CHAPTER IV

RESULTS

Cytotoxic effect of single agent on growth of HT-29 human colon cancer cells

The cytotoxic effect of CUR, HHC and 5-FU alone on the growth of HT-29 colon cancer cell lines was examined by MTT assay. This method is based on the reduction of the yellowish MTT to dark purple-color formazan by metabolically active cells. To study the effect of these agents, the HT-29 colon cancer cells were treated with various concentrations of CUR, HHC and 5-FU at 24, 48 and 72 h, respectively.

1. Cytotoxic effect of 5-FU on HT-29 human colon cancer cells

After having been treated with a 5-FU chemotherapy drug at the dose of 5, 10 and 25 μM for 24, 48 and 72 h, the MTT cytotoxic assay (Figure 19) shows that this drug can significantly inhibit the growth of HT-29 colon cancer cells when compared to a control ($P<0.05$). The inhibitory effects were concentration and time-dependent manner. Cell viability of HT-29 colon cancer cells after having been treated with 5-FU at the dose of 5, 10 and 25 μM for 24 h was 87.38±1.05%, 83.79±0.32% and 76.43±2.15%, respectively. For 48h, cell viability of HT-29 after treated with the same doses of 5-FU was 74.02±0.89%, 62.80±1.32% and 60.47±0.64%, respectively. Moreover, 59.20±0.18%, 52.83±3.03%, and 42.53±3.03% of cell viability was observed after 72 h of treatment.

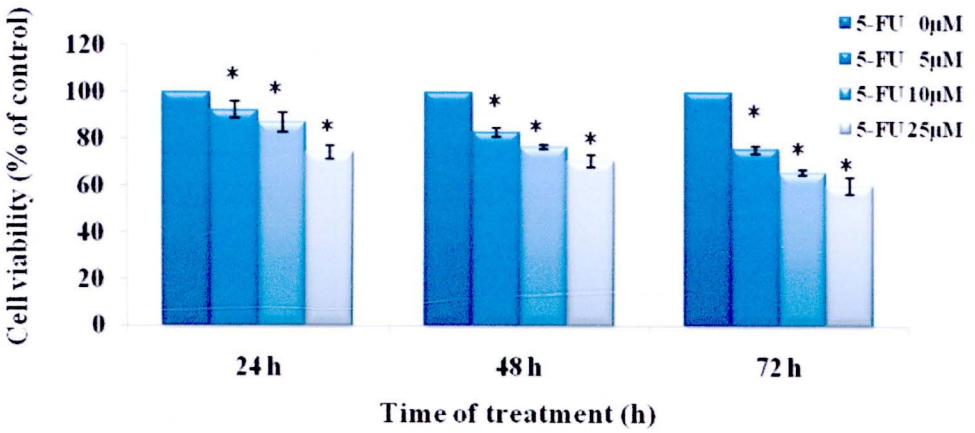

Figure 19 Cytotoxic effect of 5-FU (0, 5, 10 and 25 μM) on HT-29 cells after 24, 48 and 72 h. treatment. Each value was represented as mean ±SEM. * indicates statistically significant values when compared to a control (P<0.05).

2. Cytotoxic effect of CUR on HT-29 colon cancer cells

The cytotoxic effect of CUR was concentration and time-dependent (P<0.05). The percent of cell viability, after having been treated with CUR at 5, 10 and 25 μM for 24 h, was 98.07±1.18, 83.00±5.85% and 66.80± 2.75%, respectively. After 48 h, the viability of HT-29 colon cancer cells was 90.88±3.03, 61.17±3.39% and 34.48±3.47%. Moreover, 90.75±4.24, 60.65±4.37% and 32.61±3.82% of cell viability was observed after 72 h of treatment. The MTT results showed that CUR at 10 and 25 μM for 24-72 h could significantly inhibit the growth of HT-29 colon cancer cells when compared to a control but, no difference was noticed after low dose treatment (5 μM) of CUR (Figure 20).

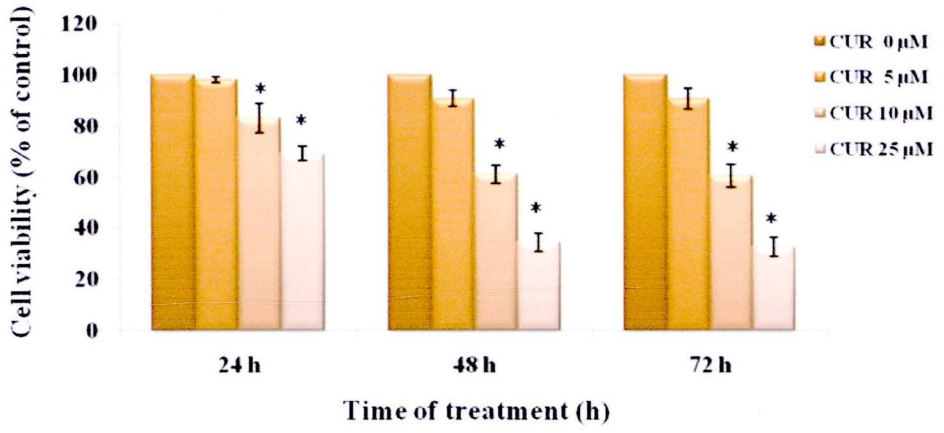

Figure 20 Cytotoxic effect CUR(0, 5, 10 and 25 μM) on growth of HT-29 after 24, 48 and 72 h. treatment.Each value was represented by mean ±SEM. * indicates statistically significance values when compared to a control (P<0.05).

3. Cytotoxic effect of HHC on HT-29 human colon cancer cells

MTT results showed that all doses of HHC (5, 10 and 25 μM) could significantly inhibit the growth of HT-29 colon cancer cells when compared to a control (P<0.05) (Figure 21). The cytotoxic effect was concentration and time-dependent. Cell viability of HT-29 colon cancer cells after having been treated with HHC at the dose of 5, 10 and 25 μM for 24 h was 85.11±2.12%, 79.17±0.68% and 69.28± 0.85%, respectively. For 48h, cell viability of HT-29 after having been treated with the same doses of HHC was 83.67±3.37%, 74.09±3.46% and 60.70±5.71%, respectively. Moreover, 64.31±3.33%, 52.26±3.21%, and 44.31±2.19% of cell viability was observed after 72 h of treatment.

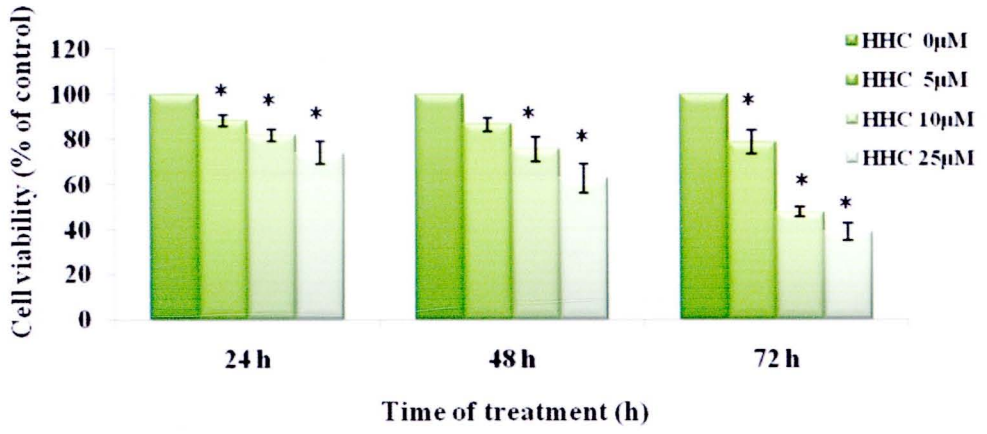

Figure 21 Cytotoxic effect of HHC (0, 5, 10 and 25 μM) on HT-29 after 24, 48and 72 h. treatment. Each value was represented by mean ±SEM. * indicates statistically significance values when compared to a control (P<0.05).

4. IC_{50} value of CUR, HHC and 5-FU on HT-29 colon cancer cells

The half maximal inhibitory concentration of 5-FU, CUR and HHC on inhibiting the growth of HT-29 colon cancer cells was represented by IC_{50}. As shown at table 2, the results indicated that HHC at 72 h of treatment was very toxic to the HT-29 colon cancer cells with an IC_{50} value of 7.63± 0.68 μM, followed by CUR (8.02±0.62 μM) whereas the toxicity of 5-FU chemotherapy drug was lower than CUR and HHC with IC_{50} values of 29.89±2.80 μM.

