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Downregulation of Wnt/β-catenin self-renewal pathway in cervical cancer cells by polyphenolic compounds

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Cervical cancer is the second most common cancer in woman of developing countries. Wnt/β-catenin self-renewal pathway is important for cervical cancer initiation and progression. Plumbagin, Pongapin and Karanjin are three plant polyphenols with known anticancer activities. Thus, this study aims to analyze the effects of these compounds on Wnt/β-catenin pathway in cervical cancer cells (HeLa), due to their high sensitivity in this cell line. The compounds significantly downregulated the co-receptor LRP6 (low density lipoprotein receptor related protein 6) expression (mRNA/ protein) in HeLa cells without any change in the expression of receptor FZD7 (Frizzled class receptor 7). The low membrane expression of LRP6 seen in the immunocytochemical analysis might be due to upregulation of its antagonist Dickkopf 1 (DKK1) protein. The compounds could also increase the expression ofFZD7 antagonists, SFRP1/2 (mRNA/protein) in HeLa cells. The upregulation of SFRPs (secreted frizzled-related protein) was due to their promoter hypomethylation through downregulation of DNMT1 (DNA methyltransferase 1) protein by the compounds. As a result, there was downregulation of effector protein β-catenin and activated phospho-β-catenin (Y654) of the pathway in HeLa cells by these compounds. Thus, the polyphenols differentially inhibit the Wnt/β-catenin pathway to restrict cervical cancer proliferation, suggesting their therapeutic importance.

Keywords: Anticancer, Frizzled class receptor, Hypomethylation, Karanjin, mRNA expression, Phospho-β-catenin, Plumbagin, *Pongamia pinnata*, Pongapin, Secreted frizzled-related protein

Cervical cancer (CC) is the fourth most common cancer in woman and ranks as third leading cause of cancer-related death in woman worldwide[1]. Wnt/β-catenin pathway is an evolutionarily conserved cascade regulating development and stemness[2,3]. Aberrant Wnt/β-catenin signalling is tightly associated with different cancers, including CC[4-7]. Wnt/β-catenin signalling contributes to different cancers including CC at multiple levels, starting from tumor initiation, progression, to invasion; and it is also responsible for therapeutic resistance[8,9]. There are reports that β-catenin are over-expressed during the development of CC[8,10]. Thus, Wnt/β-catenin signaling pathway can be targeted for effective cancer therapeutics.

Plumbagin (PL), Pongapin (PG) and Karanjin (KR) are plant polyphenolic compounds. PL is a hydroxyl naphthoquinone isolated from *Plumbago* sp. PG and KR are furanoflavanoid derivatives, isolated from *Pongamia pinnata* (L.) Pierre. It is reported that PL, PG and KR could inhibit proliferation of cervical cancer cells by inducing reactive oxygen species (ROS)-mediated DNA damage and apoptosis[10]. PL downregulates β-catenin, the effector protein of Wnt/β-catenin pathway in human ovarian cancer[12,14], colorectal cancer[13], osteosarcoma and breast cancer, etc. [15,16]. However, to the best of our knowledge, the effect of PL on Frizzled class receptor 7 (FZD7) receptor and its co-receptor low density lipoprotein receptor related protein 6 (LRP6) as well as the antagonists [Dickkopf-1 (DKK1) and secreted frizzled related protein (SFRP)1/2] of the Wnt pathway have not yet been analyzed in detail. Similarly, the effect of PG and KR on the Wnt pathway has not been studied to understand their importance in the regulation of this pathway.

Therefore, in this study, we analyzed the effect of Plumbagin, Pongapin, and Karanjin on Wnt/β-catenin pathway in cervical cancer cell line (HeLa).

