

RESEARCH ARTICLE

Pomegranate (*Punica granatum*) Peel Extract Efficacy as a Dietary Antioxidant against Azoxymethane-Induced Colon Cancer in Rat

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Abstract

Functional foods include antioxidant nutrients which may protect against many human chronic diseases by combating reactive oxygen species (ROS) generation. The purpose of the present study was to investigate the protective effect of pomegranate peel extract (PPE) on azoxymethane (AOM)-induced colon tumors in rats as an *in vivo* experimental model. Forty Sprague-Dawley rats (4 weeks old) were randomly divided into 4 groups containing 10 rats per group, and were treated with either AOM, PPE, or PPE plus AOM or injected with 0.9% physiological saline solution as a control. At 8 weeks of age, the rats in the AOM and PPE plus AOM groups were injected with 15 mg AOM/kg body weight, once a week for two weeks. After the last AOM injection, the rats were continuously fed ad-libitum their specific diets for another 6 weeks. At the end of the experiment (i.e. at the age of 4 months), all rats were killed and the colon tissues were examined microscopically for lesions suspected of being preneoplastic lesions or tumors as well as for biochemical measurement of oxidative stress indices. The results revealed a lower incidence of aberrant crypt foci in the PPE plus AOM administered group as compared to the AOM group. In addition, PPE blocked the AOM-induced impairment of biochemical indicators of oxidative stress in the examined colonic tissue homogenates. The results suggest that PPE can partially inhibit the development of colonic premalignant lesions in an AOM-induced colorectal carcinogenesis model, by abrogating oxidative stress and improving the redox status of colonic cells.

Key words: Azoxymethane model - colon cancer - pomegranate peel extract - oxidative stress - dietary antioxidants

Asian Pacific J Cancer Prev, 13, 4051-4055

Introduction

Functional foods act as antioxidant nutrients and protect against many human chronic diseases by combating reactive oxygen species (ROS) generation (Gulcin, 2012; Hu, 2011). Fruit concentrates consumption protect against oxidative stress-mediated human diseases; including cancer (Esfahani et al., 2011). In experimental animal models; azoxymethane (AOM) increases oxidative injury to colon cells and it is believed that this process play a role in the etiology of colon cancer in rats (Al-Numair et al., 2011; Rodriguez-Ramiro et al., 2011; Anilakumar et al., 2010; Bussuan et al., 2010).

Recent studies were directed to study the impact of pomegranate on cancer prevention (Lansky and Newman, 2007; Adhami et al., 2009), with a less emphasis on its role to prevent colon cancer. The proposition, that pomegranate peel extracts might prevent cancer, was based on three major domains of evidence: First; in-

vitro studies have shown that pomegranate peel has high antioxidant properties (Singh et al., 2002). Second; in-vivo animal models suggested that pomegranate peel represents an effective measure in oxidative stress-mediated diseases (Chidambara et al., 2002). Third; epidemiological studies had shown an inverse association between the risk of cancers development and consumption of fresh fruits, including pomegranate, and vegetables concentrates (Esfahani et al., 2011).

There is, however scanty information with regard to the antioxidant properties of extract isolated from pomegranate peels. This study was therefore conducted to evaluate the potential antioxidant protective effect of pomegranate (*Punica granatum*) peel extract (PPE) against Azoxymethane (AOM)-induced oxidative stress in rat colonic tissue homogenates. To our knowledge, this is the first study addresses the potential use of peel extract of pomegranate (*Punica granatum*), grown in Oman, as a functional food in chemoprevention of colon cancer.

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Materials and Methods

Pomegranate Peel Extract (PPE) Preparation

Fresh peels from pomegranate, locally grown in Oman, were cut into small pieces and homogenized in an electric mixer until a smooth texture was obtained (10 g of peels in 100 ml distilled water, i.e. 0.1 g/ml). Ten batches of extract were prepared to make 1 liter PPE extract. The mixture was then centrifuged at 6,000 g for 30 min at 3°C using Harrier 18/80 refrigerated centrifuge (SANYO, MSE, UK). Supernatant was then filtered by Whatman filter paper # 1 (150 mm) and the PPE obtained was stored at -40°C for later experiments.

