Chapter-VI

Chemopreventive effects of silibin in against DMBA-initiated and TPA-promoted two-stage skin tumorigenesis in Swiss albino mice
Introduction

Inflammation is one of the most important etiological factors associated with most of the chronic human diseases including cancer. It is well documented that chronic inflammation act as an important trigger and play key role in initiation, promotion and progression stage of cancer development (Kundu and Surh, 2008; Balkwill and Mantovani, 2002; Coussens and Werb; 2000). Various observations reveal that inflammation is one of the key events inducing cancer development by various mechanisms, such as production of ROS and RNS radical oxidants, production of growth-promoting cytokines, inducing down regulation of tumor suppressors and stimulation of signal transduction pathways (Reuter et al., 2010; Dannenberg et al., 2005; Caruso et al., 2004). Uncontrolled production of ROS or change in intracellular antioxidants level causes damage or modification of cellular macromolecules resulting in up or down expression of regulators of normal cellular physiology (Waris and Ahsan, 2006; Droge, 2000). ROS are the important tumor promotional triggers involve in multistage cancer development as they interfere with the signaling components of a number of pathways and the expression of genes (Waris and Ahsan, 2006). Aberrant production of inflammatory proinflammatory cytokines TNF-α, IL-6, IL-1β and nitric oxide have critical role in roles in multistage cancer development, including initiation, promotion, malignant conversion, invasion and metastasis (Hussain and Harris, 2007; Barker et al., 1991).

Cyclooxygenase (COX) is one of the well known enzymes catalyze the metabolism of eicosanoids and two main isoforms of COX has been identified as COX-1 and COX-2. COX-2, a rate limiting enzyme in the biosynthesis of prostaglandins, exhibit one of the best well known molecular links between inflammation and carcinogenesis as the inappropriate over expression of COX-2 implicated with various processes including inflammation, tumorigenesis and carcinogenesis (Cao and Prescott, 2002).
expression/ elevated activity of COX-2 has been observed in cells or tissue exposed with stimuli like cytokines, endotoxins, growth factors, lipopolysaccharide, ultraviolet radiation and phorbol ester (Nakadate, 1989).

TPA is the most widely used distinguished tumor promoting agent to understand the molecular and cellular alterations associated with carcinogenesis as it induces oxidative stress, ROS production, inflammation and hyperplasia (Richl et al., 2010; Ha et al., 2006; Clark et al., 1985). Research studies have revealed that it is a well-known model to understand the role of ROS, inflammation and hyperplasia in the promotion stage of carcinogenesis (Ha et al., 2006).

Inflammatory agents, carcinogens, phorbol ester, TNF-α and lipopolysaccharides (LPS) induces activation of NF-kB (Bowie and O'Neill, 2000). NF-kB, an important ubiquitous redox sensitive transcription factor known to regulate the expression of genes involved in inflammation, cellular transformation, proliferation, apoptosis suppression, invasion, angiogenesis and metastasis (Aggarwal, 2004; Karin and Delhase, 2000). Although, various signaling cascade component have been found to involve in promotional stage of cancer development but those that congregate with NF-kB has potential role in tumor promotion (Balkwill and Mantovani, 2002).

Silibinin [(2R,3R)-3,5,7-trihydroxy-2-[(2R,3R)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxyl methyl)-2,3-dihydrobenzo[β][1,4] dioxin-6-yl]chroman-4-one] is a major bioactive flavonolignan present in milk thistle (Silybum marianum) and has antioxidant, anti-inflammatory and anti-carcinogenic potential (Gu et al., 2007; Kren and Walterova, 2005, Singh and Agarwal, 2005). It has been used as a traditional medicine for the treatment of various liver disorders (Singh and Agarwal, 2005). Moreover; it induces apoptosis and cell cycle arrest in different cancer cell lines (Singh and Agarwal, 2005).

Taking this into account we postulated that Silibinin, a natural compound having both antioxidant and anti-inflammatory potential could be effective in preventing tumor promotional damage caused by TPA in mouse skin. To test this possibility, we have
studied the effects of silibinin on the TPA-induced cutaneous oxidative stress and inflammation and its anti-tumorigenic potential against DMBA-TPA-induced two-stage skin carcinogenesis.