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Materials and Methods

Drugs and Reagents

Pongapin and Karanjin (>99% purity) were extracted and purified at the National Research Institute for Ayurvedic Drug Development, Kolkata, India. Plumbagin was purchased from Sigma-Aldrich Chem. Co. , St. Louis, MO, USA (*P7262 sigma*). All the drugs were reconstituted in dimethyl sulfoxide (DMSO). DMSO, 6-diamidino-2-phenylindole, fluorescent nuclear staining dye (DAPI), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Minimal essential medium (MEM), 0. 25% trypsin-EDTA, L-glutamine, phosphate buffered saline (PBS), foetal bovine serum (FBS) as well as penicillin and streptomycin mixture (100 IU/mL; 100/100 µg/mL) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies against FZD7, LRP6, DKK1, SFRP1, SFRP2,β-catenin, phospho-β-catenin (Y654), horseradish peroxidase-conjugated/FITC-conjugated anti-rabbit or anti-mouse IgG antibodies, and α-tubulin were procured from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA. For mRNA expression analysis, TRIzol reagent, superscript III reverse transcriptase and power SYBR green PCR master mixtures were obtained from Life Technologies Corp. (Carlsbad, CA, USA).

Cell line and cell culture condition

HeLa cells (cervical cancer cell line) were obtained from National Centre for Cell Sciences, Pune, India. HeLacell line was maintained in MEM. The media was supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. All the experiments were performed within 20-40 passages in order to confirm cell population, their uniformity, and reproducibility.

Expression analyses

Protein extraction and Western blot analysis

Total protein was extracted from the HeLa cell line (both before and after the compounds' treatments) by sonication with radio-immunoprecipitation assay (RIPA) lysis buffer as described in our previous study[11]. Then, the samples were electrophoresed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8-12% gel) and Western blotting was done by wet transfer. After the transfer, the membranes were blocked by 3-5% BSA for 1 h at room temperature (22-25°C). Then the blocked membranes were incubated with primary antibody against the target protein for overnight at 4°C, followed by incubation with the secondary antibody for 1-2h at 4°C. The target protein bands were then detected using luminol reagent and autoradiographed on X-ray film (Kodak, Rochester, NY, USA). The band intensities were then quantified using densitometric scanner (GS-800, Bio-Rad Laboratories, Hercules, CA, USA), and the peak densities of the proteins of interest were normalized using the loading control (α-tubulin) peak density and then graphically the relative protein expression were represented after normalization with the control group.

Immunocytochemical analysis

HeLa cells were seeded on to cover slips and then treated with different drugs (i. e. , PG, KR or PL) at different concentrations. Subsequently, the cells were fixed in methanol for 30 min and kept at –20°C, till further use. The coverslip with HeLa cells were first blocked with 3-5% BSAfor 1h at room temperature. Then, the coverslips were incubated with primary antibody against the desired protein overnight at 4°C, followed by incubation with the FITC-conjugated secondary antibody for 1-2 h at 4°C. The coverslip was then counter stained with DAPI (4′,6-diamidino-2-phenylindole) and mounted with 10% glycerol and photographed under fluoroscence microscope[11]. Each experiment was performed at least in triplicate.

mRNA expression analysis

Total RNA was isolated from HeLa cell line using TRIzOL reagent (Invitrogen Corp. , Carlsbad, CA, USA) according to the manufacturer's protocol. Purity of RNA was checked by spectrophotometric analysis of A260/280 ratio and concentration was determined from A260 value. The 8 µg of total RNA was treated with DNaseI and then, the cDNA was synthesized from 2 µg of DNaseI treated RNA by using reverse transcriptase (Promega Corp. , Madison, WI, USA)[9,11]. Real-time quantification of the candidate genes was performed using a power SYBR-green assay (Applied Biosystems, Inc. , Beverly, MA, USA) with β2-microglobulin (B2M) as control[5]. Each sample was loaded in triplicate and run at 40 cycles on an ABI prism 7500 machine (Applied Biosystem Inc. , Beverly, MA, USA). After each run, melting curves were generated to confirm amplification of specific transcripts. To determine the relative level of gene expression, the comparative threshold cycle