Table 2 IC_{50} of CUR, HHC and 5-FU against HT-29 colon cancer cells after 24, 48 and 72h of treatment

Agents	IC_{50} (μM ± SEM)		
	24h	48h	72h
CUR	41.62±0.55	26.61±3.02	8.02± 0.62
HHC	77.05±1.53	56.95±2.75	7.63± 0.68
5-FU	38.15±2.75	37.13±2.32	29.89± 2.80

Note: Values represent mean± SEM of three independent studies

Combination effects of 5-FU with CUR and HHC on growth of HT-29 colon cancer cells

The cytotoxic effects of 5-FU in combination with CUR (5-FU+CUR) and HHC (5-FU+HHC) on growth of HT-29 colon cancer cell lines were also studied by MTT assay. The cytotoxicity of 5-FU resulted in significant reduction of the growth of HT-29 after having been treated with 5, 10 and 25 μM. However, there was not much difference between doses. To minimize the toxicity and side effect of 5-FU, we used the low doses of 5-FU (5 μM) combined with various concentrations of CUR and HHC to decrease the toxicity and side effects of 5-FU. The HT-29 colon cancer cells were incubated with 5-FU combined with CUR and HHC for 24, 48 and 72 h.

1. **Combination effectsof 5-FU with CUR on growth of HT-29 cells**

Treatment of HT-29 colon cancer cells with combination of 5-FU (5 μM) and all doses of CUR (5, 10 and 25 μM) for 24, 48 and 72 h, respectively could significantly reduce cell viability when compare to a control ($P<0.05$). However, the 5-FU+CUR did not decrease the viability of HT-29 cells than treated with 5-FU or CUR alone (Figure 22).

Percent of cell viability after having been treated with combination of 5-FU and CUR at the dose of 5, 10 and 25 μM for 24h, the percent of cell viability were 84.89± 1.75, 78.56± 4.82 and 69.19± 4.77, respectively. After 48h, the viability of HT-29 colon cancer cells was 57.29± 1.61, 52.44± 2.56 and 28.47± 7.20, respectively. Furthermore, 5-FU+CUR treatment for 72h markedly reduced viability of HT-29 cells by 37.52± 3.47, 35.26±0.61 and 21.47±2.40, respectively.

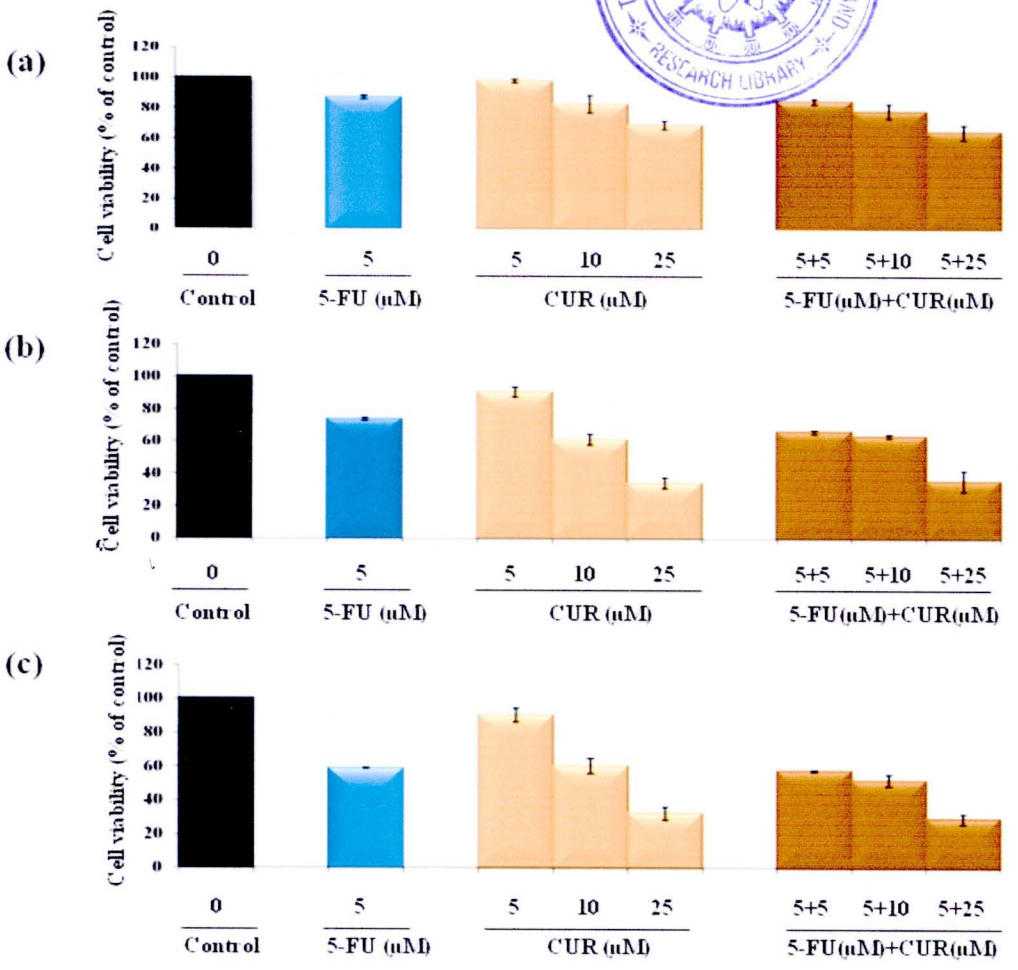

Figure 22 Growth inhibition of HT-29 after treated with 5-FU (5 μM), CUR (5, 10, 25 μM) alone and their combination for 24h (a), 48h (b), and 72h (c).Each value was represented by mean±SEM of three independent studies. * indicates statistically significance values compared to 5-FU and HHC monotherapy (P<0.05).

2. Combination of 5-FU with HHC on growth of HT-29 cells

The combination effects of 5-FU (5 μM) with all doses of HHC (5, 10 and 25 μM) for 24, 48 and 72h (Figure 23) significantly reduced the cell viability of HT-29 colon cancer cells when compared to a control (P<0.05). For 24h after treatment, the 5-FU (5 μM) combined with HHC at 10 and 25 μM showed markedly inhibited growth of HT-29 colon cancer cells as compared to 5-FU and HHC when treated alone. Percentages of cell viability of these combination treatments were 67.59±1.86

and 63.03±1.63, respectively. Furthermore, the combination of 5-FU with HHC at 5, 10 and 25 μM for 48 h significantly induced the cytotoxicity of HT-29 colon cancer cells (P<0.05) with the percent of cell viability being 57.46± 2.19, 50.81± 4.07 and 47.08± 3.02, respectively. However, 5-FU+HHC combination treatment for 72 h did not decrease the viability of HT-29 cells than 5-FU and HHC treatment alone (P<0.05). The MTT results showed the percentage of cell viability after 72 h of treatment was 54.64±2.08, 46.04±1.60 and 36.84±2.00, respectively.

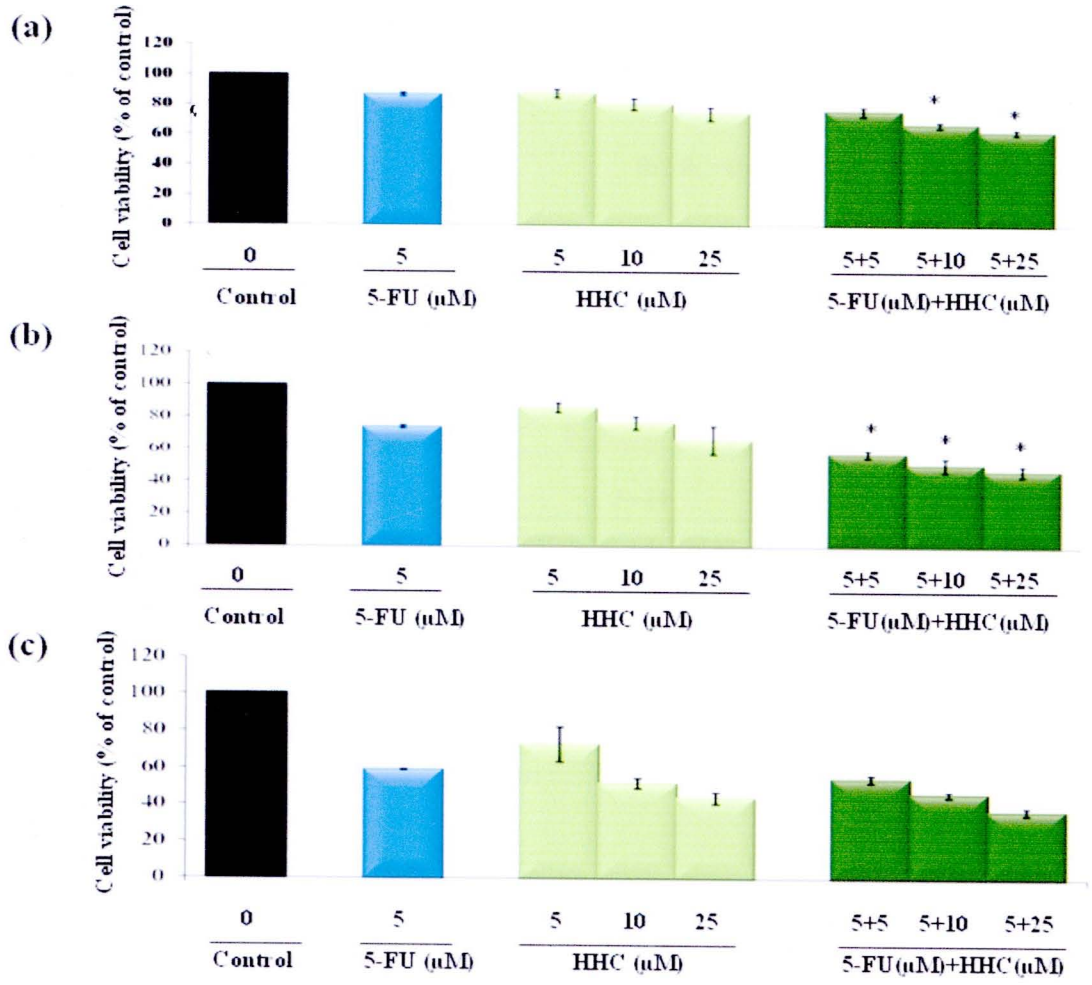

Figure 23 Growth inhibition of HT-29 after treated with 5-FU (5 μM), HHC (5,10, 25 μM) alone and their combination for 24 (a), 48 (b), and 72 h (c). Each value was represented by mean ±SEM of three independent studies. * indicates statistically significant values when compared to 5-FU and HHC monotherapy (P<0.05).

