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A polysaccharide from *Tinospora cordifolia* stem induces cell cycle arrest in human breast cancer cell lines MCF-7 and MDA-MB-231

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Abstract

Plant-based therapies are practiced for various human and veterinary ailments since time immemorial. The present study is concerned with finding the anti-breast cancer potential of a novel polysaccharide isolated from the methanolic extract of *Tinospora cordifolia* stem. The compound tested on MCF-7 and MDA-MB-231 proved to have potential to induce death of both the cell lines with IC_{50} at 100 µM, as revealed in MTT, and LDH assays, and AO/EtBr staining. DNA fragmentation studies indicated damage to DNA. Flow cytometric analysis showed polysaccharide-induced cell cycle arrest at G2/M phase in both the cell lines. Western blot studies made it evident that the polysaccharide inhibits cell cycle progression via change in the expression of cell cycle regulators such as Cyclin D1, Cyclin D3 and p18 INK4. In the i*n-silico* approach the structure of the compound was drawn using ChemSketch and the ADME/T properties of the compound were analyzed using Accord for Excel software. The compound possesses good ADME/T properties required for an active drug. The compound was found to possess anticancer efficacy via its effect on cell cycle regulatory proteins in the breast cancer cell lines and also satisfied the ADME/T properties of a drug. Hence, the novel compound isolated from *T. cordifolia* stem may be evaluated further so as to develop it as a breast cancer drug.

Keywords: Anticancer, Breast Cancer, Cell Cycle, Phytotherapy, ADME/T

1. Introduction

Breast cancer is one of the frequently occurring cancers among women and its incidence has been increasing globally[1, 2]. Etiology of cancer involves change in several regulatory processes of which alteration in cell cycle regulation is a key event. Regulation of cell cycle involves a number of protein kinases such as cyclin-dependent kinases (CDKs), which interact with specific cyclins to initiate cell cycle end regulate its progression through the different phases[3]. The interaction between these cyclins and CDKs can be altered by several CDK inhibitors (CKIs). The CKIs of INK4 family (p15 INK4b, p16 INK4a, p18 INK4c, and p19 INK4d) directly bind to specific CDKs and inhibit the interaction of cyclins with CDKs[4, 5]. The Cip/Kip family of CKIs (p21 CIP1, p27 KIP1, and p57 KIP2) are those that inhibit the formation of cyclin-CDK complex and thereby inactivate them[6-8]. Abnormal cell proliferation that is usually noticed in cancer conditions is also due to genetic change in the regulation of these proteins[9].

Tinospora cordifolia (Willd.) Miers (*T. cordifolia*) (Menispermaceae) is a medicinal herb that is used to treat several human ailments[10]. The plant has been reported as cytotoxic to tumor-associated macrophage-derived dendritic cells[11]. Stem of *T. cordifolia* is widely included

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in traditional folk medicines in India[12]. An alkaloid, palmatine, isolated from the stem of *T. cordifolia* was shown to exhibit anticancer activity against DMBA-induced tumors in Swiss albino mouse[13]. The polysaccharide fraction from *T. cordifolia* was found to be highly effective in reducing the metastatic potential of B16-F10 melanoma cells[14]. The plant extract or compounds in it acted as anticancer agents against skin carcinoma[15], hepatocellular carcinoma[16], Ehrlich ascites carcinoma[17], and breast cancer[18]. Radioprotective effect of the compounds was also demonstrated[19].

In an earlier paper we reported that methanolic extract of *T. cordifolia* stem was an excellent anti-diabetic agent against streptozotocin (STZ)-induced diabetic rats[20]. A novel polysaccharide present in the methanolic extract of *T. cordifolia* stem was shown to possess hypoglycemic activity in STZ - induced diabetic Wistar rat, and application was filed for Indian patent (4832/CHE/2012)[21]. The present study was aimed at evaluating the anticancer efficacy of a polysaccharide present in the methanolic extract of *T. cordifolia* against MCF-7 and MDA-MB-231 human breast cancer cell lines. The study was specially directed towards finding if the compound targets cell cycle regulatory proteins to induce apoptosis in the breast cancer cell lines.

2. Materials and Methods

2.1 Chemicals

Acridine orange (AO), ethidium bromide (EtBr), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Chemical Co., USA. Trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS) and antibiotics-antimycotics were purchased from Gibco, Canada. Antibodies used for western blotting were procured from Cell Signalling Technologies, USA. Phosphate buffered saline (PBS) was procured from Himedia Chemicals, Mumbai. All solvents were obtained from Fischer Scientific Ltd., India. All the chemicals used were of extra pure and culture grade.

2.2 Plant Material

T. cordifolia stem was collected from Tirunelveli District, Tamil Nadu, India. The species was identified and authenticated by Dr. Roseline, Plant taxonomist, Department of Botany, Holy Cross College, Tiruchirappalli, Tamil Nadu, India, and a reference sample is kept in the College herbarium. The stem was shade-dried, cut into small pieces and coarsely powdered. The coarse powder was used for isolation of the novel polysaccharide.

2.3 Isolation of the Novel Polysaccharide

Methanolic extract *T. cordifolia* stem was chromatographed over separate silica gel column (Acme's silica gel, 100-200 mesh size, 750gm, 3.5 i.d. x 60 cm) and successively eluted with stepwise gradient of hexane, ethyl acetate and methanol solvent system (5%, 10%, 20%, 30%, 50%, 70% and 100%). The various fractions obtained were pooled together based on similarity of Rf values obtained in thin layer chromatography to obtain fraction 3 and the fraction was further eluted with a stepwise gradient of ethyl acetate: metahnol (9:1) solvent system to obtain sub-fractions. Sub-fractions 11-69 were having similar Rf values and were pooled to acquire pure individual compound[21]. This methodology of isolation process for the novel compound has been submitted for Indian patent (4832/CHE/2012).

2.4 Structure of the Novel Polysaccharide

The structure of novel polysaccharide was drawn and validated by analyzing the properties of the structure such as molecular weight and molecular formula using ACD/ ChemSketch™ software.

