

## Identifying the Differential Effects of Silymarin Constituents on Cell Growth and Cell Cycle Regulatory Molecules in Human Prostate Cancer Cells

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### **Abstract:**

Prostate cancer (PCa) is the leading cause of cancer-related deaths in men; urgent measures are warranted to lower this deadly malignancy. Silymarin is a known cancer chemopreventive agent, but the relative anticancer efficacy of its constituents is still unknown. Here, we compared the efficacy of 7 pure flavonolignan compounds isolated from silymarin, namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin, isosilydianin, silychristin and isosilychristin, in advanced human PCa PC3 cells. Silybin A, silybin B, isosilybin A, isosilybin B, silibinin and silymarin strongly inhibited the colony formation by PC3 cells ( $p < 0.001$ ), while silydianin, silychristin and isosilychristin had marginal effect ( $p < 0.05$ ). Using cell growth and death assays, we identified isosilybin B as the most effective isomer. FACS analysis for cell cycle also showed that silybin A, silybin B, isosilybin A, isosilybin B, silibinin and silymarin treatment resulted in strong cell cycle arrest in PC3 cells after 72 hr of treatment, while the effect of silydianin, silychristin and isosilychristin was marginal (if any). Western blot analysis also showed the differential effect of these compounds on the levels of cell cycle regulators-cyclins (D, E, A and B), CDKs (Cdk2, 4 and Cdc2), CDKIs (p21 and p27) and other cell cycle regulators (Skp2, Cdc25A, B, C and Chk2). This study provided further evidence for differential anticancer potential among each silymarin constituent, which would have potential implications in devising better formulations of silymarin against prostate and other cancers.

**Keywords:** prostate cancer | chemoprevention | silymarin | flavonolignans | cell cycle

### **Article:**

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Abbreviations: Cdc25, cell division cycle 25; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; FACS, fluorescence-activated cell sorting; HRP, horseradish peroxidase; PARP, poly(ADP-ribose)

Prostate cancer (PCa) is the most common noncutaneous cancer, and is the second leading cause of cancer related deaths in American men after lung cancer.<sup>1</sup> According to American Cancer Society report, about 218,890 incidences (29% of total estimated new cases) and 27,050 deaths (9% of total estimated deaths) due to PCa are estimated in American men in 2007.<sup>1</sup> Hormonal ablation therapy is usually employed for PCa treatment but often leads to an androgen-independent stage of malignancy, which is resistant to chemotherapy and radiotherapy, and these patients have poor survival.<sup>2,3</sup> In most of these PCa cases, death occurs because of cancer metastasis to bones and other skeletal tissues.<sup>4,5</sup> Lately, the use of cancer chemopreventive strategies has emerged as a new alternative or supplementary measure to support cancer chemotherapy. In this regard, phytochemicals, which are relatively nontoxic, cost-effective, physiologically bioavailable and have multiple molecular targets, have shown promising results for the prevention and/or intervention of various cancers.<sup>6-8</sup> Naturally occurring polyphenolic compounds, including silymarin, have been shown to possess potent cancer preventive efficacy against various cancers including PCa.<sup>8-12</sup>

Silymarin (SM) is a crude extract isolated primarily from the seeds of milk thistle (*Silybum marianum*) and is comprised of various flavonolignans. Silymarin is acceptable for human consumption and has been used clinically for its hepatoprotective action for more than 3 decades in Europe and recently in Asia and the United States.<sup>13</sup> Research conducted in last decade has clearly established the efficacy of silymarin and silibinin (SB), the major active constituent of silymarin, against various epithelial cancers including PCa.<sup>8, 10, 14-20</sup> Despite these advances in milk thistle research, the anticancer efficacy of various silymarin constituents largely remains unknown. In this direction, we isolated, purified and characterized from silymarin 7 distinct flavonolignans namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin; and 1 flavonoid taxifolin,<sup>20, 21</sup> and in subsequent work, we compared the effect of these pure compounds on various antiproliferative end points, including prostate cancer cell growth suppression, cell cycle distribution, as well as the inhibition of prostate-specific antigen (PSA) secretion, and DNA topoisomerase II $\alpha$  promoter activity.<sup>20</sup> Silybin A, silybin B, isosilybin A and isosilybin B were effective in all the parameters studied, while other 4 compounds had lesser or no effect.<sup>20</sup> Based on these results here we have designed and performed more experiments with these compounds to understand their molecular targets and mechanism(s) of action(s).

Deregulated cell cycle has been known as one of the hallmark of cancer cells, which provides them unlimited replicative potential.<sup>22</sup> Cell cycle regulation involves various factors namely: cyclins, cyclin dependent kinases (CDKs), cyclin dependent kinase inhibitors (CDKIs) and cell division cycle 25 (Cdc25) phosphatases.<sup>10, 23, 24</sup> The G1/S transition is regulated by CDK4 and 6 (in association with D-type cyclins) and CDK2 (in association with cyclin E and cyclin A).<sup>10, 23</sup> The G2/M transition is positively regulated by Cdc2 kinase in association with cyclin B.<sup>25</sup> These activated CDKs phosphorylate the retinoblastoma family of proteins to release E2F transcription factors, which then regulates the expression of various genes required for cell cycle transition.<sup>26, 27</sup> However, CDKIs (Kip1/p27, Cip1/p21 and INK4 family) are known to regulate the activity of CDKs.<sup>23, 28</sup> Cdc25 phosphatases also regulate the cell cycle transition by removing

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polymerase; PCa, prostate cancer; PI, propidium iodide; RPMI, Roswell Park Memorial Institute; SB: silibinin; Skp2, S-phase kinase-associated protein 2; SM, silymarin.

the inhibitory phosphorylation present on CDKs.<sup>29</sup> These phosphatases are inactivated through their phosphorylation by checkpoint kinases (Chk1/2).<sup>10, 30</sup> Targeting the deregulated cell cycle has been suggested as one of the potential strategy for cancer prevention and/or intervention.<sup>31</sup> Here, we analyzed the effect of these pure compounds on growth, cell cycle and cell cycle regulatory molecules in PCa cells. The present work is an effort to further understand whether these compounds possess distinct or overlapping molecular actions which contributes to overall, broad anticancer efficacy of the silymarin.