3. Synergistic inhibitory effects of 5-FU with CURand HHC

The CI value was determined the quantitative interactions of these two drugs. From the results, the CI values indicated that 5-FU (5 μM) in combination with 5 μM of CUR appears to be synergistic after 24, 48 and 72 h of treatment (Figure 24). For 5-FU (5 μM) combined with CUR at 10 μM showed the synergistic effect after 24 and 48 h, whereas this combined treatment appeared to be additive effect after 72 h of treatment. Moreover, the combination effects of 5-FU and HHC showed that low dose of 5-FU add together with all doses of HHC showed the synergistic effects after 24 and 48 h but not found after 72 h of treatment (Figure 25).

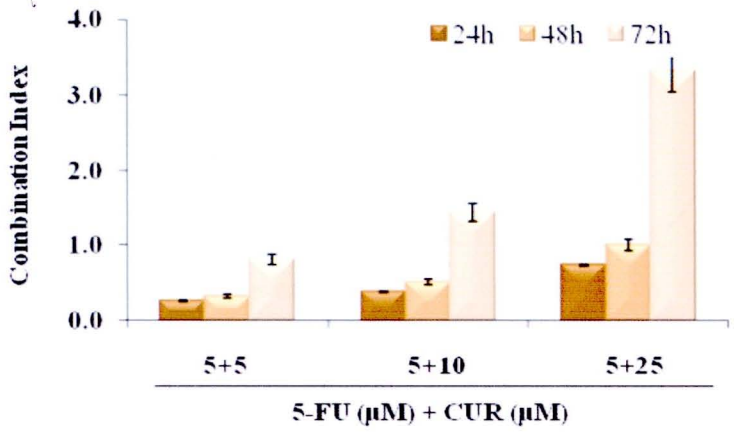

Figure 24 Combination index of 5-FU combined with CUR at 24, 48 and 72 h on HT-29 colon cancer cells. Each value was represented by mean ±SEM of three independent studies.

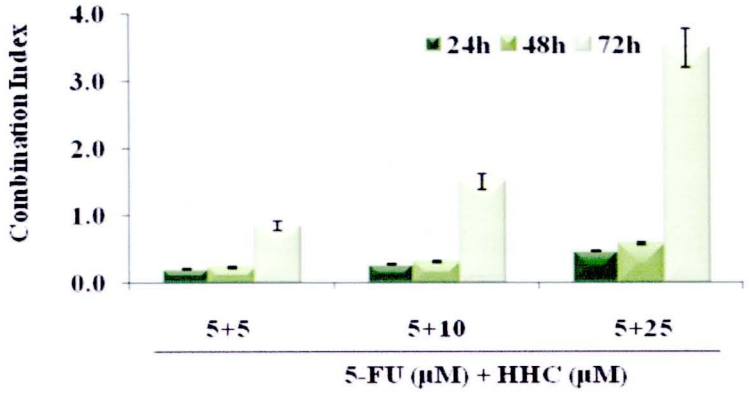

Figure 25 Combination index of 5-FU combined with HHC at 24, 48 and 72 h on HT-29 colon cancer cells. Each value was represented by mean ±SEM of three independent studies.

4. Combination effect of 5-FU with CUR and HHC on COX-2 mRNA expression of HT-29 colon cancer cells

From the previous results, the 5-FU at 5 μM combined with all doses of CUR and HHC for 24-48 h showed the quantitative synergistic inhibitory effects on HT-29 cells (CI < 1). Moreover, the preliminary study, the COX-2 mRNA showed highly expression at 24 h. Therefore, this study investigated the effect of 5-FU at 5μM in combination with high dose of HHC (25μM) on COX-2 mRNA expression in HT-29 cells for 24 h of treatment. The result showed that 5-FU did not decrease the COX-2 mRNA level, but this expression was significantly decreased after exposed to HHC as compared to a control ($P< 0.05$). Moreover, HHC in combination with 5-FU exhibited the statistically reduced the expression of COX-2 mRNA as compared to HHC and 5-FU monotherapy ($P< 0.05$) (Figure 26)

A similar effect was observed in CUR treatment alone and in combination with 5-FU. The percent of COX-2 mRNA expression after having been treated by CUR, HHC and 5-FU in combination withCUR and HHC was 83.37±3.69, 61.01 ± 0.35, 68.43 ± 8.63 and 31.93±5.69, respectively.

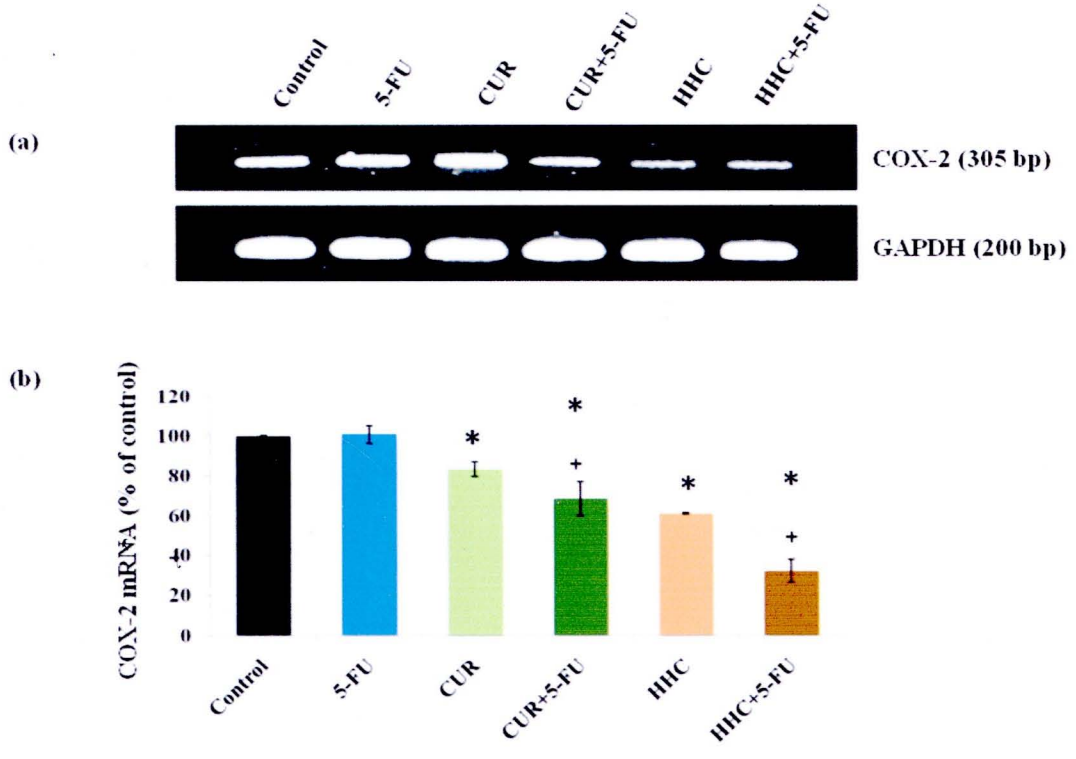

Figure 26 RT-PCR(a) illustrate the expression of COX-2 mRNA of HT-29 cells after treatedby 5-FU (5μM) combined with CUR and HHC (25 μM) for 24 h. Percent of mRNA expression (b) was determined by normalizing the band intensity of COX-2 withGAPDH. The control levels of COX-2 expression were considered as 100% and the treated levels were calculated as relative percentages for each experiment. Each bar is a mean±SEM of three experiments. * indicates statistically significance values compared to a control; $^{+}$ indicates statistically significantvalues compared to 5-FU and HHC monotherapy ($P<0.05$)

Furthermore, this study also observed the combination effects on the expression of COX-1 mRNA. The results showed that the level of COX-1mRNA was not altered by treatment with CUR, HHC, 5-FU or their combination (Figure 27).

