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Taxifolin, a natural flavonoid interacts with cell cycle regulators causes cell cycle arrest and causes tumor regression by activating Wnt/ β -catenin signaling pathway

Suhail Razak^{1,2*}, Tayyaba Afsar², Asad Ullah¹, Ali Almajwal², Musaed Alkholief³, Aws Alshamsan³ and Sarwat Jahan¹

Abstract

Background: New approaches for the prevention of colon cancer perseveres an essential necessity. Though, resistance to existing chemo-preventive drugs is moderately predominant in colon carcinogenesis. Taxifolin (dihydroquercetin) is a flavononol, have shown virile biological activities against few cancers. The current study was designed to investigate and equate antitumor activity of Taxifolin (TAX) in colorectal cancer cell lines and in HCT116 xenograft model in a comprehensive approach.

Methods: Two human colorectal cancer cell lines HCT116 and HT29, were used. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MMT) protocol was performed to elucidate the impact of TAX and β -catenin inhibitor (FH535) on the viability of HCT116 and HT29 cell lines. Apoptosis /cell cycle assay was performed. Data interpretation was done with a FACScan (Becton Dickinson, NJ). About 1×10^4 cells per sample were harvested. Histograms of DNA were analyzed with ModiFitLT software (verity Software House, ME, USA). Western blotting and RT-PCR were performed for protein and gene expression respectively in in vitro and in vivo.

Results: We found that TAX induced cytotoxicity in colorectal cells in a dose-dependent manner and time dependent approach. Further, our data validated that administration of TAX to human colorectal cancer HCT116 and HT29 cells resulted in cell growth arrest, variation in molecules controlling cell cycle operative in the G2 phase of the cell cycle and apoptosis in a concentration dependent approach. Further our results concluded that TAX administration decreases expression of β -catenin gene, *AKT* gene and *Survivin* gene and protein expression in in vitro and in vivo.

Conclusion: Our findings proposed that targeting β -catenin gene may encourage the alterations of cell cycle and cell cycle regulators. Wnt/ β -catenin signaling pathway possibly takes part in the genesis and progression of colorectal cancer cells through regulating cell cycle and the expression of cell cycle regulators.

Keywords: Colorectal cancer, Wnt/ β -catenin, Taxifolin and cell cycle

* Correspondence: ruhail12345@yahoo.com

¹Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan

²Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

Full list of author information is available at the end of the article



Background

The worldwide frequency of colorectal cancer (CRC) is third among the cancer rate of relapse in males and fourth among females [1, 2]. Additionally, colonic adenocarcinoma accounts for 37–45% of all metastatic ovarian tumors. While there is a decline in the death rates for colorectal cancers from 1990 till date. Even with advancement in screening and surgical treatment, no guaranteed therapy has been revealed for metastatic cancer and the half decade endurance rate is disappointingly little (about 9%). Such astonishing incompetence of regular anti-cancer therapies has been authorized to the survival of relatively infrequent, exceedingly drug-resistant, inert or slow propagating cells with stem-like properties: cancer stem cells (CSCs). Existing studies reflects tumors as multifarious varied organ-like systems with a hierarchical cellular organization, rather than merely as collections of homogeneous tumor cells [3, 4]. CRC rises through the gradual accumulation of alterations in oncogenes and tumor suppressor genes. The accumulation of alterations usually arise because of cumulative effects of epigenetic variations and multiple genetic mutations involving genes that control cell development and segregation [5].

Natural polyphenols have materialized as a novel approach to defend cells against oxidative stress. Besides anticarcinogenic activities, every polyphenol have a potent antioxidant activity, thus declining the jeopardy of cancer [6].

Most of the polyphenols have shown chemoprotective, antiproliferative, antioxidative and estrogenic/antiestrogenic activity besides tempting cell-cycle arrest or apoptosis and the detoxification of enzymes. Also, they control the immune system and fluctuations in cellular signaling [7]. Particularly, tannins, flavonoids, gallic acid, anthocyanins, resveratrol, proanthocyanidins, epigallocatechin-3-gallate, and various plant extracts have shown defensive activity in various cancer models [8, 9]. Dihydroquercetin (Taxifolin 3, 5, 7, 3',4'-pentahydroxy-flavanone) is an operative flavonoid, abundantly found in olive oil, grapes, in citrus fruits and onions [10]. Taxifolin (TAX), a prevalent bioactive component of foods and herbs, showed a tremendous variety of pharmacological and biochemical consequences, including hepatoprotective, anti-diabetic, cardioprotective, antitumor, neuroprotective effects, and anti-inflammatory effects, and played a wonderful role in the preclusion of Alzheimer's disease [10]. Also, TAX has an effective anti-oxidant activity which boosts apoptosis encouraged by an assortment of anti-cancer agents and is available in the market under a trade name of Venorutons (semisynthetic form) [11–13]. Recently it was reported that TAX encompasses Nrf2-dependent pathway to encouraged the expression of phase II antioxidant and detoxifying enzymes and exert a critical defensive role against DNA oxidative damage [14]. Prominently, TAX

appreciably has shown accelerated expression of heme oxygenase – 1 (HO-1) by inducing expression of Nrf2 in cytoplasm and nuclear translocation [14]. The therapeutic promise of dihydroquercetin in chief inflammatory disease states like cancer was recently evaluated [11]. Particularly, it was revealed that dihydroquercetin can proceed as a scavenger of myeloperoxidase (MPO)-derived RNS [11]. Interestingly, dihydroquercetin decreases BSO-induced injury to dermal fibroblasts [15]. In addition, a most current organized study, highlighted that dihydroquercetin was capable to downregulate the collagenase I (MMP-1) in UVB-treated skin cells [11]. Furthermore, TAX showed inhibitory action against the excess production of ROS and the oxidative enzymes, thus reorganizing cerebral the ischemia–reperfusion injury [16].

FH535 is considered as twofold small molecule inhibitor of β -catenin/TCF/LEF and peroxisome proliferator-activated receptors (PPARs) [17]. Earlier studies revealed that FH535 hampers the growth of colon, lung, breast, hepatocellular cancer cells [18], and suppresses angiogenesis and pancreatic cancer xenograft growth [19].

In spite of numerous observations signifying the cytotoxicity of TAX in numerous cancer cell lines, the mechanism of action of the TAX during tumor deterioration is chiefly unclear. Hence, in the present study, we explored the anticancer effect of TAX in a systematic manner, using in vitro and in vivo model structures. We found that TAX induces numerous fold advanced levels of cytotoxicity in cancer cells. Further, we showed growth impediment and induction of apoptosis in colorectal cancer cells induced by polyphenolic compound TAX, Down-regulation of Wnt/ β -catenin signal transduction pathway, Anti-proliferative effect of treatment of TAX, β -catenin Inhibitor (FH535) in HCT116 and HT29 cells, Flow cytometric analysis of colorectal cancer cells after TAX treatment for apoptosis and cell cycle, Inhibition of colony formation in HCT and HT29 cells after treatment with TAX and Alteration in CTNBN1 protein level after TAX treatment. Thus our data indicate that TAX could be developed further as a potential anti-cancer agent, both in conventional and combination therapy.

Methods

Ethical declaration

Athymic nude mice studies were performed according to the Institutional principles for the concern and use of animals and the experimental protocol was approved (BAS#0256) by the ethical board of Quaid-i-Azam University, Islamabad, Pakistan and Committee dealing animal care and use, college of Pharmacy, King Saud University, Kingdom of Saudi Arabia. Before starting experiment on human colorectal cancer cell lines HCT116 and HT29 (ATCC[®] CCL-247[™] and ATCC[®] HTB-38[™]

respectively) purchased in July 2017 from American Type Culture Collection (MD, USA), ethical approval was taken from ethics committee of preclinical studies, college of pharmacy, King Saud University, KSA.

Cell culture

Two human colorectal cancer cell lines HCT116 and HT29 were grown in a 5% CO₂ atmosphere at 37 °C in medium containing DMEM medium 1640 (GIBCO), 10% fetal bovine serum and 1% penicillin/streptomycin. Taxifolin (TAX) and β- catenin inhibitor (FH535) suspended in DMSO was applied for cell treatment. Cells with 70% confluency were induced with TAX and β- catenin inhibitor at 10-100 μM for 48 h in cell culture medium and the dilution of DMSO applied for each treatment was 0.1% (V/V).

MMT assay/viability assay/ cell proliferation assay

To check the effect of TAX and β- catenin inhibitor (FH535) on the viability of HCT116 and HT29 cell lines, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium-bromide (MMT) protocol was carried. 10 × 10³ cells /well were plated in 1 ml of culture medium consisting of 10-100 μM dilution of TAX and FH535 in 96-well plate. Cells were incubated for 48 h. at 37 °C, 200 μl of 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide (5-7 mg/ml PBS) was supplemented to each well and set aside for two hours, 200 μl of DMSO were added to each plate which were then spinned (1800×g for 5 min at 4 °C). The readings at 540 nm wavelength were noted on a microplate reader (Elx 800). Impact of TAX and FH535 on inhibition of growth was calculated as % cell viability as DMSO-administrated cells were considered as control. Absorbance numbers of media containing wells were subtracted from test sample values.

$$\text{Cell viability} = \left[\frac{\text{Absorbance of Sample} - \text{Absorbance of Blank}}{\text{Absorbance of DMSO} - \text{Absorbance of Blank}} \right] \times 100$$

Clonogenic assay

HCT116 cells and HT29 cells were collected subsequently to treatments with active TAX for 48 h. Cells were suspended in fresh medium, cell number was determined, and 500 cells (HCT116) or 1 × 10³ cells (HT29) were plated into cell culture dishes (35 mm) in triplicate. Following 7 days in culture, 0.5% crystal violet was used for staining colonies [20]. Cell colonies were count up by using a cubic colony counter (AO scientific) under dark field. The amount of cells in every colony was resolved by phase contrast microscopy. The colony sizes were calculated on images using Adobe Photoshop software. Data was signified as colony number in TAX group relative to expressed as mean ± SEM of three individual tests.

