

## Isosilybin B and isosilybin A inhibit growth, induce G<sub>1</sub> arrest and cause apoptosis in human prostate cancer LNCaP and 22Rv1 cells

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Deep, G.; [Oberlies, N.H.](#); Kroll, D.J.; and Agarwal, R. (2007) Isosilybin B and isosilybin A inhibit growth, induce G<sub>1</sub> arrest and cause apoptosis in human prostate cancer LNCaP and 22Rv1 cells. *Carcinogenesis* 28, 1533-1542.

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### **Abstract:**

Silymarin and, one of its constituents, silibinin exert strong efficacy against prostate cancer (PCA); however, anticancer efficacy and associated mechanisms of other components of silymarin, which is a mixture of flavonolignans, are largely unknown. Here we have assessed the anticancer efficacy of two pure compounds isosilybin B and isosilybin A, isolated from silymarin, in human prostate carcinoma LNCaP and 22Rv1 cells. Isosilybin B and isosilybin A treatment resulted in growth inhibition and cell death together with a strong G<sub>1</sub> arrest and apoptosis in both the cell lines. In the studies examining changes in cell cycle and apoptosis regulators, isosilybin B and isosilybin A resulted in a decrease in the levels of both cyclins (D1, D3, E and A) and cyclin-dependent kinases (Cdk2, Cdk4 and cell division cycle 25A), but caused an increase in p21, p27 and p53 levels, except in 22Rv1 cells where isosilybin B caused a decrease in p21 protein level. Isosilybin B- and isosilybin A-induced apoptosis was accompanied with an increase in the cleavage of poly (ADP-ribose) polymerase, caspase-9 and caspase-3 and a decrease in survivin levels. Compared with LNCaP and 22Rv1 cells, the antiproliferative and cytotoxic potentials of isosilybin B and isosilybin A were of much lesser magnitude in non-neoplastic human prostate epithelial PWR-1E cells suggesting the transformation-selective effect of these compounds. Together, this study for the first time identified that isosilybin B and isosilybin A, two diastereoisomers isolated from silymarin, have anti-PCA activity that is mediated via cell cycle arrest and apoptosis induction.

**Abbreviations:** PCA, prostate cancer; PI, propidium iodide; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; Cdc25A, cell division cycle 25A; PARP, poly (ADP-ribose) polymerase

### **Article:**

#### *Introduction*

Prostate cancer (PCA) is the leading cause of cancer incidences accounting for ~33% (234 460) of estimated new cases in 2006 excluding non-melanoma skin cancers and is the third leading cause of cancer-related deaths that accounts for ~9% (27 350 cases) of all cancer-related deaths in US males (1). Surgical ablation of androgens and anti-androgen therapy are two of the therapeutic approaches against locally advanced or metastatic PCA (2). Although most patients respond to these therapies initially, the disease ultimately progresses to hormone-refractory state (2). Thus, the surgery and current chemotherapeutic options seem to be inadequate in controlling and curing PCA, and there is an urgent need for the identification and development of alternative strategies. In this regard, prevention and therapeutic intervention by dietary and plant-based drugs have been suggested as a newer dimension in the arena of cancer management (3,4). Among various groups of cancer chemopreventive agents, extensive experimental data have been generated, which suggest the role of polyphenolic flavonoids in chemoprevention of various cancers including PCA (5–8).

Silymarin is a crude mixture of flavonolignans obtained from the seeds of milk thistle [*Silybum marianum* (L.) Gaertn.]. This group of compounds results from the peroxidase-mediated condensation in the plant between the flavonoid, dihydroquercetin and coniferyl alcohol. Silymarin and silibinin (a 50:50 mixture of silybin A and silybin B) are widely known for their hepatoprotective activity and has been in clinical use for over three

decades in Europe and recently in USA and Asia (9). Several studies by others and us in the last decade have demonstrated the pre-clinical efficacy of both silymarin and silibinin against various epithelial cancers including PCA (10–13), and their efficacy is currently being evaluated in cancer patients (14). This has led us to identify and purify specific, biologically active chemical entities present in silymarin with a goal to maximize the therapeutic utility of this botanical agent. In this direction, we have isolated, purified and characterized eight pure compounds (seven flavanolignans and a flavonoid) from silymarin, namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin and taxifolin (10,15). Distinct biological efficacy of these compounds was observed in human PCA cells on various cancer end point markers such as cell proliferation, prostate-specific antigen secretion and topoisomerase II $\alpha$  promoter activity (10). Isosilybin B and isosilybin A, which constitute about 2–4.5% and 5–6.5% of silymarin (w/w), respectively, depending upon the source, were effective in most of the parameters studied in both androgen-dependent LNCaP and androgen-independent PC3 and DU145 cells (10). For example, the IC<sub>50</sub> in DU145 cells for isosilybin B and isosilybin A was 20.5 and 32  $\mu$ M, respectively, which was lower compared with silymarin (IC<sub>50</sub> 67.9  $\mu$ M) and silibinin (IC<sub>50</sub> 55.6  $\mu$ M) (10). Though it would be premature to conclude that isosilybin B and isosilybin A were the most effective among all the constituents from this one study alone, the data provided us a strong impetus to further examine the efficacy and associated mechanisms for isosilybin isomers in PCA cells.

De-regulated cell cycle and evasion of apoptosis have been recognized as hallmark of cancer progression including PCA (16). Cell cycle is known to be regulated by various kinases, namely Cdk4, Cdk2 and Cdk1 (or cdc2), whose activity is controlled by binding of different cyclins, cyclin-dependent kinase inhibitors (CDKIs) and Cdc25 phosphatases (17,18). Apoptosis refers to programmed cell death in response to various intrinsic or extrinsic death signals, and is executed by series of cysteine proteases known as caspases (19,20). Cancer chemopreventive agents are known to exert their anticancer effect via modulating the expression of cyclins–cyclin-dependent kinases (CDKs)–CDKIs and caspases resulting in induction of cell cycle arrest and apoptosis (7,8,12,21). Accordingly, in the present study, we investigated the efficacy and mechanism of isosilybin B and isosilybin A in human PCA LNCaP and 22Rv1 cells. For the first time, our findings suggest that both these agents impart anti-PCA activity via cell cycle arrest and apoptosis induction involving modulation in cyclins–CDKs–CDKIs expression and caspase activation together with a decrease in survivin levels.

## **Materials and Methods**

### **Cell lines and reagents**

Isosilybin B and isosilybin A (Figure 1A) were isolated (purity between 98–100%) from silymarin (Madus AG, Cologne, Germany) as described previously with modifications to enhance the separation of large-scale amounts of each isomer (10,15,22). Human prostate carcinoma LNCaP and 22Rv1 cells and non-neoplastic PWR-1E cells were obtained from the American Type Culture Collection (Manassas, VA). RPMI1640, keratinocyte–SFM media and other cell culture materials were from Invitrogen Corporation (Gaithersburg, MD). Antibody for Cip1/p21 was from Upstate (Charlottesville, VA) and for Kip1/p27 and tubulin was from Neomarkers (Fremont, CA). Antibodies for Cdk2, Cdk4, cell division cycle 25A (Cdc25A), cyclin D1, cyclin D3, cyclin E and cyclin A were from Santa Cruz Biotechnology (Santa Cruz, CA). Hoechst 33342, propidium iodide (PI) and antibody for  $\beta$ -actin were from Sigma–Aldrich Chemical Co. (St Louis, MO). ECL detection system and anti-mouse peroxidase-conjugated secondary antibody were from GE Healthcare (Buckinghamshire, UK). Antibodies for cleaved poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, cleaved caspase-9 and anti-rabbit peroxidase-conjugated secondary antibody were obtained from Cell Signaling (Beverly, MA). Anti-survivin antibody was from Novus Biologicals (Littleton, CO). All other reagents were obtained in their commercially available highest purity grade.