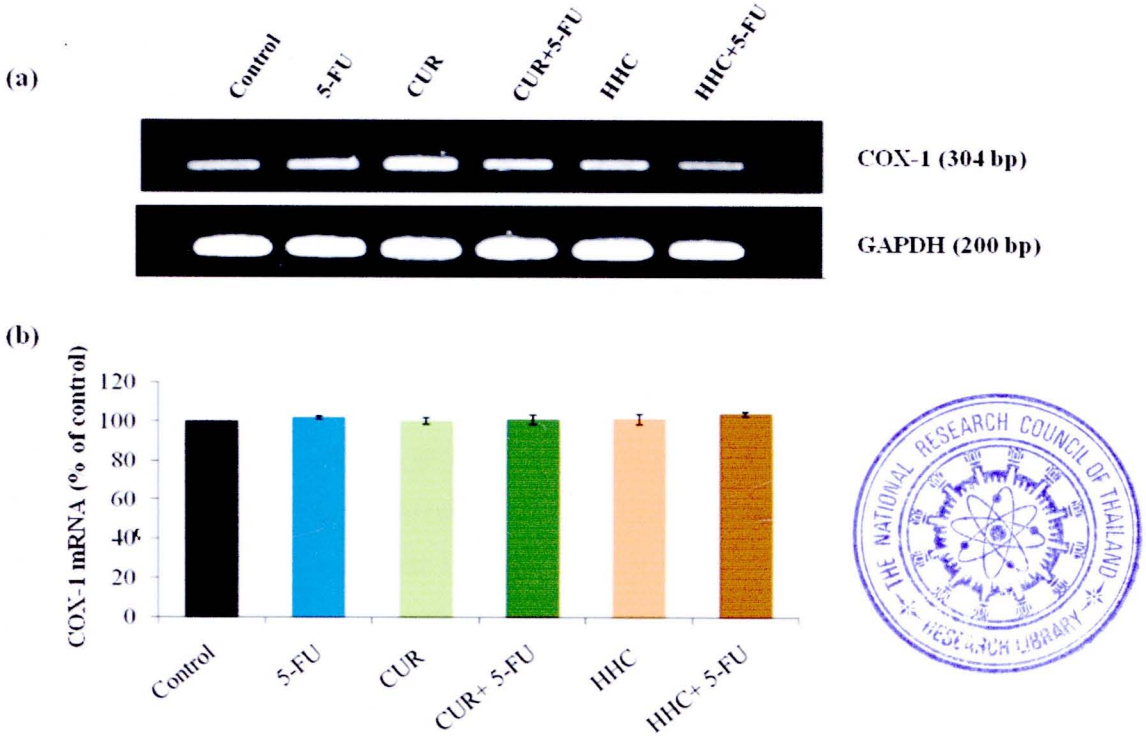

Figure 27 RT-PCR (a) illustrate the expression of COX-1 mRNA of HT-29 cells after treated with 5-FU (5μM) combined with CUR and HHC (25 μM) for 24 h. Percent of mRNA expression (b) was determined by normalizing the band intensity of COX-1 with GAPDH. The control levels of COX-1 expression were considered as 100% and the treated levels were calculated as relative percentages for each experiment. Each bar is a mean±SEM. of three experiments. * indicates statistically significance values compared to control ($P<0.05$).

5. Combination effect of 5-FU with CUR and HHC on COX-2 protein of HT-29 colon cancer cells

To investigate the inhibitory effect of 5-FU combined with CUR and HHC on the expression of COX-2 protein, the HT-29 colon cancer cells were treated with 5 μM of 5-FU combined with CUR and HHC at a dose of 25 μM for 48 h. The expression of COX-2 protein was examined by western blot analysis. Percent of protein expression was determined by normalizing the band intensity of COX-2 with

ß-actin. The control levels of COX-2 protein were considered as 100% and the treated levels were calculated as relative percentages for each experiment.

After analysis the data by pos hoc Duncan's test, the results showed that the percent of the expression after having been treated with CUR, HHC alone, 5-FU concurrently with CUR and HHC for 48 h was 89.39 ±8.31, 76.35±0.05, 63.00±2.24 and 63.52±1.56, respectively. The COX-2 protein was highest in control group and did not change after 5-FU when treated alone (Figure 28), while treatment with CUR and HHC alone for 48h did significantly reduce the expression of COX-2 protein ($P < 0.05$) as compared to a control group. Interestingly, 5-FU combined with CUR and HHC significantly reduced the expression of COX-2 protein when compared to 5-FU, CUR and HHC when treated alone ($P<0.05$).

Furthermore, this study was observed the effect of HHC alone and in combination with 5-FU on the expression of COX-1 protein by western blot analysis. The result showed that HHC alone and in combination with 5-FU did not change the COX-1 protein level and also observed in CUR alone and in combination with 5-FU (Figure 29).

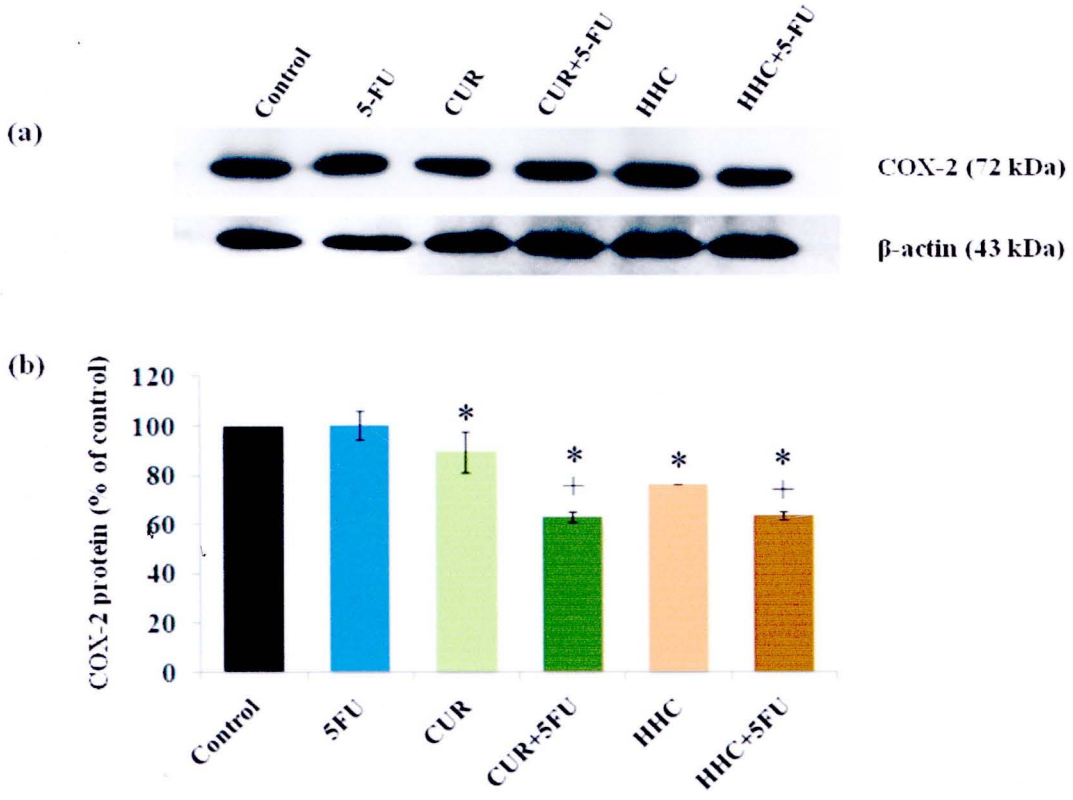

Figure 28 Western blots (a) illustrate the expression of COX-2 (72 kDa) and ß-actin (43kDa) in HT-29 colon cancer cells treated with 5-FU at 5µM combined with CUR and HHCat 25 µMfor 48 h. COX-2 protein level in HT-29 colon cancer cells after having been treated with control, 5-FU (5µM), CUR, HHC (25 µM) and their combination for 48 h. Percent of protein expression (b) was determined by normalizing the band intensity of COX-2 with ß-actin. The control levels of COX-2 expression were considered as 100% and the treatment levels were calculated as relative percentages for each experiment. Each bar is an arithmetic mean of three experiments. Statistically significant differences between the control and all treated groups are indicated by * (P<0.05); + compared with 5-FU, CUR and HHC monotherapy (P<0.05).

