Dig Dis Sci (2013) 58:2507–2517
DOI 10.1007/s10620-013-2704-z

ORIGINAL ARTICLE

Inhibitory Effect of a Standardized Pomegranate Fruit Extract on Wnt Signalling in 1, 2-Dimethylhydrazine Induced Rat Colon Carcinogenesis

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Received: 22 January 2013 / Accepted: 24 April 2013 / Published online: 31 May 2013

Abstract
Background De-regulation of Wnt signalling is increasingly being implicated in both experimental and human carcinogenesis including colon cancer.
Aims Our goal was to identify possible dietary agents that block Wnt signalling as a step toward investigating new strategies for suppression of colon cancer. Pomegranate extract has emerged as an intriguing candidate due to its polyphenolic content.
Methods We used a 1,2-dimethylhydrazine dihydrochloride (DMH)-induced rat colon carcinogenesis model to investigate the expression pattern of the main key players in Wnt signalling by reverse transcription polymerase chain reaction (RT-PCR) analysis.
Results Our results showed that many Wnt-target genes, e.g., Wnt5a, frizzled receptor (FRZ)-8, β-catenin, T cell factor/lymphoid enhancer binding protein (Tcf4/Lef1), c-myc and cyclin D1, were up-regulated whereas adenomatous polyposis coli (APC) and axin1 exhibited down-regulation in colonic tissues of our DMH-colon cancer group compared with the normal group. Standardized pomegranate extract minimised all the aberrant alterations observed in the studied Wnt genes in colonic tissues of the DMH + pomegranate group as compared with the DMH-induced colon cancer group. This effect was also confirmed by the normalization of survival rate, inhibition of tumour incidence and a reduction of serum tumour marker carcinoembryonic antigen (CEA) level. Histopathological observations provided supportive evidence for the biochemical and molecular analyses.
Conclusions Standardized pomegranate extract holds great promise in the field of colon cancer prevention by dietary agents.

Keywords Wnt signalling/β-catenin · Colon cancer · Pomegranate · Rat

Abbreviations

APC	Adenomatous polyposis coli
AOM	Azoxymethane
CK2	Casein kinase2
CEA	Carcinoembryonic antigen
DMH	1,2-dimethylhydrazine
EA	Ellagic acid
FRZ	Frizzled receptors
GAEs	Gallic acid equivalents
GSK3β	Glycogen synthase kinase 3β
JNKs	c-Jun N-terminal kinase
PKCa	Protein kinase Ca
RT-PCR	Reverse transcription polymerase chain reaction
Tcf/Lef	T cell factor/lymphoid enhancer binding protein

Introduction

Colon cancer is a leading cause of cancer-related deaths in Western societies, contributing to 10 % of all cancer deaths in the United States alone [1]. The World Health

Electronic supplementary material The online version of this article (doi:10.1007/s10620-013-2704-z) contains supplementary material, which is available to authorized users.

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Organization estimates that nearly one million people are diagnosed with colorectal cancer worldwide each year. Experimental models mimicking this disease in rodents, such as 1, 2-dimethylhydrazine (DMH) or azoxymethane (AOM)-induced carcinogenesis, provide a tool for the understanding of the molecular alterations arising in human colon cancer. Importantly, AOM/DMH-induced tumours develop almost exclusively in the colon, in contrast with other experimental models in which carcinogenesis develops mainly in the small intestine, a site rarely involved in human cancer [2].

The tumours developing after induction in these experiments are relatively uniform and show phenotypic and genotypic features similar to those observed in human sporadic colon cancers, notably activation of Wnt signalling pathway [3]. The origin of the name Wnt comes from a hybrid of Wg (wingless) and Int in Drosophila, which is the best characterized Wnt gene [4]. The Wnt genes have been identified in animals from Hydra to Human. The Wnt pathway involves a large number of proteins that can regulate the production of Wnt signalling molecules, their interactions with receptors on target cells and the physiological responses of target cells that result from the exposure of cells to the extracellular Wnt ligands. Wnt proteins are a large group of secreted glycoproteins, which play a critical role in regulating cell fate, differentiation, cell cycle, proliferation, apoptosis and potentially tumour formation [5, 6].

Aberrant activation of the Wnt signalling has been demonstrated in both experimental and human carcinogenesis [7] and has been implicated in a variety of human cancers including colon, breast, prostate, and ovary. Mutations in adenomatous polyposis coli (APC), which affect the Wnt pathway, are the most frequent mutations found in colon cancers, and such mutations, in conjunction with about 14 other various "driver mutations", lead to colon cancer [4]. The Wnt pathway activation in oncogenesis and its consequences has been extensively reviewed [8, 9]. Wnt signals are transduced through a canonical pathway (that involves β-catenin and multiple protein complexes containing glycogen synthase kinase 3β (GSK3β), axin, APC and Akt) and several non-canonical pathways (that involve calcium, protein kinase Ca (PKCa), c-Jun N-terminal kinase (JNKs), and focal adhesion kinase) [10–12]. The Wnt/β-catenin pathway is the best understood Wnt signalling pathway, and is highly conserved during evolution. β-catenin is a transcriptional cofactor and is also an essential component of cell–cell adhesion complexes. β-catenin is the central player of the Wnt signalling pathway [13] (Fig. 1). Because of an unexpectedly wide role in colon cancer processes, substances that limit Wnt signalling in cancer may have an impact for prevention and treatment [8, 14].

