately, 2 and 24 hour were performed. Figure 11 shows that quercetin induced changes of spindle shape to be round shape within 2 hours and the chemical stressor of 0 to 100 μM was well

regulated in RGM1 and Mn-SOD transfected RGM1 cells. The 4-HNE protein adducts distribution pattern was almost the same with those obtained from the series of DMSO. However at high concentration such as 200 μM, quercetin should be found in aggregated form following precipitation why very low lipid peroxidation were observed.

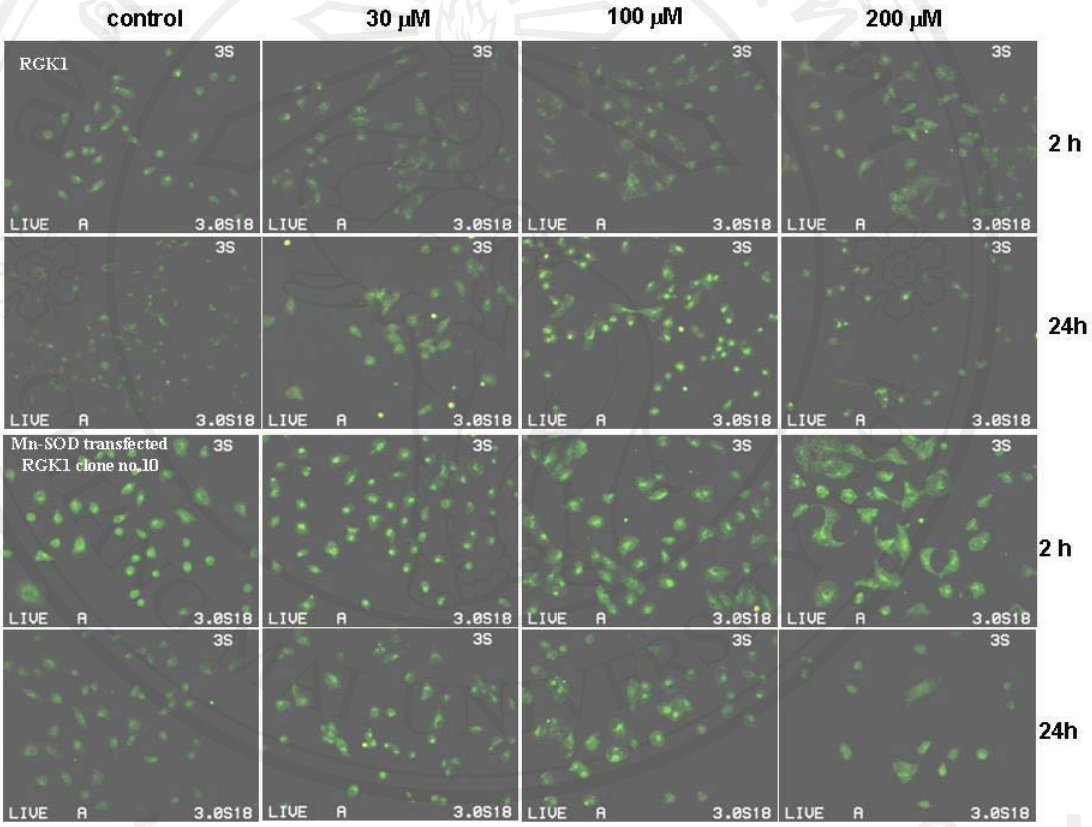

Figure 12. Confocal micrographs of RGK1 and Mn-SOD transfected RGK1 clone no. 6 cells in the presence of indicated concentration of quercetin for 2 and 24 hours.

The similar results were obtained in RGK1 and Mn-SOD transfected RGK1 cells. Figure 12 clearly showed that quercetin mediated lipid peroxidation originate the perinuclear accumulation of 4-HNE protein adducts in higher degree compared with those in normal cells. These should be caused changes of cellular spindle shape to be round shape following detachment of cells.

The confocal micrographs obtained from these series of experiments were analyzed by IPlab spectrum version 3 software. The 4-HNE protein adducts were reported as the fluorescence intensity as indicated in Figure 13.

