Secoisolariciresinol diglucoside lignan concentrate of flaxseeds exhibits chemoprotective role in non-melanoma skin cancer through inhibition of CDK4 and upregulation of p53

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Cyclin-dependent kinases (CDKs) serve as target for various cancers including skin cancer. Secoisolariciresinoldiglucoside (SDG) and lignans exert anticancer effect on colon cancer through inhibition of CDKs. However, reports of SDG lignan concentrate (SLC) of *Linum usitatissimum* (L.) on skin cancer are not available. Hence, in this study, we evaluated the effect of SLC of *L. usitatissimum* on skin cancer, and determined the mechanism of action. Cell viability studies were done using the A-375 cell line. Skin cancer was induced by dimethyl Benz(a) anthracene and croton oil in female balb/c mice. SLC (5%) was administered from the 7th to 16th week after which we evaluated serum and tissue parameters. The IC50 value of SLC was found to be 93.7 µg/mL on the A-375 cell line. Skin cancer control animals exhibited increased tumor volume and burden and an increase in non-specific serum markers and tissue markers. Treatment with SLC decreased tumor volume and burden, and serum and tissue markers. Histopathological studies also depicted protection with SLC treatment. Docking studies revealed that SDG exhibits a good binding score with CDK4. Skin cancer control mice showed significantly increased CDK4 mRNA and decreased p53 mRNA levels which were prevented by SLC treatment. SLC exhibited a chemopreventive effect in skin cancer depicted by a reduction in serum biomarker, oxidative stress, collagen levels, tumor volume, tumor burden, and histopathological studies. These effects are mediated through the inhibition of CDK4 and upregulation of p53.

**Keywords:** Croton oil, Dimethyl benz(a) anthracene, Docking, Linseed, *Linum usitatissimum*, mRNA expression, Secoisolariciresinoldiglucoside

Non-melanoma skin cancer affects 1.2 million people worldwide, about 0.064 million succumbed to this disorder in 2020. Different therapies are used for treating skin cancer viz., surgical treatments, chemotherapy, radiation therapy, and photodynamic therapy. However, the occurrence of different side effects, recurrence of diseases, and effectiveness against only early stages of skin cancer are certain limitations with current therapies. These drawbacks emphasize the need for newer molecules acting on novel targets of skin diseases. Cyclins and cyclin-dependent kinases (CDKs) are one such target.

Cyclins and CDKs are regulatory molecules governing the multiplication of the cells. CDK4 along with cyclin D causes activation and hyper-phosphorylation of regulatory protein retinoblastoma (Rb) resulting in activation of transcriptional regulators which transcribe different proteins required for cell division. Blocking the action of CDK4 stops the DNA duplication for cell multiplicity resulting in a block in the cancer cell growth. p53 is a tumor suppressor transcription factor, and loss of one or two alleles of p53 has been shown to induce tumors. p53 is also reported to inhibit Cdk4 activity through p21 and by repression of Cdk4 synthesis. Thus, targeting these CDK4 and p53 can prove to be effective in cancer. Current CDK inhibitors have several limitations viz., non-selective toxic effects like myelosuppression, thrombosis, skin rashes, and fatigue. Such limitations point out towards discovery of novel molecules for the treatment of skin cancer.

Amongst the plethora of herbal molecules, lignans are diphenolic compounds reported to inhibit cell growth by acting on CDKs. Flaxseeds, obtained from *Linum usitatissimum* (L. *usitatissimum*), are a rich source of a specific lignan called secoisolariciresinoldiglucoside (SDG). Globally in...
many parts of the world, flaxseed is cultivated for fibers and oil in addition to nutritive applications. Owing to the presence of alpha-linolenic acid, lignans, and dietary fiber, it is considered a functional food. Flaxseed lignans are reported to have an anticancer effect against colon cancer, diabetic colon cancer and diabetes induced vascular reactivity. Despite these, no reports of the effect of *L. usitatissimum* on skin cancer are not available. Hence, present study, are to study the effect of SDG lignan concentrate of *L. usitatissimum* on skin cancer and to determine its mechanism of action.