Cell cycle analysis

HCT116 and HT29 cells were treated with TAX (40-60 μM:48 h.) in complete medium were trypsinized and fixed in 1% paraformaldehyde: 1× PBS and washed with cold PBS twice and spinned. Chilled 70% ethanol was used to suspend cell pellet and incubated overnight, then the cells were spinned for 4-5 min at 800–1000 rpm. To remove ethanol, pellet was washed with chilled PBS twice then finally cells are labeled with FITC and propidium iodide (PI) using the Apo-Direct Kit (BD Pharmagen, CA). Data interpretation was done with a FACScan (Becton Dickinson, NJ). About 1 × 10⁴ cells per sample were harvested. Histograms of DNA were interpreted with ModifitLT software (verily Software House, ME, USA).

Protein extraction and Immuno-blotting analysis

SDS-PAGE and western blot investigations were executed by earlier described protocol with minor amendments [21]. Subsequent to 24 h and 48 h of administration with TAX at necessary amounts, HCT116 and HT29 cells lyses was carried out in RIPA buffer augmented with freshly added protease and phosphatase inhibitor cocktail 1:100 (Santa Cruz, CA) and concentration of protein was anticipated by Bradford assay [22]. To resolve 40-60 μg of protein, 8–12% poly acrylamide gels were used in immunoblotting. Transferred on to a nitrocellulose membrane, with candidate monoclonal primary antibodies, and perceived by super signal west Pico, Dura or Femto Chemiluminescence Reagent (Thermo scientific, USA). Protein bands were quantified by measuring band density using Image J software. The densities of the bands (normalized to actin) relative to that of the untreated control (designated as 1.00) were presented as mean ± SEM of three individual experiments.

Gene expression analysis

Whole RNA was extracted from the cells using a Genei RNA purification kit (Ambion, USA). The RNA extracted was then subjected to first stand complimentary deoxy ribonucleotide (cDNA) synthesis using RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas, Germany). RT- PCR was executed, containing 25 μl total reaction mixture. The reaction contains: SYBR® GreenER™ qPCR SuperMix Universal 12.5 μl, Forward primer (pF) 0.3 μl, Reverse primer (pF) 0.3 μl, cDNA 6 μl, Taq Polymerase 0.5 μl, Nuclease free water 5.4 μl. Thermal cycling conditions were: 4 min at 94 °C for template DNA denaturation with 35 cycles of amplification each consisting of 3 steps: 35 s at 94 °C for DNA denaturation into single strands; 35 s at 60 °C for primers to hybridize or “anneal” and one minute at 72 °C for extension and final 10 min at 72 °C. PCR was carried out in Smartcycler (Cepheid, Germany). Five

standards were run in triplicate tubes in Smartcycler for real time PCR. The gene expression levels were determined using $2^{-\Delta\Delta CT}$. Designed Primer sequences used were;

CTNNB1 Non-template: 5'-TGTGAATCCCAAGTACC
AGTGT-3'.

CTNNB1 Template: 5'- CGTCAGACAAGGAGAAA
CATT-3'.

β -Actin Non-Template: 5'- CCTCTTCCTCAATC
TCGCTC-3'.

β -Actin Template: 5'- GCTCAATGTCAAGGCAGGA
G-3'.

Immunofluorescence microscopy

HCT116 and HT29 colorectal cancer cells were cultured in a two chamber tissue culture glass slides and were administered with 40 μ M of TAX at 75% confluence for 24 h. Once the chamber was removed, Phosphate buffer was used to rinse the slides, 2% paraformaldehyde was used to fix the cells and permeabilized in methanol. Slides were rinsed with phosphate buffer and 2% serum was used as blocking agent. Primary antibody was incubated overnight. Then incubation with appropriate fluorophore tagged secondary antibody. For mounting antifade 4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Invitrogen NY) was used to apply and hematoxylin for counter staining. Analysis was done by using Bio-Rad Radiance system (2100 MP Rainbow) for imaging. The apoptotic and necrotic cells were identified by the Annexin-V-fluos staining Kit (Roche, Switzerland) according to the kit's procedure. Fluorescence was measured by confocal microscopy (Zeiss 410). Annexin V and propidium iodide was used to stain the cells. The unstained cells in a chosen field were calculated to determine the level of necrosis as well as apoptosis.

In vivo tumor xenograft model

Athymic male mice were acquired from King Faisal Hospital and research center, Riyadh, KSA, were homed under contamination free environment (12 h clock), nourished with a sterilized food ad libitum. HCT116 cells were selected for evaluating the in vivo impact of TAX and β -catenin inhibitor (FH535), as they generate fast tumors in mice. Cells were harvested, suspended in complete RPMI media 1640. Tumor xenografts HCT116 cells in mice were established by injecting cells (1×10^6) subcutaneously mixed with matrigel (Collaborative Biomedical Products, Bedford, MA) in a equal ratio.

Thirty mice were categorized into three groups.

Group1: Served as Control Group, consisting six mice, DMSO was given intra-peritoneally (i.p).

Group2: Divided into two subgroups; Group 2a and 2b consisting of six animals each. Received

TAX (15 and 25 mg/kg) intra-peritoneally (i.p) respectively, twice weekly.

Group3: Divided into two subgroups; Group 4a and 4b consisting of six animals each. Received FH535 (15 and 25 mg/kg) intra-peritoneally (i.p) respectively, twice weekly.

During the experiment, food, body weight of animals, and water expenditure were documented twice a week. Digital caliper was used to measure the Tumor volume and calculated using the formula:

$$L1 \times L2 \times H \times 0.5238$$

Where, H = height of the tumor, L1 and L2 = long diameter and short diameter respectively.

Tumor sizes were recorded two times in a week. At the end of the experiment when tumor volume reached to $\sim 1210 \text{mm}^3$ animals were sacrificed by CO_2 inhalation was used as anesthesia. Tumors were resected, weighed and frozen at -80°C for subsequent western blotting, RNA extraction and immunohistochemistry.

Immunohistochemistry

Hematoxylin and eosin were used for staining the tumor tissue section for morphological visualization. Furthermore tissues were fixed in 10% formalin. Deparaffinization was done with with EZ Prep (Ventana, Arizona, USA) at 70°C , heat pretreated in Cell Conditioning 1 (CC1; Ventana, Arizona, USA) using "standard cell conditioning" for antigen reclamation at 90°C . Overnight incubation with antibodies against β -catenin, cyclinA and cleaved caspase3 (diluted 1:75). Then incubation with ultraview universal HRP multimer (secondary antibody), diaminobenzidine/ DAB (DAKO, CA) staining and counter staining with hematoxylin. After mounting in DPX (distyrene, a plasticizer and xylene), the sections were covered with cover slips. Immunostained tissue sections were assessed at magnification of $100\times$ and $200\times$ (Olympus BX51 light Microscope and DP72 Olympus Digital Camera, Olympus America Inc., Center Valley, PA, USA).

Data analysis

Densitometry of western blot images was performed using an image analysis software (Image J 1.41). Data of in vitro assays was analyzed GraphPad Prism 5 software to determined IC_{50} values. Level of significance between different treatments groups relative to control were estimated by one way analysis of variance followed by Tukey's multiple comparison test. Comparison between more than one parameter was accomplished using two way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. $p < 0.05$ was considered

statistically significant. Where required correlation analysis was done and R values were calculated.

Results

TAX and FH535 inhibits progression and viability of colorectal cancer cells

To analyze the anti-proliferative prospective of TAX and FH535, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay against HCT116 and HT29 colorectal cancer cells was executed. It was observed that TAX and FH535 treatment (10-100 μM for 24 h and 48 h) to colorectal cancer cells caused reticence of cell growth in a dose and time dependent approach. Time course analysis illustrated that colorectal cancer cells retort to TAX and FH535 administration within 48 h. As revealed in (Fig. 1a and b), the IC_{50} values of TAX and FH535 -treated HCT116 were 51.3 and 43.5 and 29.9 and 26.2 μM at 24 h and 48 h respectively and IC_{50} values of TAX and FH535 -treated HT29 cells were 66.1 and 43.5 and 39.0 and 26.2 μM at 24 h and 48 h respectively (Table1). The data proposed that HCT 116 and HT29 cells showed better sensitivity to TAX and FH535 treatment.

To determine the effect of TAX administration on the clonogenicity of HCT116 and HT29 cells, clonogenicity assay was carried out. HCT116 and HT 29 cells were pre-administrated with TAX at 40 and 60 μM doses confirmed a dose dependent inhibition of colonogenicity with deference to untreated control cells. Results show that clonogenicity of HCT116 and HT29 cells after administration with TAX was markedly condensed in comparison with the control (Fig. 1c and d).

Taxifolin (TAX) behavior caused cell cycle arrest

Flow cytometric analysis of cell cycle arrest caused by administration of TAX

To evaluate the cell cycle sketch of TAX administrated colorectal cancer cells we carried out flow cytometric analysis and observed the effect of TAX on cell cycle circulation. An investigation was performed on HCT116 and HT29 cells. As a result of TAX treatment a patent concentration dependent raise of a cell population in the G2 state of the cell cycle was make out by flow cytometric analysis. The G2-phase cell cycle distribution for HCT116 was 44.61% and 59.52% and for HT 29 was 47.72%, and 57.72% at 40 and 60 μM doses of TAX respectively. The boost in G2 phase cell population was accompanied by a synchronized decline in the Go/G1 and S phase cell population (Fig. 2a and b).

