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In vitro investigation of genoprotective and antioxidant effects of santalol in CCD-1079Sk human skin fibroblast cells

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ABSTRACT: *Santalum album (S. album)* is a plant in the family of Santalacea that has been used in Indian traditional medicine for many years due to its pharmacological properties such as antiseptic, antipyretic, antidiabetic, diuretic, expectorant, stimulant, antibacterial and antifungal. In recent studies, apoptotic, cytotoxic, antiviral, anti-Helicobacter pylori, hepatoprotective activity of *S. album* has been found. The main molecule in *S. album* is santalol. Considering its wide variety of use in the traditional medicine and cosmetic industry, it has been considered important to clarify its genoprotective and antioxidant properties. Starting from this point of view, the aim of our study is to examine the antioxidant and genoprotective effects of santalol against H_2O_2 induced damage in healthy human skin fibroblast cell line CCD-1079Sk.

The cytotoxicity of santalol was investigated with the measuerement of intracellular ATP content and MTT cytotoxicity test. According to the results of the cytotoxicity tests, the dose of santalol that caused an increasement in cell viability was chosen. The antioxidant effect of santalol was investigated by measuring protein carbonyl levels. Comet Assay was performed to determine the genoprotective properties of santalol.

The results showed that 50 µM santalol treatment after 50 µM H_2O_2 exposure reduced the protein carbonyl level and DNA damage. This revealed the potential antioxidant and genoprotective properties of santalol. Considering the results obtained from these *in vitro* studies, it has been seen that santalol is worth continuing to study further in terms of protection from oxidative stress and genotoxicity.

KEYWORDS: santalol; santalum album; sandalwood; antioxidant; genotoxicity.

1. INTRODUCTION

One of the most significant medicinal plants in the world is *Santalum album* (*S. album*, Sandalwood). This woody tree is a root hemiparasite tropical species belonging to the Santalaceae family and is used in Ayurveda, the Indian traditional medicine system, as an antiseptic, antipyretic, antichabetic, diuretic, expectorant, stimulant, and for the treatment of bronchitis, dysuria, urinary tract infection, gonorrhea, thanks to its antibacterial and antifungal properties [1].

Sandalwood's medicinal benefits and significance in health care are related to its abundant phytochemical resources, particularly sesquiterpenes [2]. It was observed that different chemical compounds were obtained from different parts of the plant. The oil is obtained from the bark. The main component of the oil is santalol (90%). The other components of the oil are exo-norbicycloexantalal, β-santhalic, teresantalic, nortricycloeccanthalic, bicycloeccanthalic and dihydro-β-santhalic acids, urs-12-en-3β-yl-palmitate, β-sitosterol, (+) episantalic -β-santalol, (-) β-santalol, (-) trans-β-santalol, α-santalol (52%), β-santalol (23%), epi-β-santalol, cis-lanceol, cis-nuciferol , β- and epi-β-teresantalic acid, β- and epi-bnorexanthatelic acid, β- and epi-β-exanthatelic acid, 11-keto-dihydro-α-santhalic acid, bisabolenols A, B, C, D and E , tricycloecanthalol, α - and β -santanalanes, trans- α -bergamoten, α -curcumon, nuciferol. l-allohydroxyproline, betulinic acid, β-

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sitosterol, and fatty acids are obtained from the fruits, and betulinic acid (0.05%), β-sitosterol, glucose, fructose, and sucrose are obtained from the leaves [3].

Sandalwood and its oil offer a wide range of medicinal properties. In recent years, anticancer, antifungal, and anti-inflammatory activities have been documented [4]. Anticancer [5], antiviral [6, 7] and anti-Helicobacter pylori [8] bioactive substances containing santalols and lignans [9] which are obtained from the heartwood of the plant. Considering the studies, it is seen that the S. album plant can be protective and/or therapeutic, especially thanks to its antioxidant, antimicrobial, and antitumorigenic effects.

People are routinely exposed to sandalwood essential oil through numerous routes of administration, including the skin, because to the rising popularity of essential oils (EOs) for massage and aromatherapy. Because of their lipid solubility and extremely small molecular size, as well as the lipophilic nature of the skin, EOs are rapidly absorbed via the skin [10].