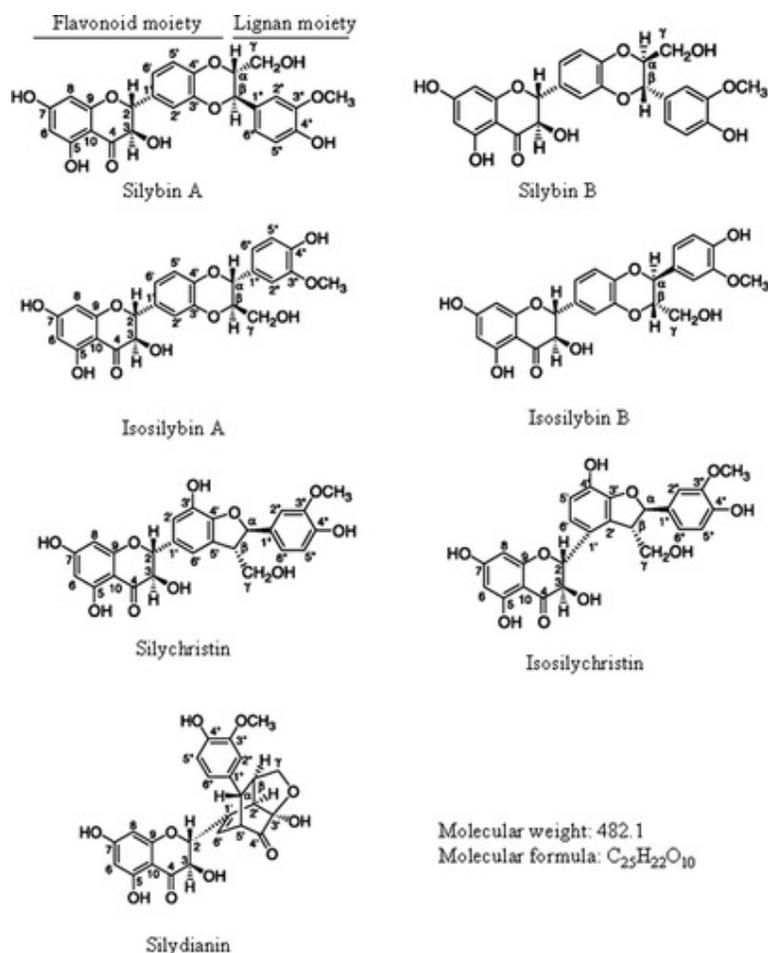
## Material and methods

### Cell lines and reagents

Silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin and isosilychristin (Fig. 1) were isolated (purity between 98 and 100%) from powdered extract (1 kg; lot 37501) of the fruits of *Silybum marianum* (L.) Gaertn. [obtained from Euromed, S.A. (Barcelona, Spain), a part of the Madaus Group (Köln, Germany)]. A preparative HPLC method was used as described previously with modifications to enhance the separation of large-scale amounts of each isomer.<sup>21, 32</sup> Silibinin was obtained from Sigma (St. Louis, MO). For both silibinin (a 1:1 diastereoisomeric mixture of silybin A and silybin B) and silymarin (crude mixture of 8 polyphenolic compounds) molar calculations were based on a formula weight of 482.1 as if they were single compounds. Human prostate carcinoma PC3 and LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). RPMI1640 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, Cdc25A, Cdc25B, Cdc25C, S-phase kinase associated protein 2 (Skp2), cyclin D1, cyclin D3, cyclin E, cyclin B1 and cyclin A were from Santa Cruz Biotechnology (Santa Cruz, CA). Propidium iodide (PI) and antibody for  $\beta$ -actin were from Sigma-Aldrich Chemical Co. (St Louis, MO). ECL detection system and anti-mouse HRP-conjugated secondary antibody were from GE Healthcare (Buckinghamshire, UK). Antibodies for Cdc2, pChk2 Thr68, Chk2, cleaved Poly (ADP-ribose) polymerase (cPARP), cleaved caspase 3, cleaved caspase 9 and anti-rabbit peroxidase-conjugated secondary antibody were obtained from Cell Signaling (Beverly, MA). All other reagents were obtained in their commercially available highest purity grade.

### Cell culture and treatments

PC3 and LNCaP cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin G and 100  $\mu$ g/ml streptomycin sulfate at 37°C in a humidified 5% CO<sub>2</sub> incubator. Cells were plated and treated at 40–50% confluency with different concentrations of compounds (60–90  $\mu$ M in medium), dissolved originally in dimethyl sulfoxide (DMSO), for the indicated time periods (12–72 hr) in complete serum conditions. 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, cell morphology was examined under a light microscope and photomicrographs were taken using Kodak DC290 digital camera (200 $\times$  magnification).



**Figure 1.** Chemical structure of pure compounds isolated from silymarin (*Silybum marianum*).

### Cell growth and death assays

In each case, cells were plated to about 40–50% confluency and treated with compounds under serum condition as detailed earlier. After the desired treatment time, 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 group and time point had 3 independent plates. The experiment was repeated at least twice under identical conditions.

### Clonogenic assay

PC3 cells ( $\sim 1 \times 10^3$ ) were plated in 6-well plates and, after being permitted to attach to the plates for 24 hr, treated with 90  $\mu$ M of isomers every 48 hr. At the end of 10th day, cells were washed twice with ice cold PBS, fixed with mixture of methanol and glacial acetic acid (3:1) for 10 min and then stained with 1% crystal violet in methanol for 15 min followed by washing with deionized water. Colonies with more than 50 cells were scored and counted under the microscope.

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

After identical treatments as detailed earlier, 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  $\mu\text{g/ml}$  PI (w/v), 0.1 mM ethylenediaminetetraacetic acid and 10  $\mu\text{g/ml}$  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 earlier, cell lysates were prepared in nondenaturing lysis buffer as reported earlier,<sup>17</sup> and 50–70  $\mu\text{g}$  of protein lysate per sample was denatured in 2 $\times$  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 hr at room temperature. Membranes were probed for the protein levels of desired molecules using specific primary antibodies followed by the appropriate peroxidase-conjugated secondary antibody and visualized by ECL detection system. To ensure equal protein loading, each membrane was stripped and reprobed with anti- $\beta$ -actin antibody, which was also used to normalize for differences in protein loading in densitometric analyses.