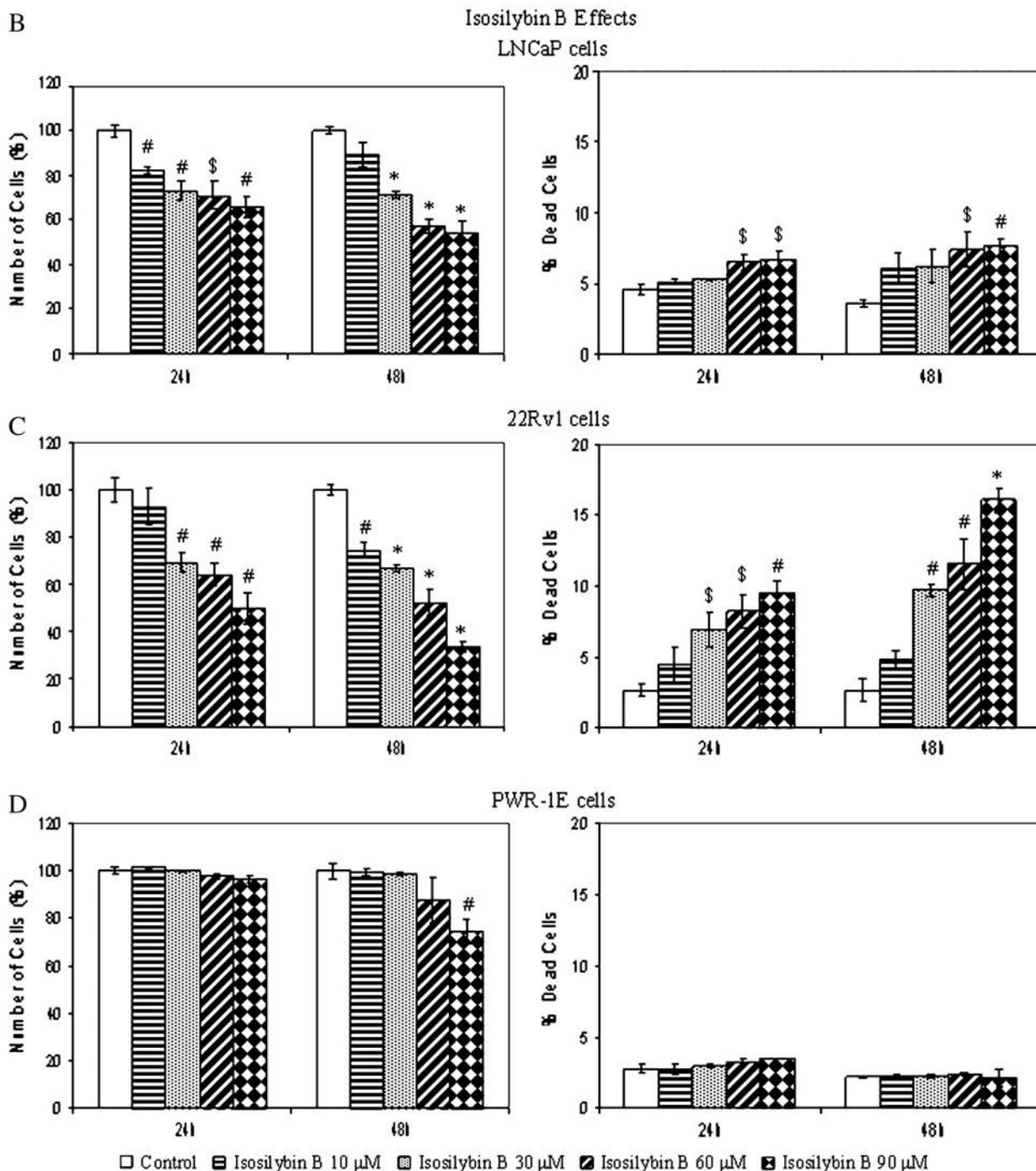
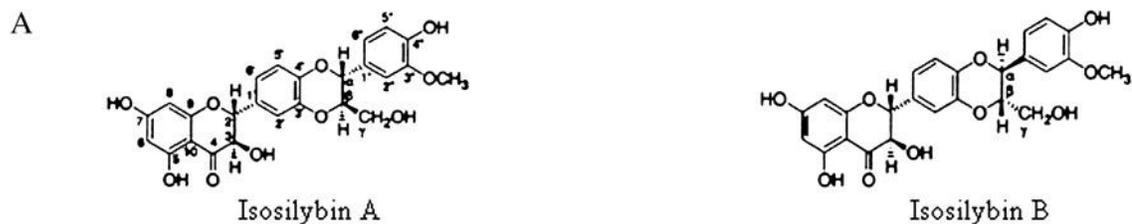


Fig. 1. Structures of isosilybin A and isosilybin B and the effect of isosilybin B on cell growth and cell death in LNCaP, 22Rv1 and PWR-1E cells. (A) The chemical structures of isosilybin A and isosilybin B. (B–D) LNCaP cells, 22Rv1 cells or PWR-1E cells were treated with DMSO (control) or various doses of isosilybin B (10–90 µM) for 24 and 48 h. At the end of each treatment time, both adherent and non-adherent cells were collected and processed for determination of total cell number and dead cells as mentioned in Materials and methods. The data shown are mean ± standard error of mean of three samples for each treatment. These results were similar in three independent experiments. \*,  $P \leq 0.001$ ; #,  $P \leq 0.01$ ; \$,  $P \leq 0.05$ .

## Cell culture and treatments

LNCaP cells (passage 5–45) and 22Rv1 cells (passage 5–30) were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate at 37°C in a humidified 5% CO<sub>2</sub> incubator. PWR-1E cells were cultured in keratinocyte–Serum Free Media supplemented with 10% fetal bovine serum, 5 ng/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract. Cells were plated and treated at 40–50% confluency with different doses of isosilybin B and isosilybin A (10–90 µM in medium) dissolved originally in Dimethyl sulfoxide (DMSO) for the desired time periods (24–48 h) in serum condition. An equal amount of DMSO (vehicle) was present in each treatment, including control; DMSO concentration did not exceed 0.1% (v/v) in any treatment. At the end of desired treatments, various analyses were done as described below.

## Cell growth and death assays

In each case, cells were plated to about 40–50% confluency and treated with isosilybin B and isosilybin A under serum condition as detailed above. After 24 and 48 h of treatments, cells were collected by brief trypsinization and washed with phosphate-buffered saline. Total cell number was determined by counting each sample in duplicate using a hemocytometer under an inverted microscope. Cell viability was determined using trypan blue exclusion method. Each treatment and time point had three independent plates. The experiment was repeated at least twice under identical conditions.

## Fluorescence-activated cell sorting analysis for cell cycle distribution

After identical treatments as detailed above, cells were collected and processed for cell cycle analysis. Briefly,  $0.5 \times 10^5$  cells were suspended in 0.5 ml of saponin/PI solution (0.3% saponin (w/v), 25 µg/ml PI (w/v), 0.1 mM ethylenediaminetetraacetic acid and 10 µg/ml RNase A (w/v) in phosphate-buffered saline) and incubated overnight at 4°C in dark. Cell cycle distribution was then analyzed by flow cytometry using fluorescence-activated cell sorting analysis core facility of the University of Colorado Cancer Center. The experiment was repeated at least twice under identical conditions.