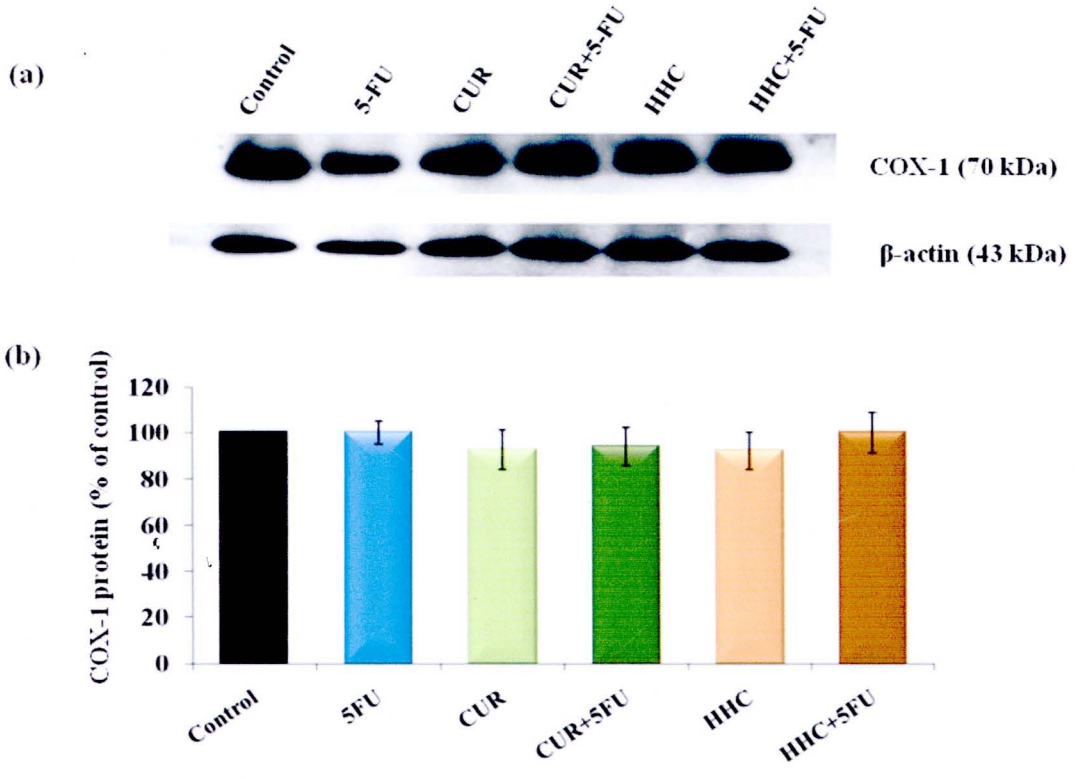

Figure 29 **Western blots (a) illustrate the expression of COX-1 (70 kDa) and ß-actin (43kDa) in HT-29 colon cancer cells treated with 5-FU at 5μM combined with CUR and HHCat 25 μMfor 48 h. COX-1 protein level in HT-29 colon cancer cells after having been treated with control, 5-FU (5μM), CUR, HHC (25 μM) and their combination for 48h. Percent of protein expression (b) was determined by normalizing the band intensity of COX-1 with ß-actin. The control levels of COX-1 expression were considered as 100% and the treatment levels were calculated as relative percentages for each experiment. Each bar is an arithmetic mean of three experiments.**

6. Combination effect of 5-FU with CUR and HHC on apoptotic induction

To investigate the inhibitory effect of 5-FU combined with CUR and HHC on apoptotic induction, the HT-29 colon cancer cells were treated with 5 μM of 5-FU combined with CUR and HHC at a dose of 25 μM for 48 h. Hoechst 33342 staining assay was performed to observe the combination effect of 5-FU with CUR and HHC on nuclear morphology. As shown in Figure 30, the control cells displayed intact nuclear construct, while nuclei with chromatic condensation and formation of apoptotic bodies were seen in treated cells. The percentage of apoptotic induction by Image J® analysis, in the control was 26.34±4.62%, while apoptosis significantly increased after having been treated with 5-FU (50.69±3.60%), CUR (51.04±0.83%), HHC (54.22±1.45%), 5-FU + CUR (61.64±0.82%) and 5-FU+HHC (67.62±5.42%) as compared to a control ($P< 0.05$). Moreover, 5-FU combination with CUR and HHC were significantly induced the apoptosis as compared with monotherapy ($P< 0.05$) (Figure 31).

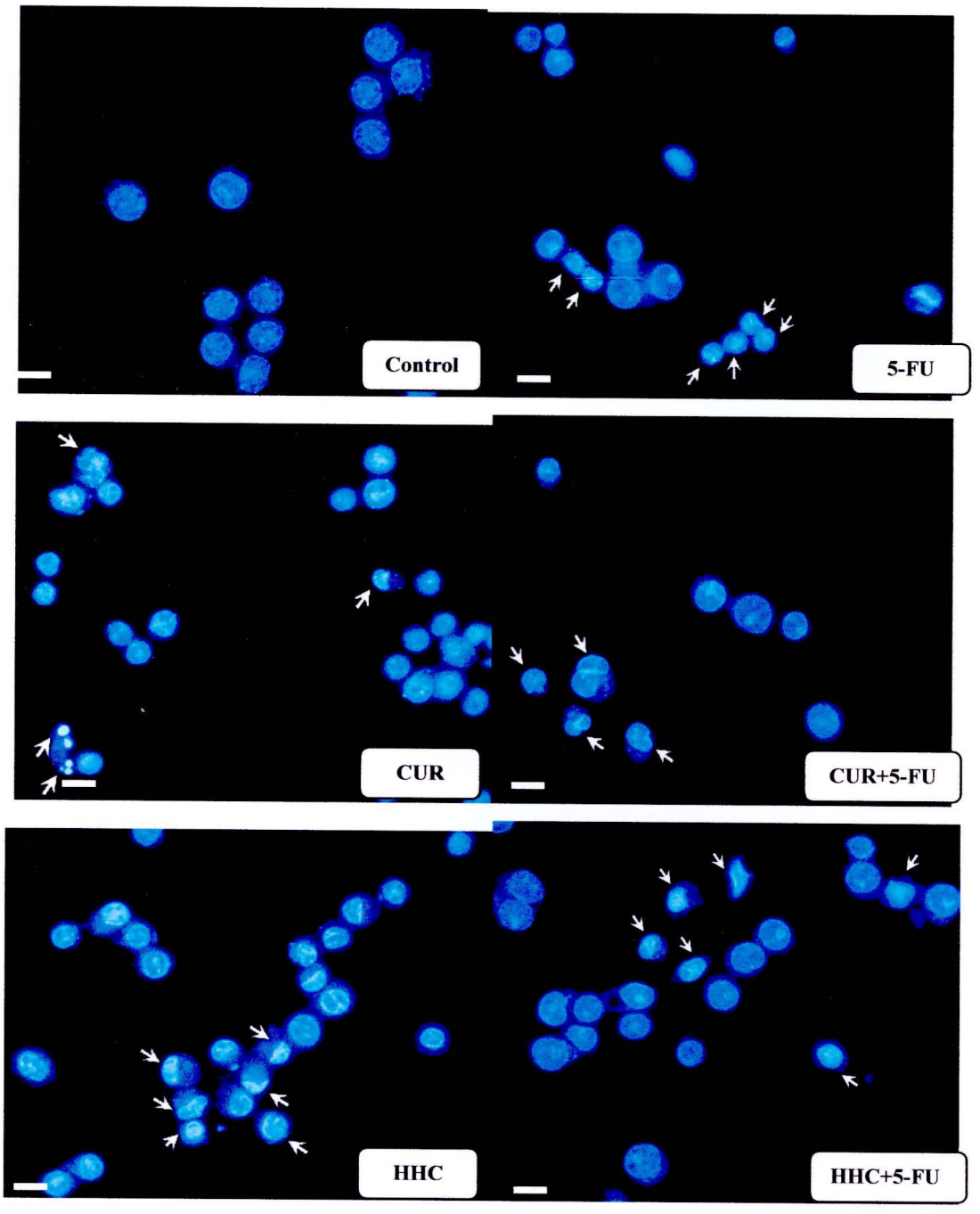

Figure 30 Fluorescence images of HT-29 colon cancer cells using Hoechst 33342 staining showed apoptotic morphological changes (→) induced by various treatment. Scale bars = 25 μm

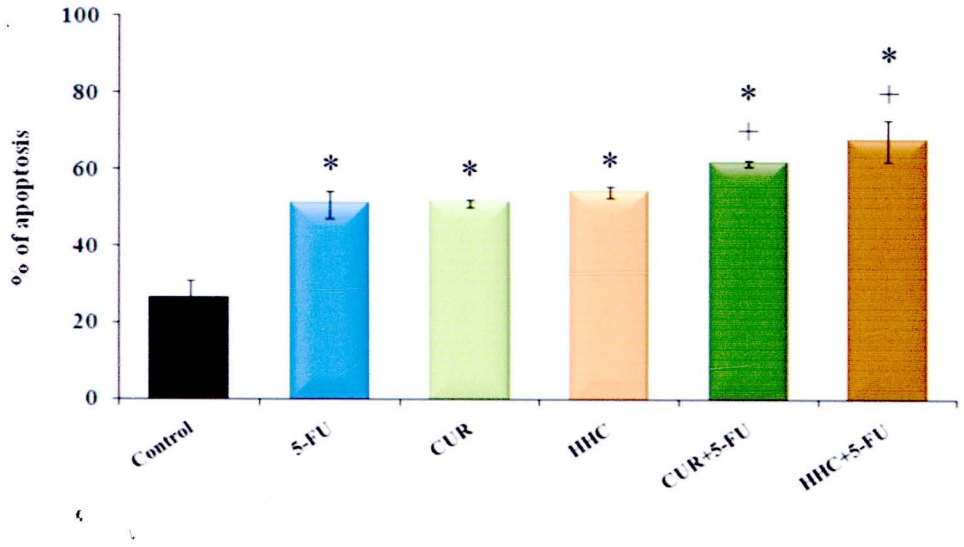

Figure 31 Apoptosis of HT-29 colon cancer cells after treated with 5-FU (5μM), CUR, HHC (25 μM) alone and their combination for 48 h. Each value was represented by mean ±SEM of three independent studies. * indicates statistically significance values when compared to a control (P<0.05); $^{+}$ indicates statistically significant values compared to 5-FU, CUR and HHC monotherapy (P<0.05).

