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Research Article

THE ANTIOXIDANT ROLE OF STORAX IN BORON COMPOUNDS INDUCED HACAT CELLS

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Abstract: *The skin is the first line of defense against microbial and chemical agents. Keratinocytes represent the major component of the skin. Storax is thought to have antioxidant, anti-inflammatory, and antimicrobial effects. some substances in storax have a cytotoxic effect and storax can be a source of oxidative stress. Boron compounds have a wide physiological effect on biological systems at low concentrations and are toxic at high concentrations. The aim of this study is to evaluate the oxidative effect of storax on boron compounds treated HaCaT keratinocytes in vitro. To determine the effect of boron compounds on cell viability and 50% lethal dose, the MTT method was employed, and the IC50 dose was found to be 1000 µg/ml borax and 250 µg/ml colemanite at the 24th hour. To determine the antioxidant activity of storax cells treated with borax and colemanite with or without storax and then the oxidative stress index, SOD, GPx, and MDA levels were evaluated with ELISA. Storax reduces the oxidative stress index through GPX, SOD, and MDA activities. When all the results are evaluated, the idea arises, that storax can be used as a possible therapeutic agent for the skin.*

Keywords*: Borax, Colemanite, Keratinocyte, Skin, Storax*

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1. Introduction

The skin is the primary interface between the environment and the body is the skin which provides the first layer of defense against chemical and microbial agents [1]. Keratinocytes are the major component of the skin that takes part in the skin's immune system by producing, constitutively or upon stimulation, numerous soluble mediators [2, 3]. The increasing oxidative stress in epidermal keratinocytes results in decreasing barrier function, the dermal-epidermal junction flattening, and reduced trans-epidermal water loss [4, 5].

There are some mechanisms to prevent cellular damage caused by oxidant molecules in the body. Antioxidants are substances that prevent the formation of free radicals, metabolize free radicals, or increase the scavenging of free radicals in order to prevent the oxidation of oxidizable substances such as proteins, lipids, carbohydrates, and DNA in living cells. Normally, there is a counterpoise among antioxidants and oxidants in our body, and oxidative stress caused by free radicals inspired by exogenous or endogenous events occurs [6]. Antioxidants are divided into endogenous (enzymes and non-enzymes) and exogenous sources. Antioxidants, which are endogenous enzymes; glutathione transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), mitochondrial oxidase and glutathione reductase system. The antioxidant enzyme Superoxide Dismutase (SOD) catalyzes the conversion of superoxide radicals to molecular oxygen and hydrogen peroxide. Catalase; breaks down hydrogen peroxide into oxygen and water. Catalase takes place mainly in peroxisomes, to a lesser extent in the cytosol and the microsomal fraction. Glutathione peroxidase: It turns hydrogen peroxide into the

water while oxidizing glutathione. Glutathione reductase converts oxidized glutathione (GSSG) to reduced (active) glutathione (GSH) [7].

Antioxidants interact with each other. Generally, these substances work as synergists. This interaction creates the total antioxidant capacity by making an effect more than the sum of the effects of the components alone. A decrease in one antioxidant can be compensated by an increase in another. Therefore, the measurement of total antioxidant capacity provides more valuable information than measuring antioxidants one by one [8]. Oxidative stress and tissue damage occur when the total oxidant status (TOS) exceeds the total antioxidant status (TAS). ROS (Reactive oxygen species) induced oxidative stress is one of the most important factors involved in the development of a wide variety of pathological conditions by altering metabolic homeostasis and regulation of cell growth and differentiation. Hence, the antioxidant production against excessive accumulation of ROS in cells and tissues has been shown to be an important pathogenic mechanism.

Lipid peroxidation is the reaction of polyunsaturated fatty acids found in mammalian cell membranes by free oxygen radicals to various products such as hydroxy fatty acids, alcohols, aldehydes, peroxides, pentane, ethane, malondialdehyde (MDA) [9]. The degree of the cell damage caused by lipid peroxidation depends on the activity levels of the defense systems inside the cell. These defense systems are free radical scavengers and antioxidants [10].

In Turkey, often known as the Anatolia sweetgum tree (*Liquidambar Orientalis Mill.*) is the *Hamamelidaceae* family, from the *Bucklandioida* subfamily, is a genus *Liquidambar* and is a kind of showing the spread of Southwestern Turkey. Liquidambar type has a balsam channel in its body. Considering its name, it is a combination of the words "liquidus", which means liquid in Latin, and "amber", which means the general name given to fragrant aromatic substances in Arabic, so it is also known as the fragrant liquid.