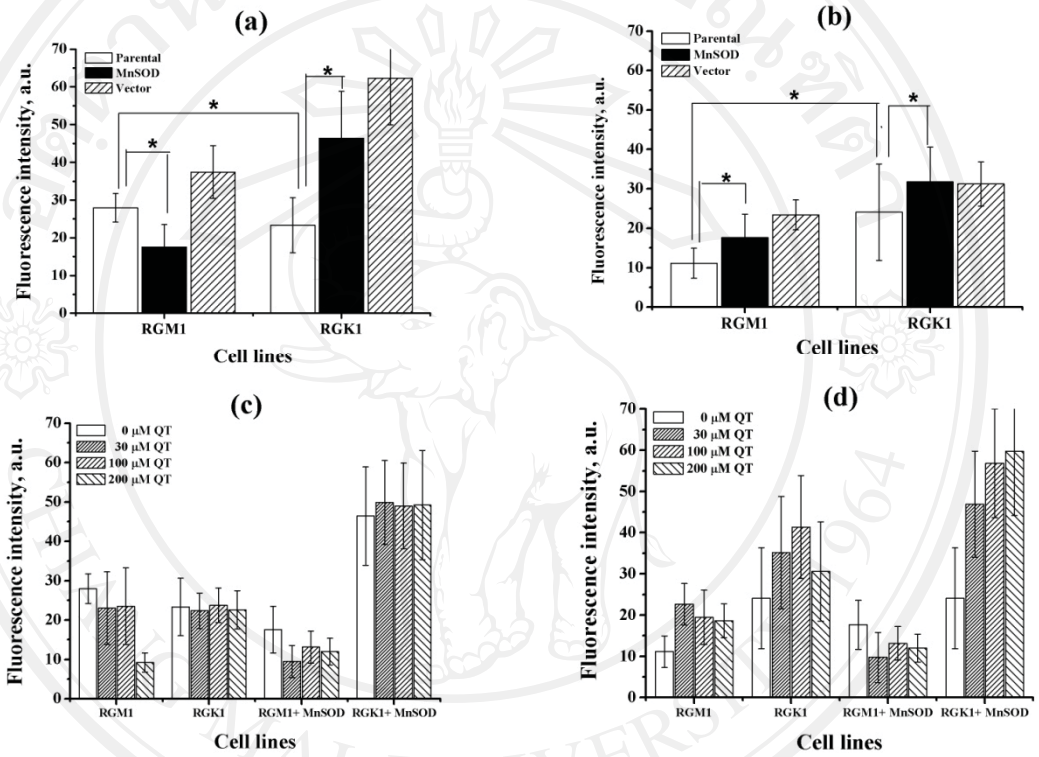

Figure 13. The 4-HNE protein adduct contents without (a, b) and with indicated the concentration of quercetin (c, d) determined at 2 and 24 hours of RGM1, Mn-SOD transfected RGM1, RGK1 and Mn-SOD transfected RGK1 cells. * Represents $P<0.05$.

5. Quercetin inhibits cell growth

Quercetin efficiently inhibited the proliferation of cells as indicated in Figure 14. These signified that the numbers of cells counted after exposing to quercetin should be contributed by the number of dead and dividing cells. It should be noted that quercetin possessed pro-oxidant activity and caused cellular damages. As mentioned in the previous section, 100 μM or 200 μM quercetin caused high degree of cell death. These results should be interpreted as 100 μM or 200 μM quercetin caused cytotoxicity in all cell lines thus decreasing in the specific growth rate.