2.5 Prediction of ADME/T

ADME/T properties of the novel polysaccharide were calculated using Accord for Excel 6.1 version software. Based on the structure of the compound all the parameters involved in analysing the ADME/T properties were calculated.

2.6 Cell Lines

Human breast cancer cell lines MCF-7 and MDA-MB-231, and normal breast epithelial cell (MCF-10A) were procured from ATCC and cultured in DMEM culture medium with 10% FBS at 5% CO_2 and 37 °C. Cells were passaged using trypsin-EDTA to 70-80 % confluence.

2.7 Sample Preparation

Stock solution of the novel compound at 1 mM concentration was prepared in DMSO. From the stock, samples were prepared at different micromolar concentrations (5,

10, 20, 40, 80, 100 and 150) in serum-free medium (SFM) for the test. The concentration of DMSO was aimed not to exceed 0.01%.

2.8 Cell Viability Assay

Viable cells were measured by a colorimetric assay composed of solutions of a tetrazolium compound MTT (dimethyl thiazolyl tetrazolium bromide). MTT is metabolized by cells into a formazan product that is soluble, and the absorbance of formazan is measured at 570 nm. The cells (MCF-7 and MDA-MB-231) were seeded at a density of 5×10^3 cells/well, in a 96-well plate and incubated for 24 h at 37 °C in CO_2 incubator at 5% CO_2. After attachment, cells were washed with 1 x PBS, and then serum-free medium (SFM) was added and incubated for 12 h. Cells were added to fresh medium containing different concentrations of the compound and incubated for 24 h. Then, the medium was removed and washed twice with 1 x PBS, and 100 µL of MTT (0.5 mg/mL) solution was added to each well and incubated for 3 h. After incubation 100 µL of DMSO was added for solubilization of cells and incubated in dark for 1 h. Intensity of the color that developed was read at 570 nm in an ELISA plate reader. The cell viability was calculated as follows:

% cell viability = absorbance of treated cells/ absorbance of control cells × 100.

Five different observations with each concentration of the compounds were performed and the concentration that gave 50% reduction in the number of live cells (IC_{50}) was determined.

2.9 Assessment of Cell Viability Adopting LDH Assay

The cytotoxic nature of the compound was determined adopting colorimetric assay. Each cell was seeded at a density of 5×10^3 cells/well, in a 96-well plate and incubated for 24 h at 37 °C in 5% CO_2 incubator. After attachment, cells were washed with 1 x PBS and then serum-free medium (SFM) was added and incubated for 12 h. Fresh medium containing different concentrations of the compound was added to the wells and incubated for 24 h. Cells were then harvested and the assay was carried out using LDH assay kit procured from Agappe Diagnostics, Kerala, India. One hundred microliters of the sample was mixed with the working reagent (10:1000) and incubated for 1 min at 37 °C and read in an ELISA plate reader at 340 nm. The percentage of cells with LDH leakage was calculated according to manufacturer's instruction.

2.10 Detection of Cell Death

The ability of the compound to induce cell death in the cell lines was determined by AO/EtBr dual staining. Cells were grown on cover slips in a 24-well plate with 1×10^5 cells/well for 24h. Cells were then treated with IC_{50} concentration (100 µM) of the compound for 24 h. After incubation, 5 µL of AO (1 mg/mL) and 5 µL of EtBr (1 mg/mL) were added and live and dead cells were recorded using a fluorescent microscope.

2.11 DNA Fragmentation Assay

The control and compound-treated (100 µM) breast cancer cells (both adherent and detached) were harvested and washed with 1 x PBS. Approximately 1.8×10^6 cells were lysed with a lysis buffer composed of 50 mM Tris–HCl, 10 mM ethylenediaminetetraacetic acid (EDTA)-4Na and 0.5% sodium-*N*-lauroyl sarcosinate (pH 7.8). The lysates were incubated in the lysis buffer containing 0.33 mg/mL RNase A at 50 °C for 30 min and then further incubated in the lysis buffer containing 0.33 mg/mL proteinase K at 50 °C for 30 min.

Equal amounts of DNA were electrophoresed on 2.0% agarose gel and visualized in UV light.

2.12 Cell Cycle Analysis

The cells were cultured at a density 5 x 10^5/well in a 24-well plate containing 1 mL of complete medium. After 24 h, the medium was changed to serum free medium and incubated for 24 h. Cells were then treated with and without 100 µM of the polysaccharide and incubated for 24 h. After incubation, both floating and adherent cells were collected and centrifuged at 1500 rpm for 5 min at room temperature. The cell pellets were gently re-suspended in 1 x PBS and fixed overnight at 4 °C in the fixing solution (15% FBS and 15% PBS in 70% ethanol). The cells were centrifuged at 1500 rpm for 5 min at room temperature. Cells pellets were washed twice with cold 1 x PBS and incubated for 1 hr at room temperature in 500 µL propidium iodide (PI) solution containing 0.05 mg/mL PI, 0.1 mm EDTA, and 0.05 mg/mL RNase A in PBS. The percentages of treated and un-treated cells in various stages of cell cycle were determined using FACSCalibur cell

sorter (BD Biosciences, San Jose, CA, USA) and analyzed by Flow Jo 7.5.5 (Tree Star, Ashland, OR, USA).

2.13 Protein Preparation

MCF-10A normal breast cells, control MCF-7 and MDA-MB-231 breast cancer cells and 40 µM polysaccharide treated MCF-7 and MDA-MB-231 cells were washed with 1 x PBS, and 600 µL of RIPA buffer containing protease inhibitor cocktail was added to the cells. The cell lysates were then subjected to centrifugation for 15 min at 10,000 rpm at 4 ºC. The supernatants were collected as protein samples and the concentration of protein was determined by Lowry's method[22].

2.14 Protein Expression Analysis

The protein samples obtained as above (50 µg) were electrophoresed in SDS polyacrylamide gel and the separated proteins were transferred to a PVDF membrane. The non-transferred sites in the membrane were blocked using 5% blocking solution for 1 h. After blocking, the membrane was washed using TBS and TBST, and incubated overnight with the primary antibodies against Cyclin D1, D3 and p18 INK4c (1:1000) in TBST, with β-actin as the loading control. After a rinse, the membrane was incubated for 1 h with the secondary antibody-HRP conjugate (1:5000) in TBST. Protein bands were detected using enhanced chemiluminescence kit and quantified using Quantity One Software.