The aim of this study is to evaluate the antioxidant and genoprotective effects of santalol which is the main active substance of *S. album*. For this purpose, human CCD-1079Sk skin fibroblast cells were used. The antioxidant and genoprotective effects of santalol was evaluated by pretreating the cells with hydrogen peroxide (H_2O_2) before santalol treatment and the protein carbonyl and DNA damage levels of the cells were measured. The genoprotective effect of santalol against DNA damage was examined using Comet Assay technique.

2. RESULTS

2.1. MTT cytotoxicity test results

Significant increase in cell viability was observed at 25, 50, 100, 400 µM (*p < 0.05) and 200 µM (**p < 0.01) concentrations after 24 h santalol exposure in CCD-1079Sk cell line (Figure1). The control group was the solvent control group that contain 1% DMSO (dimethyl sulfoxide).

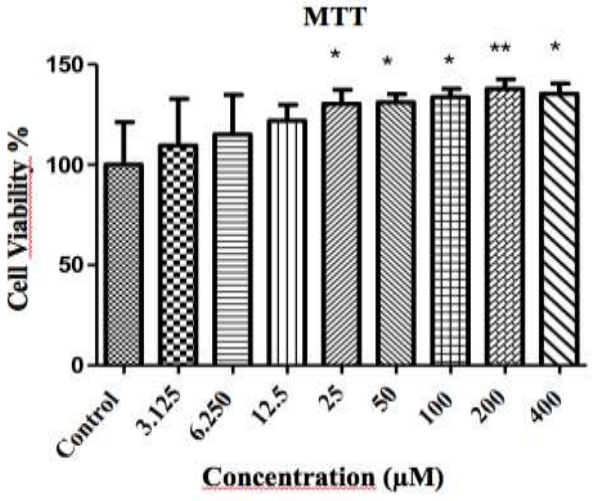

Figure 1: Effect of santalol on the MTT content of CCD-1079Sk cells after 24 h treatment. Cells were treated with 3.125-400 µM concentrations. Data are expressed as mean ± SD, *p < 0.05, **p < 0.01 versus control group.

2.2. ATP cytotoxicity test results

As seen in the Figure 2, statistically significant increase in ATP content was observed at 200 µM concentration (*p < 0.05) after 24 h of santalol exposure in CCD-1079Sk cell line (Figure2). The control group was the solvent control group that contain 1% DMSO.

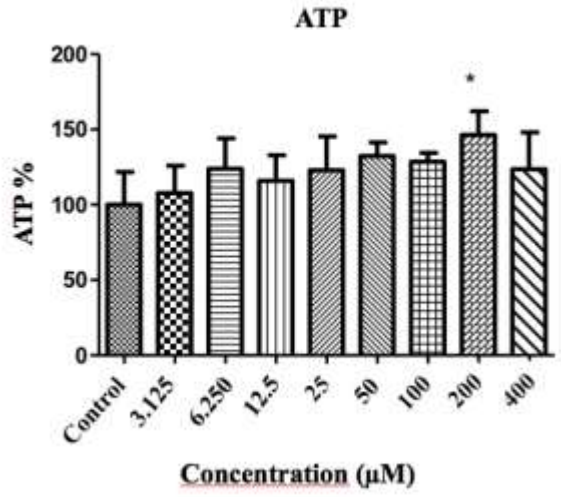

Figure 2: Effect of santalol on the ATP content of CCD-1079Sk cells after 24 h treatment. Cells were treated with 3.125-400 µM concentrations. Data are expressed as mean ± SD, *p < 0.05 versus control group.

2.3. Protein carbonyl assay (ELISA)

CCD-1079Sk cells were treated with 50 µM H_2O_2 for 4 h, and 50 µM santalol for 24 h. The control group was the solvent control group that contain 1% DMSO. Protein carbonyl levels were measured by enzyme-linked immunosorbent (ELISA) assay kit according to the manufacturer instructions (Bioassay Technology Laboratory, China). The protective effect of santalol against H_2O_2-induced protein carbonyl increasement was observed (*p < 0.05) according to the changes of protein carbonyl levels after 24 h santalol exposure which is given in Figure 3.