**Treatment regimen**

**Experiment I (short-term study)**

To assess the protective effect of silibinin against double TPA treatment of mouse skin

To observe the protective effect of silibinin against TPA induced cutaneous oxidative and inflammatory responses, animals (Swiss albino mice) were divided into four groups (I-IV) of six mice each (n=6). Dorsal skin of each animal was shaved with the electric clipper two days prior to the start of the experiment. Animals of group I (control) were treated with topical application of only 0.2 ml acetone (vehicle). Group II animals were given topical application of TPA (10 nmol) in 0.2 ml acetone. Animals of the group III were given topical pre-treatment of the silibinin at the dose of 9 mg in 0.2 ml acetone 30 min before TPA [(10 nmol) in 0.2 ml acetone] application. Group IV animals were treated with topical application of silibinin (9 mg in 0.2ml acetone) only. The treatments were carried out for 2 days at the interval of 24 hrs. All the animals were sacrificed by cervical dislocation 1 h after the last TPA treatment and skin tissue was processed for the evaluation of different parameters.

**Experiment II (long-term study)**

To assess the chemopreventive potential of silibinin against two-stage skin carcinogenesis in albino mice

For skin tumor induction, classical DMBA-TPA model was used. Animals (Swiss albino mice) were divided into four groups (I-IV) of fourteen mice each (n=14). Dorsal skin of each animal was shaved with the electric clipper two days prior to the start of the experiment.
To see the anti-tumorigenic potential of silibinin, the following treatment schedule was used.

**Group I (Control)** - Mice in this group were treated with acetone (0.2 ml) only.

**Group II (DMBA + TPA)** - Group II mice were treated on their shaved backs with a single topical application of DMBA (50 μg) dissolved in 0.2 ml acetone. One week after the initiation, TPA (2.0 μg) in 0.2 ml acetone was applied twice a week for 20 consecutive weeks.

**Group III (Anti-initiation group)** - Group III mice were pre-treated with silibinin at a dose of 9.0 mg/0.2 ml acetone/mouse/day for 14 days. On day 15 they were given topical application of DMBA (50 μg) and then after one week TPA (2.0 μg) in 0.2 ml acetone was topically applied twice a week until the termination of the experiment at 20 weeks.

**Group IV (Anti-promotion group)** - Group IV mice were given topical application of DMBA (50 μg). One week after the initiation, animals were pre-treated with silibinin at the dose of 9 mg in 0.2 ml acetone 30 minutes prior to the each TPA (2.0 μg) application twice a week until the termination of the experiment at 20 weeks.

Animals in all groups were watched for any apparent signs of toxicity and mortality during the entire period of study. The data are expressed in percentage of tumors bearing mice and number of tumors per mouse.

**Results**

**Short Term:**

**Effect of silibinin on the cutaneous NO production**

Topical application of TPA resulted in significantly elevated cutaneous NO production in the group II as compared with the acetone treated group I (p<0.001). We observed that pre-treatment with silibinin was significantly effective in reducing NO production in
group III when compared with the group II (p<0.01). There was no significant difference observed between group I and IV as far as NO production is concern (Fig. 1).

**Effect of silibinin on TPA induced oxidative stress in mouse skin**

Silibinin inhibits lipid peroxidation caused by TPA application in terms of TBARS (MDA), a well known biomarker of oxidative stress. Application of TPA leads to significant elevation in the level of MDA in the group II as compared to acetone treated group I (p<0.001). Pre-treatment with silibinin half an hour before TPA application was found significantly (p<0.01) effective in the inhibition of MDA formation. There was no significant change observed in the level of MDA between control and only silibinin treated animals (Fig. 2).

**Effect of silibinin on the cutaneous GSH content**

Pre-treatment of silibinin before the TPA application was found effective in restoring the endogenous anti-oxidant GSH. There was significant depletion in the level of GSH content in group II when compared with group I (p<0.001). Pre-treatment with silibinin in group III shows significant increase in the level of GSH content (p<0.01) as compared to group II. There was no significant difference in the GSH content between group I and IV (Fig. 3).

**Effect of silibinin on the cytoprotective enzymes**

The effect of silibinin pre-treatment on TPA induced depletion in the activity of different antioxidant enzymes was examined and the results were shown in table 1. We have observed that there was a significant (p<0.001) decrease in the activity of different antioxidant enzymes in TPA treated group II as compared to acetone treated group I. However pre-treatment with silibinin in the group III before TPA application significantly (p<0.05, p<0.01) restored the activity of antioxidant enzymes when compared with the TPA treated group II. There was no significant difference observed between the group I and IV.
Effect of silibinin on cutaneous TNF-α, IL-6 and IL-1β

We have assessed the effect of silibinin on TPA induced cutaneous TNF-α, IL-6 and IL-1β level (Fig. 4). We found that there was a significant elevation in the level of proinflammatory cytokines in TPA treated group II as compared to only acetone treated group I (p<0.001). Pre-treatment with silibinin significantly inhibit their production in the group III when compared with the only TPA treated group II (p<0.05, p<0.01, p<0.001). There was no significant difference found between group I and IV.