Since AOM/DMH tumours are affected by the same dietary variations known to affect human colon carcinogenesis; this model is among the most used and reliable for the identification of potentially chemopreventive agents [15]. Interestingly, the consumption of a phytochemical-rich diet, including fruits and vegetables, has been correlated with a reduced risk of colon cancer incidence [16].

The pomegranate (*Punica granatum* L., Punicaceae) tree, originating in the Middle East and India, has been used for centuries in Greco-Arab medicine for the treatment of a variety of ailments, including sore throat, inflammation, and rheumatism [17]. Major areas of pomegranate cultivation are India, Spain, Egypt, and the United States, where it has recently gained economic importance due to increased consumption. Pomegranate is consumed both fresh and in processed forms as juice, wines and extracts. Pomegranates feature prominently in Judaism, Christianity and Islam. In Ayurvedic medicine practiced in India, the pomegranate is considered "a pharmacy unto itself." It is commonly used in the context of cancer by patients in the Middle East [17–19].

It is well documented that the high antioxidant and anti-inflammatory properties [19–24] of the edible part of pomegranate are due to the high content of polyphenolic compounds, particularly ellagitannins, the major ones being punicalagins [25–27]. The potent bioactivity of punicalagins and other ellagitannins can be due to its ability to hydrolyze into ellagic acid (EA) and other smaller polyphenols in vivo [28]. EA concentration above 5 % in the juice and extracts of pomegranate may be explained by hydrolysis of ellagitannins formed during processing and extraction [29]. Pomegranate juice and extracts have been found to provide similar levels of plasma and urinary ellagitannin metabolites in human subjects [30].

Recent research has shown that several dietary phenolics which target Wnt may have potential for colon cancer prevention and treatment [31, 32]. Unfortunately, data on the effects of dietary extract of pomegranate on Wnt are scarce. Therefore, the present study was designed to evaluate the effects of a standardized pomegranate extract on the main key players of canonical Wnt signalling using a DMH-model of colon carcinogenesis in rat.

Materials and Methods

Chemicals

DMH was obtained from Sigma Chemicals Company, St. Louis, MO, USA. All primers were synthesized by the laboratories of the Midland Certified Reagent Company Inc. (Midland, TX, USA).

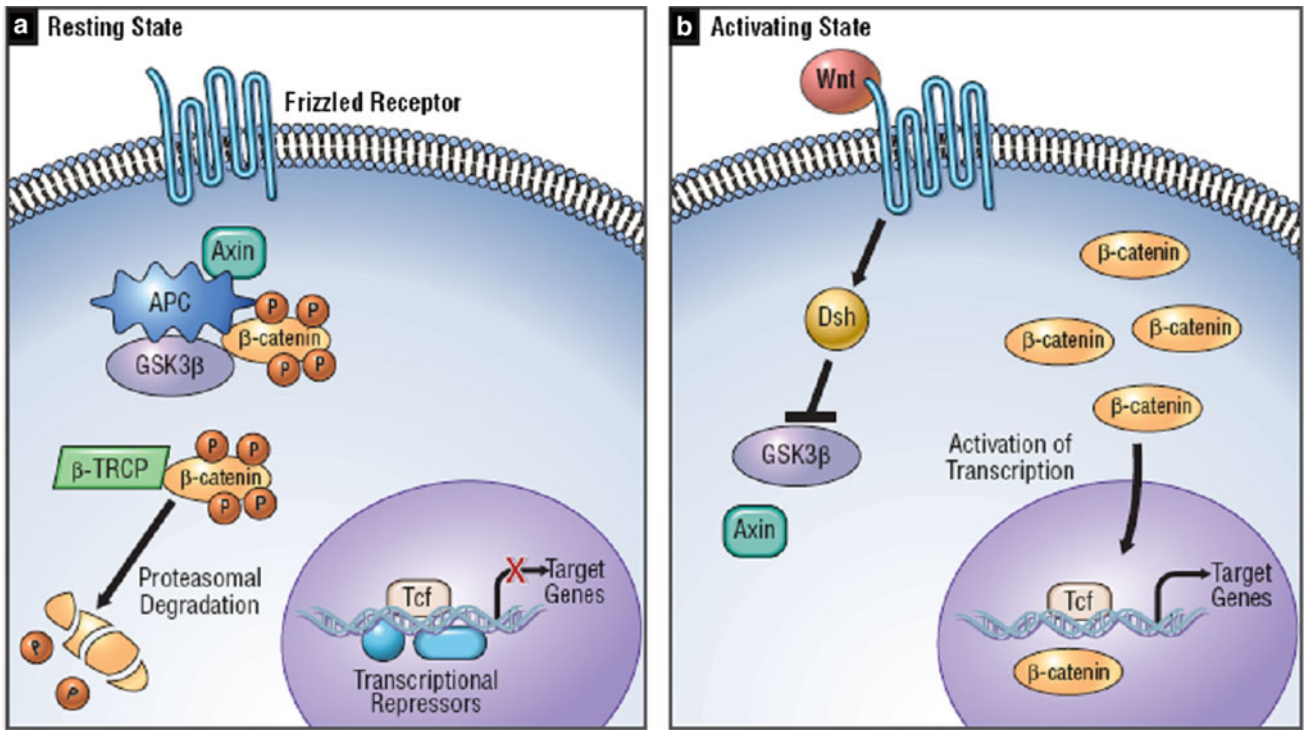

Fig. 1 The Wnt signaling pathway in the resting (**a**) and activated (**b**) states. The pathway is in the resting state in the absence of a Wnt signal, or in the presence of normal APC. The pathway is activated if a Wnt signal is present, or in the presence of mutant APC. *APC* adenomatous polyposis coli, *β-TrCP* beta transducin repeat-containing protein, *Dsh* disheveled proteins, *GSK3β* glycogen synthase kinase-3β, *Tcf* T-cell factor. Adapted with permission from Clements et al. [13]

Standardized Pomegranate Extract

The pomegranate fruit extract powder was prepared as previously described [33] and was provided by Verdure Sciences (Noblesville, IN, USA). The extract is HPLC-standardized using validated standards and methods to the major pomegranate ellagitannins (not less than 30 % punicalagin R and punicalagin β) and approximately 5 % of EA (Supplementary Table S1).