Flow cytometric analysis of apoptosis induced by treatment of TAX

As cell cycle arrest and apoptosis is allied with each other, a manifested concentration dependent increase in

population of dead cells was determined by flow cytometric analysis as a result of TAX administration in HCT 116 and HT cells. The percentage apoptosis in HCT cells observed was 34.9% and 53% and for HT 29 17.2% and 30.9% at concentration of 40 and 60 μM of TAX (Fig. 2c, d and e).

TAX administration induced reticence of cyclins, cdks and activation ofWAF1/p21, KIP1/p27 in colorectal cells

Cell division is outgoing and cdks/cyclins operate as check points or targets in cancer cells. Development of cell cycle is extremely controlled by communication of cyclins, inhibitor proteins WAF1/p21 and KIP1/p27 and cdks. We evaluated the effect of TAX on molecules that control cell cycle execution in the G2 phase of the cell cycle. We determined the effect of TAX on the activation of WAF1/p21 and KIP/p27, which are supposed to regiment the access of cells at the G1-S state transition check points and cause cell death. The Immunoblot analysis showed that the TAX treatment to HCT 116 and HT 29 cells resulted in a prominent upregulation of P21 and P27 in a concentration dependent manner (20, 40 and 60 μM) as compared to basal levels (Fig. 3a). The effect of TAX administration on protein expressions of cyclins B1, D1, A and E and cdks 2, 4 and 6 was determined, which are prominently regulated by p21 and involved in G2 phase of cell cycle. Immunoblot analysis showed a dose dependent down regulation of both cyclins and cdks which support cell cycle arrest in G2 phase (Fig. 3b).

TAX administration tempts apoptosis via commencement of intrinsic and extrinsic pathway

TAX administration caused initiation of active caspase 3, 7 and 9 in colorectal cancer cells

Caspases are cysteine proteases that have a vital role in various forms of apoptosis. Usually they occur in an inactive proenymes which are triggered to active form by oligomerization to form a large multimeric complex, which inturn trigger caspases 7 and 9. Caspase 3 is triggered by proteolytic cleavage. The caspase once active, are responsible for cleavage of various substrates, both in nucleus or cytoplasm which in turn forms distinguishing morphological features of cell death by apoptosis. To explore the TAX induced apotosis through the triggering of intrinsic or extrinsic pathway the expression of cleaved caspases 3/7 and 9 were analyzed by western blotting (Fig. 4). A prominent increased induction of cleaved caspase 3/7 and 9 after administration of TAX in a concentration dependent approach to HCT116 and HT29 cells was seen. Also, truncation of Bid which further supports apoptosis (Fig. 4a).

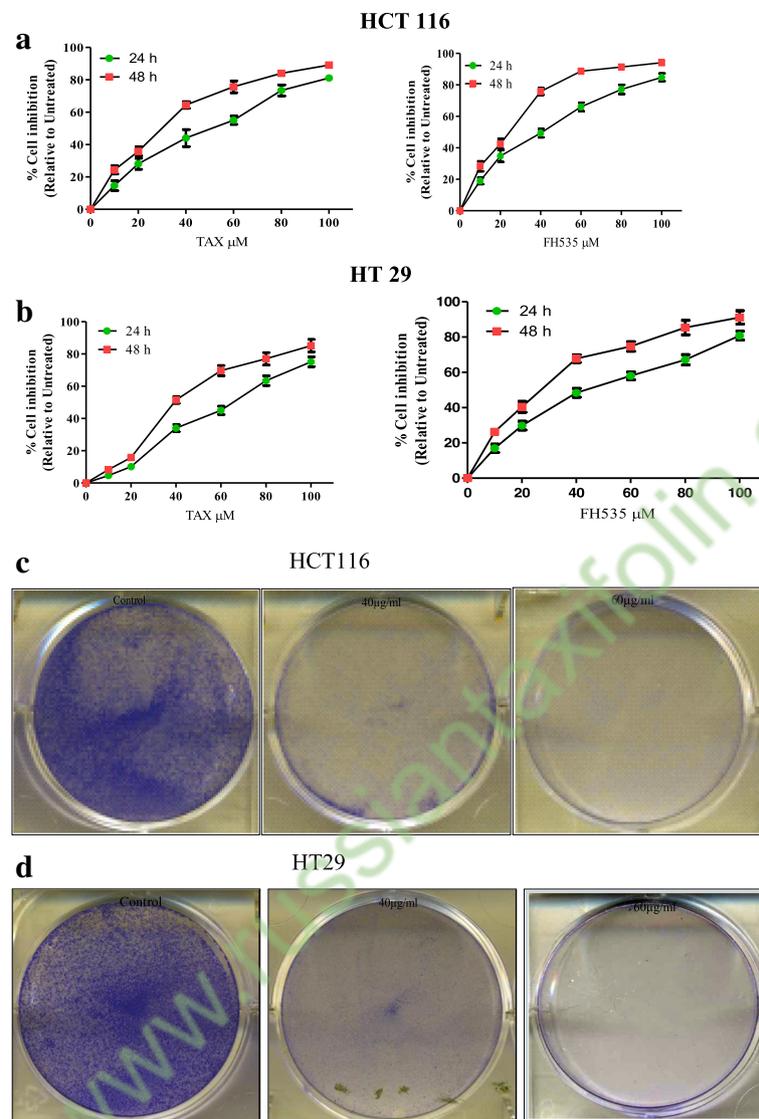


Fig. 1 **a** and **b** Inhibition of cell growth in colorectal cancer. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide assay was carried out on two colorectal cancer cell lines HCT116 and HT29 cells in order to gauge the effect of TAX and FH535 administration on growth of these colorectal cancer cell lines ***, $P < 0.001$ and **, $P < 0.01$ vs. control. % cell viability of HCT116 and HT29 cells were determined. **c** and **d** Inhibition of colony formation in colorectal cancer cells by TAX (Clonogenic assay; 7 days). TAX administration (40 and 60 μM) of HCT116 and HT29 cells inhibiting colony formation. Each value represents a mean \pm SD ($n = 3$)

TAX induces apoptosis through cleavage of poly (ADP-ribose) polymerase (PARP) expression in colorectal cancer

PARP is hallmark of primary proceedings in apoptosis. On administration of TAX to HCT116 and HT29 cells, an induction in 89 kDa fragment of PARP protein expression, a reduction in full size PARP (116 KD) protein expression was seen (Fig. 5).

TAX treatment amends Bax, Bcl2, Bak and Bcl-XL protein expression in HCT116 and HT29 cell lines

Bax and Bcl2 are vital in apoptosis, their expression gives prominent picture of deregulation of apoptotic

process. Bax and Bcl2 working antagonist, Bax act as apoptotic promoting marker and Bcl2 works as antiapoptotic element. There was a prominent boost in bax protein expression with a concentration dependent decline in Bcl2 protein expression on administration of taxifolin on HCT116 and HT29 cells. Also Bak, a proapoptotic protein and Bcl2-X_L, a antiapoptotic showed prominent increase and decrease respectively in protein expression on treatment with TAX in a concentration dependent manner in both HCT116 and HT 29, which effectively confirm generation of apoptosis (Fig. 5).