Table 1 — Different doses of the three compounds selected for further treatment(s) in HeLa cell lines during the present study

Compound (Represented as)	Dose inhibiting 30% cell viability (IC_{30}) (μg/mL)	Dose inhibiting 50% cell viability (IC_{50}) (μg/mL)	Dose inhibiting 70% cell viability (IC_{70}) (μg/mL)
Pongapin (PG)	8. 45	16. 9	23. 66
Karanjin (KR)	23. 5	47. 05	65. 8
Plumbagin (PL)	0. 17	0. 34	0. 48

(ddCT) method was employed after normalization of the respective gene of interest expression level against that of B2M (dCT)[5,17]. After the treatments with the compounds in HeLa cells, the relative gene expression of the genes in terms of fold expression change (2^{-ddCT}) were calculated from the ddCT values obtained using the formula:

ddCT = [dCT(Target) − dCT(B2M)] (compound treated HeLa cells)

− [dCT (Target) − dCT(B2M)] (untreated control HeLa cells)

Promoter methylation analysis

Promoter methylation status of SFRP1, SFRP2 and DKK1were analyzed in CpG-rich islands in the promoter region of the respective genes by polymerase chain reaction (PCR)-based methylation sensitive restriction analysis (MSRA) using HhaI (Promega Corp. , Madison, WI, USA), enzyme. The β-3A adaptin gene (K1) and RARβ2 (K2) were used as digestion and integrity controls, respectively. The primer sequences are listed as Supplementary data (Suppl. Tables S 1-3. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr. niscpr. res. in*). Quantification of promoter methylation was done by real-time PCR using a power SYBR-Green assay (Applied Biosystems Inc. , Foster City, CA, USA)[18]. Each sample was loaded in triplicate and run at 40 cycles on an ABI prism 7500 machine (Applied Biosystems, Inc. , Foster City, CA, USA). The percentage of hypomethylation was calculated by taking the ratio of the cycle threshold (Ct) values of the digested DNA sample with its respective undigested DNA, presuming the undigested DNA as 100% methylated.

The percentage of hypomethylation is a comparative term which was plotted as the differences in the methylation percentages among the samples. Higher the percentage of hypomethylation, lower the frequency of methylation in the sample and *vice versa*. The percentage of hypomethylation of a particular gene was calculated using the following formula:

(Ct [digested]/Ct [undigested] ×100) – 100)[18].

Statistical analysis

Data obtained from the PG, KR, and PL treated groups were compared with HeLa control group. Statistical analysis was performed using t-test. P value <0. 05 and P <0. 001 were considered as statistically significant and highly significant, respectively. Data are expressed as mean with standard deviation (SD). All the experiments were repeated at least three times.

Results

Treatment of HeLa cells with PL, PG and KR

In our previous study[19], utilizing cytotoxicity assay, the different doses for the compounds were determined to be useful for the treatment and further experiments. It was also found that the compounds showed high sensitivity against cervical cancer cells (i. e. , HeLa). Concentration of the compounds inhibiting cell viability by 30% (IC_{30})/50% (IC_{50})/70% (IC_{70}), were chosen for the study. The doses selected for different compounds for the studies are provided in Table 1.

Downregulation of LRP6 expression by PL, PG and KR

In Wnt/β-catenin pathway, the interaction of FZD7 and LRP6 through ligand Wnt leads to activation of the pathway resulting in upregulation of its effector protein β-catenin[20]. The compounds showed no significant change in the mRNA expression of FZD7after the treatment (Fig. 1A). However, they could downregulate the mRNA expression of LRP6 in a dose-dependent manner. PL showed comparatively higher inhibition followed by PG and KR (Fig. 1B).

Like mRNA expression, the Western blot analysis showed significant decrease in LRP6 protein expression after treatment with the compounds (Fig. 1 C and D), but there was no such change seen in the protein expression of FZD7 (Fig. 1 C and E).

