CHAPTER V

DISCUSSION AND CONCLUSION

This study aimed to determine the synergistic effect of HHC and 5-FU in inhibiting human colon cancer cells and to explore potential molecular mechanisms using *in vitro* cell culture and *in vivo* rat model.

Cytotoxic effect of 5-FU on growth of HT-29 colon cancer cells

The effect of 5-FU on HT-29 cells was examined by using a MTT reduction assay. HT-29 cells were exposed to various concentrations of 5-FU (0, 5, 10 and 25 μM) for 24, 48 and 72 h. The results showed that all doses of this drug significantly decreased cell viability when compared to a control. However, there was not significantly difference between doses. It seemed that the HT-29 cells were relatively resistant to 5-FU, in agreement with other (Du, et al., 2006). In addition, many studies confirmed that systemic administration of this drug would generate unacceptable levels of toxicity to normal cells, especially bone marrow and gastrointestinal tract, resulting in server adverse effects (Houghton, et al., 1979; Choi, et al., 2007). These side effects restrict extensive clinical applications of 5-FU to patients and affect on quality of patient life. Moreover, cancerous cells develop drug resistant overtime have been major obstacles in successful colorectal cancer chemotherapy. Various mechanisms of resistance have been proposed, including gene mutation, increased level of TS expression (Bunz, et al., 1999; Brody, et al., 2009). Therefore, from the MTT results, we used 5 μM of 5-FU in combination with CUR and HHC to minimize the toxicity and enhance its therapeutic effectiveness of 5-FU.

Effect of HHC alone and in combination with 5-FU on growth of HT-29 cells

To further investigate the combination effects of HHC and 5-FU treatment, the first we characterized ability of HHC to play a role as a potential chemotherapeutic agent for human colorectal cancer. We evaluated the HHC cytotoxic effect*invitro* study and compared its effects with a progenitor CUR and 5-FU standard chemotherapy.

Treated with all doses of HHC alone for 24, 48 especially 72h could significantly decreased cell viability of HT-29 colon cancer cells when compared to a control. For comparative studies, the results indicated that HHC exhibited cytotoxic effect to HT-29 colon cancer cells on time and concentration-dependent manner. The IC_{50} value indicated that the cytotoxic activity of CUR at 24h and 48h were highest activity against the HT-29 cells. The cytotoxic effect remarkable inhibited in the order: CUR> HHC > 5-FU. In agreement with this finding, several studies demonstrated that CUR have been shown to inhibit cell proliferation in a wide variety of human cancer cells, including colon cancer cells (Siwak, et al., 2005; Johnson, et al., 2009; Sahu, et al., 2009). However, for long-time exposure (72 h) of these agents showed no differences observed in the effectiveness of CUR and HHC in inhibited the proliferative of HT-29 human colon cancer cell line. Our results confirm that CUR and HHC are potent inhibitor of growth in HT-29 cells.

The combination effect of HHC and 5-FU on growth of HT-29 colon cancer cells was also studied by MTT reduction assay. HT-29 cells were exposed to low dose (5 μM) of 5-FU concurrently with various doses of HHC for 24, 48 and 72h. The results showed that HHC at 10 and 25 μM in combination with low dose of 5-FU for 24 and 48 h significantly decreased the cell viability of HT-29 when compared to HHC and 5-FU monotherapy. Especially, this combined schedules exhibited a synergistic effect (CI < 1) on anti-proliferation against HT-29 human colon cancer cells. However, this combined treatment for long-time exposure (72 h) showed no difference observed in decreased the cell viability of HT-29 human colon cancer cells when compared to monotherapy and also exhibited additive effect (CI > 1) on anti-proliferation against HT-29 human colon cancer cells. From this results, we suggested that long-time exposure of 5-FU may resulting the HT-29 human colon cancer cells were resistant to 5-FU chemotherapy.

Furthermore, this study compared the efficacy of this combined treatment with CUR in combination with 5-FU. The results showed that CUR in combination with 5-FU treatment for 24 and 48 h exhibited a quantitative synergistic inhibitory effect (CI < 1) on growth of HT-29 cells which in agreement with previously studied (Du, et al., 2006).However, the efficacy of this combined treatment did not significantly difference from HHC in combination with 5-FU.