**Material and Methods**

**Preparation of SDG lignan concentrate (SLC)**

Flaxseeds were identified, authenticated by the Department of Pharmacognosy, Institute of Pharmacy, Nirma University, Ahmedabad, India. The coarse powder of the dried seeds was defatted using n-hexane followed by preparation of ethanolic extract using a soxhlet extractor. The SLC was prepared from the ethanolic extract.

**Quantitative analysis using chromatography**

The SDG standard was separated using preparative thin-layer chromatography (TLC). HPTLC was performed on a precoated silica gel HPTLC60 (Merck), F 254 (5cmx10cm) for the quantification of SDG in SLC. The sample was sprayed with nitrogen gas on the HPTLC plates using Linomat 5. After application, the plates were developed in CAMAG horizontal development using a solvent system (ethyl acetate:methanol:water:formic acid - 7.7:1.3:1.0:0.5). Densitometric evaluation was performed with help of CAMAG TLC SCANNER 3 along with winCATS software (version 3.5). Scanning parameters were set in reflectance mode at 282 nm.

**In vitro cell viability studies**

A-375 cell culture was derived from National Centre for Cell Science (NCCS), Pune. Stock cells of these cell lines were cultured in Dulbecco's Modified Eagle's medium, low glucose with glutamine (DMEM), supplemented with 10% fetal bovine serum (FBS), 5% Hank's Balanced Salt Solution (HBSS), penicillin, streptomycin, and amphotericin-B, in a humidified atmosphere of 5% CO₂ at 37°C until confluence was reached. The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay.

**In vivo pharmacological studies**

**Induction of skin cancer**

Female Balb/c mice weighing 25-30 g were chosen for the study and were maintained under controlled conditions viz., temperature at 25±2°C, and relative humidity at 55±5%. All the animals had access to standard laboratory mice chow and UV-filtered water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (Protocol number: IP/PCOL/MPH/13-1/010 dated 08/08/2013).

Animals were divided into eight groups: Normal control (CON), Control animals treated with 2% 5-FU solution (COF), Control animals treated with 5% suspension of SLC (COS5), Skin cancer control (SCC), Skin cancer treated with 2% 5-FU solution (SCF), Skin cancer treated with 5% suspension of SLC (SCS5). 2% 5-FU is the standard dose used for skin cancer and the dose of SLC at 5% was selected based on preliminary studies. 50 µL of the dimethyl benz (a) anthracene (DMBA) (1Mg/mL) solution was sprayed twice weekly for one week. About 1% croton oil solution was applied twice weekly from 3rd week to 16th week. The drug treatment was started from the 7th week and continued till the 16th week. About 100 µL of respective drugs were applied on the back region with help of a paint brush (size 1). Tumor volume and tumor burden were determined using the following formula:

Tumor volume = (maximal length of tumor × {width of tumor}²)/2

Tumor burden = Tumor volume × Number of tumors

**Analysis of parameters**

After 16 weeks, blood samples were collected, serum was separated and analyzed for gamma-glutamyltransferase(GGT), lactic acid dehydrogenase (LDH), and C-reactive protein levels(CRP) spectrophotometrically (Shimadzu UV-1601, Japan) using available biochemical diagnostic kits (Labcare Diagnostics Pvt. Ltd., India). Following blood collection, all the animals were sacrificed, dorsal skin from the mice was carefully removed. The skin portion in which the tumor was developed was excised and was subjected to estimation of malondialdehyde(MDA), reduced glutathione(GSH), superoxide dismutase(SOD) and collagen levels. The skin was also subjected to histopathological studies for hematoxylin and eosin (HE) staining. The sections were observed and
photographed using Olympus (trinocular-CX21FS1) photomicroscope under 40X magnification.