2.15 Statistical Analysis

The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to access the significance between groups at a level of $p<0.05$ using SPSS 17.0 version.

3. Results

3.1 ADME/T Properties of the Novel Polysaccharide

The structure of the novel polysaccharide (Figure 1), drawn and validated using Chemsketch, was used for its ADME/T property calculation. The ADMET property of the compound thus calculated is given in Table 1. The novel compound was found to possess a satisfactory level of absorption and good solubility with no blood-brain barrier (BBB) penetration and hepatotoxicity.

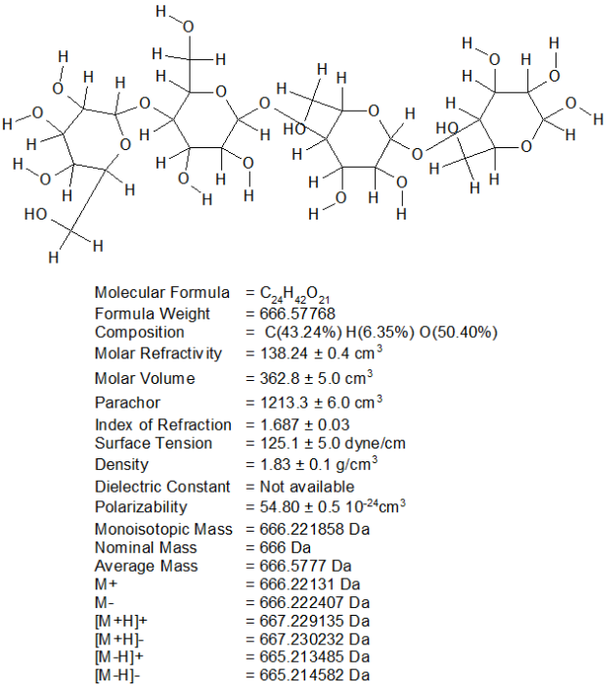

Figure 1. Structure and physiochemical properties of the novel polysaccharide drawn using ChemSketch

Table 1. ADME/T properties of the novel polysaccharide

Components of ADME/T studies	Novel polysaccharide	Properties of novel polysaccharide
Aqueous solubility level	1	Good solubility
BBB penetration	4	No penetration
CYP2D6	0	Non-inhibitor
Hepatotoxicity	0	Non-toxic
Human Intestinal Absorption	3	Satisfactory absorption
Protein binding level	0	No binding

3.2 Antiproliferative Effect of Polysaccharide

The effect of the novel polysaccharide on viability of MCF-7 and MDA-MB-231 breast cancer cell lines was analyzed using MTT assay (Figure 2). Increase in the concentration of the compound caused a sequential decrease in the percentage of cell viability compared to the untreated cells. The cytotoxic potential of the compound on breast cancer cell lines was identified adopting LDH assay (Figure 3). The compound-treated cells were found to exhibit an increase in LDH leakage in a dose-dependent manner. Both the assays confirmed the compound-induced death of MCF-7 and MDA-MB-231 cells and 50% decrease of cell viability, IC_{50}, was found at 100 µM concentration of the compound.

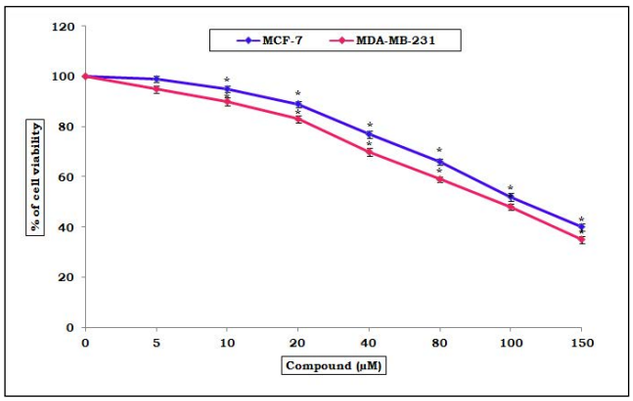

Figure 2. Effect of the novel polysaccharide on viability of breast cancer cells. Each bar represents the mean ± SEM of six independent observations. '*'represents statistical significance between control and compound-treated groups at $p < 0.05$.

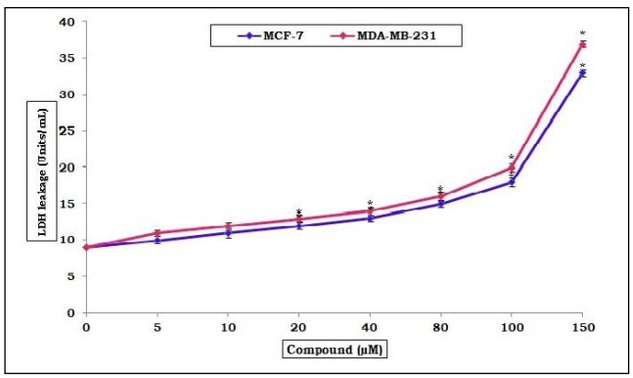

Figure 3. Cytotoxic effect of the compound on breast cancer cells. Each bar represents the mean ± SEM of six independent observations. '*'represents statistical significance between control and compound-treated groups at $p < 0.05$.

3.3 Compound-Induced Breast Cancer Cell Death

The ability of the polysaccharide to induce death of the breast cancer cells was confirmed by AO/EtBr dual staining. The fluorescent microscopic analysis revealed that the untreated MCF-7 and MDA-MB-231 cells were found to be stained in uniform green color indicating viable cells and the compound-treated cells were found to fluoresce in green and orange, where the presence of orange-colored cells indicated induction of cell death (Figure 4).

