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Original Article

Cytotoxic dose of Saffron's (*Crocus sativus L.*) Stigma Total Extract in Experimental Colon Cancer Model in rats induced by "Azoxymethane/Dextran Sodium Sulfate"

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Abstract:

Background: In this 22-weeks study, cytotoxic dose of Saffron(*Crocus sativus L.*) stigma total extract on five structural abnormalities(based on histopathology evaluation) of crypts in colon carcinoma initiated by azoxymethane (20 mg/kg once in the week of 4 of study) and followed by DSS(1%) were studied. **Methods:** Saffron at concentration of 300, 150, 75, and 20 ppm/day were be given by gavage for twenty-two weeks. Seventy-two 6-weeks-old male rats were randomly group as follow: Control (10 rats), cancer (12 rats), saffron (10 rats), and four cancerous-saffron treated groups: saffron's High Dose (300ppm), saffron's Medium Dose (150ppm), saffron's Low Dose (75ppm) and saffron's Very Low Dose (20ppm) (10 rats per group). **Result:** Cancerious rats fed medium dose of saffron (150 ppm) had significant fewer loss of nuclear polymorphism, less Epithelial stratification, less nuclear dispolarity, fewer loss of goblet cell's number and fewer structural abnormality of crypt($P<0.05$) than rats fed the other doses of saffron total extract. **Conclusion:** the "dietary"-and also "cytotoxic" dose of saffron extract, which capable of inhibiting azoxymethane/Dextran Sodium Sulfate-induced crypt's abnormalitiesin cancerous colon tissue in rats is 150 ppm. Saffron may exert its anticarcinogenic activity by modulating oxidative equilibrium in dose dependent manner in an invivo colon cancer model.

Key words: Colon, Saffron, Dysplasia, Animal model, Invivo model

INTRODUCTION

As it sounds, the number of synthetic drugs for chemotherapy in the colon cancer - the fourth cancer-related death in the world(1)- has been augmented, but the outcomes of consumption of these drugs didn't lead to high quality of life and survival of patients with colon cancer (2, 3). A useful approach not only for colon cancer treatment but also for its prevention is cancer chemoprevention(4). Based on this approach, natural herbs and plants may lead to reduce the use of synthetic drug, interfere with the mutagenesis, and have antiproliferative/antiprogressive activity (2, 5-9).

Based on the second *Avicenna*'s book (Canon of medicine), saffron was a curative and edible herb which was used in traditional medicine (10, 11). Saffron is the richest source of large number of phytochemicals(12) including a) flavonoids, b) carotenoids (such as crocin, picrocrcin, safranal, and lycopene), and c) several volatile components derived from oxidative cleavage of carotenoids(13-16). Based on

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many *Invivo* and *Invitro* studies the original carotenoids of saffron have anti-neoplastic properties (17, 18). Different proposed hypothesis for anti-neoplastic effect of saffron are a) induction of apoptosis (19-23) (b) hindering effect on synthesis of cellularnucleotides: DNA and RNA (19); (c) having provitamin A activity (24); (d) the interaction with topoisomerase II (25) and e) activation of detoxification systems(13, 26, 27). Other related cellular and molecular mechanisms and also organelle-involved dysfunction such as changing mitochondrial membrane potential (MMP) ($\Delta\psi$)or the extent of ROS production which may contribute to cell death, and arrestment in different phases of cell cycle(G1, S, and G2/M) were not studied well.

The colon carcinogenesis model is the best known animal model for investigating the efficiency of chemopreventive agents (28-31). The microscopic lesions in colonic crypt -which are named "aberrant crypt foci"- have morphological abnormalities such as nuclear polymorphism, nuclear strafication, nuclear dispolarity and shrinkage of goblet cells. These lesions are considered premalignant in colon adeno-carcinoma progression (32-35). Azoxymethane is one of the best colon-specific carcinogen that causes morphological abnormalities in the colonic crypts of rats or mice which are histopathologically similar to human's colon cancer crypts(36-38).