**mRNA expression studies**

CDK4 and p53 mRNA levels were determined\(^1\). Briefly, 200-300 mg of skin specimen were taken and homogenized. Total ribonucleic acid (RNA) was extracted using FastRNA® Pro Green Kit (M.P. Biomedicals) following the instructions of the user-manual. First-strand cDNA was synthesized from 1.0 µg of RNA using First Strand cDNA Synthesis Kit (Novagen) and incubated at 42°C for 60 min followed by 70°C for 10 min. PCR was carried out to check gene expression using primers specific for rat β actin (Forward: 5'-ATCCGTAAAGACCTCTATGC-3'; Reverse: 5'-AACGCAGCTCAGTAACAGTC-3'), CDK4 (Forward: 5'-CTTCCGTCAGCAGTTCC-3'; Reverse: 5'-GGTCAGCATTTCCAGTAG C-3') and p53 (Forward: 5'-GGGACAGCCAAGTCTGTTATG-3'; Reverse: 5'-GGAGTCTTCCAGTGTGATGAT-3') according to the user manual. The quantification of gene expression was done by Phroetix 1D v 11.4:1DPRO-J3K9-RGSZ-AN (Total lab software)

**Docking Studies**

Sybyl × 1.3 was used for molecular docking\(^2\). The co-crystallized structure of CDK4 [PDB id: 2W96] was taken from Protein Data Bank (PDB) and modified for docking calculations. Co-crystallized ligand was removed from the structure, water molecules were removed, H atoms were added, and side chains were fixed during protein preparation. SDG and standards of CDK4 viz., purvanalol, roscovitine, and staurosporine were docked and the docking score and number of hydrogen bonds were determined.

**Statistical analysis**

Results are presented as Mean ± SEM. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey’s test. Data were considered statistically significant at P-value <0.05.

**Results**

**HPTLC**

The peak of SDG was obtained at 0.26 Rf, using the separated SDG as an internal standard. The standard curve was plotted and \( r^2 \) value obtained was 0.991 (Fig. 1A). Using the linearity equation it was found that 17.57 µg of SDG was present in 1Mg of SLC (Fig. 1B).

Fig. 1 — (A) Calibration curves in linear in the range of 500, 700, 900, 1000, 1100, 1300 and 1500 ng for SDG standard; and (B) HPTLC densitogram of SLC

**Cell viability**

Fig. 2 represents the % cell inhibition of SLC and 5-FU on A-375 cells. The IC 50 value of SLC was found to be 93.7 µg/mL, while that for 5-FU was 89.9µg/mL.

**Tumor volume and Tumor burden**

The tumors started appearing from the 10\(^{th}\) week. There was a significant increase in tumor volume and tumor burden in the skin cancer control group from the 10\(^{th}\) to the 16\(^{th}\) week (Table 1). Treatment with 2% 5-FU significantly reduced the tumor volume and burden from the 12\(^{th}\) week and continued reduction till the 16\(^{th}\) week. Treatment with 5% SLC significantly reduced the tumor volume and burden from the 14\(^{th}\) week and 12\(^{th}\) week respectively and continued reducing till the 16\(^{th}\) week (Table 1).
Fig. 2 — A375 cells were treated with various concentrations of SLC and 5-FU for 24 h. [Cell viability was measured using the MTT assay. The IC50 values of SLC and 5-FU were determined. Results shown are representative of three independent experiments]