In immunocytochemical analysis, reduced membrane expression of LRP6 was observed at IC_{50} doses of the compounds (Fig. 1F). However, no such effect was seen in the expression of FZD7 after treatment with the compounds (Fig. 1G).

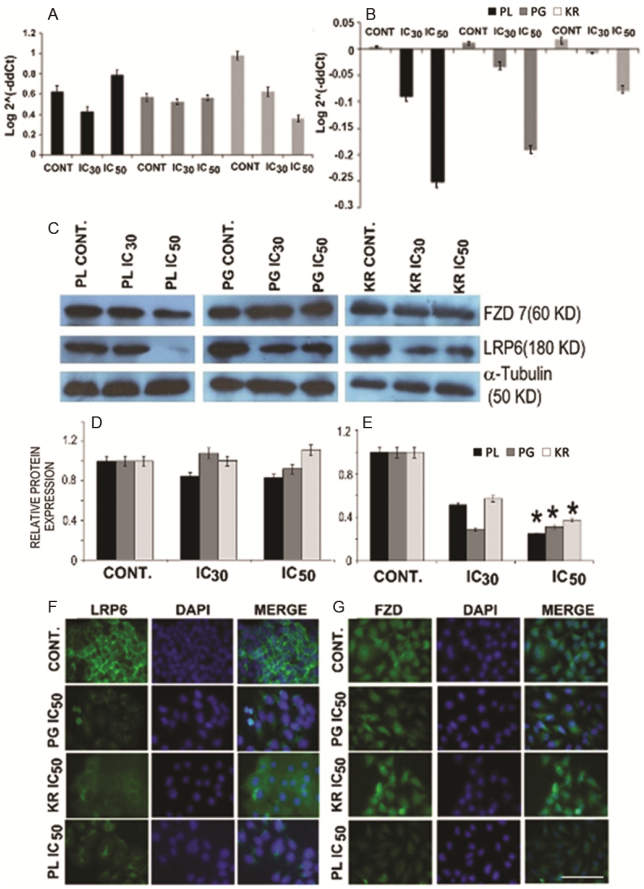

Fig. 1 — Effect of PL, PG and KR on Wntsignalling receptor FZD7 and co-receptor LRP6. Relative mRNA expression of (A) FZD7 and (B) LRP6. The relative gene expression [2^(-ddCt)] was calculated by normalization with average expression of control cells followed by β2-microglobulin gene as internal control. Western blot analysis of (C) FZD7 and LRP6 protein expression, and α-tubulin for loading control after treatment with PG (IC_{30} and IC_{50}), KR (IC_{30} and IC_{50}), and PL (IC_{30} and IC_{50}), respectively, in HeLa cells for 48 h. Histograms show the relative protein expression levels of (D) FZD7 and (E) LRP6 in HeLa cells after the treatment with the compounds. Relative peak density was normalized with loading control. For immunocytochemical analysis, HeLa cells were photographed under fluorescence microscope for (F) FZD7 and (G) LRP6 protein expressions after treatment with the compounds at their IC_{30} and IC_{50} doses, respectively, for 48 h in HeLa cells. [Cont. = control; PG =pongapin; KR =karanjin; and PL = plumbagin. All the significance level was calculated relative to the respective control. * P <0. 01]

