

## Chemopreventive effects of combination of honokiol and magnolol with $\alpha$ -santalol on skin cancer developments

Chandeshwari Chilampalli<sup>1</sup>, Xiaoying Zhang<sup>1</sup>, Radhey S. Kaushik<sup>2,3</sup>, Alan Young<sup>3</sup>, David Zeman<sup>3</sup>, Michael B. Hildreth<sup>2</sup>, Hesham Fahmy<sup>1</sup>, Chandradhar Dwivedi<sup>1,\*</sup>

<sup>1</sup> Departments of Pharmaceutical Sciences, South Dakota State University, Brookings, SD, USA;

<sup>2</sup> Departments of Biology and Microbiology, South Dakota State University, Brookings, SD, USA;

<sup>3</sup> Departments of Veterinary and Biomedical Science, South Dakota State University, Brookings, SD, USA.

**ABSTRACT:**  $\alpha$ -Santalol is active component of sandalwood oil and has been shown to have chemopreventive effects against chemically and UVB-induced skin cancer development in mice.  $\alpha$ -Santalol is also shown to have skin permeation enhancing effects. Honokiol and magnolol isolated from *Magnolia officinalis* bark extract have also been shown to have chemopreventive effects against chemically and UVB-induced skin cancer in mice. This study was conducted to investigate the combination effects of  $\alpha$ -santalol, honokiol and magnolol to study any additive/synergistic effects to lower the doses required for chemoprevention. Pretreatment of combinations of  $\alpha$ -santalol with honokiol and magnolol significantly decreased tumor multiplicity upto 75% than control,  $\alpha$ -santalol, honokiol and magnolol alone in SKH-1 mice. Combination of  $\alpha$ -santalol with honokiol and magnolol also decreased cell viability, proliferation, and enhanced apoptosis in comparison to  $\alpha$ -santalol, honokiol and magnolol alone in Human epidermoid carcinoma A431 cells. Overall, the results of present study indicated combinations of  $\alpha$ -santalol with honokiol and magnolol could provide chemoprevention of skin cancer at lower doses than given alone.

**Keywords:** Honokiol, magnolol, chemoprevention, UVB photocarcinogenesis, apoptosis

### 1. Introduction

Human non melanoma skin cancer including basal

\*Address correspondence to:

Dr. Chandradhar Dwivedi, Department of Pharmaceutical Sciences, College of Pharmacy Box 2202 C, South Dakota State University, Brookings, SD 57007, USA.

E-mail: Chandradhar.Dwivedi@sdstate.edu

cell carcinoma and squamous cell carcinoma is most common type of cancer in the United States for over 1.3 million new cases each year (1). Both experimental and epidemiological evidences suggest UVB acts as complete carcinogen in skin cancer (2,3). Carcinogenesis induced by UV is a multistep process which involves initiation, promotion and progression by which genetic events accumulated with in a cell leading to dysplastic cellular appearance, deregulated cell growth and finally to carcinoma development (4). UVB in mouse models can act as complete carcinogen by initiating and promoting skin cancer.

Chemoprevention involves the administration of natural or synthetic compounds to prevent or reverse the process of carcinogenesis. Cancer chemoprevention by naturally occurring agents, especially the phytochemicals, minerals and vitamins, has shown promising results against various malignancies (5,6). It is promising strategy for carcinoma inhibition before development of invasive tumor (7). In recent years to inhibit or reverse the multistage process, considerable efforts are focused towards natural occurring compounds (5,6). Over 1,000 phytochemicals have shown chemopreventive effects against cancer (6-9). Among such phytochemicals, chemopreventive effects of  $\alpha$ -santalol, honokiol and magnolol have been investigated against skin cancer and have shown excellent chemopreventive effects.

$\alpha$ -Santalol is active component of sandalwood oil which constitutes about 61% (w/w) of oil. Studies conducted in our laboratory have shown the topical application of 5% (w/v) of  $\alpha$ -santalol inhibited skin tumorigenesis against both chemical (10) and UVB (11) induced animal cancer models. In addition to this, mechanistic studies conducted in human epidermoid carcinoma A431 cells have shown that possible mechanisms involved in chemopreventive effects of  $\alpha$ -santalol is by induction of apoptosis through extrinsic and intrinsic pathway and inhibition of cell proliferation through cell cycle arrest at G2/M phase (7,12,13).