Animals

Male Sprague-Dawley rats were used in this experiment. The rats were housed in individual polypropylene cages and were provided with standard laboratory chow diet (Oman Mills, Muscat, Oman) and normal tap water ad-libitum. Rats were housed under standard conditions of temperature (22 ± 2 °C), humidity (60%) and a 12 hr light: dark cycle. The study was approved by the Sultan Qaboos University Animal Ethics Committee, and was conducted in accordance to international laws and policies (EEC Council directives 86/609, OJL 358, 1 December, 12, 1987; NIH Guidelines for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Experimental Procedure

Forty weanling male rats were used in this study. They were randomly divided into 4 groups (10 rats per group) and were fed the same experimental diet for the whole experimental period, starting at 4 weeks of age with an average body weight of 50 ± 5 g. Two groups were control and AOM-injected group; and the other two groups were fed the experimental diet plus PPE (1.5 ml/day) via oral route at 47 mg (in terms of gallic acid equivalents)/kg of body weight (1 week dose) with or without AOM injections starting at 8 weeks of age (100 ± 20 g). After the last AOM injection, the rats were continuously fed ad-libitum their specific diets for another 6 weeks.

The rats in the control group were given 1 ml intra peritoneal injection of 0.9% physiological saline solution once a week for 2 weeks and the rats in the AOM-injected group were given 2 intra peritoneal injections of AOM (Sigma Chemical Co., St. Louis, MI) dissolved in physiological saline once a week (15 mg/kg body weight) for 2 weeks. Body weight and food intake were recorded weekly for whole duration of the experiment. After 6 weeks from the last AOM injection, the animals were sacrificed by decapitation under diethyl ether anesthesia after an overnight fast and the colon tissues were removed for subsequent analysis.

Colon Preparation

The colons were carefully removed from rats and were

kept on a glass plate in ice jackets. The colons were then opened longitudinally, rinsed with ice-cold physiological saline solution, and sectioned longitudinally into two halves of equal width and were spread out with flat mucosal side up. The mucosal layer from one half was removed by scraping and immediately homogenized. The other half was fixed flat in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ) between two filter papers for one week before Aberrant Crypt Foci (ACF) enumeration.

Aberrant Crypt Foci (ACF) Enumeration

ACF are commonly accepted precursor lesions for colonic tumors and the method used for ACF enumeration was followed as described in previous studies (Al-Numair et al., 2011; Bird, 1995). Fixed colons were stained with 0.2% methylene blue in Krebs's ringer bicarbonate buffer for 20 minutes in a Petri dish and rinsed with physiological saline solution. After staining, the colons were placed with the mucosal surfaces up on a slide, to be examined with a light microscope under 40X magnification and scored for ACF. In brief, the ACF were distinguished from normal crypts by their darker stain, enlarged and slightly elongated size, thick epithelial lining, slightly elongated cryptal opening and often slit shaped. The total number of ACF was recorded for all examined colons.

Scraped Colonic Mucosal Tissue Homogenization

The colon mucosal layer scrapings of each rat (~50 mg) were immediately homogenized in 1 mL of 100 mM potassium phosphate buffer (pH 7.2) by a glass-Teflon homogenizer with an ice-cold jacket and centrifuged at 10,000 g at 4°C for 60 minutes. The resulting supernatant was used for the determination of protein content and oxidative stress indices (antioxidant enzymes, glutathione and total antioxidant capacity).

Oxidative Stress Biochemical Measurements

Total antioxidant concentration (TAC) and reduced glutathione (GSH) were measured spectrophotometrically, using Total Antioxidant Status Assay Kit (Calbiochem, Darmstadt, Germany). and a Glutathione Assay Kit (BioVision, California, USA) respectively. The following antioxidant enzymes were also measured; [catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) and superoxide dismutase (SOD)]. All antioxidant enzymes assay kits were purchased from Sigma Chemical Co; GST assay kit (Catalog Number CS0410), GPx assay kit (Catalog Number CGP1), GR assay kit (Catalog Number GRSA), SOD assay kit (Catalog Number 19160), CAT assay kit (Catalog Number CAT 100).

Analysis of Protein Content

Protein content of colonic mucosal homogenates was assayed by the method of Lowry et al (1951), using bovine serum albumin as a standard and the protein content was expressed as mg/ml of sample.