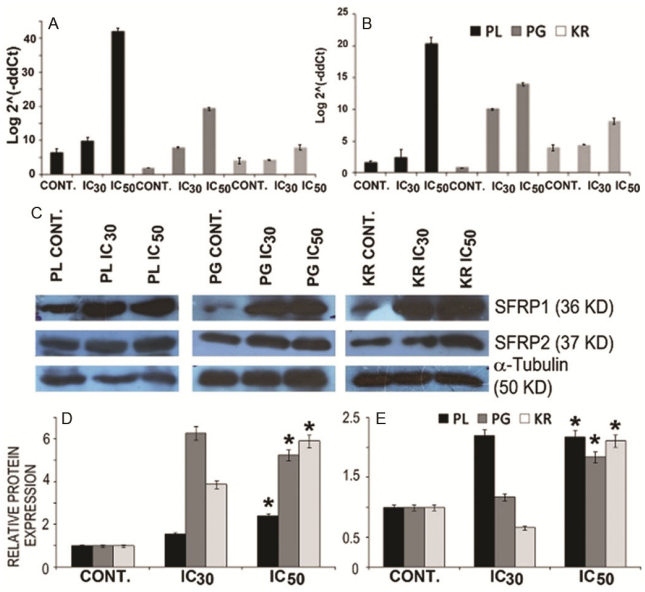

Fig. 2 — Effect of PL, PG and KR on FZD7 inhibitor- SFRP1 and SFRP2. Relative mRNA expression of (A) SFRP1 and (B) SFRP2, after treatment with PG (IC_{30}, IC_{50} and IC_{70}), KR (IC_{30}, IC_{50} and IC_{70}), and PL (IC_{30}, IC_{50} and IC_{70}), respectively, in HeLa cells for 48 h. The relative gene expression [2^(-ddCt)] was calculated by normalization with average expression of control cells followed by β2-microglobulin gene as internal control. Western blot analysis of (C) SFRP1 and SFRP2 protein expression, and α-tubulin as the loading control after treatment with the compounds at their respective IC_{30} and IC_{50} doses in HeLa cells for 48 h. Histograms show the relative protein expression levels of (D) SFRP1 and (E) SFRP2 expression in HeLa cells after the treatment with the compounds. Relative peak density was normalized with loading control. [Cont. = control; PG =pongapin; KR =karanjin; and PL = plumbagin. All the significance level was calculated relative to the respective control. * P <0. 01]

Upregulation of Wnt pathway antagonists byPG, KR and PL

DKK1 is a secreted antagonist of WNT pathway that inhibits the functioning of LRP6[21,22]. SFRP1 and SFRP2 bind to FZD7 receptor to form a non-functional complex inhibiting Wnt-FZD7 interaction[23]. In mRNA expression analysis, concentration-dependent upregulation of SFRP1 and SFRP2 expression was seen after treatment with the compounds in HeLa cells (Fig. 2 A and B). Similar trend has also been seen in case of SFRP1 and SFRP2 protein expression by Western blot analysis (Fig. 2C, D and E).

In case of DKK1, its mRNA expression did not change considerably (Fig. 3A). However, in Western blot analysis, its protein expression was significantly increased at IC_{50} concentrations of the compounds (Fig. 3 B and C). This has been validated in immunocytochemical analysis of DKK1 expression at IC_{50} concentrations of the compounds (Fig. 3D).

Effect of PG, KR and PL on promoter methylation of antagonists

To better understand the reason behind the increased expression of the antagonists, their promoter methylation status was determined in the presence of the compounds in HeLa cells. By quantitative promoter methylation analysis, gradual increase in promoter hypomethylation frequencies of SFRP1 and SFRP2 was seen with increasing