Estimation of Total Phenolics

Total phenolics were determined according to the Folin-Ciocalteu method and were measured as gallic acid equivalents (GAEs) as previously reported [34]. Briefly, the extract was diluted 1:100, or as appropriate, with methanol/H_2O (1:1, v/v), and 200 µL of sample was incubated with 3 mL of methanol/H_2O (1:1, v/v) and 200 µL of Folin–Ciocalteau reagent for 10 min at 25 °C. After this, 600 µL of 20 % Na_2CO_3 solution was added, vortexed and further incubated for 20 min at 40 °C. After incubation, sample was immediately cooled in an ice bath to room temperature. Sample and standards (gallic acid) were processed identically. The absorbance was determined at 755 nm, and final results were calculated from the standard curve.

Animals

Male Wistar albino rats weighing 100–120 g (4 weeks old) were purchased from the Egyptian organization for biological products and vaccines (Cairo, Egypt). The experiments were conducted in accordance with the approval of Ethics Committee of Faculty of Pharmacy, Cairo University, Cairo, Egypt. Animals were fed commercial pellet diet and water ad libitum throughout the experimental period.

Experimental Design

After a 2-week acclimatization period, the animals were randomly divided into four groups. For induction of colon cancer, group 1 rats ($n = 23$) received a dosage of 30 mg/kg body weight DMH (dissolved freshly in 1 mM EDTA, adjusted to pH 6.5 with 1 mM NaOH) subcutaneously once weekly for 30 weeks [35] and were termed the DMH group. Group 2 ($n = 7$) received similar injections of EDTA solution (DMH vehicle) and was termed the normal control group. Group 3 ($n = 13$) rats received DMH as previously mentioned simultaneously with their diet supplemented with 3 % (wt/wt) standardized pomegranate extract [36] and were termed the DMH + pomegranate group whereas group 4 ($n = 7$) received only standardized pomegranate extract-supplemented diet and was termed the

pomegranate group. The animals were weighed once a week.

At the end of 32 weeks (including 2 weeks of acclimatization), all rats were sacrificed by cervical decapitation. Blood was collected and serum was separated and stored at −30 °C. The entire colon was excised, longitudinally opened, taking care not to disturb the tumours, cleaned of residue, rinsed with ice-cold saline and blotted dry on filter paper. Tumour incidence was calculated using the following formula:

$$\text{Tumour incidence } \% = \text{number of tumour bearing rats} / \text{number of tested rats} \times 100$$

For histopathological study, specimen of the colonic (tumour and tumour adjacent) tissues were fixed in 10 % neutral buffered formalin, embedded in paraffin wax, cut at 5-μm thickness and stained with hematoxylin and eosin (H&E) after processing. The rest of the colonic tissues were stored at −30 °C for subsequent molecular and biochemical analysis.

Determination of Serum Carcinoembryonic Antigen (CEA) Level

Serum CEA level was determined using enzyme-linked immunosorbent assay (Enzymun-Test CEA, Boehringer Mannheim, Germany) rat kit. Level of serum CRP (ng/mL) was calculated by interpolation from a sigmoid-shaped standard curve generated in the same assay with reference standards of known concentrations. All assays were performed in duplicate according to the manufacturer's instructions.

Molecular Biology Assays in the Colon

RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total cellular RNA was isolated from colonic (tumour and tumour adjacent) tissue specimens based on the method of Chomczynski and Sacchi [37]. Quantitative RT-PCR analysis was performed. Two micrograms of total RNA were reverse transcribed into first strand cDNA in a volume of 25 μL at 37 °C for 60 min using a first strand cDNA synthesis kit (Novagen, Madison, WI, USA) and heated at 95 °C for 5 min to terminate the reverse transcription reaction. Wnt5a, frizzled receptor (FRZ)-8, β-catenin, APC, axin1, T cell factor/lymphoid enhancer binding protein (Tcf4/Lef1), c-myc, cyclin D1 and β-actin (housekeeping gene) genes were amplified from 2 μL cDNA mixtures in a final volume of 20 μL PCR reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM each of dNTPs, 0.25 μM each of sense and antisense primers and 1.25 U Taq DNA polymerase. Reaction mixtures were first denatured at 95 °C for 5 min, and amplification was performed for 35 cycles, at 95 °C for 45 s, annealing (57 °C for Wnt5a, 53 °C for FRZ-8, 51 °C for β-catenin, 54 °C for APC, 50 °C for axin1, 55 °C for Tcf4, 53 °C for Lef1, 58 °C for c-myc, 55 °C for cyclin D1, and 58 °C for β-actin) for 1 min, and at 72 °C for 1 min, followed by an extension for 7 min at 72 °C. Primer sets for the PCR amplification of Wnt5a [38], β-catenin, APC, β-actin [39] and c-myc [40] genes were selected based on published sequences. The rest of the primer sets were designed with the Primer3-Blast software (NCBI, USA). The PCR primer pairs used were enlisted in Table 1.