Liquidambar genus have 4 different species. The *Liquidambar Orientalis* (LO) is the most common species, which is called Turkish sweetgum or sığla. The medicinal products of LO are mostly obtained by damaging the outer surface of the tree. Storax has been used for treating, coughs, dysentery, infections, and wounds. Storax has had medicinal use since ancient times [11]. Storax has been described to contain numerous compositions (α -terpinol, terpinene-4-ol, γ –terpinene, sabinene, etc.) which have anti-microbial and antioxidant effects [12].

Boron (B) is a nonmetal element that is in the IIIA group in the periodic table and has an oxidation state of +3. Its atomic number is 5, its atomic weight is 10.81. Boron is an essential and important element for the plant, animal, and human health. This element has toxic effects at high concentrations and wide physiological effects at low concentrations. Although there are many studies on the toxicity and biological effects of boron, more studies are needed to understand its mechanism of action [13].

Boron does not exist in the form of a pure element in nature. Borax and colemanite are different forms of boron structures. Usage areas of boron compounds include glass, ceramics, cleaning, bleaching, cosmetics, metallurgy, nuclear, computer, and aircraft industry, energy sector, agriculture, and health. It is stated that there are more than 250 boron compounds in the air, soil, and water. Due to the high affinity of boron for oxygen, there are a wide variety of boron-oxygen compounds called borates [14]. Boron oxide (B2O3) and boric acid (H3BO3) are these compounds. They are the simplest structured ones [15]. In addition, boron has calcium, magnesium, and sodium elements and compounds. Some of the important ones are borax (Na2B4O7.10H2O) and colemanite (Ca2B6O11.5H2O). Borax and colemanite are widely used as antiseptics, bactericides, soaps, and detergents such as cleaning agents, preservatives, fire retardants, fertilizers, insecticides, and herbicides [16].

The aim of this study is to evaluate the oxidative effect of storax on boron compounds treated HaCaT keratinocytes in vitro and try to figure out the oxidative damage of boron compounds on keratinocytes and the potential antioxidative effect and mechanism of storax on treated keratinocytes.

2. Material and Methods

2.1. Cell Culture

DMEM-high glucose (Gibco) with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) used for HaCaT (RRID: CVCL_0038) keratinocyte cell culture. Cells were grown in a 5% CO2, 95% air-humidified incubator at 37 °C. The medium was removed, and fresh medium was added every 2-3 days. After reaching 80 to 90% confluence, the cells were trypsinized with 0.25% trypsin-EDTA-solution and recultured.

2.2. Storax Analysis Method

Gas Chromatography Mass Spectrometry was used for essential oil and a Gas Chromatography Flame Ionization Detector is used to determine percentages. For sample preparation, a 5% solution of essential oil in n-hexane dissolved. The analysis was done at Bezmialem Vakıf University Phytotherapy Education Research and Application Center (BITEM).

2.3. MTT Assay

The effects of the following agents were tested: borax (Na2B4O7·10H2O, CA S N o.1303-96-4), colemanite (Ca2B6O11·5H2O, CA SNo. 1318-33-8). The compounds were purchased from Eti Mine Works General Management (Turkey). The Effects of borax and colemanite on cell proliferation in HaCaT keratinocytes were detected by MTT assay according to manufacturer methodology (MTT Cell Viability Assay Kit; Biotium cat no: 30006). HaCaT cells were seeded into 96-well plates at a concentration of 1×10^4 cells per well. After 24 hours of incubation, the cells were treated with 250 µg/ml, 500 µg/ml, 750 µg/ml, and 1000 µg/ml concentrations of borax, and colematine dissolved in dH2O during 24 and 48hr. Untreated cells were used as control cells. After the incubation period, the MTT mixture was added and then formazan formation was determined at 570 nm (reference wavelength 630 nm) by a microplate reader (Biotek). Background absorbance was subtracted from signal absorbance to obtain normalized absorbance values.

Viability (%) = Absorbance of experiment well / Absorbance of control well × 100

2.4. Total Antioxdant Status (TAS) and Total Oxidant Status (TOS)

HaCaT cells were seeded into T-25 flasks (Sarstedt) at a concentration of 1×10^6 cells. After 24 hours of incubation, the cells were treated with 1000 µg/ml borax and 250 µg/ml colemanite at 24 hr with or without 50 µg/ml storax. After 24 hours the medium of culture was stored for the subsequent experiments.