Effect of silibinin on the TPA-induced cutaneous immunohistochemical expression of NF-kB (p65), COX2 and iNOS

Cutaneous expression of NF-kB (p65), COX2 and iNOS are shown in the figures 5, 6 and 7 respectively. Brown colour clearly indicates the more number of cells having NF-kB, COX-2 and iNOS expression in the group II as compare to that of group I. Pretreatment with silibinin in the group III reduced the number of cells showing expression of NF-kB, COX-2 and iNOS. However there was no significant difference observed in the expression of these proteins in group IV as compared to group I. For immunohistochemical analysis, brown colour indicates specific immunostaining of NF-kB, COX-2 and iNOS and light blue colour indicates haematoxylin staining. Original magnification: x10 and x40; Scale bar 50 μm.

Long Term:

Effect of silibinin on DMBA initiated and TPA promoted skin VEGF expression, tumor incidence, multiplicity and histopathology

Cutaneous expression of the VEGF is shown in the figure 8. The unregulated expression of both VEGF is critically associated with tumor development. Darkly stained brown colour VEGF immunopositive cells were present in DMBA+TPA treated group II (Fig. 8 B) as compared to vehicle treated group I (Fig.8 A). Silibinin treated groups III (anti-initiation) Fig. 8 C, and group IV (anti-promotion) Fig. 8 D, has less immunopositive
cells with weak staining as compared with the group II. Original magnification: x40; scale bar-50μm

Topical application of silibinin resulted in the strong protection in both, tumor initiation and tumor promotion, experimental animals (Fig 9, Table 2). In the group II, 100 % tumor incidence was found as shown in Table 2, Fig 9 B and tumor multiplicity was 11.71. Silibinin pretreatment in the both anti-initiation and anti-promotion group effectively suppressed the tumor incidence as well as tumor multiplicity as compared to group II.

In case of anti-initiation group III, application of silibinin for 14 days before DMBA application resulted in effective suppression in both tumor incidence and multiplicity. At the termination of the experiment at 20 weeks, compared to 100% animals with skin tumors in the group II, only 71 % of the animals in the silibinin pre-treated group III, exhibited skin tumors accounting for 29 % inhibition in tumor incidence (Table 2). The gross tumor multiplicity in the group III was much less than those with group II (Table 2).

In case of anti-promotion experimental group IV, application of silibinin prior to each TPA application resulted in strong preventive effect against TPA-induced tumor promotion in DMBA-initiated mouse skin with reference to both tumor incidence and multiplicity (Table 2).

On the termination of the experiment at 20 weeks, compared to 100% animals with skin tumors in the group II, only 78 % of the animals in the silibinin pre-treated group IV, exhibited skin tumors accounting for 22 % inhibition in tumor incidence (Table 2). The gross tumor multiplicity in the group IV was much less than those with group II (Table 2).

The histological observations revealed that in case of DMBA+TPA treated mice tumors (group II), intense infiltration of inflammatory cells, dyskeratosis of the epidermis with
deposition of keratinocyte pearls was observed in dermis and epidermis (Fig.10 B). While, in case of silibinin treated group III and IV the damage was less as compared to group II (Fig.10 C and D).

Discussion

The findings of the present study revealed that topical application of silibinin inhibits phorbol ester induced tumor promotional triggers like expression of COX-2, i-NOS, activation of NF-kB, proinflammatory cytokine production, markers of inflammation and oxidative stress. Further, evaluation of anti-tumorigenic (anti-initiation and anti-promoting) potential of silibinin on DMBA initiated and TPA promoted mouse skin carcinogenesis showed that pretreatment with silibinin significantly reduced the tumor incidence and multiplicity.

Cancer development is a multistep process viz., initiation, promotion and progression. Topical application of different tumor promoters has been shown to induce oxidative and inflammatory responses which have close intimacy with the development of skin tumor. Therefore, TPA induced oxidative stress, inflammation and their mediators are recognised as important regulator of tumor promotion.