Honokiol and magnolol are isomers isolated from bark and seed cones of *Magnolia officinalis* which

are used in traditional Chinese medicine (14). Studies conducted recently in our laboratory have shown that honokiol and magnolol have shown chemopreventive effects against skin cancer development (15,16). Furthermore, the study conducted in our laboratory have shown that  $\alpha$ -santalol acts as a permeation enhancer for certain drug such as 5-fluorouracil. This study was conducted to investigate if reduced doses of  $\alpha$ -santalol produce similar effects in combination of honokiol and magnolol, may enhance the chemopreventive effects of honokiol and magnolol, and may provide additive/synergistic effects against UVB-radiation induced skin cancer development in SKH-1 mice and *in vitro* in A431 cells.

## 2. Materials and Methods

### 2.1. Reagents

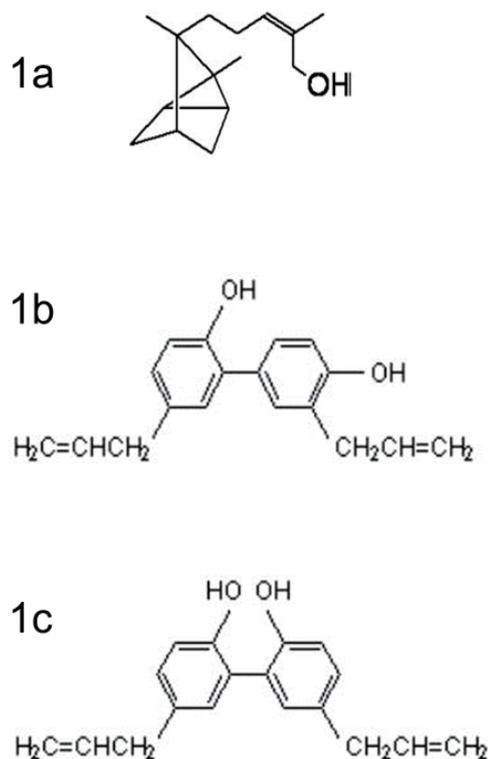
$\alpha$ -Santalol was isolated from sandalwood oil and characterized as reported (18). Thiazolyl blue tetrazolium bromide (MTT) and other chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell proliferation ELISA kit and In Situ Cell Death Detection Kit were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Vibrant Apoptosis Kit 2 and APO-BrdU TUNEL assay kit were purchased from Molecular Probes (Eugene, OR, USA).

### 2.2. Isolation characterization and purity of $\alpha$ -santalol

$\alpha$ -Santalol (Figure 1a) was isolated from sandalwood oil by distillation under vacuum as described in detail (18). On the basis of the NMR spectrum and the boiling point of the distillate, the major component of sandalwood oil is  $\alpha$ -santalol. Further, GC-MS analysis, NMR data and mass spectrum of the isolated agent were consistent with the structure of  $\alpha$ -santalol. Honokiol (Figure 1b) and magnolol (Figure 1c) were purchased from Nacalai tesque (Kyoto, Japan).

### 2.3. UVB exposure source and animals

UVB light source was four FS-40-T-12-UVB sunlamps (Daavlin, Bryan, OH) emitting 80% radiation within 280-340 nm with a peak at 314 nm. The UVB exposure dose was controlled using two Daavlin Flex Control Integrating 305 Dosimeters. Female SKH-1 hairless mice (five-weeks-old) were purchased from Charles River Laboratories (Wilmington, MA). All animal protocols were approved by the Institutional Animal Care and Use Committee. Mice were housed in the College of Pharmacy, South Dakota State University, animal room facilities (temp.  $22 \pm 1^\circ\text{C}$ , humidity, 40-60%, light 6.00-18.00 h), and given food and water *ad libitum*. Mice were acclimatized for 2 weeks before starting the experiment.