TAS levels were measured using TAS assay kits (Cat.No: RL0017, Relassay, Turkey). The novel automated method is based on the bleaching of the characteristic color of a more stable ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants. The results were expressed as mmol Trolox equivalent/L [17].

TOS levels were measured using TOS assay kits (Cat.No: RL0024, Relassay, Turkey). The oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to the ferric ion. The oxidation reaction was enhanced by glycerol molecules abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (µol H2O2 equivalent/L) [18].

OXIDATIVE STRESS INDEX (OSI)

The ratio of TOS to TAS was accepted as the oxidative stress index (OSI). For calculation, the resulting unit of TAS was converted to µol/L, and the OSI value was calculated according to the following Formula :

OSI (arbitrary unit) = TOS (µol H2O2 equivalent/L) / TAC (µol Trolox equivalent/L) [19, 20, 21].

2.5. 2.5 SOD, GPx, MDA ELISA

$1x10^6$ HaCaT cells were seeded per well of a 6-well plate. After 24 h, cells were treated with 250 µg/ml Colemanite, 1000 µg/ml Borax, and with or without 50 µg/ml Storax. The culture medium was collected at the 24th hour, and the amount of SOD, GPx, and MDA was determined with ELISA. The collected media at the specified times were kept at -20 °C until ELISA measurement was performed. ELISA assay was performed according to the manufacturer's instructions (Cat. No: E4502Hu, E3921Hu, SH0020 BT-Lab, China).

2.6. Statistical Analysis

IBM SPSS Version 23 (SPSS Inc., Chicago, IL, USA) analysis program was used to perform the parametric and nonparametric analysis of dose and control groups. ELISA results were evaluated with a one-way ANOVA test. $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Storax Analysis

According to the GC-MS data and chromatogram (Figure 1). Cinnamyl cinnamate constitutes the bulk of the essential oil composition (64.708 %). Then the second most abundant substance was p-ethlyphenol (8.632%). The other compounds were Alpha pinene (3.259%), 3-Phenylpropanol (3.183%), Cinnamyl Alcohol (2.428%), Ethyl Cinnamate (1.374%), Beta Pinene (1.126%), Acetophenone (0.933%), and the compounds with an amount less than 0.1% (14.357%).

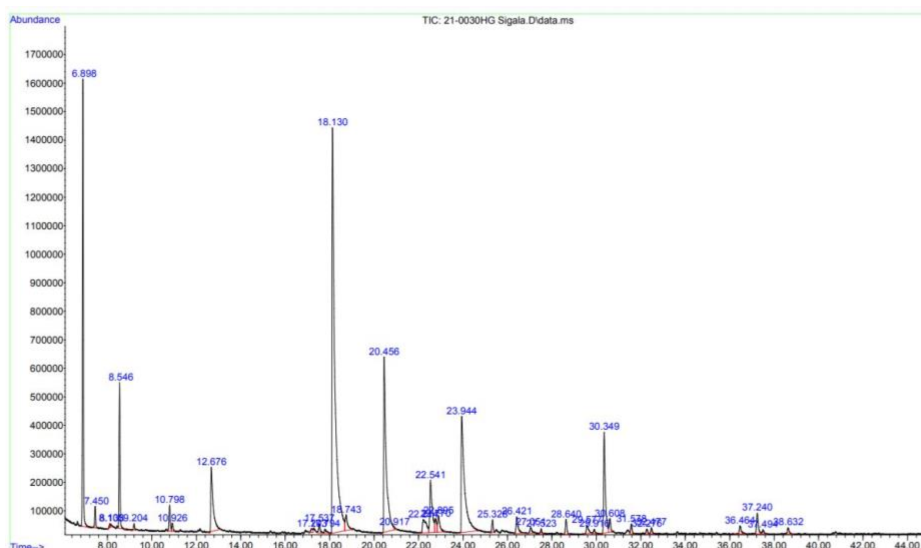


Figure 3.1. Gas chromatography-mass spectrometry (GC-MS) profiles of Storax essential oil

3.2. Effect of Borax and Colemanite on HaCaT keratinocyte cells

The effect of borax and colemanite on HaCaT keratinocytes was determined by MTT assay. In this way, the effect of borax and colemanite on HaCaT keratinocytes was investigated depending on time and dose. The IC_{50} dose of borax was found to be 1000 µg/ml in the 24th hour while the IC_{50} dose of colemanite was found to be 250 µg/ml in the 24th hour.