## Statistical analysis

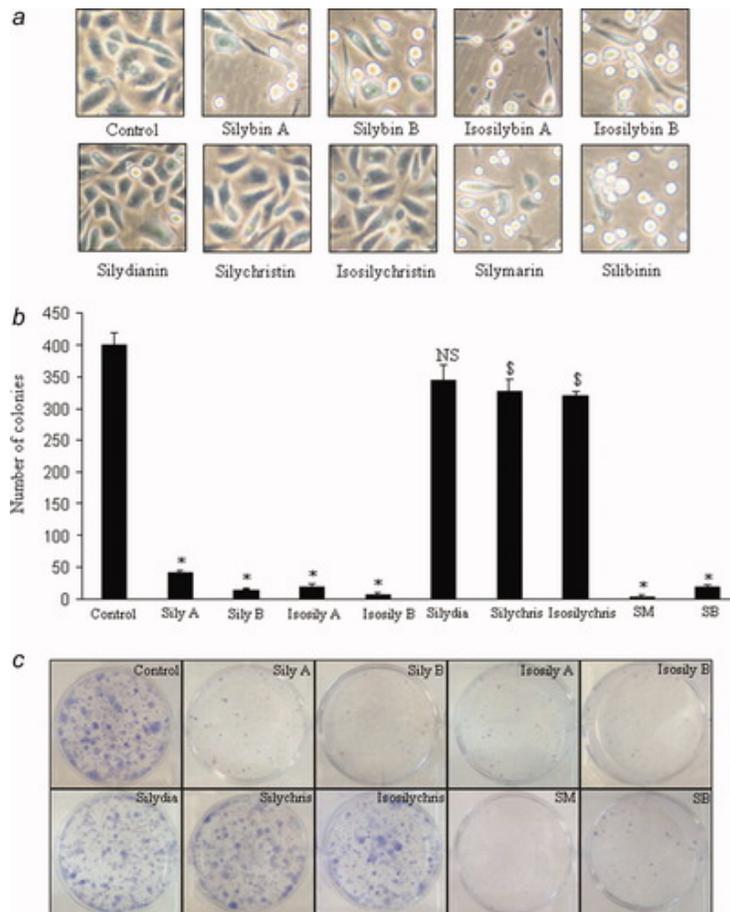
Statistical analysis was performed using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA). Data was analyzed using *t*-test and a statistically significant difference was considered to be at  $p < 0.05$ . The autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, 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 (DMSO treated) after normalization with respective loading controls ( $\beta$ -actin).

## Results

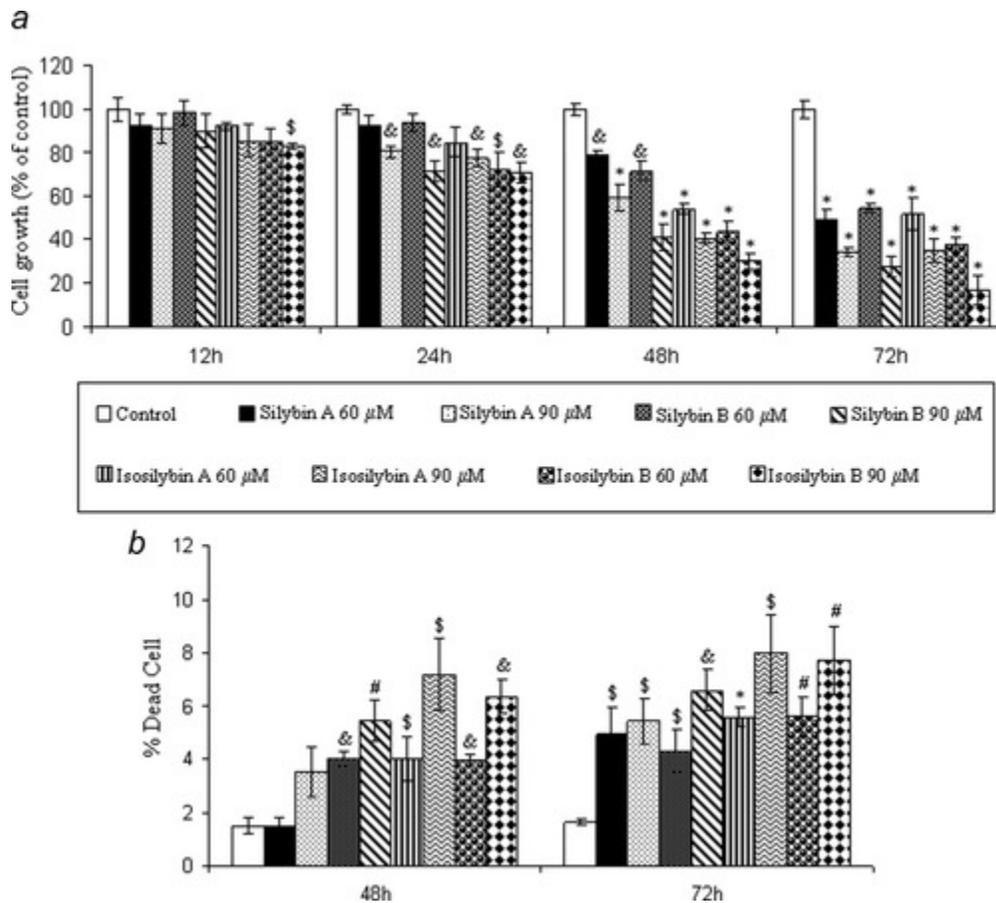
### Effect of pure compounds, SM and SB on the morphology and clonogenicity of PC3 cells

The effect of pure compounds (at 90  $\mu\text{M}$ ) silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin and silymarin and silibinin was analyzed after 72 hr of treatment. As shown in Figure 2a, treatment of these compounds except silydianin, silychristin and isosilychristin resulted in decreased cell density accompanied with significant morphological changes in PC3 cells. We observed a trend toward increased rounding off cells after treatment with these compounds, but further studies are needed to understand the nature of morphological changes. Next we assessed the long-term effect of these compounds on PC3 cells growth using

clonogenic assay. The aim for this experiment was to evaluate whether the compounds silydianin, silychristin and isosilychristin, which relatively had no effect on the cell density and morphological changes in short term treatment (72 hr), could affect PC3 cell growth following longer exposure. Treatment of silybin A, silybin B, isosilybin A and isosilybin B for 9 days significantly inhibited the colony formation by 89.8%, 96.1%, 94.9% and 97.8%, respectively ( $p < 0.001$ ) (Figs. 2b and 2c), while the effect of silydianin (14.3% inhibition), silychristin (18.2% inhibition) and isosilychristin (19.9% inhibition) was comparatively lesser (Figs. 2b and 2c). The rank order of potency of isomers was: isosilybin B > silybin B > isosilybin A > silybin A > isosilychristin > silychristin > silydianin. Both silymarin and silibinin were effective with 98.6% and 95% inhibition of colony formation in PC3 cells ( $p < 0.001$ ) (Figs. 2b and 2c).



