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Original Article

The Selective Cytotoxicity of The Hydroalcoholic Extract of *Santalum Album* Linn Wood on A375 and SK-MEL-3 Human Malignant Melanoma Cells

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Abstract

Melanoma is the cause of most skin cancer all around the world because of its high proliferation rate, metastatic nature, and limited effective therapies. According to the rapid increment in its incidence compared to other types of skin cancers, new therapies are seemed to be essential. Natural remedies can be used to treat many diseases, including cancer, so more research is needed. The wood of Santalum album Linn, known as "white sandalwood", is one of the herbs which is a rich source of antioxidants that can be used as a therapeutic medication in different types of cancers, especially skin cancer. This research aimed to verify the cytotoxic effects of white sandalwood on A375 and SK-MEL-3 melanoma and AGO-1522 normal human fibroblast cell lines. At first, the ethanolic extract was prepared. Then, cell viability and cytotoxic activities were evaluated. Furthermore, ROS formation, lipid peroxidation, and release of cytochrome-c were also assessed. Herb extract significantly increased the death of A375 and SK-MEL-3 melanoma cells ($p < 0.001$), lipid peroxidation ($p < 0.01$), and reactive oxygen species ($p < 0.01$) and cytochrome c concentration ($p < 0.001$). Meanwhile, the same amount was ineffective and safe on AGO-1522 normal fibroblast cells. The extract of white sandalwood has considerable cytotoxic effects on the human melanoma cell line. Further studies are required to demonstrate the therapeutic effects of white sandalwood on melanoma cancer.

Keywords: Melanoma, White sandalwood, Melanoma cell line, Cytotoxicity, Santalum album Linn wood, Skin cancer.

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1. Introduction

Malignant melanoma is a highly malignant and invasive skin tumor, which is the most fatal skin cancer [1]. In recent decades, the worldwide incidence of that cancer has increased rapidly. Researches indicate that due to the aging of the population, melanoma prevalence is doubled every 10 to 20 years [2,

3]. Environmental factors including exposure to sunshine (specially type B ultraviolet radiation), occupational and nutritional factors, as well as multiple and abnormal nevi and immunosuppression are melanoma risk factors [4, 5]. Besides, genetic and epigenetic factors have an important role in this disease [6-8]. There are many efforts for reaching effective treatment for melanoma. Routine therapies include surgery, chemotherapy, hormone therapy, radiation therapy, gene therapy, and immunotherapy. Surgery is the first-line treatment for local lesions, especially in the early stage. However, in advanced cases, due to the metastatic nature of this cancer, response to treatment is weak. Melanoma is resistant to common medications such as single-drug chemotherapy regimens and radiotherapy, therefore, suggesting novel, effective, and low-risk therapies [9].

The use of medicinal plants in cancer therapy regimens has always been a favorite topic for research [10]. However, the use of herbal compounds and antioxidants for cancer therapy is still an important issue. But research has shown that nutritional advice provided to patients at the right time and under the supervision of a specialist can be somewhat helpful to treatment during the course of the disease and reduce drug side effects [11, 12]. In a study conducted by Abu Hazafa and his colleagues, the use of herbal compounds and especially polyphenols could be helpful in various cancers therapy through molecular mechanisms [13]. Previous studies reported the effectiveness of natural products in the treatment of malignant melanoma [14, 15].

Santalum album Linn, the family of Santalaceae, is the tree attains a height of 60-65 feet and is actually an obligate hemiparasite plant on various hosts. Trees more than 30 years old may have circumference from 18 to 38 inches. The bark and sapwood do not have any odor but the roots and heartwood contains the essential oil [16]. This herb is native to Coorg, Chennai and Mysore of southern India. The major components of sandalwood are santalol (α-santalol and β-santalol) as well as tannins, terpenes, resins and waxes [17-19]. α-santalene, trans-β-bergamotene, β-santalene, α-curcumine, α-santalol, β-santalol, nuciferol, α-santalal and β-santalal are another important constituent [20, 21].

Santalum album Linn is famous in traditional Persian medicine for treating different types of Owram (old Persian word means Cancers) [22]. Sandalwood has been reported to have the many pharmacological effects like: antifungal activities [23], Antibacterial Activities [24], Antiviral activities [24], antianginal attacks activities [25], Anti-ulcerogenic activities [26], Anti-inflammatory activities [27], Antipyretic activities [28], Antioxidant activities [29, 30], antihypertension activities [31], and antineoplastic activities [32].