## Western immunoblotting

At the end of the desired treatments detailed above, cell lysates were prepared in non-denaturing lysis buffer as reported by us (23), and 50–70 µg of protein lysate per sample was denatured in 2x sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8, 12 or 16% Tris–glycine gel. The separated proteins were transferred on to nitrocellulose membrane followed by blocking with 5% non-fat milk powder (w/v) in Tris-buffered saline (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Membranes were probed for the protein levels of desired molecules using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualized by ECL detection system. To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin or anti-tubulin antibody to normalize for differences in protein loading.

## Apoptosis assay

Quantitative apoptotic cell death by isosilybin B and isosilybin A in LNCaP and 22Rv1 cells was measured by Hoechst assay as described previously (21). Briefly, after desired treatments, cells were collected and then stained with DNA-binding dye Hoechst 33342 and PI. Apoptotic cells were quantified using fluorescent microscope (Axioskope 2 plus-HBO 100, Zeiss, Jena, Germany) by counting 100 cells per microscopic field (at 100x magnification) in five fields for each sample in triplicate.

## Statistical analysis

Statistical analysis was performed using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA). Data were analyzed using *t*-test and a statistically significant difference was considered to be at  $P < 0.05$ . For all the results where applicable, the autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA), and the mean density of each band was analyzed by the Scion Image program (National Institutes of Health, Bethesda, MD). In each case, blots were subjected to multiple exposures on the film to make sure that

the band density is in the linear range. Densitometry data presented below the bands are ‘fold change’ as compared with control for both isosilybin B and isosilybin A treatments in each case.

## **Results**

### **Pure isosilybin diastereoisomers inhibit growth and induce death of LNCaP and 22Rv1 cells**

We first examined the effect of isosilybin B and isosilybin A on cell growth and cell death in human prostate carcinoma LNCaP and 22Rv1 cells. Cells were treated with various doses of these isosilybin isomers and cell number and cell death was analyzed by trypan blue exclusion assay. Treatment with isosilybin B (10–90  $\mu$ M) for 24 and 48 h resulted in a decrease in cell number in both LNCaP and 22Rv1 cells in both dose- and time-dependent manner. In case of LNCaP cells, 24 and 48 h treatments with isosilybin B resulted in 18–35% and 11–46% decrease in cell number, respectively (Figure 1B). Relatively, a somewhat greater effect was observed in 22Rv1 cells where identical isosilybin B treatments for 24 and 48 h exhibited 7–50% and 26–66% decreases in cell number, respectively (Figure 1C). In LNCaP cells, there was a slight but significant increase in cell death after 24 and 48 h of isosilybin B treatment at 60 and 90  $\mu$ M concentrations (Figure 1B); however, the effect was more remarkable in 22Rv1 cells where compared with both 24 and 48 h controls showing 2.7% dead cells, isosilybin B treatment for 24 and 48 h at 30–90  $\mu$ M doses resulted in 7–10% and 10–16% dead cells, respectively (Figure 1C).

Isosilybin A treatment also resulted in a decrease in cell number and an increase in cell death in both LNCaP and 22Rv1 cells, and the results obtained here were comparable with those for isosilybin B. In case of LNCaP cells, identical isosilybin A treatment for 24 and 48 h resulted in 17–28% and 19–52% decrease in cell number, respectively (Figure 2A). In 22Rv1 cells, relatively better effect was observed showing 20–42% and 41–62% decrease under identical isosilybin A treatments for 24 and 48 h, respectively (Figure 2B). Regarding cell death effect, similar to isosilybin B, isosilybin A treatment resulted in only a slight increase in dead cells in LNCaP cells (Figure 2A), whereas the effect in 22Rv1 cells was much more pronounced where the dead cells significantly increased to 3–11% and 8–16% of the total number of cells with 30–90  $\mu$ M doses of isosilybin A treatment for 24 and 48 h, respectively, whereas in control, the dead cells were 1.6% after 24 h and 1.8% after 48 h (Figure 2B).

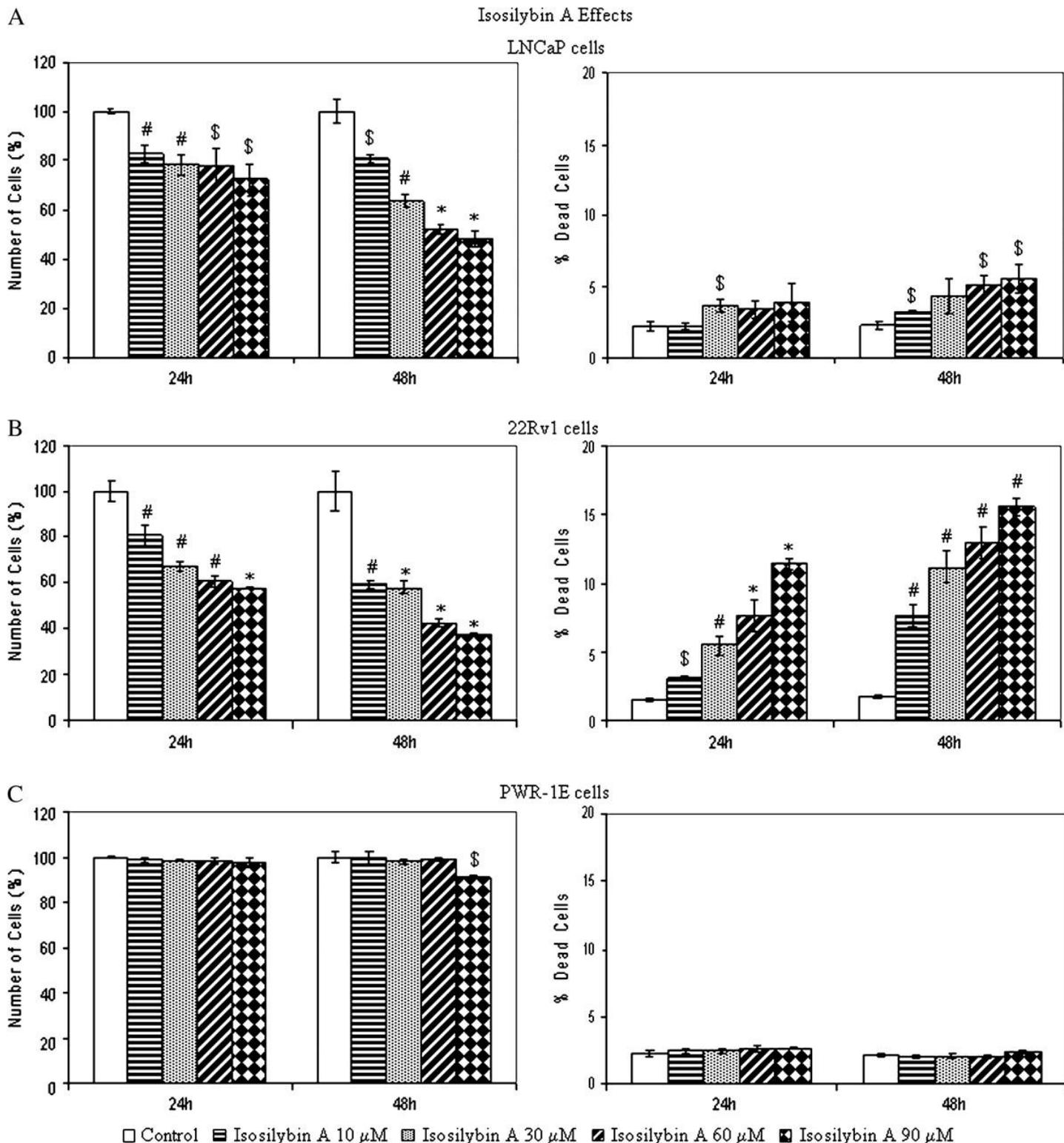


Fig. 2. Effect of isosilybin A on cell growth and cell death in LNCaP, 22Rv1 and PWR-1E cells. (A–C) LNCaP cells, 22Rv1 cells or PWR-1E cells were treated with DMSO (control) or various doses of isosilybin A (10–90  $\mu$ M) for 24 and 48 h. At the end of each treatment time, both adherent and non-adherent cells were collected and processed for determination of total cell number and dead cells as mentioned in Materials and methods. The data shown are mean  $\pm$  standard error of mean of three samples for each treatment. These results were similar in three independent experiments. \*,  $P \leq 0.001$ ; #,  $P \leq 0.01$ ; \$,  $P \leq 0.05$ .