**Figure 2.** Comparative effect of pure compounds, SM and SB on human PCa cell morphology and growth. (a) PC3 cells were treated at about 40% confluency with DMSO or 90  $\mu\text{M}$  of silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, silymarin and silibinin for 72 hr. Pictures were taken by Kodak DC290 digital camera ( $\times 200$  magnification). Each picture is an average representation of 3 plates. (b) PC3 cells ( $\sim 1 \times 10^3$ ) were plated in 6-well plates and 24 hr later cells were treated with DMSO or 90  $\mu\text{M}$  of silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, silymarin or silibinin. Fresh media with DMSO or compounds were added every 48 hr. After 9 days of treatment, cells were processed as mentioned in “Material and methods” and colonies were counted. The data shown are mean  $\pm$  standard error of mean of 3 plates with similar treatment. These results were almost similar in 2 independent experiments. \*,  $p \leq 0.001$ ; &,  $p \leq 0.005$ ; #,  $p \leq 0.01$ ; \$,  $p \leq 0.05$ . (c) Pictures were taken with a digital camera. Each picture is an average representation of three plates. Abbreviations: sily A, silybin A; sily B, silybin B; isosily A, isosilybin A; isosily B, isosilybin B; silydia, silydianin; silychris, silychristin; isosilychris, isosilychristin; SM, silymarin; SB, silibinin.



**Figure 3.** Comparative effect of silybin A, silybin B, isosilybin A and isosilybin B on PC3 cells growth and death. PC3 cells were treated with DMSO or 60–90  $\mu$ M concentrations of silybin A, silybin B, isosilybin A and isosilybin B for 12, 24, 48 and 72 hr. At each treatment time, both adherent and non-adherent cells were collected and processed for determination of total cell number (a) and dead cells (b) as mentioned in “Material and methods.” Each bar represents the mean  $\pm$  standard error of mean of three samples. These results were almost similar in two independent experiments. \*,  $p \leq 0.001$ ; &,  $p \leq 0.005$ ; #,  $p \leq 0.01$ ; \$,  $p \leq 0.05$ .

#### Effect of silybin A, silybin B, isosilybin A and isosilybin B on PC3 cell growth

Since silybin A, silybin B, isosilybin A and isosilybin B were observed as most effective in long term growth assay, next we assessed their efficacy (at 60 and 90  $\mu$ M) on cell growth and death as a function of their treatment time points (12, 24, 48 and 72 hr). After 12 hr treatment with these compounds, only isosilybin B (90  $\mu$ M) was effective in decreasing the cell number significantly (17.1%,  $p < 0.05$ ) (Fig. 3a). After 24 hr of treatment, only higher concentrations of silybin A, silybin B and isosilybin A, that is, 90  $\mu$ M inhibited the PC3 cell growth by 19.8, 29 and 22.9%, respectively ( $p < 0.005$ ), while both concentrations of isosilybin B (60 and 90  $\mu$ M) effectively inhibited the PC3 cell growth (28.2 and 29.7%, respectively,  $p < 0.05$  to  $p < 0.005$ ) (Fig. 3a). At 48 hr of treatment, all the compounds significantly inhibited the cell growth in a dose-dependent manner with isosilybin B having most evident effect. All the compounds except silybin A (41.1% inhibition) inhibited the cell growth by more than 50% at 90  $\mu$ M with 59.2%, 59.5% and 70.1% inhibition by silybin B, isosilybin A and isosilybin B, respectively (Fig. 3a). After 72 hr of treatment, all the 4 compounds significantly inhibited the cell growth by more than 50% even

at 60  $\mu\text{M}$  ( $p < 0.001$ ) (Fig. 3a), and cell growth inhibition with 90  $\mu\text{M}$  was 66.1% (silybin A), 72.32% (silybin B), 65.3% (isosilybin A) and 83.3% (isosilybin B) (Fig. 3a). The rank order of potency for growth inhibition with the 90  $\mu\text{M}$  concentration at 72 hr was: isosilybin B > silybin B > silybin A  $\approx$  isosilybin A.

#### Effect of silybin A, silybin B, isosilybin A and isosilybin B on PC3 cell death

We also measured the effect of silybin A, silybin B, isosilybin A and isosilybin B on cell death in PC3 cells using the trypan blue exclusion assay. None of the isomers significantly affected the cell death after 12 hr of treatment (data not shown). After 24 hr of treatment, only isosilybin A and B resulted in significant cell death ( $p < 0.05$ ) (data not shown), while after 48 hr of treatment, silybin B, isosilybin A and isosilybin B caused significant cell death ( $p < 0.05$  to  $p < 0.005$ ) (Fig. 3b). At the 72 hr time point, all the compounds significantly induced the cell death ( $p < 0.05$  to  $p < 0.001$ ) (Fig. 3b). Silybin A treatment resulted in 3.0- and 3.4-fold increases in cell death with 60 and 90  $\mu\text{M}$ , respectively. Similarly, silybin B caused increases of 2.7- and 4.0-fold; isosilybin A caused 3.4- and 4.9-fold increases and isosilybin B caused 3.4- and 4.8-fold increase in cell death at 60 and 90  $\mu\text{M}$ , respectively (Fig. 3b). At 72 hr with 90  $\mu\text{M}$  of each compound, the rank order of efficacy was isosilybin A  $\approx$  isosilybin B > silybin B > silybin A. But overall, maximum cell death because of these compounds remained less than 8% (Fig. 3b), even at highest dose and at 72 hr time point, which suggest that cell death is not the major reason for strong decrease in cell number observed. In addition, the effect of these compounds was analyzed on PARP cleavage (a sensitive indicator of apoptosis) and expression of the activated form of caspases (caspase 3 and 9) to determine their apoptotic effect. Treatment with these compounds only slightly increased cleaved PARP, while activated form of caspases (caspase 3 and 9) were not detected by western blotting (data not shown), suggesting minimal or no apoptosis with these compounds in PC3 cells.

