RESEARCH ARTICLE

D-Pinitol Promotes Apoptosis in MCF-7 Cells via Induction of p53 and Bax and Inhibition of Bcl-2 and NF-ϰB

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Abstract

Development of drugs from natural products has been undergoing a gradual evoluation. Many plant derived compounds have excellent therapeutic potential against various human ailments. They are important sources especially for anticancer agents. A number of promising new agents are in clinical development based on their selective molecular targets in the field of oncology. D-pinitol is a naturally occurring compound derived from soy which has significant pharmacological activitites. Therefore we selected D-pinitol in order to evaluate apoptotic potential in the MCF-7 cell line. Human breast cancer cells were treated with different concentrations of D-pinitol and cytotoxicity was measured by MTT and LDH assays. The mechanism of apoptosis was studied with reference to expression of p53, Bcl-2, Bax and NF-kB proteins. The results revealed that D-pinitol significantly inhibited the proliferation of MCF-7 cells in a concentration-dependent manner, while upregulating the expression of p53, Bax and down regulating Bcl-2 and NF-kB. Thus the results obtained in this study clearly vindicated that D-pinitol induces apotosis in MCF-7 cells through regulation of proteins of pro- and anti-apoptotic cascades.

Keywords: Breast cancer - D-pinitol - MCF-7 - MTT assay - apoptosis

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Introduction

Breast cancer is the most commonly diagnosed cancer in women in the US and is one of the leading causes of mortality. Epidemiological studies have consistently suggested that the inverse association between cancer risk and intake of fruits and vegetables. These health benefits have been linked to the additive and synergistic combination of phytochemicals in fruits and vegetables. The National Cancer Institute has highlighted a number of foods for which there is evidence from epidemiology or experimental studies of an association with a reduced risk of cancer, amongst which soyabeans are most important (Zuo et al., 2008).

A number of studies have reported that consumption of soy and their products are associated with some degree of protection against either induced or spontaneous cancers in animals as well as reduced cancer risk in several human epidemiological studies (Andres et al., 2011). Soy diet has been associated with various beneficial effects in human beings. It contains various biologically active components and has received much attention for their potential beneficial effects in cancer prevention, osteoporosis, diabetes and obesity, menopausal symptom relief and reducing risks in cardiovascular disease, as well as concerns of toxicity due to their potential to act as endocrine disruptors (Adlercreutz et al., 2002).

D-pinitol is a compound of the soybean and the methyl ether of D-chiro-inositol found as large quantities in soy foods and it occurs in about 1% of dry weight of soybean meal (Phillips et al., 1982). It is an active low-molecular cyclitol isolated from the seed coat, cotyledon and embryo axis of soybean seeds (Kuo et al., 2009). Moreover, as a natural therapeutic agents it has gained much attention due to their diverse biological activities such as inhibition of the T-helpercell-1 response, antiviral, larvicidal, anti-inflammatory, antihyperlipidemic, cardioprotective, inhibition of ovalbumin-induced airway inflammation and antioxidant (Sethi et al., 2008; Sivakumar et al., 2010).

All of these functions are connected with the ability of pinitol to attenuate or suppress oxidative stress and the inflammatory process both in *in vitro* and *in vivo*. NF- ϰB may play an important role in the etiology of breast cancer. In this context, Sato et al. (2011) are of the opinion that NF-ϰB has recently become a major target for cancer drug development, as shown by the many non-specific natural and synthetic compounds shown to exert their therapeutic effects at least in part through the inhibition

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of NF-κB. Therefore considering the available data in the literature, we have designed the present study to evaluate the apoptosis promoting activity of D-pinitol in the MCF-7 cell line and assess underlying mechanisms.

Materials and Methods

Chemicals and antibodies

Acrylamide, bis-acrylamide, ammonium per sulfate and TEMED were purchased from Bio Rad Chemicals, USA. Dulbecco's Modified Eagles medium (DMEM) sodium bicarbonate, trypsin-EDTA were purchased from Biochrome, Germany, antibiotic/antimycotic solution and Fetal Bovine Serum (FBS) were purchased from GIBCO, Invitrogen, USA. All other chemicals including solvents were of high purity and of analytical grade marketed by Glaxo Laboratories, Mumbai and Sisco Research Laboratories Pvt, Ltd, Mumbai, India. All antibodies used in this study were purchased from Cell Signaling Technologies, USA.