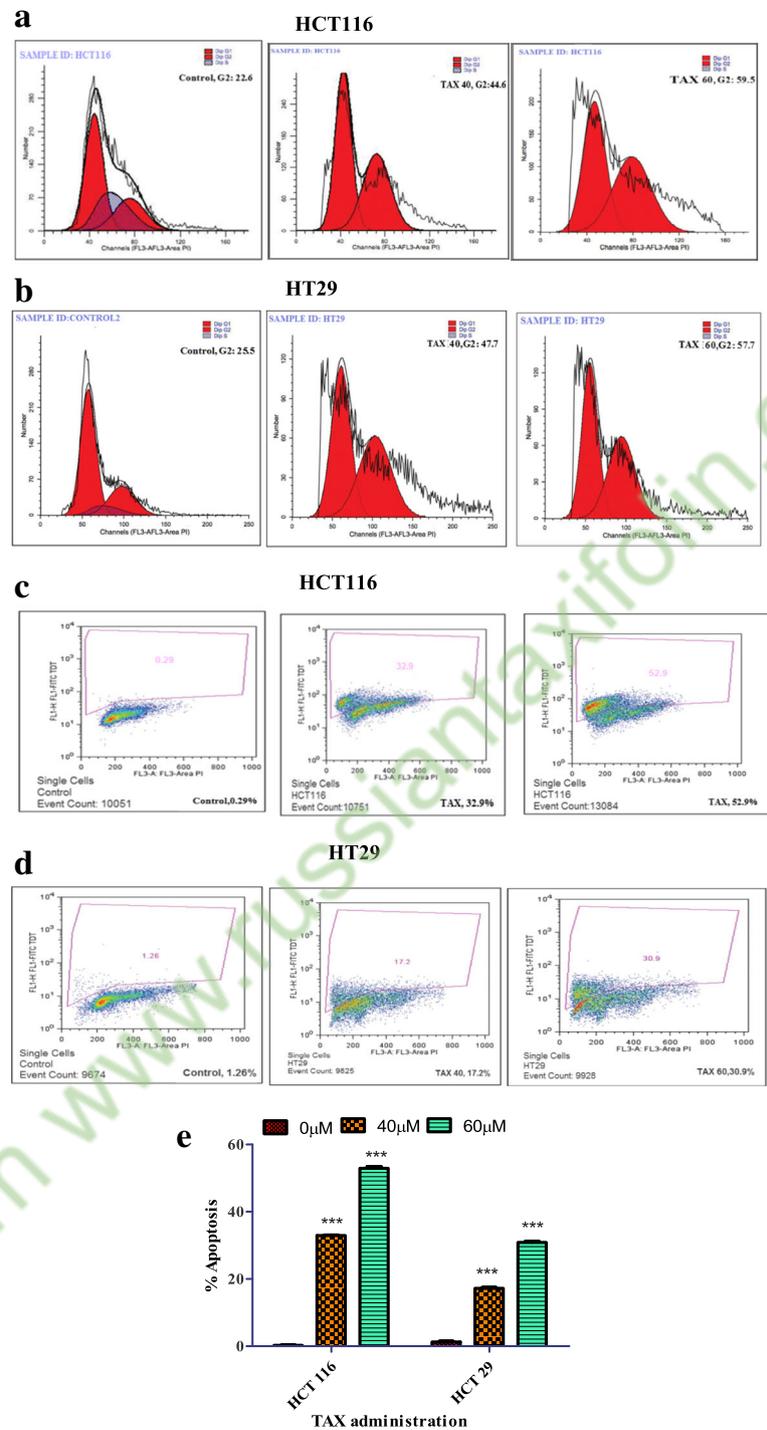


Fig. 2 **a** and **b** TAX treatment on HCT116 and HT29 cells resulted in accumulation of cells at the G2-phase. After 24 h incubation of TAX treated cells and staining with propidium iodide, DNA content was analyzed by flow cytometry. Percentage of cell population in G2-phase of the cell cycle is shown. **c**, **d** and **e** TAX treatment on HCT116 and HT29 cells resulted in growth inhibition and apoptosis, after 24 h incubation of TAX treated cells and staining with propidium iodide, Cells were analyzed by flow cytometry. Percentage of apoptotic cells is shown. Experiments were performed in triplicate

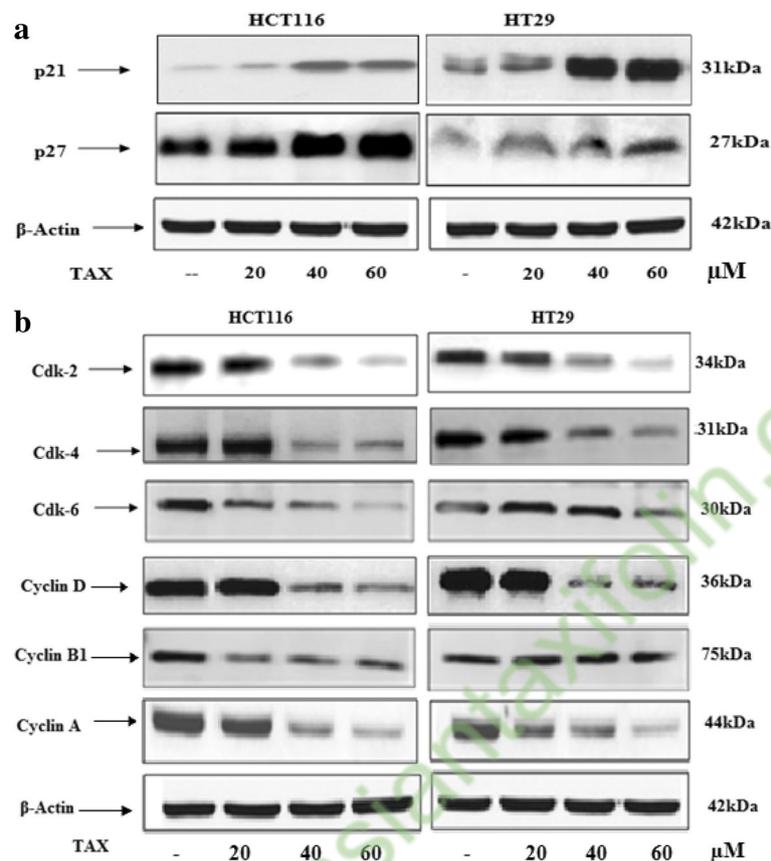


Fig. 3 Effect of TAX treatment of HCT116 and HT 29 cells on protein expression of WAF1/p21 and KIP1/p27, cdk 2, 4 and 6, cyclin D, B1 and A. Experiments were performed in triplicate

TAX reduces the β -catenin expression in colorectal cancer

Results revealed that β -catenin have a crucial task in succession and propagation of colorectal cancer. Decrease in β -catenin expression in concentration dependent fashion was observed on colorectal cancer cells (HCT116 and HT29) after administration of TAX for 24 h by employing immunoblotting (Fig. 6a). The HCT116 and HT29 cells show high protein content of β -catenin. The concentration dependent effect of TAX on HCT116 and HT29 cells showed a marked reduction in β -catenin protein levels at 20, 40 and 60 μ M doses.

To elevate, either the experimental diminish in β -catenin protein was due to decreased transcription of β -catenin gene, alteration of β -catenin expression by TAX induction in HCT116 and HT29 cells, a prominent decline in mRNA expression by employing RT-PCR, was seen to be in concentration dependent manner. At 20, 40 and 60 μ M of TAX a momentous reduced in β -catenin expression was seen (Fig. 6b and c).

Immunofluorescence staining of HCT116 and HT29 cells illustrated reduction in β -catenin expression at concentration of 40 μ M of TAX administrated as compared to control (untreated). As our study evidently illustrated

that TAX administration induced apoptosis and cell cycle arrest by modulation of expression of β -catenin in colorectal cancer cell lines (HCT116 and HT29 cells) as revealed earlier by cleavage of Caspase 3 and PARP, also by modulation of cyclin dependent kinases in HCT116 and HT29 cells after administration of TAX in a dose dependent manner. Significant Alexa fluor staining of β -catenin (nucleus) of both cell lines (green fluorescence) (counter stain used was DAPI, blue fluorescence) were observed in control, although the expression of β -catenin as indicated by staining was markedly reduced in TAX administrated cells as shown in the Fig. 7.

TAX administration induced reticence of PI3K and Akt protein phosphorylation expression in colorectal cells

The PI3K regulates major signaling networks in malignancy cells which results to cell succession, survival, apoptosis and angiogenesis. PI3Ks transduce signals from assorted cytokines and growth factors into intracellular communications by engendering phospholipids, which in sequence activate the serine/threonine kinase AKT, and further downstream effector pathways. Administration of HCT116 and HT29 cells with TAX

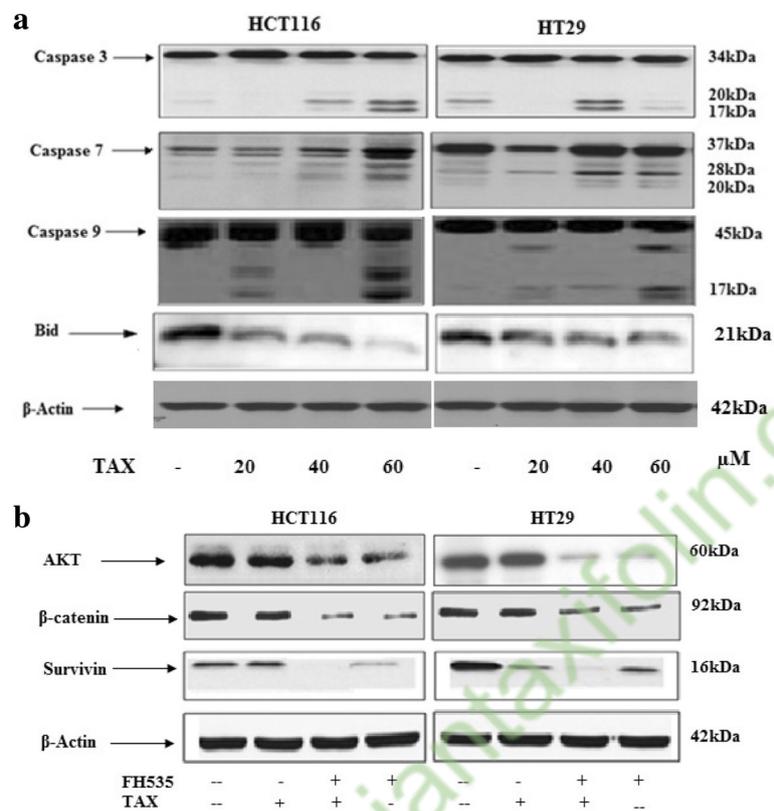


Fig. 4 Effect of TAX on induction of apoptosis. Effect of TAX on protein expression of active Caspase 3, 7 and 9 and truncation of Bid in HCT116 and HT29 cells. Cells were treated with TAX (20, 40 and 60 μ M), total cell lysates were prepared and 40 μ g proteins was subjected to SDS page followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was verified by stripping the Immunoblot and again probing it for Actin. The immunoblots shown here are representative of three individual experiments with similar results

induced a dose dependent reticence in the expression of PI3K. Akt is crucial key of signaling cascades for cell subsistence and development throughout development and carcinogenesis. It maintains cell cycle and growth by activating cdk inhibitors WAF1/p21 and KIP1/p27 and extensively shifting levels of cyclin D1. As reduction in the concentrations of cdk inhibitors and cyclin D1 on induction of cells with TAX was seen, the consequence on phosphorylation of Akt was elevated. The administration of TAX to HCT116 and HT29 cells activated a dose dependent inhibition in the Akt phosphorylation.

Immunofluorescence staining of TAX treated HCT116 and HT29 cells showed reduction in p-Akt expression at concentration of 40 μ M, as compared to control (untreated). Significant Alexa fluor staining of p-akt (cytoplasm) of both cell lines (green fluorescence) (counter stain used was DAPI, blue fluorescence) were observed in control, although the expression of p-Akt as indicated by staining was markedly reduced in TAX treated cells as shown in the Fig. 8.

