

Chrysin-Enhanced Cytotoxicity of 5-Fluorouracil-Based Chemotherapy for Colorectal Cancer in Mice: Investigating its Effects on Cyclooxygenase-2 Expression

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5-fluorouracil (5-FU) has been recognized as an effective medication used to treat colorectal cancer (CRC); however, its administration is facing limitations due to some complications reported. It is also generally accepted that combination therapy is among strategies to improve chemotherapy efficiency. Therefore, chrysin, with its anticancer effects, in combination with 5-FU was investigated in the present study. Azoxymethane (AOM) as a carcinogenic substance along with dextran sodium sulfate (DSS) was additionally utilized to induce CRC in mice. The anticancer effects of chrysin were then evaluated using aberrant crypt foci (ACF) counting and percentage of pathologic lesions in epithelial tissues from distal colon. In this study, cyclooxygenase (COX-2) protein expression was correspondingly explored through immunohistochemistry (IHC). The results revealed that chrysin alone or in combination with 5-FU could decrease ACF counting and percentage of pathologic lesions in comparison with AOM ($p < 0.05$). Moreover, the combination of chrysin (at a dose of 50 mg/kg) with 5-FU reduced COX-2 expression compared with 5-FU alone ($p < 0.001$) or 5-FU in combination with chrysin at a dose of 100 mg/kg ($p < 0.05$). Furthermore, the combined chrysin boosted 5-FU efficiency, so it was suggested as an auxiliary therapy for CRC.

Keywords: Colon cancer. Combination treatment. Chrysin. Cyclooxygenase-2.

INTRODUCTION

Colorectal cancer (CRC) has been acknowledged as the third most common type worldwide and also one of the most important causes of mortality. Despite development of diagnostic and therapeutic approaches, CRC incidence is still on an increasing trend (Loomans-Kropp, Umar, 2019).

As well, 5-fluorouracil (5-FU) has been introduced as one of the common medications used to treat CRC; however, its efficacy has been limited due to some side effects, toxicity, as well as drug resistance (Srimuangwong

et al. (2012). Accordingly, a new strategy to augment efficacy of synthetic drugs in chemotherapy is their administration with natural phytochemicals such as polyphenols (Fantini *et al.*, 2015).

Chrysin is also known as a natural flavonoid found in many herbs, honey, and especially propolis, with anticancer, antioxidant and anti-inflammatory activities. (Khan, Devaraj, Devaraj, 2011; Lim *et al.*, 2017). As reported, presence of this constituent in the intestinal mucosa of rats exposed to the carcinogenic substance of 1, 2-dimethylhydrazine has already reduced frequency and severity of preneoplastic lesions (Sequetto *et al.*, 2013). Chrysin has further led to inhibiting proliferation and inducing apoptosis in U937 leukemia cells (Woo

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et al., 2004) and has moderated cyclooxygenase (COX-2) expression in RAW 264.7 macrophage-like cells (Harris *et al.*, 2006). It should be noted that the Cox-2 as an enzyme plays an important role in development and progression of malignant diseases including CRC (Sharma *et al.*, 2003; Su, Zhang, Zhu, 2016). Hence, elevation in Cox-2 expression can be associated with a growth in levels of anti-apoptotic protein of B-cell lymphoma 2 (Bcl-2), which causes resistance to apoptosis in the intestinal epithelial cells (Sun *et al.*, 2002). Improving COX-2 expression and its end product, i.e. prostaglandin E2 (PGE2), has thus a significant contribution to carcinogenesis, and, on the other hand, reducing or inhibiting its expression leads to cancer prevention or treatment (Harris, 2007).

