Full Research Paper

Chemopreventive Effects of Sarcotriol on Ultraviolet B-induced Skin Tumor Development in SKH-1 Hairless Mice

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Abstract: Sarcotriol (ST) has been shown to be chemopreventive on 7,12-dimethyl-benz(a)anthracene (DMBA) initiated and 12-O-tetradecanoylphorbol-13-acetate (TPA)-promoted skin tumor development in CD-1 mice in recent studies from our laboratory. The objective of this study was to determine the chemopreventive effects of ST on ultraviolet B (UVB)-induced skin tumor development in female SKH-1 hairless mice, an experimental model relevant to human skin cancer development, and its possible mechanisms of action. Female SKH-1 mice were divided into two groups: Control and ST treated. Control was topically treated with 100 µL acetone and ST treated group administered with 30 µg ST in 100 µL acetone one hour before UVB exposure. For UVB-induced tumorigenesis, carcinogenesis was initiated and promoted by UVB (180 mJ/cm^2). Group weights and tumor counts were taken once every week. After 30 weeks, mice were sacrificed and dorsal skin samples were collected. The proteins from the skin sample were further used for SDS-PAGE and Western blotting using specific antibodies against caspase-3, caspase-8, caspase-9 and p53. Tumor multiplicity was found 19.6, 5.2 in the control and ST treated groups respectively. Caspase-3, -8, -9 and p53 were significantly ($P < 0.05$) upregulated in ST treated group compared to Control group. Together, this study for the first time identifies the chemopreventive effects of ST in UVB-induced carcinogenesis possibly by inducing apoptosis and upregulating p53.
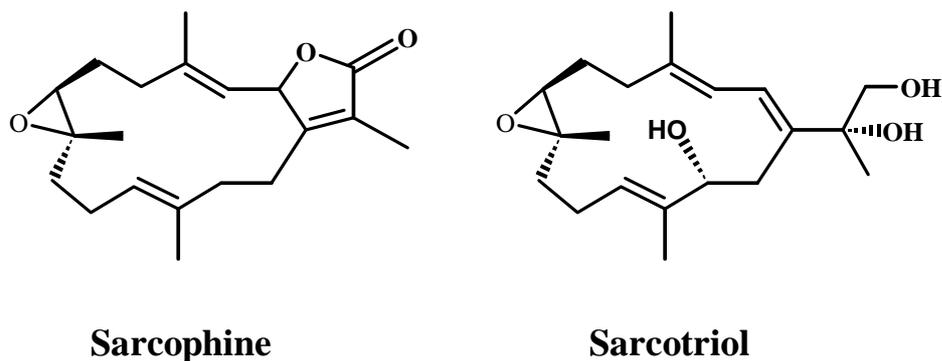
1. Introduction

Skin cancer is the most common of all cancer types, leading to the diagnosis of more than 1 million cases each year in the United States. An estimated 10,850 deaths will occur in 2007 [1], resulting from repeated sunlight exposure in which ultraviolet B (UVB) radiation is a major environmental carcinogen that induces nonmelanoma skin cancer [2-4].

Chemical and UVB radiation-induced carcinogenesis in murine and humans is a multistep process and has been divided into defined stages of initiation, promotion and progression [5]. UVB acts both as an initiator as well as promoter. UVB radiation is absorbed by DNA which causes damage to the DNA, primarily at sites of adjacent pyrimidines in the form of dimers [3, 4, 6]. The generation of DNA photoproducts has been shown to initiate tumorigenesis. Mechanism(s) of the UVB-induced promotion are not been fully understood. Several factors such as generation of reactive oxygen species causing oxidative stress and activation of various signaling cascades including the synthesis of prostaglandins (PG) play a major role in clonal expansion of UVB-initiated cells into visible skin tumors [7-9].