Effect of HHC alone and in combination with 5-FU on COX-2 mRNA and protein expression in HT-29 cells

The present study found that HHC significantly decrease the level of COX-2 mRNA in HT-29 human colon cancer cells when compared to a control (data not shown). These results correlated with previous study, which suggested that HHC inhibits the biosynthesis of prostaglandin (PGE_2) in LPS-stimulated macrophages (Shao, et al., 2003) and could sensitize the cancer cells to chemotherapeutic drugs by decreasing PGE_2 levels on phorbol ester-induced PGE_2 production in human colonic epithelial cells (HCECs) (Ireson, et al., 2001). PGE_2 is a major product of COX-2 enzymes implicates in colorectal carcinogenesis and has been shownto stimulate the growth of human colorectal carcinoma cells (Williams, et al., 1996; Sunayama, et al., 2002; Janssen, et al., 2006). These results in agreement with a progenitor CUR in the present and previously studies which demonstrated that CUR directly down-regulated the expression of COX-2 mRNA and protein in several gastrointestinal cell lines and also in colon cancer cell lines (Goel, et al., 2001; Lev-Ari, et al., 2005; Binion, et al., 2008). Moreover, CUR suppressed COX-2 mRNA and protein in human intestinal microvascular endothelial cells (HIMECs), stimulated with vascular endothelial growth factor (VEGF) resulted in inhibits microvascular endothelial cell angiogenesis(Binion, et al., 2008).

Furthermore, HHC (25 μM) combined with low dose of 5-FU for 24 h of treatment could significantly down-regulated the COX-2 mRNA and protein expression when compared to either 5-FU or HHC alone.

Based on the cytotoxic effect, the expression of COX-2 mRNA and protein results, the cytotoxic effect of CUR and HHC were moderately decreased cell viability of HT-29 colon cancer cells after 24 h of incubation, whereas highly cytotoxicity of these two agents were found after 48 and 72 h of incubation. Therefore, we suggested that combined treatment of HHC and low dose of 5-FU for appropriate time resulted in synergistically inhibited the growth of HT-29 colon cancer cells. This study correlated with previous reports which demonstrated that CUR usually used in combination with traditional chemotherapeutic drugs or other regimens both *invitro*, *invivo* and also in clinical studies of colorectal cancer. CUR enhances the cytotoxic effect of celecoxib in several human colon cancer cell lines. They found that this combined treatment

synergistically inhibit on growth of colon cancer cells associate with suppressed the synthesis of PGE_2 and down-regulated COX-2 mRNA expression (Lev-Ari, et al., 2005).

Furthermore, the present study was compared the combination effects of 5-FU with CUR and HHC on COX-2 mRNA and protein expression of HT 29 human colon cancer cells. The result showed that the expression of COX-2 mRNA and protein were not difference between treated with 5-FU combined with HHC or CUR. However, it is reasonable to assume that HHC alone or in combination with 5-FU are effective agent for inhibit the expression of COX-2 mRNA and protein of human colorectal cancer.

Effect of HHC alone and in combination with 5-FU on COX-1 mRNA and protein expression in HT-29 cells

This study further investigated the HHC alone and in combination with 5-FU on COX-1 mRNA and protein expression of HT-29 human colon cancer cells. The study found that HHC alone and in combination with 5-FU did not alter the level of COX-1 mRNA and protein. Several studies reported that COX-1 is a housekeeping enzyme that plays an essential homeostatic role in gastrointestinal (GI) cytoprotection(Kargman, et al., 1996; Capone, et al., 2010), while COX-2 plays dominant roles in pathophysiologic process. Non-steroidal anti-inflammatory drugs (NSAIDs) are beneficial, and adverse effects due to the inhibition of COX-1. Inhibition of constitutively expressed COX-1 in the GI tract and in platelets by NSAIDs seems to play a role in the increased risk of upper GI bleeding or perforation (Patrono, et al., 2001).

Based on these results, it is reasonable to assume that this therapeutic approach may have no toxic effect and may enable the use of 5-FU chemotherapy drug at a lower and safer concentration which would be highly desirable for long-term treatment of colon cancer.From these results may suggested that HHC is a specific COX-2 inhibitor same as CUR. Several studies demonstrated that CUR specifically inhibited COX-2 mRNA and protein expression which highly expressed in human colorectal cancer but did not alter the level of COX-1 (Plummer, et al., 1999; Goel, et al., 2001). CUR is considered as a possible, safe and non-toxic chemopreventiveand

chemotherapeutic agent for human colorectal cancer (Goel, et al., 2001; Rowe, et al., 2009; Teiten, et al., 2010). As a result, CUR has already been used as chemopreventive treatment in some preclinical trials. They found that CURdid not cause adverse effects even at high dosages (Sharma, et al., 2004; Dhillon, et al., 2008). Based on their successful completion suggests that the use of CUR may increase in the future (Du, et al., 2006). In addition, thisstudy confirmed the previous results that HHC or CUR alone and in combination with 5-FU did not suppressed COX-1 level.