Maintenance of michigan cancer foundation-7 (MCF-7) cell lines

Human mammary carcinoma cell lines MCF-7 was obtained from NCCS, Pune, India. These cells were grown as mono layers in Dulbecco's modified eagle medium (DMEM) supplemented with 10% v/v heat inactivated fetal bovine serum (FBS), antibiotics, such as penicillin 50 U/mL, streptomycin 50μg/mL and 1 mmol/L sodium pyruvate under standard conditions containing in a humidified incubator with 5% CO_2 at 37ºC. The medium was changed for every three days.

Viability assay

Cell viability was assessed as per the standard protocol by MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) method of Mossmann (1983). The cell viability is calculated using the formula: ***%growth inhibition=(A570nm of treated cells/A570nm of control cells)×100.***

Lactate dehydrogenase (LDH) leakage assay

Lactate dehydrogenase (LDH) leakage assay was performed by the method of Grivell and Berry, (1996). From the growth medium of experimental cultures, 100μl of sample were added to 1ml cuvette containing 0.9ml of a reaction mixture to yield a final concentration of 1mM/L pyruvate, 0.15mM/L NADH and 104mM/L phosphate buffer, (pH 7.4). The incubation mixture was mixed thoroughly and the absorbance was measured at 340nm for 45 seconds. LDH activity was expressed as micromoles of NADH liberated/minute/well.

Estimation of glutathione (GSH)

Total reduced glutathione (GSH) was determined by the method of Moron et al. (1979). Five percent of TCA was added to MCF-7 cells (1×10^6 cells). The precipitate was removed by centrifugation at 3000 rpm. To an aliquot of the supernatant, 2ml of DTNB reagent was added to make final volume of 3 ml. The absorbance was read at 412nm against a blank containing TCA instead of sample. Aliquots of standard solution were treated similarly. The amount of GSH was expressed as n moles/10^6 cells.

DNA fragmentation analysis

DNA fragmentation was performed followed by the method of Chen et al. (1997). After 24-hour treatment 10×10^5 cells with 750μl 1× suspension buffer, mixed well and taken in 2 ml microcentrifuge. Five microliters RNase solutions were added (10 mg/ml) and mixed 5-6 times by inverting the vial and incubated at 65ºC for 10 minutes with intermittent mixing. After incubation, 1 ml lysis buffer was added and mixed 5-6 times and then incubated at 65ºC for 15 minutes and then the lysate was cooled at room temperature (RT). After incubation, the lysate was centrifuged at 13,000× g for 1 minute at RT. To the supernatant in a 2 ml microcentrifuge tube, equal volume of isopropanol was added to each vial and mixed well centrifuged at 13,000× g for 15 minutes at RT and discarded the supernatant, To the pellet, 1 ml of 70% ethanol was added and centrifuged at 13,000× g for 15 minutes at RT and supernatant was discarded. Again to the pellet, 1 ml of 70% ethanol was added and centrifuged at 13,000× g for 15 minutes at RT and the supernatant was discarded. The pellet was dried at 37ºC for 10 minutes, 50μl of autoclaved milliQ water was added to each vial and the DNA was suspended by placing the vial at 65ºC for 15 minutes or at 4ºC over night. The DNA was quantified by UV-vis spectroscopy and 10μg of DNA was electrophoresed in a 1.2% agarose gel containing ethidium bromide in a mini gel tank containing Tris-Boric acid-EDTA buffer for 2 hours under 90V. Then the gel was visualized under UV transilluminator.

Western blot analysis

After the 24-h treatment period the cells were lysed in RIPA buffer containing 1× protease inhibitor cocktail and the concentrations of protein were determined by the method of Lowry (1951). Cell lysates (20-50μg) were electrophoresed in 12% SDS polyacrylamide gel and then transferred into PVDF membranes. The membranes were incubated with primary antibodies against Bax, p53, Bcl-2, NF-κB (1: 2000). After washing, the membranes were incubated with HRP conjugated antimouse IgG (1: 5000) or HRP conjugated goat-antirabbit IgG (1: 5000). Protein bands were detected using chemiluminescence system (ECL kit) and quantified in Chemi Doc XRS Imaging System, Bio-Rad (USA).