**Figure 1. Structures of a)  $\alpha$ -santalol, b) honokiol, and c) magnolol.**

### 2.4. Effects of $\alpha$ -santalol and honokiol on UVB-induced skin cancer development

Female SKH-1 mice were divided in four groups having 20 mice in each group. Group assignment was as follows: group 1, control (200  $\mu\text{L}$  acetone); group 2,  $\alpha$ -santalol (5 mg in 200  $\mu\text{L}$  acetone); group 3, honokiol (30  $\mu\text{g}$  in 200  $\mu\text{L}$  acetone); group 4,  $\alpha$ -santalol (5 mg in 100  $\mu\text{L}$  acetone) and honokiol (30  $\mu\text{g}$  in 100  $\mu\text{L}$  acetone). The mice in all groups were irradiated with 30  $\text{mJ}/\text{cm}^2$  dose of UVB one hour after the topical application as outlined above. UVB-irradiation was given 5 days a week and the experiment was continued for 30 weeks. Animals were monitored for food and water consumption, and any apparent signs of toxicity such as weight loss or mortality during the entire study period. Skin tumor formation, as evident by an outgrowth  $> 1$  mm in diameter and persisting for  $\geq 2$  weeks was recorded. Tumor incidence and multiplicity were recorded weekly until the end of the experiment at 30 weeks.

### 2.5. Effects of $\alpha$ -santalol and magnolol on UVB-induced skin cancer development

Female SKH-1 mice were divided in four groups having 20 mice in each group. Group assignment was as follows: group 1, control (200  $\mu\text{L}$  acetone); group 2,

$\alpha$ -santalol (5 mg in 200  $\mu$ L acetone); group 3, magnolol (30  $\mu$ g in 200  $\mu$ L acetone); group 4,  $\alpha$ -santalol (5 mg in 100  $\mu$ L acetone) and magnolol (30  $\mu$ g in 100  $\mu$ L acetone). Experimental protocol similar to honokiol was carried out for 30 weeks.

### 2.6. Cell culture

Human epidermoid carcinoma A431 cells were purchased from American Type Culture Collection (Manassas, VA). A431 cells were cultured in DMEM supplemented with 10 % FBS, 100  $\mu$ g/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>.

### 2.7. MTT assay for cell viability

Cells (9,000 cells/well) were plated in 96 well plates. After 24 h, cells were treated with different concentrations of control,  $\alpha$ -santalol, honokiol, magnolol for 48 h using control as cells treated with growth medium alone. At the end of each treatment, cells were incubated with 10% of MTT stock solution (5 mg/mL) for 4 h. Thereafter medium was aspirated and 150  $\mu$ L of DMSO (dimethyl sulfoxide) was added to dissolve crystal dye formazan for 1 h. Absorbance was measured at 570 nm with absorbance at 650 nm to correct background for blank (media without cells) using Spectra Max M2 microplate reader.

### 2.8. BrdU assay for cell proliferation

Bromodeoxyuridine incorporation assay is carried out using ELISA kit using manufactures protocol. Briefly 9,000 cells/well were plated in 96 well plate and treated with different concentrations of control,  $\alpha$ -santalol, honokiol, magnolol for 48 h. At the end of each treatment period, cells were labeled with BrdU by incubating for 3 h at 37°C. Then cells were fixed by using FixDenat solution for 30 min followed by incubating fixed cells with anti-BrdU peroxidase solution for 90 min. Then cells were rinsed with washing solution and incubated with substrate solution for 20 min. Reaction was stopped using 1M H<sub>2</sub>SO<sub>4</sub> and absorbance of samples were measured using microplate reader using 450 nm with absorbance at 690 nm as reference.

### 2.9. Apoptosis assay

Apoptosis was quantified by using Vibrant Apoptosis Kit 2 as per manufactures protocol. Briefly A431 cells ( $2 \times 10^5$ ) were treated with control,  $\alpha$ -santalol, honokiol, magnolol for 48 h. At the end of treatment cells were washed with ice cold PBS and incubated with annexin V labeled with Alexa 488 and PI for 15 min at room temperature. The stained cells were analyzed by FACS using Cell Quest 3.3 software. The early apoptotic cells

were stained with Alexa 488 represented in lower right (LR) quadrant that gave green fluorescence, and late apoptotic cells were stained with both Alexa 488 and PI that gave green and red fluorescence represented in upper right (UR) quadrant.