3.3. Antioxidant effect of storax in borax and colemanite-induced keratinocytes

For detecting the antioxidant activity of storax on boron compounds treated keratinocytes, the total oxidant, and antioxidant status were evaluated, and the oxidative stress index was calculated. OSI levels of the borax-treated cells were statistically different than the control group (2.016 vs 0.407, $p<0.05$) and the group which is treated both with borax and storax (2.016 vs 1.470, $p<0.05$). Also, OSI levels of the colemanite-treated cells were statistically different than the control group (2.142 vs 0.407, $p<0.05$) and the group which is treated both with colemanite and storax (2.142 vs 1.099, $p<0.05$) (Figure 2).

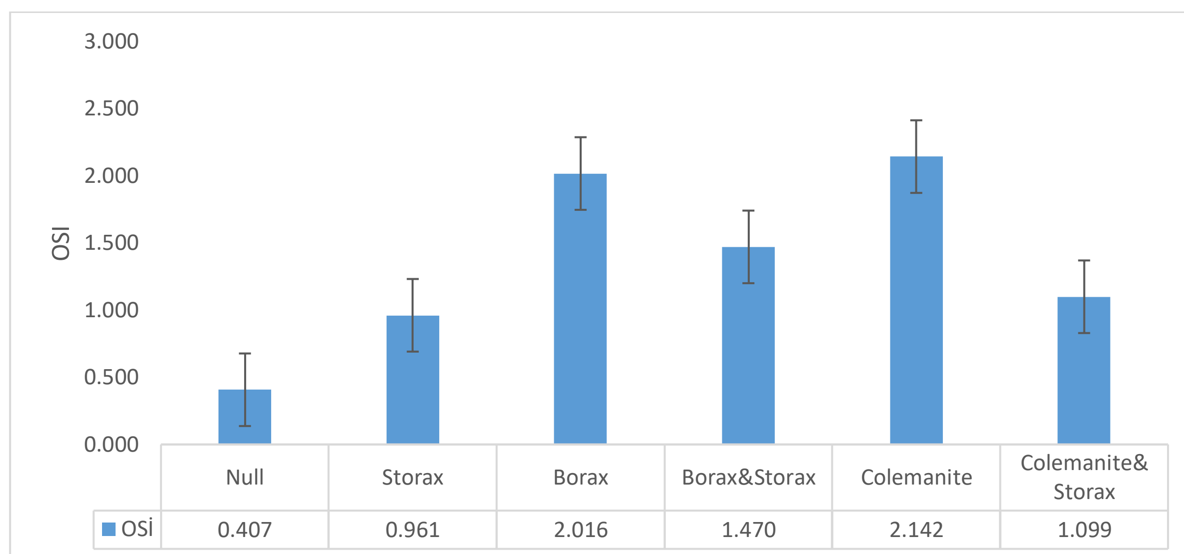
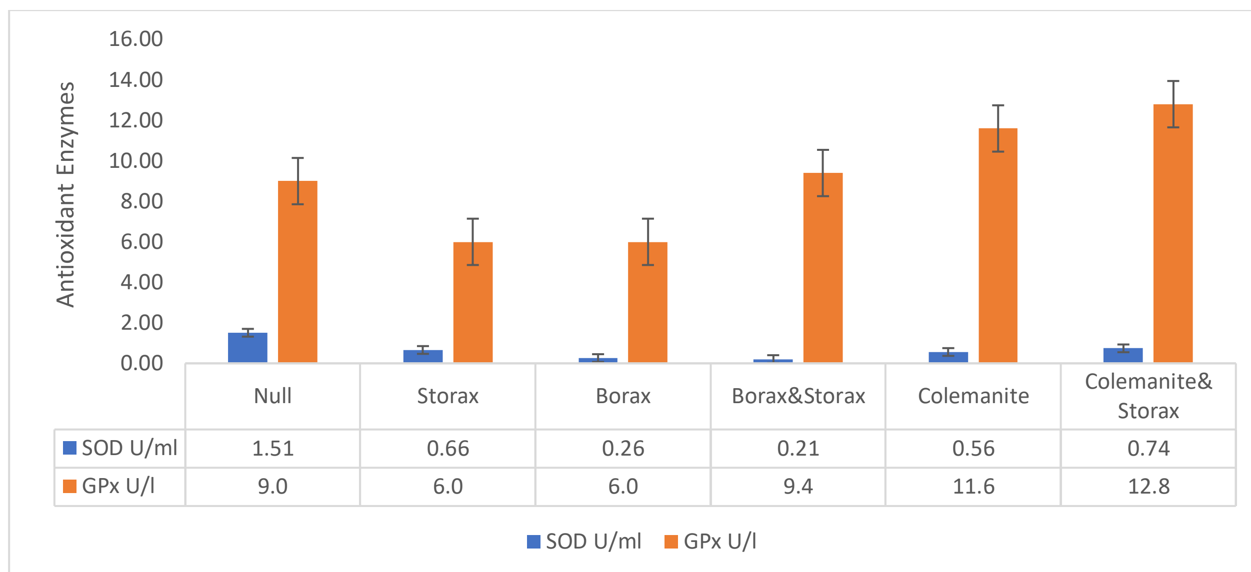