#### Effect of pure compounds, SM and SB on cell cycle distribution

Cancer chemopreventive agents have been reported to retard the growth of cancer cells via inducing cell cycle arrest.<sup>10, 33, 34</sup> Accordingly, next we compared the effect of these pure compounds on cell cycle by FACS analysis. Treatment with 60 and 90  $\mu\text{M}$  concentrations of silybin A, silybin B, isosilybin A, silymarin and silibinin for 72 hr resulted in G1 arrest in a concentration-dependent manner ( $p < 0.005$  to  $p < 0.001$ ), and silybin A was most effective in inducing G1 arrest (Table I). Silydianin treatment also induced moderate G1 arrest in PC3 cells but without any dose correlation ( $p < 0.05$  to  $p < 0.01$ ) (Table I). Overall, the increase observed in G1 cell population with these compounds was at the expense of S and G2/M cell population. Silychristin treatment at both concentrations did not affect the cell cycle, while isosilychristin treatment caused only a slight increase in G2/M population at 60  $\mu\text{M}$  ( $p < 0.005$ ) (Table I). Isosilybin B was unique among all the pure compounds in its effect on cell cycle. At 60  $\mu\text{M}$  isosilybin B, a moderate increase in G1 population ( $p < 0.005$ ) was observed and there was a moderate increase in G2M population which was statistically insignificant (Table I). At 90  $\mu\text{M}$ , isosilybin B treatment resulted in a strong increase in G2/M population ( $p < 0.001$ ) (Table I). Isosilybin B treatment caused a strong decrease in S-phase population in a concentration-dependent manner ( $p < 0.001$ ) (Table I). Overall, silydianin, silychristin and isosilychristin seem to contribute less to silymarin induced cell cycle arrest. It is also interesting to note that silymarin

treatment caused only a G1 arrest in PC3 cells, suggesting that the effect of isosilybin B on cell cycle (G2/M arrest) have been totally masked by other constituents of silymarin. Isosilybin B comprises only 2.1% to 4.4% of commercial silymarin extracts.<sup>20</sup>

**Table I.** Comparative Effect of Silymarin and its Constituents on Cell Cycle Distribution in Human PCa PC3 Cells

Treatment	Concent ration	G1 (mean ± s.e.m)	S (mean ± s.e.m)	G2/M (mean ± s.e.m)
Control	DMSO	55.24 ± 0.93	29.36 ± 0.61	15.40 ± 0.43
Silybin A	60 µM	78.16 ± 0.89*	14.35 ± 1.02*	7.49 ± 0.34*
	90 µM	84.35 ± 1.82*	8.58 ± 1.06*	7.08 ± 0.90*
Silybin B	60 µM	73.86 ± 2.79&	16.74 ± 1.30*	9.40 ± 1.50\$
	90 µM	76.47 ± 0.75*	14.10 ± 0.96*	9.42 ± 0.61*
Isosilybin A	60 µM	67.14 ± 1.41&	21.84 ± 0.88&	11.02 ± 0.54
	90 µM	73.69 ± 0.79*	16.62 ± 1.06*	9.69 ± 0.30*
Isosilybin B	60 µM	61.50 ± 1.17\$	19.02 ± 0.78*	19.48 ± 1.79 <sup>NS</sup>
	90 µM	50.42 ± 0.78\$	17.36 ± 0.86*	32.22 ± 0.79*
Silydianin	60 µM	60.45 ± 1.10\$	25.66 ± 0.78\$	13.89 ± 1.03 <sup>NS</sup>
	90 µM	60.99 ± 0.67#	24.17 ± 0.52&	14.83 ± 0.17 <sup>NS</sup>
Silychristin	60 µM	53.83 ± 2.00 <sup>NS</sup>	30.77 ± 1.09 <sup>NS</sup>	15.40 ± 1.03 <sup>NS</sup>
	90 µM	54.35 ± 1.03 <sup>NS</sup>	30.78 ± 0.39 <sup>NS</sup>	15.28 ± 0.74 <sup>NS</sup>
Isosilychristin	60 µM	51.77 ± 0.28\$	30.78 ± 0.03 <sup>NS</sup>	17.45 ± 0.30\$
	90 µM	55.68 ± 3.58 <sup>NS</sup>	27.85 ± 2.05 <sup>NS</sup>	16.48 ± 1.54 <sup>NS</sup>
Silymarin	60 µM	74.26 ± 0.62*	15.29 ± 0.73*	10.45 ± 0.54&
	90 µM	76.27 ± 0.88*	13.07 ± 0.66*	10.66 ± 0.48&
Silibinin	60 µM	74.94 ± 0.59*	13.65 ± 0.68*	8.39 ± 0.437*
	90 µM	77.97 ± 0.91*	13.74 ± 0.72*	11.31 ± 0.66#

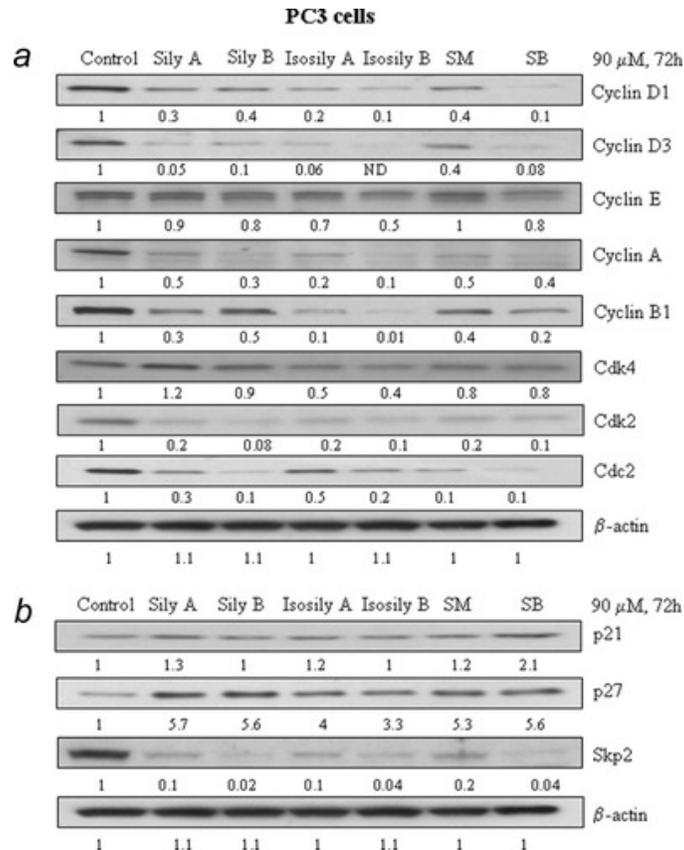
\*  $p \leq 0.001$ . &  $p \leq 0.005$ . #  $p \leq 0.01$ . \$  $p \leq 0.05$ . NS, Not Significant.