Combination effects of 5-FU with CUR and HHC on DMH-induced colon cancer rats

1. General observation after combined treatment on DMH-induced colorectal cancer rats

During the experimental period, no clinical signs of toxicity were observed in any of the groups. The body weight of all rats was recorded weekly throughout the experimental period (Figure 32).

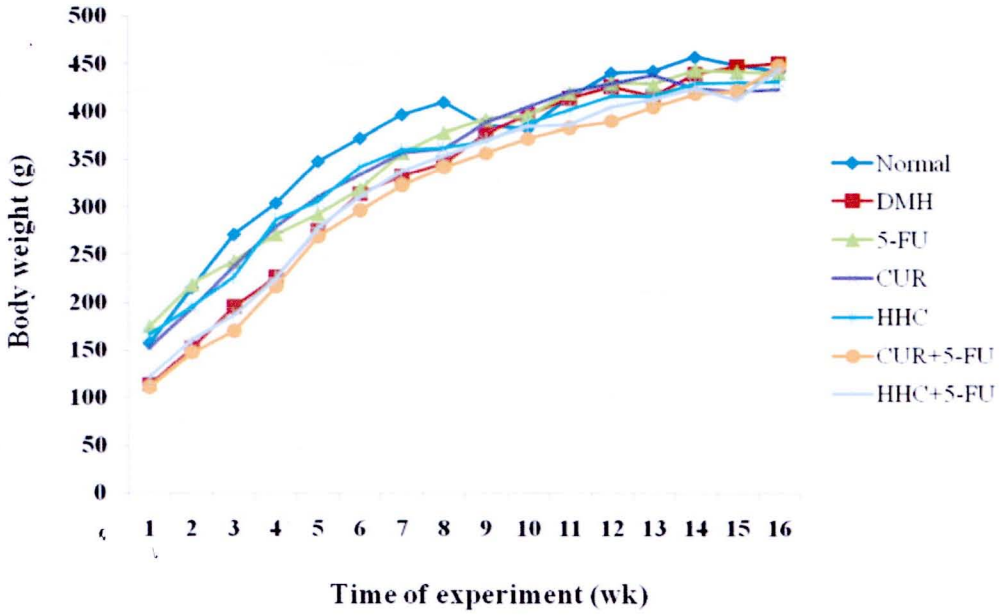

Figure 32 Mean body weight of rat in all experimental groups. Values are mean±SEM

2. Combination effects of 5-FU with CUR and HHC on ACF formation in colon of rats exposed to DMH

To observe the ACF in the colon tissue of DMH treated rats, most parts of colon were stained with 0.2% methylene blue and observed under a light microscope. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, darker epithelial lining than normal crypt and large luminal opening (Figure 33). This study assessed ACF formation in colon epithelium of experimental models by recording two parameters of ACF: (1) number of crypt and (2) large ACF containing 4 crypts or more (>3 crypts/ ACF).

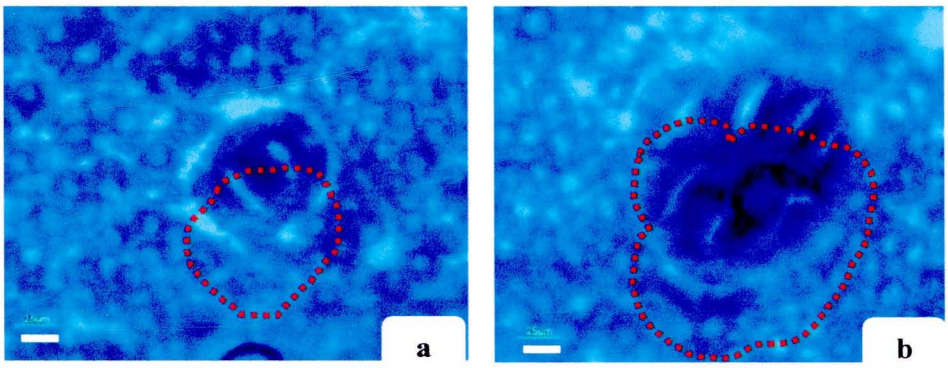

Figure 33 Topographical view of aberrant crypt foci, small ACF with 2 crypts (a) and large ACF containing more than 3 crypts per ACF (b) in whole-mount colon of rats exposed to DMH. Scale bars = 25 μm.

At the end of 16 weeks of experimentation, the formations of small and large ACFs were detected (Figure 33). The results showed that the ACF formation were not present in normal rats. All rats developed ACF in the colon after 2 weeks of DMH induction, particularly in the middle and distal part of the colon. The first parameter; the total numbers of aberrant crypts was THE highest in the vehicle group (1558.20±17.37) as shown in table 3. ACF formations were markedly reduced after having been treated with 5-FU (1231.20±25.62), CUR (1284.20±25.47), HHC (1086.80±53.47), 5-FU+CUR (880.20±13.67) and 5-FU+HHC (665.80±16.64) as compared to the vehicle group ($P< 0.05$). Furthermore, HHC treatment alone could markedly inhibit the ACF formation better than CUR treatment ($P< 0.05$). Interestingly, 5-FU +HHC treatment significantly decrease the number of ACF more than HHC and 5-FU monotherapy($P< 0.05$).

The second parameter; the large ACF, early lesions, having capable progression to colon cancer, were significantly reduced after having been treated with 5-FU (111.00±7.88), HHC (186.60±21.51) alone and their combination as compared to the vehicle (262.20±10.18) group. Furthermore, 5-FU+HHC (119.00±17.92) could significantly reduced the formation of large ACF when compared to HHC treatment alone ($P<0.05$), but did not reduce formation any better than 5-FU treatment alone. In addition, this inhibition was also observed in 5-FU+CUR (122.00±5.94) treatment.

Table 3 Effect of CUR, HHC, 5-FU and their combined treatment on rats exposed to DMH on ACF formation

Groups	Total ACF	Large ACF
Vehicle	1558.20±17.37	262.20±10.18
5-FU	1231.20±25.62 (*)	111.00±7.88 (*)
CUR	1284.20±25.47 (*)	178.00±7.33 (*)
HHC	1086.80±53.47 (*)	186.60±21.51 (*)
5-FU+CUR	880.20±13.67 (*,+)	122.00±5.94 (*)
5-FU+HHC	665.80±16.64 (*,+)	119.00±17.92 (*)

Each value was represented by an arithmetic means of seven rats in each group. Statistically significant differences between the vehicle and treated groups are indicated by * (P<0.05), + compared with monotherapy (P<0.05).

3. Combination effects of 5-FU with CUR and HHC on expression of COX-2 in colon of rats exposed to DMH

The COX-2 protein was observed under a light microscope and found in the cytoplasm of colon mucosa of all rats exposed to DMH (Figure 34). The numbers of COX-2 positively stained cells of distal colon were counted by image analysis. These studies revealed a COX-2 over-expression in the colon tissues of the vehicle group. The up-regulations of COX-2 protein expression was not decreased after having been treated with 5-FU alone. However, the expression was significantly suppressed by CUR, HHC, 5-FU+CUR and 5-FU+HHC (P< 0.05) as compared to the vehicle group (Figure 35). Moreover, HHC+5-FU could significantly reduce the COX-2 protein more than 5-FU when treated alone, but not any differently than HHC when treated alone. However, 5-FU+CURtreatment markedly decreased the level of COX-2 protein when compared with 5-FU and CURmonotherapy (P<0.05). Percentages of COX-2 protein expression after treatment with 5-FU, CUR, HHC, 5-FU+CURand HHC+5-FU were 99.42±2.75, 80.01±8.92, 85.35±8.91, 69.70±9.03 and 77.33±8.22, respectively.