Effect of HHC alone and in combination with 5-FU on apoptotic induction of HT-29 cells

This study evaluated the potential benefits of HHC treatment alone or in combination with 5-FU on apoptotic induction of HT-29 cells by Hoechst 33342 staining assay. This assay was performed to observe the combination treatment on cell nuclear morphology. The microscopic observations showed that the control cells displayed intact nuclear structure, while the nuclei of treated cells composed of chromatin condensation and formation of apoptotic bodies similar to treatment by CUR alone and in combination with 5-FU. Importantly, this study found that the number of apoptotic cells markedly increased when these two drugs were combined as compared to monotherapy with the drugs.

Based on these results, this study suggested that addition of HHC could enhance the effects of 5-FU increased apoptotic of HT-29 human colon cancer cells. This synergism was mediated through a mechanism that probably involves inhibition of the COX-2 pathway. These results in agreement with previously study (Du, et al., 2006) considered that the systemic assessment of the pharmacological modulation of COX-2 might be a useful biomarker of drug efficacy and provide a surrogate measure of COX-2 in inhibitory effects in the target tissue (Plummer, et al., 2001).

Recent reports have demonstrated that overexpression of COX-2 is also present in several human cancer types including colorectal cancer (Tsujii and DuBois, 1995; Oshima, et al., 1996; Tsujii, et al., 1997). Furthermore, up-regulation of COX-2 could confer resistance to apoptosis induction (Tsujii and DuBois, 1995) or stimulates the production of angiogenic factors, which increases metastatic potential of cancer cells (Song, et al., 2001). In addition, lack of COX-2 expression results in decreased

growth and number of tumor cells that develop in $APC^{\Delta 716}$ knockout mice (Oshima, et al., 1996). Previously study demonstrated that CUR treatments alone or in combination with celecoxib inhibit proliferation and induction of apoptosis in human colon cancer cells which correlated with inhibition of PGE_2 synthesis and down-regulation of COX-2 (Lev-Ari, et al., 2005; Lev-Ari, et al., 2006). However, the apoptotic induction after treated with 5-FU and HHC not differ from 5-FU combined with CUR.

In summary, this study's findings illustrate that HHC is specific COX-2 inhibitor, which plays an important role in carcinogenesis.These results have demonstrated that HHC treatment alone could sensitize the HT-29 human colon cancer cells to chemotherapeutic drug by inhibiting the expression of COX-2 and induction of apoptosis. Although previous study reported that HHC exhibits stronger antioxidant activity than CUR. They suggested that the hydrogenation at the conjugated double bonds of the central seven carbon chain and a keto group of the ß-diketone of CUR to HHC markedly enhances antioxidant activity (Somparn, et al., 2007). However, this study found that the anti-carcinogenic effect of HHC did not differencefrom a progenitor CUR. Thus it could be suggested that HHC exhibits stronger antioxidant activity than CUR but not difference in inhibited the growth of HT-29 human colon cancer cells.

HHC augments the growth inhibitory effect of 5-FU. This synergism was mediated through a mechanism that probably involves inhibition of the COX-2 pathway which mediates the apoptosis of HT-29 human colon cancer cells. However, the inhibitory effect of this combination treatment was not higher than CUR together with 5-FU.