Electrophoresis and Quantification

The PCR products were electrophoretically resolved on 2 % agarose–TBE gel containing 0.5 mg/mL ethidium bromide and run for 1.5 h at 8 V/cm. The gel image was captured using the Eagle Sight Software (Stratagene) system. The PCR products were then quantified using a quantification kit (Promega Corporation, Madison, WI, USA). This method depends on the purification of the PCR product using a Promega Wizard PCR preps DNA purification kit (Promega Corporation, Madison, WI, USA). The mixture for quantification consisted of DNA quantification buffer, sodium pyrophosphate, NDPK enzyme solution, T4 DNA polymerase and DNA. This mixture was incubated at 37 °C for 10 min, at which point 100 μL of Enliten L/L reagent was added. The reaction was immediately read using a luminometer. The same steps were performed on DNAs of known concentration provided by the kit, and a standard curve was performed by plotting the readings of the luminometer against the known concentrations. The readings of the amplified PCR products of the ten different genes after using the luminometer were determined based on the standard curve. The results were expressed as μg/mg wet tissue [41].

Statistical Analysis

Statistical analysis was performed using the InStat 2.04 statistical package (GraphPad InStat). Ratio comparison was determined by the chi-square test. Data are expressed as mean ± standard error (S.E.M). Numbers of observations are indicated in tables and figures. Differences among groups were assessed by one-way analysis of variance. Tukey–Krammer's test was performed for inter-group comparisons. The minimum level of statistical significance used was at $P < 0.05$. Significance at P values <0.001, <0.01 and <0.05 have been given respective symbols.

Table 1 Sequence of all primers used in the experiment

Parameter	Primer sequence	PCR product size (bp)
Wnt5a	F: CATCTGCCAGGTTGTATACTGTCC	324
	R: TGATGCAAATAGGCAGCCGAGAGA	
FRZ-8	F: TTGCTAACAGTTCAAGAATTAGAGG	166
	R: AAATGATTTAAGTACTACCCTGTCCC	
β-catenin	F: ACAGCACCTTCAGCACTCT	168
	R: AAGTTCTTGGCTATTACGACA	
APC	F: CGGAACATGCATGACTGAGAC	310
	R: GTCACGAGGTACGACCTCAGAT	
axin1	F: TGCAGAGTCCCAAAATGAATG	658
	R: GAGCCTGTCCTTGTGTAC	
Tcf4	F: CAGAATGAAGACAGAGAGTGGCA	160
	R: CCTATGCATTATTGCCTTGCAG	
Lef1	F: CAAGGCCCTTAGCTTCCTG	175
	R: CCCCCTGTCCTTTATCTTGC	
c-myc	F:-AGCGTCCGAGTGCATCGACC	538
	R: ACGTTCCAAGACGT TGTGTG	
cyclin D1	F: CAGAAGTGCGAAGCTTAGGTCT	470
	R: GTAGCAGGAGAAGTTGTTGG	
β-actin	F: CATGTGCAAGGCCGGCTTCG	665
	R: GTAGCAGGAGAAGTTGTTGG	

Results

Survival and Tumour Incidence

During the 30 weeks of tumour induction, six rats survived out of 23 before completion of carcinogen exposure (survival rate, 26 %, $P < 0.05$). Normalization of survival rate was revealed in the DMH group fed with pomegranate as 12 rats survived out 13 completed the experimental period (survival rate, 92 %). No mortality was detected in the normal group or the control group supplemented with the standardized pomegranate extract diet (Table 2). Tumour incidence (66.77 %) observed in the DMH group was totally inhibited in the DMH group fed with pomegranate.

Effect of a Standardized Pomegranate Fruit Extract on DMH-Induced Colon Carcinogenesis

In the DMH-colon cancer group, the activated Wnt pathway was revealed by the significant overexpression levels of the secreted Wnt ligand Wnt5a, Wnt receptor FRZ-8, β-catenin, β-catenin-activated Tcf4/Lef1 transcription factors, c-myc and cyclin D1 (Fig. 2; Table 3; each, $P < 0.001$) and down regulations of APC ($P < 0.001$) and axin1 (Fig. 2; Table 3; $P < 0.01$) in colonic tissues compared with the normal group. A significant increase in serum CEA level was also observed in the DMH-colon cancer group compared with the normal group (Fig. 3; $P < 0.001$). Results showed that standardized pomegranate extract significantly minimised all aberrant alterations in the Wnt pathway observed in colonic tissues of the DMH + pomegranate group as compared with the DMH-induced colon cancer group. This was revealed by inhibiting the overexpression levels of Wnt5a, FRZ-8, β-catenin, Lef1, Tcf4, c-myc and cyclin D1 and increasing the expression levels of APC and axin1 in colonic tissue along with a significant reduction of serum CEA level (Fig. 2; Table 3; each, $P < 0.001$ except axin1, $P < 0.05$). Overall, no statistically significant differences were found between the group of normal rats supplemented with pomegranate extract and the normal group.