Saffron as a natural cancer-chemopreventive agents could be promising for use in future if it is non-toxic or having minimal toxicity and interfered with the process of cancer development. Identification of toxic dose, molecular targets, cellular events, intracellular organelles dysfunction and also cellular signal transduction are necessary to demonstrate whether saffron is suitable edible plant for using of cancer chemoprevention.

This study was done to find the cytotoxic dose of saffron's stigma total extract based on five structural abnormalities (based on histopathology evaluation) of crypts in rats with colon carcinoma which induced by colon-specific carcinogeneazoxymethane and dextran sodium sulfate.

MATERIALS AND METHODS

Chemicals and Reagent Kits

Azoxymethane(AOM) was obtained from Sigma Chemical Co. (St. Louis, MO, USA)(39). Dextran Sulfate Sodium (DSS) salt (42867-5G) (molecular weight 40,000) was purchased from Sigma Chemical Co.

Isolation of Saffron (Crocus Sativus L.) Stigma Extract

The part of saffron.that is being used as additive is the stigma. Dry stigmas of pure saffron were purchased from Golpeech Ltd.(St. Mash'had, Iran), and keep in a dark and cold (4°C)place. *Mash'had* the capital of *Khorasan razavi* provincehas mild and dry climate which has proper environment for saffron cultivation.Much of saffron in Iran is exported from this province. Saffronwas identified by "the Pharmacological Research Center" of Medicinal Plants ofSchool of Pharmacy with the herbarium code of (PM 832). Extraction of saffron's stigma were done in medicinal & natural products chemistry research center of Shiraz University of Medical Sciences. Dried stigmas of saffron were milled mechanically by mortargrinding machine. Light-color liquid extracts of saffron's stigma were obtained by soxhlet apparatus in which ethanol (80%) were used as a solvent. Ethanol was then removed by rotary evaporator in 42°C with vacuum pressure of 62 hPa (pascal/hour). The dark-red thick liquid extract were dried by freeze dryer in -42.5°C with vacuum pressure of 180 pascal. Extract of saffron stored at -20°C (21) till further use.

Animal Experimental Protocol and Model

Seventy-two (6-weeks-old) male "Sprague-Dawley rats"(40) were housed in the "Experimental and Comparative Medical Center" of Shiraz University of Medical Sciences. All ethical use of laboratory animals were taken into consideration. All rats were maintained under controlled temperature (21 ± 2°C), humidity (50 ±10 %), and lighting (12hour light/ dark cycle) condition. All rats were fed a standardized diet(*Behparvar* odent chow) and ad libitum tap water.

Based on TANAKA model(37), a single intraperitoneal (i.p) injection of azoxymethane (20 mg/kg) were used to 52 male rats, in the fourth week of study(37, 41). Four days before injection, rats were on fasting(42). In the starting of fifth week, rats were drunk a DSS (1%)-dissolved drinking water for one week. The DSS powder is used as carcinogen promoter in colon cancer experimental model after the injection of azoxymethane. A basal diet was contained with different dose of saffron extract (as describe in next section) until the experiment was terminated 19wk after exposure to azoxymethane. The experimental design was shown in Figure 1. The standard operating procedure for azoxymethane use in animals was considered for researchers and animal laboratory's staff(43, 44).

Animal Treatment Schedule

We considered 10 rats in each group of study but the cancer group had 12 rats (because this is for the first time that colon cancer model was studied in Shiraz University of Medical Sciences, we followed the colon carcinogenesis at 8th weeks after azoxymethane injection by scarifying one rat to ensure that the process of colon carcinogenesis was initiated). As shown in Figure 1, this study had seven group with the following treatments: 1) Control group which were normal