**Lack of isosilybin isomer effects on cell growth in non-neoplastic human prostatic epithelial PWR-1E cells**  
 PWR-1E is an immortalized cell line developed from the non-neoplastic adult human prostatic epithelium using

adenovirus-12/simian virus-40 (Ad12-SV40) hybrid virus (24). In the studies examining the effect of isosilybin B and isosilybin A on PWR-1E cells, neither of the agent showed any significant change in cell growth at 24 h and cell viability after 24 and 48 h; however, isosilybin B and isosilybin A treatment at 90  $\mu$ M dose for 48 h decreased the cell number by 25 (Figure 1D) and 9% (Figure 2C) compared with their controls, respectively.

#### **Isosilybin isomers induce G<sub>1</sub> arrest in LNCaP and 22Rv1 cells**

LNCaP and 22Rv1 cells were treated with different doses of isosilybin B and isosilybin A and analyzed for cell cycle distribution by fluorescence-activated cell sorting analysis. There was a significant G<sub>1</sub> arrest in LNCaP cells after 24 h (30, 60 and 90  $\mu$ M) and 48 h (60 and 90  $\mu$ M) of treatment with isosilybin B, which was accompanied by a significant decrease in S phase population (Figure 3A). Further, there was a slight but significant increase in G<sub>2</sub>-M population at 90  $\mu$ M dose of isosilybin B in LNCaP cells after 48 h of treatment (Figure 3A). In 22Rv1 cells, isosilybin B treatment for 24 and 48 h increased the G<sub>1</sub> population even at 30  $\mu$ M dose, which increased further in a dose-dependent manner (Figure 3A). This increase in G<sub>1</sub> population at both the time points was at the expense of S and G<sub>2</sub>/M phase cells which decreased in a dose-dependent manner. Similarly, isosilybin A treatment also resulted in an increase in G<sub>1</sub> population after 24 h (10, 30, 60 and 90  $\mu$ M) and 48 h (90  $\mu$ M) of treatment in LNCaP cells (Figure 3B). Further, there was a slight increase in the G<sub>2</sub>-M population after 48 h of treatment. In 22Rv1 cells, the treatment of isosilybin A increased the G<sub>1</sub> population after 24 and 48 h, which was accompanied by a significant decrease in S phase population (Figure 3B).

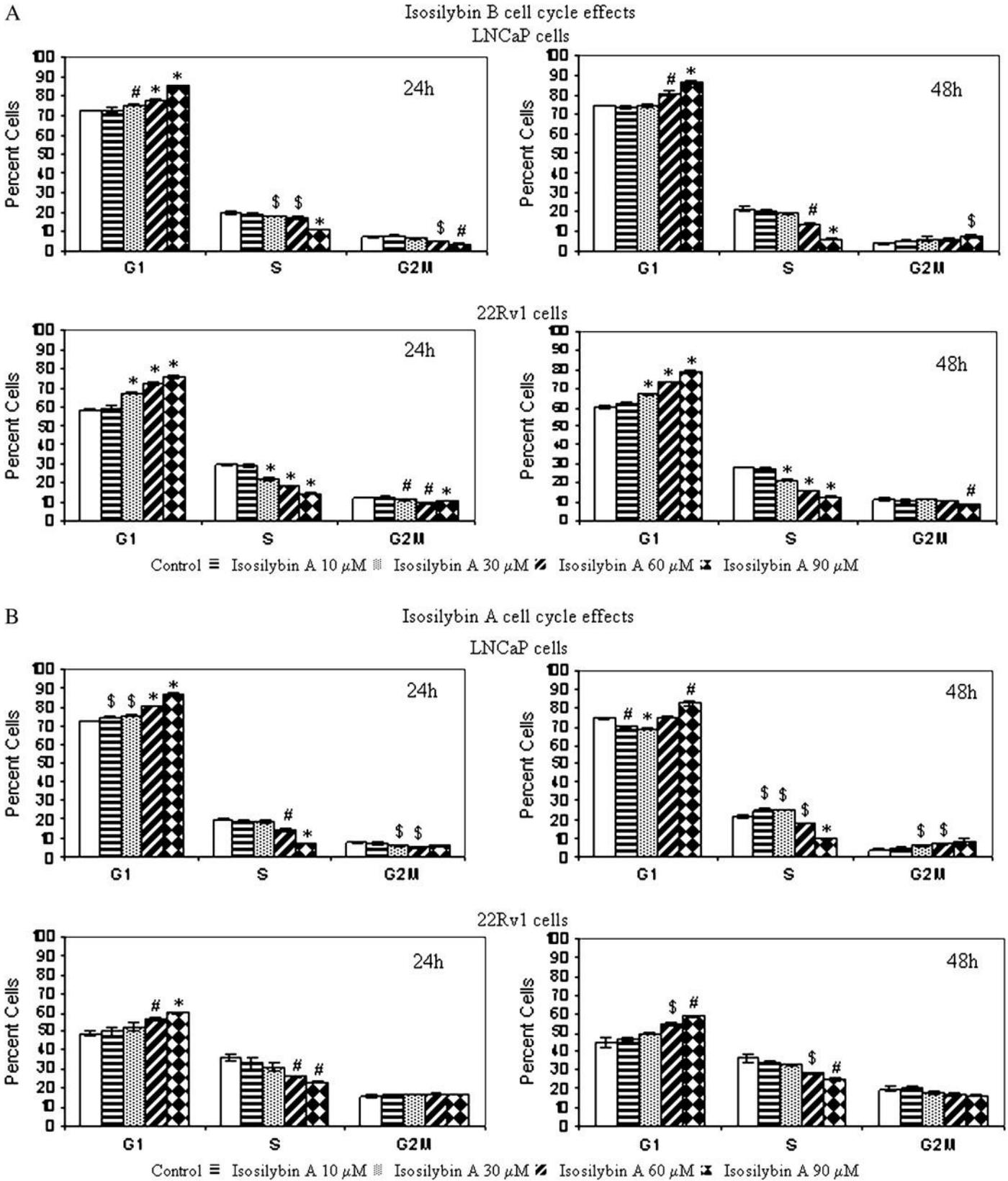


Fig. 3. Effect of isosilybin B and isosilybin A on cell cycle distribution in human PCA LNCaP and 22Rv1 cells. LNCaP or 22Rv1 cells were treated with DMSO or various doses (10–90  $\mu$ M) of isosilybin B and isosilybin A for 24 and 48 h. At the end of each treatment, cells were collected and incubated overnight with saponin/PI solution at 4°C and subjected to fluorescence-activated cell sorting analysis as detailed in Materials and methods. (A) Quantitative cell cycle distribution data for isosilybin B in LNCaP and 22Rv1 cells after 24 and 48 h of treatment. (B) Quantitative cell cycle distribution data for isosilybin A in LNCaP and 22Rv1 cells after