Table 2 Survival (%) and tumour incidence (%) in the different studied groups

Parameter	Normal	DMH	DMH + pomegranate	Pomegranate
Survivors/total	7/7	6/23	12/13	7/7
Survival %	100	26[a]	92[b]	100
Tumour incidence %	0	66.77[a]	0	0

DMH 1, 2-dimethylhydrazine

[a] Significant difference from the normal group

[b] Significant difference from the DMH group. Chi-square test. $P < 0.05$

Histopathological Analysis

Tissue sections of the normal group and pomegranate group displayed normal crypts and colonic architecture with no signs of apparent abnormality (Fig. 4a–c). Irregular crypts, lined with atypical cells invasive to deep muscle layers with ulcerated mucosa and infiltrating adenocarcinoma were observed in the DMH-induced rats (Fig. 4d). Mucus producing malignant acini, shown as indicated by arrows (Fig. 4e), was also noted. In the DMH + pomegranate rats, histology revealed restoration of nearly colonic architecture with little lymphocytic infiltration and mild atrophy of acini (Fig. 4f).

Discussion

The objective of our study was to identify possible new colon cancer prevention strategies. We sought to identify natural compounds that may be used for blocking cancers with Wnt signalling. De-regulated Wnt signalling by genetic or biochemical means triggers an oncogenic gene expression program that contributes to colon tumourigenesis in both experimental models and humans [7]. Characterizing

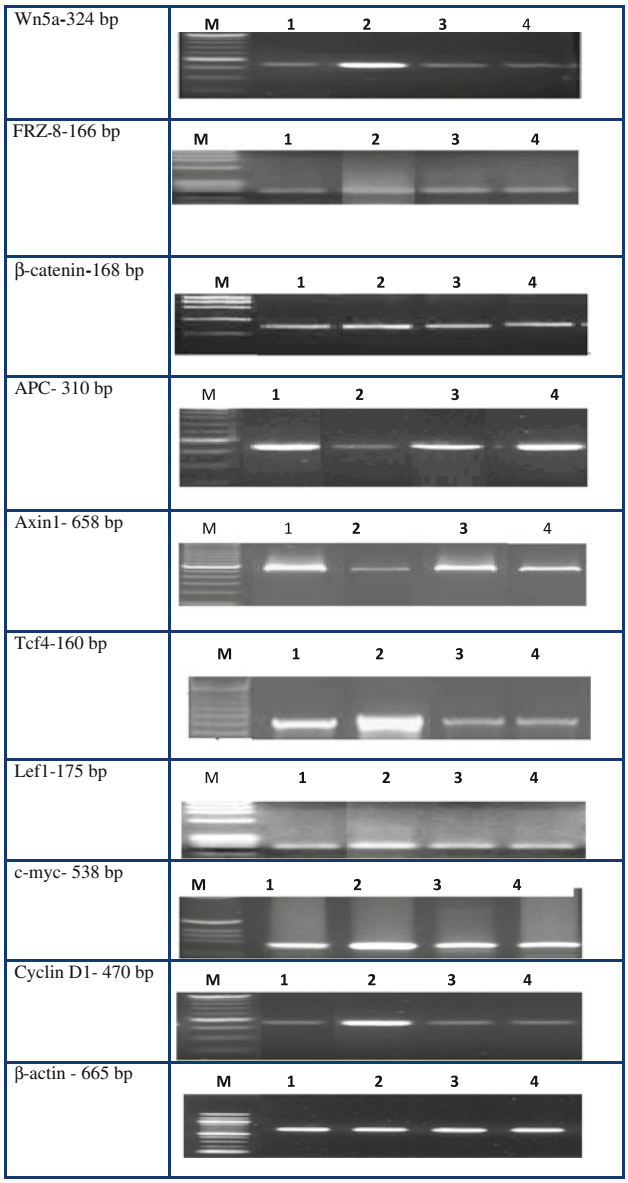

Fig. 2 Representative agarose gel electrophoresis profiles of mRNA amplification of Wn5a, FRZ-8, β-catenin, APC, axin 1, Tcf4, Lef1, c-myc, cyclin D1 and β-actin obtained by RT-PCR of colon tissues from *1* normal ($n = 7$), *2* DMH ($n = 6$), *3* DMH + pomegranate ($n = 12$), and *4* pomegranate ($n = 7$)

proteins that regulate the Wnt pathway have revealed important insights into Wnt-dependent processes and potential directions towards novel therapies [42]. The best understood of the major Wnt pathways is the canonical/β-catenin pathway.

The essential event in Wnt signalling is the stabilization of β-catenin (Fig. 1) [13]. In the absence of Wnt ligands, β-catenin levels are suppressed by the APC-axin-GSK3β protein complex via phosphorylation and subsequent degradation by the proteasome [8]. Activation of the canonical Wnt signalling pathway requires the binding of Wnt ligands to FRZ receptors [43]. Activation leads to the phosphorylation of disheveled proteins which in turn inactivate GSK3β [44]. Inactivated GSK3β cannot recognize β-catenin to phosphorylate serine and threonine residues, thus preventing the targeting of β-catenin for proteolytic destruction [45]. Consequently, unphosphorylated β-catenin accumulates in the cytosol and binds to members of the Tcf/Lef family of architectural high-mobility group domain transcription factors forming complexes that are translocated into the nucleus. On their own, Tcf/Lef proteins are weak transcriptional activators; binding to β-catenin induces a significant increase in transcriptional activity that can activate certain genes that ultimately establish the oncogenic phenotype [46, 47]. Both Tcf and β-catenin orchestrate the transcription of Wnt target genes which promote proliferation and survival of affected cells such as c-myc and cyclin D1 and others [13].