Table 1 — Effect of SLC on tumor burden and tumor volume

<table>
<thead>
<tr>
<th>No of week</th>
<th>CON</th>
<th>SLC</th>
<th>SCS2</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10th week</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor burden</td>
<td>39.63±0.62*</td>
<td>36.02±0.47</td>
<td>24.60±0.41</td>
<td></td>
</tr>
<tr>
<td>12th week</td>
<td>53.64±0.59*</td>
<td>27.95±0.32a</td>
<td>40.72±0.66a</td>
<td></td>
</tr>
<tr>
<td>14th week</td>
<td>61.71±0.73a</td>
<td>15.52±0.32a</td>
<td>57.77±0.90a</td>
<td></td>
</tr>
<tr>
<td>16th week</td>
<td>185.75±1.09a</td>
<td>7.91±0.1a</td>
<td>75.79±0.99a</td>
<td></td>
</tr>
<tr>
<td>Tumor volume</td>
<td>6.93±0.62a</td>
<td>7.20±0.47a</td>
<td>5.62±0.41a</td>
<td></td>
</tr>
<tr>
<td>12th week</td>
<td>7.08±0.59a</td>
<td>5.58±0.32a</td>
<td>8.57±0.66a</td>
<td></td>
</tr>
<tr>
<td>14th week</td>
<td>12.65±0.73a</td>
<td>3.19±0.32a</td>
<td>10.27±0.90a</td>
<td></td>
</tr>
<tr>
<td>16th week</td>
<td>23.21±1.09a</td>
<td>1.82±0.18a</td>
<td>14.10±0.99a</td>
<td></td>
</tr>
</tbody>
</table>

Values represent Mean ± SEM of 6 mice. *Significantly different from the normal control group (P <0.05). 5Significantly different from the skin cancer control group (P <0.05). CON, Normal control; SCC, Skin cancer control group; SCF, Skin cancer treated with 2% 5-Flourouracil; SCS2, Skin cancer treated with 2% SLC; and SCS5, Skin cancer treated with 5% SLC.

Non-specific serum markers

At the end of 16th week, there was a significant increase in the serum GGT, LDH, and CRP levels in the skin cancer control group as compared to normal control group. Treatment with 2% 5-FU and 5% SLC significantly reduced these levels in disease-treated groups as compared to the skin cancer control group (Table 2).

Tissue markers

DMBA and croton oil produced a significant increase in MDA and a significant decrease in SOD and GSH levels in skin tissue of skin cancer control groups as compared to the normal control group. Treatment with 2% 5-FU and 5% SLC significantly reduced the tissue collagen levels and tissue levels in disease-treated groups as compared to the skin cancer control group (Table 2).

Histopathological studies

Histopathological analysis of skin tissues from control mice depicted well distribution of the epidermis, dermis and subcutis layers; the presence of normal sebaceous glands, hair follicles, epidermal thickness and fatty layer distribution (Fig. 3 A-C). In the case of the skin cancer control group, there was disruption of the fatty layer, an increase in epidermal thickness and presence of keratin pearls, rete ridges extending through the connective tissue, dysplastic, and hyperplastic epithelial cells (Fig. 3D). Treatment with 5-FU showed decreased rete ridges, absence of dysplastic epithelial cells, and unscathed fatty layer with the reduced epidermal thickness (Fig. 3E). Histopathological sections of skin from mice treated with 5% SLC also showed a complete absence of
keratin pearls, dysplastic epithelial cells, and rete ridges with a decrease in epidermal thickness and undisrupted fatty layer; although an increased number of hyperplastic cells was observed (Fig. 3F).

Docking study

The docking score of SDG with CDK4 was 7.80 while that of its standards purvanalol, roscovitine, and staurosporine was 7.41, 7.80, and 4.53 respectively (Fig. 4A). Purvanalol, roscovitine, and staurosporine showed respectively 2, 3, and 2 hydrogen bonding while SDG showed 5 hydrogen bonding with the proteins (Table 3). Amongst several binding pockets, SDG exhibited hydrogen bonding with B/ARG62 and B/ARG163 which are common binding sites for staurosporine and roscovitine respectively (Fig. 4 A-D).