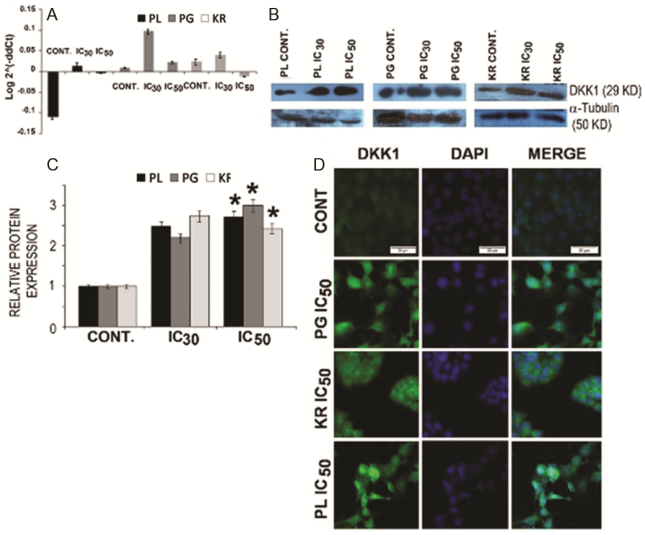

Fig. 3 — Effect of PL, PG and KR on LRP6 inhibitor- DKK1. Relative mRNA expression of (A) DKK1. The relative gene expression [2^(-ddCt)] was calculated by normalization with average expression of control cells followed by β2-microglobulin gene as internal control. Western blotanalysis of (B) DKK1 protein expression, and α-tubulin is taken as loading control after treatment with PG (IC_{30} and IC_{50}), KR (IC_{30} and IC_{50}), and PL (IC_{30} and IC_{50}), respectively, in HeLa cells for 48 h. Histograms showing the relative protein expressions of (C) DKK1 expression in HeLa cells after the treatment with the compounds. Relative peak density was normalized with loading control. The cells were photographed in fluorescence microscope for (D) DKK1 protein expression by immunocytochemistry after treatment with the compounds at their respective IC_{30} and IC_{50} doses for 48 h in HeLa cells. [Cont. = control; PG =pongapin; KR =karanjin; and PL = plumbagin. All the significance level was calculated relative to the respective control. * P <0. 01]

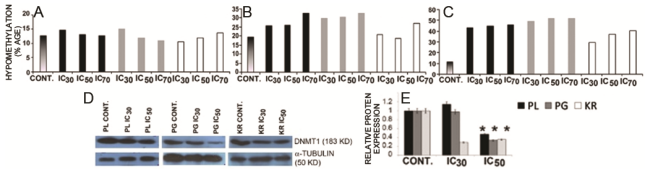

Fig. 4 — Effect of PL, PG and KR on promoter methylation status of the inhibitors- DKK1, SFRP1, and SFRP2. Quantitative methylation analysis of (A) DKK1; (B) SFRP1; and (C) SFRP2 genes in HeLa cell line after the treatment with PL (IC_{30}, IC_{50} and IC_{70}), KR (IC_{30}, IC_{50} and IC_{70}) and PG (IC_{30}, IC_{50} and IC_{70}), respectively, for 48 h. Representative histogram of qRT-PCR analysis showing percentage of hypomethylation of the genes while digested with HhaI enzyme. (D) Western blot analysis of DNMT1 protein expression; and (E) the histograms show the relative protein expression of DNMT1, where α-tubulin is taken as loading control after treatment with the compounds at their respective IC_{30} and IC_{50} doses for 48 h in HeLa cells. [control; PG =pongapin; KR =karanjin; and PL = plumbagin]

concentrations of the compounds, but no such effect was seen in the promoter methylation status of DKK1 (Fig. 4). This is in accordance with the mRNA