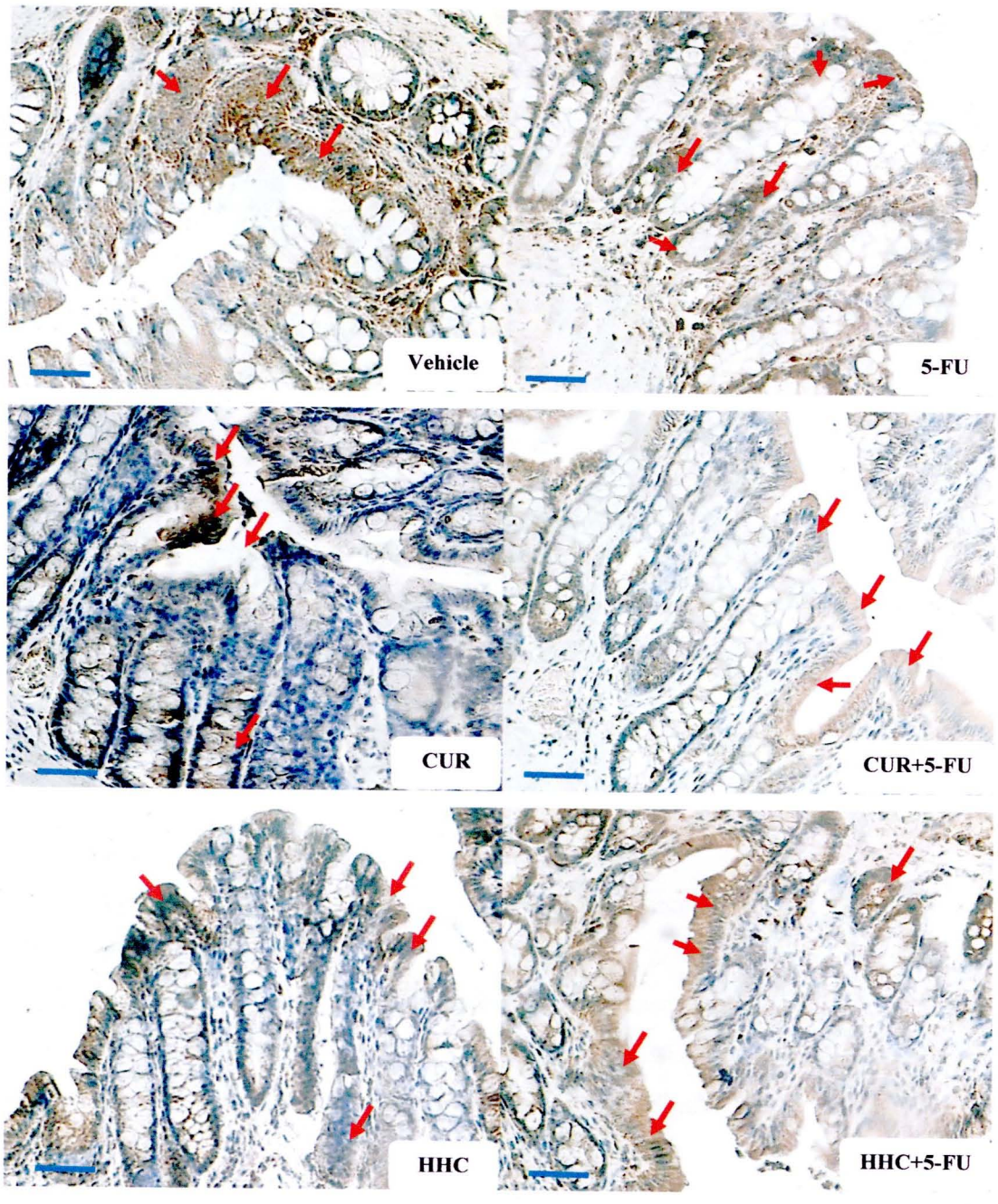

Figure 34 Immunohistochemical staining of COX-2 protein labeled cell in colon mucosa of rat model. Arrow (→) indicated the COX-2 protein labeled cell. Scale bars = 50 μm.

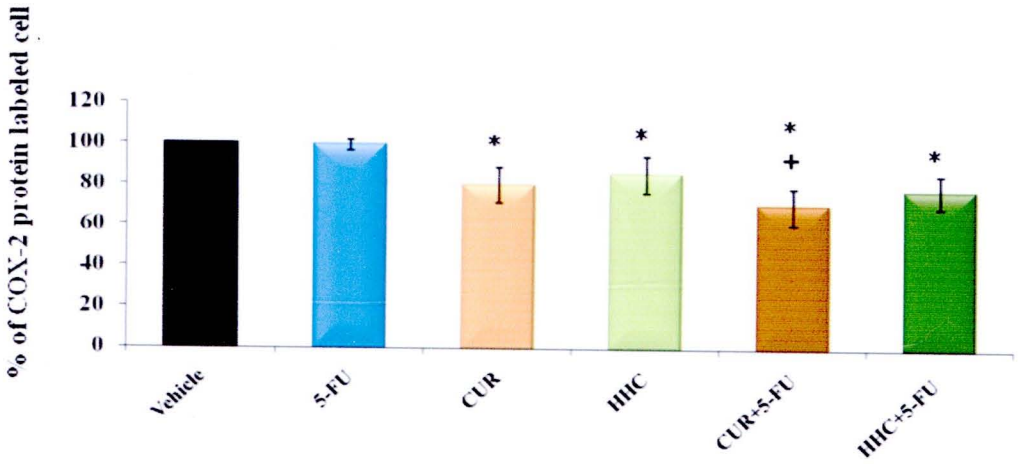

Figure 35 Percent of COX-2 proteinlabeled cell in colon mucosa of rat exposed to DMH. Each value was represented by mean±SEM. Statistically significant differences between the vehicle and treated groups are indicated by * ($P<0.05$); + compared with monotherapy ($P<0.05$).

4. Combination effects of 5-FU with CUR and HHC on expression of COX-1 in colon of rats exposed to DMH

The COX-1 expression was most observed in cytoplasm of cells in coloniccrypt of all animals (Figure 36). The COX-1 protein labeled cell was analyzed by image analysis. These studies revealed a COX-1 protein labeled in the colon tissues of all rats, including normal rats. The resultfound that the level of COX-1 protein of all treated rats was not different from the normal group (Figure 37).

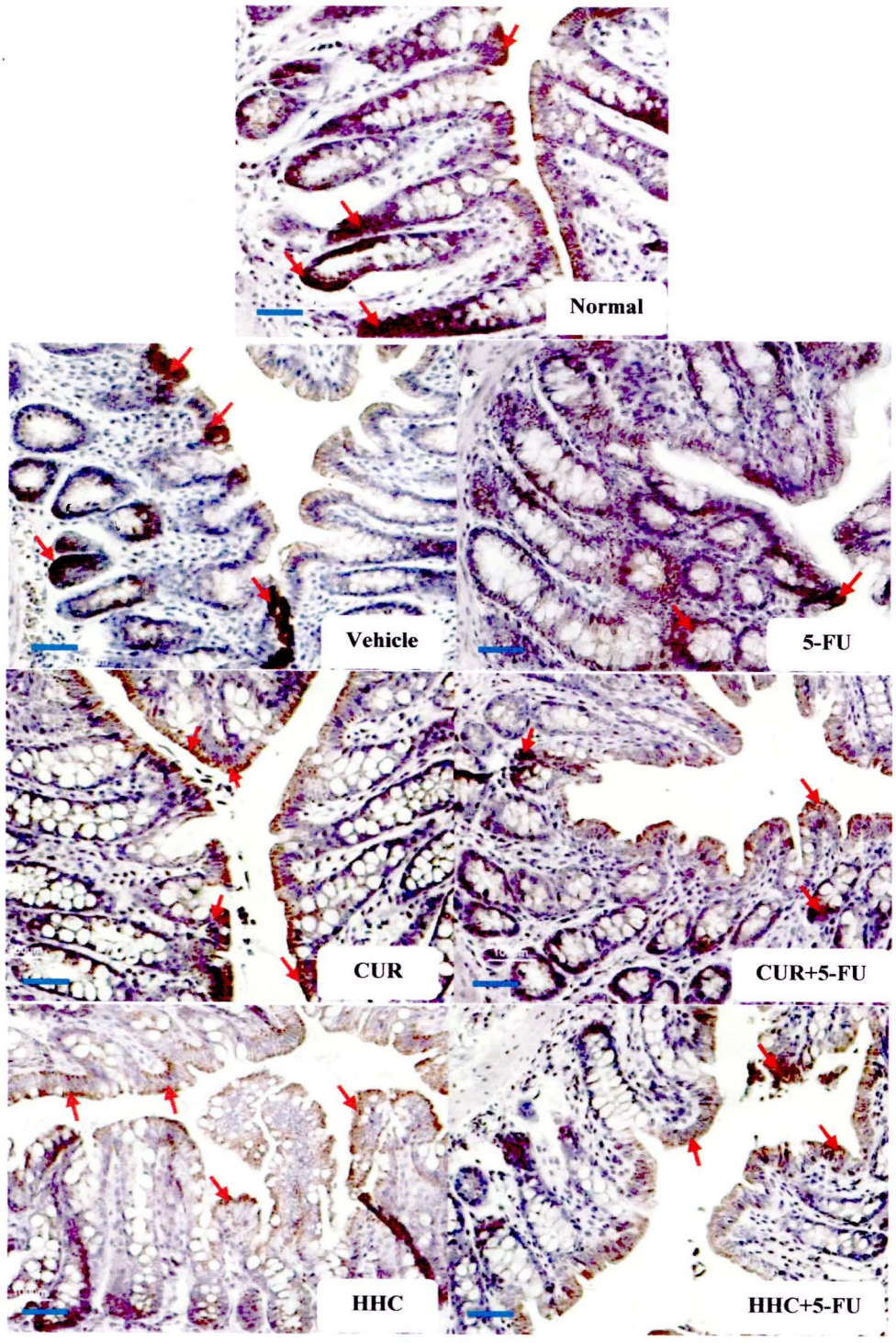

Figure 36 Immunohistochemical staining of COX-1 protein labeled cell in colonmucosa of all experimental groups. Arrow (→) indicated the COX-1 protein labeled cell. Scale bars = 100 μm.