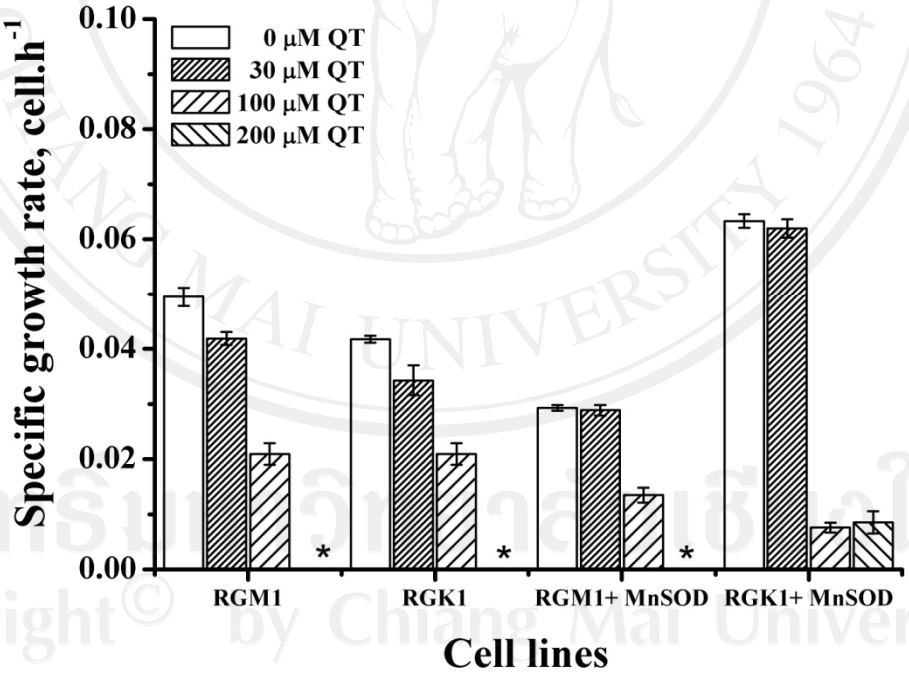

Figure 14. The effects of quercetin on the specific growth rate in RGM1, Mn-SOD transfected RGM1 clone no. 6, RGK1 and Mn-SOD transfected RGK1 clone no. 10 cells. * completely inhibitory of cell growth.QT = quercetin

6. Quercetin induced autophagy

Autophagy is characterized by the formation of double-membrane autophagosomes that fuse with lysosomes to form autolysosomes. Lysosomes and autophagosomes are forms of acidic vesicular organelles. Autophagosome formation also involves the microtubule-associated protein light chain 3 (MAP-LC3). In this study, RGM1, RGK1 and Mn-SOD transfected cells were incubated with 100 and 200 μM quercetin for 24h. The morphology of the cells under inverted microscope was shown in the Figure 15.

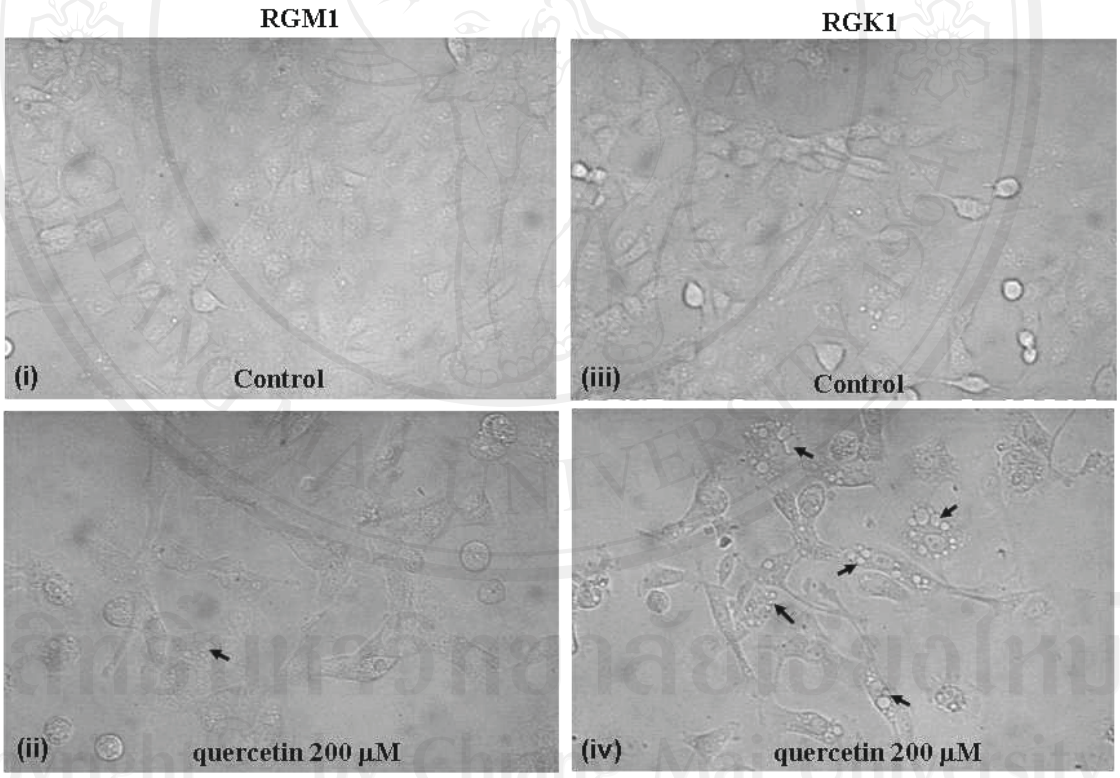

Figure15. Light micrographs of RGM1 and RGK1 cells without (i, iii) and treated with quercetin 200 μM (ii, iv). The arrows show vacuole-like structures in the cytoplasm.

