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Original Article

Screening of β-Catenin/TCF Transcription Factor Inhibitors in Medicinal Herb Extracts

Yedang Choe[1], Byungjo Na[2], Seyeon Park[1*]

[1]Department of Applied Chemistry, Dongduk Women's University Seoul 136-714, Korea
[2]Weedam Korean Hospital

Objectives: This study was performed to screen target-specific inhibitors of β-catenin/TCF signaling whose functional activation plays an important role in early events in carcinogenesis.
Methods: To investigate the activation or suppression of β-catenin/TCF transcription, we established a transiently transfected cell line with a constitutively active β-catenin mutant gene whose product is not degraded. This cell line was also co-transfected with luciferase reporter gene constructs containing either an optimized (TOPflash) or mutant (FOPflash) TCF-binding element. We investigated cytotoxic effects using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay. To find effective inhibitors of β-catenin/TCF signaling from medicinal herbs, the crude extracts of 99 types of medicinal herbs were screened using a luciferase assay system in HEK-293 and SH-SY5y cells.
Results: At a concentration of 50 µg/ml, extracts of *Angelica koreanae* radix, *Cannabis sativa* semen, *Ephedrae intermedia Schrenk* radix, and *Vitis rotundifolia* fruit showed the following inhibitory effects on β-catenin/TCF signaling: 40 ± 5.6%, 23 ± 6.1%, 8 ± 5.1%, and 22 ± 9.8% in β-catenin-activated HEK-293 cells and 9 ± 4.7%, 39 ± 8.1%, 39 ± 6.4%, and 42 ± 10.1% in β-catenin-activated SH-SY5y cells, respectively. Crude extracts of *E. radix* were isolated by silica gel column chromatography, and two non-polar fractions of these extracts showed inhibitory effects on β-catenin/TCF signaling.
Conclusions: In this study, we established a transiently transfected cell line as a screening system and found that various medicinal herb extracts had inhibitory effects on β-catenin/TCF signaling.

Key Words : β-catenin/TCF signaling, carcinogenesis, screening, medicinal herb extract

Introduction

More than 20 target genes of the β-catenin signaling pathway have been identified. Among them, axin-2, TCF, c-myc, cyclin D1, metalloproteinases, and vascular endothelial growth factor are regulators of cell fate, proliferation, migration, polarity, developmental control, and carcinogenesis[1-2]. β-catenin is ordinarily involved in cell-cell adhesion in cooperation with the cytoplasmic domain of E-cadherin[3]. Cytosolic β-catenin is phosphorylated by axin/adenomatous polyposis coli (APC)/glycogen synthase kinase (GSK)-3β complex and is recognized by a degradation system composed of ubiquitin and the proteasome[4]. Therefore, cytoplasmic β-catenin is rapidly degraded by the proteasome in normal cells[5]. However, β-catenin accumulation may result from either the mutational inactivation of the APC gene or β-catenin mutations at regulatory amino-terminal serine residues that block its degradation[4]. When a failure in degradation occurs

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・Correspondence to : Seyeon Park
Department of Applied Chemistry, Dongduk Women's University, 23-1 Wolgok-dong, Sungbuk-ku, Seoul 136-714, Korea
Tel : +82-2-940-4514, Fax : +82-2-940-4193, Email : sypark21@dongduk.ac.kr

in mutant cells, increased amounts of β-catenin accumulate in the cytoplasm and translocate into the nucleus[4]. In the nucleus, β-catenin has transcriptional activity in cooperation with T-cell factor (TCF) and the lymphoid enhancer factor (LEF) transcription factor.

The oncogenic role of β-catenin was shown by the discovery that β-catenin-activated mutations are detected in approximately 50% of colorectal cancers that contain wild-type APC[6]. The APC and β -catenin genes are mutated in many types of cancer, including colorectal cancer, melanoma, hepatocellular carcinoma, and gastric carcinoma, and the transcriptional activity of β-catenin is up-regulated in these cancers as well[7-9]. Several studies have reported that β-catenin nuclear localization occurs in one-third of gastric cancers and that β-catenin mutations occur in both diffuse- and intestinal-type gastric cancers at a high rate[10], suggesting that activated β-catenin/TCF signaling via the accumulation of β-catenin in the nucleus is related to human carcinogenesis. Therefore, if β-catenin's transcriptional activity can be markedly down-regulated, tumor growth may be suppressed in a β-catenin-activated types of cancer.