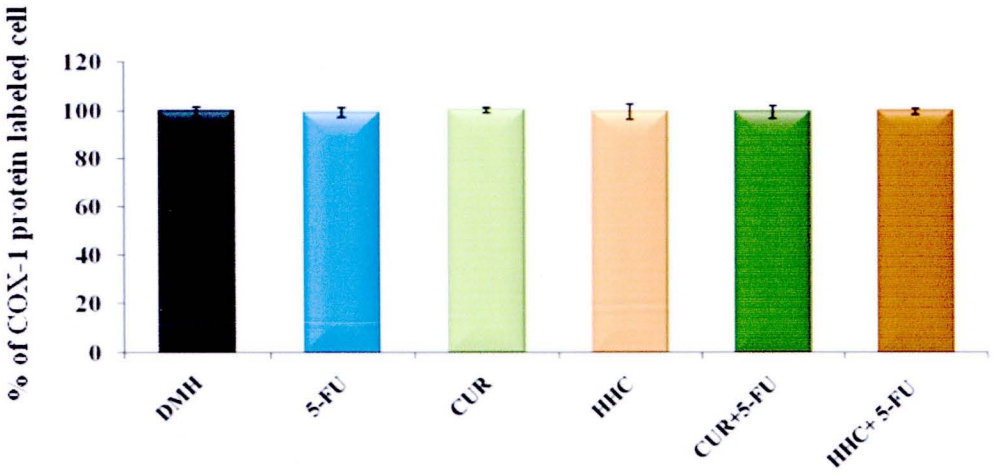

Figure 37 Percent of COX-1 protein labeled cell in colon tissues of rats treated with various agents. Each value was represented by mean±SEM of five animals per group.

5. Combination effect of 5-FU with CUR and HHC on induction of apoptosis in colon of rats exposed to DMH

Apoptotic cell death in colon tissues of rats was determined by the TUNEL technique. After observing under light microscope, the apoptotic positive stained cells were observedon colon mucosa of all rats (Figure 38). As shown in figure 36, a high apoptotic index (AI) was presented in the normal group (75.71±3.59) and a significantly less AI in all DMH-induced colon cancer groups ($P< 0.05$). After treatment with CUR, HHC, 5-FU+CUR and 5-FU+HHC, the AI significantly increased as compared to vehicle group ($P< 0.05$). The AI of rats in vehicle, 5-FU, CUR, HHC, 5-FU+CUR and 5-FU+HHC treated group was 23.55±2.12, 38.85±4.73, 41.78±6.92, 41.06±4.81, 49.05±6.75 and 53.69±8.59 respectively. However, the AI of 5-FU+HHC treatment group did not reach significant levels as compared with 5-FU and HHC treatment alone.

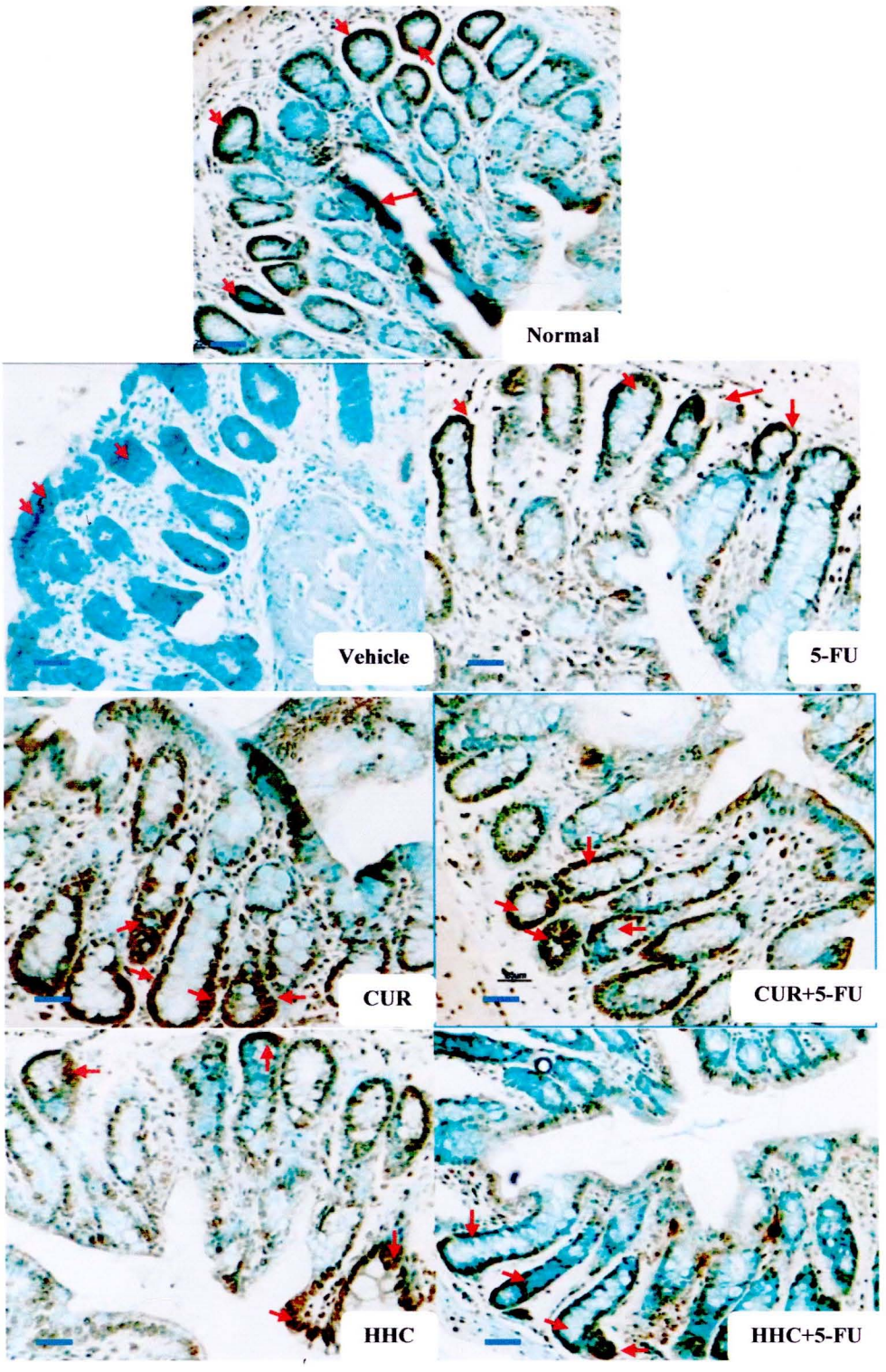

Figure 38 Apoptosis labeled with TUNEL method. Arrow (→) indicated the apoptosis labeled cell. Scale bar = 25μm.

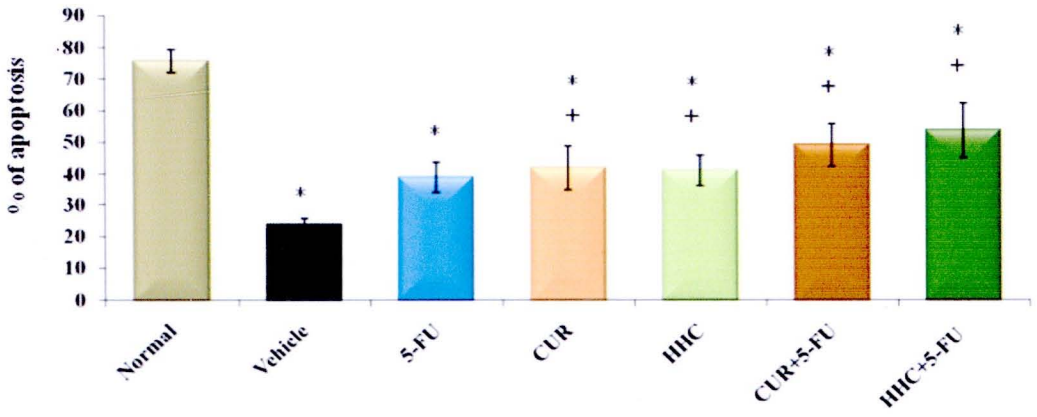

Figure 39 Apoptosis in colon of rat after exposed to different treatments. Each value was represented by mean±SEM. * indicates statistically significance values compared to normal ($P<0.05$); $^{+}$ compared with vehicle ($P<0.05$).