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ORIGINAL CONTRIBUTION

Ellagic acid coordinately attenuates Wnt/β-catenin and NF-κB signaling pathways to induce intrinsic apoptosis in an animal model of oral oncogenesis

Prabukumar Anitha · Ramamurthi Vidya Priyadarsini · Krishnamurthy Kavitha · Paranthaman Thiyagarajan · Siddavaram Nagini

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Abstract
Purpose Constitutive activation of the Wnt signaling pathway and its downstream effectors plays a key role in neoplastic transformation. The objective of this study was to investigate the effect of ellagic acid, a plant-derived polyphenol on Wnt/β-catenin signaling and its downstream circuits- NF-κB and mitochondrial apoptosis in the 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis model.
Methods Hamsters were divided into six groups. The right buccal pouches of animals in groups 1–4 were painted with 0.5% DMBA three times a week for 14 weeks. Animals in groups 2–4 received in addition basal diet containing ellagic acid at a concentration of 0.1, 0.2, and 0.4% in the diet. Group 5 animals were given 0.4% ellagic acid alone. Group 6 animals served as control. The expression of the members of Wnt and NF-κB signaling and intrinsic apoptosis was evaluated by western blot analysis.
Results Dietary supplementation of 0.4% ellagic acid suppressed the development of HBP carcinomas by preventing the constitutive activation of Wnt pathway through the downregulation of Fz, Dvl-2, GSK-3β and nuclear translocation of β-catenin. Abrogation of Wnt signaling by ellagic acid was also associated with inactivation of NF-κB and modulation of key components of the mitochondrial apoptotic network.
Conclusions Our findings suggest a functional crosstalk between Wnt and NF-κB signaling pathways in HBP carcinomas that is blocked by ellagic acid supplementation. Dietary ellagic acid that targets the Wnt/β-catenin pathway as well as its downstream signaling mediators is a unique candidate for cancer chemoprevention.

Keywords Apoptosis · Chemoprevention · Ellagic acid · Hamster buccal pouch · NF-κB · Wnt

P. Anitha · R. V. Priyadarsini · K. Kavitha · P. Thiyagarajan · S. Nagini (✉)
Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar, Chidambaram, Tamil Nadu 608 002, India
e-mail: s_nagini@yahoo.com; snlabau@gmail.com

Background

Wnt signaling plays a pivotal role in diverse cellular processes such as cell fate determination, proliferation, and motility [1]. A growing body of evidence suggests that deregulation of the Wnt signaling cascade contributes to the development of various human malignancies [2–4]. Activation of the canonical Wnt signaling pathway involves binding of Wnt ligands to the frizzled receptor (Fz), recruitment of the cytoplasmic phosphoprotein dishevelled (Dvl), and disruption of the multiprotein complex containing glycogen synthase kinase 3β (GSK-3β). Inactivation of GSK-3β in turn results in the stabilization and accumulation of free cytosolic β-catenin. Consequently, β-catenin translocates to the nucleus, interacts with TCF/lymphoid enhancer factor (LEF), a family of high mobility group (HMG) box proteins, and regulates the expression of several downstream target genes involved in tumorigenesis [1].

More recently, activation of Wnt/β-catenin signaling has been recognized to trigger signaling pathways associated with cell survival and cell death, especially the canonical nuclear factor kappa B (NF-κB) pathway by promoting the phosphorylation and degradation of IκB-α by IκB kinase (IKK) [5–7]. Consequently, free cytosolic NF-κB translocates to the nucleus, binds to the κB elements in DNA, and

regulates the transcription of over 400 genes that eventually establish the oncogenic phenotype [8, 9].

Wnt/β-catenin activation also plays a crucial role in restraining the apoptotic machinery [10]. Apoptosis, a form of programmed cell death, is tightly regulated by the pro- and anti-apoptotic members of the Bcl-2 family, and inhibitor of apoptosis proteins (IAPs) [11, 12]. The pro-apoptotic Bcl-2 proteins such as Bax facilitate mitochondrial membrane permeabilization, with release of cytochrome *c* from the mitochondria and activation of the caspase cascade, whereas the anti-apoptotic members Bcl-2 and Bcl-xL inhibit mitochondrial apoptosis thereby favoring cell survival [13]. Survivin, a multifunctional IAP, inhibits caspase-3 and inactivates Smac. Smac/DIABLO, a mitochondrial protein upon efflux into the cytosol, potentiates apoptosis by neutralizing survivin and other IAP molecules. Opa-1, a nuclear-encoded mitochondrial protein, involved in cristae remodeling functions as a key component of the mitochondrial apoptotic network [14, 15].

Identification of dietary phytochemicals that can block Wnt/β-catenin pathway and its associated signaling circuits has assumed significance in recent years as a promising strategy for cancer chemoprevention [16]. Ellagic acid, a plant-derived polyphenol found abundantly in raspberries, strawberries, grapes, blackcurrants, and nuts, exhibits a wide array of biological properties including antioxidant, antiproliferative, antimutagenic, and anticarcinogenic effects [17–20]. However, the effect of ellagic acid supplementation on Wnt/β-catenin signaling and its downstream signaling circuits has not been explored. The present study was designed to investigate the modulatory effects of ellagic acid on Wnt/β-catenin signaling and its associated networks- NF-κB and intrinsic apoptosis in the 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis model. A panel of markers reflecting Wnt signaling (Fz, Dvl-2, GSK-3β, and β-catenin), NF-κB signaling (NF-κB p50 and p65, IκB-α, p-IκB-α and IKKβ), and intrinsic apoptosis (Bcl-2, Bax, survivin, Smac/DIABLO, Opa-1, cytochrome *c*, Apaf-1, cleaved caspase-3 and PARP) was evaluated.

Materials and methods

Chemicals

Acrylamide, bovine serum albumin (BSA), bromophenol blue, 7,12-dimethylbenz[a]anthracene (DMBA), ellagic acid, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), and N,N,N′,N′-tetramethylene diamine (TEMED) were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other reagents used were of analytical grade. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology, USA.

Animals and diet

The experiment was carried out with male Syrian hamsters aged 8–10 weeks weighing between 100 and 110 g obtained from the Central Animal House, Annamalai University, India. The animals housed four to a polypropylene cage were provided with standard pellet diet (Kamadhenu Agencies, Bangalore, India) and water ad libitum. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12 h light/dark cycle. The animals were maintained in accordance with the guidelines of the Indian Council of Medical Research and approved by the ethical committee of Annamalai University. The experimental diet was prepared every day by mixing ellagic acid to preweighed standard pellet diet containing crude protein (22.12%), crude oil (4.12%), crude fiber (3.18%), ash (5.17%), and sand silica (1.13%) with energy value of 3625 kcal/kg. The diet was replenished everyday, and the food consumption was recorded.