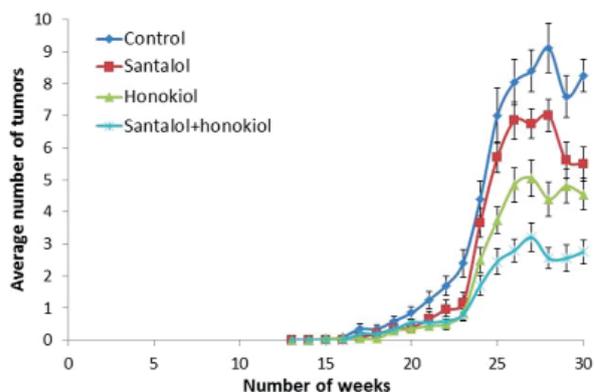
### 2.10. Statistical analysis

ANOVA and Tukey test were performed on sample means using INSTAT software (Graph Pad, Sand Diego, CA). Significance was considered at  $p < 0.05$ .

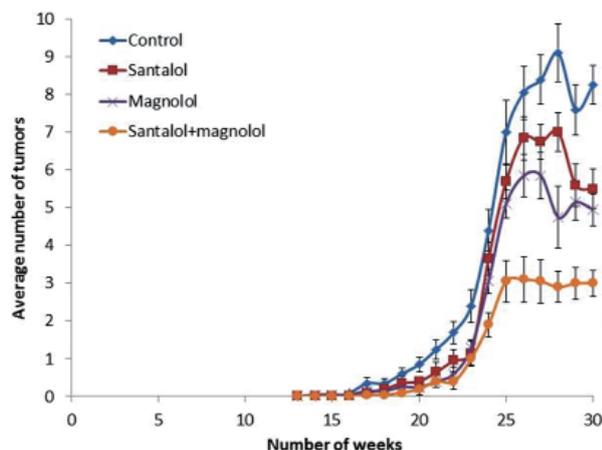
## 3. Results

### 3.1. $\alpha$ -Santalol and honokiol combination decreased tumor multiplicity

The effects of  $\alpha$ -santalol, honokiol and combination of  $\alpha$ -santalol and honokiol on UVB-induced tumor multiplicity is shown in Figure 2. In the present study we found that topical application of  $\alpha$ -santalol and honokiol combination significantly decreased tumor multiplicity compared to control and mice treated with  $\alpha$ -santalol and honokiol alone. We found topical application of  $\alpha$ -santalol and honokiol combination resulted in strong protection throughout our study where UVB act as complete carcinogen. At the end of 30 weeks experiment, average number of tumors in various groups was found to be 8.3, 5.6, 4.6, and 3. Furthermore, the combination of  $\alpha$ -santalol and honokiol not only provided about 75% decrease in number of tumors as compared to control mice but significantly higher decrease than  $\alpha$ -santalol and honokiol alone. These results clearly provide the



**Figure 2. Effects of  $\alpha$ -santalol, honokiol and combination of  $\alpha$ -santalol and honokiol on tumor multiplicity in SKH-1 mice.** Mice were irradiated chronically with UVB, 1 h after drug treatment. Group 1 served as control received acetone and group 2, 3, 4 are  $\alpha$ -santalol, honokiol, and combination of  $\alpha$ -santalol and honokiol. Combination significantly decreased tumor numbers compared to control and mice treated with individual drugs alone. Each data point represents mean tumor number  $\pm$  SE.



**Figure 3. Effects of  $\alpha$ -santalol, magnolol, and combination of  $\alpha$ -santalol and magnolol on tumor multiplicity.**  $\alpha$ -Santalol and magnolol combination significantly decreased tumor number compared to control,  $\alpha$ -santalol, and magnolol alone. Each data point represents mean tumor number  $\pm$  SE.