In this mechanistic cellular study, we aimed to investigate the selective cytotoxicity of hydroalcoholic extract of sandalwood against human malignant melanoma cells.

2. Materials and Methods

2.1. Chemicals

2,4,6-Trinitrobenzene sulfonic acid (TNBS) and rhodanine from Sigma-Aldrich

Chemie (GmbH, Munich, Germany), thiobarbituric acid (TBA), trichloroacetic acid (TCA), n-butanol, hexadecyl trimethyl ammonium bromide (HETAB), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), diphenyl-2-picryl hydrazyl (DPPH), methanol, hydrochloric acid (HCl), malondialdehyde (MDA), ethylenediamine tetra-acetic acid (EDTA), O-di anisidine hydrochloride, hydrogen peroxide, acetic acid, sodium acetate, Coomassie reagent, bovine serum albumin (BSA), ferric chloride (FeCl3 · 6 H2O,(sodium sulfate (Na2SO4), sulfuric acid (H2SO4,(phosphoric acid (H3PO4), potassium dihydrogen phosphate (KH2PO4), potassium hydrogen diphosphate (K2HPO4), peroxide hydrogen (H2O2) and sodium carbonate (Na2CO3) from Merck (Germany) and wood of *Santalum album* Linn. was purchased from Iran.

2.2. Preparation of Plant Sample

The wood of *Santalum album* Linn. were purchased from the market of medicinal plants in Tehran, Iran, in December 2020. The wood grated well. A voucher specimen was preserved in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Science (TUMS), Tehran, Iran (No. PMP-939).

The sandalwood was finely powdered by milling. The extract was prepared using 30 g of powder of the gall with a mixture of ethanol/water (70:30) (21.8 g dry weight corresponding to 73% w/w) [33].

2.3. Cell Culture

Human malignant melanoma cell lines, A375 and SK-MEL-3 cell lines and normal human fibroblast, AGO-1522 cell line, was obtained from the Iranian biological resource center (Tehran, Iran). In this study, The AGO-1522 cell line, was used as a healthy control cell line.

These cell lines have cultured as a monolayer culture in RPMI medium supplemented with 1% nonessential amino acids, 1% L-glutamine, 100 IU per mL penicillin, 100 IU per mL streptomycin, 20 mg/mL glutamine, and 10% fetal bovine serum at 37°C in a 5% CO2 humidified atmosphere and 95% air in a CO_2 incubator. Cells were passaged twice/week under sterile conditions.

Cell lines at exponential growth phase were washed, trypsinized, and resuspended in fresh medium. Cells were seeded at a concentration of 10^4 cells/well in 96-microtitre plate. The cells were treated with different concentration of the sandalwood extracts for 24 h.

2.4. Cytotoxicity

The anti-proliferation effect is the first indication to be assessed when investigating novel antitumor agents. For this purpose, cell viability was evaluated using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), at 24h. The cells were seeded in 96-well plates (2.0×10^3 cells/well). Cells were allowed to attach overnight, and cell viability was evaluated by measuring the conversion of

tetrazolium salt MTT to formazan crystals. Then, 10 µL of MTT reagent (5 mg/mL in phosphate buffered saline) was dissolved in 120 µL complete medium and added to the cells (100 µL/well). After incubation for 4 h at 37 °C, the medium was discarded and 200 µL of 2-propanol was added. The reaction product was quantified by measuring the absorbance at 540 nm using an ELISA plate reader. Thus the cell growth inhibitory activity of plant extract was initially assessed on the melanoma cell lines and normal human fibroblast 24 h after treatment with different concentrations of the extract and IC50 which 50% of the cells in the plate lost their proliferation and viability, was defined by regression analysis and related models with probit regression model procedure using PRISM program. Then, the cytotoxicity effects of sandalwood extract were evaluated on the melanoma cell lines in comparison with normal human fibroblast using an MTT assay [34].

2.5. Lipid Peroxidation

Cellular lipid peroxidation was investigated through the measurement of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides. The absorbance measured spectrophotometrically [35].

2.6. Reactive Oxygen Species

For determination the free radical scavenging activity of galls, dichlorofluorescin diacetate (1.6 µM) was added to cell plates. ROS were determined spectrofluorometrically by the measurement of highly florescent DCFH. The results were expressed as fluorescent intensity per million cells [36].