In our study we tried to investigate the altered Wnt signalling at three main levels: the cell membrane (the ligand Wnt5a and receptor FRZ-8), the cytosol (APC-axin1-β-catenin and Tcf4/Lef1) and the nucleus (c-myc and cyclin D1). Accordingly, our study revealed that many Wnt-target genes, e.g., Wnt5a, FRZ-8, β-catenin, Tcf4, Lef1, c-myc and cyclin D1, were up-regulated whereas APC and axin1 exhibited down regulation in colonic tissues of our DMH-colon cancer group compared with the normal group.

Wnt5a is one of the 16 Wnt-type ligands that have been described as secreted protooncogenic glycoproteins whose overexpression and binding to frizzled cell-surface receptors has been reported previously in human tumour cells [48, 49]. Loss of function mutations in APC and axin, and gain-of-function mutations in β-catenin have been reported in numerous cancers [4, 9]. In fact, many cancer types have high β-catenin levels with an apparently genetically intact Wnt pathway [50]. About 60–80 % of colon cancers develop due to dysregulation of the Wnt/β-catenin signalling pathway which is indicated by an accumulation of β-catenin in the tumour cells [51].

The APC gene product, known as APC, is a phosphoprotein that functions as a tumour suppressor through its effects on several cellular processes including cell cycle control, migration, differentiation, DNA replication and apoptosis [52]. The role of APC in the Wnt pathway is to regulate cytoplasmic levels of β-catenin through the control of β-catenin degradation through the association with the protein complex formed by β-catenin, axin, and GSK3β. In a previous study, the introduction of the β-catenin–binding domain into colorectal tumour cell lines with mutant APC resulted in growth arrest and apoptosis [53].

Another proposed mechanism of tumour suppression by APC is the nuclear shuttling of β-catenin. It has been proposed that APC can bind to β-catenin in the nucleus and transport it to the cytoplasm for degradation, thus acting as

Table 3 Gene expression levels (μg/mg tissue) of the main studied key players of the Wnt pathway in colonic tissues in the different studied groups

Parameter	Normal	DMH	DMH + pomegranate	Pomegranate
Wnt5a	646.3 ± 37.7	2657.2 ± 41.6[a]*	868.5 ± 32.6[a]*[b]*[c]*	622.3 ± 24.2
FRZ-8	514.6 ± 18.8	1813 ± 40.5[a]*	868.8 ± 32.6[a]*[b]*[c]*	507.7 ± 13.9
β-catenin	242.2 ± 7.4	741.2 ± 38.8[a]*	380.5 ± 26.3[a##,b*,c##]	249.7 ± 10.3
APC	749.9 ± 15.2	270.0 ± 14.1[a]*	505.8 ± 10.5[a]*[b]*[c]*	741.4 ± 7.7
Axin1	899.1 ± 35.7	492.8 ± 63[a##]	665.5 ± 24.5[a##,b#,c##]	869.4 ± 31.5
Tcf4	302.5 ± 22.8	954.3 ± 17.8[a]*	483 ± 18.7[a]*[b]*[c]*	312.7 ± 20
Lef1	265.3 ± 14.2	834.7 ± 29.5[a]*	456 ± 31.1[a]*[b]*[c]*	290.1 ± 15.7
c-myc	526.6 ± 8.0	1567.0 ± 24.7[a]*	661.3 ± 13.8[a]*[b]*[c]*	525.7 ± 6.7
Cyclin D1	653.3 ± 24.8	1699.3 ± 42.5[a]*	912.2 ± 17.5[a]*[b]*[c]*	666.0 ± 28.4
n	7	6	12	7

Data are represented as mean ± SEM; *n* number of observations for each group

[a] Significant difference from the normal group

[b] Significant difference from the DMH group

[c] Significant difference from the pomegranate group

* $P < 0.001$; ## $P < 0.01$; # $P < 0.05$

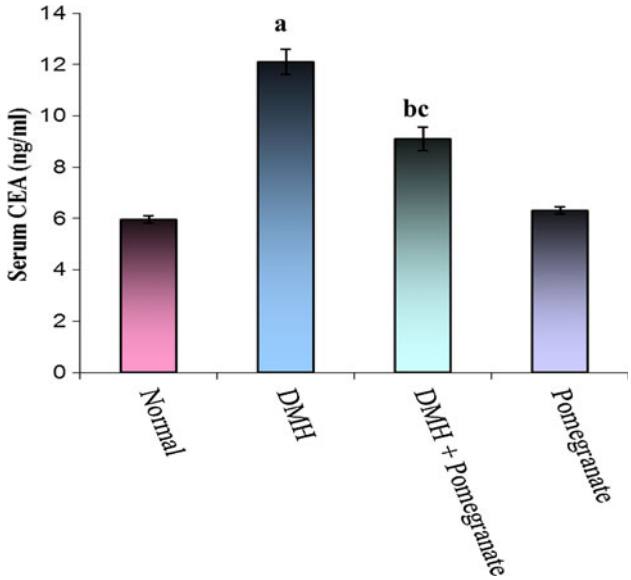

Fig. 3 Serum level of CEA (ng/mL) in different studied groups. Data are represented as means ± SEM. **a** Significant difference from normal group; **b** Significant difference from DMH group; **c** Significant difference from pomegranate group at $P < 0.001$

a shuttle to carry β-catenin from one compartment of the cell to another [54, 55]. Thus, "APC may act to control the relative concentrations of β-catenin in the nucleus and in the cytoplasm by mediating the transport of nuclear β-catenin to the cytoplasm and through the degradation of cytoplasmic β-catenin. Truncations in APC that lead to a loss of the β-catenin-binding domain cause the protein to lose the ability to regulate cytoplasmic levels of β-catenin with subsequent accumulation of β-catenin in the cytosol and translocation to the nucleus, where it activates Tcf4/Lef family members to initiate downstream gene transcription" [13]. The expression of the Tcf isoform is such that Tcf4 is highest in organ sites with active Wnt signalling like colonic epithelium [56].