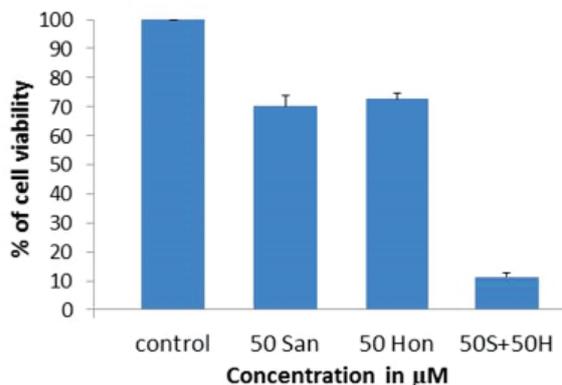
evidence that  $\alpha$ -santalol significantly enhances the chemopreventive effects of honokiol on UVB-induced cancer development in SKH-1 mice.

### 3.2. $\alpha$ -Santalol and magnolol combination decreased tumor multiplicity

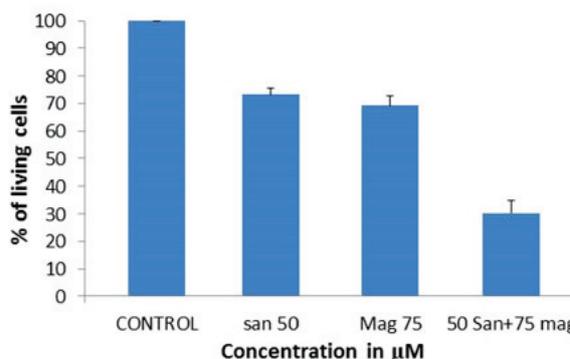
The effects of  $\alpha$ -santalol, magnolol and combinations of  $\alpha$ -santalol and magnolol on UVB-induced tumor multiplicity are shown in Figure 3.  $\alpha$ -Santalol and magnolol significantly decreased the average number tumors when compared with control. A combination of  $\alpha$ -santalol and magnolol topical treatment again provided about 75% decrease in the number of tumors per mice. Furthermore, the combination of  $\alpha$ -santalol and magnolol provided significantly higher decrease in average number of tumors than both mice treated with  $\alpha$ -santalol and magnolol alone. The data again clearly demonstrates the enhancing effects of  $\alpha$ -santalol on the chemopreventive effects of magnolol.

### 3.3. Effects of $\alpha$ -santalol combination on the effects of honokiol and magnolol on cell viability of A431 cells

The effects of  $\alpha$ -santalol combination on the effects of honokiol and magnolol on cell viability of A431 cells is shown in Figure 4 and 5.  $\alpha$ -Santalol and honokiol treatment (50  $\mu$ M) alone resulted about 30% decrease in the cell viability of A431 cells (Figure 4). However combination of  $\alpha$ -santalol (50  $\mu$ M) with honokiol (50  $\mu$ M) caused a 90% decrease in cell viability of A431 cells. This combination of  $\alpha$ -santalol with honokiol significantly enhances the cytotoxic effects in A431 cells. Similarly, combination of  $\alpha$ -santalol (50  $\mu$ M) with magnolol (100  $\mu$ M) significantly enhances the cytotoxic effects in A431 cells (Figure 5).



**Figure 4. Effects of  $\alpha$ -santalol, honokiol, and combination of  $\alpha$ -santalol and honokiol on cell viability in A431 cells.** A431 cells were treated with  $\alpha$ -santalol, honokiol, and combination of  $\alpha$ -santalol and honokiol for 48 h. By using method as described in materials and methods, MTT assay was performed. Values are expressed as mean  $\pm$  SE of eight replicates in each experiment in terms of percentage relative to control cells.



**Figure 5. Effects of  $\alpha$ -santalol, magnolol, and combination of  $\alpha$ -santalol and magnolol on cell viability in A431 cells.** A431 cells were treated with  $\alpha$ -santalol, magnolol, and combination of  $\alpha$ -santalol and magnolol for 48 h. By using method as described in materials and methods, MTT assay was performed. Values are expressed as mean  $\pm$  SE of eight replicates in each experiment in terms of percentage relative to control cells.