Although there have been many studies using the screening of medicinal herbs for drug development, β-catenin/TCF signaling has rarely been used as a target. In this study, we investigated the inhibitory effects of medicinal herbs using HEK-293 and SH-SY5y cells transiently transfected with a constitutively active mutant β-catenin gene and luciferase reporter gene constructs containing either an optimized (TOPflash) or mutant (FOPflash) TCF-binding element.

These medicinal herbs may be considered cancer chemopreventive agents and facilitate the standardization of combined components that are capable of interrupting the β-catenin signaling target, which has been implicated in oncogenic transformation.

Materials and Methods

1. Cell culture

Human embryo kidney (HEK)-293 and human neuroblastoma (SH-SY5y) cell lines were purchased from Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Eagle's Minimum Essential Medium with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) antibiotics in an incubator at 37°C and 5% (v/v) CO_2.

2. Sample preparation

The crude extracts of 99 types of medicinal herbs (Table 1) were obtained from the Plant Extract Bank (Korean Research Institute of Bioscience and Biotechnology, Daejeon, Korea). The medicinal herbs were extracted with 95% ethanol, dried, and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO).

3. Cell cytotoxicity assay

HEK-293 and SH-SY5y cells (5×10^3 cells/well) were transfected with a constitutively active mutant of β-catenin (pcDNA S33Y). The cells were treated with medicinal herb extracts (50 µg/ml) or DMSO [2.5×10^{-4}% (v/v)] as a blank control. After 24 hours, cell cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay, using the CellTiter 96®AQueous One Solution Assay (Promega, Madison, WI). The reagents were applied to the cultures, incubated for 3 hours, and the absorbance was recorded at 490 nm using a multi reader (Zenyth 1100, ANTHOS, Austria).

4. DNA preparation for transfection

JM109 bacteria cells were transformed with a luciferase reporter plasmid containing an optimal Tcf-4-binding site (TOPflash), mutant Tcf-4-binding site (FOPflash), renilla reporter gene (pGL4.74), and

Table 1. List of Medicinal Herbs.
(Some extracts were isolated from different parts of the same plant and are indicated as such in Latin.)