Reverse transcription-polymerase chain reaction

The total RNA was isolated from frozen tissues using TRI (TRIZOL) reagent according to the method of Chomczynski and Sacchi (1987). The total RNA obtained was free from protein and DNA contamination. The diluted RNA sample was quantified spectrophotometrically by measuring the absorbance at 260nm. The reverse transcription (RT) and polymerase chain reaction (PCR) kits were purchased from Invitrogen and KAPATaq ReadyMix DNA Polymerase respectively. The cDNA products were amplified for Bcl-2, Bax, NF-kB gene expression via PCR with Bcl-2,Bax,NF-kB specific primers as mentioned below, GAPDH as internal standard, GAPDH (Forward

primer: TTTCTTTGCAGCAATGCCTCC; Reverse primer: CCATGAGTCCTTCCACGATACC),Bcl-2 (Forwardprimer: GGGAGAACGGGGTACGATA; Reverse primer: CATCTCCCGCATCCCACTC),Bax (Forward primer: TTCATCCAGGATCGAGCAGG; Reverse primer: TGAGACACTCGCTCAGCTTC), NF-ϰB (Forward primer: CCCCGCAGACTATCAATCCC; Reverse primer: ACTTACTGCCCCCTTCCAGA) for 30 cycles (30s at 95ºC, 2 min at 60ºC, 1 min at 72ºC) using a GeneAmp PCR System. The amplified PCR products were analyzed by electrophoresis in 2% agarose gels and visualized under UV illumination after ethidium bromide staining.

Statistical analysis

Values are expressed as mean±SD. The results were statistically evaluated using independent

Results and Discussion

The antiproliferative effect of D-pinitol on MCF-7 cells were performed by the MTT method, which is reliable to detect proliferation of cells. The results of the MTT assay are presented in Figure 1. The results clearly confirmed that the exposure of D-pinitol at different concentrations such as 20, 40, 60, 80, 100 and 120μM for 24hr resulted in decrease of cell proliferation in a dose dependent manner. It exhibits 47.3% reduced cell viability at 40μM, and the viability was decreased more than 52% (cell proliferation) at 60μM. MTT assay is a non-radioactive, fast and economical assay widely used to quantify cell viability and proliferation. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. The reduction of MTT is proportional to the number of active mitochondria in the live cells (Prasanna et al., 2009). From the present study the % of inhibition was reduced in D-pinitol treated cells. D-pinitol inhibits above 50% of the cell population at the concentration 60µM for 24 h in and it clearly indicated that D-pinitol strongly inhibits the MCF-7 cells in culture and exhibits its anti-proliferative and cytotoxic nature.

The levels of lactate dehydrogenase (LDH) released into the medium of control and D-pinitol treated at the concentration of 40 and 60µM (MCF-7) cells are presented in Figure 2. From the results it was observed that LDH activity was found to be significantly elevated after 24 hr of exposure in the medium containing D-pinitol when

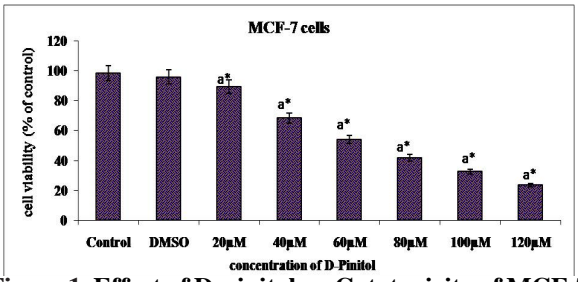

Figure 1. Effect of D-pinitol on Cytotoxicity of MCF-7 Cells after 24hr Exposure

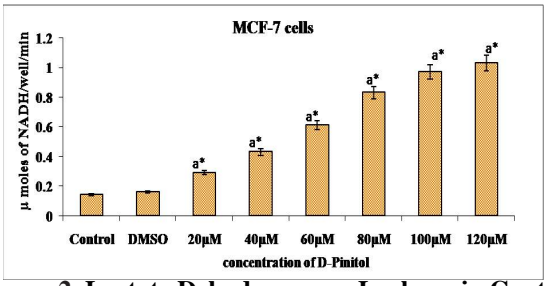

Figure 2. Lactate Dehydrogenase Leakage in Control and D-pinitol Treated MCF-7 Cells after 24hr Exposure