24 and 48 h of treatment. The data shown are mean  $\pm$  standard error of mean of three samples for each treatment. These results were similar in two independent experiments. \*,  $P \leq 0.001$ ; #,  $P \leq 0.01$ ; \$,  $P \leq 0.05$

### **Isosilybin isomers modulate expression of G<sub>1</sub> phase cell cycle regulators**

We observed a strong G<sub>1</sub> arrest with isosilybin B and isosilybin A in LNCaP and 22Rv1 cells after 24 and 48 h of treatment; therefore, we next examined the effect of these compounds on the levels of cyclins (D1, D3, E and A), CDKs (Cdk2 and Cdk4), CDKIs (p27 and p21) and Cdc25A, which are known to regulate the G<sub>1</sub> phase of cell cycle (17,18). Isosilybin B treatment (10–90  $\mu$ M) of LNCaP cells resulted in a moderate to strong decrease in the levels of cyclins D3, D1, A and E along with a decrease in the level of Cdk4, Cdk2 and Cdc25A (Figure 4). Isosilybin B treatment, however, increased the levels of p27 and p21 in LNCaP cells (Figure 4). In 22Rv1 cells also, treatment of isosilybin B (60 and 90  $\mu$ M doses) for 24 and 48 h resulted in a decrease in the levels of cyclins D3, D1, E and A along with a decrease in the levels of Cdk2, Cdk4 and Cdc25A in a dose- and time-dependent manner (Figure 5). Isosilybin B treatment also resulted in a slight to moderate increase in the levels of p27, whereas the levels of p21 decreased under similar treatment conditions (Figure 5). Similar to isosilybin B, isosilybin A treatment resulted in a decrease in the levels of cyclins D3, D1, E and A along with decrease in the levels Cdk2 and Cdk4 in LNCaP cells (Figure 4). In 22Rv1 cells, isosilybin A treatment caused only a slight to moderate decrease in the levels of cyclins D3, E, A and Cdk2 (Figure 4). Isosilybin A treatment also increased the levels of p21 and p27 in both LNCaP and 22Rv1 cells (Figures 4 and 5). Further, there was a decrease in the level of Cdc25A in both the cell lines with isosilybin A treatment (Figures 4 and 5). Membranes were stripped and reprobed with anti- $\beta$ -actin antibody for protein loading correction.

LNCaP cells

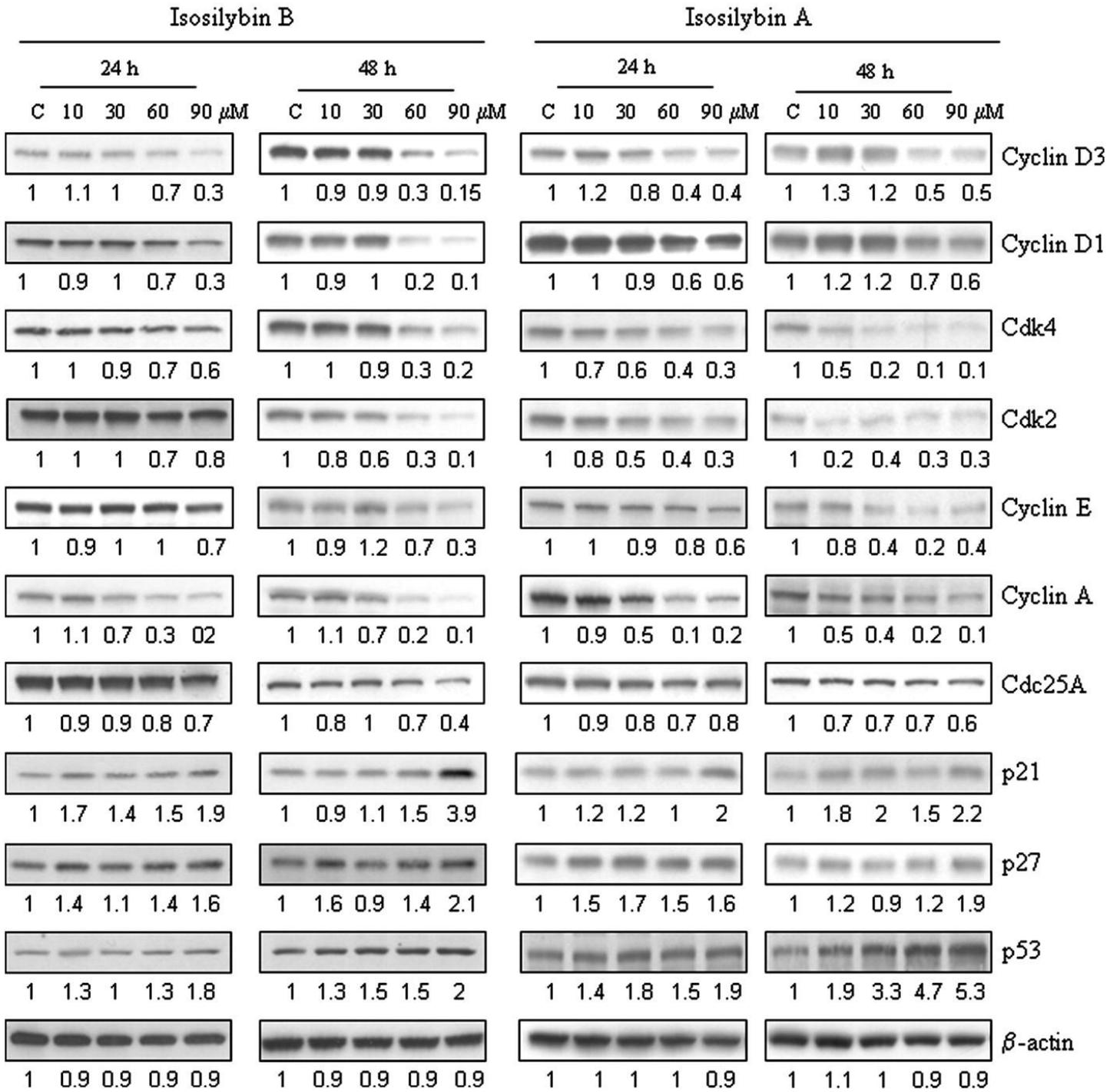


Fig. 4. Effect of isosilybin B and isosilybin A on G<sub>1</sub> cell cycle regulatory molecules in LNCaP cells. LNCaP cells were treated with DMSO or various doses of isosilybin B and isosilybin A (10, 30, 60 and 90  $\mu$ M) for 24 and 48 h. At the end of each treatment time, cell lysates were prepared in non-denaturing lysis buffer as mentioned in Materials and methods. For each sample, 50–60  $\mu$ g of protein lysate was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western immunoblotting, and membranes were probed for cyclin D3, cyclin D1, Cdk4, Cdk2, cyclin E, cyclin A, Cdc25A, p21, p27 and p53. Membranes were also stripped and re-probed with anti- $\beta$ -actin antibody for protein loading correction. Blots shown are representative of at least two independent experiments in each case out of the experiments done at different cell passage number. The densitometry data presented below the bands are ‘fold change’ as compared with control after normalization with respective loading control ( $\beta$ -actin).