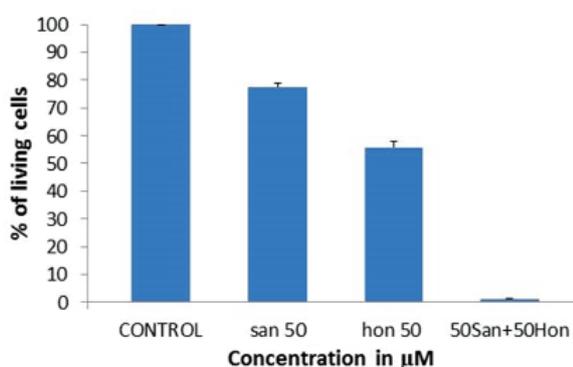
### 3.4. Effects of $\alpha$ -santalol combination on the effects of honokiol and magnolol on cell proliferation of A431 cells

Effects of  $\alpha$ -santalol combination on the effects of honokiol on cell proliferation of A431 cell is presented in Figure 6.  $\alpha$ -Santalol (50  $\mu$ M) treatment caused a reduction of 20 and 40% respecting cell proliferation of A431 cells. However, a combination of  $\alpha$ -santalol (50  $\mu$ M) with honokiol (50  $\mu$ M) almost completely stopped the cell proliferation of A431 cells. Although, combination of  $\alpha$ -santalol with magnolol significantly enhances the effects on cell proliferation but not completely as with honokiol (Figure 7).

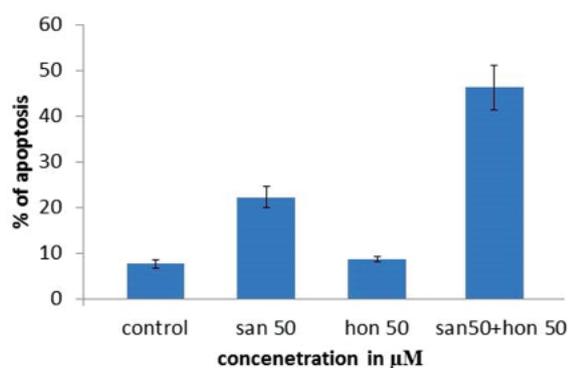
### 3.5. Effects of $\alpha$ -santalol combination on the effects of honokiol and magnolol on apoptosis of A431 cells

The effects of  $\alpha$ -santalol combination on the effects

of honokiol and magnolol on apoptosis in A431 cells is shown in Figures 8 and 9. To investigate whether cell death resulted is in response to apoptosis, we determined it by annexin V/PI staining which detects early stages of apoptosis and results were analyzed through FACS. The cells were treated with  $\alpha$ -santalol, honokiol, combination of  $\alpha$ -santalol and honokiol (50, 50, and 50 + 50  $\mu$ M) concentrations respectively for 48 h. The results showed that compared to DMSO treated control, apoptotic cells in  $\alpha$ -santalol, honokiol, and combination of  $\alpha$ -santalol and honokiol were increased. These results suggested that combination of  $\alpha$ -santalol and honokiol significantly increased apoptosis compared to cells treated with  $\alpha$ -santalol and honokiol alone (Figure 8).



**Figure 6. Effects of  $\alpha$ -santalol, honokiol, and combination of  $\alpha$ -santalol and honokiol on cell proliferation in A431 cells.** A431 cells were treated with  $\alpha$ -santalol, honokiol, and combination of  $\alpha$ -santalol and honokiol for 48 h. BrdU assay is performed for determining cell proliferation. Values are expressed as mean  $\pm$  SE of four replicates in each experiment in terms of percentage relative to control cells.

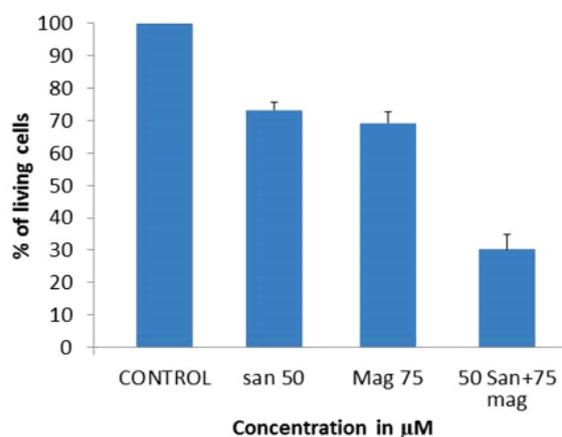


**Figure 8. Effects of  $\alpha$ -santalol, honokiol, and combination of  $\alpha$ -santalol and honokiol on apoptosis in A431 cells.** A431 cells were treated with  $\alpha$ -santalol (san 50) and honokiol (hon 50) at 50  $\mu$ M and combination of  $\alpha$ -santalol and honokiol (san 50 + hon 50) for 48 h. Cells were stained with annexin V/PI by using vibrant kit as described under materials and methods and results were analyzed by flow cytometry. Data represent mean  $\pm$  SE of three observations.  $p < 0.05$  indicates statistical significance in drug treated groups.