#### Effect of pure compounds, SM and SB on the expression of cyclins, CDKs and CDKIs

Next, we compared the effect of these compounds on the expression of cyclins, CDKs and CDKIs, which are known to regulate the cell cycle progression.<sup>23, 25, 26, 28</sup> Treatment with 90 µM of silybin A, silybin B, isosilybin A and isosilybin B, SM and SB for 72 hr resulted in a moderate to strong decrease in the expression of cyclin D1, D3, E, A and B1 as well as decrease in the expression of Cdk2, 4 and Cdc2 in PC3 cells (Fig. 4a). These compounds were observed to have similar effects on modulation of cell cycle regulatory molecules but differed in the degree of modulation. In general, isosilybin B was most effective in decreasing the levels of cyclins (D1, D3, E, A and B1) and CDKs (4, 2 and Cdc2) (Fig. 4a). No significant changes were observed in the expression of aforementioned cyclins and CDKs expression with silydianin, silychristin and isosilychristin treatment (90 µM) in PC3 cells after 72 hr of treatment (data not shown). In all cases, membranes were stripped and re probed with  $\beta$ -actin antibody for protein loading correction.

CDKIs are known to regulate the cell cycle via controlling the activity of CDKs.<sup>23, 28</sup> Our results showed that silybin A, silybin B, isosilybin A and isosilybin B, SM and SB significantly induced the levels of p27, while the effect on p21 expression was relatively less or absent (Fig. 4b). The cellular levels of CDKIs especially p27 is known to be regulated by Skp2 (S-phase kinase-associated protein 2).<sup>35, 36</sup> Skp2 is an E3-ligase and is known to be overexpressed in many cancers including PCa.<sup>37, 38</sup> Therefore, we next examined the effect of these compounds on

cellular Skp2 level. Treatment with silybin A, silybin B, isosilybin A and isosilybin B, SM and SB for 72 hr significantly reduced the expression of Skp2 (Fig. 4b). Under similar exposure conditions, there was no effect of silydianin, silychristin and isosilychristin on the expression levels of p21, p27 and Skp2 (data not shown). Again, in all cases the membranes were stripped and reprobed with  $\beta$ -actin antibody for protein loading correction.

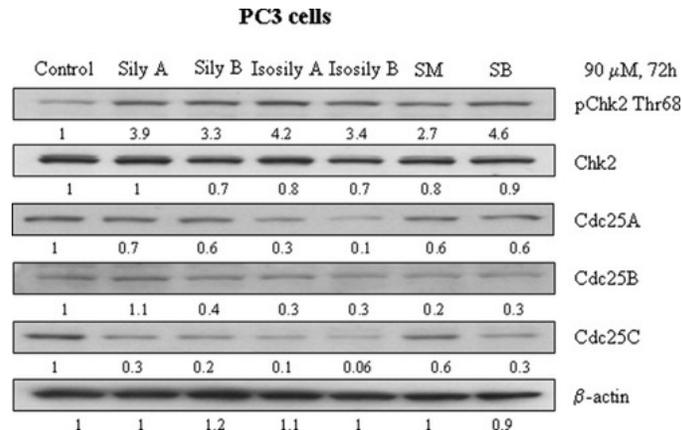


**Figure 4.** Comparative effect of pure compounds, SM and SB on cell cycle regulatory molecules in human PCa PC3 cells. PC3 cells were treated with DMSO or 90  $\mu$ M doses of silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, silymarin and silibinin for 72 hr. After the treatment time, cell lysates were prepared in nondenaturing lysis buffer as mentioned in “Material and methods.” For each sample, 50–60  $\mu$ g of protein lysate was used for SDS-PAGE and western immunoblotting, and membranes were probed for (a) cyclin D1, cyclin D3, cyclin E, cyclin A, cyclin B1, Cdk4, Cdk2, and Cdc2, (b) p21, p27 and Skp2. In all cases membranes were also stripped and reprobed with anti- $\beta$ -actin antibody for protein loading correction. The densitometry data presented below the bands are “fold change” as compared with control after normalization with the respective loading control. Abbreviations: sily A, silybin A; sily B, silybin B; isosily A, isosilybin A; isosily B, isosilybin B; SM, silymarin; SB, silibinin; ND, not detectable.

#### Effect of pure compounds, SM and SB on cellular check point and Cdc25 phosphatases

Various studies have shown that cellular checkpoints are activated in response to DNA damage, which causes phosphorylation and inactivation of Cdc25 phosphatases resulting in cell cycle arrest.<sup>10, 30, 39</sup> A similar phenomenon was observed earlier where silibinin and silymarin treatment resulted in Chk2 activation and cell cycle arrest.<sup>10</sup> So, we next compared the effect of these compounds on checkpoint activation. Treatment with silybin A, silybin B, isosilybin A and isosilybin B, SM and SB strongly increased the phosphorylation of Chk2 at Thr68 site, with