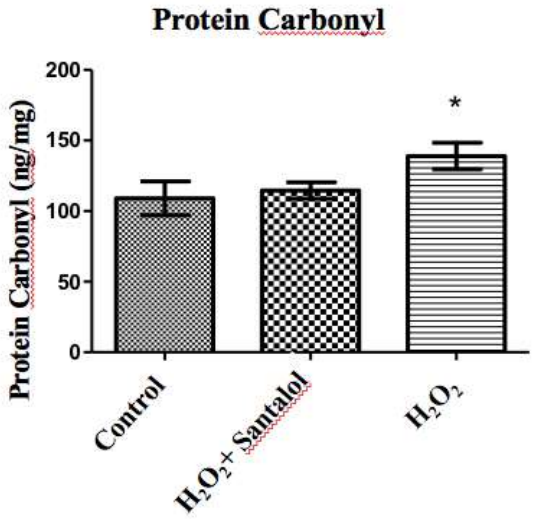

Figure 3: Effect of santalol on protein carbonyl level of the CCD-1079Sk cells following 24 h treatment. Cells were treated with 50 µM santalol for 24 h after 50 µM H_2O_2 pretreatment for 4 h. Data are expressed as mean ± SD, *p < 0.05 versus control group.

2.4. Comet assay

CCD-1079Sk cells were treated with 50 µM H_2O_2 for 4 h, and after that cells were exposed to santalol for 24 h at a concentration of 50 µM. The control group was the solvent control group that contain 1% DMSO. DNA damage was measured as tail intensity. The amount of genotoxicity is directly proportional to the length of the DNA tail. Santalol treatment was observed to protect the cells from DNA damage which is caused by H_2O_2. According to the results, there were statistical significance between H_2O_2 only group and H_2O_2+santalol

group and control group for the lenth of the DNA tail. (*p < 0.05). The results were given in the Figure 4 and 5.

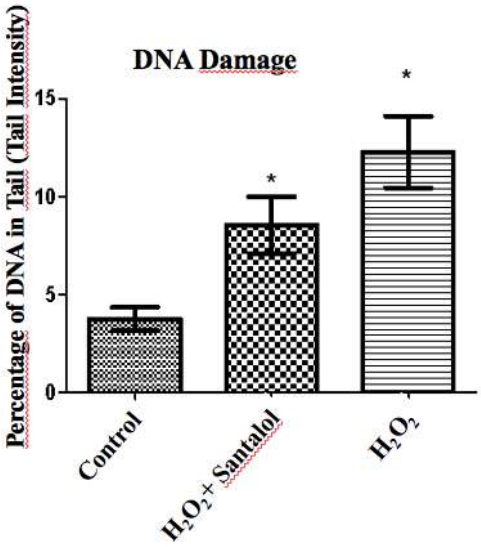

Figure 4: Effect of santalol on percentage of DNA tail of the CCD-1079Sk cells following 24 h treatment. Cells were treated with 50 µM santalol for 24 h after 50 µM H_2O_2 pretreatment for 4 h. Data are expressed as mean ± SD, *p < 0.05 versus control group.

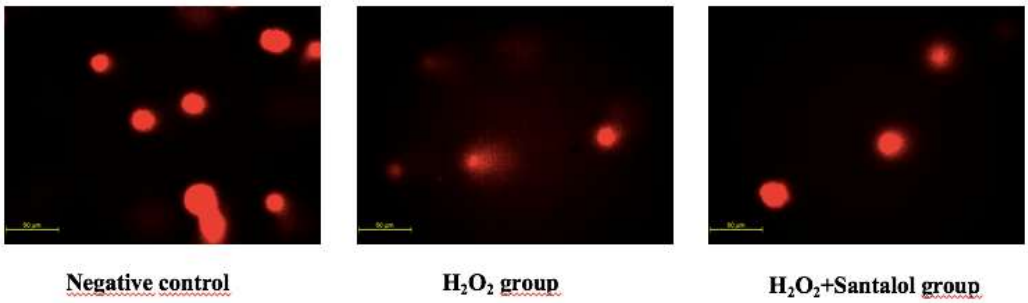

Figure 5: Comet images of the of the CCD-1079Sk cells following 24 h treatment. Cells were treated with 50 µM santalol for 24 h after 50 µM H_2O_2 pretreatment for 4 h.