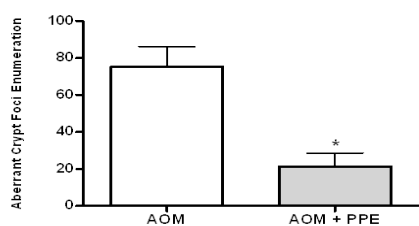


Figure 1. PPE-administration Inhibited the AOM-Induced ACF Development. *significantly lower than AOM-injected group, $P < 0.05$, based on Student's unpaired t-test.

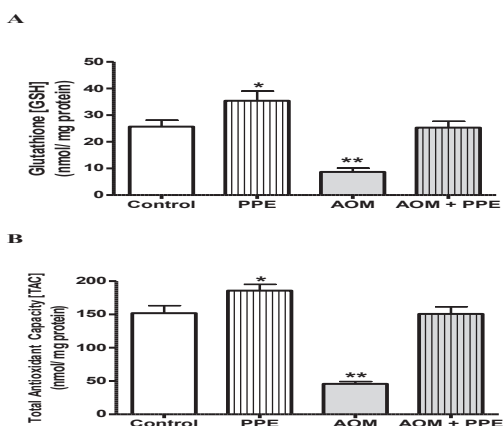


Figure 2 (A & B). PPE-administration attenuated the AOM-induced GSH depletion and TAC-suppression to the normal level of the control group. PPE augmented the basal level of GSH and TAC. *significantly higher as compared to control group. **significantly lower as compared to control group, $P < 0.05$, based on Student's Unpaired t-test.

Polyphenols Analysis

Total phenolic compound was analyzed using Folin-Ciocalteu method (Singleton and Rossi, 1965). In this method, 70 μ l of peel extract was placed into 10 ml test tube and 250 μ l of Folin-Ciocalteu reagent and 750 μ l of 1.9 M sodium carbonate were added. The volume was made up to 5 ml and test tube was placed on vortex equipment for one minute and then incubated for 2 hours in dark. The absorbance was then measured at 765 nm using UV-visible Spectrophotometer. Gallic acid was used as a standard and a calibration curve was prepared using standard solution of gallic acid. Results were expressed as gallic acid equivalents (GAE) in mg/100 g fresh-peel. All experiments were done in triplicate.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 5.03; GraphPad Software Inc. San Diego, CA). The results are expressed as means \pm standard deviation (SD). The statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's test, Student's unpaired t-test for means comparisons, and a P-value of < 0.05 is considered significant.

Results

Polyphenols

Water extract from fresh peel contained polyphenol as 223 ± 3 mg GAE/100 g fresh peel. Similarly Ramadan et al., (2009) found phenolic contents in pomegranate peel as 667 mg GAE/g dry-peel solids (i.e. 175 mg GAE/100 g fresh peel). However, Cuccioloni et al. (2009) observed higher phenolic contents 1690-2710 mg GAE/100 g dry-peel solids (i.e. 444-470 mg GAE/100 g fresh peel) depending on the variety. Similarly Pande and Akoh (2009) observed higher values 344-381 mg GAE/100 g fresh peel when six varieties were considered.

Food Consumption of Animals

The average daily food intake for the rats in all the four groups was similar with no statistical significant differences and the mean value was 12.7 ± 3.5 g/day, ($P > 0.05$), indicating that AOM-injection has no effect on the food intake.

Body Weight Gain of Animals

For all experimental groups, there was no difference between the initial body weight, and the final body weight at the end of the experiment indicating that rats from all groups grew at a similar rate and the average weight gain was 11.8 ± 1.7 g per week. No mortality was reported in any group.

Aberrant Crypt Foci (ACF) Development

The AOM dose (30 mg/kg rat body weight) was deliberately chosen as such a dose was used in previous studies as a potent carcinogenic dose that induced ACF and colon tumors in rats (Al-Numair et al., 2011; Hangen and bennink, 2002; Leonardi et al., 2010). In the present study we investigated the effect of PPE administration on the AOM-induced colon tumors by counting ACF. The rats from both the control and PPE groups did not show any development of ACF and therefore have not been depicted in Figure 1. The AOM-injected rats developed the ACF with an average 75.40 ± 10.9 which was significantly higher than the PPE plus AOM-injected group (21.51 ± 7.2), $t = 4.13$, $P = 0.002$ (Figure 1).