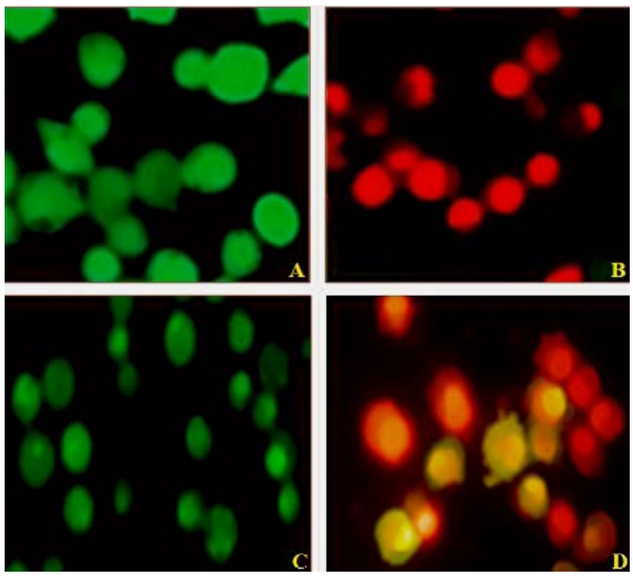

Figure 4. Ability of the novel polysaccharide to induce cell death. (A) Untreated MCF-7 cells; (B) Compound-treated MCF-7 cells; (C) Control untreated MDA-MB-231 cells; (D) Compound-treated MDA-MB-231 cells. Control cells were uniformly in green fluorescent live cells and the compound-treated cells fluoresced in bright red indicating cell death (x200).

3.4 Effect of the Polysaccharide on DNA

The polysaccharide-mediated induction of apoptosis was analyzed in terms of its action on the DNA of breast cancer cells (Figure 5). When treated with the polysaccharide (100 µM) for 24 h, the DNA pattern of the MCF-7 and MDA-MB-231 cells was found to be altered, with the occurrence of fragmentation, whereas no such fragmentation pattern was observed in the DNA of untreated cancer cells. Such DNA fragmentation in the compound-treated cells revealed that the polysaccharide induced cell death via apoptosis.

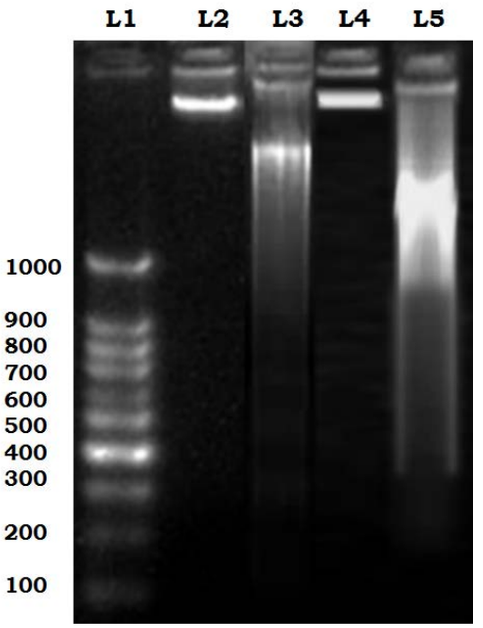

Figure 5. DNA fragmentation induced by the novel polysaccharide. L1-DNA ladder; L2-DNA of untreated MFC-7 cells; L3-DNA of compound-treated MCF-7 cells; L4-DNA of untreated MDA-MB-231 cells; L5-DNA of compound-treated MDA-MB-231 cells.

3.5 The Polysaccharide-Mediated Cell Cycle Arrest

The mechanism of action of the polysaccharide in inducing breast cancer cell death via the induction of cell cycle arrest was analyzed using flow cytometry (Figure 6). The polysaccharide-treated cells were found to show cell cycle arrest with 20.15% cells remaining at G2/M phase compared to only 9.07% untreated MCF-7 cells. Similar was the trend in the case of MDA-MB- 231 cells treated with the polysaccharide, as 27.2% of the treated cells were struck in cell cycle arrest in the G2/M phase whereas only 9.8% untreated cells were arrested at this phase. The polysaccharide induced accumulation of cells at G2/M phase with significant arrest of cells at this phase compared to untreated breast cancer cells.

3.6 Effect of the Compound on Cell Cycle Regulators

The compound-mediated change in the expression of cell cycle regulatory proteins (Cyclin D1, D3 and p18INK4c) in MCF-7 and MDA-MB- 231 cells was analyzed and compared with their expression in normal breast epithe-