3. DISCUSSION

Sandalwood oils possess plenty of usages for therapeutic applications in traditional medicine such as Chinese traditional medicine and Ayurveda. These essential oils are also used in aromatherapy, cosmetics, incense, personal care products, fragrance material. They are also approved by the FDA (United States Food and Drug Administration) for human consumption as a natural flavoring agent. The commercially available sandalwood oils are obtained from two different species of sandalwood trees named as East Indian sandalwood (*Santalum album*) and Western Australian sandalwood (*Santalum spicatum*). The contents and quality of the oils that are obtained from these two different species change and East Indian sandalwood oil (EISO) is indicated as a better quality than Western Australian sandalwood. EISO is composed of many chemical constituents but santalols -a mixture of sesquiterpenoids- are the most abundant ones [1, 11, 12].

Since these essential oils have plenty of usages in cosmetic industry, dermal exposure route is an important way to investigate how is their effect against dermal toxicity. So, the aim of our study was to examine in vitro protective effects of sandalwood oils against dermal toxicity in human skin fibroblastic cell line in genotoxicity, cytotoxicity and oxidative stress injury context. According to the results of our present study, significant increase in intracellular ATP content was observed at only 200 µM concentration after 24 h of santalol treatment in CCD-1079Sk cell line (*p < 0.05). Also, there is an increasement of cell viability % assessed via MTT cytotoxicity test after 24 h santalol treatment at 25, 50, 100, 200 and 400 µM concentrations (Fig. 1). Starting from this point of view 50 µM concentration was chosen for investigating the antioxidant and

genoprotective properties of santalol, since significant increasement of cell viability was observed at this concentration (*$p < 0.05$).

One of the important bioindicators of oxidative stress is known as protein carbonylation levels since oxidative stress causes formation of protein carbonyl. We investigated the protective effect of santalol against H_2O_2 induced damage and our results showed that after 50 µM H_2O_2 exposure for 4 h, treatment with santalol at 50 µM for 24 h decreased protein carbonyl levels compared with H_2O_2 treatment alone group (Fig. 3). This data proved the protective effect of santalol against oxidative stress damage induced by H_2O_2 in human healthy skin fibroblast cells.

There are various studies that shows different pharmacological activities such as antioxidant, antiviral, anti-inflammatory activities of EISO in the literature in different cell line models. Santalol is also being studied as a chemopreventive agent for skin cancer. Dickinson's et al investigated the chemopreventive mechanism of EISO in keratinocytes. They studied the influence of EISO on cell proliferation, cell death and apoptosis, inhibition of some important signalling pathways. It is found that EISO treated cells induce autophagy by disrupted membrane integrity and cleavage of LC3 [12]. Even though our study was about the protective effects of EISO against healthy skin cells, results prove the positive effects of these essential oils in a different manner.

Evaluation of genotoxic potential of plants allows to identify whether they might have anticarcinogenic effects or not. A study which is conducted by Ortiz et al evaluated the cytotoxicity and genotoxicity of sandalwood essential oil in human breast cell lines. According to this, they found that sandalwood oil causes single and double strand breaks of DNA selectively in MCF-7 human breast cancer cells and has genotoxic effects compared with healthy breast cell line MCF-10A. This data provides evidence that the cytotoxic activity of sandalwood oil via genotoxic effects is only in cancer cells [13, 14]. Accordingly, our study also showed that santalol is not genotoxic in healthy human fibroblast cells and it provides genoprotective effect against H_2O_2 induced DNA damage (Figure 4 and 5).