rats without any intervention (even saffron or carcinogen) throughout the experimental period. 2) Saffron group were only fed 300 ppm saffron extract throughout the experimental time. 3) Cancer group were injected azoxymethane followed by DSS as described before. 4) Saffron's High Dose group (AOM-DSS+ saffron 300 ppm)orally fed 300 ppm, 5) Saffron's Medium Dose (AOM-DSS + saffron 150ppm) orally fed 150 ppm, 6) Saffron's Low Dose group (AOM-DSS + saffron 75ppm) orally fed 75 ppm, 7) Saffron's Very Low Dose group (AOM-DSS + saffron 20 ppm) orally fed 20 ppm. Rats in groups 4 to 7 were treated by azoxymethane-DSS and fed different doses of saffron (Figure 1).

Saffron total extract was dissolved in water and was administered orally once a day via gavage needle from 21 days before azoxymethane injection and going on for 19 weeks. Selected doses of saffron in this study were based on *InVivo* study of GI accessory organ cancer model(45). At the end of study in week 22, all animals were anesthetized and colon were removed and cut for histopathology analysis.

Statistical Analysis

The frequency of mild to moderate dysplasia between the experimental groups were analyzed by SPSS software (version19). The between-groups differences of five structural features of colonic crypts of rats was analyzed by Mann–Whitney U test. The accepted level of significancy was set at *P-Value*<0.002 based on bonferroni's correction and was determined by the Kruskal-Wallis test.

RESULT

General Observation

One rat in cancer group and one rat in saffron's medium dose group died two-to-three weeks after azoxymethane injection. Feeding improperly gavage lead to death of one rat in "saffron's low dose" group and one rat in "saffron's very low dose" group. The rats in "saffron" group (only received saffron), "saffron's high dose" group and "control" group (no saffron, no azoxymethane) were alive until the end of the study.

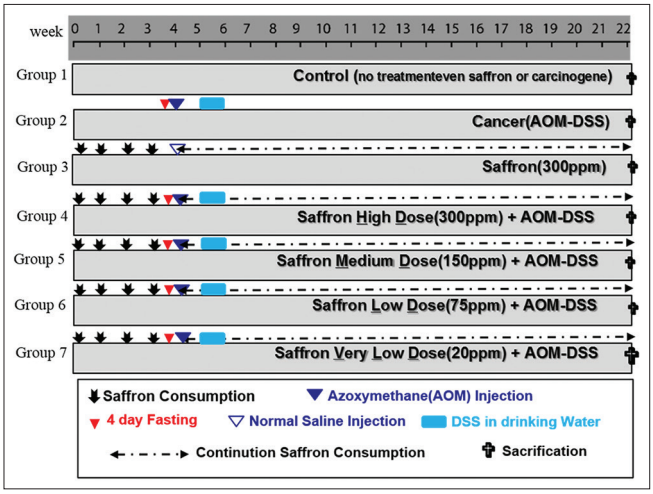

Figure 1: Experimental design

Lesions Histopathology and Type

The semi-quantitative scoring of five structural abnormalities (based on histopathology assessment) of colonic crypts was as shown in Table 1. (36, 46, 47).

At least five sections were examined for grading. Pathologist was blinded during reading the slides. The total score of abnormalities in each rat ware calculated. Almost all severe dysplasia were scored as 8 to 10 points while normal samples earned zero. Mild and Moderate dysplastic colonic mucosa got respectively the score 2 and 5 points(48).

The mild dysplasia samples had nuclear polymorphism and reduction in goblet cell. In moderate dysplastic colonic crypts, more crowded and polymorphic nuclei were seen; the number of goblet cells reduced more than in mild dysplasia and epithelial stratification was presented mildly. Severe dysplastic crypts of colon lost severely their nuclear polymorphism, stratified epithelial was presented, and completely lost or markedly reduction in the goblet cell's number were seen.