Developmrnt of novel strategies to prevent skin cancer represents a desirable goal due to the increasing rise in incidence of skin cancer patients throughout the world [10, 11]. Chemoprevention is a means of cancer control in which the occurrence of the disease can be entirely prevented, slowed down or reversed by the administration of one or more naturally occurring and/or synthetic compounds [12, 13]. Nowadays, the supplementation or topical application of synthetic agents viz. retinoids, vitamins, inhibitors of ornithine decarboxylase, cyclooxygenase, lipooxygenase and other antioxidant compounds including thiols and minerals have gained much attention on one hand, while the use of natural agents like polyphenols, monoterpenes, flavonoids, organosulphides, indoles etc have shown promise for their development as chemopreventive agents against skin cancer [14, 15].

Recent studies have focused on the potential applications of marine organisms, particularly in the treatment of human diseases [16]. Several marine natural products are in pre-clinical and clinical evaluation, one of which is sarcophytol A [17]. This is a hydroxylated cembranoid isolated from the soft coral *Sarcophyton glaucum* that has shown cancer chemopreventive activity [18]. Another related cembranoid is sarcophine, obtained from the Red Sea soft coral *Sarcophyton glaucum*, which was found to possess diverse biological activity [19]. Sarcotriol (ST), a semi-synthetic derivative of sarcophine has shown higher activity than the known chemopreventive agent sarcophytol A [18] The structures of sarcophine and sarcotriol are shown in Figure 1. Recent studies from our laboratory have shown significant chemopreventive effects of ST on 7,12-dimethylbenz(a)anthracene (DMBA)-initiated and 12-O-tetradecanoylphorbol-13-acetate (TPA)-promoted skin tumor development in female CD-1 mice by inducing apoptosis and decreasing COX-2 levels [20]. These promising results prompted this investigation to study the effects of ST on UVB-induced tumorigenesis in female SKH-1 hairless mice model.

Figure 1. Structures of sarcophine and sarcotriol.

Sarcophine   **Sarcotriol**

The objective of this study was to determine the chemopreventive effects of ST on UVB-induced skin tumor development in female SKH-1 hairless mice, an experimental model relevant to human skin cancer development. One of the biological responses for tumorigenesis is the loss of apoptotic death. Caspase-3, -8 and -9 are proapoptotic proteins which could induce apoptosis, whereas, p53, the tumor suppressor gene is involved in many cellular functions including cell cycle inhibition, regulation of differentiation, transcription, DNA repair, and apoptosis of cells sustaining DNA damage [3,4]. Therefore, the effects of ST on the expressions of caspase-3, -8, -9 and p53 after UVB exposure to SKH-1 mice also were determined to elucidate the possible mechanisms of action of ST.

2. Results and Discussion

The effects of ST treatment one hour before the UVB exposure on the tumor incidence and multiplicity are shown in Figures 2a and 2b, respectively.