Treatment schedule

The animals were randomized into experimental and control groups and divided into 6 groups of 8 animals each. In group 1, the right buccal pouches of hamsters were painted with 0.5% DMBA in liquid paraffin as previously described [21, 22]. In groups 2–4, the right buccal pouches painted with DMBA as in group 1 received in addition basal diet containing ellagic acid at a concentration of 0.1, 0.2, and 0.4% in diet corresponding to 57, 113, and 227 mg/kg body weight, respectively [23]. Animals in group 5 were fed basal diet containing ellagic acid (0.4% in diet). Group 6 animals served as control. The experiment was terminated at 14 weeks, and all animals were sacrificed by cervical dislocation after an overnight fast. Before an animal was sacrificed, the right pouch was grossly inspected to evaluate premalignant lesions or tumor development and photographed. The mean tumor burden was determined by multiplying the number of tumors in each group by the mean tumor volume in millimeters. Tumor volume was calculated using $4/3\pi r^3$, where r represents ½ tumor diameter in mm. The buccal pouch tissues were subdivided and variously processed for distribution to each experiment.

SDS–PAGE and western blot analysis

Approximately 50 mg of each tissue sample was subjected to lysis in a sample buffer containing 62.5 mM Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol,

10% glycerol, and bromophenol blue. Nuclear and cytoplasmic extracts were prepared as described by Legrand-Poels et al. [24]. Tissue samples were homogenized with 1 mL of a buffer containing 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10 mM potassium chloride (KCl), 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μL protease inhibitor cocktail. The lysate was then centrifuged at 8,000×*g* for 2 min at 4 °C, the cytoplasmic supernatant was removed, aliquoted, and frozen at −80 °C. The nuclear pellet was reconstituted in 1 mL of buffer containing 20 mM HEPES (pH 7.9), 0.4 M sodium chloride (NaCl), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 μL protease inhibitor cocktail, followed by vigorous vortexing for 20 min at 4 °C. The nuclear lysate was then centrifuged at 14,000×*g* for 5 min, and nuclear extracts were aliquoted and stored at −80 °C. For the isolation of the mitochondrial fraction, the tissue lysate was centrifuged at 1,000×*g* for 5 min to remove the nucleus and unbroken cells. The supernatant was centrifuged at 23,100×*g* for 30 min at 4 °C, and the resulting pellet containing the mitochondria was resuspended in lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris, 20 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, and protease inhibitor cocktail) [25]. The protein concentration of the lysate and extracts was determined by the method of Bradford [26].

SDS–PAGE was performed using equivalent protein extracts (55 μg) for each sample according to Laemmli [27]. Proteins separated by SDS–PAGE were electrophoretically transferred to polyvinylidene difluoride membranes. The blots were incubated in 1× PBS containing 5% non-fat dry milk for 2 h to block non-specific binding sites. The blots were incubated with primary antibodies diluted as per the instructions from the manufacturer and incubated overnight at 4 °C. The blots were washed thrice with high salt buffer followed by low salt buffer. The blots were then incubated with 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. After extensive washes with high and low salt buffers, the immunoreactive proteins were visualized using enhanced chemiluminescence (ECL) detection reagents. Densitometry was performed on IISP flat bed scanner and quantitated with Total Lab 1.11 software.

Colorimetric estimation of caspase-3 and caspase-9 activity

The activities of caspases were assayed using caspase-3 (Sigma, St. Louis MO, USA) and caspase-9 (Calbiochem, USA) colorimetric assay kits as per the manufacturer's instructions. Cytosolic extracts were prepared by homogenizing tissues in lysis buffer containing 50 mM HEPES (pH 7.4), 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 5 mM DTT. The supernatant was collected as an enzyme source. The assays are based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-nitroanilide (Ac-DEVD-pNA) by caspase-3, and Leu-Glu-His-Asp-nitroanilide (LEHD-pNA) by caspase-9 and subsequent release of the chromophore p-nitroaniline (pNA). The concentration of pNA released from the substrate was calculated from the absorbance values at 405 nm.

Statistical analysis

Statistical analysis was carried out using a nonparametric Mann–Whitney test (Statsdirect, United Kingdom). A probability value of less than 0.05 was considered significant.

Results

Body weight and food consumption

Table 1 shows the body weight gain, food consumption, and intake of ellagic acid in different experimental groups. The average weight gain was significantly decreased in group 1 animals compared to control (group 6). Dietary administration of ellagic acid increased the final body weight in groups 2–4 animals compared to group 1. No significant differences in the body weights were observed in groups 5 and 6. The average food intake per day was approximately 7.7 g. Although the amount of diet consumed by hamsters in groups 1 through 6 was not significantly different, the water intake was significantly decreased in group 1 (DMBA) animals compared to control. Water intake in all the other groups was recorded to be approximately 80 mL/day. The intake of ellagic acid was 57, 113, 228, and 227 mg/kg body weight respectively in groups 2–5, directly reflecting the dose levels.

Tumor incidence, multiplicity, burden, and histopathological observations

Table 2 shows the tumor incidence, multiplicity, burden, and histopathological changes in control and experimental groups. In DMBA-painted animals, the tumor incidence was 100%. These tumors were large and exophytic with a mean tumor burden of 82.25 mm^3 histologically identified as well-defined squamous cell carcinoma. Ellagic acid administration effectively suppressed the development of HBP carcinomas. In group 2 and 3 animals, ellagic acid

Table 1 Body weight gain, food consumption, and intake of ellagic acid in control and experimental animals

Group	Treatment	Average weight gained (g)	Food consumed (g/hamster/day)	Daily intake of ellagic acid	
				mg/hamster/day	mg/kg bw/day
1.	DMBA	50.0 ± 4.9	7.52 ± 0.7	–	–
2.	DMBA + ellagic acid (0.1% in diet)	58.0 ± 5.6	7.64 ± 0.8	7.64 ± 0.7	57.01 ± 5.1
3.	DMBA + ellagic acid (0.2% in diet)	62.0 ± 5.9	7.69 ± 0.6	15.38 ± 0.9	113.08 ± 8.2
4.	DMBA + ellagic acid (0.4% in diet)	63.7 ± 6.2	7.80 ± 0.9	31.20 ± 2.5	227.70 ± 14.4
5.	Ellagic acid alone (0.4% in diet)	65.0 ± 6.3	7.84 ± 0.8	31.36 ± 2.3	227.24 ± 13.8
6.	Control	66.0 ± 6.4	7.92 ± 0.7	–	–