Chrysin in A549 lung cancer cell line has been likewise shown to increase efficacy of Docetaxel, as an anticancer chemotherapy drug (Lim *et al.*, 2017) which has induced apoptosis with cisplatin in Hep G2 cells (Li *et al.*, 2015c). This natural flavonoid has also amplified the sensitivity of DO-7402 cells resistant to doxorubicin (Gao *et al.*, 2013). In this respect, the findings of a previous study had suggested that hexahydrocurcumin (HHC) in combination with 5-FU had resulted in improved CRC induced in rats (Srimuangwong *et al.*, 2012). In another research, the combination of 5-FU and Paclitaxel (PTX) had been reported to be effective in treating advanced gastric adenocarcinoma (Murad *et al.*, 1999). It has been shown that the combination of 5-FU with celecoxib increased COX-2 inhibition in the CRC animal model (Zhang *et al.*, 2013). The combination of Weichang'an (WCA) pill, as a traditional Chinese formula, with 5-FU could also suppress CRC in an animal model (Tao *et al.*, 2015).

Therefore, the present study examined the effects of chrysin alone and in combination with 5-FU, for the first time, on aberrant crypt foci (ACF) formation as a risk marker for CRC development and histopathologic lesions with a focus on COX-2 protein expression in colon epithelium of a CRC animal model.

MATERIAL AND METHODS

Animals and chemicals

This study was conducted on a total number of 42 adult male Balb/C mice with an average weight of 15-18 grams prepared from the Pasteur Institute of Iran (PI). The animals were also kept under standard conditions with free access to water and food. All of the experimental procedures were conducted in agreement with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. This study was approved by the Ethics Committee of the University (code of ethics: 1394.99).

The chrysin (Sigma-Aldrich Corp., USA) was first dissolved in tris-buffered saline (TBS) (pH=9) and then reached pH=7.2 with one normal solution of hydrochloric acid (1 N HCl), and then stored in a refrigerator. The required concentration of 5-FU (Ebewe Pharma GmbH, Austria) was correspondingly prepared in normal saline.

Experimental protocol

To induce the cancer model, the mice were adapted to the environment (day zero) for one week, and then injected intraperitoneally with 10 mg/kg of azoxymethane (AOM) (Sigma-Aldrich Corp., USA). After one week, 1.5% dextran sodium sulfate (DSS) (Sigma-Aldrich Corp., USA) was added to drinking water of mice for one week (Miyoshi *et al.*, 2011). The treatment started with 5-FU (50 mg/Kg) simultaneously with AOM injected intraperitoneally once a week (Srimuangwong *et al.*, 2012). The treatment with chrysin was further commenced at the desired doses (Li *et al.*, 2015b; Rashid *et al.*, 2014; Sequetto *et al.*, 2013) as intraperitoneal injection concurrently with AOM, and continued for eight weeks and five days a week. The animals were thenceforth allocated randomly to seven groups (that is 6 mice per group). Group 1 (normal control, N-Con) received intraperitoneal saline solution, and groups 2 to 7 experienced the cancer. Group 2 (i.e. AOM control) took only saline solution after induction of cancer. Group 3 (namely, 5-FU) was treated with 5-FU, and Group 4 (that is, chrysin50; Ch50) was administered with 50

mg/kg of chrysin. Moreover, Group 5 (i.e. Ch100) was treated with 100 mg/kg of chrysin, Group 6 (that is, 5-FU+Ch50) received 5-FU+50 mg/kg of chrysin, and Group 7 (namely, 5-FU+Ch100) took 5-FU+100 mg/kg of chrysin.

Preparation of tissue samples

Once the animals were euthanized, the colon was dissected, washed with normal saline, incised longitudinally, and cut into three equal parts (i.e. proximal, middle, and distal colon with rectum).

The colon was subsequently fixed in formalin buffer 10% (pH=7.2) for 24 hours. Distal colon was also used to assess aberrant crypt foci (ACF) counting and to perform histopathology and immunohistochemistry.

ACF analysis

After 24 hours, the distal colon was transferred onto the slide with the mucosal side upward, stained with toluidine blue dye 0.1% for 2 minutes, and washed with phosphate-buffered solution (PBS) (pH=7.2). A stereo microscope (magnification of 60×) equipped with an OLYMPUS DP11-N digital camera was also used to detect and count ACF at the level of the intestinal mucosa. ACF was then classified with reference to the frequency of aberrant crypts in each focus as Grade 1: a focus with an aberrant crypt, Grade 2: a focus with 2 to 3 aberrant crypts, Grade 3: a focus with 4 to 10 aberrant crypts, and Grade 4: a focus with over 10 aberrant crypts (Srimuangwong *et al.*, 2012).