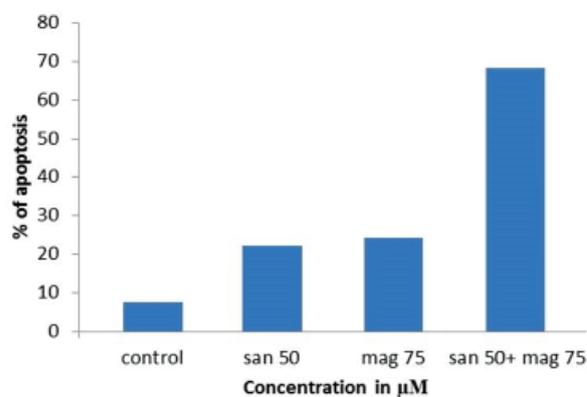
In case of  $\alpha$ -santalol and magnolol combination cells were treated with varying concentrations of  $\alpha$ -santalol, magnolol, and combination of  $\alpha$ -santalol and magnolol (50, 100, and 50 + 100  $\mu$ M) respectively for 48 h. Similar to  $\alpha$ -santalol and honokiol combination,  $\alpha$ -santalol and magnolol combination significantly ( $p < 0.05$ ) increased apoptosis (Figure 9).

#### 4. Discussion

Emulsion, paste, or essential oil of sandalwood (SW) has been used for centuries in India for treatment of inflammatory and eruptive skin diseases (18). Ayurvedic physicians (traditional medical practitioners in India) treat numerous skin lesions in patients with



**Figure 7. Effects of  $\alpha$ -santalol, magnolol, and combination of  $\alpha$ -santalol and magnolol on cell proliferation in A431 cells.** A431 cells were treated with  $\alpha$ -santalol, magnolol, combination of  $\alpha$ -santalol and magnolol for 48 h. BrdU assay is performed for determining cell proliferation. Values are expressed as mean  $\pm$  SE of four replicates in each experiment in terms of percentage relative to control cells.



**Figure 9. Effects of  $\alpha$ -santalol, magnolol, and combination of  $\alpha$ -santalol and magnolol on apoptosis in A431 cells.** A431 cells were treated with  $\alpha$ -santalol (san 50) and magnolol at 100  $\mu$ M (mag 100) and combination of  $\alpha$ -santalol and magnolol (san 50 + mag 100) for 48 h. Cells were stained with annexin V/PI by using vibrant kit as described under materials and methods and results were analyzed by flow cytometry. Data represent mean  $\pm$  SE of three observations.  $p < 0.05$  indicates statistical significance in drug treated groups.

SW oil (18). The essential oil of SW is distilled from the small chips and billets cut out of the heartwood of SW. The oil is extremely viscid, of a light yellow color, and possesses a characteristic pleasant odor. The major constituent of oil is santalol, a mixture of two isomers,  $\alpha$ - and  $\beta$ -santalol. Other constituents of oil are aldehydes, ketones, isovaleric aldehyde, santanone, esters, and free acids (19). SW oil inhibits the replication of Herpes simplex viruses-1 and 2 *in vitro* (19). SW oil treatment (100  $\mu$ L, 5% in acetone, twice a week, topically) significantly decreased papilloma incidence by 67% and multiplicity by 96% in 7, 12-dimethyl benzanthracene (DMBA)-initiated and 12.0-tetradecahydro phorbol-13-acetate (TPA)-promoted CD-1 mice. SW oil treatment also decreased TPA-induced epidermal ODC activity in CD-1 mice (20). SW oil treatment decreased papilloma incidence and multiplicity in a time- and concentration-dependent manner in CD-1 mice (20). The pretreatment with 5% SW oil 1 h before DMBA and TPA treatment provided a maximum reduction in papilloma incidence and multiplicity (21). Our laboratory isolated  $\alpha$ -santalol from SW oil by distillation and characterized it by NMR and GC-MS. NMR and GC-MS indicated that  $\alpha$ -santalol is a major component (~61%) of SW oil.