	Korean name	Latin Name		Korean name	Latin Name
1	갈화	Puerariae Flos	52	목적	Equiseti Herba
2	감국	Chrysanthemi Flos	53	목진피	Fraxini Cortex
3	감초	Glycyrrhizae Radix	54	목향	Saussureae Radix
4	강활	Angelicae koreanae Radix	55	목향	Saussureae Radix
5	맥문동	Liriopis Tuber	56	미후도	Actinidiae Fructus
6	원지	Polygalae Radix	57	미후등	Actinidiae Caulis
7	건율	Castaneae Semen	58	밀몽화	Buddleiae Flos
8	건지황	Rhemaniae Radix	59	박하	Menthae Herba
9	계지	Cinnamomi Ramulus	60	반대해	Sterculiae Scaphigerae Semen
10	고련피	Meliae Cortex	61	반지련	Potulacae Grandiflorae Herba
11	골쇄보	Drynariae Rhizoma	62	목방풍	Peucedani Japonici Radix
12	광곽향	Pogostemonis Herba	63	비자	Torreyae Semen
13	괴각	Sophorae Fructus	64	백개자	Sinapis Semen
14	괴화	Sophorae Flos	65	백굴채	Chelidoni Herba
16	구맥	Dianthi Herba	66	백두구	Amomi Cardamomi Fructus
17	구절초	Chrisanthemi sibirici Herba	67	백두옹	Pulsatillae Radix
18	귀전우	Euonimi Lignum Suberalatum	68	모근	Imperatae Rhizoma
19	귤피	Citri unshiu Pericarpium	69	백미	Cynanchi Radix
20	귤핵	Citri tangerinae Semen	70	백부근	Stemonae Radix
21	구척	Cibotii Rhizoma	71	백렴	Ampelopsis Radix
22	금전초	Desmodii Herba	72	백작약	Paeoniae Radix alba-roof
23	길경	Platycodi Radix	73	백작약	Paeoniae Radix alba
24	낙석등	Trachelospermi Cauilis	74	백지	Angelicae Dahuricae Radix-root
25	남성	Arisaematis Rhizoma	75	백질려	Tribuli Semen
26	노근	Phragmitis Rhizoma	76	백질여	Tribuli Semen-leaf
27	노회	Aloe	77	백축	Pharbitidis Semen
28	단삼	Salviae Radix	78	백출	Atractylodis Rhizoma alba
29	당귀	Angelicae Sinensis Radix-stem	79	백출	Atractylodis Rhizoma alba-root
30	당귀	Angelicae Gigantis Radix-leaf	80	백출	Atractylodis Rhizoma alba-leaf
31	당귀	Angelicae Gigantis Radix-root	81	백편두	Dolichoris Semen
32	당귀미	Angelicae Gigantis Radix	82	백편두	Dolichoris Semen-leaf
33	부자	Aconiti Tuber	83	백수오	CynanchiWilfordiiRadix
34	대복피	Arecae Pericarpium	84	백수오	CynanchiWilfordiiRadix-root
35	도인	Persicae Semen	85	백합	Lilie Bulbus
36	도인	Persicae Semen-end	86	복령	Hoelen
37	독활	Araliae Cordatae Radix	87	복분자	Rubi Fructus
38	두충	Eucomiae Cortex	88	복분자	Rubi Fructus-root
39	두충엽	Eucomiae Folium	89	봉출	Zedoariae Rhizoma
40	두충지	Eucomiae Ramulus	90	부소맥	Tritici Immatri Semen
41	두충	Eucomiae Cortex-leaf	91	부평	Spirodelae Herba
42	옥죽	Polygonati Officinalis Rhizoma	92	비해	Tokoro Rhizoma
43	마두령	Aristolchiae Fructus	93	빈랑	Arecae Semen
44	마자인	Cannabis Semen	94	사간	Belamcandae Rhizoma
45	마전자	Nux-Vomica	95	사과락	Luffae Fructus Retinervus
46	마황	Ephedrae Herba	96	사군자	Quisqalis Fructus
47	마황근	Ephedrae Radix	97	더덕	Codonopsitis Lanceolatae Radix
48	만삼	Codonopsitis Radix	98	사삼	Adenophorae Radix
49	만형자	Viticis Fructus	99	사상자	Torilidis Fructus
50	맥문동	Liriopis Tuber	100	산사	Crataegi Fructus
51	모과	Chaenomelis Fructus			

pcDNA S33Y. DNA was prepared using a QIAGEN® Plasmid Midi Kit (Qiagen, Manchester, UK).

5. Transfection and luciferase assay

Transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). HEK-293 cells (5×10^4) and SH-SY5y cells (5×10^4) were transfected with 120 ng TOPflash or FOPflash as a negative control and 120 ng pGL4.74 for signal normalization. The cells were co-transfected with a constitutively active mutant of β-catenin (pcDNA S33Y). After 4 hours, media and reagents were displaced with fresh 10% FBS-containing media. Tranfected cells were treated with the medicinal herb extracts (50 µg/ml) or DMSO (2.5×10^{-4}%) diluted in fresh serum-free media. After 24 hours, firefly and renilla luciferase activities were determined using the Dual-Glo™ Luciferase Assay System (Promega) with a luminescence multi reader (Zenyth 1100).