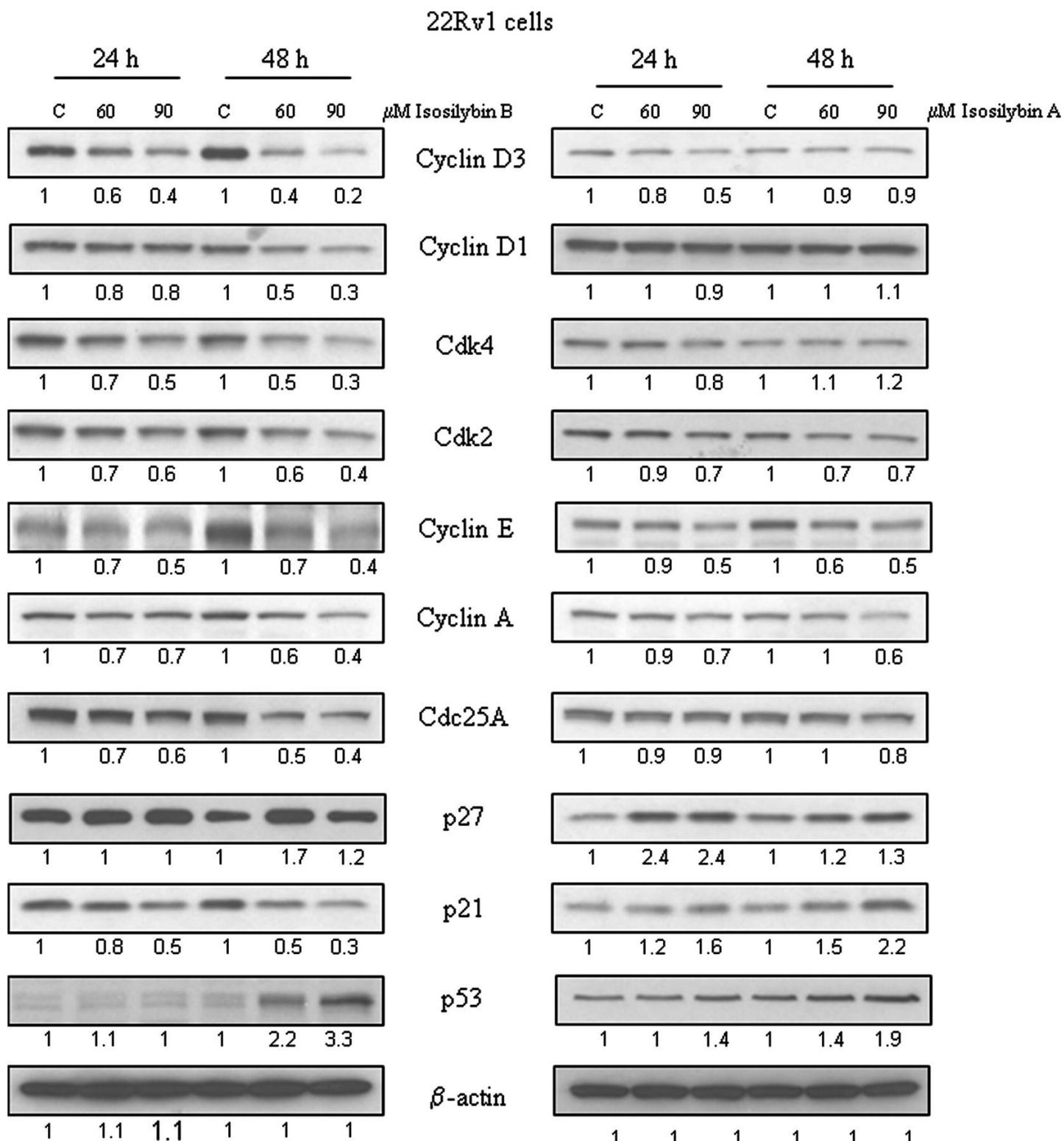


Fig. 5. Effect of isosilybin B and isosilybin A on G<sub>1</sub> phase cell cycle regulatory molecules in 22Rv1 cells. 22Rv1 cells were treated with DMSO or various doses of isosilybin B and isosilybin A (60 and 90  $\mu\text{M}$ ) for 24 and 48 h. At the end of each treatment time, cell lysates were prepared in non-denaturing lysis buffer as mentioned in Materials and methods. For each sample, 50–60  $\mu\text{g}$  of protein lysate was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western immunoblotting, and membranes were probed for cyclin D3, cyclin D1, Cdk4, Cdk2, cyclin E, cyclin A, Cdc25A, p21, p27 and p53. Membranes were also stripped and re-probed with anti- $\beta$ -actin antibody for protein loading correction. Blots shown are representative of at least two independent experiments in each case out of the experiments done at different cell passage number. The densitometry data presented below the bands are ‘fold change’ as compared with control after normalization with respective loading control ( $\beta$ -actin).

### **Isosilybin B and isosilybin A treatment increases p53 level**

P53 is known as a tumor suppressor gene important for the maintenance of genome integrity. In response to stress signal, the p53 protein is accumulated in a specific manner by post-translational modifications leading to either cell cycle arrest or apoptosis (25). Since we observed an induction of cell cycle arrest and apoptosis with isosilybin B and isosilybin A treatment in LNCaP and 22Rv1 cells both of which harbor wild-type p53, we also examined the effect of these compounds on p53 level. Both isosilybin B and isosilybin A treatment increased the total p53 levels in LNCaP and 22Rv1, which was more prominent after 48 h of treatment (Figures 4 and 5).

### **Isosilybin isomers induce apoptotic death in LNCaP and 22Rv1 cells**

Since we observed cell death with these agents together with a strong G<sub>1</sub> arrest that might lead the cell to apoptotic exit from the cell cycle, we next examined the effect of these compounds on the apoptotic death of LNCaP and 22Rv1 cells after 48 h of treatment. As shown in Figure 6A, apoptotic population increased significantly by 2-folds with 90 μM isosilybin B in LNCaP cells as measured by Hoechst staining. The increase was about 2- to 3-fold with 60 and 90 μM dose of isosilybin A after 48 h of treatment in LNCaP cells. In 22Rv1 cells, treatment of 60 and 90 μM dose of isosilybin B for 48 h resulted in an increase in apoptotic cell population by 3- to 4-fold, whereas with isosilybin A, apoptotic cell population increased by about 4- to 5-fold at identical doses (Figure 6A).