mRNA expression of CDK4 and p53

Skin cancer control group exhibited a significant increase in CDK4 mRNA and a significant decrease in p53 mRNA levels as compared to the normal control group. Treatment with 5% SLC significantly reduced CDK4 mRNA and significantly increased the p53 mRNA levels in disease-treated groups as compared to the skin cancer control group (Fig. 5).
Fig. 5 — Effect of SLC on (A) CDK4 mRNA levels; and (B) p53 mRNA levels. [Each bar represents Mean ± S.E.M of six rats, Significantly different from *Normal control group (P <0.05), and # skin cancer control group (P <0.05), CON, Normal control; SCC, Skin cancer control group; SCF, Skin cancer treated with 2% 5-Fluouracil; SCS2, Skin cancer treated with 2% SLC; and SCS5, Skin cancer treated with 5% SLC]

Discussion

A-375 cells lines have characteristic features of growing tumors at subcutaneous region which are of amelanotic melanomas type. In the present study, the IC50 value of SLC and 5-FU was 93.7 and 89.9 µg/mL, respectively. The IC50 value of SLC being close to that of 5-FU indicates that SLC exhibits potent chemoprotective actions in skin cancer.

DMBA and croton oil induced skin cancer model is a two-stage chemical method that produces skin papillomas at first, which are further transformed into deep squamous cell carcinoma in the span of 16 weeks. In the present investigation, there was a significant increase in tumor volume and burden in the skin cancer control group from the 10th to 16th week which was reduced by treatment with 2% 5-FU and 5% SLC. Histopathology studies of the skin tissue also support the data of the chemoprotective role of SLC. Histopathological sections from the 5% SLC treated group showed a complete absence of keratin pearls, dysplastic epithelial cells, and rete ridges. These results suggest the chemoprotective role of SLC of *L. usitatissimum*.

Higher levels of GGT, a non-specific tumor marker, indicate the appearance of a more aggressive and resistant tumor. For accelerated growth of tumor cells, a constant amount of nutrient flow is required which is accomplished by an excessive supply of cysteine. In support to supply more cysteine molecules into the cells, there is increased activity of GGT. It is also reported that the application of DMBA and croton oil causes an increase in GGT levels. In the present study, GGT levels were reduced by 5% SLC treatment. An increase in GGT levels is directly linked with an increase in the Ras pathway which is increased in skin cancer. Ras signaling has been reported to down-regulate the CDK inhibition and helps in cell cycle progression. It has been reported that lignans inhibit the CDK molecules either by increasing the levels of p21 or p16 (CDK inhibitory proteins). Thus, it can be proposed that incongruence to other lignans, SLC might have inhibited CDK which might have altered the Ras pathway and thereby reduced the levels of GGT.

In the case of cancer cells, there is an increased formation of pyruvate in glycolysis, the level of LDH also increases. In the present study, there was a decrease in levels of LDH 5% SLC treatment. It has been observed that with an increase in expression of CDK and cyclin there is an increase in levels of LDH. Hence, SLC might inhibit the levels of CDK which might reduce the levels of LDH.

An increased level of CRP is associated with an increased risk of cancer in a healthy individual. In the current study, treatment with 5% SLC decreased CRP levels. CRP is shown to increase the levels of p53, and subsequently increase the levels of p21 and induce cell arrest. Hence, a decrease in CRP levels by SLC suggest that through the restoration of p53, SLC might prevent inflammation of the tumor environment. Increase in non-specific markers are due to formation of tumors as host-tumor interaction occurs during cancer. In current study, SLC reduced tumor formation and thereby it might have altered the host-tumor interaction and thereby reduced the markers, although they have been applied locally.
In cancer, reactive oxygen species have been shown to have an important role in the cancer development phase. With application of DMBA and croton oil there is increase in lipid peroxidation levels and decrease in GSH and SOD activity\textsuperscript{32}. In the current investigation, 5\% SLC controlled oxidative stress. Lignans, including SLC, are potent antioxidants\textsuperscript{33} that might act directly against the radicals. From the ongoing discussion, it appears that SLC controls oxidative stress and thus might serve to exhibit a chemoprotective role.