Figure 2. Oxidative stress index in borax, storax, and colemanite treated HaCaT keratinocytes.

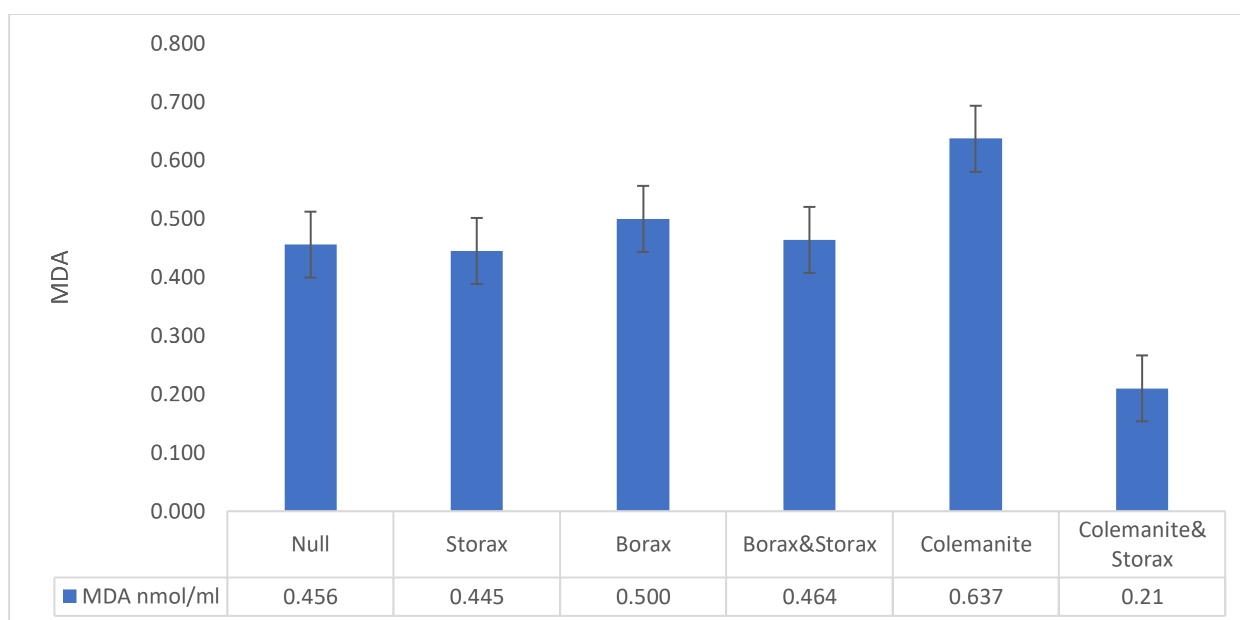
To explain the changes in the oxidative stress index the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), lipid peroxidation product malondialdehyde (MDA) levels were measured and statistically evaluated. Twenty-four-hour borax treatment caused alterations in the antioxidant enzyme activities of keratinocytes. GPx level decreased with borax treatment (6.0 vs 9.0, $p<0.05$) but treatment with borax and storax restore the GPx level in the control group (9.4 vs 9.0, $p>0.05$). SOD level decreased with borax treatment (0.26 vs 1.51, $p<0.05$) and storax treatment decreases the SOD level with or without borax treatment (0.66 vs 0.21, $p<0.05$). Twenty-four-hour colemanite application caused alterations in the antioxidant enzyme activities of keratinocytes. GPx level increased with colemanite treatment (11.6 vs 9.0, $p<0.05$), and also treatment with colemanite and storax increased the GPx level more (12.8 vs 11.6, $p=0.238$). SOD level decreased with colemanite treatment (0.56 vs 1.51, $p<0.05$) but storax treatment decreases the SOD level (0.56 vs 0.74, $p<0.05$) (Figure 3).