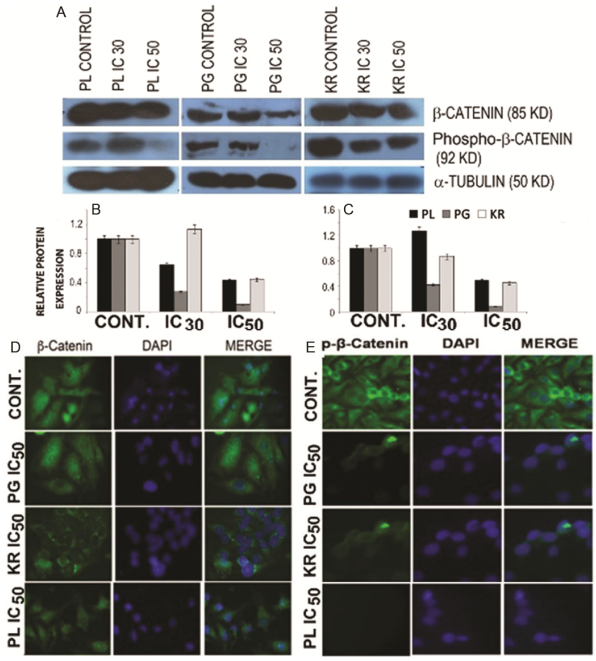

Fig. 5 — Effect of PL, PG and KR on effector protein of Wnt- β-catenin. Western blot analysis of (A) β-catenin and phospho-β-catenin protein expression, and α-tubulin for loading control after treatment with PL (IC_{30} and IC_{50}), KR (IC_{30} and IC_{50}), and PG (IC_{30} and IC_{50}), respectively, in HeLa cells for 48 h. Histograms showing the relative protein expressions of (B) β-catenin and (C) phospho-β-catenin expression in HeLa cells after the treatment with PL, KR and PG. Relative peak density was normalized with loading control. The cells were photographed in fluorescence microscope for (D) β-catenin and (E) phospho-β-catenin expression by immunocytochemistry after treatment with the compounds at their respective IC_{30} and IC_{50} dosesfor 48 h in HeLa cells. [control; PG =pongapin; KR =karanjin; and PL = plumbagin. All the significance level was calculated relative to the respective control. * P <0. 01]

expression profile of the antagonists in HeLa cells after the treatment with the compounds (Fig. 2).

To understand the mechanism underlying promoter hypomethylation of the antagonists, DNMT1 expression was analyzed in the presence of the compounds in HeLa cells. Interestingly, in Western blot analysis, significant downregulation of DNMT1 expression was seen at IC_{50} concentration of the compounds (Fig. 4).

Downregulation of β-catenin expression byPG, KR, and PL

To understand the ultimate effect of altered expression of LRP6 and the antagonists on the self-renewal pathway, expression of the effector protein of the pathway, β-catenin was analyzed after the treatment with the compounds in HeLa cells. In Western blot analysis, significant decrease in the expression of β-catenin and activated nuclear β-catenin (Phospho-Y654) were seen after the treatment with the compounds (Fig 5 A-C). Comparatively, PL and PG

showed higher inhibition of β-catenin than KR. This was also validated by immunocytochemical analysis of β-catenin and activated nuclear β-catenin (Phospho Y654) at IC_{50} concentrations of the compounds in HeLa cells (Fig. 5D).

Discussion

Plumbagin (PL), Pongapin (PG) and Karanjin (KR) are three plant polyphenolic compounds. The efficacy of these compounds as potential anticancer compound has already been studied in different human cancer cells. In this study, it was found that these compounds were most effective against cervical cancer cells, HeLa[11,24]. To understand the antitumor mechanism of the compounds against HeLa cells, their effect on Wnt/β-catenin self-renewal pathway was analyzed.

At first, expression profiles of the receptor FZD7and co-receptor LRP6 were checked in the presence of the compounds in HeLa cells. The expression (mRNA/ protein) of LRP6 was significantly decreased in the presence of these compounds (Fig. 1 B, C, E and F), but the expression (mRNA/ protein) of FZD7 did not change considerably (Fig. 1 A, C, D and G). Among the three compounds studied, PL showed high inhibiting potential followed by PG and KR. This indicates that the compounds could inhibit the LRP6 expression transcriptionally and also at protein level. It seems that the transcriptional inhibition might be due to some indirect pathway. The reduced membrane expression of LRP6 by the compounds might be due to the upregulation of its antagonist DKK1 protein resulting in cytoplasmic internalization of LRP6 for its proteasomal degradation as seen in other studies[24,25].