The above results indicated that TAX administration of HCT116 and HT29 cells resulted in apoptosis via inhibition of β -catenin, survivin and p-Akt. Since FH535

β -catenin inhibitor inhibits Akt phosphorylation which resulted in decrease in protein expression of β -catenin and survivin. Compared with TAX treatment (40 μ M), pre-incubation of HCT116 and HT29 cells with FH535 β -catenin inhibitor (20 μ M) for 2 h before TAX treatment resulted in significant decrease in the protein expression of p-Akt, Survivin and beta catenin as observed by Immunoblot analysis. TAX administration (40 μ M) to β -catenin inhibitor administrated cells further augmented the decrease of β -catenin expression and survivin, suggesting that these effects are mediated in part through Akt (Fig. 4b).

In vivo studies

TAX restrains the tumorigenic ability in vivo in alliance with inhibition of Wnt signaling.

TAX hampers growth of HCT116 xenografts in athymic nude mice

TAX infused intraperitoneally to nude mice results in inhibition of colorectal carcinoma HCT116 tumor xenografts growth. Appearance of little solid tumors was seen after eighth day of cell inoculation in nude mice, getting

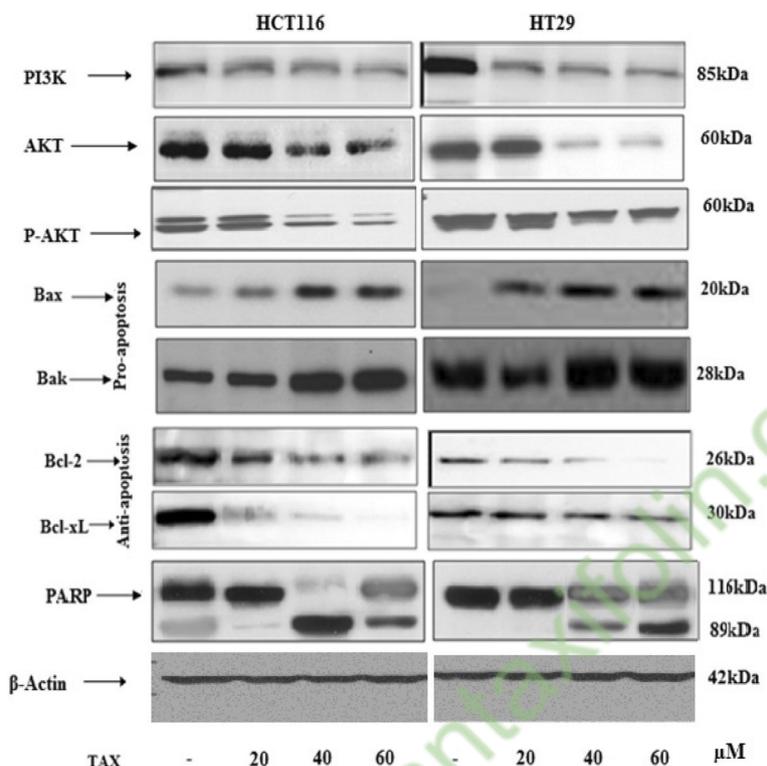


Fig. 5 Effect of TAX on protein expression of PI3K and phosphorylation of Akt at Ser473 in HCT116 and HT29 cells. Also the effect of treatment of TAX on HCT116 and HT29 cell lines on protein expression of Bax, Bcl2, Bak, Bcl-X_L and PARP. Total cell lysate were prepared and 40 μg of protein was subjected to SDS-page followed by Immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for Actin

water as drinking fluid. This incubation phase was comprehensive to 14–18 days in animals administrated with TAX intraperitoneally. TAX was infused intraperitoneally ad libitum to these animals a week after tumor implantation. Tumor growth inhibition was momentous in mice receiving 15 and 25 mg/kg of TAX. More prominent inhibition was witnessed in animals administrated with 25 mg/kg of TAX. At the end of experiment, all mice were killed when the tumor implanted attained ~ 1210 mm³ in volume. The mean tumor volume of 800mm³ was seen after ~ 35 ± 4 days succeeding to tumor cell inoculation. Meanwhile, the mean tumor volumes of 15 and 25 mg/kg TAX administrated groups were 68 and 48mm³, respectively. The prominent tumor hampering reaction was seen in the 25 mg/kg administrated group (Fig. 9A1). The endurance probability of tumor volume data was investigated by Kaplan-Meier analysis, specifying that continuous TAX injection to athymic mice confirmed amplified endurance ($P < 0.0001$, log-rank test), with a norm endurance of 5 and 6 weeks (15 and 25 mg/kg of TAX, respectively) as compared with vehicle group ($P < 0.0001$, log-rank test).

H & E staining of xenograft tumors illustrated that TAX administration results in contraction of nucleus,

apoptosis and inflammatory cell infiltration in TAX administrated group when compared with control group (Fig. 9b).

No difference in body weight and no organ toxicity was observed in TAX administrated mice

There was no observed difference in body weight between vehicle and TAX treated group throughout the experiment. The animals were monitored daily; no signs of stress throughout the experiment were witnessed. Before sacrificing, all mice were observed for gross pathology and nothing was seen adverse, there was no edema or a typical organ size enlargement in non-candidate regions (Additional file 1).

Cyclin D1 and caspases arbitrates resistance to apoptosis by administration of TAX

TAX induces apoptosis in xenograft tumors

Caspases are an exceedingly conserved cysteine proteases family that functions as widespread effector molecules in apoptotic progression. Caspases are typically synthesized as inactive proenzymes and gets activated either by oligomerization to form a large multimeric complex or by proteolytic cleavage that pertains for effector

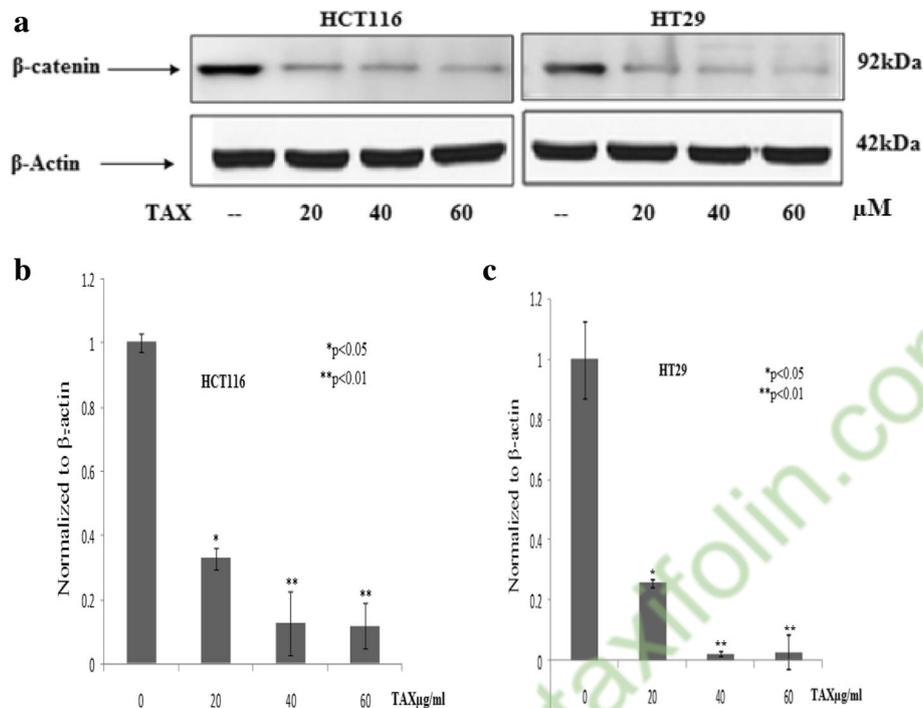


Fig. 6 **a** Immunoblot analysis of β -catenin expression of HCT116 and HT29 in TAX administrated group as compared to control group **(b, c)** qPCR analysis of TAX administrated HCT116 and HT29 cells for changes in β -catenin mRNA levels. The data expressed as fold change represent the mean \pm standard errors experiment performed in triplicate where * $p < 0.05$, ** $p < 0.01$ was considered significant vs control

caspases like Caspase 3. Numerous substrates are cleaved once caspases get activated in the cytoplasm or nucleus, causing several discrete transformations for apoptotic process.

The result of TAX on cleavage of Caspase 3 was evaluated in order to distinguish whether the growth inhibition of cells is due to initiation of apoptosis (Fig. 9c and d). The induction of cleaved Caspase 3 in HCT116 xenograft tumors after administration with TAX. The morphology of TAX administrated cells also showed encouragement of cell death when observed under fluorescence microscope after staining (immunohistochemistry) the xenograft tumors with cleaved Caspase 3 (Fig. 9d). This method was used to identify the brown stained cells for apoptosis. Results demonstrated that administration with TAX induced apoptosis as the dead cells number increases in a dose dependent approach.

TAX inhibits xenograft tumor growth

Cyclin is a family of proteins that organize the development of cells during the cell cycle by activating cyclin-dependent kinase (Cdk) enzymes. Cyclin D is one of the chief cyclins formed in terms of its functional importance and is a vital watchdog of cell proliferation, apoptosis and tumourigenesis.

Protein expression of cyclin D was assessed in both TAX administrated and vehicle group. Results demonstrated

induction of Cyclin D protein expression in control group as compared to TAX administrated group (25 and 15 mg/kg). Down regulation of Cyclin D protein expression in TAX administrated group, favors inhibition of proliferation. The expression of cyclin D in staining (immunohistochemistry) of group administrated with TAX and vehicle group was also investigated. A prominent difference was seen between TAX administrated and vehicle. The expression of cyclin D was found to be high in vehicle group while TAX administrated show low expression of cyclin D (Fig. 10a and b).