	Null	Storax	Borax	Borax&Storax	Colemanite	Colemanite& Storax
SOD U/ml	1.51	0.66	0.26	0.21	0.56	0.74
GPx U/l	9.0	6.0	6.0	9.4	11.6	12.8

Figure 3. Antioxidant enzyme status in borax, storax, and colemanite treated HaCaT keratinocytes.

The MDA level increased with borax treatment (0.5 vs 0.456, $p<0.05$) but treatment with borax and storax restore the MDA level in the control group (0.5 vs 0.464, $p>0.05$). The MDA level increased with colemanite treatment (0.637 vs 0.456, $p<0.05$) but treatment with borax and storax increased the MDA level lower than the control group (0.21 vs 0.456, $p<0.05$) (Figure 4).



	Null	Storax	Borax	Borax&Storax	Colemanite	Colemanite& Storax
MDA nmol/ml	0.456	0.445	0.500	0.464	0.637	0.21

Figure 4. Lipid peroxidation status in borax, storax, and colemanite treated HaCaT keratinocytes.

4. Discussion

Oxidative stress is caused by an imbalance between the production of the reactive oxygen system's ability to detoxify the reactive products or easily repair the resulting damage. The reactive oxygen species (ROS) can trigger the signaling processes and lead to cytotoxicity in many disorders, as the role of the antioxidant system.

Storax obtained from *Liquidambar orientalis Mill (Hamamelidaceae)* has been used as an antiulcerogenic in Turkish folk medicine for centuries [22]. Antibacterial activity of storax has been reported by in vitro techniques performed by Sağdıç et al. [23,24]. Antioxidant activity was determined by the DPPH test by Topal et al. [25]. Suzek et al. have extensively investigated the antioxidant activity of storax in vivo. They concluded that storax, a resinous exudate obtained from the injured body of Liquidambar Orientalis and locally named "Sığla Yağı", has a protective property and antioxidant activity [26].

Storax contains 65% Cinnamyl cinnamate, a phenolic compound. Antioxidant, antibacterial, and anti-inflammatory properties of this substance have been demonstrated in studies with plant extracts containing Cinnamyl cinnamate and propolis. It was also shown that Cinnamyl cinnamate protects some cells from lipid peroxidation and damage, caused by various oxidative toxins. Storax is thought to have antioxidant, anti-inflammatory, and antimicrobial effects with Cinnamyl cinnamate even though there is no detailed information about its pharmacokinetics in humans [27].

It has been shown that some substances in storax have a cytotoxic effect and that storax can be a source of oxidative stress. It was emphasized that storax carries out these properties through DNA damage, and this is the source of the antimicrobial effect of storax. Therefore, it has been stated that storax is a plant oil that has oxidant properties as well as an antioxidant effect [28]. In our study, storax treatment increased the oxidative stress index and the level of the antioxidant enzyme (SOD and GPx) decreased. These results are parallel with the literature which point to the oxidative effects of storax.

The lipid peroxide radicals formed to cause the formation of new lipid radicals by affecting other polyunsaturated fatty acids in the membrane structure. The most important breakdown product resulting from lipid peroxidation, MDA causes cell damage by reacting with functional groups of various compounds in the cell [29]. In our study, MDA levels did not change with the storax treatment. While storax and bor compounds co-treatment the MDA levels decrease.

SOD has a central role in oxidative damage. This enzyme catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide [30]. Türkez et al. indicated that not only borax but also colemanite have caused significant increases in the SOD activity in blood plasma. In our study, storax and bor compounds treatment decreases the SOD level [31]. Storax could reverse the SOD activity at co-treatment with colemanite, but it is still significantly different from than control group. Furthermore, storax could reverse the GPx activity at co-treatment with borax. Interestingly, the GPx levels of colemanite and storax-colemanite co-treatment were higher than the control group.

5. Conclusion

In conclusion, the oxidant properties of storax were also confirmed by the significant difference in OSI values with the control group versus storax treated group. However, when a secondary oxidative stress source (borax and colemanite) is co-treated, it has been shown that storax reduces the oxidative stress index through GPX, SOD, and MDA activities. When all the results are figured out, the idea appears that storax can be used as a possible therapeutic agent for skin. In this respect, it is important to expand and accelerate in vitro studies and to start in vivo studies using experimental animals.

Declarations

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