It has been observed that the compounds could increase the mRNA expression of FZD7 antagonists SFRP1/2 in HeLa cells (Fig. 2 A and B), resulting increase in their protein expression. Unlike SFRPs, the compounds did not change mRNA expression of DKK1 considerably (Fig. 3A). This is in accordance with the low variation in promoter methylation profile of DKK1 by the compounds (Fig. 4A). The transcriptional activation of SFRP1/2 might be due to the increase in their promoter hypomethylation frequencies as seen in our study. The increase in promoter hypomethylation of SFRP1/2 by the compounds (Fig. 4 B and C) was highly co-related with the reduced DNMT1 protein expression (Fig. 4 D and E), as already reported in case of other

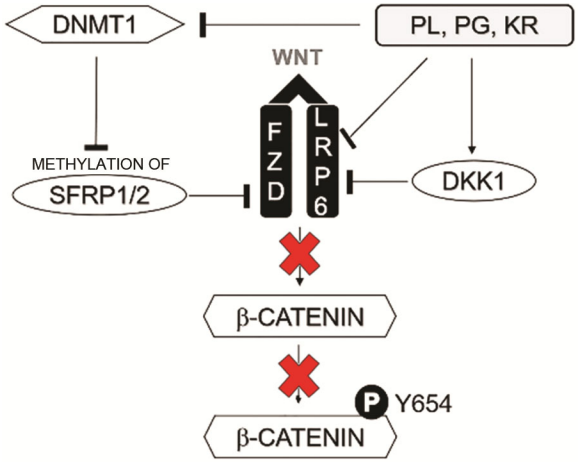

Fig. 6 — Schematic representation of the effect of the three compounds (PG, KR and PL) on Wnt/β-catenin pathway in HeLa cells. On one hand, PL, PG and KR led to downregulation of DNMT1 expression resulting in hypomethylation of SFRP1/2, as a result the expression of SFRP1/2 is increased. On the other hand, the compounds upregulated DKK1 expression resulting in downregulation of LRP6 restricting the FZD7-LRP6-WNT complex formation which ultimately led to the downregulation of its effector protein β-catenin and activated β-catenin. Thus, the compounds could attenuate the Wnt/β-catenin pathway in cervical cancer cell. [Cont. = control, PG = Pongapin, KR = Karanjin, and PL = Plumbagin. All the significance level was calculated relative to the respective control. * P <0. 01]

polyphenols like catechins in different cancer cell lines[19,26-28].

Thus, downregulation of FZD7 and LRP6 through upregulation of their antagonists (SFRP1/2 and DKK1)[29,30] by the compounds in HeLa cells might downregulate the β-catenin protein expression[31-33] as seen in our study (Fig. 5 A, B and D). The downregulation of β-catenin protein might be due to its reduced cytoplasmic stabilization resulting in lower expression of the activated nuclear phosphor-β-catenin (Y654) expression (Fig. 5 A, C and E). Thus, inactivation of β-catenin attenuates the Wnt/β-catenin pathway[34-36] (Fig. 6) and its downstream target genes in CC[37-40].

Conclusion

Based on experimental observations, it is evident that the three polyphenolic compounds [i. e. , Plumbagin (PL), Pongapin (PG) and Karanjin (KR)] could differentially inhibit the self-renewal Wnt/β-catenin pathway in cervical cancer cells. Their action is mediated through downregulation of DNMT1. DNMT1 downregulation has led to promoter hypomethylation of the two Wnt antagonists — SFRP1 and SFRP2, along with upregulation of

DKK1, due to its protein stabilization. Altogether, upregulation of the antagonists has led to downregulation of Wnt co-receptor LRP6, thereby reducing the activity of FZD7-LRP6-Wnt complex. This ultimately leads to attenuation of the downstream Wnt signalling pathway and inactivation of its effector protein, β-catenin. This study thus, proves the potential of these three natural compounds as anticancer agents in controlling the cervical cancer.

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Conflict of Interest

Authors declare no competing interests.

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