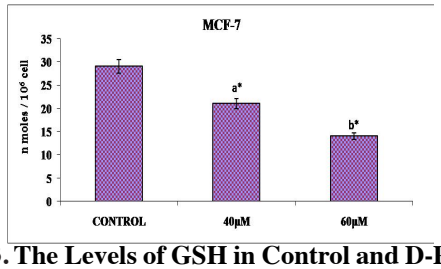

Figure 3. The Levels of GSH in Control and D-Pinitol Treated MCF-7 Cells. Values are expressed as Mean±SD; N=6, *p<0.05 vs Control

compared to the control. LDH leakage is considered as a marker of cytoxicity. LDH leakage assay monitors the integrity of the plasma membrane and is sensitive and easy to perform (Sivalokanathan et al., 2006). Leakage of LDH correlates well with available data and has been shown to be quantifiably more reliable than dye exclusion for measuring cellular damage or impairment in cytotoxicological studies (Kumaravel et al., 2012). LDH is known to be released from cells due to damage in cell membrane, which indicated cell death either due to necrosis or apoptosis. The LDH levels increased significantly in D-pinitol treated MCF-7 cells when compared to control cells. The LDH leakage in MCF-7 cells may be due to the cytotoxic nature of the D-pinitol. The levels of GSH content in control and D-pinitol treated MCF-7 cells are presented in Figure 3.

From the present study it is inferred that a significant ($p<0.05$) depletion of GSH content was observed in D-pinitol treated at the concentration of 40 and 60μM (MCF-7) when compared to the control cells. Reduced glutathione (GSH) most abundant cellular thiol and the major determinant of cellular redox equilibrium have been shown to be an important factor in apoptosis (Ojeswi et al., 2010). Glutathione contributes to an extraordinary range of metabolic process including the maintainace of intracellular redox status and plays an important role in detoxification of intracellular H_2O_2, lipid peroxides and also in the metabolism of exogenously derived compounds, mutagens and carcinogens. Depletion of intracellular glutathione renders the cancer cells more sensitive to apoptotic agents. Nandakumar et al. (2011) are of the opinion that inhibition of glutathione synthesis or modulation of glutathione storages in tumors to reduce anticancer drugs resistance may comprise a novel anticancer strategy. The GSH levels were significantly lower when compared to control cells. Cellular GSH plays an important role in protection against oxidative stress

induced injury. Depletion of GSH levels has been shown to enhance susceptibility to oxidative stress-induced cytotoxicity. This result was consistent with inositol derivatives (Norhaizan et al., 2011).

The depletion of endogenous GSH sensitized the cells to D-pinitol treatment and thereby increased the apoptotic cells, thus suggesting inactivation by the formation of GSH-Pinitol conjugate. The DNA fragmentation by agarose gel electrophoresis pattern of DNA of normal and D-pinitol treated MCF-7 cells are presented in Figure 5. In the control, there was no fluorescence signal observed in agarose gel. However, the signal appeared in D-pinitol treated at the concentration of 40, 60μM showed the fragmented laddering pattern of DNA and increased fragmentation of D-pinitol treated cells were also observed. This could be due to the chromatin condensation and nuclear fragmentation. This observation strongly supports that the cytotoxicity of D-pinitol might have occurred via apoptosis.

The protein and gene expression of p53, Bcl-2, Bax and NF-kB in control and D-pinitol treated MCF-7 cells are presented in Figure 6 and Figure 7. In order to determine the pathway by which D-pinitol induces apoptosis in MCF-7 cells, the western blot and RT-PCR analysis were performed. D-pinitol promotes the expression of p53 and inhibits the expression of Bcl-2. The expression of p53 was increased in a dose dependent manner, whereas the expression of Bcl-2 was decreased in D-pinitol treated MCF-7 cells. Administration of D-pinitol increase the band intensity of Bax mRNA compared to control. The accumulation of p53 protein indicates the expression of tumor suppressor protein which is involved in the apoptosis of D-pinitol treated MCF-7 cells.