2.7. Cytochrome-c Release

The concentration of cytochrome-c was determined by using the cytochrome-c Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn) and the optical density of each well was determined by spectrophotometer set to 450 nm [37].

2.8. Statistical Analysis

Statistical Analysis Results are reported as mean ± SD. Assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance (all tests) was determined using the one-way ANOVA test followed by the post hoc Tukey. Statistical significance was set at $p < 0.05$.

3. Results and Discussion

3.1. Cytotoxicity

Due to MTT assay results, normal fibroblast and melanoma cancer cell lines had acted differently when exposed to sandalwood extract in a dose-dependent manner. The IC50 of the sandalwood extract for A375 was 0.0253 mg/ml and for SK-MEL-3 was 0.0211 mg/ml and for normal fibroblast cell line was 0.819 mg/ml. Therefore, all of the tests were done at 0.02 mg/ml concentration (Fig. 1).

As shown in figure 2, extract-treated Human melanoma cell lines after 24 showed a significant decrease in cell viability ($p < 0.001$). On the other hand, there were not a significant decrease in normal fibroblast cell line at this dose and duration (Fig. 2).

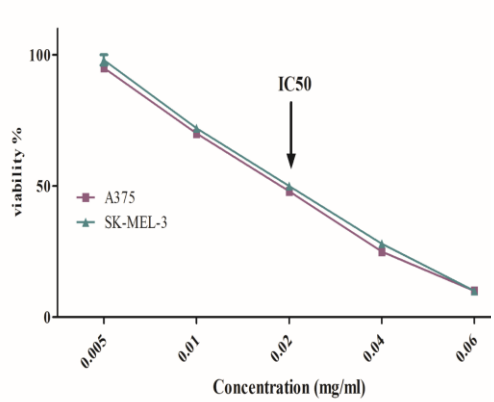

Figure 1. Measurement of IC50 values of Sandal extract on and SK-MEL-3 melanoma cell lines during 24h.

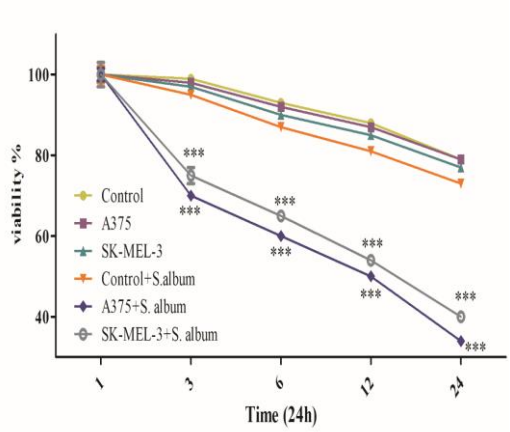

Figure 2. Effect of sandalwood extract at concentration of 0.02 mg/ml on induction of cytotoxicity using human melanoma cell lines (A375 and SK-MEL-3) and normal fibroblast cell line (control). Cytotoxicity was measured using MTT dye. Values are shown as mean ± SD of three separate experiments (n=3). ***p<0.001, significant difference in comparison with control cells.

3.2. Lipid Peroxidation

The addition of sandalwood extract (0.02 mg/ml) to human melanoma cell lines, significantly increased MDA formation compared to their corresponding control normal fibroblast cell ($P < 0.001$) (Fig. 3).

3.3. Reactive Oxygen species

As shown in Figure 4, sandalwood extract at a concentration of 0.02 mg/ml induced significant ROS formation in human melanoma cell lines ($p < 0.001$). However, there were not a significant increase in ROS formation in normal fibroblast cell.

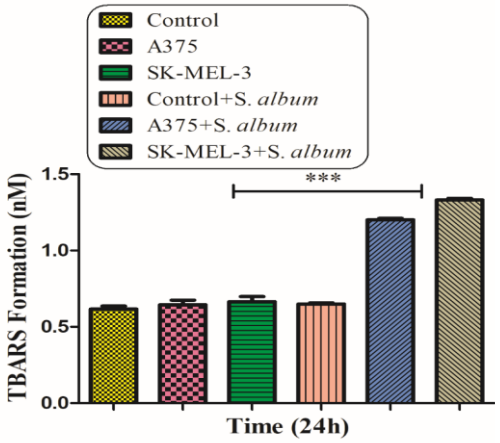

Figure 3. Effect of sandalwood extract at concentration of 0.02 mg/ml on induction of lipid peroxidation using human melanoma cell lines (A375 and SK-MEL-3) and normal fibroblast cell line (control). TBARS formation was measured spectrophotometrically and expressed as µM concentrations. Values are shown as mean ± SD of three separate experiments (n = 3). *** $p < 0.001$, significant difference in comparison with control cells.