Table 2 Tumor incidence, tumor multiplicity, tumor burden, and histopathological changes in control and experimental animals

Group	Treatment	Tumor incidence	Tumor multiplicity[a]	Tumor burden[b] (mm^3)	Hyperplasia	Dysplasia	SCC (%)
1.	DMBA	8/8 (100)	2.6 ± 1.1	82.3 ± 8.1	+++	+++	8/8 (100)
2.	DMBA + ellagic acid (0.1% in diet)	5/8 (62.5)	0.6 ± 0.4	13.7 ± 1.3	+++	+++	5/8 (62.5)
3.	DMBA + ellagic acid (0.2% in diet)	3/8 (37.5)	0.3 ± 0.2	10.5 ± 1.0	+++	++	3/8 (37.5)
4.	DMBA+ellagic acid (0.4% in diet)	–	–	–	++	–	–
5.	Ellagic acid alone (0.4% in diet)	–	–	–	–	–	–
6.	Control	–	–	–	–	–	–

+ mild, ++ moderate, +++ severe, – no change, *SCC* squamous cell carcinoma

[a] Tumor multiplicity = number of tumors per hamster

[b] Mean tumor burden was calculated by multiplying the mean tumor volume ($4/3\pi r^3$) with the mean number of tumors (r = 1/2 tumor diameter in mm)

decreased the incidence of SCC to 62.5 and 37.5% with a mean tumor burden of 13.65 and 10.52 mm^3, respectively. Histopathological examination of the pouches in group 2 and 3 animals revealed varying degrees of preneoplastic and neoplastic lesions. Although no tumors were observed in group 4 animals, histopathological examination revealed mild to moderate hyperplasia. In groups 5 and 6 animals, the epithelium was normal, intact, and continuous.

Dose–response effect of ellagic acid administration on key proteins involved in Wnt pathway, NF-κB signaling, and intrinsic apoptosis

Western blot analyses of key proteins involved in Wnt pathway, NF-κB signaling, and intrinsic apoptosis revealed increased expression of GSK-3β, β-catenin, NF-κB (p50 and p65), and Bcl-2 associated with downregulation of Bax in DMBA-painted hamsters (group 1) compared to control. Although administration of ellagic acid at 0.1% did not show any significant differences in the expression of the above-mentioned proteins, a dose response effect was observed in the expression of these markers in groups 3 and 4, with 0.4% ellagic acid exerting more significant effects (Fig. 1). Since the higher dose of ellagic acid (0.4% in diet) showed significant tumor inhibitory effects as well as modulation of protein expression, we used only this group for further analysis.

Effect of ellagic acid administration on Wnt/β-catenin signaling

We first analyzed the effect of ellagic acid supplementation on the expression of the members of Wnt signaling pathway. We found that the expression of Fz, Dvl-2, and GSK-3β was significantly increased in DMBA-painted animals compared to control. Dietary administration of ellagic acid to DMBA-painted animals significantly decreased the expression of these markers. In addition, ellagic acid supplementation also reduced the expression of nuclear β-catenin and simultaneously increased cytosolic β-catenin expression. Administration of ellagic acid alone did not induce any significant alterations in the expression of Wnt family members (Fig. 2).

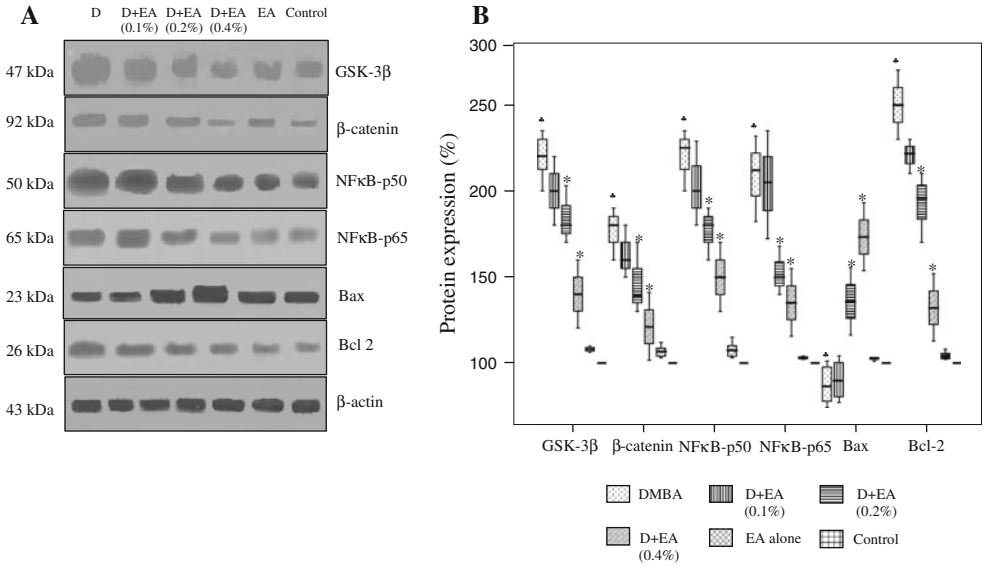

Fig. 1 Western blot analysis of the dose-responsive effect of ellagic acid on proteins involved in Wnt pathway, NF-κB signaling, and apoptosis. **a** Representative immunoblots of GSK-3β, β-catenin, NF-κB (p50 & p65), Bax and Bcl-2. Protein samples (50 μg/lane) resolved on SDS–PAGE were probed with corresponding antibodies. **b** Densitometric analysis for western blots. The protein expression from control lysates for eight determinations was designated as 100% in the graph. Each *box* plot represents the protein expression of eight determinations. β-actin was used as loading control. ♣Significantly different from control ($p < 0.05$) by Mann–Whitney test. *Significantly different from group 1 ($p < 0.05$)