6. Extraction and purification of the *Ephedrae radix*

Dried *E. radix* (500 g, China) was purchased from the Korean Pharmacopeia [KP (standard), choice goods (grade), 04. 30. 09' (date of manufacture), Dongui Hanbang, Korea] and extracted with 95% ethanol (1.0 L) under reflux conditions for 1 hour. The ethanol extract was concentrated under reduced pressure to yield a powder (13.6 g). The extract powder was suspended in chloroform (30 mL), subjected to a silica gel column chromatography (400 g, silica gel 60, 0.040-0.063 mm, Merck, Germany), and eluted with chloroform containing increasing amounts of methanol. Each eluate was concentrated under reduced pressure, yielding greater than 40 fractions, which were eluted with methanol (1 mL) and analyzed by thin-layer chromatography (TLC: Silica gel 60 F_{254}, Merck) using a chloroform-methanol system at room temperature. The fractions represented the same TLC spots were combined, and were concentrated to 18 fractions and dissolved in DMSO.

Results

1. S33Y mutant is not degraded by axin/APC/GSK-3b complex

To determine the transcriptional activation of β-catenin/TCF, we performed a luciferase assay using HEK-293 and SH-SY5y cells transiently transfected with the S33Y β-catenin mutant. The S33Y mutant product was not induced for degradation by the axin/APC/GSK-3b complex. Luciferase activity increased approximately 20-fold by S33Y gene transfection as shown in Fig. 1.

2. Plant extracts inhibit β-catenin/TCF signaling

In β-catenin-activated HEK-293 cells, luciferase activity was inhibited more than 50% by 25 different medicinal herb extracts as shown in Fig. 2. Twelve different extracts inhibited luciferase activity more than 50% in β-catenin-activated SH-SY5y cells (Fig. 3). Each value was normalized for transfection efficiency to renilla activity. Percent activity was determined and compared to the value determined with vehicle DMSO. Extracts showing both inhibitory activity toward β-catenin/TCF signaling and cytotoxicity greater than 50% are represented in Table 2.

The radix of *A. koreana* (number 4 in table 1), the semen of *C. sativa* L (number 44), the radix of *E. intermedia* (number 47), and the fruit of *Vitis rotundifolia* L. fil (number 49) showed inhibitory activity toward β-catenin/TCF signaling in both β-catenin-activated cell lines.

4. Some medicinal herb extracts show cytotoxicity

The cytotoxicity of the medicinal herb extracts (50

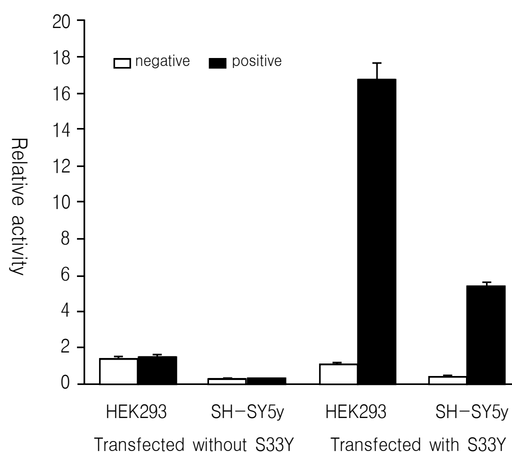

Fig. 1. Cells were transfected with or without the β-catenin mutant S33Y. The numerical values are the activity ratios of firefly/renilla activity. Negative values represent cells transfected with FOPflash. Positive values represent cells transfected with TOPflash.