3.4. Determination of Cytochrome-c Release

As shown in Figure 5, the cytochrome-c release which is the result of collapse of the mitochondrial membrane potential and disruption of mitochondrial outer membrane integrity occurred in human melanoma cell lines after treatment with sandalwood extract. But, there were not a significant increase in the release of cytochrome-c in normal fibroblast cells.

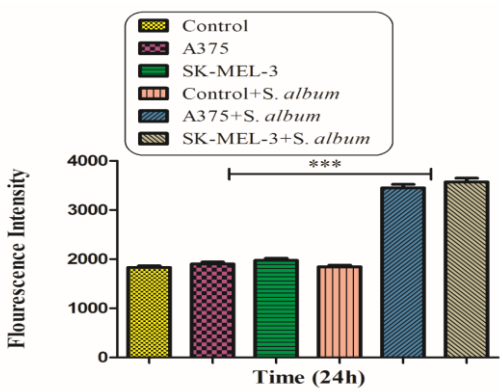

Figure 4. Effect of sandalwood gall extract at concentration of 0.02 mg/ml on the formation of reactive oxygen specious using human melanoma cell lines (A375 and SK-MEL-3) and normal fibroblast cell line (control). Reactive oxygen specious were determined spectrofluorometrically by the measurement of highly florescent DCF. Values are shown as mean ± SD of three separate experiments (n=3). ***p<0.001, significant difference in comparison with control cells.

Values are shown as mean ± SD of three separate experiments (n = 3). *** p < 0.001, significant difference in comparison with control cells.

3.5. Discussion

After heart diseases, cancer still is a moment driving reason for death worldwide [38]. Therefore, this situation put a force on scientists to study and examine new medications for cancer treatment. Hence, many laboratories start working on natural products and their anticancer effects. Natural products can induce anticancer effects through many mechanisms such as the modulation of cell cycle signaling, neutralizing free radicals, removal of cancerous agents, increased antioxidant enzyme activity, induction of apoptosis, and cell cycle arrest in tumor cells [39]. Natural products can modulate the Nrf2 and NF-κB [40]. An in vitro study demonstrated herbal constituents significantly affect the MAPK and PI3K cells that revealed their participation in cancer cell proliferation [41]. Furthermore, plant constituents like crocin, quercetin, apigenin, genistein, and luteolin can induce apoptosis in different types of malignant cells [42-44]. Oxidative stress and ROS formation and destructive reactions in DNA are the most important cause that activates the mitochondrial apoptotic pathway, depolarization of mitochondrial membrane which results in the cytochrome-c release [35, 41].

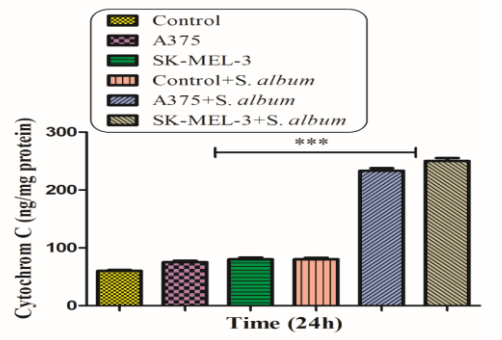

Figure 5. Effect of sandalwood extract at concentration of 0.02 mg/ml on the Cytochrome-c release using human melanoma cell line (A375 and SK-MEL-3) and normal fibroblast cell line (control). Cytochrome-c released was assayed spectrophotometrically (450 nm) by ELISA kit.

In this study, we evaluated the anticancer effect of sandalwood extract on human malignant melanoma cells. MTT study demonstrated that sandalwood extract has a beneficial effect on the cytotoxicity of human malignant melanoma cell lines. Furthermore, evaluation of lipid peroxidation shows a significant increase in TBARS formation in the human malignant melanoma cell line. Besides, ROS formation in mentioned cell line showed a significant increase. Finally, the cytochrome-c release which is the result of the collapse of the mitochondrial membrane potential and depolarization of the mitochondrial membrane was happened in human malignant melanoma cells. All these results

indicate that the extract has a very good and abundant effect in the treatment of malignant melanoma.