Histopathological analysis

The colon pieces fixed in formalin 10% were molded in paraffin blocks. From each block, five sections with a thickness of 5 µm were prepared with microtome (Leitz Co; Germany) and stained with hematoxylin-eosin stain. For each mouse, 10 microscopic fields were selected randomly and the histopathology of each crypt was also investigated using an optical microscope connected to a digital camera (OLYMPUS DP11-N, Sony, Japan) based on the following parameters (Perse, Cerar, 2011):

(1) hyperplasia (enhanced staining, nucleolus presence, and enlarged nucleus in the lower two-thirds of the crypt and reduced levels of mucin), (2) dysplasia at three mild, moderate and severe grades, and (3) carcinoma..

Immunohistochemistry analysis

The sections of the distal colon with a thickness of 5 µm were prepared for IHC staining. The slides were then deparaffinized with xylene, and hydrated using ethanol descent concentrations. Afterwards, antigen retrieval was carried out through the thermal process of TBS (pH=9). In the next step, the slides were rinsed with TBS+polysorbate 20 (also known as Tween 20) and blocked with 1% bovine serum albumin (BSA)+1% fetal bovine serum (FBS) solution in TBS for 2 hours. The slides were subsequently incubated by primary antibody (ab62331) of Cox-2 (at a dilution of 1 to 100) for 16 hours at 4°C. Endogenous peroxidase activity was further inhibited by 3% hydrogen peroxide (H₂O₂) in TBS for 15 minutes. Next, the slides were incubated by the secondary antibody (Goat Anti-Rat IgG H&L, ab9705) of Cox-2 (at a dilution of 1 to 200) for one hour at room temperature. After that, the slides were incubated by 3,3'-diaminobenzidine (DAB) as a widely used chromogen (ab94655) for 10-15 minutes and differential staining was completed with hematoxylin.

The image of each section was taken under a microscope by an OLYMPUS DP11-N digital camera (OLYMPUS DP11-N, Sony, Japan) with a magnification of 400× and 200×. Then, ten microscopic fields (with an area of 2000 µm²) were randomly explored to count Cox-2 positive cells in each section of the distal colon. Cox-2 expression was also reported as a percentage of the counting of positive staining cells divided by the total count of cells computed by Histopathology Image Analysis software. In this study, the liver tissues were used as positive control, and all immunohistochemical steps were performed for negative control and only the initial antibody incubation step was removed.

Statistical analysis

Data were collected, tabulated and were presented as mean ± SD. Attained data from different groups were

analyzed by SPSS version 22 software using one-way analysis of variance (ANOVA) and Tukey's post hoc test at a significance level of $P < 0.05$.

RESULTS

Effects of 5-FU and Chrysin and their Combined Treatments on ACF Formation

AOM and DSS caused ACF formation in the distal colon of mice (Figure 1). Moreover, no aberrant crypts were identified in the distal colons of N-CON mice. ACF counting in different groups also indicated that the AOM control group had a higher ACF with Grades 4 and 3

compared with the treatment groups (Figure 2). Moreover, treatment with 5-FU and chrysin alone (50 and 100 mg/kg) reduced the counting of ACF in the colon mucosa of cancerous mice compared with the AOM control group, but ACF with Grades 4 and 3 showed a significant decrease only in the 5-FU group compared with the AOM group ($p < 0.05$).

Besides, co-administration of 5-FU with any of the 50 and 100 doses of chrysin resulted in a significant drop in ACF counting compared with the 5-FU, Ch50, and Ch100 groups ($p < 0.05$). The number of crypts with Grade 3 in the 5-FU+Ch50 group was also lower than that of the 5-FU+Ch100 one ($p < 0.05$).

