RESEARCH ARTICLE

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An Evaluation of Damages Caused by Doxorubicin in Liver Tissue and Potential Protective Effect of Propolis on These Damages

ABSTRACT

Objective: Doxorubicin (DOX), one of the chemotherapeutic drugs utilized in cancer treatment, has limited clinical use due to its serious toxic effects on non-target organs. The purpose of this study is to reveal the harmful effects of DOX in rat liver and the possible protective effect of propolis (PRPLS), a mixture of various herbal products collected by honeybees, on these damages by Attenuated Total Reflection-Fourier Transformation Infrared (ATR-FTIR) spectroscopy.

Methods: *Sprague dawley* rats were separated into 4 groups; control, DOX (cumulative dose: 15 mg/kg), PRPLS (200 mg/kg) and DOX + PRPLS. The rats were given 200 mg/kg PRPLS by oral gavage daily for 20 consecutive days and 2.5 mg/kg DOX intraperitoneally on days 10, 12, 14, 16, 18 and 20 of the experiment. 24 hrs after the last administrations, liver samples were collected and examined by ATR-FTIR spectroscopy.

Results: DOX caused a decrease in the amount of glycogen and nucleic acids, an increase in the amount of lipids and proteins and some important changes in the metabolism, structure and conformation of these molecules in the liver. DOX also induced lipid peroxidation, an increase in membrane fluidity, a decrease in membrane order and protein denaturation. PRPLS did not induce any toxic effect on the liver when it was given alone and PRPLS administered before DOX was not effective to eliminate these harmful effects of DOX.

Conclusions: DOX caused significant structural and compositional changes in liver tissue and PRPLS was inadequate to prevent these changes at the dose and time used here.

Keywords: Doxorubicin, Propolis, ATR-FTIR Spectroscopy, Liver, Chemotherapy.

Doksorubisinin Karaciğer Dokusunda Oluşturduğu Hasarın ve Propolisin Bu Hasar Üzerindeki Potansiyel Koruyucu Etkisinin Değerlendirilmesi

ÖZET

Amaç: Kanser tedavisinde kullanılan kemoterapötik ilaçlardan birisi olan doksorubisin (DOX), hedef dışı organlar üzerindeki ciddi toksik etkileri nedeniyle sınırlı klinik kullanıma sahiptir. Bu çalışmanın amacı, DOX'un sıçan karaciğerindeki zararlı etkilerini ve bal arıları tarafından toplanan çeşitli bitkisel ürünlerin bir karışımı olan propolis (PRPLS)'in bu zararlar üzerindeki olası koruyucu etkisini Azaltılmış Toplam Yansıma-Fourier Dönüşüm Kızılötesi (ATR-FTIR) spektroskopisi ile ortaya çıkarmaktır.

Gereç ve Yöntem: *Sprague dawley* sıçanlar 4 gruba ayrılmıştır; kontrol, DOX (kümülatif doz: 15 mg/kg), PRPLS (200 mg/kg) ve DOX + PRPLS. Sıçanlara ardışık 20 gün boyunca oral gavaj yoluyla günlük 200 mg/kg PRPLS ve 10, 12, 14, 16, 18 ve 20. günlerde intraperitoneal olarak 2,5 mg/kg DOX verilmiştir. Son uygulamalardan 24 saat sonra karaciğer örnekleri alınmış ve ATR-FTIR spektroskopisi ile incelenmiştir.

Bulgular: DOX karaciğerde glikojen ve nükleik asit miktarında azalmaya, lipid ve protein miktarında artışa ve bu moleküllerin metabolizması, yapısı ve konformasyonlarında bazı önemli değişikliklere sebep olmuştur. DOX, ayrıca lipid peroksidasyonuna, membran akışkanlığında bir artışa, membran düzeninde bir azalmaya ve protein denatürasyonuna neden olmuştur. PRPLS tek başına verildiğinde karaciğer üzerinde herhangi bir toksik etki oluşturmamış ve DOX'tan önce verilen PRPLS, DOX'un zararlı etkilerini ortadan kaldırmada etkili olamamıştır.

Sonuç: DOX, karaciğer dokusunda önemli yapısal ve kompozisyonel değişikliklere neden olmuş ve PRPLS bu çalışmada kullanılan doz ve zamanda bu değişiklikleri önlemekte yetersiz kalmıştır.

Anahtar Kelimeler: Doksorubisin, Propolis, ATR-FTIR Spektroskopisi, Karaciğer, Kemoterapi.

INTRODUCTION

Doxorubicin (DOX), also known as adriomycin, is an efficient chemotherapeutic drug belonging to the group of antracycline antibiotics. It has a very broad spectrum and is extensively used in the therapy of numerous cancers such as leukemias, lymphomas and various solid tumors (1). However, the clinical use of DOX is restricted due to its significant toxic side effects in some non-target organs including the liver, testis and heart (2-5). It has been known that the liver is one of the most vulnerable organs to DOX damage and liver damage is a common DOX-related side effect observed in the treatment of other types of cancer using this drug (3). Even though the mechanism of DOX-mediated liver injury is not known exactly, results from previous studies suggest that it induces the formation of reactive oxygen species (ROS), nucleic acids strand breakage and apoptosis (3, 4).

Liver is the principle organ responsible for the detoxification of chemicals, excretion of wastes in bile and various critical roles such as the synthesis, storage and redistribution of carbohydrates, lipids and proteins (6). Like other drugs, DOX is metabolized in the liver and then excreted in bile. If liver function is impaired, the elimination of DOX slows down and it begins to accumulate in the body and causes adverse effects on other tissues. Thus, understanding the mechanisms of DOX-induced toxicity in the liver is very important to eliminate these toxic effects, to increase efficiency of this drug and to reduce the damages to other tissues.