p53 is critical regulator in many cellular processes including cell signal transduction, cellular response to DNA damage, genomic stability, cell cycle control and apoptosis (Feng et al., 2010). It can function as a master regulator of the apoptotic program, capable of coordinating the process at multiple levels via several mechanisms. It is the most frequently mutated gene in human tumors and loss of p53 function can both disable apoptosis and accelerate tumor development in transgenic mice (Martin Brown and Laura, 2005). Further, the frequent mutations in p53, which appears to play a critical role in response to DNA damage indicates that apoptosis is dependent on p53 and has been associated with DNA damage (Xianglin et al., 2009). In this connection, Xuea et al. (2005) have reported that an increase in cell cycle arrest is an activation of p53 protein which induced the apoptotic pathway. Therefore, D-pinitol may possibly enhance the MCF-7 cells to undergo apoptosis. Hence, it seems that apoptotic modulators may be promising agents for the treatment of breast cancer. In the present investigation, expression of p53 is up regulated at the concentration of 60μM after 24hr incubation.

Bcl-2 family members can be functionally divided into pro-apoptotic factors (eg. Bax) and anti-apoptotic factors (eg. Bcl-2). Bcl-2 family members are capable of forming homo and heterodimers with one another suggesting that the overall ratio of pro to anti-apoptoti regulators may determine whether a cell is condemned to die of apoptosis or not (Li et al., 2013). Extensive research into the molecular mechanisms of the apoptotic stimulus has uncovered the Bcl-2 protein family that is thought to function as apoptosis regulators (Ma et al., 2009). Many anticancer drugs trigger mitochondria mediated apoptosis in cancer cells through downregulation of Bcl-2 and up regulation of Bax. Moreover the subcellular location of Bax protein has been found to be important in the apoptotic process (Guo-Ping et al., 2003).

The expression of Bax was increased in a dose dependent manner in D-pinitol treated MCF-7 cells. Bax is generally sequestered in the cytosol into mitochondria with large aggregates during apoptosis (Kumar et al., 2012). Bax translocation into mitochondria targets the mitochondrial intermembrane contact sites and release cytochrome C. Bax is then packaged into large aggregates

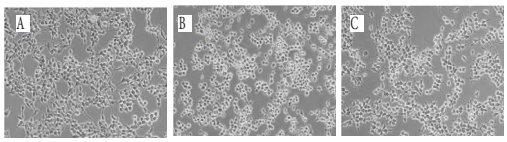

Figure 4. A Light Microscopic Observation of Control and D-pinitol Treated MCF-7 Cells. A) The Control Cell Shows a higher Confluency of Monolayer and Normal Cells ; the Structural Alterations and Reduction of MCF-7, Cell Populations Were Observed in both 40µm **B)** And 60µm **C)** Concentrations

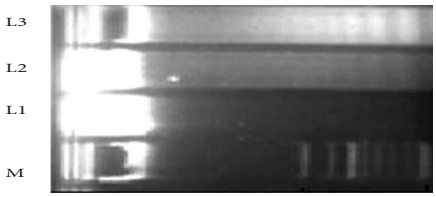

Figure 5. DNA fragmentation Analysis of Control and D-Pinitol Treated MCF-7 Cells. M-DNA Marker (100bp DNA ladder); Lane 1 - Control; Lane 2 - D-pinitol (40 µM); Lane 3 - D-pinitol (60µM)

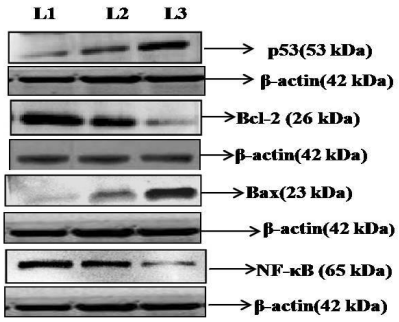

Figure 6. Expressions of p53, Bcl-2, Bax, NF-κB Protein in Control and D-pinitol Treated MCF-7 Cells. Lane 1- control; Lane 2- 40µm of D-pinitol; Lane 3- 60µm of D-pinitol