One of the findings of the present study is that silibinin inhibits TPA induced cutaneous lipid peroxidation, a well known marker of oxidative stress, associated with tumor promotion. Peroxidation of major cellular macromolecules lead to abnormal cellular integrity and functioning which can results in inappropriate functioning of various inter and intracellular communication pathways (Bartsch and Nair, 2006; Gerber et al.,1997). Further, it was observed that silibinin also up-regulate the level and activity of the major endogenous antioxidants and cytoprotective enzymes (GSH, catalase, GR, GPx, G6PD, and SOD) that confirms its antioxidant potential. Endogenous antioxidants defense system comprised of antioxidant enzymes responsible for the inactivation or elimination
of most of the potential cell damaging moieties implicated with promotion stage of cancer development. Present study data suggest the critical role of antioxidants and detoxifying enzymes in phorbol ester-induced early tumor promotional events and pre-treatment with silibinin suppress the oxidative damages possibly by boosting the endogenous antioxidant defense machinery via triggering their transcriptional up-regulation which is in accordance with previous observation (Katiyar et al., 1997; Zhao et al., 1999).

Silibinin inhibited TPA induced inflammatory responses, such as (leucocytes infiltration, edema formation, and hyperplasia) proinflammatory cytokines production expression of COX-2, i-NOS and activation of NF-kB.

TNF is the most prominent inflammatory mediator and plays a central role in inflammatory reaction of the innate immune system including cytokine production, activation, immune-cell proliferation, expression of adhesion molecules and induction of inflammatory processes.

TNF-α, IL-1β, IL6 and NO are the well known proinflammatory agents’ and the major signaling messengers play key roles in the promotion stage of carcinogenesis. Their role in the production of adhesion molecules, growth factors, eicosanoids, nitric oxide, angiogenic factor like VEGF, and activation of the NF-kB is also well established (Kundu and Surh, 2008).

Inappropriate expression/ production of TNF-α, IL-1β and IL-6 have been associated with promotion phase of skin carcinogenesis which was confirmed by the previous observations that mouse deficient in TNF-α, IL-6 and IL-1β are resistant to skin tumor formation (Moore et al., 1999; Tricot, 2000; Vidal-Vanalocha et al., 2000). Our data suggest that silibinin effectively suppress the production of these cytokines that implicates its possible role in chemoprevention.
Different observations indicate that inappropriate expression / activity of COX-2 is implicated particularly in promotion stage of carcinogenesis (Chun et al., 2004; Mohan and Epstein, 2003). COX-2, a key enzyme play important role in inflammatory signaling has been sorted out as one of the prime targets for chemoprevention and targeted inhibition of COX-2 expression or activity is critical for both alleviating inflammation and preventing tumor promotion (Shrottiya et al., 2010). A number of stimuli like pro-oxidant /proinflammatry cytokines, endotoxins, oncogenes and phorbol ester cause anomalous regulation of signaling regulated by kinases and transcription factors results in abnormal expression of COX-2 (Cao and Prescott, 2002). Here the inhibitory effect of silibinin on TPA-induced COX-2 expression in mouse skin delineates its anti-inflammatory and antitumor-promoting properties which is in good accordance with the observation of Gu et al., that silibinin inhibited the radiation induced COX-2 expression in mouse epidermis (Gu et al., 2007). Thus the inhibition of COX-2 expression/ activity by silibinin not only reflects its anti-inflammatory but also antitumor promoting potential.

TPA induced expression of COX-2 in mouse skin has been reported to be regulated by array of redox sensitive transcription factors, including NF-kB (Kim and Fischer, 1998) as the promoter region of COX-2 gene contain consensus sequence for binding with NF-kB. In resting cells NF-kB resides along with its cytosolic repressor inhibitory protein IkB. Exposure with different stimuli like oxidative stress/phorbol esters causes extensive modification of IkB that result in the nuclear translocation of NF-kB. Our study demonstrates that silibinin strongly suppress the activation of NF-kB that might be mediated via inhibition of the phosphorylation or degradation of IkB as previous findings implicate that natural antioxidants inhibit NF-kB activation by blocking the modification of IkB (Surh, and Na, 2008).
Angiogenesis is one of the important phenomonons that help in tumor development. Chronic inflammation play critical role in the tumor angiogenesis. Proinflammatory mediators, such as cytokines, chemokines, growth factors, prostaglandins and others secreted by inflammatory cells (macrophages, mast cells and neutrophils) play vital role in the angiogenesis (Lu et al., 2006; Kundu and Surh, 2008). Further, NO also play important role in tumor angiogenesis. Recent studies reveal that persistent activation of inflammatory mediators induces tumor angiogenesis by up-regulation of vascular endothelial growth factor (VEGF). Over expression of VEGF causes angiogenesis, vascular hyperpermeability and enhanced tumorigenesis (Larcher et al., 1998). We have observed that silibinin suppressed the activation of VEGF which is in agreement with previous observations (Gu et al., 2007).