TAX administration decreases β -catenin and survivin expression in xenograft tumors

Effect of TAX (15 and 25 mg/kg) on β -catenin and survivin expression in HCT116 xenograft tumors was confirmed by immunoblotting, immunohistochemical staining and RT-PCR.

A prominent decline in expression was observed (Fig. 10a). The concentration dependent impact of TAX on HCT116 xenograft tumors demonstrated a prominent decline in β -catenin and survivin protein levels at 2.5 and 1.25 mg concentrations (Fig. 11a). Immunohistochemical staining of HCT116 xenograft tumors in athymic nude mice illustrated decline in β -catenin expression in TAX administrated as compared to vehicle ones (Fig. 10b).

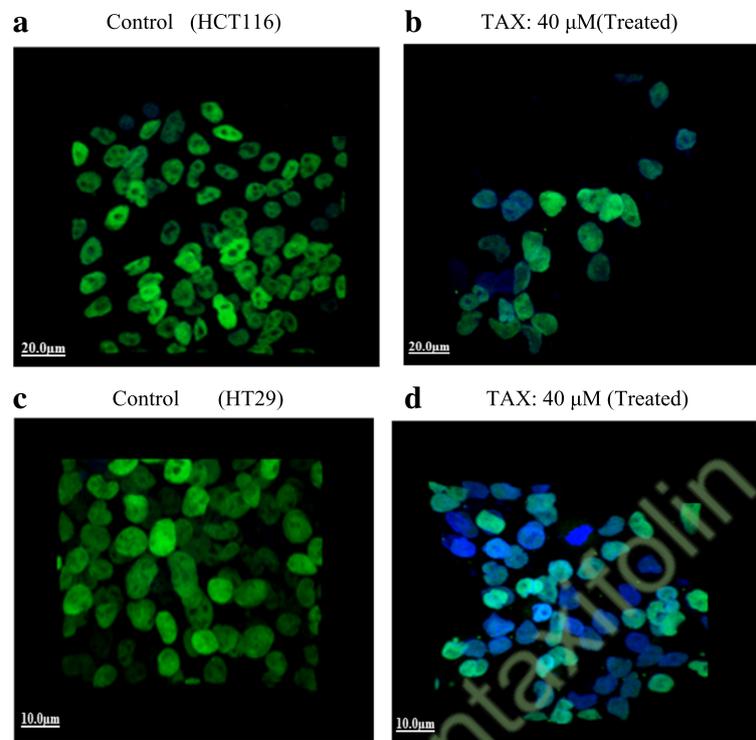


Fig. 7 Immunofluorescence staining of HCT116 (b) and HT29 cells (d) demonstrating expression of β -catenin in both TAX treated (40 mol/L) as compared to control HCT116 (a) and HT29 (c) cells (untreated). Alexa fluor staining of TAX of both cell lines (green fluorescence) and counter stained with DAPI (blue fluorescence) were seen

To elevate, either the observed decrease in β -catenin protein was due to decreased transcription of β -catenin gene, alteration of β -catenin expression by TAX induction in HCT116 xenograft, a prominent decline in mRNA expression by employing RT-PCR, was seen to be in concentration dependent approach. At 15 and 25 mg/kg of TAX a momentous reduction in β -catenin expression was seen (Fig. 11b).

TAX induced inhibition of Akt phosphorylation in HCT116 xenografts in athymic nude mice

Phosphorylation of β -catenin by AKT boosts its transcriptional activity and encourages tumor cell invasion, demonstrating that AKT-dependent regulation of β -catenin plays a crucial part in tumor incursion and progression. Animals implanted with HCT116 xenografts with TAX administration induced a concentration dependent reticence of AKT phosphorylation. AKT is essential player of signaling cascades for cell growth and survival during formation and cancer. The protein expression of AKT was investigated by immunohistochemical staining and immune blotting. The down regulation of AKT protein expression by TAX (15 and 25 mg/kg) administration, favors the inhibition of proliferation and there was prominent difference between expressions of vehicle and treated group. The AKT expression was

observed diminished in TAX administrated group as compared to vehicle group. Also p-AKT expression was investigated but no expression of p-Akt protein was seen (Fig. 11a).

Discussion

The assorted molecular outlines of CRC and the necessity to categorize patients which could efficiently take clinical benefit from combined chemotherapies ignited the categorization of the mechanisms accountable for sensitivity and confrontation to treatments. The common of sporadic types of CRC are distinguished by deregulation of Wnt/ β -Catenin signaling which results in amplified transcriptional activity of the β -Catenin. Regardless of the complexities in to dissect the association of Wnt/ β -Catenin pathway in the initiation and development of CRC, the study of these mechanisms is now rising as a potent platform to recognize budding objectives of incursion for CRC cure.

Countless natural compounds have been recommended to operate as anti-cancer agents or lessen the side effects of additional anti-cancer therapeutic drugs by mounting the therapeutic effects of the vital anti-cancer agent or act as “enhancers” [23, 24].

Taxifolin, a flavanonol plagiaristic of flavonoids, is copious in foods and sages [25]. It displays an extensive

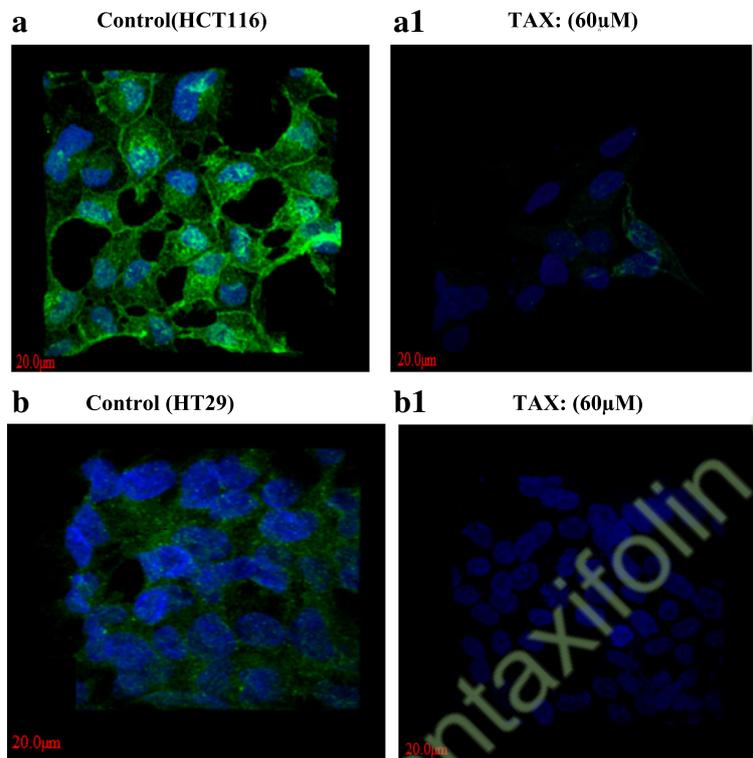


Fig. 8 Immunofluorescence staining of HCT116 (A1) and HT29 cells (B1) demonstrating expression of p-AKT in both TAX treated as compared to control HCT116 (a) and HT29 (b) cells (untreated). Alexa fluor staining of p-AKT of both cell lines (green fluorescence) and counter stained with DAPI (blue fluorescence) were observed

range of bioactivities, amongst which the antioxidant activity is relatively distinctive [26]. Numerous studies have confirmed TAX demonstrates advantageous chemopreventive activity on colon carcinogenesis, but the specific mode of action by which it obtains its anti-carcinogenic effect remains veiled [27]. Studies also revealed the therapeutic behavior of TAX confined on in vivo and in silico-mediated tendency of Nrf2, Wnt/ β -catenin, and inflammatory signaling pathways [28]. Also studies illustrated that TAX defends RPE cells against oxidative strain by hindering the H₂O₂-induced decline in the intracellular generation of ROS, cell viability and cell apoptosis. The effective mechanism emerges to encompass the stimulation of NRF2 and the upregulation of the phase II antioxidant enzyme system.

Cellular proliferation significant in tumor formation may arise due to amendment in cell cycle regulation [29, 30]. A chief principal cause of cancer progression is accredited to immediate and uninhibited proliferation results to succession and upsurge of tissue accumulation. Results of MMT assay quantified that TAX are specific in their action and competent against cell cancer cell lines from diverse origin. Cell growth in both HCT116 and HT29 cells is altered by TAX. Administration with TAX resulted in arrest of cellular propagation in a dose dependent approach, amplification in

hammering of cell viability was observed with increase in the concentration of dose.

Apoptosis is a dominant event to suitable explicit components of cells while shunning inflammatory effect usually escorting necrosis, thus no disparaging effect to the adjacent healthy cells will occur meanwhile the cells undergoes apoptosis. Thus apoptosis is an event ratifying chemotherapy efficiency, believed as an endpoint of anti-cancer drug therapy [31, 32].