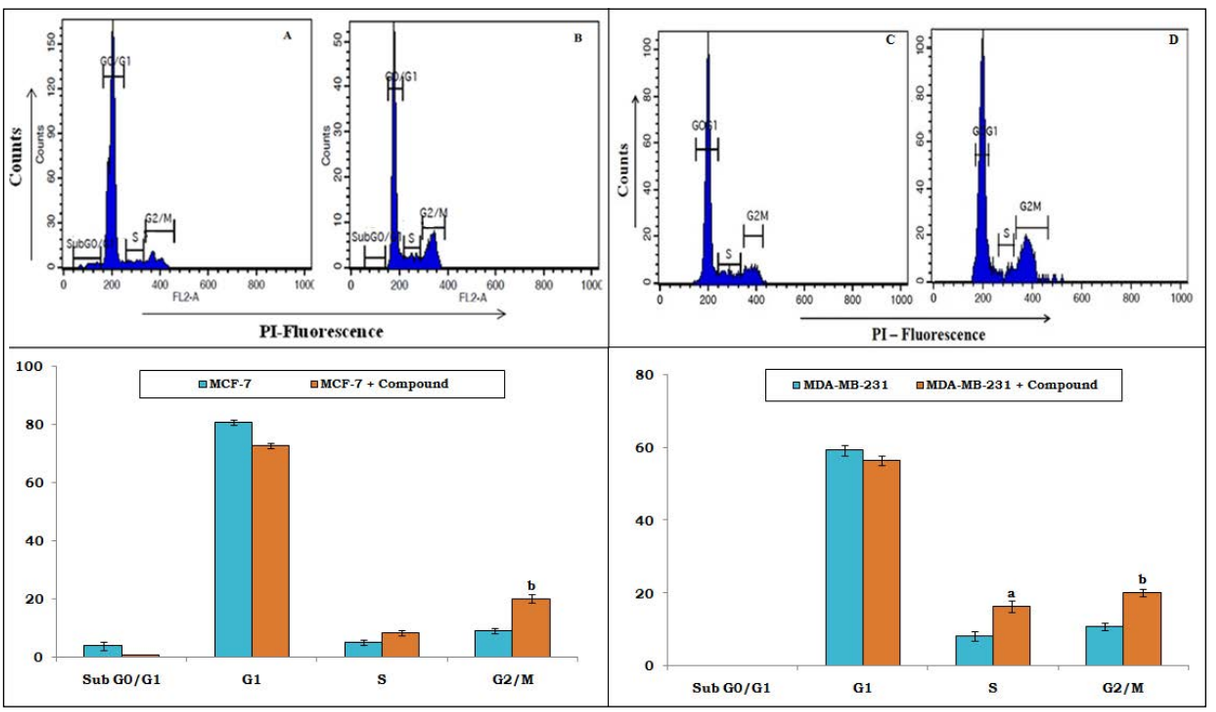

Figure 6. The polysaccharide-mediated cell cycle arrest. (A) Untreated MCF-7 cell; (B) Compound-treated MCF-7 cell; (C) Untreated MDA-MB-231 cell; (D) Compound-treated MDA-MB-231 cell. Each value is mean ± SEM of three independent observations, with statistical significance between control and treated groups. a: $p<0.05$ by comparison with G1 phase of control cells, b: $p<0.05$ by comparison with G2/M phase control cells.

lial cells (Figures 7, 8 and 9). Cyclin D1 and D3 proteins were found to be upregulated in both the breast cancer cells compared to normal cells and it was found that the polysaccharide caused a decrease in the expression of these proteins. The expression level of p18 INK4c was found to be decreased in breast cancer cells compared to MCF-10A cells. The compound treatment caused significant increase in the expression of proteins in the cancer

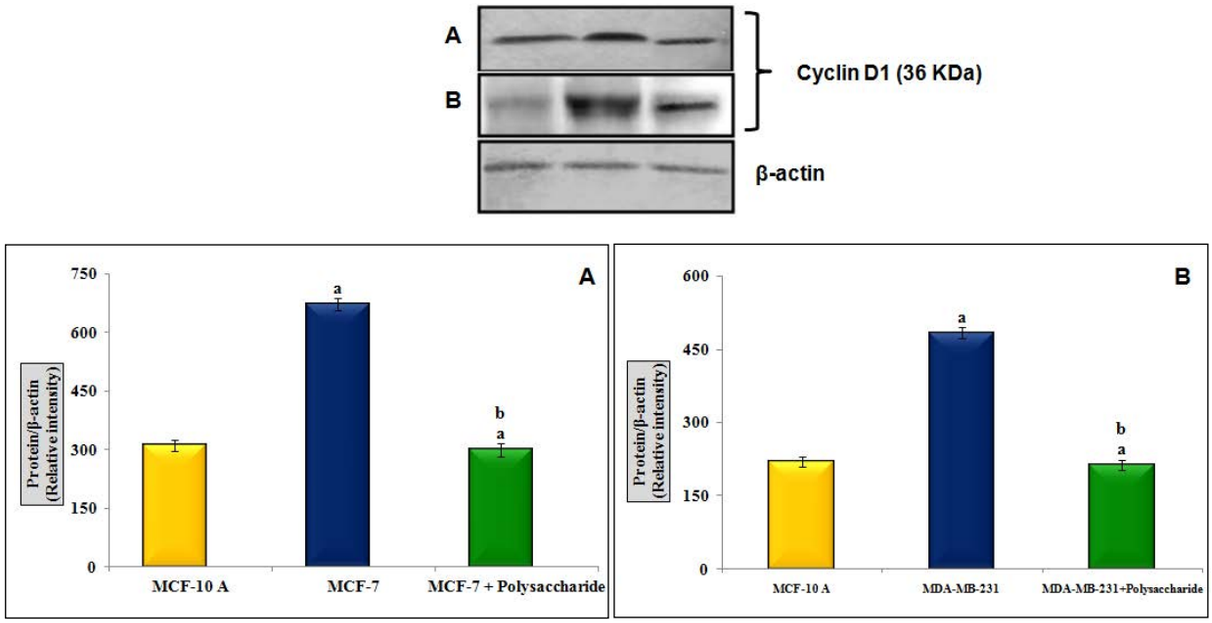

Figure 7. Effect of the polysaccharide on cell cycle regulator Cyclin D1 on (A) MCF-7, and (B) MDA-MB-231 cells. Each bar represents mean ± SEM of three independent observations, with statistical significance between control and the treated groups at $p < 0.05$ – 'a' compared with MCF-10A normal control; 'b'-compared with respective cancer control.