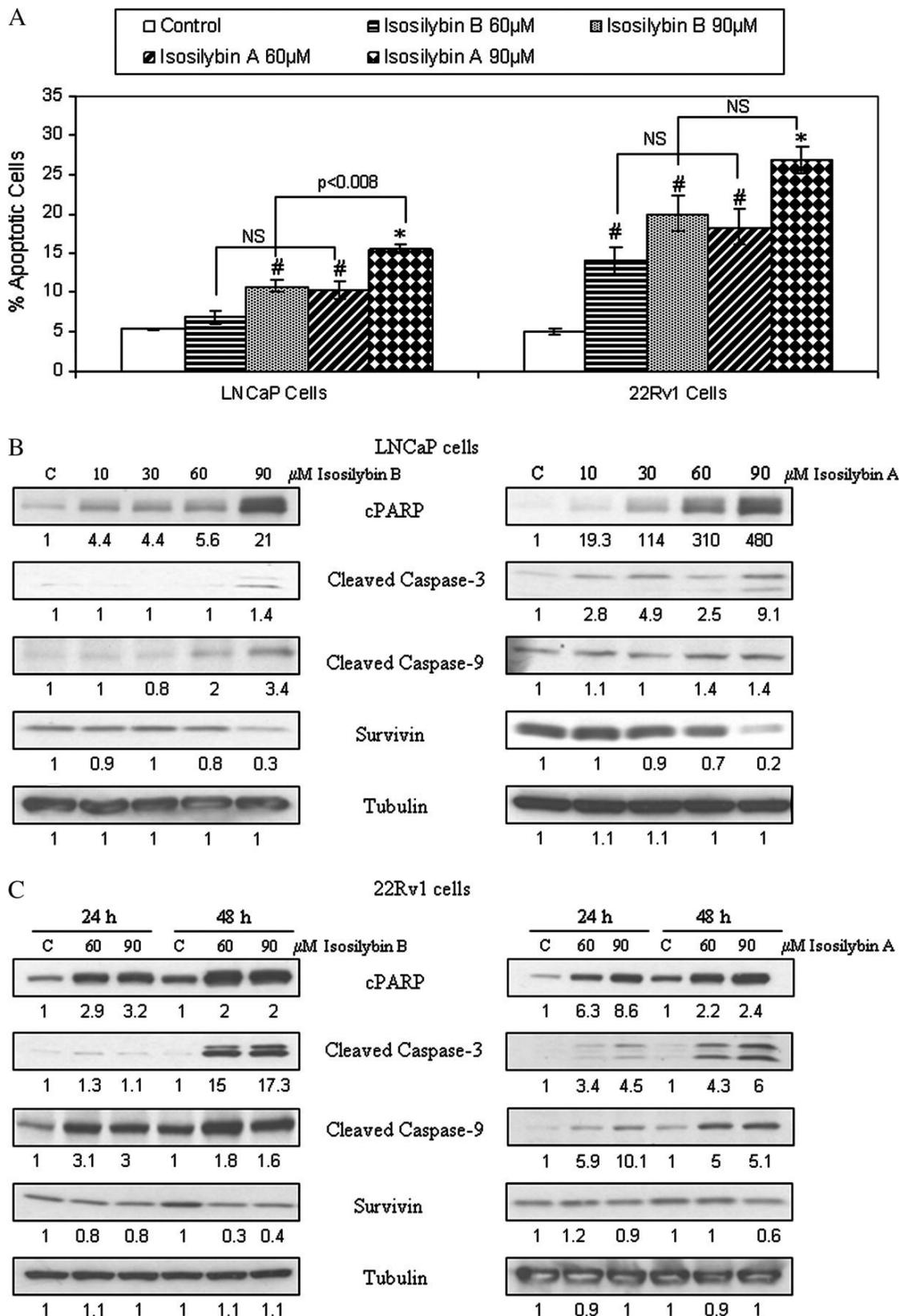


Fig. 6. Isosilybin B and isosilybin A induce apoptosis in human prostate carcinoma LNCaP and 22Rv1 cells. (A) LNCaP and 22Rv1 cells were treated with DMSO or 60 and 90 μM doses of isosilybin B and isosilybin A for 48 h. Both adherent and non-adherent cells were collected and stained with Hoechst and apoptotic cell population was measured as mentioned in Materials and methods. Data shown are mean ± standard error of mean of three samples for each treatment. These results were similar in two independent experiments. \*,  $P \leq 0.001$ ; #,  $P \leq 0.01$ , NS, non-significant. (B) LNCaP cells were treated with DMSO or various doses of isosilybin B and isosilybin A (10, 30, 60 and 90 μM) for 48 h. At the end of each treatment time, cell lysates were

prepared in non-denaturing lysis buffer as mentioned in Materials and methods. For each sample, 60–70 µg of protein lysate was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western immunoblotting, and membranes were probed for cleaved PARP, cleaved caspase-3, cleaved caspase-9 and survivin. Membranes were also stripped and reprobed with anti-tubulin antibody for protein loading correction. (C) 22Rv1 cells were treated with DMSO or 60 and 90 µM doses of isosilybin B and isosilybin A for 24 and 48 h. At the end of each treatment time, western blot analysis was performed for cleaved PARP, cleaved caspase-3, cleaved caspase-9 and survivin. Membranes were also stripped and reprobed with anti-tubulin antibody for protein loading correction. Blots shown are representative of two independent experiments in each case. The densitometry data presented below the bands are ‘fold change’ as compared with control after normalization with respective loading control (tubulin).

### **Isosilybin isomers-induced apoptosis involves caspase-3, caspase-9 and PARP cleavage and a decrease in survivin level**

We observed a significant increase in apoptotic population by both isosilybin compounds; therefore, we next examined their effect on the molecules that are known to regulate and execute apoptosis. Treatment of LNCaP and 22Rv1 cells with isosilybin B and isosilybin A resulted in a dose-dependent increase in the PARP cleavage (Figure 6B and C), which is a known marker for apoptosis (26). Since caspases are known as executors of apoptosis (19,27), we also examined the effect of isosilybin B and isosilybin A on the levels of cleaved caspase-9 and cleaved caspase-3. As shown in Figure 6B, there was a slight increase in the levels of these active fragments in LNCaP cells after 48 h of treatment with these agents; however, in 22Rv1 cells, similar treatments resulted in a much more prevalent increase in the levels of cleaved caspase-3 and cleaved caspase-9 (Figure 6C).

Survivin belongs to the family of inhibitor of apoptosis proteins and is generally over-expressed in cancer cells (28). Since it is known to counter apoptosis by inhibiting the activation of caspases (29,30), we next examined the effect of isosilybin B and isosilybin A on the survivin level in LNCaP and 22Rv1 cells, and found that there was a slight to moderate decrease with both agents in both the cell lines (Figure 6B and C).

### **Discussion**

Pure preparations have only become available recently of the milk thistle flavonolignan diastereoisomers, isosilybin A and isosilybin B. Isosilybin A and isosilybin B share the same chemical formula and structure with the exception of the stereochemical positions of the terminal aryl (3-methoxy-4-hydroxyphenyl) and hydroxymethyl groups at the  $\alpha$  and  $\beta$  carbons (Figure 1A). Further, both possess the same 1' linkage between flavonoid and lignan moiety, which has been earlier suggested to be responsible for the overall growth inhibitory action of flavonolignans isolated and purified from silymarin (10). The findings of the present study suggest that isosilybin B and isosilybin A exert strong antiproliferative effects against human prostate carcinoma LNCaP and 22Rv1 cells and that this effect involves alterations in cell cycle regulators, causing both G<sub>1</sub> arrest as well as induction of cell death via apoptosis. Importantly, we found that these two compounds exhibited very little to moderate antiproliferative effect in immortalized non-neoplastic human prostate epithelial PWR-1E cells depending upon the duration of treatment, suggesting that these compounds exhibit cytotoxicity selectively against cancer cells. Furthermore, this is the first detailed comparative mechanistic study for the diastereoisomers isosilybin B and isosilybin A for their apoptotic and cell cycle effects in human PCA cells.

Progression through the various phases of cell cycle is mediated by CDKs in complex with specific cyclins (31). Cyclin D1 and cyclin D3 are known to form complex with Cdk4, whereas cyclin E and cyclin A bind to and activate Cdk2. These activated kinases then phosphorylate Rb protein to release E2F transcription factors to transcribe genes needed for the G<sub>1</sub> to S transition (31). Molecular analyses of human cancers have revealed that cell cycle is abnormally regulated by aberrant expression of key cyclins and CDKs (32–35). For example, cyclin E is over-expressed in human breast, gastric, colorectal, leukemia, genitourinary tract, lung and skin cancers (32), and cyclin D1 is also over-expressed in many cancers, including PCA (33). In the present study, both

agents resulted in a decrease in cyclin D1, D3, E and A levels along with a decrease in Cdk4 and Cdk2 protein levels, which might have a direct relevance to the observed G<sub>1</sub> arrest in both LNCaP and 22Rv1 cells by these agents.