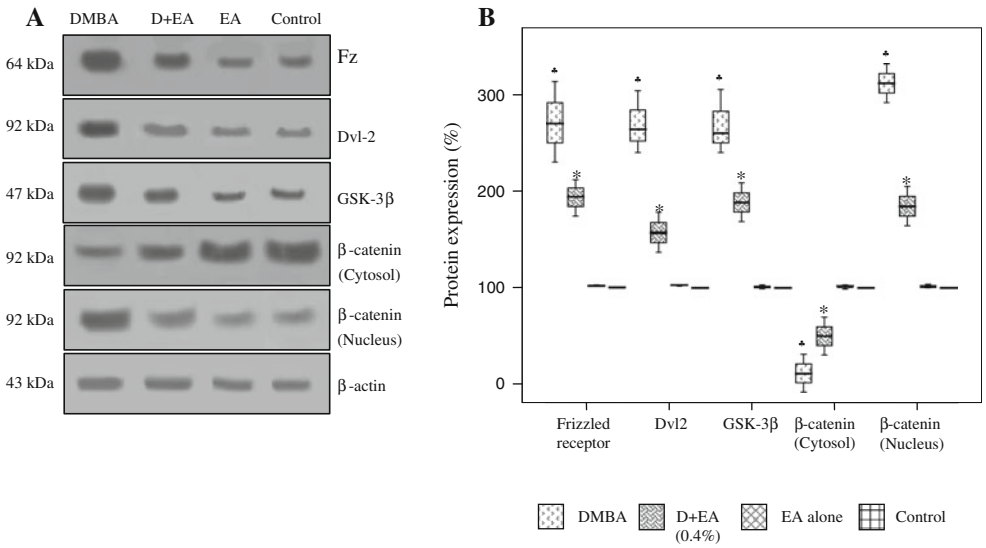

Fig. 2 Western blot analysis of proteins involved in Wnt signaling. **a** Representative immunoblots of frizzled receptor (Fz), Dvl-2, GSK-3β and β-catenin (cytosol and nucleus). Protein samples (50 μg/lane) resolved on SDS–PAGE were probed with corresponding antibodies. **b** Densitometric analysis for western blots. The protein expression from control lysates for eight determinations was designated as 100% in the graph. Each *box* plot represents the protein expression of eight determinations. β-actin was used as loading control. ♣Significantly different from control ($p < 0.05$) by Mann–Whitney test. *Significantly different from group 1 ($p < 0.05$)

Effect of ellagic acid administration on NF-κB signaling

In view of the fact that canonical Wnt and NF-κB signaling pathways are reciprocally activated in several malignancies, we next evaluated the effect of ellagic acid on NF-κB activation. Our results demonstrated increased expression of p-IκB, and IKKβ, with downregulation of IκB in DMBA-painted animals. In addition, DMBA-painted animals showed increased expression of nuclear NF-κB (p50 and p65). Dietary supplementation of ellagic acid to DMBA-painted animals significantly downregulated p-IκB

and IKKβ with increase in IκB expression, associated with decreased expression of NF-κB (both p50 and p65) in the nucleus. However, ellagic acid administration alone did not significantly alter the expression of NF-κB signaling proteins compared to control (Fig. 3).

Effect of ellagic acid administration on intrinsic apoptosis

Since modulation of aberrant Wnt and NF-κB signaling by phytochemicals is generally accompanied by apoptosis induction, we next examined the effect of ellagic acid on intrinsic apoptosis. Analysis of Bcl-2 family members and survivin revealed increased expression of Bcl-2 with decreased expression of bax associated with an increase in cytosolic survivin relative to nuclear survivin in DMBA-painted animals. Ellagic acid supplementation significantly downregulated Bcl-2 and upregulated bax and enhanced nuclear survivin with concomitant downregulation of cytosolic survivin expression compared to group 1 animals (Fig. 4).

To confirm apoptosis induction by ellagic acid supplementation, we next investigated the expression of apoptogenic molecules, caspases, and PARP cleavage. We found overexpression of Opa-1 with downregulation of Smac/DIABLO associated with an increase in mitochondrial cytochrome *c* relative to the cytosolic fraction in DMBA-painted animals. Dietary supplementation of ellagic acid significantly decreased Opa-1 and mitochondrial cytochrome *c* with concomitant increase in the expression of Smac/DIABLO and cytosolic cytochrome *c* compared to group 1 animals. In addition, ellagic acid significantly increased the expression of Apaf-1, and cleaved caspase-3 and induced PARP cleavage compared to group 1 animals. Assay of caspase-3 and -9 enzyme activities further confirmed the results of western blot analysis (Fig. 5).

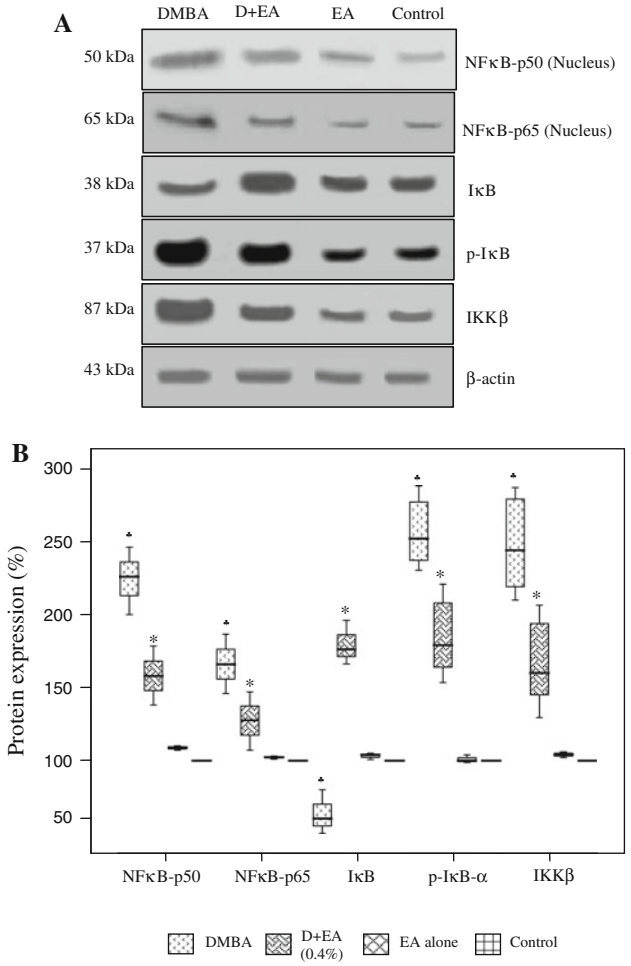

Fig. 3 Western blot analysis of NF-κB family members. **a** Representative immunoblots of NF-κB (p50 and p65), IκB, p-IκB, and IKKβ. Protein samples (50 µg/lane) resolved on SDS–PAGE were probed with corresponding antibodies. **b** Densitometric analysis for western blots. The protein expression from control lysates for eight determinations was designated as 100% in the graph. Each *box* plot represents the protein expression of eight determinations. β-actin was used as loading control. ♣Significantly different from control ($p < 0.05$) by Mann–Whitney test. *Significantly different from group 1 ($p < 0.05$)