Effect of HHC alone or in combination with 5-FU on ACF formation in DMH-induced colorectal cancer rat

The DMH-induced colorectal cancer model has previously been applied in the prediction of the CUR ability to reduce the ACF formation (Kwon and Magnuson, 2009). Therefore, this study was assessed the ACF formation in colon epithelium of DMH-induced colon cancer rats by recording two parameters of ACF: (1) number of ACF and (2) large ACF. The present results showed that all rats developedACF

formation in the colon tissues especially in the middle and distal part of colon (data not shown) but was not observed in normal rats which similar to previous study. DMH is an alkylating carcinogen induced colon tumors which currently the most popular models to study the morphology, prevention and treatment of colorectal cancer. DMH is metabolized P450 to methyldiazonium to exert its carcinogenic effect in the colon (Fiala, et al., 1987; Barth, et al., 2005). In the rodent DMH model, DNA alkylation leading to DNA damage has been shown to be closely associated with increased epithelial proliferation, which taken together result in enhanced tumor induction in the distal colon (Barth, et al., 2005). Several studies reported that administrated of DMH develop aberrant crypt foci (ACF) in colon of rodents and increase with time (Caderni, et al., 1995; Cameron, et al., 1996; Park, et al., 1997). Furthermore, ACF has been described as single or clusters of abnormally large crypts of the colon mucosal surface after stained with methylene blue. They have been generally accepted as precancerous lesions both in rodents and human colorectal cancer (McLellan, et al., 1991; Cheng and Lai, 2003) and proved to be a reliable biomarker in short-term screening assay for colon carcinogenesis in laboratory rodents (Velmurugan, et al., 2008). They represent lesions that can also be characterized by genetic and biochemical alterations (Pretlow, et al., 1992; Cheng and Lai, 2003). The number of crypts per ACF also termed "crypt multiplicity" increase in advanced stages of carcinogenesis and correlates particularly well with the incidence of colorectal adenomas (McLellan, et al., 1991; Magnuson, et al., 1993). In particularly, ACFs in colon of rodents treated with carcinogens were closely resembled aberrant crypts seen in human colorectal cancer. Moreover, ACF in human colon were more often located in the distal parts than in the proximal parts, which was verified in rodent treated with carcinogens(Shpitz, et al., 1998; Bouzourene, et al., 1999). Therefore, crypt multiplicity would be an important parameter for evaluating ACF progression.

In the present study, the ACF formation markedly reduced after treated with HHC alone when compared to a vehicle group and also reduced in 5-FU treated group. Importantly, HHC administration significantly reduced the large ACF as compared to vehicle group but did not differ from CUR treated alone. Several studies reported that ACF are recognized as early preneoplastic lesions in colorectal carcinogenesis while crypt multiplicity may correspond to the promotion step of colon carcinogenesis (Bird,

1995; Roncucci, et al., 1998; Zhang, et al., 1992). Based on these results, we suggested that HHC has ability to inhibit both initiation and promotion steps of colorectal carcinogenesis which similar to CUR property. This result was confirmed the previous results that CUR could reduce the formation and progression of ACF toward colorectal cancer (Wargovich, et al., 1996; Wargovich, 2001).

HHC together with 5-FU showed markedly inhibited the formation of ACF and specially reduced the number of large ACF when compared to vehicle group. Interestingly, this combined treatment showed significantly decreased the formation of ACF when compared either HHC or 5-FU treated alone. Moreover, the number of large ACF in the DMH-treated rats that received both HHC and 5-FU treatment was significantly decreased when compared to vehicle treatment but did not difference when compared either HHC or 5-FU monotherapy. Moreover, this study found that the formation of ACF and number of large ACF did not difference between two combined treatments. Therefore, it is reasonable to assume that HHC augments the growth inhibitory effect of 5-FU chemotherapy by inhibited both initiation and promotion steps of colorectal carcinogenesis but these effects did not difference from CUR together with 5-FU treatment. These results confirmed previous studied that CUR together with 5-FU significantly decreased the formation ACF more than either CUR or 5-FU treated alone in DMH-induced rat model. Therefore, this study suggested that CUR together with 5-FU synergistically inhibited colorectal carcinogenesis both initiation and promotion step in rat colorectal cancer model induced by DMH chemical carcinogen. Combination therapy of anticancer agents is an attractive trend in chemoprevention strategies. Previous study demonstrated that CUR augment growth inhibitory effect of celecoxib, selective COX-2 inhibitor(Shpitz, et al., 2006)and 5-FU chemotherapy. CUR combined with 5-FU shown quantitative synergistic (CI <1) growth inhibition of HT-29 human colon cancer cells (Du, et al., 2006) and also inhibit growth of HCT-116 human colon cancer cells than 5-FU or CUR treated alone (Patel, et al., 2008).