The in vitro study revealed that the N-terminal of p21 contains 1–82 amino acids, is crucial for the reticence of cyclin–CDK, and involvement with cyclin A, cyclin E, or CDK2 in an uncoupled form or an intricate form [33]. Observations illustrated that cyclin E allies with p21 via the Cy motif (17). Also studies signify that the Cy-deleted mutant-p21 precipitate the cyclin A–CDK2 and p21 administrated with anti-Cy motif antibody co-precipitated with cyclin A–CDK2. The communication of the p21-allied CDK inhibitor, p27, and cyclin A is mediated by an RNLFG sequence in p27 that communicates with a hydrophobic groove on the surface of cyclin A [34]. In the present study, assessment of apoptosis tempted in HCT116 and HT29 cells illustrated that TAX are very efficient inducers of apoptosis in concentration dependent approach. Results of our study showed decreased expression of Cyclin B1, D1, E

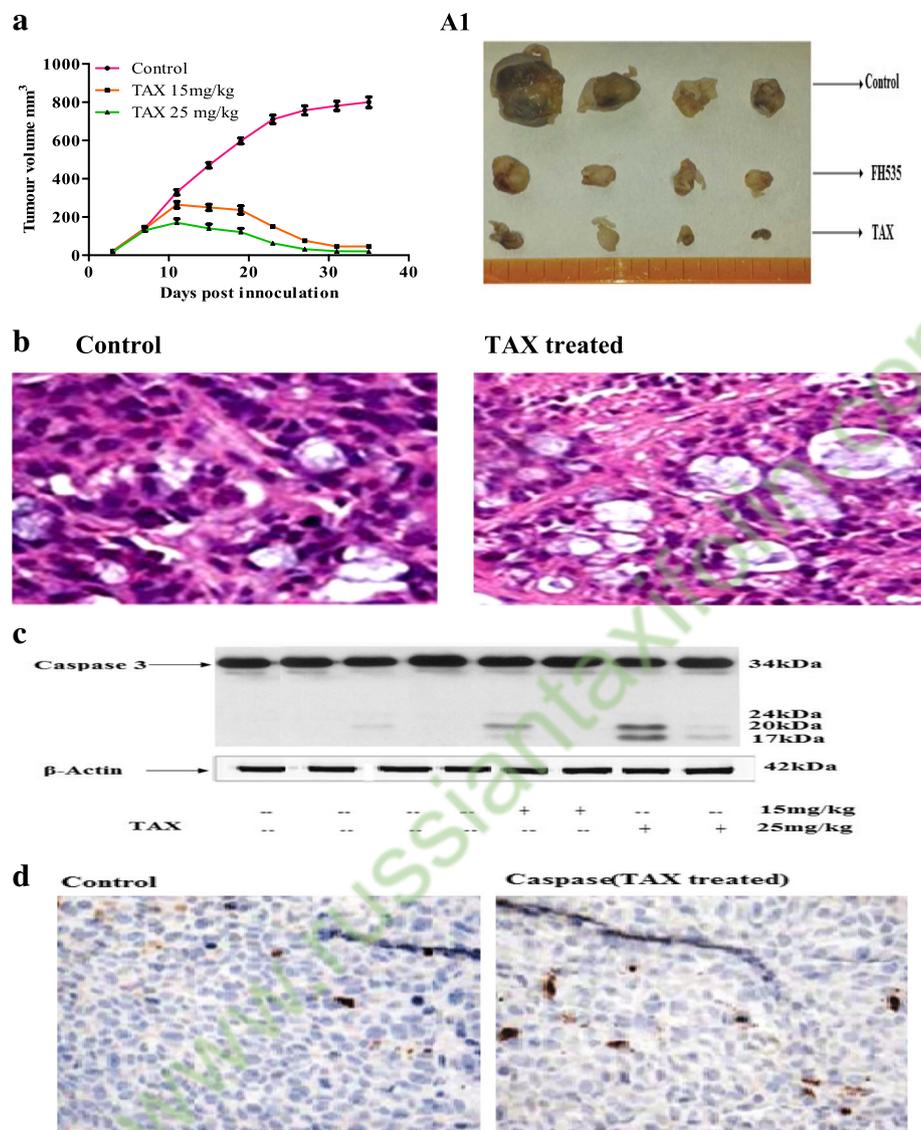
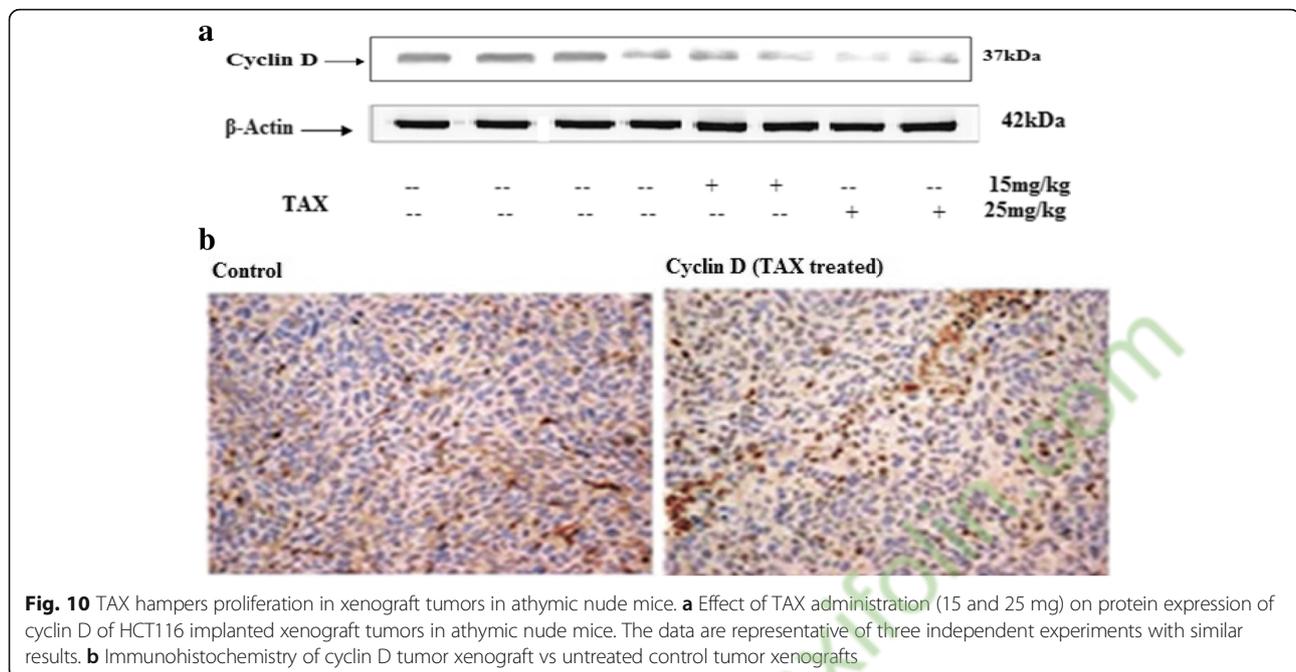


Fig. 9 Consequence of TAX administration on HCT116 tumor growth in athymic nude mice. **a** Average tumor volume of water fed, 15 & 25 kg/mg taxifolin injected mice plotted over days after tumor cell inoculation. Values represent mean \pm SD of six mice. *, $p < 0.01$ (25 mg/kg); **, $p < 0.05$ (15 mg/kg) vs water fed normal control mice ***, $p < 0.001$. **b** H&E staining of taxifolin administrated xenograft tumors vs control. **c** and **d** Immunoblotting and Immunohistochemistry of cleaved Caspase 3 of TAX administrated tumor xenograft vs untreated control tumor xenografts. The data are representative of three independent experiments with similar results

and A, Cdk-2, 4 and 6 and accompany with an upsurge in expression of cdk inhibitors p21 and p27 in TAX administrated cells. Furthermore TAX colorectal cancer cells exhibited arrest in the G2 phase of the cell cycle. These findings are significant since cell cycle regulation is a central goal for anticipation against colorectal cancer. Zhang et al. [35], illustrated that TAX boosts the anti-proliferation and apoptotic effects of Andrographolide and suggested that TAX might function as an enhancer in andro-induced cell cycle arrest and cell death of DU145 cells, but our study revealed that TAX alone tempted cell-cycle arrest and cell death of HCT116 and HT29 cells.

PARP, a typical caspase substrate, is a crucial competitor of DNA repair against ecological strain and in the continuation of cell viability. Cleavage of PARP is considered as a trademark of apoptosis [36]. PARP cleavage results concurrently with cleavage of procaspase 3, 7, and 9 in a dose-dependent approach, demonstrating that TAX induced apoptosis in HCT116 and HT29 cells is arbitrated in the passage of an intrinsic apoptosis pathway. Numerous studies have revealed that PARP-1 is overexpressed in different human malignancies [37–39]. In addition, studies revealed that PARP-1 has a part in colon cancer growth [40, 41], as its expression was



considerably increased in colon cancer and was associated with tumor size and histopathology [41]. Above results strengthened our results showing increased expression of PARP in TAX administrated cells as compared to control.

Bcl-2 family associates are the chief watchdogs of apoptosis. Their overexpression constantly hinders apoptosis due to cytotoxic injuries by abolition of free radicals, deterrence of mitochondrial canal formation, and the discharge of c cytochrome [42, 43]. In the anti-apoptotic subfamily, Bcl-2 and Bcl-xL, showed decreased expression in TAX administrated cells in concentration dependent approach. In contrast, the expression of Bax and Bak, pro-apoptotic proteins, showed increased expression in TAX treated HCT116 and HT29 cells as compared to control cells. Our results proposed that TAX mediated upsurge in the expression of Bax and decreased regulation of Bcl2 expression may be a promising direction through which TAX induce apoptosis in colorectal cancer. This signifies its pleiotropic influence on the apoptotic signaling pathway.