Axin1 is another tumour suppressor in the Wnt pathway that acts to downregulate signalling. Axin are negative regulators of the Wnt signalling pathway, which promote the phosphorylation and degradation of β-catenin [57]. The protein acts as a scaffold to bind β-catenin, GSK3β, and APC, and allows the phosphorylation of β-catenin. Axin mutations have been reported in hepatocellular carcinomas and correlate with the presence of nuclear β-catenin. Growth suppression and apoptosis in cancer cell lines with an activated Wnt pathway was observed upon the introduction of normal axin in the study of Satoh et al. [58].

To corroborate the signalling results, we investigated the growth-promoting c-myc and the cell-cycle regulator cyclin D1 as relevant Wnt target genes. Increased expression of c-myc and cyclin D1 observed in our DMH group is linked to colon cancer [59, 60]. c-myc has a key role in human cell transformation in oncogene complementation studies and in the regulation of the telomerase gene, which is a critical component in transformation [61]. Increased expression of cyclin D1 has been reported in various human tumours [62] and several carcinogen-induced mouse and rat tumour models including a DMH-rat colon carcinogenesis model [63, 64]. Interestingly, c-myc was found to increase hepatic glucose metabolism by increasing glycolytic enzyme gene transcription in rat hepatoma cells [65]. This near universal glycolytic phenotype of cancer cells is a phenomenon known to have a potential role in

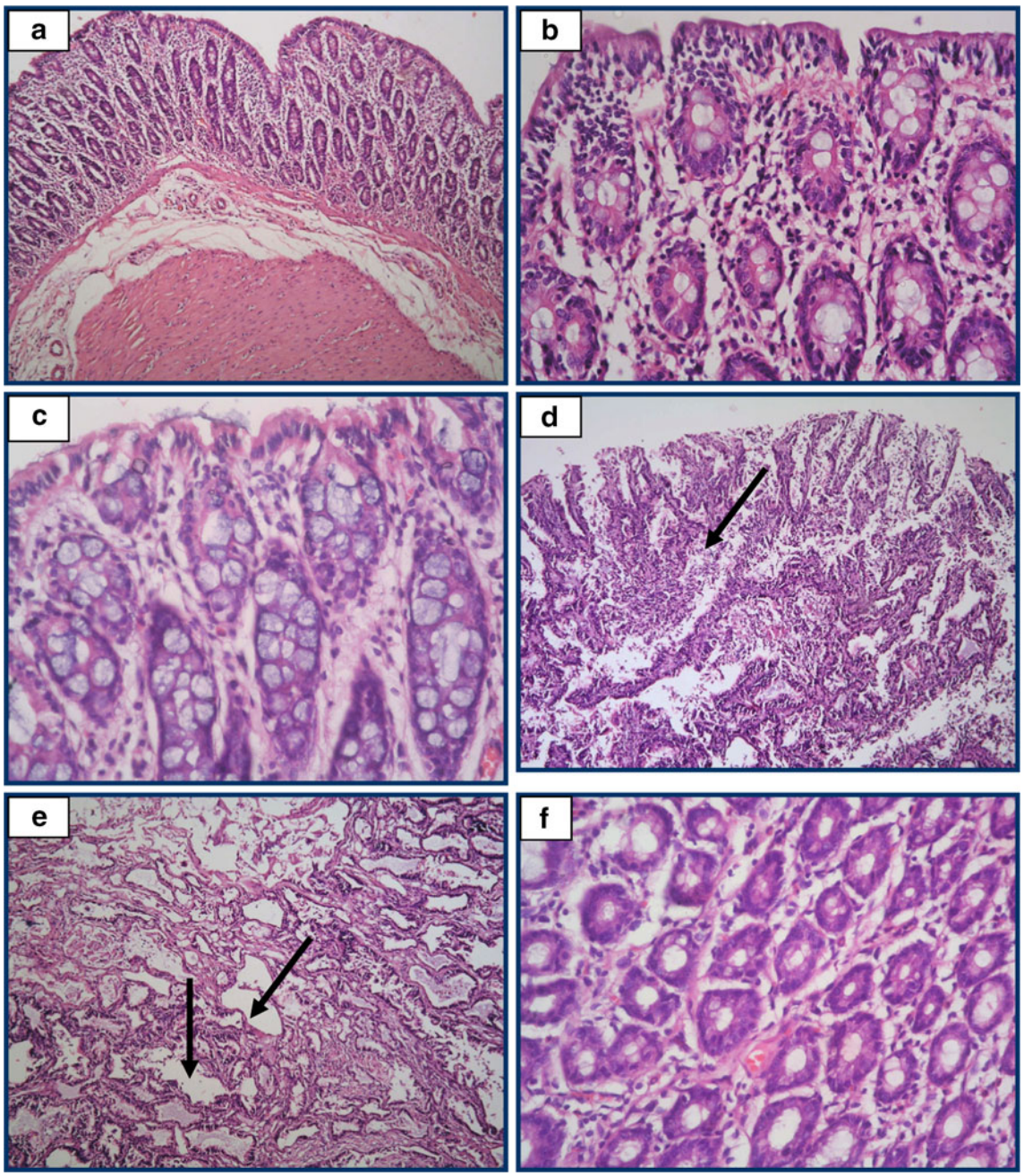

Fig. 4 Photomicrographs (H&E) of colon of normal (**a** ×100 and **b** ×400) and pomegranate group (**c** ×400) rats showing normal colonic architecture. Colon of 1, 2-dimethylhydrazine (DMH) rats showing adenocarcinoma (**d** ×100) and mucus producing malignant acini as indicated by *arrows* (**e** ×100). Colon of DMH + pomegranate rats showing nearly normal mucosa (**f** ×400)