The Cip/Kip CDKI family represents another group of cell cycle regulators, which are known to regulate G<sub>1</sub>-S and G<sub>2</sub>-M phase transitions (17). p21 (Cip1) is known to interact with cyclin D-Cdk4 complex and p27 (Kip1) suppresses the activity of cyclin E-Cdk2 complex (17). The loss of p21 and p27 expression has been found to be associated with a poor prognosis in a number of different human tumors including laryngeal, breast, gastric, non-small-cell lung and prostate carcinomas (36). Our results showed that both isosilybin B and isosilybin A increase Cip1/p21 and Kip1/p27 protein levels except in 22Rv1 cells, where isosilybin B treatment resulted in a decrease in the levels of p21. Further studies are needed to examine the role of isosilybin B-mediated decrease in p21.

Cdc25A belongs to dual specificity phosphatase family and controls cell cycle progression by dephosphorylating and activating CDKs at positions Thr14 and Tyr15 (18). Cdc25A has been described as an oncogene and is over-expressed in a wide variety of tumors (37). Microinjection of antibodies against Cdc25A arrests cells before S phase (38). Isosilybin A and isosilybin B treatment resulted in a decrease in the levels of Cdc25A, which might have a potential role in inactivation of CDK and thus contributing to the observed G<sub>1</sub> arrest.

Several lines of evidence indicate that progression of the cell cycle and control of apoptosis are intimately linked processes, even though both have separate regulatory molecular mechanisms (39,40). The G<sub>1</sub> phase arrest of cell cycle progression provides an opportunity for cells to either undergo repair mechanisms or follow the apoptotic pathway. Our results showed that both isosilybin isomers induce apoptosis in LNCaP and 22Rv1 cells. Apoptosis is known to be tightly regulated by pro-apoptotic and anti-apoptotic effector molecules and can be mediated by several different pathways in response to various intrinsic and extrinsic signals (20,41). These signals affect the membrane potential of mitochondria and resulting in release of cytochrome c, which binds with Apaf-1, resulting in activation of caspase-9 and subsequently caspase-3 (21,42). The activation of caspase-3 leads to the cleavage and inactivation of key cellular proteins, such as PARP, resulting in disassembly of the cell (27). We found that isosilybin B and isosilybin A increased the cleavage of caspase-9 and caspase-3 followed by PARP cleavage. These results suggest the potential role of caspases activation by both the compounds in both LNCaP and 22Rv1 cells.

Activation of caspases is regulated by various proteins, including the inhibitory proteins of the inhibitor of apoptosis protein family (43). Survivin belongs to inhibitor of apoptosis protein family and is over-expressed in vast majority of cancers including esophageal, lung, ovarian, breast, colorectal, bladder, gastric, prostate, pancreatic, laryngeal, uterine etc. (28). Survivin is known to act as death suppressor as a result of caspase inhibition (29,30). Additionally, survivin has been shown to interact directly with Cdk4 and plays a role in Cdk2/cyclin E activation resulting in G<sub>1</sub> transition (44). Isosilybin B and isosilybin A treatment resulted in a decrease in the levels of survivin, which might contribute to both cell cycle arrest and induction of apoptosis as observed in LNCaP and 22Rv1 cells.

As mentioned earlier, p53 is a tumor suppressor gene and is activated in response to various intrinsic and extrinsic stress signals (25). Activated p53 is a sequence specific DNA-binding transcriptional factor and some of the target genes of its transcriptional activity are important for cell cycle arrest or for inducing apoptosis (45). For example, p53 regulated genes (Apaf-1, bax, noxa, puma etc.) are known to initiate a protein cascade leading to the activation of caspase-9 and then caspase-3 followed by apoptosis (45,46). Similarly, role of p53 in cell cycle arrest has been shown via regulating the expression of p21, cdc25A and 14-3-3 sigma (37,45,46). In the present work, we observed an increase in the level of p53 with isosilybin B and isosilybin A treatment, which might contribute to the observed cell cycle arrest and apoptosis; however, more studies are needed in future to establish such a cause and effect relationship.

In conclusion, the results of the present study indicate that isosilybin B and isosilybin A inhibit proliferation and induce G<sub>1</sub> phase arrest and apoptosis in human PCA cells. Isosilybin B was more effective in its effect on reducing cyclin D1 and cyclin D3 levels compared with isosilybin A in LNCaP cells. Similarly, in 22Rv1 cells, isosilybin B was more effective in down-regulating the levels of cyclins (D1, D3, E and A) and CDKs (Cdk4 and Cdk2) and Cdc25A, whereas isosilybin A was more effective in the induction of CDKIs (p21 and p27). These results point toward a difference in the mechanism for induction of G<sub>1</sub> phase arrest by isosilybin B and isosilybin A. Similarly, we found higher apoptotic cell population with isosilybin A compared with isosilybin B in both LNCaP and 22Rv1 cells suggesting that isosilybin A might be more potent in its apoptotic action. In non-tumorigenic PWR-1E cells where both compounds had minimal effects, isosilybin A showed lesser potency compared with isosilybin B in inhibiting cell proliferation. These results suggest that these two diastereoisomers do have distinct cellular targets, which might be due to the difference in the positioning of functional groups in their lignan moieties (Figure 1A). However, further studies are needed in future to address these issues as both compounds also have many overlapping effects as summarized in Table 1. Overall, this study provided the first detailed account of anticancer efficacy of isosilybin B and isosilybin A against human prostate carcinoma cells.

Table I. Summary of the results of isosilybin B and isosilybin A in LNCaP and 22Rv1 cells

Molecules studied	Isosilybin B		Isosilybin A		Significance
	LNCaP	22Rv1	LNCaP	22Rv1	
Cyclin D3	↓	↓	↓	↓	Cell cycle arrest
Cyclin D1	↓	↓	↓	—	Cell cycle arrest
Cdk4	↓	↓	↓	—	Cell cycle arrest
Cdk2	↓	↓	↓	↓	Cell cycle arrest
Cyclin E	↓	↓	↓	↓	Cell cycle arrest
Cyclin A	↓	↓	↓	↓	Cell cycle arrest
Cdc25A	↓	↓	↓	↓	Cell cycle arrest
p21	↑	↓	↑	↑	Cell cycle arrest
p27	↑	↑	↑	↑	Cell cycle arrest
Cleaved PARP	↑	↑	↑	↑	Apoptosis
Cleaved caspase-3	↑	↑	↑	↑	Apoptosis
Cleaved caspase-9	↑	↑	↑	↑	Apoptosis
Survivin	↓	↓	↓	↓	Cell cycle arrest, apoptosis
p53	↑	↑	↑	↑	Cell cycle arrest, apoptosis

↑Increases with isosilybin B or isosilybin A treatment.

↓Decreases with isosilybin B or isosilybin A treatment.

— No change with isosilybin B or isosilybin A treatment.

## Acknowledgements

This work was supported by U.S. Public Health Service grants RO1 CA102514 and CA104286 from the National Cancer Institute, National Institutes of Health. Special thanks to Tyler N.Graf of Research Triangle Institute for his hard work in isolating these diastereoisomers.

*Conflict of Interest Statement:* None declared.

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