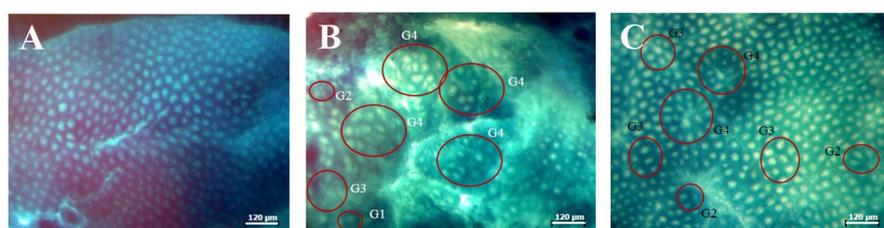


FIGURE 1 - Aberrant crypt foci with different grades in the longitudinal sections of the distal colon region stained with Toluidine blue. A: normal, the distribution of crypts is regular and uniform; B and C: Aberrant crypt foci with grades 1 (G1), 2 (G2), 3 (G3) and 4 (G4).

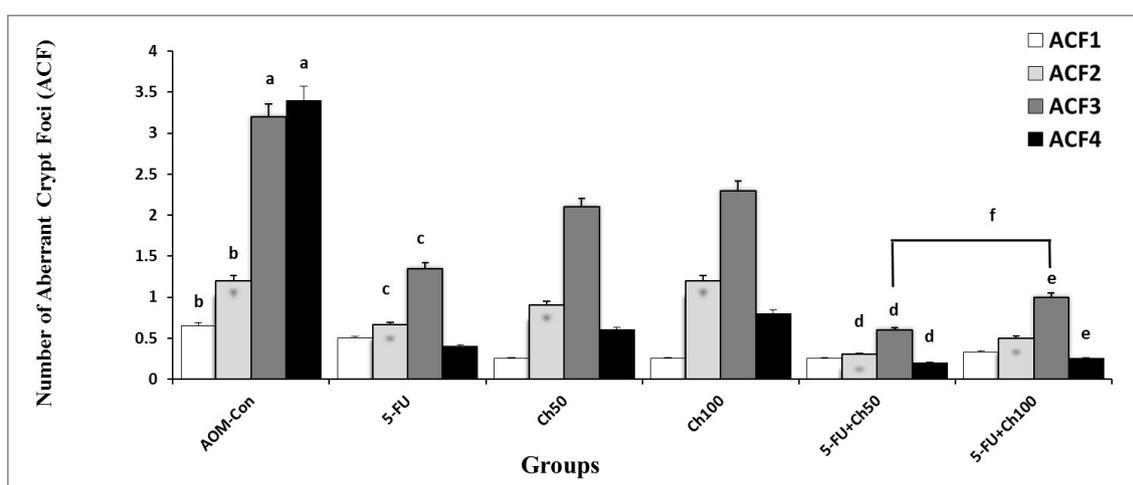


FIGURE 2 - The effects of 5-FU and chrysin and their combined treatments on the aberrant crypt foci (ACF) formation of the distal colon region in mice receiving azoxymethane. Significance level was $p < 0.05$. ACF1 = crypt with grade 1, ACF2 = crypt with grade 2, ACF3 = crypt with grade 3, ACF4 = crypt with grade 4. a: versus all treated groups, b: versus Ch50 + 5-FU and Ch100 + 5-FU, c: versus Ch100, d: versus Ch50, 5-FU and Ch100, e: versus Ch50, 5-FU and Ch100 groups, f: Significant difference of ACF3 between 5-FU + Ch50 and 5-FU + Ch100.

Effects of 5-FU and Chrysin and their Combined Treatments on Histopathological Parameters

The H&S-stained colon sections revealed that AOM had caused hyperplasia, dysplasia, and carcinoma in the distal colons of the mice (Figure 3). The pathologic lesions had further indicated that treatment with 5-FU, Ch50, and Ch100 alone had diminished the number of

lesions compared with that in the AOM group (Table I). Dysplasia and carcinoma were not, however, observed in the groups treated with 5-FU or Ch50 alone as well as 5-FU+Ch50 group. The percentage of lesions in the 5-FU+Ch50 and 5-FU+Ch100 groups was additionally lower than that in other treatment groups. As well, the percentage of lesions in the 5-FU+Ch50 group was found lower than that in the 5-FU+Ch100 group.