Figure 15 shows that the RGM1 and RGK1 were attached cells when they attached to the bottom surface of the culture disc differences of cell morphology including irregular, spindle and round flat shapes were observed. It should be noted that there were round shape cells suspended in the medium. As can be seen in Figure 15 (iv) the vacuole-like structures in cytoplasm (arrows) in RGK1 after treatment with quercetin for 24 h was clearly identified that was not present in untreated cells. In addition, the vacuole-like structures were also found in RGM1 after treatment with quercetin but less than in RGK1. Surprisingly, vacuole-like structures in almost RGK1 cells were found after treatment with quercetin. We also confirmed the presence of MAP-LC3 using an antibody against that is a marker for autophagosome (autophagy). We observed an increase of the relative MAP-LC3 staining intensity after treatment with quercetin up to 200 μM in RGM1 and RGK1 (Figure 16). In addition, the Mn-SOD transfected RGK1 cells show the high fluorescence intensity after treatment with quercetin compared with Mn-SOD transfected RGM1 cells (Figure 17).

The confocal micrographs were quantitatively measured the fluorescence intensity by using IPLab spectrum version 3.0 as indicated in Figure 18. Once again the results clearly shown that quercetin might be considered as a chemical stressor caused dramatically autolysosomes in Mn-SOD transfected RGK1 cells.

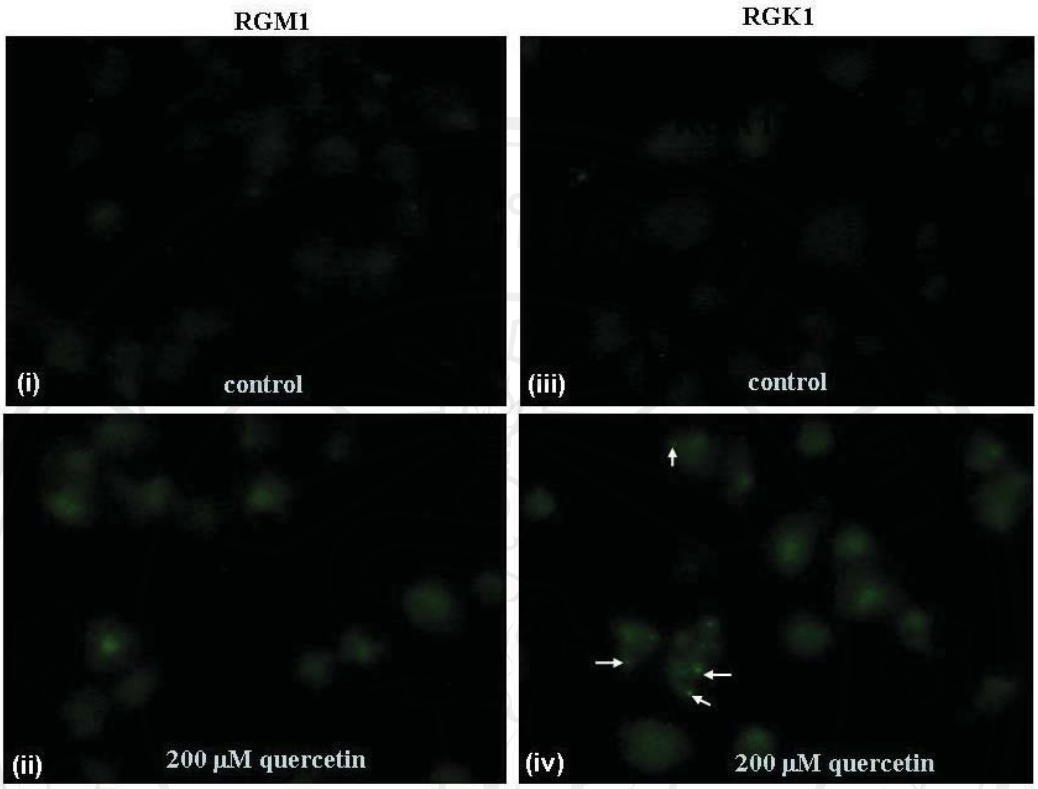

Figure 16. Characterization of autophagic cells by immunofluorescence technique, using goat anti-MAP-LC3 IgG as primary antibody and Alexa Fluor 488 donkey anti goat IgG as secondary antibody. Confocal micrographs of RGM1 and RGK1 without (i, iii) and treated with quercetin 200 µM (ii, iv), respectively.