Impairment of Redox Status of Colonic Cells by AOM-Injection

Figure 2 (A & B) represent the intracellular GSH and TAC measurements in colonic mucosal tissue homogenates in control, PPE, AOM and PPE plus AOM groups. AOM-injection caused GSH depletion (8.7 ± 1.4 nmol/mg protein) as compared to control group (25.7 ± 2.4 nmol/mg protein), the difference was significant, $t = 2.24$, $P = 0.04$ (Figure 2, A). The same trend was observed for TAC measurements, where AOM caused a significant impairment (45.8 ± 3.3 nmol/mg protein) as compared to the control group (151.9 ± 11.4 nmol/mg protein), $t = 2.29$, $P < 0.05$ (Figures 2A and 2B).

PEE administration augmented the GSH and

Table 1. Pomegranate Peel Extract (PPE) Protects against Azoxymethane (AOM) -Induced Antioxidant Enzymes Inhibition in Rat Colonic Tissue Homogenates

Group	GST	GPx	GR	SOD	CAT
Control	22.3± 2.7	16.9 ± 1.2	11.1± 1.4	88.7 ± 6.8	123.4 ± 11.5
AOM	10.7 ± 1.1 ^a	8.8 ± 1.1 ^a	5.8 ± 0.8 ^a	41.9 ± 5.1 ^a	64.8 ± 6.9 ^a
PPE	25.3± 3.1 ^b	20.1 ± 4.7 ^b	13.4 ± 1.3 ^b	92.5 ± 8.1 ^b	134.6 ± 9.4 ^b
AOM+PPE	21.7± 1.3	15.9 ± 1.6	11.4 ± 1.2	84.5 ± 7.4	121.5 ± 10.3

Results are the means ± SD. Data with no superscripts are not significantly different from control. ^aSignificantly lower than Control; ^bSignificantly higher than control group, (P< 0.05). Based on one-way ANOVA analysis. Antioxidant enzymes: GST, Glutathione Transferase; GPx, Glutathione peroxidase; GR, Glutathione reductase; SOD, Superoxide dismutase; CAT, Catalase; Unit of enzymes activities is $\mu\text{mol}/\text{min}/\text{mg}$ protein.

TAC basal level and attenuated the AOM-induced GSH depletion (25.3 ± 2.4 nmol/mg protein) and TAC impairment (150.7 ± 10.9 nmol/mg protein) as compared to AOM-injected groups, ($t=6.12$, $t= 8.92$, $P<0.05$ respectively). Table 1 shows that the measured antioxidant enzymes were inhibited in the AOM group., On the other hand PPE administration augmented the basal antioxidant enzymes level as compared to control group and restored the AOM-inhibition to a comparable level to the control group, $P<0.05$

Discussion

The PPE dose used in this study was selected on the basis of LD_{50} value of polyphenols (Chidambara et al., 2002). PPE was effective in reducing the ACF number and restoring the redox status of the colon cells. Our data suggests that PPE-administration improves the redox status of the colonic cells and this has a primary prevention impact against AOM-induced colon cancer in the studied experimental model. The results of this study support the notion from the previous studies about the potential application of dietary antioxidant foods as a chemopreventive measure against colon cancer, since ACF appears in the early stages of colon cancer and may sequentially lead to the development of polyps and adenomas.

The observed GSH depletion, TAC impairment and inhibition of antioxidant enzymes are in line with reported by previous studies that documented the involvement of oxidative stress and low redox capacity of the cells in the pathogenesis of colon cancer (Shiraishi et al., 2009; Tsunadu et al., 2003). In conclusion, the AOM-injected rats showed significantly lower GSH, TAC and antioxidant enzymes levels and higher ACF as compared to control group. PPE administration abrogated the AOM observed suppression effects. These findings suggest that adopting a specific PPE dietary regimen (pre-, during and post-AOM treatment) can attenuate the AOM-suppression effect on GSH and TAC levels in colonic mucosal tissue homogenates in a mechanism that indicate the effectiveness of the antioxidant properties in counter balancing and quenching the ROS and its related oxidative damage in the colonic cells.

PPE may therefore be useful in the chemoprevention of colorectal carcinogenesis. However, further studies

are required to investigate the molecular mechanism underlying the PPE protective effects against AOM-mediated colon cancer pathogenesis.