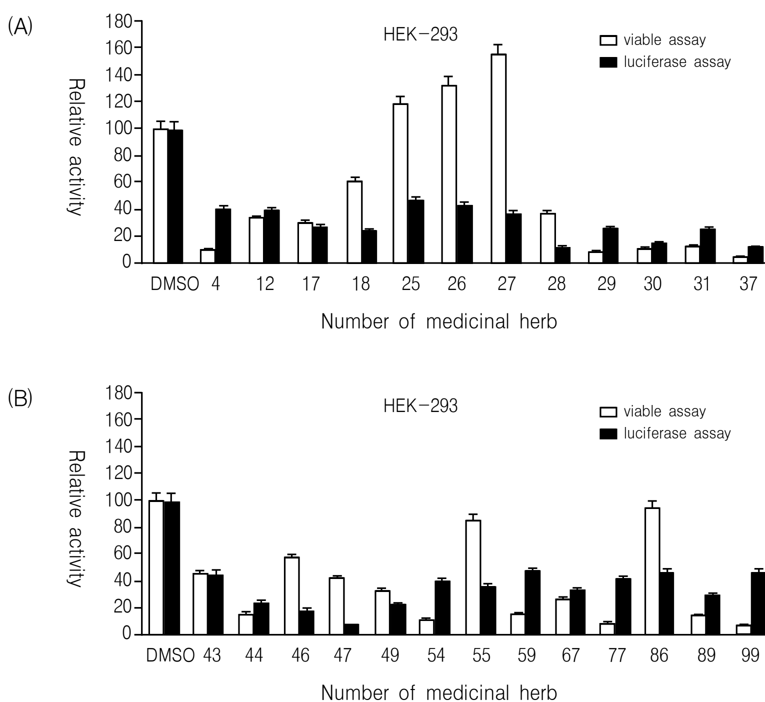

Fig. 2. Viability and luciferase activity in HEK-293 cells transfected with the β-catenin mutant S33Y.

μg/mL) was determined by MTS assay and results are shown in Fig. 2 and 3. Percent viability was determined and compared to the value determined with vehicle DMSO. Some medicinal herbs showed inhibitory activity of β-catenin/TCF signaling and cytotoxicity greater than 50% in both cases (numbers

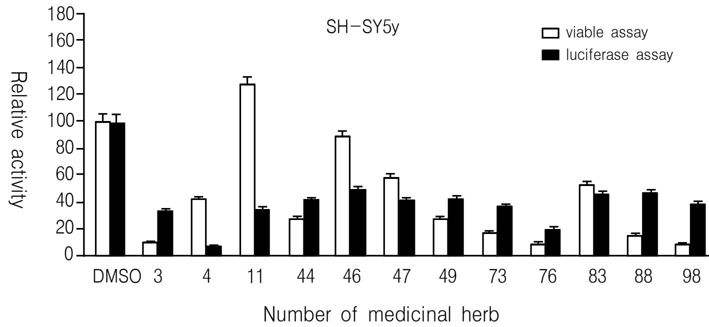

Fig. 3. Viability and luciferase activity in SH-SY5y cells transfected with the β-catenin mutant S33Y.

4, 12, 17, 28, 29, 30, 32, 37, 43, 44, 47, 49, 54, 59, 67, 77, 89 and 99 in β-catenin-activated HEK-293 cells; numbers 3, 4, 44, 47, 49, 73, 76 and 98 in β-catenin-activated SH-SY5y cells). It seems that the cytotoxicity seems to be compatible with inhibitory activity of β-catenin/TCF signaling in this case.

5. Extracts of Ephedrae radix inhibit β-catenin/TCF signaling

Extracts of *Ephedrae* radix were separated into 18 fractions by silica gel chromatography. Luciferase activity was determined using these fractions (Fig. 4). Fractions 7 and 16 at a concentration of 50 µg/ml

Table 2. Medicinal Herbs Inhibited β-catenin/TCF Signaling Greater Than 50% in HEK-293 and SH-SY5y Cells. The numbers 4, 44, 47, and 49 were inhibitory in both HEK-293 and SH-SY5y cells.

HEK-293		SH-SY5y	
		3	Glycyrrhizae Radix
4	Angelicae koreanae Radix	4	Angelicae koreanae Radix
12	Pogostemonis Herba		
17	Chrisanthemi sibirici Herba		
28	Salviae Radix		
29	Angelicae Sinensis Radix-stem		
30	Angelicae Gigantis Radix-leaf		
31	Angelicae Gigantis Radix-root		
37	Araliae Cordatae Radix		
43	Aristolchiae Fructus		
44	Cannabis Semen	44	Cannabis Semen
47	Ephedrae Radix	47	Ephedrae Radix
49	Viticis Fructus	49	Viticis Fructus
54	Saussureae Radix		
59	Menthae Herba		
67	Pulsatillae Radix		
		73	Paeoniae Radix alba
		76	Tribuli Semen-leaf
77	Pharbitidis Semen		
		83	Cynanchi Wilfordii Radix
		88	Rubi Fructus-root
89	Zedoariae Rhizoma		
		98	Adenophorae Radix
99	Torilidis Fructus		