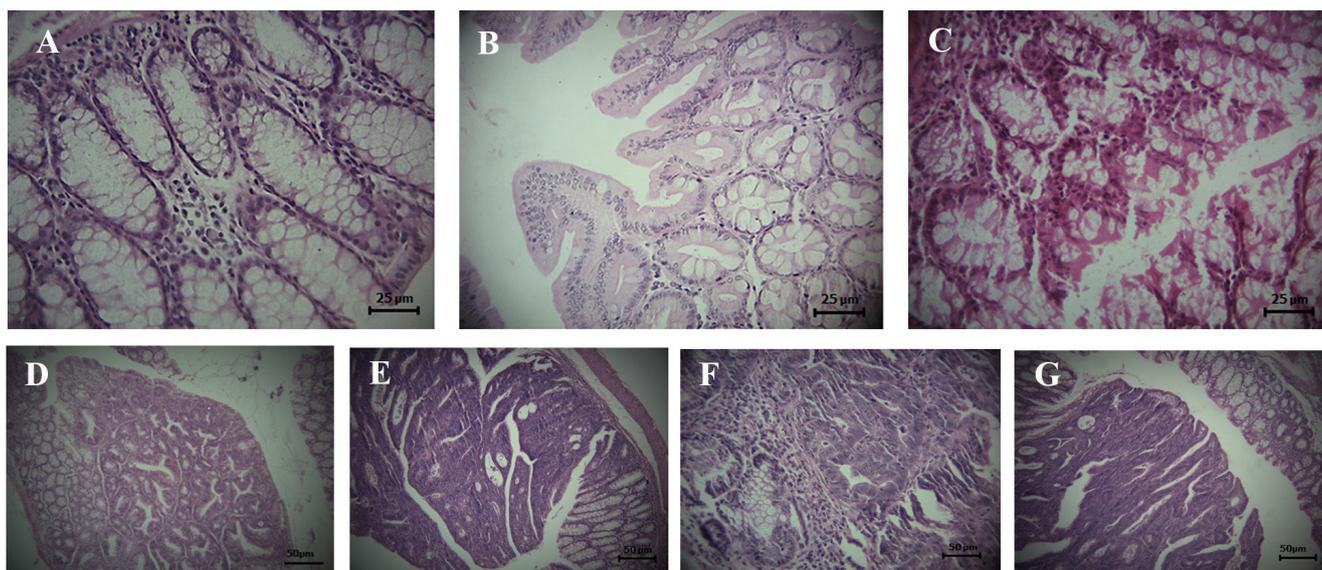


FIGURE 3 - Histopathologic indexes of distal colon after hematoxylin-eosin staining **A: Normal Control, B: Hyperplasia, C: Mild Dysplasia, D: Moderate Dysplasia, E: Severe Dysplasia, F: Insitu Carcinoma, G: Invasive Carcinoma.**

TABLE I - The effects of 5-FU and chrysin and their combined treatments on histopathologic indexes of the colon tissue in mice receiving azoxymethane

Animal Groups	Total No. of Samples	Total Lesions No. / %	Normal No. / %	Hyperplasia No. / %	Dysplasia No. / %	Carcinoma No. / %	Lesion Burden
Normal Control	36	0/0	100/36	0/0	0/0	0/0	0
AOM Control	36	77.8/28	22.2/8	16.7/6	38.9/14	22.3/8	4.66
5-FU	36	22.22/8	77.78/28	23.33/7	0/1	0/0	1.33
Ch50	36	27.78/10	72.22/26	26.66/9	0/1	0/0	1.66
Ch100	36	36.11/13	63.89/23	16.67/6	11.11/4	8.33/3	2.16

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TABLE I - The effects of 5-FU and chrysin and their combined treatments on histopathologic indexes of the colon tissue in mice receiving azoxymethane