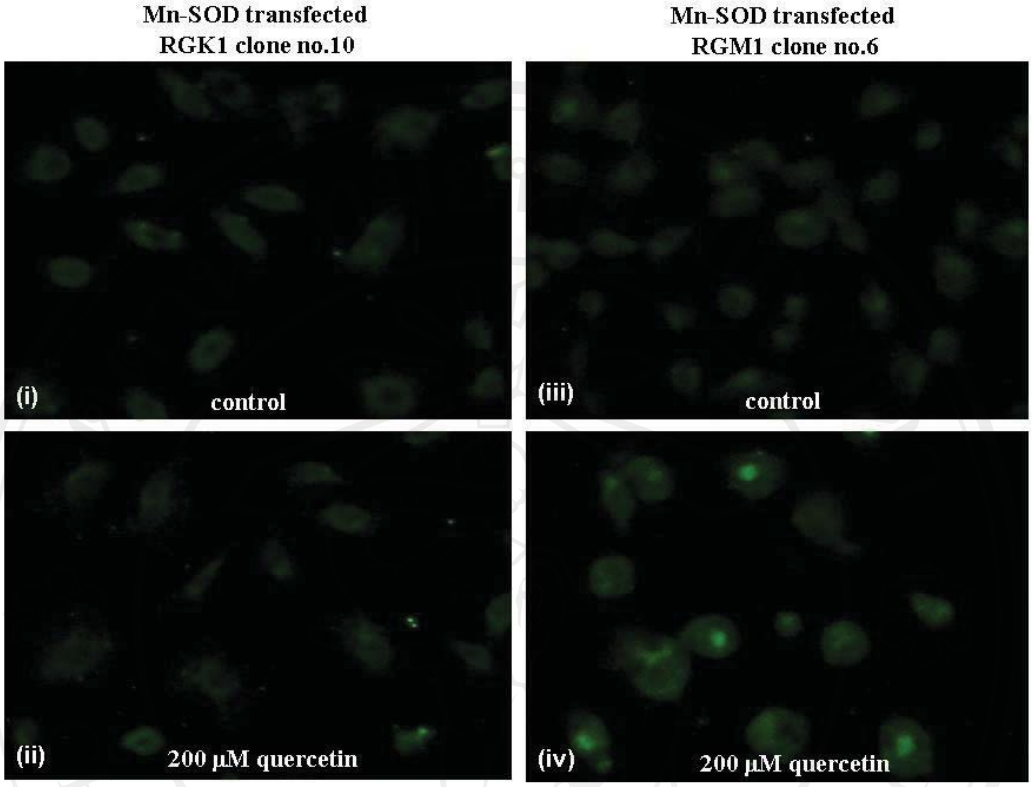

Figure 17. Determination of autophagic cells by using goat anti-MAP-LC3 IgG as primary antibody and Alexa Fluor 488 donkey anti goat IgG as secondary antibody. Confocal micrographs of Mn-SOD transfected RGM1 and Mn-SOD transfected RGK1 cells without (i, ii) and treated with quercetin 200 µM (iii, iv)), respectively.

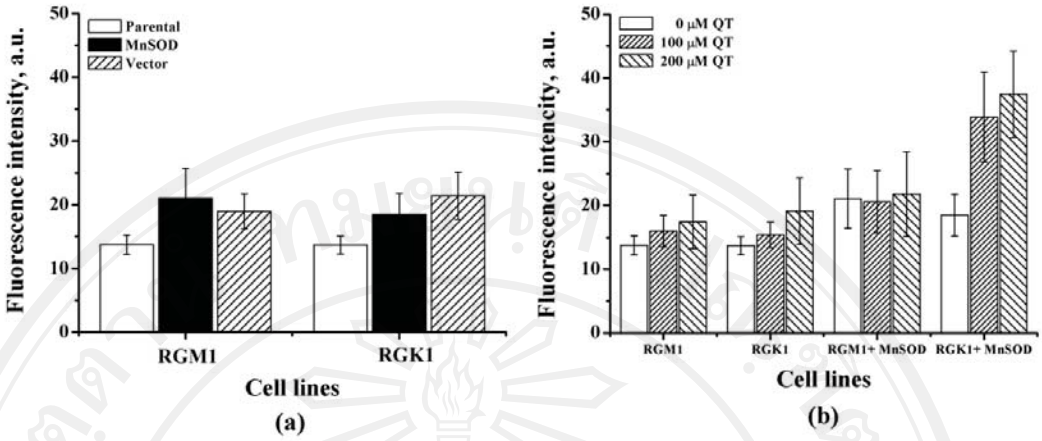

Figure 18. The relative MAP-LC3 staining intensity in RGM1, RGK1 and their corresponding Mn SOD transfected cells (a) and the presence of quercetin for 24h (b) using goat anti-MAP-LC3 IgG as primary antibody and Alexa Fluor 488 donkey anti goat IgG as secondary antibody.

7. Determination of pycnotic nuclei in apoptotic cells

The pycnotic nuclei were found < 1% in the control series and < 2% in the treated series. These results were well along with the previous series confirmed that the treated cells using quercetin have the cytoplasmic changes without DNA damage signify the autophagy or the cell death type II.