Discussion

Ellagic acid has been documented to exert antimutagenic and anticarcinogenic effects against a wide variety of carcinogens and functions as both a blocking and suppressing agent in carcinogenesis [20, 28–31]. In particular, ellagic acid inhibits the metabolism of polycyclic aromatic hydrocarbons such as DMBA [32]. Despite the large body of evidence demonstrating the chemopreventive potential of ellagic acid, a few studies have shown that intravenous injection of ellagic acid induces a hypercoagulable state and cerebral thromboembolism in some animal models [33, 34]. However, no adverse effects have been reported when ellagic acid was administered in the diet [31, 35]. The no observed-adverse-effect level (NOAEL) of ellagic acid was found to be 5% (3,254 mg/kg/day) based on a subchronic toxicity study performed in F344 rats, a dose that is more than ten times used in this study [36]. Thus, both the route of administration and the dose levels of ellagic acid supplementation used in the present study may be considered safe for chemopreventive purpose.

In the present study, we report for the first time the inhibition of DMBA-induced HBP carcinogenesis by dietary ellagic acid based on the modulation of Wnt pathway and its downstream signaling cascades- NF-κB and mitochondrial apoptosis. Constitutive Wnt signaling has been documented as a key factor in the development of various cancers [37–41]. Recently, we have demonstrated the aberrant activation of Wnt/β-catenin signaling during the

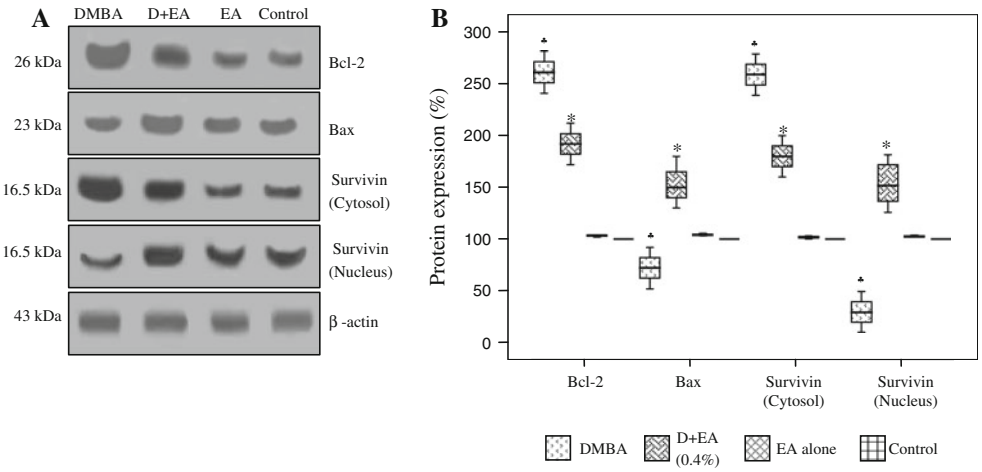

Fig. 4 Expresssion of Bcl-2 family proteins and survivin. **a** Representative immunoblots. Protein samples (50 µg/lane) resolved on SDS–PAGE were probed with corresponding antibodies. **b** Densitometric analysis for western blots. The protein expression from control lysates for eight determinations was designated as 100% in the graph. Each *box* plot represents the protein expression of eight determinations. β-actin was used as loading control. ♣Significantly different from control ($p < 0.05$) by Mann–Whitney test. *Significantly different from group 1 ($p < 0.05$)

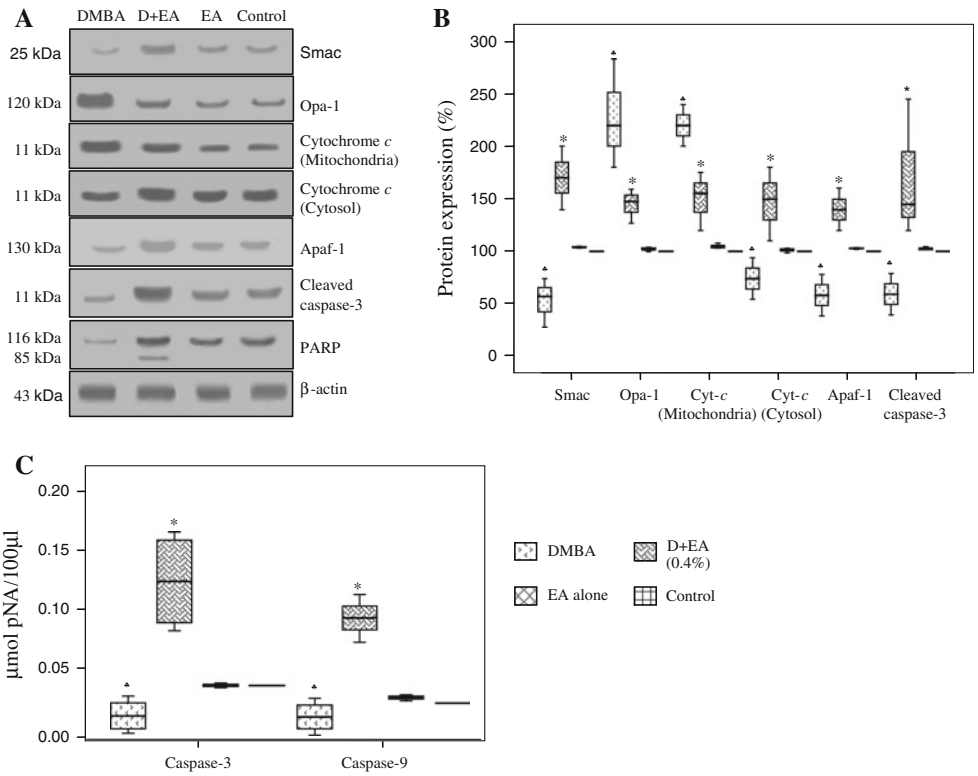

Fig. 5 Protein expression of apoptogenic molecules and activities of caspase-3 and -9 in control and experimental animals. **a** Representative immunoblots. Protein samples (50 µg/lane) resolved on SDS–PAGE were probed with corresponding antibodies. **b** Densitometric analysis. β-actin was used as loading control. ♣Significantly different from control ($p < 0.05$) by Mann–Whitney test. *Significantly different from group 1 ($p < 0.05$). **c** The activity of caspase -3 and -9 in control and experimental animals. ♣Significantly different from control ($p < 0.05$) by Mann–Whitney test. *Significantly different from group 1 ($p < 0.05$)

stepwise evolution of DMBA-induced HBP carcinogenesis [42].