Figure 2a. The effects of ST pretreatment on tumor incidence in UVB-induced tumorigenesis in female SKH-1 hairless mice. ST pretreatment delayed the onset of tumors from 12th week till 15th week when compared to the control group.
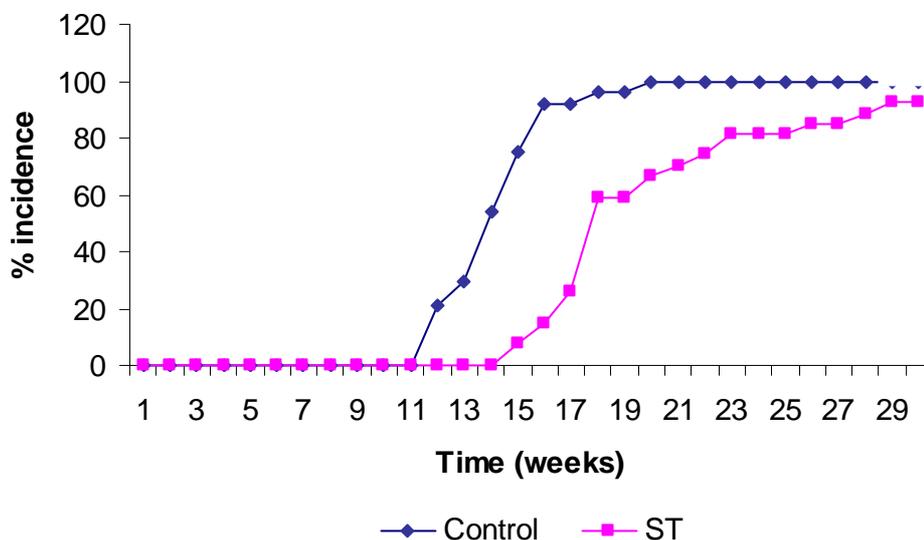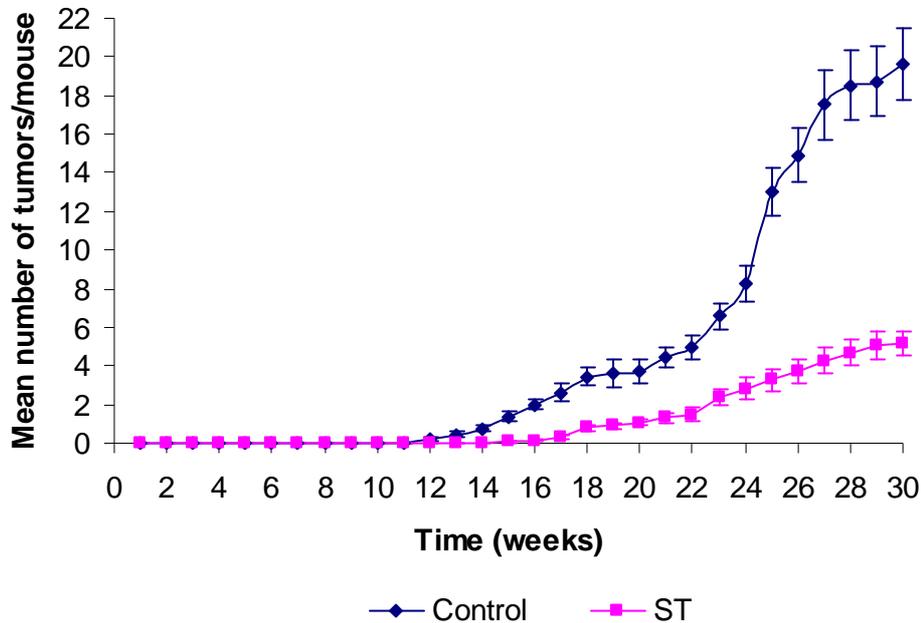