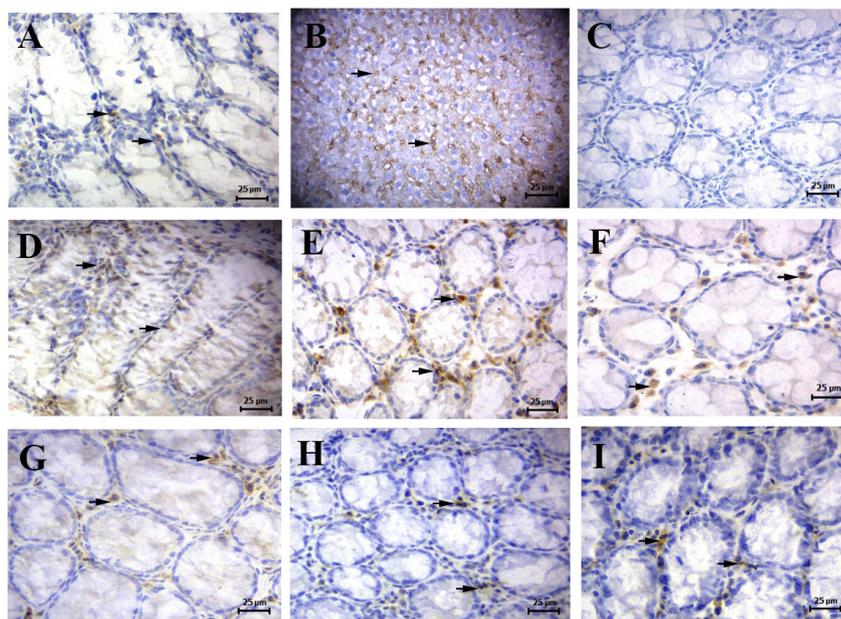
Animal Groups	Total No. of Samples	Total Lesions No. / %	Normal No. / %	Hyperplasia No. / %	Dysplasia No. / %	Carcinoma No. / %	Lesion Burden
5-FU+Ch50	36	11.11/4	88.89/32	11.11/4	0/0	0/0	0.66
5-FU+Ch100	36	16.67/6	83.33/30	11.11/4	5.56/2	0/0	1.0

Total Lesions: The number and percentage of total mice with lesions. Lesion burden: The ratio of the number and percentage of total lesions in each group to the total number of mice (n = 6). Normal: The number and percentage of samples with normal and healthy status compared to total samples. Hyperplasia, Dysplasia and Carcinoma: The number and percentage of samples with pathologic lesions compared to total samples.

Effects of 5-FU and Chrysin and their Combined Treatments on Cox-2 Expression

In IHC, cells with brown granules represent Cox-2 expression (Figure 4). Counting the number of Cox-2 positive cells also showed that Cox-2 protein expression was very low in the N-CON group, and AOM had then increased it (Figure 5). Cox-2 expression level in all treatment groups was also found lower than that in AOM

control group ($p < 0.01$). Treatment with chrysin (both doses) alone and in combination with 5-FU had correspondingly reduced Cox-2 expression level in comparison with 5-FU group ($p < 0.001$ or $p < 0.05$). In addition, the combination of Ch50 with 5-FU had resulted in a further reduction of Cox-2 expression in comparison with chrysin (both doses) alone ($p < 0.001$). The effect of Ch50 combined with 5-FU was also greater in terms of diminution of Cox-2 expression compared with that in Ch100+5-FU group ($p < 0.05$).



FIGURES 4 - Immunohistochemical staining of mice colonic tissue using Cox-2 antibody (brown, arrows) in different groups. All slides were counterstained with hematoxylin. A: Normal control, B: Positive control, C: Negative control, D: AOM control, E: 5-FU, F: Ch50, G: Ch100, H: 5-FU+Ch50, I: 5-FU+Ch100.