It is indicated that santalol -the active ingredient of sandalwood oil- also activates caspase-3 and inhibits the growth of human prostate cancer cells. Furthermore, it is shown that sandalwood oil causes tumour growth inhibition via different mechanisms involved G2/M cell cycle arrest, induction of apoptosis and autophagy. The protective effects of santalol against proteotoxic stress is also reported in C.*elegans* model for Parkinson's disease [15, 16]. These data show that sandalwood oil has many different activities by affecting various pathways in different in vitro and in vivo models, and further research is needed to elucidate its mechanisms.

There are plenty of natural antioxidants that are used to prevent accumulation and formation of free radicals. Like many other Indian plants with etnopharmacological usages for antioxidant potentials, EISO is also studied for it's antioxidant property. Misra et al evaluated the *in vivo* anti-hyperglycemic and antioxidant potential of sandalwood oil. They observed that oxidative stress induced mice has higher levels of antioxidant enzymes after treatment with sandalwood oil [11]. Mohankumar et al also evaluated the neuroprotective and geroprotective effect of EISO in C. *elegans* model via antioxidant properties. They found that EISO have high antioxidant capacity in a comparative study with vitamin c and epigallocatechin gallate as a positive control. In another study conducted by the same research group reported that santalol has antiaging effects via preventing oxidative stress and maintaining protein homeostasis [15, 17]

According to the data from the literature, it is proven that EISO has many different activities and traditional folk usages. In this study, we evaluated the antioxidant and genoprotective effects of santalol using the healthy skin fibroblastic cell line CCD-1079Sk. To investigate these effects, the non-cytotoxic concentrations of santalol were chosen for the experiments. The results of our study are found consistent with literature that santalol has protective effect against H_2O_2-induced oxidative stress and genotoxic effects in CCD-1079Sk cells.

4. CONCLUSION

There are various known therapeutic and cosmetic usages of sandalwood oils. Especially, since aromatherapy have became very popular lately, studies about sandalwood oils in different organisms and cell models can be encountered frequently. In addition to these studies, our study revealed the genoprotective and antioxidant properties of santalol in human skin fibroblast cell line. It was observed that santalol is a protective constituent of sandalwood oil against oxidative stress and genotoxicity induced by H_2O_2 exposure in CCD-1079Sk cells. This study can be considered a valuable preliminary study of a more detailed *in vivo* study investigating santalol's effects after the absorbtion from the skin which is an important route for exposure.

5. MATERIALS AND METHODS

5.1. Cell culture studies

Human fibroblast cells CCD-1079Sk were obtained from American Type Culture Collection (ATCC, VA, USA) and cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum and 1 % penicillin–streptomycin (Gibco, USA). Sub-culturing was done every 2–3 days.

5.2. Santalol treatment

Santalol was purchased from Sigma and dissolved in DMSO. The studied concentrations were chosen according to the results of the cell viability tests which the treatment was done in 400-3.125 µM concentration range. 50 µM concentration was chosen for investigating the antioxidant and genoprotective properties of santalol, since significant increasement of cell viability was observed at this concentration ($^*p < 0.05$). For protein carbonyl and comet assay, CCD-1079Sk cells were treated with 50 µM H_2O_2 for 4 h, after that cells were exposed to santalol for 24 h at a concentration of 50 µM.

5.3. MTT cytotoxicity test

The use of MTT tetrazolium compound to measure the number of viable cells *in vitro* was first described by Mosmann in 1983. It is based on the conversion of the tetrazolium ring in the MTT compound into water-insoluble formazan crystals by the mitochondrial succinate dehydrogenase enzyme of living and mitochondrial intact cells. MTT compound dissolves in the cell culture media and it has a structure to pass into the cell membrane. MTT was prepared in PBS at concentration of 5 mg/mL and added directly to the cells in culture. Before calculating the absorbance at 570 nm, formazan crystal precipitates were dissolved in DMSO. The amount of absorbance is proportional to the number of viable cells.