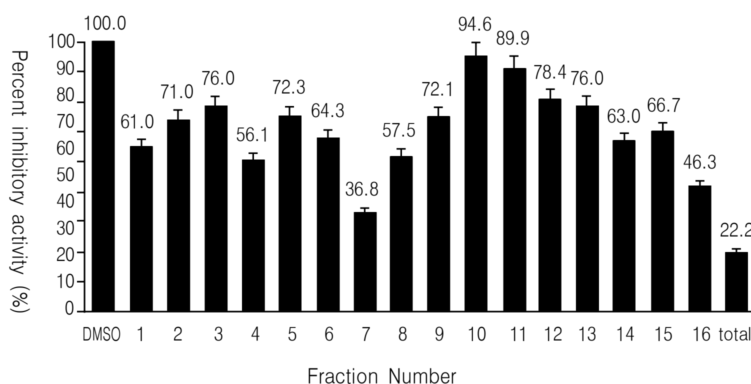

Fig. 4. Fractions of *Ephedrae* radix inhibited β-catenin/TCF signaling.

inhibited β-catenin/TCF signaling by 36.8% and 46.3%, respectively.

Discussion and Conclusions

We screened various Chinese herbs and found that ethanolic extracts of the radix of *A. koreana*, the semen of *C. sativa* L, the radix of *E. intermedia*, and the fruit of *V. rotundifolia* L. fil inhibit β-catenin/TCF transcriptional activity.

Medicinal herbs can serve as anti-cancer drugs[11]. As the importance of β-catenin as a cause of tumorigenesis increases, many more studies on β-catenin inhibitors and their mechanisms of action are underway[12]. Elevated β-catenin/TCF levels via the accumulation of β-catenin in the nucleus are involved in a number of human cancers, including colon cancer, gastric cancer, and melanoma[7-9]. Activation of an abnormal APC/β-catenin/TCF signaling pathway and alterations in cellular adhesion mediated through changes in β-catenin homeostasis within the colonic epithelium are crucial factors in the development of the majority of colorectal cancers[7].

Various studies on inhibitory agents toward β-catenin/TCF signaling in cancer cell lines have been performed. Non-steroidal anti-inflammatory drugs, nitric oxide-generating aspirin, Gleevec, and epigallocatechin-3-gallate have been reported to inhibit β-catenin/TCF signaling[13-16]. Recently, it was suggested that a combination of tea plus sulindac inhibits intestinal neoplasia in mice via effects on the β-catenin/APC pathway[17]. We have previously reported inhibitory effects of curcumin and the curcumin analogs, quercetin and flavonoids, against β-catenin/TCF signaling.

We therefore investigated the effects of medicinal herb extracts on β-catenin/TCF signaling. The results presented here demonstrate that four types of medicinal herbs, *A. koreana*, C. *sativa* L, E. *Intermedia*, and *V. rotundifolia* L. fil, inhibit β-catenin/TCF transcriptional activity.

Ephedrae (Ma Huang) is one of the most extensively studied Chinese herb medicines. *Ephedrae* has been used clinically to disperse a cold, control wheezing, promote urination, and reduce edema[18]. *Ephedrae* has been used for the treatment of asthma, allergies, and immune diseases[19]. Moreover, *Ephedrae* has shown not only cytotoxicity but also inhibition of multidrug-resistance system in HeLa cells[20]. However, the effect and mechanism of *Ephedrae* on cancer cells have rarely been investigated.

Medicinal herbs possess a variety of biological activities at non-toxic concentrations. The role of

medicinal herbs in cancer prevention has been widely discussed, as have the various mechanisms by which medicinal herbs may affect tumorigenesis, including anti-oxidation and scavenging of activated mutagens and carcinogens. However, concrete and inclusive molecular mechanisms have not been completely elucidated. Given their function in inhibiting β-catenin/TCF signaling, these medicinal extracts may be valuable as a chemotherapeutic agents against β-catenin-activated tumors.