Ellagic acid-mediated suppression of the Wnt pathway during DMBA-induced HBP carcinogenesis is evidenced by downregulation of Fz, Dvl-2, GSK-3β, and β-catenin expressions. An in vitro study on a panel of human cancer cells has demonstrated that cancer cells secrete Wnt family ligands that subsequently activate the Wnt pathway through the frizzled receptors [43]. Thus, the downregulation of frizzled receptors and Dvl-2 observed in the present study implies that ellagic acid prevents the constitutive activation of Wnt pathway by averting the activation of frizzled receptors and subsequent relay of Wnt signals from Fz receptors to downstream effectors through Dvl-2. Furthermore, downregulation of GSK-3β and β-catenin expression by ellagic acid could inhibit the

dissociation of GSK3β from the multiprotein complex, thereby preventing the accumulation of free cytosolic β-catenin and its nuclear translocation, a critical event essential for the transactivation of downstream target genes and signaling cascades. Consistent with this observation, ellagic acid is also documented to inhibit canonical Wnt signaling in HEK 293T colon cancer cells [44].

Suppression of the constitutive activation of Wnt by ellagic acid was associated with inhibition of NF-κB signaling. The mechanism underlying NF-κB inactivation involved downregulation of IKKβ with consequent decrease in p-IκB that prevents nuclear translocation of NF-κB. Several chemopreventive agents such as resveratrol, curcumin analog, and eugenol are known to inactivate NF-κB via direct inhibition of IKKβ in a manner similar to ellagic acid [45–47]. Our findings are in line with recent reports of modulation of NF-κB expression by ellagic acid in 1,2-dimethylhydrazine (DMH)-induced rat colon carcinogenesis and in human umbilical vein endothelial cells (HUVECs) exposed to oxidized low-density lipoprotein [29, 48]. The results of the present study also suggest a functional crosstalk between Wnt and NF-κB signaling pathways in HBP carcinomas that is blocked by dietary ellagic acid. Reduced nuclear beta-catenin expression has been documented to be associated with inhibition of NF-κB signaling [49]. GSK-3β, a key intermediate in Wnt signaling as a multiprotein complex, is reported to block NF-κB activation by preventing the degradation of IκB [7].

Attenuation of canonical Wnt and NF-κB pathways is recognized to facilitate apoptotic cell death [50, 51]. In the present study, dietary administration of ellagic acid was associated with an augmentation of mitochondrial apoptosis, as evidenced by modulation of the expression of pro-/anti-apoptotic, and apoptogenic molecules. An increase in the expression of Bax relative to Bcl-2 facilitates conformational changes in Bax, thereby initiating oligomerization and integration of Bax into the outer mitochondrial membrane. Opa-1, a dynamin-related protein present in the inner mitochondrial membrane, functions as a molecular staple between the adjacent membranes of the cristae, preventing cristae widening and cytochrome *c* release from the mitochondria [52]. Downregulation of Opa-1 together with increased expression of Smac/DIABLO, mitochondrial cytochrome *c*, Apaf, and caspases observed in the present study indicates that loss of Opa-1 expression facilitates mitochondrial apoptosis by promoting Smac/DIABLO and cytochrome *c* release into the cytosol, formation of the casposome complex and subsequent activation of the caspase cascade. Recently, ellagic acid was shown to modulate Bcl-2, Bax, and cytochrome *c* and activate caspase-3 in DMH-induced rat colon carcinomas through inhibition of AKT-phosphoinositide-3 kinase pathway substantiating our observations [53].

In addition to the Bcl-2 family proteins, IAPs, predominantly survivin, play a pivotal role in determining mitochondrial apoptosis. While cytosolic survivin inhibits intrinsic apoptosis by directly or indirectly interfering with the function of caspases, nuclear survivin is believed to control cell division and displays pro-apoptotic activity [12, 54]. Thus, the antiapoptotic activity of survivin essentially depends on its cytosolic or nuclear compartmentalization. Synchronized upregulation of nuclear survivin together with downregulation of cytosolic survivin observed in the present study on ellagic acid treatment demonstrates that enforced nuclear localization of survivin could be a key factor in apoptosis induction.

The results of the present study provide evidence that ellagic acid effectively attenuates the Wnt/β-catenin pathway and its downstream events- NF-κB signaling and apoptosis evasion to inhibit the development of DMBA-induced HBP carcinomas. Interestingly, the results also demonstrate the differential sensitivity of HBP carcinomas and normal pouch to ellagic acid supplementation. Consistent with these findings, Losso et al. [55] demonstrated that ellagic acid selectively induced apoptosis in Caco-2, MCF-7, Hs578T breast, and DU 145 human prostatic cancer cells without exerting any cytotoxic effects on the viability of normal human lung fibroblast cells.

Of late, agents that coordinately target canonical Wnt/β-catenin and NF-κB signaling pathways and induce apoptosis have emerged as effective chemopreventive agents [22, 45, 50, 51]. Recently, the curcumin analog GO-Y030 was reported to induce apoptosis in PK-1, 8505c, and HuCCT-1 cancer cells and suppress nuclear translocation of NF-κB p65 by inhibition of IKKβ [46]. Dimethoxyflavone, a chrysin derivative, and the plant flavonoid fisetin were found to induce apoptosis in cancer cells by simultaneously attenuating Wnt and NF-κB signaling pathways [50, 51]. Taken together, these studies provide evidence that agents such as ellagic acid that target Wnt/β-catenin pathway- a hub in intracellular signaling, as well as its downstream signaling mediators NF-κB family members and proteins involved in intrinsic apoptosis offer ample opportunities for new anti-cancer drug development. Although 5 portions a day containing ellagic acid-rich berries, pomegranates, grapes, and nuts could theoretically provide 0.2–0.4% ellagic acid, factors such as environmental conditions, extent of ripeness, site of cultivation, cultivar, processing and storage that could influence ellagic content in foods must be taken into consideration while extrapolating the results of studies using free ellagic acid to human nutrition [56, 57].