facilitating tumour invasion and perturbation of the tumour microenvironment [66].

Recently, Yamada et al. [67] revealed the crucial role of microRNA-145 in inhibiting nuclear β-catenin translocation and consequent down-regulation of Lef/Tcf, c-myc and cyclin D1.

From the previous discussion, the Wnt signalling pathway may be a logical focus for novel chemopreventive and therapeutic strategies because of a likely role as one of the "driver pathways" in colorectal tumourigenesis. The development of new cancer chemopreventive agents that could be employed to inhibit tumour development without causing systemic toxicity is a major research goal. Phytochemicals from pomegranate have been shown to inhibit colon cancer cell proliferation and apoptosis through the suppressing of Wnt signalling in the cell-based assay

[68, 69]. Separation of individual constituents from a whole entity may decrease overall activity due to an unanticipated requirement for other components. In this respect, pomegranate juice proved to be more potent than its purified ellagitannins, in inhibiting proliferation and inducing apoptosis in HT-29 colon cancer cells [69]. "The superior bioactivity of pomegranate juice compared to its purified polyphenols illustrated the multifactorial effects and chemical synergy of the action of multiple compounds compared to single purified active ingredients" [26].

Our study showed that standardized pomegranate extract improved all aberrant alterations in the Wnt pathway observed in colonic tissues the DMH + pomegranate group as compared with the DMH-induced colon cancer group. By inhibiting the overexpression of Wnt5a, FRZ-8, β-catenin, Lef1, Tcf4, c-myc and cyclin D1 and increasing the expression levels of APC and axin1, standardized pomegranate extract showed potent chemopreventive efficacy. The chemopreventive effect was also confirmed by the normalization of survival rate, inhibition of tumour incidence, reduction of serum tumour markers CEA level and the nearly normal colonic architecture revealed by the histopathological investigation. CEA is the best marker in colorectal cancer patients and also most thoroughly characterized tumour-associated antigens, in both biochemical and clinical aspects [70].

There is considerable evidence that the anticarcinogenic effect of pomegranate ellagitannins is mainly due to their hydrolysis product, EA, which induces apoptosis in human colon cancer cell line via the intrinsic pathway with the release of cytochrome c into the cytosol, activation of initiator caspase 9 and effector caspase 3 and down-regulation of Bcl-XL. Also, pomegranate treated Caco-2 cells showed arrest in the S phase of the cell cycle, as down-regulation of cyclins A and B1. Interestingly, neither EA nor punicalagin induced apoptosis in normal colon CCD-112CoN cells [28]. In addition, data provided by previous studies has shown that EA is an inhibitor of the casein kinase CK2 [71]. CK2 is a positive regulator of Wnt signalling and can modulate the interaction of β-catenin with members of the β-catenin destruction complex [72]. Moreover, ellagitannins are converted by gut microbiota to urolithin metabolites, which may persist in the colon through enterohepatic circulation [19, 27, 73]. Tissue disposition studies reveal that urolithins are enriched in prostate, intestinal, and colon tissues in mouse [74]. Urolithins inhibit the proliferation of colon cancer cells, induce cell cycle arrest, and modulate key cellular processes associated with colon cancer development, suggesting that urolithins may be relevant bioactives in the colon and may contribute to the colon cancer chemopreventive properties resulting from the consumption of ellagitannins-rich foods. Sharma et al. [69] reported that standardized pomegranate extract, ellagitannins and urolithins inhibited Wnt signalling in vitro, suggesting that ellagitannins-rich foods have potential against colon carcinogenesis.

Our results support the role of standardized pomegranate extract in suppressing Wnt signalling and colon cancer and provide a framework for testing other nutrient and pharmacological compounds for blocking Wnt signalling for cancer prevention or therapeutics. Our study with standardized pomegranate extract opens up future work for combining it with other inhibitors of Wnt signalling that have been reported with differing mechanisms. A future question is additivity and/or synergism with other possible compounds. A future possible combination study may be reduced levels of Cox-2 inhibitors and standardized pomegranate extract to limit possible side effects in the Cox-2 inhibitors. The increasing detection of colon cancer highlights a major need for future design of safer prevention and suppression strategies.

In conclusion, standardized pomegranate extract inhibits colon carcinogenesis by regulating the Wnt/β-catenin signalling pathway. Overall, the data indicate that standardized pomegranate extract holds great promise in the field of colon cancer chemoprevention by dietary agents.

Acknowledgments The authors acknowledge Dr Osama Helmy, Pathology Department, National Cancer Institute, Cairo University, Cairo, Egypt for performing the histopathological examinations of this study. The present work was supported by the financial assistance provided by Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Conflict of interest None.

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