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References

1. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature. 1999; 398(6726):422-6.
2. Zhang F, White RL, Neufeld KL. Cell density and phosphorylation control the subcellular localization of adenomatous polyposis coli protein. Mol Cell Biol. 2001; 21(23):8143-56.
3. Mareel M, Boterberg T, Noe V, Van Hoorde L, Vermeulen S, Bruyneel E, *et al*. E-cadherin/catenin/cytoskeleton complex: a regulator of cancer invasion. J Cell Physiol. 1997; 173: 271-274.
4. Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S , Polakis P. Binding of GSK3b to the APC?β-catenin complex and regulation of complex assembly. Science. 1996; 272:1023-1026.
5. Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. β-catenin is a target for the ubiquitin proteasome pathway. EMBO J. 1997; 16:3797-3804.
6. Luu HH, Zhang R, Haydon RC, Rayburn E, Kang Q, Si W, *et al*. Wnt/beta-catenin signaling pathway as a novel cancer drug target. Curr. Cancer Drug Targets. 2004; 4(8):653-71.
7. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, *et al*. Activation of β-catenin-TCF signaling in colon cancer by mutations in β-catenin or APC. Science. 1997; 275:1787-1790.
8. Fujie H, Moriya K, Shintani Y, Tsutsumi T, Takayama T, Makuuchi M, *et al*. Frequent β-catenin aberration in human hepatocellular carcinoma. Hepatol Res. 2001; 20:39-51.
9. Woo DK, Kim HS, Lee HS, Kang YH, Yang HK, Kim WH. Altered expression and mutation of β-catenin gene in gastric carcinomas and cell lines. Int. J Cancer. 2001; 95:108-113.
10. Clements WM, Wang J, Sarnaik A, Kim OJ, MacDonald J, Fenoglio-Preiser C, *et al*. β-catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. Cancer Res. 2002; 62:3503-3506.
11. Ho JW, Leung YK, Chan CP. Herbal medicine in the treatment of cancer. Curr. Med. Chem. Anticancer Agents. 2002; 2(2):209-14.
12. Clapper ML, Coudry J, Chang WC. β-catenin-mediated signaling: a molecular target for early chemopreventive intervention. Mutat Res. 2004; 555:97-105.
13. Dihlmann S, Siermann A, Doeberitz MK. The nonsteroidal anti-inflammatory drugs aspirin and indomethacin attenuate bcatenin/TCF-4 signaling. Oncogene. 2001; 20:645-653.
14. Nath N, Kashfi K, Chen J, Rigas B. Nitric oxide-donating aspirin inhibits β-catenin/T cell factor (TCF) signaling in SW480 colon cancer cells by disrupting the nuclear β-catenin-TCF association. Proc. Natl. Acad. Sci. U S A. 2003; 100:12584-12589.

15. Zhou L, An N, Haydon RC, Zhou Q, Cheng H, Peng Y, *et al*. Tyrosine kinase inhibitor STI-57/ Gleevec down-regulates the β-catenin signaling activity. Cancer Lett. 2003; 193:161-170.
16. Dashwood WM, Orner GA, Dashwood RH. Inhibition of β-catenin/TCF activity by white tea, green tea, and epigallocatechin-3-gallate (EGCG): minor contribution of H2O2 at physiologically relevant EGCG concentrations, Biochem. Biophys. Res. Commun. 2002; 296:584-588.
17. Orner GA, Dashwood WM, Blum CA, Diaz GD, Li Q, Dashwood RH. Suppression of tumorigenesis in the Apcmin mouse: down-regulation of β-catenin signaling by a combination of tea plus sulindac. Carcinogenesis. 2003; 24:263-267.
18. Dan B, Andrew G. 1992. Chinese Herbal Medicine. Materia Medica. Eastland Press: Seattle. 1992:32-34.
19. Bielory L. Complementary and alternative interventions in asthma, allergy, and immunology. Annals of allergy, asthma & immunology 93. 2004:S45-S54.
20. Takara K, Horibe S, Obata Y, Yoshikawa E, Ohnishi N, Yokoyama T. Effects of 19 herbal extracts on the sensitivity to paclitaxel or 5-fluorouracil in HeLa cells. Biol Pharmaco Bull. 2005: 28:138-142.