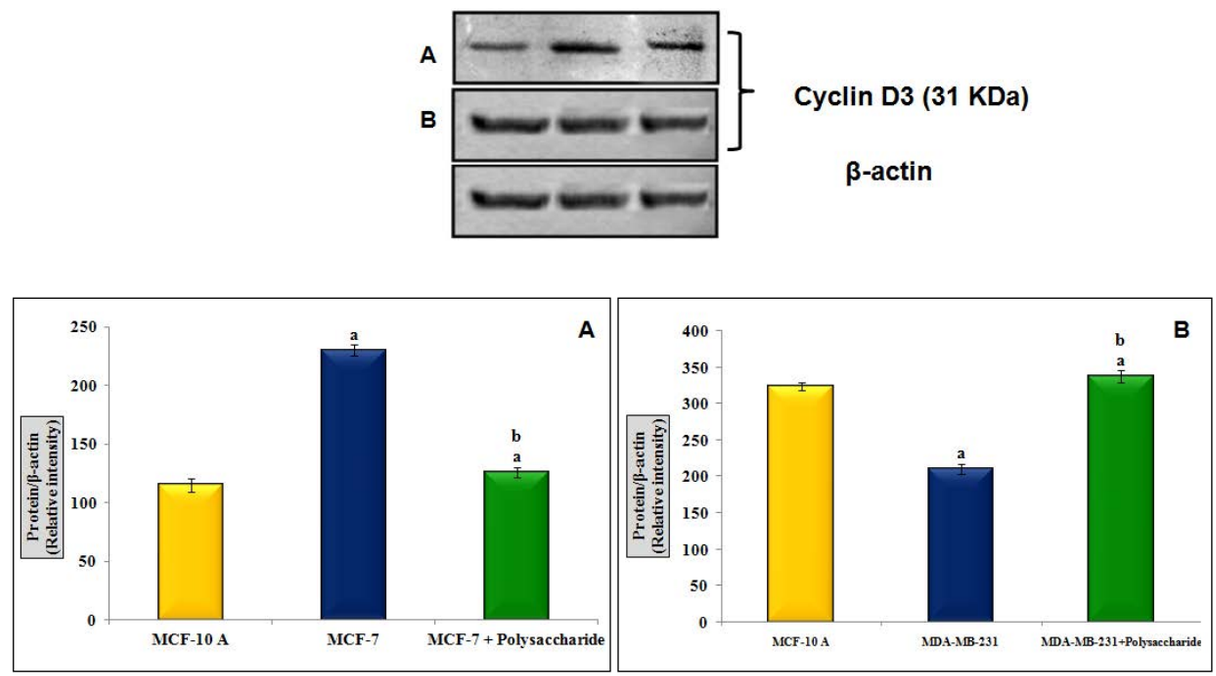

Figure 8. Effect of the polysaccharide on cell cycle regulator Cyclin D3 on (A) MCF-7 and (B) MDA-MB-231 cells. Each bar represents mean ± SEM of three independent observations, with statistical significance between control and the treated groups at $p < 0.05$ – 'a' compared with MCF-10A normal control, 'b'-compared with respective cancer control.

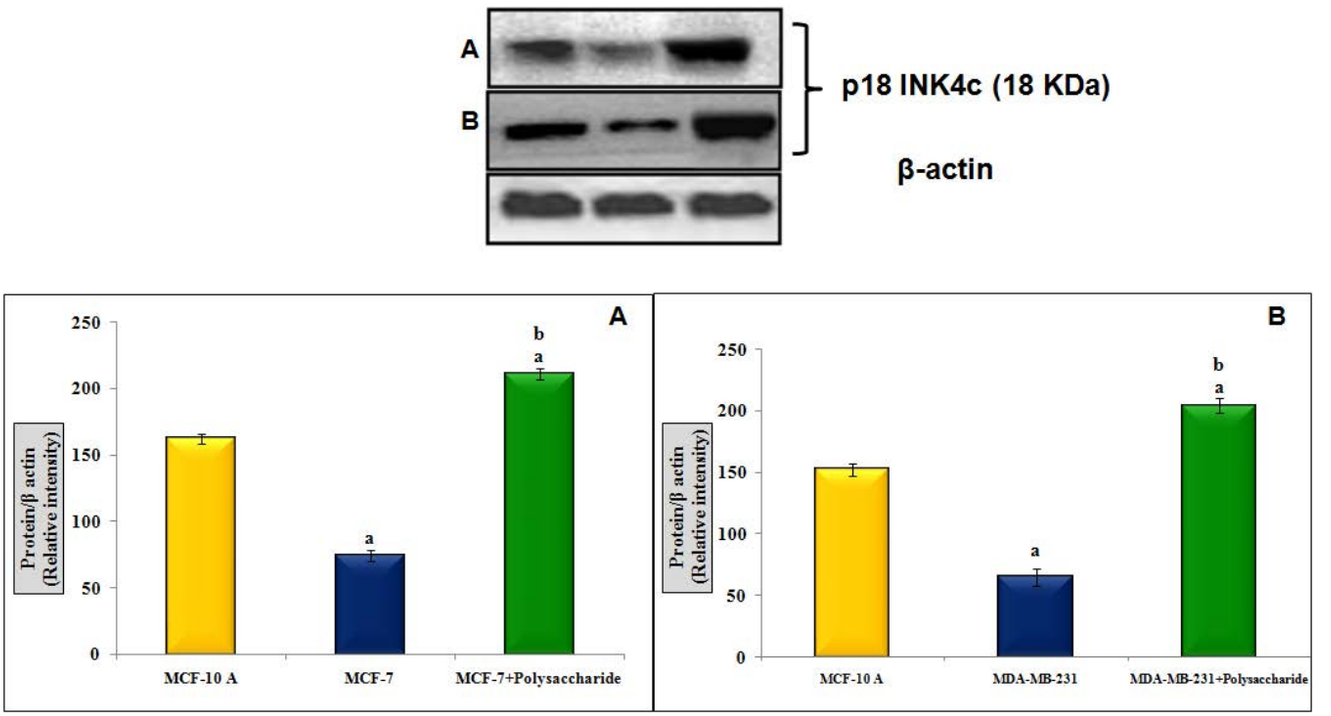

Figure 9. Effect of the polysaccharide on cell cycle regulator p18 INK4c on (A) MCF-7 and (B) MDA-MB-231 cell. Each bar represents mean ± SEM of three independent observations, with statistical significance between control and the treated groups at $p < 0.05$ – 'a' compared with MCF-10A normal control, 'b'-compared with respective cancer control.

cells. The compound-mediated alteration in the expression of proteins was found to be similar to the protein expression levels in normal breast epithelial cells.

4. Discussion

In general, there is a great emphasis towards research on complementary and alternative medicines for cancer management[23]. Biologically active molecules from plants are found to be non-toxic and are used as alternative cancer therapeutic agents[24]. In the present study, a novel polysaccharide was isolated from the methanolic extract of *T. cordifolia* stem and its anticancer efficacy against a human breast cancer cell line (MCF-7 & MDA-MB-231) was explored. Screening and identification of lead molecules via computational tools lessen the burden of laboratory experiments to a great extent. ADME/T property prediction of a molecule is one of the most critical stages in drug discovery, and the molecule can survive in Phase I clinical trial only if it satisfies these properties[25]. Computational prediction of ADME/T studies involves the analysis of properties of a compound related to its absorption, distribution, metabolism, excretion and toxicity levels, which includes HIA, aqueous solubility, BBB, CYP2D6 inhibition, hepatotoxicity properties and protein binding levels of a drug. In the present study, ADME/T properties of the novel polysaccharide were predicted and it is evident that the novel polysaccharide possesses appropriate ADME/T properties with satisfactory levels of absorption and no BBB penetration and hepatotoxicity.