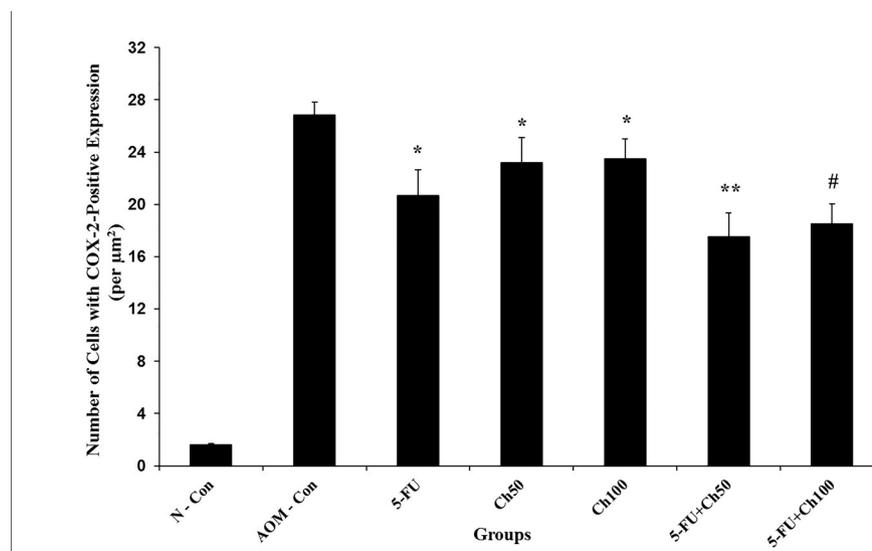


FIGURE 5 - Effects of Chrysin 50 mg/kg (Chr50), Chrysin 100 mg/kg (Chr100), 5-FU, and their combinations on the expression of cyclooxygenase-2 (Cox-2) protein in AOM-induced mice colorectal tumors (n=6). * $p < 0.01$ versus AOM-Con; ** $p < 0.001$ versus AOM - Con, Ch50, Ch100 and $p < 0.05$ versus 5-FU; # $p < 0.001$ versus Aom-Con, Ch50, Ch100.

DISCUSSION

5-FU alone or in combination with other chemotherapy medications is considered as a standard drug in treatment of human CRC. Use of 5-FU in clinical settings is, however, facing limitations due to drug resistance, some side effects, toxicity, and low dose-response rate (Srimuangwong *et al.*, 2012). Accordingly, combination therapy provides a new strategy for treating cancer through increased efficacy and reduced drug toxicity (Li *et al.*, 2015a). A combination of natural phytochemicals can thus reduce the required dose of drugs in chemotherapy and thereby minimize complications and drug resistance (Shakibaei *et al.*, 2013).

Chrysin has been thus introduced as a natural flavonoid with anti-inflammatory and anticancer activities. This constituent in cancer cell lines also enhances efficacy and augments sensitivity of anticancer drug-resistant cells (Gao *et al.*, 2013; Li *et al.*, 2015c; Lim *et al.*, 2017) As well, chrysin induces autophagy via increasing reactive oxygen species (ROS) level, thus inhibiting cell viability in CRC cells (Lin *et al.*, 2018). It additionally down-regulates COX-2 enzyme through activating peroxisome proliferator-activated receptor gamma (Liang *et al.*, 2001).

Therefore, a model of colorectal tumorigenesis was induced by AOM and DSS in mice to investigate the effects of chrysin in combination with 5-FU. In many ways, this model is similar to the human CRC and is being used in in-vitro studies. The present study was thus designed to evaluate the efficacy of chemopreventive chrysin alone and in combination with 5-FU with a focus on COX-2 protein expression in an animal model.