5.4. ATP cell viability assay

Adenosine triphosphate (ATP) is a molecule found in all metabolically active cells and serves as an energy source in biological systems. After cell death, ATP synthesis does not occur in the cell and the existing ATP in the cell is rapidly destroyed. The amount of intracellular ATP is a sensitive indicator of cell viability. ATP content of the cells was determined by using the ATP Bioluminescence kit CellTiter-Glo® 2.0 (Promega, USA). Opaque-walled 96-well plates were used. Cells were seeded in culture medium as 10^4 in each well, and santalol treatment was conducted after a 24 h incubation. Following santalol treatment, cells were shaken for 2 min with CellTiter-Glo® 2.0 Reagent equal to the volume of cell culture medium present in each well. Then the plate was incubated for 10 min in room temperature to stabilize the luminescence signal. Finally, the luminescent signal was recorded.

5.5. Protein carbonyl assay

The presence of oxidative damage is determined by measuring the protein carbony levels, which is released as a result of the effects of free radicals and oxidative damage of macromolecules, by biochemical methods. Protein carbonyl levels were measured by enzyme-linked immunosorbent (ELISA) assay kit according to the manufacturer instructions (Bioassay Technology Laboratory, China). According to the kit description, briefly, cells were seeded in 6-well plates. The cells were incubated for 24 h for adherence to the plate. After that, santalol treatment was conducted then it was incubated for another 24 h and collected by trypsinization into centrifuge tubes. The supernatant formed after centrifugation was discarded and the precipitate was dispersed in PBS. The newly formed mixture was centrifuged. The supernatant formed after centrifugation was discarded and the precipitate was dispersed in PBS again. Cell-PBS mixtures were freeze-thawed 3 times so that the cells were burst. Then they were centrifuged again. The supernatant obtained as a result of centrifugation was used during the experiment according to the kit description. The absorbance of the resulting color was measured at 450 nm absorbance using a BioTek Synergy H1 microplate reader. The protein concentrations were measured using the BCA Protein Assay kit (Intron Biotechnology, Korea) and the results are given as ng/mg protein. The statistical analysis of oxidative damage in cells was done according to the standard curve created for the previously prepared standard solutions.

5.6. Comet assay

The genoprotective effect of santalol against DNA damage was examined using the Comet Assay technique. Cells were seeded in 6-well plates and were incubated for 24 h for adherence to the plate. 50 µM

H_2O_2 pretreatment was conducted for 4 h, then the cells were incubated for 24 h with 50 µM santalol. The supernatant formed after centrifugation was discarded and the precipitate was dispersed in PBS. Comet agarose was used to cover comet slides to create a base layer. Cell samples were combined 1/10 (v/v) with comet agarose, mixed well by pipetting, and immediately transferred onto 75 µL/well comet agarose base layer. The cells were spread on the slides with a pipette tip. The slides were transferred to 4°C for 15 minutes keeping them horizontal. The slides were carefully transferred to a small basin/container containing pre-chilled lysis buffer (10mM Tris, 2.5M NaCl, 100mM EDTA, distilled water ~25 mL/slide) and immersed in buffer for 30-60 minutes at 4°C in the dark. The lysis buffer was carefully aspirated from the container and replaced with pre-chilled alkaline solution (3% NaOH, 0.5 %EDTA~25 mL/slide). The slides were immersed in the solution for 30 minutes at 4°C in the dark. Maintaining the slides horizontally, they were transferred carefully from the alkaline solution to a horizontal electrophoresis chamber. The chamber was filled the with cold alkaline electrophoresis solution until the buffer level covers the slides. Voltage was applied to the chamber for 15-30 mins at 1 volt/cm. The chamber set to 300mA. Maintaining the slides horizontally, they were transferred carefully from the electrophoresis chamber to a clean, small basin/container containing pre-chilled distilled H_2O (~25 mL/slide). The slide was immersed for 2 mins, water was aspirated, and then repeat twice more. The final water was aspirated rinse and replace with cold 70% ethanol for 5 mins. The slides were removed from the 70% ethanol and allowed to air dry. When the agarose and the slides were completely dry, 100 µL/well of diluted Vista Green DNA Dye was added. It was incubated for 15 minutes at room temperature. Slides were viewed by epifluorescence microscopy using a FITC filter. The DNA damage was assessed from the percentage of DNA in the tail (TI %). 100 cells/slide were assessed using a Comet assay IV image analysis system (Perceptive Instruments, UK).

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