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References

1. Sethi JK, Vidal-Puig A (2010) Wnt signalling and the control of cellular metabolism. Biochem J 427:1–17
2. Queimado L, Obeso D, Hatfield MD, Yang Y, Thompson DM, Reis AM (2008) Dysregulation of Wnt pathway components in human salivary gland tumors. Arch Otolaryngol Head Neck Surg 134:94–101
3. Fu L, Zhang C, Zhang LY, Dong SS, Lu LH, Chen J, Dai Y, Li Y, Kong KL, Kwong DL, Guan XY (2011) Wnt2 secreted by tumour fibroblasts promotes tumour progression in oesophageal cancer by activation of the Wnt/{beta}-catenin signalling pathway. Gut 60:1635–1643
4. Nojima M, Suzuki H, Toyota M, Watanabe Y, Maruyama R, Sasaki S, Sasaki Y, Mita H, Nishikawa N, Yamaguchi K, Hirata K, Itoh F et al (2007) Frequent epigenetic inactivation of SFRP genes and constitutive activation of Wnt signaling in gastric cancer. Oncogene 26:4699–4713
5. Nusse R (2005) Wnt signaling in disease and in development. Cell Res 15:28–32
6. Ma Y, Wang M, Li N, Wu R, Wang X (2009) Bleomycin-induced nuclear factor-kappaB activation in human bronchial epithelial cells involves the phosphorylation of glycogen synthase kinase 3beta. Toxicol Lett 187:194–200
7. Sanchez JF, Sniderhan LF, Williamson AL, Fan S, Chakraborty-Sett S, Maggirwar SB (2003) Glycogen synthase kinase 3beta-mediated apoptosis of primary cortical astrocytes involves inhibition of nuclear factor kappaB signaling. Mol Cell Biol 23: 4649–4662
8. Chaturvedi MM, Sung B, Yadav VR, Kannappan R, Aggarwal BB (2011) NF-kB addiction and its role in cancer: 'one size does not fit all'. Oncogene 30:1615–1630
9. Sung B, Pandey MK, Ahn KS, Yi T, Chaturvedi MM, Liu M, Aggarwal BB (2008) Anacardic acid (6-nonadecyl salicylic acid), an inhibitor of histone acetyltransferase, suppresses expression of nuclear factor-kappaB-regulated gene products involved in cell survival, proliferation, invasion, and inflammation through inhibition of the inhibitory subunit of nuclear factor-kappaBalpha kinase, leading to potentiation of apoptosis. Blood 111:4880–4891
10. Pećina-Slaus N (2010) Wnt signal transduction pathway and apoptosis: a review. Cancer Cell Int 10:22
11. Leibowitz B, Yu J (2010) Mitochondrial signaling in cell death via the Bcl-2 family. Cancer Biol Ther 9:417–422
12. Altieri DC (2010) Survivin and IAP proteins in cell-death mechanisms. Biochem J 430:199–205
13. Heiser D, Labi V, Erlacher M, Villunger A (2004) The Bcl-2 protein family and its role in the development of neoplastic disease. Exp Gerontol 39:1125–1135
14. Adrain C, Creagh EM, Martin SJ (2001) Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J 20:6627–6636
15. Frezza C, Cipolat S, De Brito OM, Micaroni M, Beznoussenko VG, Rudka T, Bartoli D, Polishuck RS, Danial NN, Strooper BD, Scorrano L (2006) OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell 126:177–189
16. Kundu JK, Surh YJ (2005) Breaking the relay in deregulated cellular signal transduction as a rationale for chemoprevention with anti-inflammatory phytochemicals. Mutat Res 591:123–146
17. Festa F, Aglitti T, Duranti G, Ricordy R, Perticone P, Cozzi R (2001) Strong antioxidant activity of ellagic acid in mammalian cells in vitro revealed by the comet assay. Anticancer Res 21:3903–3908
18. Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, Heber D (2005) In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem 16:360–367
19. Rao CV, Tokumo K, Rigotty J, Zang E, Kelloff G, Reddy BS (1991) Chemoprevention of colon carcinogenesis by dietary administration of piroxicam, α-difluoromethylornithine, 16α-fluoro-5-androsten-17-one, and ellagic acid individually and in combination. Cancer Res 51:4528–4534
20. Loarca-Pina G, Kuzmicky PA, de Mejia EG, Kado NY (1998) Inhibitory effects of ellagic acid on the direct-acting mutagenicity of aflatoxin B_1 in the *Salmonella* microsuspension assay. Mutat Res 398:183–187
21. Shklar G (1999) Development of experimental oral carcinogenesis and its impact on current oral cancer research. J Dent Res 78:1768–1772
22. Vidya Priyadarsini R, Vinothini G, Senthil Murugan R, Manikandan P, Nagini S (2011) The flavonoid quercetin modulates the hallmark capabilities of hamster buccal pouch tumours. Nutr Cancer 63:218–226
23. Singletary K, Liao CH (1989) Ellagic acid effects on the carcinogenicity, DNA-binding and metabolism of 7, 12-dimethylbenz(a)anthracene (DMBA). In Vivo 3:173–175
24. Legrand-Poels S, Schoonbrodt S, Piette J (2000) Regulation of interleukin-6 gene expression by pro-inflammatory cytokines in a colon cancer cell line. Biochem J 349:765–773
25. Chan SH, Wu CA, Wu KL, Ho YH, Chang AY, Chan JY (2009) Transcriptional upregulation of mitochondrial uncoupling protein 2 protects against oxidative stress-associated neurogenic hypertension. Circ Res 105:886–896
26. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein-dye binding. Anal Biochem 72:248–254
27. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
28. Chen SC, Chung KT (2000) Mutagenicity and antimutagenicity studies of tannic acid and its related compounds. Food Chem Toxicol 38:1–5
29. Umesalma S, Sudhandiran G (2010) Differential inhibitory effects of the polyphenol ellagic acid on inflammatory mediators NF-kappaB, iNOS, COX-2, TNF-alpha, and IL-6 in 1, 2-dimethylhydrazine-induced rat colon carcinogenesis. Basic Clin Pharmacol Toxicol 107:650–655
30. Tanaka T, Kojima T, Kawamori T, Wang A, Suzui M, Okamoto K, Mori H (1993) Inhibition of 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic and ferulic acids. Carcinogenesis 14:1321–1325
31. Lesca P (1983) Protective effects of ellagic acid and other plant phenols on benzo[a]pyrene-induced neoplasia in mice. Carcinogenesis 4:1651–1653
32. Harttig U, Hendricks JD, Stoner GD, Bailey GS (1996) Organ specific, protocol dependent modulation of 7, 12-dimethylbenz[a]anthracene carcinogenesis in rainbow trout (*Oncorhynchus mykiss*) by dietary ellagic acid. Carcinogenesis 17:2403–2409
33. Na L, Jun-tian L, Qiang-zong Z (2010) Ellagic acid-induced hypercoagulable state in animals: a potentially useful animal hypercoagulable model for evaluation of anticoagulants. Chin Med Sci 25:237–242
34. Hara T, Iwamoto M, Ishihara M, Tomikawa M (1994) Preventive effect of argatroban on ellagic acid-induced cerebral thromboembolism in rats. Haemostasis 24:351–357