Towards predicting the properties of the novel compound as a drug molecule adopting *in-silico* tools, the compound was experimentally explored for its anticancer potential to induce apoptosis. Apoptosis is referred to as programmed cell death which involves loss of plasma membrane integrity, fragmentation of DNA and formation of membrane-bound apoptotic bodies[26]. Analysis of induction of apoptosis in cancer cells is a valuable tool for development of a cancer therapy, and the agents that are capable of inducing apoptosis could be potentially developed as cancer chemotherapeutic drugs. In the present study, the novel polysaccharide was found to inhibit the growth and proliferation of breast cancer cells with decrease in cell population. Cytotoxicity is marked by LDH leakage, which depends on loss of membrane integrity[27]. The novel compound mediated a significant level of LDH leakage in MCF-7 cells, indicating its cytotoxic efficacy. Induction of cell death by the novel compound was studied using AO/EtBr dual staining. In accordance with the staining principle of Spector et al.[28], the present study revealed

uniformly green fluorescing viable untreated cancer cells and the cells treated with the novel compound were found to be red-fluorescing, indicating the loss of membrane integrity with induction of apoptosis upon compound treatment[29]. Apoptosis is correlated with induction of cleavage of DNA at the internucleosomal linker sites to produce DNA fragments, which occurs through the action of endogenous endonucleases[30]. Fragmentation of DNA by the novel compound indicates the targeted action of the compound to induce apoptosis in breast cancer cells.

Cell cycle regulation is another key phenomenon involved in inducing apoptosis, where deregulation of normal cell cycle is observed in cancer cells. Induction of cell cycle arrest in such abnormal cells allows the cells to repair their defect and thereby prevent transmission to daughter cells[31]. Several anti-cancer drugs are known to block cell cycle at G2/M phase and induce apoptosis[32]. In the present study, treatment with the novel compound caused accumulation of cells in G2/M phase and thereby arrested the cell cycle. This may be attributed to alteration in activation and expression of cell cycle regulatory proteins that contribute to change in cell cycle progression. In cancer conditions the positive cell cycle regulators are found to be upregulated and the negative regulators are downregulated. Expression of cyclin D1 and D3 proteins was elevated in several cancers, where the condition enhanced tumor progression and metastasis[33, 34]. It was also reported that apoptosis is induced when there is an upregulation of p18 INK4c and inhibition of binding of CDK-4 with cyclin D and thereby inhibition of cell cycle progression[35]. Hence, the reduction in the expression of cyclin D1 and D3 proteins and the upregulation of p18 INK4c protein induced by the polysaccharide in MCF-7 and MDA-MB-231 cells in the present study show that change in the expression of these proteins inhibits cell cycle progression in breast cancer cells. Thus, the present study shows the action of polysaccharide isolated from *T. cordifolia* on cell cycle regulations to induce apoptosis in breast cancer cells.

Thus, from the present study it is evident that the novel polysaccharidic compound isolated from *T. cordifolia* stem is an effective and active principle against breast cancer under *in-vitro* condition. The compound was found to be potential at 100 µM concentration, with considerable cell death due to leakage of LDH. Fluorescent staining studies also reiterated the efficacy of the compound to induce breast cancer cell death. Fragmentation induced by the compound in the DNA of these cells and the cell cycle arrest at G2/M phase explain the targeted action of the novel compound. The mechanism of action of the compound in inducing apoptosis via its action to alter the regulation of cell cycle regulators indicates the mode of action of the compound. The compound was also found to satisfy the ADME/T proper of a drug. Thus, the study reveals the anti-cancer potential of the novel compound and it can be further explored for use in for breast cancer therapy.

5. Acknowledgment

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6. References

1. Murthy NS, Agarwal UK, Chaudhry K, Saxena S. A study on time trends in incidence of breast cancer – Indian scenario. European Journal of Cancer Care. 2007. 16:185–6. https://doi.org/10.1111/j.1365-2354.2006.00761.x PMid:17371429
2. Hortobagyi GN, De la Garza Salazar J, et al. The global breast cancer burden: variations in epidemiology and survival. Clinical Breast Cancer. 2005. 6: 391–401. https://doi.org/10.3816/CBC.2005.n.043 PMid:16381622
3. Sherr CJ. Mammalian G1 cyclins. Cell. 1993. 73:1059–65. https://doi.org/10.1016/0092-8674(93)90636-5
4. Hirai H, Roussel MF, Kato JY, Ashmun RA, Sherr CJ. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin d-dependent kinases CDK4 and CDK6. Molecular and Cellular Biology. 1995. 15: 2672–81. https://doi.org/10.1128/MCB.15.5.2672 PMid:7739547 PMCid:PMC230497
5. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes and Development. 1999. 13:1501–12. https://doi.org/10.1101/gad.13.12.1501 PMid:10385618
6. El-Diery WS, Harper JW, O'Connor PM, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Research. 1994. 54: 1169–74.
7. Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell. 1994. 78: 67–74. https://doi.org/10.1016/0092-8674(94)90573-8
8. Sa G, Stacey DW. p27 expression is regulated by separate signalling pathways, downstream of Ras, in each cell cycle phase. Experimental Cell Research. 2004. 300: 427–39. https://doi.org/10.1016/j.yexcr.2004.07.032 PMid:15475007
9. Weinert T, Lydall D. Cell cycle checkpoints, genetic instability and cancer. Seminars in Cancer Biology. 1993. 4: 129–40. PMid:8513148
10. Rao EV, Rao MV. Studies on the polysaccharide preparation (Guduchi Satwa) derived from Tinospora cordifolia. Indian Journal of Pharmaceutical Sciences. 1981. 43:103–6