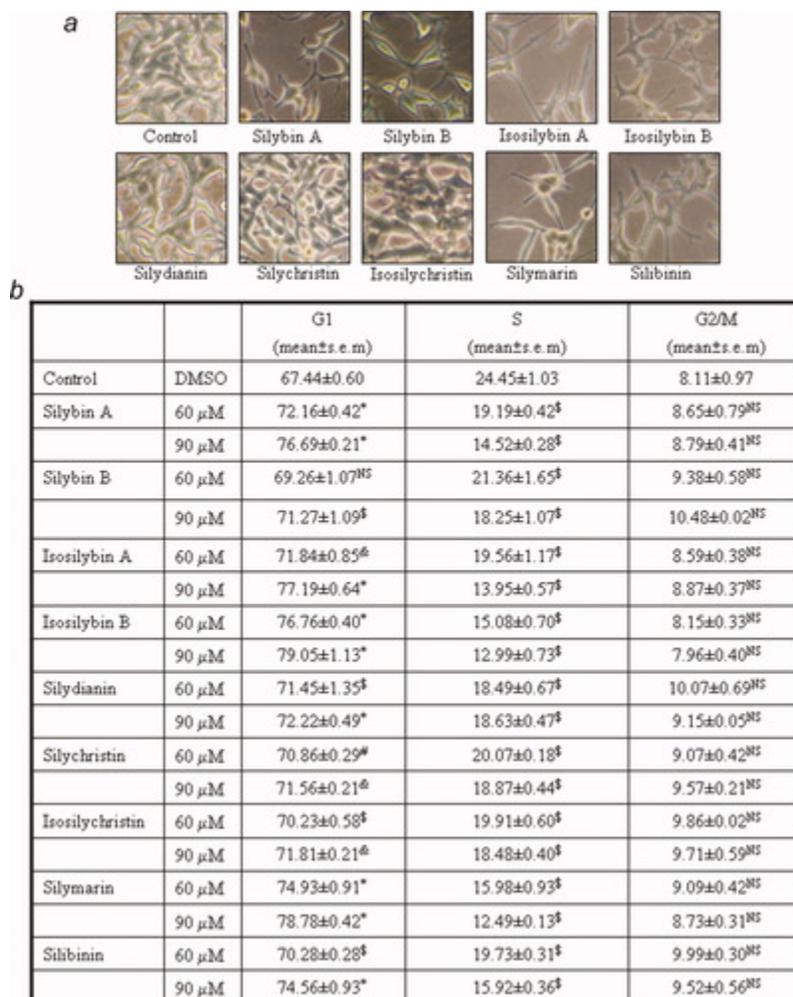
slight or no decrease in the total Chk2 levels (Fig. 5). These results were associated with a strong decrease in the levels of Cdc25A, B and C after treatment with silybin A, silybin B, isosilybin A and isosilybin B, SM and SB (Fig. 5). Under similar treatment conditions, the effect of silydianin, silychristin and isosilychristin on these molecules was not significant (data not shown).



**Figure 5.** Comparative effect of pure compounds, SM and SB on checkpoint activation and Cdc25 phosphatase inhibition in human PCa PC3 cells. PC3 cells were treated with DMSO or 90  $\mu$ M doses of silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, silymarin or silibinin for 72 hr. After the treatment time, cell lysates were prepared in nondenaturing lysis buffer as mentioned in “Material and methods”. For each sample, 50-60  $\mu$ g of protein lysate was used for SDS-PAGE and western immunoblotting, and membranes were probed for pChk2 Thr68, Chk2, Cdc25A, Cdc25B and Cdc25C. In all cases membranes were also stripped and reprobed with anti- $\beta$ -actin antibody for protein loading correction. The densitometry data presented below the bands are “fold change” as compared with control after normalization with respective loading control. Abbreviations: sily A, silybin A; sily B, silybin B; isosily A, isosilybin A; isosily B, isosilybin B; SM, silymarin; SB, silibinin.

#### Effect of pure compounds, SM and SB in androgen dependent human PCa LNCaP cells

Results in androgen-independent cancer PC3 cells clearly suggest a differential effect of these compounds on growth, cell cycle and cell cycle regulatory molecules. We also compared the efficacy of these compounds in androgen-dependent LNCaP cells. Figure 6a shows the effect of these compounds on LNCaP cells morphology. As with PC3 cells, the effect of silydianin, silychristin and isosilychristin on LNCaP cells appears minimal, while silybin A, silybin B, isosilybin A, isosilybin B, silymarin and silibinin treatment (90  $\mu$ M for 72 h) resulted in decreased cell density and morphological changes (Fig. 6a). In general, LNCaP cells appeared more elongated after treatment with these compounds, but further studies are needed to understand the nature of morphological changes. In another experiment, FACS analysis showed that all the compounds (60 and 90  $\mu$ M for 72 hr) cause G1 arrest in a concentration-dependent manner, albeit to different extent (Fig. 6b). In general, silybin B, silydianin, silychristin, isosilychristin were least effective, and isosilybin B was most effective in inducing the G1 arrest ( $p < 0.001$ ), while the effect of silibinin, silybin A, isosilybin A and silymarin was intermediate (Fig. 6b). Further, the observed increase in G1 cell population was mainly at the expense of the S phase population (Fig. 6b). We also compared the effect of these compounds on cell cycle regulatory molecules. Isosilybin B was most effective in decreasing the levels of cell cycle regulatory molecules-cyclin D1, cyclin D3, cyclin E, cyclin A, Cdk4 and Cdk2, and in inducing p27 levels (data not shown).



**Figure 6.** Comparative effect of pure compounds, SM and SB on morphology and cell cycle distribution in human PCa LNCaP cells. (a) LNCaP cells were treated at 40% confluency with DMSO or 90 μM concentrations of silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, silymarin and silibinin for 72 hr. Pictures were taken using Kodak DC290 digital camera (×200 magnification). (b) LNCaP cells were treated with DMSO or 60–90 μM concentrations of silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, silymarin and silibinin for 72 hr. After the treatment time, both adherent and nonadherent cells were collected and incubated overnight with saponin/PI solution at 4°C then subjected to fluorescence-activated cell sorting analysis as detailed in “Material and methods.” The data shown are mean ± standard error of mean of three samples for each treatment. The results were similar in two independent experiments. \*,  $p \leq 0.001$ ; &,  $p \leq 0.005$ ; #,  $p \leq 0.01$ ; \$,  $p \leq 0.05$ .