Figure 2b: The effects of ST pretreatment on tumor multiplicity in the UVB-induced tumorigenesis in female SKH-1 hairless mice. ST decreased tumor multiplicity throughout the experiment. Each point represents mean number of tumors per mice ± SE derived from 27 mice.
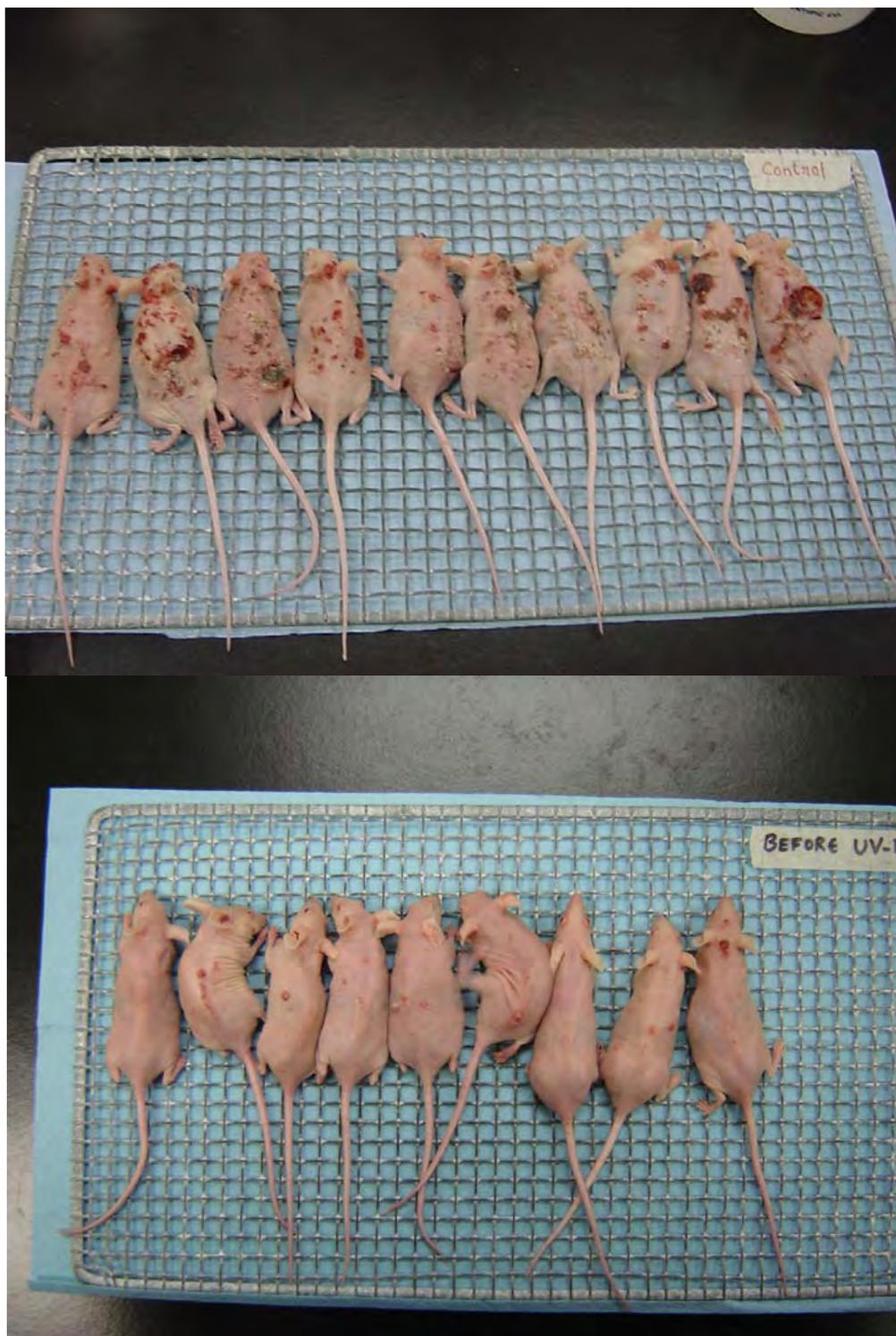