35. Mandal S, Stoner GD (1990) Inhibition of N-nitrosobenzylmethylamine-induced esophageal tumorigenesis in rats by ellagic acid. Carcinogenesis 11:55–61
36. Tasaki M, Umemura T, Maeda M, Ishii Y, Okamura T, Inoue T, Kuroiwa Y, Hirose M, Nishikawa A (2008) Safety assessment of ellagic acid, a food additive, in a subchronic toxicity study using F344 rats. Food Chem Toxicol 46:1119–1124
37. Serra R, Easter SL, Jiang W, Baxley SE (2011) Wnt5a as an effector of TGFβ in mammary development and cancer. J Mammary Gland Biol Neoplasia 16:157–167
38. Uraguchi M, Morikawa M, Shirakawa M, Sanada K, Imai K (2004) Activation of WNT family expression and signaling in squamous cell carcinomas of the oral cavity. J Dent Res 83:327–332
39. Barbolina MV, Burkhalter RJ, Stack MS (2011) Diverse mechanisms for activation of Wnt signalling in the ovarian tumour microenvironment. Biochem J 437:1–12
40. Najdi R, Holcombe RF, Waterman ML (2011) Wnt signaling and colon carcinogenesis: beyond APC. J Carcinog 10:5
41. Gaston-Massuet C, Andoniadou CL, Signore M, Jayakody SA, Charolidi N, Kyeyune R, Vernay B, Jacques TS, Taketo MM, Le Tissier P, Dattani MT, Martinez-Barbera JP (2011) Increased wingless (Wnt) signaling in pituitary progenitor/stem cells gives rise to pituitary tumors in mice and humans. Nutr Cancer 63:558–564
42. Vidya Priyadarsini R, Senthil Murugan R, Nagini S (2011) Aberrant activation of Wnt/β-catenin signaling pathway contributes to the sequential progression of DMBA-induced HBP carcinomas. Oral Oncol (in press)
43. Bafico A, Liu G, Goldin L, Harris V, Aaronson SA (2004) An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. Cancer Cell 6:497–506
44. Sharma M, Li L, Celver J, Killian C, Kovoor A, Seeram NP (2010) Effects of fruit ellagitannin extracts, ellagic acid, and their colonic metabolite, urolithin A, on Wnt signaling. J Agric Food Chem 158:3965–3969
45. Bhardwaj A, Sethi G, Vadhan-Raj S, Bueso-Ramos C, Takada Y, Gaur U, Nair AS, Shishodia S, Aggarwal BB (2007) Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells. Blood 109:2293–2302
46. Sato A, Kudo C, Yamakoshi H, Uehara Y, Ohori H, Ishioka C, Iwabuchi Y, Shibata H (2011) Curcumin analog GO-Y030 is a novel inhibitor of IKKβ that suppresses NF-κB signaling and induces apoptosis. Cancer Sci 102:1045–1051
47. Manikandan P, Vinothini G, Vidya Priyadarsini R, Prathiba D, Nagini S (2011) Eugenol inhibits cell proliferation via NF-κB suppression in a rat model of gastric carcinogenesis induced by MNNG. Invest New Drugs 29:110–117
48. Kuo MY, Ou HC, Lee WJ, Kuo WW, Hwang LL, Song TY, Huang CY, Chiu TH, Tsai KL, Tsai CS, Sheu WH (2011) Ellagic acid inhibits oxidized low-density lipoprotein (OxLDL)-induced metalloproteinase (MMP) expression by modulating the protein kinase C-α/extracellular signal-regulated kinase/peroxisome proliferator-activated receptor γ/nuclear factor-κB (PKC-α/ERK/PPAR-γ/NF-κB) signaling pathway in endothelial cells. J Agric Food Chem 59:5100–5108
49. Rajakangas J, Misikangas M, Paivarinta E, Mutanen M (2008) Chemoprevention by white currant is mediated by the reduction of nuclear beta-catenin and NF-kappaB levels in Min mice adenomas. Eur J Nutr 47:115–122
50. Khan MS, Halagowder D, Devaraj SN (2011) Methylated chrysin induces co-ordinated attenuation of the canonical Wnt and NF-kB signaling pathway and upregulates apoptotic gene expression in the early hepatocarcinogenesis rat model. Chem Biol Interact 193:12–21
51. Suh Y, Afaq F, Johnson JJ, Mukhtar H (2009) A plant flavonoid induces apoptosis in colon cancer cells by inhibition of COX2 and Wnt/EGFR/NF-kappaB-signaling pathways. Carcinogenesis 30:300–307
52. Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, Youle RJ (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 1:515–525
53. Umesalma S, Sudhandiran G (2011) Ellagic acid prevents rat colon carcinogenesis induced by 1, 2 dimethyl hydrazine through inhibition of AKT-phosphoinositide-3 kinase pathway. Eur J Pharmacol 660:249–258
54. Stauber RH, Mann W, Knauer SK (2007) Nuclear and cytoplasmic survivin: molecular mechanism, prognostic, and therapeutic potential. Cancer Res 67:5999–6002
55. Losso JN, Bansode RR, Trappey A 2nd, Bawadi HA, Truax R (2004) In vitro anti-proliferative activities of ellagic acid. J Nutr Biochem 15:672–678
56. Häkkinen SH, Kärenlampi SO, Mykkänen HM, Törrönen AR (2000) Influence of domestic processing and storage on flavonol contents in berries. J Agric Food Chem 48:2960–2965
57. Del Rio D, Borges G, Crozier A (2010) Berry flavonoids and phenolics: bioavailability and evidence of protective effects. Br J Nutr 3:67–90