## Discussion

The higher incidences of prostate cancer in the Western world compared with South and East Asian countries have been attributed mainly to lifestyle and dietary habits. In general, the high fat, high meat and lesser plant-based diet in the western countries pose a higher risk for prostate cancer.<sup>40, 41</sup> Therefore, lifestyle modifications and preventive measures are needed for lowering the burden of this malignancy. In this regard, the role of phytochemicals in prostate cancer management has been suggested and numerous studies have shown a beneficial role of

phytochemicals, especially polyphenolic compounds, in prostate cancer prevention and/or intervention.<sup>8-12, 42</sup>

Silymarin is a mixture of polyphenolic compounds and has a long history of human use.<sup>13</sup> There are numerous *in vivo* and *in vitro* reports showing the efficacy of silymarin and silibinin against PCa.<sup>10, 14, 16, 18, 43, 44</sup> Silymarin treatment was shown to inhibit the 3,2-dimethyl-4-aminobiphenyl (DMAB)-induced prostate carcinogenesis in male F344 rats.<sup>45</sup> Silibinin treatment also inhibited the human prostate cancer PC3 and DU145 cells xenograft growth in athymic nude mice via inhibiting proliferation, angiogenesis and by inducing apoptosis.<sup>43, 44, 46</sup> The anticancer effects of silymarin and silibinin have also been related to their inhibitory effect on androgen receptor mediated signaling, EGFR-signaling and NF- $\kappa$ B-signaling in human PCa cells.<sup>14, 47, 48</sup> Further, both silymarin and silibinin have been shown to induce cell cycle arrest in human PCa LNCaP, PC3 and DU145 cells via modulating the expression of key cell cycle regulatory molecules.<sup>10, 14, 34, 48</sup> Silymarin and silibinin treatment also inhibited the growth and survival of human umbilical vein endothelial cells (HUVEC).<sup>49, 50</sup> In our completed phase I clinical trials in prostate cancer patients, oral administration of silibinin-phytosome (siliphos) resulted in biologically relevant serum levels of free silibinin (about 100  $\mu$ M) without any major toxicity.<sup>51</sup> Despite this progress, the relative contribution of individual silymarin constituents to its anticancer efficacy largely remains unknown. Therefore, the aim of the present study was to define whether the constituents of silymarin have distinct molecular targets that act in tandem or each compound exhibited similar activities with variable potency. Cell growth data from the present study suggest that these compounds exhibit similar but variable potency in their anti-cancer action. Their effect on molecular targets suggests that these compounds might have subtle differences in their action and might contribute differentially in determining the anticancer effect of silymarin.

Deregulated cell cycle and abnormal expression of cell cycle regulators (cyclins-Cdks-Cdc25 overexpression or loss of CDKIs and cellular checkpoints) are known to be essential elements in prostate carcinogenesis.<sup>52</sup> The present study showed that silybin A, silybin B, isosilybin A and isosilybin B were effective in modulating the expression of cell cycle regulators and inducing cell cycle arrest, while there was marginal or no effect of silydianin, silychristin and isosilychristin on cell cycle or cell cycle regulators. Together, silydianin, silychristin and isosilychristin constitute 30% of silymarin and might lower the effect of silymarin on cell cycle and cell cycle regulatory molecules.

The present study also reveals isosilybin B as the most potent flavonolignan in the following: (i) inhibiting colony formation, (ii) inhibiting cell growth, (iii) causing cell cycle arrest, (iv) altering the expression of cell cycle regulators in PC3 cells. Isosilybin B composes only 2.1% to 4.4% of silymarin depending upon the source and formulation and, therefore, the potency of silymarin may be enhanced by enriching it with isosilybin B and/or by decreasing the ratio of inactive constituents. Further, the present work also suggests that the anticancer efficacy of isosilybin B, as a single isomer, should be examined. In this direction, we reported that isosilybin B was effective in inhibiting the growth of prostate cancer LNCaP and 22Rv1 cells by causing cell cycle arrest and apoptosis.<sup>42</sup> Further, isosilybin B seems to be relatively nontoxic, like silymarin and silibinin, as it exerted very little cytotoxicity against non-neoplastic prostate epithelial PWR-

1E cells.<sup>42</sup> Further *in vitro* and *in vivo* studies with isosilybin B are in progress and might be helpful in drawing future clinical use of silymarin.

All the 7 pure compounds used in the present study have same chemical formula (C<sub>25</sub>H<sub>22</sub>O<sub>10</sub>) and molecular mass (482.1), and also possess same flavonoid nucleus but differ in the conformation lignan moiety (Fig. 1). In the plant, these various conformations are catalyzed by a peroxidase reaction between the flavonoid, dihydroquercetin (taxifolin) and coniferyl alcohol.<sup>53</sup> Silybin A, silybin B, isosilybin A and isosilybin B contain the lignan moiety with identical functional groups, but differ with respect to the stereochemical positions of the terminal aryl(3-methoxy-4-hydroxyphenyl) and hydroxymethyl groups at the  $\alpha$  and  $\beta$  carbons (Fig. 1). So, the overall inactivity of silydianin, silychristin and isosilychristin could be related to their different condensation products as compared to silybin A, silybin B, isosilybin A and isosilybin B. These differences in the structure, however little, might determine their biological efficacy. Further studies are needed to clearly understand the structure-activity relationships of these compounds.

Over the last few decades, silymarin has been extensively researched and reported to have impressive bioactivity, albeit limited by poor bioavailability.<sup>54</sup> Therefore, numerous attempts have been aimed at increasing its bioavailability by complexing silymarin or silibinin with phospholipids mainly phosphatidylcholine (PC).<sup>54</sup> However, till recently, not such effort has been made toward analyzing the pharmacokinetics and metabolism of individual flavonolignans. In a recent study, Wen *et al.* analyzed the plasma concentrations of free, conjugated (sulfated and glucuronidated), and total levels of silymarin flavonolignans after a single oral dose of 600 mg standardized milk thistle extracts to 3 healthy volunteers.<sup>55</sup> Pharmacokinetic analysis indicated that silymarin flavonolignans were rapidly eliminated with short half-lives (1 to 3 and 3 to 8 hr for the free and conjugated, respectively).<sup>55</sup> The mean  $AUC_{0 \rightarrow \infty}$  (area under the plasma concentration-time curve from time 0 to infinity) values of the free and conjugated silymarin flavonolignans were as follows: silybin A [63 and 208 ( $\mu\text{g h}/\text{l}$ )], silybin B [51 and 597 ( $\mu\text{g h}/\text{l}$ )], isosilybin A [30 and 734 ( $\mu\text{g h}/\text{l}$ )], isosilybin B [26 and 355 ( $\mu\text{g h}/\text{l}$ )].<sup>55</sup> This study clearly suggested that the individual silymarin flavonolignans are quickly metabolized and exhibits quite different plasma profiles for both the free and conjugated fractions.<sup>55</sup> However, more *in vivo* studies are warranted to understand the stability and bioavailability differences of these flavonolignans.

In conclusion, present study further shows the differential effects of these pure compounds on various molecular targets and also identifies isosilybin B as a valuable anticancer agent. This study might be helpful to prepare a better and more effective silymarin formulation. Further, *in vitro* and *in vivo* studies are warranted to shed more light on the potential clinical utility of these compounds against prostate cancer.

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