The results of the present investigation revealed a delay in the onset of tumorigenesis in the animals pretreated with ST. In the Control group, the onset of tumors commenced at 12th week of promotion and reached 100% by the 20th week, whereas pretreatment with ST one hour before UVB, the onset of tumors were delayed until the 15th week of promotion and, moreover, it was only 75 % until the 23rd week. Tumor incidence was significantly ($p < 0.05$) lower in ST treated group than Control group until 28th weeks of promotion. By the end of the experiment, tumor incidence was found to be 100 and 92 % in the Control and ST treated group respectively. As shown in Figure 2b, ST treatment resulted in a significant ($P < 0.05$) reduction in the number of tumors per mouse compared to the Control group throughout the duration of experiment. The mean number of tumors at the end of experiment in Control and ST treated group was found to be 19.6 and 5.2 respectively. These results indicated that ST inhibited skin tumor development in both tumor incidence, to a lesser extent, and tumor multiplicity in the ST treated groups (Figure 3). There was no significant difference in the average mouse weight between the Control and ST treated group throughout the experiment (data not shown). Moreover, the skin of mice that were treated with ST appeared to be healthy and no toxic symptoms were observed.

One biological event responsible for tumorigenesis is the loss of apoptotic death of transformed cells [21]. In most cancers, the apoptotic pathway of tumor cells is compromised with a survival advantage over the normal cells [22]. Induction of apoptosis is a key event, which controls the outcome of chemopreventive efficacy of an agent under investigation [22, 23]. A fundamental biochemical event that starts apoptosis is the activation of caspases [24]. Apoptosis occurs through two pathways; extrinsic and intrinsic [25]. The extrinsic pathway is activated by the ligation of death receptors, which ultimately leads to the activation of caspase-8 [25]. In the intrinsic pathway of
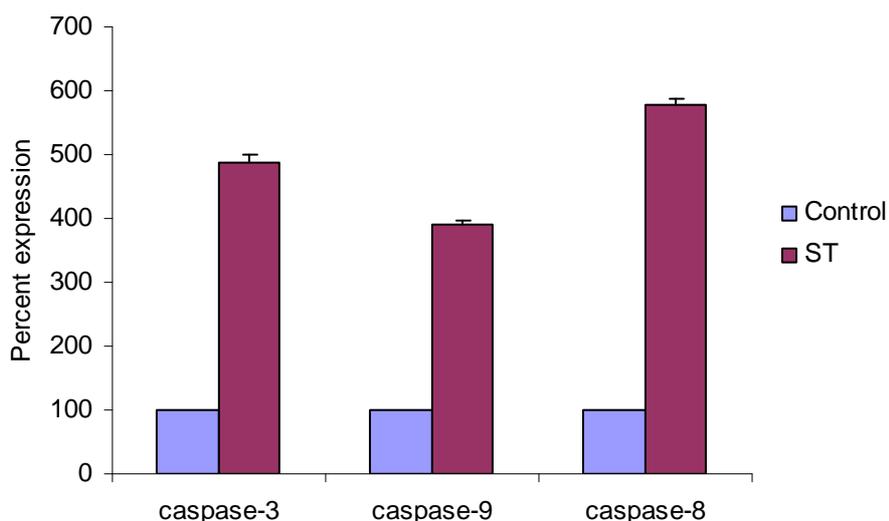
apoptosis, mitochondrial dysfunction takes place causing the release of cytochrome *c* into the cytosol, which finally leads to the activation of caspase-9 [26]. Finally the upstream caspases-9 and -8 converge to caspase-3 leading to apoptosis [25].

Figure 3: The skin cancer protective effect of sarcotriol: skin tumor incidence and multiplicity were significantly reduced in mice pretreated with sarcotriol (bottom) compared to the untreated control (top).
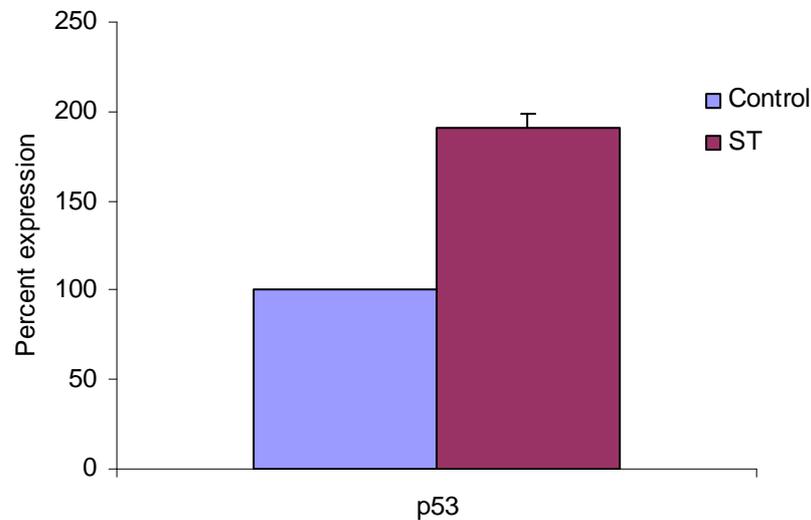
Based on the hypothesis that ST induces apoptosis by upregulating the caspase levels as its possible mechanism of chemoprevention against skin tumor development in CD-1 mice [16], the effects of ST on caspase-3 were analyzed. As shown in Figure 4, ST significantly ($P < 0.05$) upregulated the levels of caspase-3. We observed that the value of caspase-3 as compared in ST treated group was found to be 4.9 fold higher than Control group. In order to assess the mechanism of caspase-3 activation, a further study was carried out to find out whether caspase-9 and -8 are activated as upstream effectors leading to caspase-3 activation. We observed a significant ($P < 0.05$) increase in caspase-9 and -8 activities as shown in Figure 5. The levels of caspase-8 in ST treated group were found to be 5.7 fold higher than Control. Whereas the values of caspase-9 in ST treated group were 3.9 fold higher than Control. These results suggest that ST induced apoptotic cell death might be mediated by both extrinsic and intrinsic pathways of apoptosis in tumor cells in UVB-induced carcinogenesis.