Further, the findings of the present study demonstrate that silibinin has strong anti-tumorigenic potential against DMBA initiated and TPA promoted mouse skin tumorigenesis and that the underlying mechanism may involve inhibition of TPA-induced inflammatory responses, hyper proliferation, oxidative stress and endogenous proinflammatory cytokines. Induction of various antioxidant and phase II cytoprotective enzymes also provides substantial evidence for the anti-tumorigenic potential of silibinin.

In conclusion the findings of the present study show that silibinin strongly suppressed cutaneous tumorigenesis via boosting of endogenous antioxidant defense system, inhibition of oxidative stress and inflammation. Theses finding suggest the strong anticarcinogenic potential of silibinin and hence its use may serve as one of the important strategies for the prevention of cancer.
Fig. 1. Effect of silibinin on TPA-induced cutaneous NO level

Values are expressed as mean ± SEM. \((n = 6)\). \(* * * p < 0.001\) shows significant difference in TPA treated group II [TPA (10 nmol)] as compared to vehicle treated control group I (0.2 ml acetone). 
\(##p < 0.01\) shows the significant difference in the silibinin pre-treated group III [S (9 mg) + TPA (10 nmol)] as compared to TPA treated group II [TPA (10 nmol)]. S- Silibinin

Fig. 2. Effect of silibinin on TPA-induced cutaneous lipid peroxidation

Values are expressed as mean ± SEM. \((n = 6)\). \(* * * p < 0.001\) shows significant difference in TPA treated group II [TPA (10 nmol)] as compared to vehicle treated control group I (0.2 ml acetone). 
\(##p < 0.01\) shows the significant difference in the silibinin pre-treated group III [S (9 mg) + TPA (10 nmol)] as compared to TPA treated group II [TPA (10 nmol)]. S- Silibinin
Fig. 3. Effect of silibinin on TPA-induced cutaneous GSH content

Values are expressed as mean ± SEM. \((n = 6)\). **\(p<0.001\) shows significant difference in TPA treated group II [TPA (10 nmol)] as compared to vehicle treated control group I (0.2 ml acetone). ###\(p<0.01\) shows the significant difference in the silibinin pre-treated group III [S (9 mg) + TPA (10 nmol)] as compared to TPA treated group II [TPA (10 nmol)]. S- Silibinin

Fig. 4. Effect of silibinin on TPA-induced proinflammatory cytokines

Values are expressed as mean ± SEM. \((n = 6)\). ***\(p<0.001\) shows significant difference in TPA treated group II [TPA (10 nmol)] as compared to vehicle treated group I (0.2 ml acetone). #\(p<0.05\), ##\(p<0.01\), ###\(p<0.001\) shows significant difference in the silibinin pre-treatment group III [S (9 mg) + TPA (10 nmol)] when compared with TPA treated group II [TPA (10 nmol)]. S- Silibinin
Table 1. Effects of silibinin and TPA on the activities of different cytoprotective enzymes