This study also evaluated the prevalence and frequency of colon polyps in different groups. Empirical evidence in this respect confirmed that ACF lesions had appeared in the stages of hyperplasia and dysplasia of the intestinal epithelium, and they had high potentials for being converted into intestinal carcinoma (Sequetto *et al.*, 2013). The results of the present study also demonstrated that the highest number of ACF and pathologic lesions were in the AOM control group. Reducing the number of ACF in mere chrysin-treated groups further reflected the effects of chrysin on inhibiting CRC onset and progression, which was consistent with the results of an investigation reflecting on the inhibitory effect of chrysin (Sequetto *et al.*, 2013). Besides, the co-administration of 5-FU with Ch50 or Ch100 compared with Ch50 or Ch100 alone showed a synergistic effect with a significant drop in ACF, which was similar to that of hexahydrocurcumin (Srimuangwong *et al.*, 2012),

as well as WCA pill in combination with 5-FU in CRC treatment (Tao *et al.*, 2015). In the present study, the effect of chrysin (at the dose of 50) in combination with 5-FU was better than that in the combination of chrysin (at the dose of 100) with 5-FU in terms of reducing the number of ACF, suggesting that the synergistic effects of these two drugs could be dose-dependent.

The pathologic lesions in this study denoted that although 5-FU or chrysin alone had reduced the number of lesions, the combination of chrysin with 5-FU had caused a further decline in pathologic lesions compared with each one alone. The fall in the percentage of pathologic lesions in different treatment groups was thus consistent with reduction in the number of ACF in the study groups.

In an animal model of dimethylhydrazine-induced CRC, chrysin also increased the number of mucin-secreting cells and decreased dysplasia crypt, mitosis, and argyrophilic nucleolar organizing regions (AgNORs/nucleus). It should be noted that AgNORs/nucleus is a marker of tumor severity and growth rate. The chrysin additionally improved CRC by inhibiting cell proliferation, retrieving antioxidant levels, and reducing oxidative stress (Sequetto *et al.*, 2013).

In the present study, AOM and DSS increased COX-2 protein expression, which was in line with previous research evaluating dimethylhydrazine-induced CRC in rats (Srimuangwong *et al.*, 2012; Takahashi *et al.*, 2000). Increasing COX-2 activity was also associated with CRC risk, in a way that its specific inhibitors had protective effects against CRC (Ren *et al.*, 2018). The cytoplasmic incidence of this enzyme was consequently observed in 85-90% of colorectal adenocarcinomas and 40-90% of colorectal adenomas. By producing prostaglandin E2 epithelial, COX-2 had further caused epithelial proliferation, apoptotic inhibition, and angiogenesis (Singh, Lucci, 2002; Wang, Dubois, 2010). In addition, PGE2 could bind to several prostaglandin (PG) E2 receptors, which could interfere with intracellular calcium or cyclic adenosine monophosphate (cAMP) levels and accordingly induce inflammatory responses (Su, Zhang, Zhu, 2016).

Therefore, reducing Cox-2 expression can be regarded as a strategy for CRC prevention and treatment (Ren *et al.*, 2018).

In this study, chrysin alone and in combination with 5-FU could reduce Cox-2 expression. In one other study, chrysin had also prevented the development of hepatocarcinoma by diethylnitrosamine in rats through reduction of Cox-2 expression (Khan, Devaraj, Devaraj, 2011). The results of this study showed that chrysin had a synergistic or incremental effect on 5-FU in terms of reducing COX-2 protein expression, which was consistent with the effect of hexahydrocurcumin in combination with 5-FU (Srimuangwong *et al.*, 2012). This flavonoid can thus obstruct cell division in G2 phase of the mitotic cycle and induce apoptosis in human CRC cells via activating caspases (Wang *et al.*, 2004). This natural constituent also inhibits cell growth by inhibiting PCNA expression in HeLa cells (Zhang *et al.*, 2004).

CONCLUSION

The results of this study revealed, for the first time, that chrysin had reduced levels of COX-2 protein expression, ACF counting, and percentage of pathological lesions in an animal model of CRC, and it also had a synergistic effect with 5-FU in the early stages of CRC. As chrysin is a natural flavonoid with fewer side effects than 5-FU, 5-FU/chrysin therapy may lower the required dose of 5-FU and thus decrease complications, toxicity, or increased sensitivity of cells to medications. Therefore, it is suggested to administer different doses of chrysin combined with lower doses of 5-FU for CRC chemotherapy in an in-vivo study.

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CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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