Macroscopically, more rats in cancer group (6 vs. 11) and in saffron's high dose group (7 vs. 10) revealed aberrant crypt with histopathology feature as severe dysplasia. Cancerous animal treated with low dose- and very low dose- of saffron have a lesser severe dysplastic lesions than two other groups mentioned above. The cytotoxic dose of saffron's extract was 150 ppm which lead to reduction in structural abnormalities in colonic crypt (Table 2, 3 and Figure 2).

The cytotoxic effect of other doses(very low, Low and high) of saffron's extract on all five histopathology features of aberrant crypt of animals subjected to colon cancer were not seen when compare with medium dose; however there are significant differences among all saffron-treated groups

Table 1: The semi-quantitative scoring of five structural abnormalities (based on histopathologyassessment) of dysplastic crypts of colon

Structural abnormalities	Score 0	Score 1	Score 2
Loss of nuclear polymorphism	None	Mild	Severe
Existence epithelial stratification	None	Mild	Severe
Nuclear dispolarity	None	Mild	Severe
Change in number goblet depletion	Null to mild	Moderate	Severe
Mtructural abnormality	None	Mild	Severe

Table 2: Effect of different doses of saffron total stigma's extract on five structural abnormalities (based on histopathology evaluation) of dysplastic crypts of colon in rats subjected to colon cancer

Groups	Loss of nuclear polymorphism1					Existence epithelial stratification1					Nuclear dispolarity1					Reduction in number goblet depletion1					Structural abnormality of crypt1				
	2	1	0	Mean rank	P value*	2	1	0	Mean rank	P value*	2	1	0	Mean rank	P value*	2	1	0	Mean rank	P value*	2	1	0	Mean rank	P value*
Cancer[2] (n=11)	63	36	0	2.00		63	36	0	2.00		63	36	0	2.0		73	27	0	2.00		64	26	0	2.00	0.00
Saffron (n=10)	0	0	0	0		0	0	0	0		0	0	0	00.00		0	0	0	0.00	0.00	0	0	0	00.00	
Saffron's HD [3](n=10)	50	50	0	1.50	0.00	50	50	0	1.50	0.00	50	50	0	1.50	0.00	70	30	0	2.00		50	50	0	1.50	
Saffron's MD[4](n=9)	11	44	44	1		0	33	66	0		11	22	67	00.00		11	22	68	0.00		11	33	56	0.00	
Saffron's LD[5](n=9)	22	78	0	1		22	66	11	1.00		22	67	11	1.00		22	78	0	1.00		22	68	0	1.00	
Saffron's VLD6(n=9)	22	78	0	1		22	78	0	1.00		22	78		1.00		56	44	0	2.00		33	67	0	1.00	

The structural feature which was assessed histopathologically in aberrant colonic crypt and was pointed as 2, 1, and 0. For each structural features point 2 means severe, point 1 : mild, and point 0: none), Induced by colonic carcinogen azoxymethane (20mg/kg) followed byDSS (1%). HD: High Dose of saffron fed : 300 ppm, MD: Medium Dose of saffron fed : 150 ppm, LD: Low Dose of saffron fed : 75 ppm, VLD: Very Low Dose of saffron fed: 20 ppm

Table 3: Frequency of different categories of dysplastic crypts in cancerous rats treated by saffron total stigma's extract

Rats	Mild dysplasia	Moderate dysplasia	Severe dysplasia	Normal
Cancer 1	0% (0/11)	36% (4/11)	63% (6/11)	0% (0/11)
Saffron's HD 2	10% (1/10)	20% (2/10)	70% (7/10)	0% (0/10)
Saffron's MD3	11% (1/9)	11% (1/9)	11% (1/9)	66% (6/9)
Saffron's LD 4	0% (0/9)	66% (6/9)	22% (2/9)	11% (1/9)
Saffron's VLD5	0% (0/9)	66% (6/9)	33% (3/9)	0% (0/9)
	4% (2/48)	42% (20/48)	39% (19/48)	15% (7/48)

Induced by colonic carcinogen azoxymethane (20mg/kg) followed by DSS (1%), HD: High Dose of saffron fed : 300 ppm, MD: Medium Dose of saffron fed : 150 ppm, LD: Low Dose of saffron fed : 75 ppm, VLD: Very Low Dose of saffron fed: 20 ppm

when compared with cancer group($p<0.002$) (Table 2). Table2 shows the results of the histopathology examination of five structural features of aberrant crypt in different groups which evaluated descriptively as the degrees of normal=0, mild to moderate =1, and severe =2.