11. Singh N, Singh SM, Shrivastava P. Effect of Tinospora cordifolia on the antitumor activity of tumor-associated macrophages-derived dendritic cells. Immunopharmacology and Immunotoxicology. 2005. 27:1–14. https://doi.org/10.1081/IPH-51287 https://doi.org/10.1081/IPH-200051287 PMid:15803856
12. Sangeetha MK, Raghavendran Balaji HR, Gayathri V, Vasanthi HR. Tinospora cordifolia attenuates oxidative stress and distorted carbohydrate metabolism in experimentally induced type 2 diabetes in rats. Journal of Natural Medicines. 2011. 65:544–50 https://doi.org/10.1007/s11418-011-0538-6 PMid:21538233
13. Ali H, Dixit S. Extraction optimization of Tinospora cordifolia and assessment of the anticancer activity of its alkaloid palmatine. Scientific World Journal. 2013. 376216. doi:10.1155/2013/376216 https://doi.org/10.1155/2013/376216
14. Leyon PV, Kuttan G. Inhibitory effect of a polysaccharide from Tinospora cordifolia on experimental metastasis. Journal of Ethnopharmacology. 2004. 90:233–7 https://doi.org/10.1016/j.jep.2003.09.046 PMid:15013186
15. Chaudhary R, Jahan S, Goyal PK. Chemopreventive potential of an Indian medicinal plant (Tinospora cordifolia) on skin carcinogenesis in mice. Journal of Environmental Pathology, Toxicology and Oncology. 2008. 27:233–43 https://doi.org/10.1615/JEnvironPatholToxicolOncol.v27.i3.70 PMid:18652570
16. Dhanasekaran M, Baskar AA, Ignacimuthu S, Agastian P, Duraipandiyan V. Chemopreventive potential of epoxy clerodane diterpene from Tinospora cordifolia against diethylnitrosamine-induced hepatocellular carcinoma. Investigational New Drugs. 2009. 27:347–55. https://doi.org/10.1007/s10637-008-9181-9 PMid:18853103
17. Thippeswamy G, Salimath BP. Induction of caspase-3 activated DNase mediated apoptosis by hexane fraction of Tinospora cordifolia in EAT cells. Environmental Toxicology and Pharmacology. 2007. 23:212–20. https://doi.org/10.1016/j.etap.2006.10.004 PMid:21783760
18. Maliyakkal N, Udupa N, Pai KSR, Rangarajan A. Cytotoxic and apoptotic activities of extracts of Withania somnifera and Tinospora cordifolia in human breast cancer cells. International Journal of Applied Research in Natural Products. 2013. 6:1–10.
19. Sharma P, Parmar J, Sharma P, Verma P, Goyal PK. Radiation-induced testicular injury and its amelioration by Tinospora cordifolia (An Indian medicinal plant) extract. Evidence Based Complementary and Alternative Medicine. 2011. Article ID 643847.
20. Rajalakshmi M, Eliza J, Priya CE, Nirmala A, Daisy P. Antidiabetic properties of Tinospora cordifolia stem extracts on streptozotocin- induced diabetic rats. African Journal of Pharmacy and Pharmacology. 2009. 3: 171–80
21. Manikkam R, Roy A. β-cell regenerative efficacy of a polysaccharide isolated from methanolic extract of Tinospora cordifolia stem on streptozotocin-induced diabetic Wistar rats. Chemico Biological Interactions. 2016. 243: 45–53 https://doi.org/10.1016/j.cbi.2015.11.021 PMid:26616445
22. Lowry OH, Rosebrough NJ, Farr AL, Randall R (1951). Protein Measurement with the Folin Phenol reagent. Journal of Biological Chemistry, 193: 265–275. PMid:14907713
23. Sawadogo WR, Schumacher M, Teiten MH, Dicato M, Diederich M. Traditional pharmacopoeia, plants and derived compounds for cancer therapy. Biochemical Pharmacology. 2012. 84:1225–40. https://doi.org/10.1016/j.bcp.2012.07.021 PMid:22846603
24. Duffy R, Wade C, Chang R. Discovery of anticancer drugs from antimalarial natural products: a MEDLINE literature review. Drug Discovery Today. 2012. 17:942–53 https://doi.org/10.1016/j.drudis.2012.03.013 PMid:22504324
25. Johnson DE, Wolfgang GH. Predicting human safety: screening and computational approaches. Drug Discovery Today. 2000. 5:445–54 https://doi.org/10.1016/S1359-6446(00)01559-2
26. Cotter TG. Apoptosis and cancer: the genesis of a research field. Nature Review: Cancer. 2009. 9:501–7 https://doi.org/10.1038/nrc2663 PMid:19550425
27. Molders P, Hogberg J, Orrenirs S. Methods in Enzymology. Vol. 52, Eds. Fleischer S., Packer L., Academic Press, New York, pp. 60–71. 1978.
28. Spector DL, Goldman RD, Leinwand LA. Cell: a Laboratory Manual, Vol. 1, Chap. 15, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 1–24. 1998.
29. Compton MM. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer Metastasis Review. 1992. 11:105–19 https://doi.org/10.1007/BF00048058 PMid:1327565
30. Williams GH, Stoeber K. The cell cycle and cancer. Journal of Pathology. 2012. 226: 352–64 https://doi.org/10.1002/path.3022 PMid:21990031
31. Stewart ZA, Westfall MD, Pietenpol JA. Cell-cycle dysregulation and anticancer therapy. Trends in Pharmacological Sciences. 2003. 24:139–45. https://doi.org/10.1016/S0165-6147(03)00026-9
32. Russell A, Thompson MA, Hendley J, Trute L, Armes J, Germain D. Cyclin D1 and D3 associate with the SCF complex and are coordinately elevated in breast cancer. Oncogene. 1999. 18: 1983–91. https://doi.org/10.1038/sj.onc.1202511 PMid:10208420
33. Wang C, Li Z, Fu M, Bouras T, Pestell RG. Signal transduction mediated by cyclin D1: from mitogens to cell proliferation: a molecular target with therapeutic potential. Cancer Treatment and Research. 2004. 119: 217–237. https://doi.org/10.1007/1-4020-7847-1_11 PMid:15164880
34. Franklin DS, Godfrey VL, Lee H, Kovalev GI, et al. CDK inhibitors p18INK4c and p27Kip1 mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. Genes and Development. 1998. 12: 2899–911. https://doi.org/10.1101/gad.12.18.2899 PMid:9744866 PMCid:PMC317173