$\alpha$ -Santalol (5% topical) inhibited skin papilloma development during the promotion phase of DMBA and TPA protocol in CD-1 and SENCAR strains of mice. Induction of epidermal ODC activity and DNA synthesis are some of the prominent effects of TPA treatment on skin. As expected,  $\alpha$ -santalol treatment (5% topical) significantly decreased ( $p < 0.05$ ) TPA-induced ODC activity and incorporation of 3H-thymidine in DNA in the skin of CD-1 and SENCAR strains of mice. The effects of  $\alpha$ -santalol on skin papilloma incidence and multiplicity are very similar to the effects of SW oil as reported earlier from our laboratory (20,21).  $\alpha$ -Santalol has a pleasant fragrance, does not produce any stain, and appears to be nontoxic at concentrations used in our study. The effectiveness of  $\alpha$ -santalol as chemopreventive agents appears to be very promising in skin cancer control.

Since UV radiations are the major cause of skin cancer in humans the effects of  $\alpha$ -santalol on ultraviolet B (UVB) radiation-induced skin tumor development and UVB-caused increase in epidermal ornithine decarboxylase (ODC) activity in female hairless SKH-1 mice were studied (10). For the tumor studies, 180 mice were divided into three groups of 60 mice each and each group was divided into two subgroups of 30 mice. The first subgroup served as control and was treated topically on the dorsal skin with acetone. The second subgroup served as experimental and was treated topically on the dorsal skin with  $\alpha$ -santalol (5%, w/v in acetone). The tumorigenesis in the first group was initiated with UVB radiation and promoted with TPA; in the second group it was initiated with DMBA and promoted with UVB radiation; and in the third

group it was both initiated and promoted with UVB radiation. UVB radiation was at a dose of 180 mJ/cm<sup>2</sup>. Topical application of  $\alpha$ -santalol significantly ( $p < 0.05$ ) decreased tumor incidence and multiplicity in all the three protocols, suggesting chemopreventive efficiency against UVB radiation-induced skin cancer.  $\alpha$ -Santalol also significantly inhibited UVB-induced epidermal ODC activity. These findings suggest that  $\alpha$ -santalol could be a potential chemopreventive agent against UVB-induced skin tumor development.

Honokiol and magnolol isolated from the bark of *Magnolia officinalis* have been shown to decrease chemically-induced skin cancer development in mice (22). We investigated the chemopreventive effects of honokiol and magnolol on UVB-induced skin cancer development in mice, a model more relevant to human. Both honokiol and magnolol provided a significant protection against UVB-induced skin cancer development in mice (15,16). Furthermore, mechanistic studies conducted *in vivo* in SKH-1 mice and in A431 cells showed that honokiol and magnolol inhibited skin carcinogenesis by inducing apoptosis and inhibiting cell proliferation by causing cell cycle arrest (16,17).

Since  $\alpha$ -santalol, honokiol and magnolol are isolated from plants and are relatively expensive,  $\alpha$ -santalol, honokiol and magnolol have a great potential as chemopreventive agent for the skin cancer development. We investigated the effects of  $\alpha$ -santalol pretreatment on the effects of honokiol and magnolol on UVB-induced skin cancer development in mice to evaluate if pretreatment with  $\alpha$ -santalol enhances the effects of honokiol and magnolol.

The present study, for the first time demonstrated chemopreventive effects of topical applications of  $\alpha$ -santalol and honokiol and  $\alpha$ -santalol and magnolol combinations on skin tumor development in SKH-1 mice and in A431 cells. Pretreatment of combinations of  $\alpha$ -santalol with honokiol and magnolol significantly decreased tumor multiplicity upto 75% than control,  $\alpha$ -santalol, honokiol and magnolol alone. Overall, the results of present study indicated combinations of  $\alpha$ -santalol with honokiol and magnolol at lower doses than alone provides protection against UVB-induced skin tumorigenesis in SKH-1 mice possibly by inducing apoptosis.

#### Acknowledgements

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