Figure 4. The effects of ST pretreatment on proapoptotic proteins, caspase-3, 8 and 9, on UVB-irradiated skin tumors of SKH-1 hairless mice. Values represent mean ± SE derived from at least three mice. Values of ST are percentages of control values quantitated by densitometry.

The gene p53 is one of the most frequently mutated in UVB-induced carcinogenesis [27]. It plays an important role in the induction of apoptosis through caspase dependent as well as caspase independent pathways [3]. The p53 tumor suppressor gene is essential in maintaining the genomic integrity of cells through its role in allowing repair of DNA damage or apoptosis [22]. A relationship between exogenous tumor induction and p53 expression has been reported in UVB-induced skin papillomas and carcinomas of the skin [28, 29]. The p53 tumor suppressor gene induces apoptosis in response to cellular stresses such as DNA damage, hypoxia, and oncogene activation [30]. In order to unravel the mechanism behind the induction of apoptosis by ST treatment we further assessed the levels of p53 in UVB-induced carcinogenesis. As expected there was a significant ($P < 0.05$) upregulation of p53 levels in ST treated group compared to Control. The value of p53 was found to be 1.9 fold higher in ST treated group when compared to the Control; data are shown in Figure 5.

Figure 5. The effects of ST pretreatment on p53 on UVB-irradiated skin tumors of SKH-1 hairless mice. Values represent mean ± SE derived from at least three mice. Values of ST are percentages of control values quantitated by densitometry.

3. Conclusions

Taken together from the above results, the effectiveness of ST as chemopreventive agent appears to be very promising in UVB-induced skin cancer control. The mechanism behind its chemoprevention might be possibly by inducing apoptosis, upregulating p53, contributing to its overall cancer chemopreventive effects in the UVB-induced mouse skin cancer model.

4. Experimental

4.1. General

Sarcophine was isolated from the soft coral *Sarcophyton glaucum* collected from several locations of the Red Sea in Egypt. Phenylmethylsulfonylfluoride (PMSF), Tween 20, sodium chloride, ammonium persulphate, sodium lauryl sulphate, acetic acid, leupeptin and pepstatin were obtained from Sigma Chemical Company (St. Louis, MO). Acrylamide was purchased from Bio Rad Laboratories, (Hercules, CA), glycine, sodium dodecyl sulphate (SDS) and Tris from USB Corporation (Cleveland, OH), and nitrocellulose membrane from Bioexpress (Kaysville, UT). Primary antibodies against caspase-3 were purchased from Cayman Chemical Company (Ann Arbor, MI), primary antibody against caspase-8, -9, and horseradish peroxidase conjugated goat anti-rabbit secondary antibodies were purchased from BD Biosciences (Rockville, MD) and p53 from Abcam Inc. (Cambridge, USA). ECL Kit was bought from Amersham Biosciences (Piscataway, NJ). Other reagents were obtained in their highest purity grade available commercially.

4.2. Materials

Sarcophine was isolated from the soft coral *Sarcophyton glaucum* by multiple extraction with petroleum ether at room temperature following the reported procedure [19] at the laboratories of the Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt. The dried extract was evaporated under reduced pressure and chromatographed on silica gel column using hexane: ethyl acetate (1:2) as eluent. Pure sarcophine was obtained by crystallization from ethanol. ST was synthesized according to the folowing procedure: Sarcophine was reduced to its lactone ring-opened analogue (50 mg, 0.16 mmol) to which selenium dioxide (35.5 mg, 0.32 mmol) in dry 1,4-dioxane (15 mL) was added and the reaction mixture was stirred at room temperature for 4 h. Water was then added, and the product was extracted with CH_2Cl_2. Saturated $NaHCO_3$ solution was used to wash the CH_2Cl_2 layer which was dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was chromatographed on silica gel using hexane - acetone (1:1) as an eluent to obtain ST (12 mg, 23%) [18].