Effect of HHC alone or in combination with 5-FU on COX-2 in DMH-induced colorectal cancer rat

This study found the expression of COX-2 protein in the cytoplasm of colorectal mucosa of all rats exposed to DMH which agree with previous reports (Brosens, et al., 2008). The COX-2 positively stained cells in distal colon were counted by image analysis. The result showed that the COX-2 level was high in colon tissues of vehicle and 5-FU treated group. After treated with HHC alone could significantly decrease the expression of COX-2 protein as compared to a vehicle treatment group and also decreased after treated with CUR alone. Based on this result, it is suggested that HHC plays a role in inhibited the expression of COX-2 protein in colorectal carcinogenesis of rat model which similar found in a progenitor CUR(Goel, et al., 2001) and decreased the COX-2 protein in adenoma tissue of Apc^{Min+} mouse model (Tunstall, et al., 2006). Furthermore, HHC also inhibited the expression of COX-2 protein in LPS-stimulated macrophages (Shao, et al., 2003).

The combined effect of HHC and 5-FU showed statistically decreased the level of COX-2 protein when compared to a vehicle treatment group,but this effect did not different from HHC or 5-FU monotherapy which not correlated with our *in vitro* study. Based on these result, it may be suggested that this combined treatment could reduced the ACF formation in the early stage of colorectal carcinogenesis by difference in their mode of actions. In order to stop the growth of early colorectal carcinogenesis, it is important to arrest cell cycle which similar to progenitor CUR mechanism. Previous study reported that CUR significantly decrease the cell numbers in the G1 phase and significantly increase in the percentage of cells in the G2/M phase of the cell cycle of HT-29 colon cancer cells (Van Erk, et al., 2004) and other cancer types (Saha, et al., 2010; Lee, et al., 2011). In addition,CUR induced G2/M phase in cancer cells by down-regulate cyclin D1 expression in cancer cells, and by enhancing the p53 phosphorylationthrough the activation of caspase-3 followed by PARP degradation(Weir, et al., 2007).

Effect of HHC alone or in combination with 5-FU on COX-1 in DMH-induced colorectal cancer rat

COX-1 was the most observed in cytoplasm of colonic crypt of all animals. In the present study, the level of COX-1 protein after treated with HHC was not different from normal rats which correlated with our *in vitro* HHC and a progenitor CUR and also agreement with previous report (Goel, et al., 2001). Therefore, HHC can be classified as a selective COX-2 inhibitor as compared to some NSAIDs, such as meloxicam, diclofenac, and indomethacin (Engelhardt, et al., 1996) which inhibits both COX-2 and COX-1 expression cause injury to the gastric mucosa. In addition, the final body weight and organ weight ratio of colon, liver, spleen and kidney (data not shown) in all experimental groups were no significant differences between groups. Based on these results, it is suggested that HHC treated alone not produced unwanted side effects both *in vitro* and colorectal cancer rat model. Therefore, HHC administration suitable for prevent and treatment of human colorectal cancer in long-term period.

In the combined treatment, HHC and 5-FU did not alter the COX-1 protein level which correlated with this*in vitro* study. Moreover, the rats treated with HHC alone or in combination with 5-FU showed no adverse effects on growth rate including organ weight ratio. Therefore, this study suggested that HHC combined with 5-FU suitable for prolong treatment of human colorectal cancer.

Effect of HHC alone or in combination with 5-FU on apoptotic induction in DMH-induced colorectal cancer rat

In the present study, the colonic cell apoptosis in DMH-injected group were significant less than normal rats especially very low in vehicle treated group. Previous study reported that up-regulation of COX-2 causes increased binding of cells attach to basement membrane and survive on it and make them resistant to apoptosis (Tsujii and DuBois, 1995). Based on these results, the damaged colonic cells in DMH-injected groups risk develops to colorectal cancer which confirmed by increased the number of ACFs. In addition, the colonic cells elevated proliferation and reduced apoptosis are characteristics of colorectal cancer. Apoptosis provides a protective mechanism against neoplasia by moving genetically damaged stem cells from the epithelium

before they can undergo clonal expansion.Therefore, apoptosis has a profound effect on the progression from a benign to a malignant phenotype and can be targeted for treatment of various malignancies including colorectal cancer (Velmurugan, et al., 2008).