On the other hand, this study has another important finding. The normal fibroblast cell line was not affected by sandalwood extract. This golden finding means that sandalwood extract can safely use in patients with malignant melanoma without any worries about possible damage to normal healthy cells. However, further studies are needed to introduce a new anticancer drug candidate among these extract constituents.

There are some studies on the anticancer effects of sandalwood constituents, for example, in a study, it was revealed that α-santalol is an effective inhibitor of angiogenesis by acting on vascular endothelial growth factor and its receptor which decreases the growth of prostate cancer cells. Studies on prostate cancer cell lines revealed that α-santalol reduced the protein kinase B pathway, extracellular signal-related-kinase, and other kinases in prostate cancer cells. It also demonstrated cell viability decrease. Furthermore, a decrease in the number and mass of solid tumors was reported in a tumor xenograft model in immune-deficient nude mice [45]. In another study, Ortizet et al. evaluated the genotoxicity and cytotoxicity of sandalwood oil in breast cancer cell lines. The results of the study suggest that sandalwood oil show both genotoxic and cytotoxic activity in breast cancer (MCF-7). It was reported that sandalwood oil is effective in breaks in single- and double-stranded DNA in MCF-7 cells. Using LC/MS-based quantitative proteomics approach, the proteins such as EPHX1, Ku70, Ku80, and 14–3-3ζ were found to be associated with sandalwood oil genotoxicity [46]. In other study on α-santalol on skin and nipples of the breast cancer of small animals lead to breast cancer prevention [47]. East Indian sandalwood oil and α-santalol and β-santalol inhibited tubulin polymerization by directly binding to tubulin and also showed cytotoxic effects in head and neck squamous cell cancer (HNSCC) cells [48]. Sandalwood oil induced apoptosis on both UROtsa and J82 human bladder cells. It could also activate GPCR (G proteincoupled receptors) [49]. Treatment with α-santalol prevented breast tumor growth through the β-catenin pathway, in which β-catenin translocation from the cytoplasm to the nucleus was hindered in MDA-MB-231 cells [50].

In a study by Rachita Jain and colleagues Sandalwood oil and α-santalol showed beneficial effects in primarily skin cancer. They revealed the molecular mechanism for anticancer effects such as changes in cancer signaling pathways like MAPK, AP-1, β-catenin, and PI3K/Akt pathways. They could also activate caspases/PARP and upregulation of p21. Sandalwood oil also exerts anti-inflammatory activity via PGE2, IL-1β, and inhibition of the NF-κB pathway and 5-lipoxygenase. In addition, sandalwood oil and its constituents exhibit other therapeutic effects [51].

These studies confirmed the anticancer effects of sandalwood oil and its constituents, but there is no study on the hydroalcoholic extract of this plant. Considering that this plant is in danger of extinction, it might be more economical to use hydroalcoholic extract, which requires less plant for extraction. In addition, the cost of extraction in this method is much lower. Therefore, in this study, we decided to investigate the anticancer effect of the total hydroalcoholic extract of this plant on melanoma cells. It is suggested to

investigate the anticancer effect of different fractions of this plant extract on melanoma in the future studies.

4. Conclusion

In conclusion, natural products especially herbal products are important in the treatment of cancer because of their reachability, safety, inequity, inexpensiveness, proficiency, and being hostile to malignant capacity. Phytochemicals have revealed a significant therapeutic role in various malignancies, however, natural polyphenol compounds (flavonoids) represent a broad and assorted group in the treatment of different types of cancers and even other diseases [13, 52-54]. Natural polyphenols have their own anticancer efficacy through various pathways. For instance, inhibition of enzymatic function, cellular cycle arrest (S/G2, G1, S, and G2 stages), by regulation of the Nrf2 and NF-κB cells, which prevent the cellular proliferation, induction of apoptosis, modulation of MAPK signaling pathway, etc. The anticancer effects of natural polyphenols could be changed by doses, cancer types, and cell lines. The clinical trials are limited to these natural compounds. Therefore, it is strongly recommended to examine useful medicinal herbs like *Santalum album* in the clinic concurrent with standard therapies to determine their benefit in patients who suffered from cancer. As it is clear, the synthesis of new formulations of natural phenolic compounds which is well-targeted and well-designed can lead to the development of clinically beneficial medications with efficiency and selectivity.

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Conflict of interest

None

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