The canonical Wnt (or Wnt/ β-catenin) signaling pathway is crucial in progression, tissue renewal and stem cell continuance. In addition boosted signaling via this pathway is allied with the expansion and succession of various cancers [44]. Initiation of the pathway is allied to hypophosphorylation, cytosolic alleviation and growth of β-catenin in the nucleus pursued by intensified candidate gene expression [45, 46]. Increased cytosolic β-catenin levels and consequent nuclear import authorize its union with transcription players of the Tcf/

Lef family that organize the expression of genes, including cyclin D1, vegf, and survivin, all of which promote to cancer progression [47–50]. In this study AKT and β-catenin showed down regulated expression by administration of TAX in HCT116 and HT29 colorectal cancer cells as compared to control cells. Immunofluorescence staining of HCT116 and HT29 cells demonstrated decline in p-AKT expression and β-catenin at dose of 40 μM of TAX treated as compared to control. Significant Alexa fluor staining of p-AKT (cytoplasm) of both cell lines (green fluorescence) were pragmatic in control, while the expression of p-AKT as specified by staining was prominently decreased in TAX administrated cells. Further our findings illustrated that TAX induction resulted in apoptosis through inhibition of β-catenin and p-AKT. While FH535 β-catenin inhibitor baskets AKT phosphorylation which inturn decrease β-catenin protein expression. TAX administration to β-catenin inhibitor induced cells supporting amplified decrease of β-catenin expression, sustaining the fact that these alterations are mediated by protein kinase B (AKT). Our results support the involvement of AKT in encouraging the transcriptional activity of β-catenin.

AKT is the most crucial downstream effector of CK2 and phosphatidylinositol 3 kinase (PI3-K). Phosphorylated AKT encourages cell endurance through hampering pro-apoptotic proteins [51]. The commencement of PI3K/ Akt can trigger the canonical Wnt signaling through the phosphorylation of GSK-3β by the phosphorylated Akt1/2, jamming the configuration of β-catenin destroying complex (28). Therefore, the upregulation of PTEN may

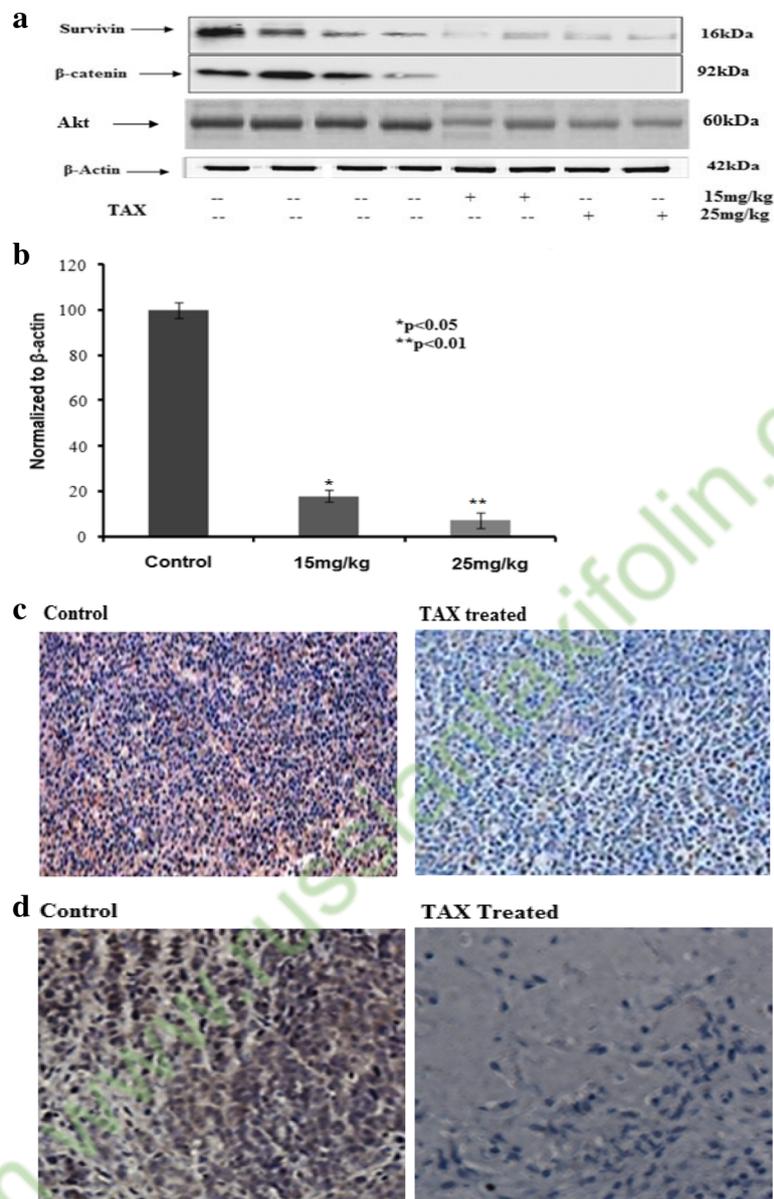


Fig. 11 Effect of TAX administrated treatment on HCT116 xenograft tumors for protein expression of β -catenin. **a** Expression of β -catenin and survivin protein by immunoblotting in TAX administrated and control group, experiment performed in triplicate. **b** qPCR analysis of TAX administrated for changes in β -catenin mRNA levels. The data expressed as fold change represent the mean \pm standard errors experiment performed in triplicate where * $p < 0.05$, ** $p < 0.01$ was considered significant v/s control. **c** Effect of TAX administrated as detected by immunohistochemical staining. DAB staining of β -catenin (brown) and counter stained with hematoxylin (blue). **d** Effect of TAX administrated on protein expression of Akt and control as detected by immunohistochemical staining. DAB staining of Akt (brown) and counter stained with hematoxylin (blue)

hamper the canonical Wnt signaling by encouraging the deprivation of β -catenin. While our results confirmed that administration of TAX to HCT116 and HT29 colon cells reduces the phosphorylation of PI3K, AKT and decreased the protein expression of β -catenin. The recent study revealed that it may not result from the reduced phosphorylation of GSK-3 β by PTEN/PI3K/Akt signaling. As it is prone to mutation of β -catenin in HCT116 cells, the β -catenin can't be tarnished by the

destruction complex in this colon cancer cells [52]. Further we investigated that administration of TAX hindered the mRNA expression of β -catenin and thus our study strongly illustrated that TAX has compelling anti-proliferation activity in human colon cancer cells, promoting apoptosis and the anti-proliferation effect of TAX may be arbitrated by PI3K/Akt signaling by jamming Wnt/ β -catenin signaling transduction, through hampering the β -catenin expression.

Recently studies confirmed that the substantial relations and efficient reticence of NF- κ B by β -catenin in colorectal cancer cells requires phosphatidylinositide 3-kinase (PI3K) [53]. Obstruction of PI3K by compound inhibitors rescinds the configuration of β -catenin and NF- κ B protein complexes. In quiescent colorectal cancer cells, β -catenin and NF- κ B restricted in the cytoplasm, and administration with PI3K inhibitor give rise to nuclear translocation of NF- κ B and membrane retention of β -catenin. Conversely, being not clear whether PI3K directly acts as an association player between β -catenin and NF- κ B or otherwise parting a role in β -catenin-mediated suppression of NF- κ B triggered by diverse stimuli. Our studies proposed that inhibition of overexpression of β -catenin by administration of TAX in HCT116 and HT29 results in reduced protein expression of NF- κ B and PI3K, hence this may be one of the possible mechanism cell death and apoptosis.

Survivin (BIRC5), an associate of the inhibitor of apoptosis (IAP) protein family, is a delegate anti-apoptotic protein which enhance tumor cell expansion [54]. Survivin is of growing curiosity as a probable therapeutic target to hamper cancer intensification [55]. Survivin enhances tumor propagation by renovating various significant cell signaling pathways. Our results clearly confirmed a noteworthy inhibition of an anti-apoptotic protein survivin expression in both HCT116 and HT29 cells by administration of TAX.

Conclusion

Administration of human colorectal cancer cells with TAX encouraged apoptosis and effects on a range of endurance signals proposing the potential of the TAX as chemopreventive agent for colorectal cancer. Briefly, our results recommend that TAX might be promising candidates for molecular target-based cancer prevention and adjuvant therapy. The novel information generated in the present work on the TAX provides important preliminary information that may be useful in designing further studies to ascertain the chemo-preventive and chemotherapeutic efficacy of the TAX.

Additional file

Additional file 1: Figure showing Organs with no toxicity. (DOCX 11616 kb)

Abbreviations

(CRC): Colorectal cancer; (CSCs): Cancer stem cells; (DAB): Diamminobenzidine; (DAPI): 4', 6-Diamidino-2-Phenylindole, Dihydrochloride; (DMEM): Dulbecco's Modified Eagle's; (DMSO): Dimethyl sulfoxide; (PI): Propidium iodide; (TAX): Taxifolin

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Author' contributions

SR designed the study, conceived the study and analyzed the results., TA, AU and AA conceived an initial part of the study, performed the experiment, histology and helped in compiling the results. SR and TA performed experiment. AA, AU and AwsA helped in writing the results. SR, TA and SJ wrote the paper with input from all other authors SJ, SR, MA, sAwsA and AA made substantial contribution in interpretation of data and revising the manuscript for intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

All the data is contained in the manuscript.

Ethics approval and consent to participate

Athymic nude mice studies were performed according to the Institutional principles for the concern and use of animals and the experimental protocol was approved (BAS#0256) by the ethical board of Quaid-i-Azam University, Islamabad, Pakistan and Committee dealing animal care and use, college of Pharmacy, King Saud University, Kingdom of Saudi Arabia. Before starting experiment on human colorectal cancer cell lines HCT116 and HT29 (ATCC® CCL-247™ and ATCC® HTB-38™ respectively) purchased in July 2017 from American Type Culture Collection (MD, USA), ethical approval was taken from ethics committee of preclinical studies, college of pharmacy, King Saud University, KSA.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author details

¹Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. ²Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia. ³Nanomedicine research unit, Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

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