Rats in cancer group showed significantly more histopathology features of destruction in crypt's structure which considered as severe dysplasia (63.36 %) compared with saffron's medium dose group (11.11%), saffron's low dose group (22.22%) and saffron's very low dose group (33.33%) ($p<0.002$). As seen in table 3, "low dose"- and "very low dose" of saffron total extract was less cytotoxic thanthe medium dose.The medium dose of saffron was the most cytotoxic dose in reducing severe dysplasia when compared with cancer- and saffron's high dose- group. The macroscopic aspect of dysplastic crypts which was categorized as mild, moderate and severe is observed in Figure 2.

Relatively more dysplastic lesions were as moderate (20 vs. 48; 42%) and were more frequent in cancerous rats who

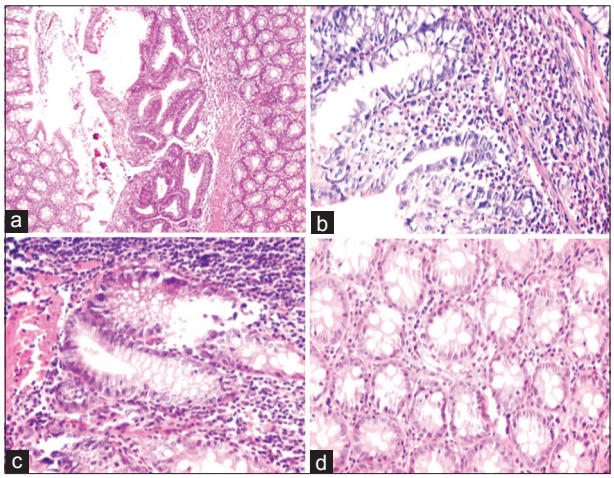

Figure 2: The macroscopic aspect of dysplastic crypts which was categorized as mild, moderate and severe based on total scores of the fivehistological feature (nuclear polymorphism, epithelial stratification, nuclear dispolarity, reduction in number of goblet cell and structural abnormality) of crypt's structure in colon tissue. (a) Rat with severely dysplastic lesions(score10), (b) Rat with moderately dysplastic lesions(score 5), (c) Rat with mild dysplastic lesions(score2), and (d) Rat with normal crypt(score 0); (H & E ×400).

feed "low dose"- and "very low dose"- of saffron (6 vs. 9; 66%) and (6 vs. 9; 66%) (Table 3). A majority of cancerous rats who feed medium dose of saffron showed no evidence of dysplastic lesions and were normal (6 vs. 9, 66 %), whereas there were a few or no rats in other group with normal histopathology features. 63 % (6/11) of rat in cancer group showed severe dysplastic crypt (Table 3).

DISCUSSION

The present short-term *in vivo* study's results show that the morphology and five structural abnormalities (based

on histopathologyassessment) of colonic crypts in colon carcinogenesis model induced by azoxymethane injection followed by DSS were normal in saffron group treated by medium dose (150 ppm). Lower frequency of severely dysplastic crypt was reached when medium dose (150 ppm) of saffron total stigma extract were fed. Based on current study's result,feeding 150 ppm saffron total stigma extract was considered as a dietary and cytotoxic dose for colon cancer in which shown to keep structural morphology of colonic crypts as normal.

There are many studies shows that the morphologic and genetic features characteristic of aberrant crypt were seen in the tumorigenic process of colon carcinoma(38, 49). Histologic-structural abnormalities of colonic crypts are valid and authentic indications of colonic tumor development(34).