Collagen levels, in cancer induced by DMBA and croton oil application, have been seen to increase in comparison to the normal skin. In the current study, there was an increase in collagen levels in the skin cancer control group which was reduced by 5\% SLC treatment. An increase in TNF-\(\alpha\) is reported to elevate the expression of matrix metalloproteinases(MMP), which further supports the spread of cancer cells\textsuperscript{34}. CDK inhibition has been reported to inhibit TNF-\(\alpha\) induced expression of MMP\textsuperscript{35}. In conjunction with the CDK inhibitory property of lignans, there can be reduced levels of TNF-\(\alpha\) which reduces the level of MMP expression. Due to lower levels of MMP, there might be a reduction in the degradation of collagen fibers by SLC treatment.

To determine the mechanism behind the chemoprevention we carried out the docking studies. Various lignans have been reported in inhibiting the levels of CDK4\textsuperscript{36}. Hence, we carried out the docking of SDG on CDK4 protein and compared the results with the docking of standards viz. roscovutine, staurosporine, and purvanalol B on CDK4. Data revealed that the binding affinity of SDG is similar to the binding score of roscovutine, while in comparison to staurosporine, and purvanalol B the docking score of SDG was higher. Also, the interaction between SDG and CDK4 molecule produced 5 hydrogen bonds, which were higher in comparison to the interaction between standards with CDK4. Hence, it can be proposed that the binding affinity of the SDG is more in comparison to purvanalol and staurosporine. Amongst many binding pockets found in SDG, binding sites like B/ARG62 and B/GLY160 were seen to be common binding sites of staurosporine and roscovutine respectively. The similarity in binding interaction of SDG and staurosporine i.e. B/ARG62 is due to the presence of lone pair of electrons on oxygen atoms in both the ligands. The interaction with the B/GLY160 is due to the presence of the hydrogen atom in roscovutine as well as in SDG. It has been shown that ATP binding pocket in all types of kinases is considered to be the conserved region\textsuperscript{37}. This suggests that SDG interacts with the conserved region of the CDK4.

Considering the docking studies which depicted that SDG might act through CDK4, we carried out mRNA expression studies of CDK4 from the skin tissue to confirm its mechanism of action. In the present study, the skin cancer control group showed increased levels of CDK4 mRNA expression which was reduced by treatment with 5\% SLC. Raised levels of CDK4 along with the decreased levels of p53 indicate the accelerated cell cycle. It has been reported that there is an increase in expression of specifically CDK4 in cancer cells\textsuperscript{38}. Thus, this confirms that SLC inhibits CDK4 expression in skin cancer and thereby interrupts the cell cycle and heralds the accelerated cell growth.

It has been reported that there is a decrease in mRNA expression of p53 in skin cancer\textsuperscript{39}. Moreover, an increase in the level of p53 causes CDK inhibition via p21 CDK inhibitory proteins\textsuperscript{40}. Hence, to corroborate the data of mechanism of action of SLC, we also studied the mRNA expression of p53 from skin tissue which revealed a decreased level of p53 in skin cancer control animals. 5\% SLC treatment exhibited an increase in the level of p53. These results confirm that SLC increases mRNA expression of p53 along with decreasing the mRNA expression of CDK4.

Despite having carried out a detailed study, this study holds some limitations. We were unable to carry out a cell cycle study using flow cytometry and could not determine the entire downstream pathway through which the drug acts. Nevertheless, future studies could be directed towards the determination of a detailed mechanism of action. This can then be taken to next level for isolation of SDG and designing novel scaffolds that are safe and more potent.

**Conclusion**

SLC of *Linum usitatissimum* (L.) exhibited a chemopreventive effect as depicted through reduction in tumor volume, tumor burden, serum and tissue markers. The docking and mRNA expression studies indicate the possible mechanism of action could be through the inhibition of CDK4 and upregulation of p53.
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Conflict of Interest
Authors declare no competing interests.

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