4.3. UVB-induced Tumorigenesis Protocol:

4.3.1. *UVB Source:*

The UVB irradiation unit, manufactured by Daavlin Corporation (Ohio, USA) consists of four UVB lamps. The exposure dose can be controlled by using two Daavlin flex control integrating dosimeters. The dose of UVB exposure is expressed in millijoules/cm^2.

4.3.2. *Animals:*

Female SKH-1 mice (5 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed ten per cage at 22 ± 1^0C and $50 \pm 10\%$ relative humidity and subjected to a 12h light/ 12h dark cycle in the College of Pharmacy animal facility. They were acclimatized for 1 week before use and provided food and water *ad libitum*.

4.3.3. *Experiment:*

The tumorigenesis protocol as described by Dwivedi *et al.* [31] was used. Female SKH-1 mice randomly divided into two groups, Control and ST treated. Both initiation as well as promotion was induced by UVB radiation (180mJ/cm^2). During the initiation phase, Control was treated with acetone (100 µL) and ST treated with ST (100 µL, 30 µg/100 µL of acetone) one hour prior to UVB treatment. This was done every day and was continued until 14 days. Ten days after the initiation phase, promotion phase was started. During the promotion phase, both Control and ST treated groups were treated in the same way as they were treated during the initiation phase. But during the promotion phase, the treatment was done only twice a week (Tuesday and Friday) and was continued until the next 30 weeks. Tumor counts and group weights were taken once every week.

4.4 Lysate Preparation

Mice from UVB-induced tumorigenesis protocol were sacrificed after 30 weeks by cervical dislocation and epidermis was collected from mice of Control and ST Treated groups respectively. The fat and tumors in the skin of these three groups were scraped by scalpel, and then epidermis was homogenized by an OMNI GLH-115 homogenizer in 0.1 mM Tris-HCl (pH 7.4) containing 0.15 M sodium chloride. The epidermal homogenate was filtered by cheesecloth and then filtrate was centrifuged at 10,000 g for 20 min in the Beckman J2-21 Centrifuge. This pellet combined with 5% SDS containing 1% 100 mM PMSF, 0.5% leupepth and 0.5% pepstatin was allowed to pass through 25G needle and centrifuged at 13,000 g for 20 min. The supernatant was collected, allowed to pass through 25G needle again and heated in heating block (100 °C) for 5 min. and epidermis was collected from mice of Control and ST treated groups respectively. The cell pellet of mice obtained as a result of ultracentrifugation is taken in an Eppendorf tube and lysed in 5% SDS containing protease inhibitors leupeptin, pepstatin and phenyl methyl suphonyl floride (PMSF) as described by Kundoor *et al.* [16] and Zhang *et al.* [32].

4.5. Western Blot Analysis of Caspase -3, -8, -9 and p53

Protein concentration was measured in each cell lysate by the protein assay (Pierce, Illinois) with albumin as a standard. Equal amounts of protein lysates (60 µg) were resolved on 12.5% SDS-poly-acrylamide gels and transferred to nitrocellulose membrane. Membranes were blocked for 1 h in 5% skim milk in TBS (10 mM Tris, 100 mM NaCl), and then probed with primary antibodies against caspase-3, -8, -9 and p53. The secondary antibodies conjugated to horseradish peroxidase were used for development with the enhanced chemiluminescence detection (ECL) kit. The Western blots were quantified using a UVP Biochem Gel Documentation system (UVP, Inc., Upland, California). Caspase -3, 8- ,-9 and p53 data were normalized using beta-actin as the housekeeping protein used routinely in our laboratories.

4.6. Statistical Analysis

The INSTAT software package (Graph Pad, San Diego, CA) was used to analyze the data. Chi Square was used for analyzing the data on tumor incidence. Student's t test was used to compare the tumor multiplicity, weight gain, caspase-3, -8, -9 and p53 levels in UVB-induced carcinogenesis. Significance in all the cases was considered at $P < 0.05$.

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