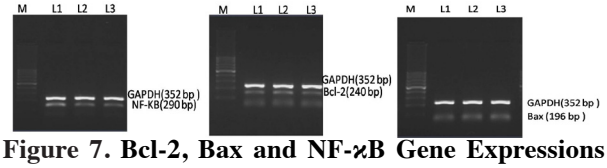

Figure 7. Bcl-2, Bax and NF-κB Gene Expressions in Control and D-pinitol Treated MCF-7 Cells. Lane 1- Marker (1000bp); Lane 2- Group I control; Lane 3- Group II 40µM of D-pinitol; Lane4- Group III 60µM of D-pinitol

on mitochondria. When Bax is in excess, cells execute a death command but when Bcl-2 dominates, the program is inhibited and the cells survive (Dai et al., 2013). Ratio of anti-apoptotic Bcl-2 and pro-apoptotic Bax determines the setpoint of cells to triggering apoptosis. Significantly this setpoint can modulate cells response to chemotherapy (Ping-Chi et al., 2004). Bax can heterodimerize with Bcl-2, this heterodimerization nullifies the channel forming activity thus, promoting cell survival. As Bax can activate Caspase and induce mitochondria1 dysfunction, the expression of Bcl-2 blocks both events (Tathagata et al., 2002). Elumalai et al. (2012) reported that the up regulation of Bax and down-regulation of Bcl-2 in breast cancer cells. In the present study, the expression of Bcl-2 was found to be decreased and incontrast the Bax expression was increased which in turn could be a sign of induction of apoptosis by the D-pinitol in MCF-7 cells. An increased ratio of Bax: Bcl-2 is expected to be associated with apoptosis.

The expression of NF-κB was down regulated in D-pinitol treated MCF-7 cells. There is evidence to support the role of NF-κB in the protection against programmed cell death. Inhibition of NF-κB /Rel proteins induces apoptosis of murine B cells, whereas over expression of c-Rel protein makes cells resistant to apoptosis (Papanikolaou et al., 2011). Several studies have shown the absence or inhibition of NF-κB subunits in cultured cells resulting in potentiation of apoptosis in response to TNF-α, ionizing radiation and anticancer agents (Williams et al., 2008). Concurrently, increasing evidence indicates that resistance to apoptosis mediates resistance to anticancer therapies. Moon et al. (2011) demonstrated that anticancer agents may be less effective in cell killing because of their activation of NF-κB. Therefore, anticancer agents that inhibit NF-κB activity may be beneficial in the prevention or treatment of cancer. NF-κB suppress apoptosis by inducing expression of a number of genes and their products inhibit apoptosis, including inhibitors of apoptosis (IAPs), caspase 8 and TNF receptor associated factor 1 (Veronique et al., 2009).

These anti-apoptotic proteins have been found to work in a coordinated fashion to block apoptosis at multiple steps along the apoptotic cascade or to regulate other pro or anti-apoptotic pathways (Liu-Qin et al., 2004). NF-κB could also regulate the expression of several members of the Bcl-2 family (Wang et al., 1998). The transcriptional factor nuclear factor-kappaB (NF-κB) plays a crucial role in human breast cancer progression and its constitutive activity is associated with cancer cell invasion and metastasis (Giacinti et al., 2012). This suggests that NF-kB inhibitors could offer a therapeutic intervention to counteract the invasion and metastatic progression of cancer.

Pinitol is an effective blocker of the NF-κB pathway and thus may have potential in treatment of a wide variety of NF-κB linked proinflammatory diseases (Kumar et al., 2004). Sethi et al. (2008) also reported that Pinitol exerts its anti-inflammatory effects through suppression of the NF-κB pathway and found that pinitol inhibited activation of NF-κB through abrogation of IKK activation and IκBα phosphorylation. Consequently, this polyalcohol blocked p65 phosphorylation, nuclear translocation and gene transcription.

Expression of gene products involved in cell proliferation, antiapoptosis and invasion were all inhibited thus enhancing apoptosis and reducing cellular invasion in many cancer cell lines. Our results are not only well in accordance with the earlier reports but also which is highly consistence as the NF-κB expression was down regulated in MCF-7 cells since it is considered as an important factor and target in the development anticancer agents. Therefore, it can be concluded that the induction of apoptosis may be an index for new antitumor drug selection and an important method of assessment for the clinical applications of many plant derived anti-cancer agents. Furthermore, the results obtained from our studies provides the basis for further investigation using animal models of carcinogenesis in order to judge the chemotherapeutic efficacy of D-pinitol.

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