Experimental studies of colon cancer indicated that histopathology abnormalities of crypt can serve as a substitute biomarker for chemopreventive efficacy of plant-base dietary agents(50).

In this study, the reduction of structural abnormalities of colonic crypts (based on histopathology evaluation)of rats in the azoxymethane-DSS model was seen by consumption of a dietary and cytotoxic dose (150ppm) of saffron'sstigma total extract. The anticancer activity of saffron is likely due to its pattern of phytochemicals and bioactive nutrients(14). Aphytochemical that is found in abundance in saffron's total stigma extract is carotenoids. Saffron's total stigma extract -in its natural form with all of its phytochemical and carotenoids(crocin, picrocrocin and saffranol) have been involved cellular metabolism, survival and signaling pathways(10, 22) related to colon cancer.

The indian scientist S.C. Nair in 1991 studied the anti-tumor properties of saffron(19, 51). Abdullaev authenticated nair's finding(52-55) through *InVitro* studies by focusing on biological effects and mechanism of action of saffron in colon cancer cell lines. Later, Petros A. Tarantilis et al showed that saffron's carotenoids have inhibitory effect on proliferation of HL-60 cell line(non-colon cancer cell line) in *invitro* study(56). Julio A. Escribano, 1996(57)et al. revealed that saffron and its useful carotenoids could be promising agents in cancer therapy.

Saffron could be used as chemopreventive agent for colon cancer treatment or prevention when dietary and cytotoxic dose were known. In current study (Table 2) the dietary dose which was also cytotoxic was 150 ppm. A higher dose (300 ppm) didn't exert cytotoxic effect on cryptal abnormalities (Table 3). We also saw pancreatic adhesion in rats of "saffron group" who feed only high dose (300ppm) of saffron and in cancerous rats who feed the high dose (300ppm). So high dose (300ppm) was not safe, even for normal cells.

Different proposed hypothesis for anti-neoplastic effect of saffron are a) induction of apoptosis (19-23) (b) hindering effect on synthesis of cellular nucleotides: DNA and RNA (19); (c) having provitamin A activity (24); (d) the interaction with topoisomerase II (25) and e) activation of detoxification system(13, 26, 27)It is difficult to identify which phytochemical of saffron's total stigma extract may responsible for anti-tumor properties because it is a rich source of both dietary carotenoids and numerous other unknown phytochemicals (58, 59). Future studies should be done to find phytochemical and biological active component of saffron, apart from its carotenoids to clarify the signaling pathway related to theanti-tomur action of saffron.

The outcome of experimental design of current study is in settlement with the Takuji Tanaka (37)studies using a similar experimental design. Although the two injection of azoxymethane(15 mg/kg body weight) is the common experimental colon cancer model(60-63); our procedure is little modified which exposing male Sprague-Dawley rats(40, 64) with one injection of 20 mg/kg azoxymethane followed byDSS (1 %) in their drinking water for seven days(41). DSS induces inflammation in the colonic mucosa through which the formation of dysplastic crypts were augmented(65, 66).

In our procedure the dose (20 mg/kg rather than 30 mg/kg) and frequency (once rather than twice) of azoxymethane's injection reduced which indicated that this methodology has good specificity for colon carcinogenesis based histopathology evaluation of structural abnormality in colonic crypts in which the frequency of moderately- and severely- dysplastic lesions were 42% (20 of 48) and 39%(19 of 48) respectively.

In conclusion, saffron's total stigma extract administered with the medium dose of 150 ppm significantly decrease structural abnormalities of colonic crypts in AOM/DSS-induced colon carcinoma model but a higher dose of 300 ppm failed to show these responses. This result illustrates that "supradietary dose" may have very different effects when compare with "safe", "dietary"-and "colon cancer-specific cytotixic"- dose. To better understand the cytotoxic effects of dietary dose (150ppm) of saffron's total stigma extract, more detailed cellular and molecular analyses should be carried out.

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