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glutathione reductase</th>
<th>Glutathione peroxidase</th>
<th>Glucose-6 phosphate dehydrogenase</th>
<th>Superoxide dismutase</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol NADPH oxidized / min / mg protein</td>
<td>nmol NADPH oxidized / min / mg protein</td>
<td>µmol NADP reduced/min/mg protein</td>
<td>units/mg protein</td>
<td>nmol H₂O₂ consumed/min/mg protein</td>
</tr>
<tr>
<td>I</td>
<td>120.6 ± 2.89</td>
<td>323.12 ± 8.28</td>
<td>142.34 ± 6.34</td>
<td>28.35 ± 4.0</td>
<td>99.23 ± 2.01</td>
</tr>
<tr>
<td>II</td>
<td>74.66 ± 2.41***</td>
<td>164.82 ± 5.23***</td>
<td>83.98 ± 3.23***</td>
<td>10.87 ± 1.89***</td>
<td>57.± 3.4***</td>
</tr>
<tr>
<td>III</td>
<td>92.0 ± 2.33##</td>
<td>245.43 ± 6.56 ##</td>
<td>119.87 ± 5.03#</td>
<td>19.01 ± 3.27#</td>
<td>80.4± 3.32##</td>
</tr>
<tr>
<td>IV</td>
<td>119.5±3.17</td>
<td>328.67±8.45</td>
<td>144.34 ± 8.21</td>
<td>30.03 ± 1.53</td>
<td>97.4± 3.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. (n = 6). ***p<0.001 shows the significant depletion in the activities of different cytoprotective enzymes in TPA treated group II [TPA (10 nmol)] as compared to vehicle treated group I (0.2 ml acetone). #p<0.05, ##p<0.01 shows the significant restoration in the activities of these enzymes in silibinin pre-treatment group III [S (9 mg) + TPA (10 nmol)] as compared to TPA treated group II [TPA (10 nmol)]. S- Silibinin
Fig. 5. Effect of silibinin on TPA-induced NF-kB expression

Representative photomicrographs (magnification ×40), (A) only acetone (Group I), (B) TPA (Group II), (C) Silibinin + TPA (Group III) and (D) Only silibinin (Group IV).

Brown color indicates NF-kB specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more NF-kB immunopositive staining as compared with vehicle treated group (Group I). Silibinin pretreatment (Group III) reduces NF-kB expression as compared to Group II. However there was no significant difference in the NF-kB immunostaining in Group IV as compared to Group I.
Fig. 6. Effect of silibinin on TPA-induced COX-2 expression

Representative photomicrographs (magnification ×10), (A) only acetone (Group I), (B) TPA (Group II), (C) Silibinin + TPA (Group III) and (D) Only silibinin (Group IV). Brown color indicates COX-2 specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more COX-2 immunopositive staining as compared with vehicle treated group (Group I). Silibinin pretreatment (Group III) reduces COX-2 expression as compared to Group II. However there was no significant difference in the COX-2 immunostaining in Group IV as compared to Group I.
Fig. 7. Effect of silibinin on TPA-induced iNOS expression

Representative photomicrographs (magnification ×10), (A) only acetone (Group I), (B) TPA (Group II), (C) Silibinin + TPA (Group III) and (D) Only silibinin (Group IV).

TPA treated group (Group II) shows more iNOS immunopositive staining as compared with vehicle treated group (Group I). Silibinin pretreatment (Group III) reduces iNOS expression as compared to Group II. However there was no significant difference in the iNOS immunostaining in Group IV as compared to Group I.
Fig. 8. Effect of silibinin pre-treatment on DMBA-TPA-induced skin VEGF expression

Representative photomicrographs (magnification ×40), (A) only acetone (Group I), (B) DMBA +TPA (Group II), (C) Anti-initiation (Group III) and (D) Anti-promotion (Group IV). DMBA +TPA treated group (Fig. 8 B) shows more VEGF immunopositive staining as compared with vehicle treated group (Fig. 8 A). Silibinin pre-treatment in Group III (Fig. 8 C) and Group IV (Fig. 8 D) reduces VEGF expression as compared to Group II.
Fig. 9 and 10, Effect of silibinin on tumor development and tumor histopathology

![Image of tumors and histopathology](image)

Table 2. Effect of silibinin on tumor incidence and multiplicity

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>No. of tumor-bearing mice</th>
<th>Tumor incidence (%)</th>
<th>Gross tumor multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I, Control</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>nil</td>
</tr>
<tr>
<td>Group-II, DMBA + TPA</td>
<td>14</td>
<td>14</td>
<td>100</td>
<td>11.71</td>
</tr>
<tr>
<td>Group-III Anti-initiation</td>
<td>14</td>
<td>10</td>
<td>71</td>
<td>6.81</td>
</tr>
<tr>
<td>Group-IV Anti-promotion</td>
<td>14</td>
<td>11</td>
<td>78</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Tumor incidence is expressed as the percentage of animals with one or more confirmed tumors. Apparent tumor multiplicity was calculated by dividing the total number of tumors observed grossly by the total number of tumor-bearing mice.
Graphical representation of the mechanism of action of silibinin on DMBA/TPA induced mouse skin tumorigenesis. The blue circles show the putative intracellular targets of silibinin.