Treated with 5-FU, CUR and HHC alone significantly induced apoptosis when compared to a vehicle group, although the differences did not reach significance between group. These results demonstrated the ability of 5-FU, CUR and HHC to induce apoptosis correlates with their ability to inhibit the ACF formation. Based on these results, it is suggested that HHC may suppress the promotion of colorectal carcinogenesis through its ability to induced apoptosis. This effect similar to a progenitor CUR treatment either the current study or previous reports. The apoptosis as one of the mechanisms of CUR-induced tumor inhibition which mediated through the inhibition of COX metabolites (Rao, et al., 1995). Moreover, CUR induce the apoptosis in human colon cancer cells by activated p53 and regulated apoptotic-related proteins (Song, et al., 2005; Wang, et al., 2009) and down-regulated NF-κB anti-apoptotic gene (Collett and Campbell, 2006). CUR decreased the amount of ACFs and induced apoptosis in the colorectal mucosa of rat model by regulated apoptotic-related proteins (Volate, et al., 2005). In addition, the ability to inducing apoptosis of colorectal cancer cells was also observed after treated by a CUR analogue, dimethoxycurcumin(Tamvakopoulos, et al., 2007). Although the precise mechanism by which HHC inhibits the DMH-induced colon carcinogenesis has not been established, these results would appear that possible action involves inhibit the expression of COX-2 similar to a progenitor CUR.

The combined treatment of HHC and 5-FU showed significantly induced apoptosis when compared to vehicle control group but not difference from HHC and 5-FU treated alone. Moreover, the effect of this combined treatment did not better than 5-FU combined with CUR. These findings suggested that HHC combined with 5-FU did not show a synergistic effect to induced apoptosis in colorectal carcinogenesis of DMH-induced rat model that correlated with our COX-2 result. Therefore, the combination effects of these agents in inhibit the ACF formation may be exhibit through other mode of actions, such as arrest cell cycle.

In summary, the evidences presented in this *in vivo* study give new insight into the anti-carcinogenesis mechanism of HHC in colorectal cancer model. The finding of this study indicating that addition of DMH caused the COX-2 overexpression resulting in resistance to apoptosis induction in colorectal tissues of animal model. Daily administration of HHC was shown to suppress the growth of colorectal cancer at the initiation step mediated though down-regulated the expression of COX-2 and induction of apoptosis. Moreover, the lack of toxicity and side effects of HHC, as well as its availability in large quantities as natural products that has been used in population groups and encouraging for further study the other mechanism and investigation in pre-clinical and clinical trial although, the inhibitory effects of HHC were not higher than a progenitor CUR. However, the down-regulation of COX-2 and induction of apoptosis was not enhanced by the combined treatment of HHC and 5-FU.

In conclusion, results from *in vitro* and *in vivo* assay illustrate that HHC is a specific COX-2 inhibitor (Figure 40), which plays an important role in colorectal carcinogenesis. It has been demonstrated for the first time that HHC could decrease cell viability, down-regulated COX-2 mRNA and protein and induced apoptosis in HT-29 human colon cancer cells and DMH-induced colorectal cancer rats. Moreover, HHC did not exhibit unwanted side effects both *in vitro* and *in vivo* studies.

The addition of HHC to 5-FU standard chemotherapy exhibited a quantitative synergistic inhibitory effect by decreasing cell viability of HT-29 human colon cancer cells though down-regulated COX-2 mRNA and protein and induced apoptosis but these effects did not differentfrom a progenitor CUR. *In vivo* study, HHC together with 5-FU treatment exhibited a synergistic growth inhibitory by decreasing ACF formation. However, the mechanism of action of this combined treatment should be further study.

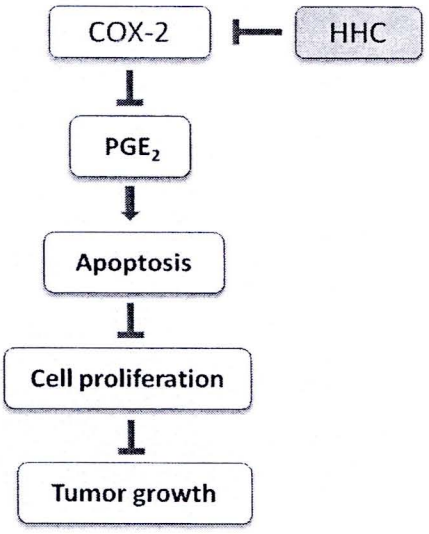

Figure 40 The possible mechanism of HHC on inhibition of colorectal carcinogenesis