Acknowledgement

The authors merit the financial assistance provided under His Majesty's Strategic Research Fund (SR/AGR/FOOD/11/01) to carry out this research project. The authors acknowledge the assistance of Mr. Sultan Al-Maskari, from Small Animal Experimental Unit, Sultan Qaboos University, for his dedicated effort in looking after the animals in this study.

References

- Adhami VM, Khan N, Mukhtar H (2009). Cancer chemoprevention by pomegranate: laboratory and clinical evidence. *Nutr Cancer*, **61**, 811-5.
- Al-Numair KS, Waly MI, Ali A, et al (2011). Dietary folate protects against azoxymethane-induced aberrant crypt foci development and oxidative stress in rat colon. *Exp Biol Med*, **236**, 1005-1.
- Anilakumar KR, Sudarshanakrishna KR, Chandramohan G, et al (2010). Effect of Aloe vera gel extract on antioxidant enzymes and azoxymethane-induced oxidative stress in rats. *Indian J Exp Biol*, **48**, 837-42.
- Bird RP (1995). Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett*, **93**, 55-71.
- Bussuan LA, Fagundes DJ, Marks G, et al (2010). The role of Fas ligand protein in the oxidative stress induced by azoxymethane on crypt colon of rats. *Acta Cir Bras*, **25**, 501-6.
- Chidambara-Murthy KN, Jayaprakasha GK, Singh RP (2002). Studies on antioxidant activity of pomegranate (*Punica Grantum*) peel extract using in vivo models. *J Agric Food Chem*, **50**, 4791-5.
- Cuccioloni M, Mozzicafreddo M, Sparapani L, et al (2009). Pomegranate fruit components modulate human thrombin. *Fitoterapia*, **80**, 301-5.
- Esfahani A, Wong JM, Truan J, et al (2011). Health effects of mixed fruit and vegetable concentrates: a systematic review of the clinical interventions. *J Am Coll Nutr*, **30**, 285-94.
- Gulcin I (2012). Antioxidant activity of food constituents: an overview. *Arch Toxicol*, **86**, 345-91.
- Hangen L, Bennink MR (2002). Consumption of black beans and navy beans (*Phaseolus vulgaris*) reduced azoxymethane-induced colon cancer in rats. *Nutr Cancer*,

- 44, 60-5.
- Hu ML (2011). Dietary polyphenols as antioxidants and anticancer agents: more questions than answers. *Chang Gung Med J*, **34**, 449-60.
- Lansky EP, Newman RA (2007). *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *J Ethnopharmacol*, **109**, 177-206.
- Leonardi T, Vanamala J, Taddeo SS, et al (2010). Apigenin and naringenin suppress colon carcinogenesis through the aberrant crypt stage in azoxymethane-treated rats. *Exp Biol Med*, **235**, 710-7.
- Lowry OH, Rosebrough NJ, Falt AL, et al (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem*, **193**, 265-75.
- Pande G, Akoh CC (2009). Antioxidant capacity and lipid characterization of six Georgia-grown pomegranate cultivars. *J Agric Food Chem*, **57**, 9427-36.
- Ramadan A, El-Badrawey S, Abd El-Ghany M, et al (2009). Rind and juice of pomegranate as natural antioxidants in cotton seed oil. The 5th Arab Annual Scientific Conference, Egypt, 8-9 April.
- Rodriguez-Ramiro I, Ramos S, Lopez-Oliva E, et al (2011). Cocoa-rich diet prevents azoxymethane-induced colonic preneoplastic lesions in rats by restraining oxidative stress and cell proliferation and inducing apoptosis. *Mol Nutr Food Res*, **55**, 1895-9.
- Shiraishi R, Fujise T, Kuroki T, et al (2009). Long-term ingestion of reduced glutathione suppressed an accelerating effect of beef tallow diet on colon carcinogenesis in rats. *Gastroenterol*, **44**, 1026-35.
- Singh RP, Chidambara Murthy KN, Jayaprakasha GK (2002). Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* models. *J Agric Food Chem*, **50**, 81-6.
- Singleton L, Rossi A (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *Am J Enology Viticulture*, **16**, 144-58.
- Tsunada S, Iwakiri R, Noda T, et al (2003). Chronic exposure to subtoxic levels of peroxidized lipids suppresses mucosal cell turnover in rat small intestine and reversal by glutathione. *Dig Dis Sci*, **48**, 210-22.