Propolis (PRPLS), a bee product formed by honey bees to protect the hive from intruders, contains hundreds of compounds with powerful antioxidant properties including phenols, flavonoids, terpenoids and vitamins. Among these compounds polyphenols and flavanoids have been suggested to be responsible for the biological activities of it (7). On the other hand, these chemical compounds vary according to flora, geographical origin, honeybee subspecies, collection season and PRPLS extraction method (8). In previous studies, it has been shown that PRPLS has antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory and immunomodulatory effects on biological systems (9). Based on these studies, it can be considered that the use of PRPLS together with chemotherapy may be effective in reducing or stopping the toxicity of DOX on healthy tissues. Previously, the protective effects of polyphenolic extracts from Algerian PRPLS, aqueous extract of Egyptian PRPLS and ethanolic extract of Indian PRPLS on different tissues against the toxical impacts of DOX have been demonstrated (2, 4, 5, 10). However, in the literature, there is no study regarding the protective effect of Turkish PRPLS (Yığılca) against the toxicity of DOX on liver tissue.

The proper functioning of a tissue is related to its structure, while the structure and function of a tissue depend on the physicochemical properties and compositions of the biomolecules in it. Thus, compositional and structural changes in a tissue can be indicators of various metabolic disorders. Fourier Transform Infrared (FTIR) spectroscopy is a high-tech product that enables the examination of structural and compositional changes in tissues. It measures the vibrations of molecules at different wavelengths, enables the visualization and characterization of different vibrational groups and therefore provides valuable information about biological systems. Using FTIR spectroscopy, molecular alterations in cellular constituents such as carbohydrates, proteins, nucleic acids and lipids can be determined at the functional group level. In FTIR spectrometers by utilizing the Attenuated Total Reflectance (ATR) unit, it is possible to determine such alterations more quickly and accurately by minimizing sample preparation or by examining samples directly, regardless of sample thickness. Detection of alterations in the band area ratios, wavenumbers and bandwidths of ATR-FTIR bands gives information about the amounts of biomolecules, their interactions with each other, the level of lipid peroxidation and secondary structures of proteins in a tissue (11-13).

Although it has been shown that DOX has many toxic impacts on the liver tissue, the studies investigating the toxic effects of this drug on the liver are generally at histological or biochemical level (3, 4, 14) and do not provide enough information about the structural, compositional and functional changes that may have caused the pathology in the liver tissue. The purpose of the current study is to reveal the damages caused by DOX in liver tissue at molecular level and to evaluate the protective effect of Turkish PRPLS against the damaging effect of DOX on this tissue by using ATR-FTIR spectroscopy.

MATERIAL AND METHODS

Propolis: PRPLS was obtained from the Düzce University Beekeeping Research and Application Centre, Düzce, Turkey. Since ethanol is a good solvent for PRPLS and not toxic for organisms, ethanolic extraction of PRPLS was preferred (5, 10). Ethanolic extracts of PRPLS (completing the volume of 20 g of PRPLS to 100 ml with 96% ethanol) were prepared, left for 5 days in the dark under moderate shaking and filtered utilizing filter paper. The filtrate was evaporated by utilizing a vacuum evaporator. Then the residual was dissolved in 70% ethanol and stored at +4 oC until use.

Animal Experiments: Animal experimental design of the current study was approved by the Düzce University Experimental Animals Ethics Committee (2019/1/9). Male Sprague-Dawley rats

(10-12 weeks old, 250-300 g) were housed in a 12-h light:12-h dark photo period with standard rat diet and water ad libitum at room temperature (22 ± 2 °C). Animals were administered 200 mg/kg PRPLS ethanolic extract by oral gavage daily for 20 days (2) and DOX (Adrimisin, Saba Pharma, İstanbul) intraperitoneally (i.p.) in six divided doses (2.5 mg/kg) with a total cumulative dose of 15 mg/kg (15). The rats were separated into 4 groups: 1. Control group (n=7): Animals were given the solvent of PRPLS (ethanol) for 20 days by oral gavage and the solvent of DOX i.p. (saline) on days 10, 12, 14, 16, 18 and 20. 2. DOX group (n=7): Animals were given the solvent of PRPLS for 20 days and DOX (2.5 mg/kg; i.p.) on days 10, 12, 14, 16, 18 and 20. 3. PRPLS group (n=6): Animals were given PRPLS daily for 20 days and the solvent of DOX on days 10, 12, 14, 16, 18 and 20. 4. PRPLS + DOX group (n=7): Animals were given PRPLS daily for 20 days and DOX (2.5 mg/kg; i.p.) on days 10, 12, 14, 16, 18 and 20. 24 hrs after the last administrations, the rats were euthanized; the liver tissues were removed and stored at -80 °C until use.

Spectroscopic Studies: The spectra of liver tissues were obtained with a Spectrum Two FTIR spectrometer (Perkin-Elmer Ltd., UK) attached with an ATR accessory. 0.5 X 0.5 X 0.1 cm sized samples cut from three different parts of the rat liver were put on the diamond/zinc-selenite crystal of the ATR and treated with nitrogen gas (N2) for five minutes to get rid of the water in the environment. The tissue was compressed by applying 100 force gauge pressure to ensure a smooth surface contact. The spectra were collected with a scanning number of 64 at a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} wavenumber range at room temperature. In order to improve the validity of the results, 3 replicates were scanned from the neighboring portions of these chosen parts of each sample and the averages of these spectra were taken and analyzes and statistical tests were performed on the average spectra (11).

Spectral analyses were performed using Perkin Elmer Spectrum 100 and OPUSNT (Bruker Optics, Reinstetten, Germany) softwares. Raw mean spectra were used for detailed analysis. Bandwidth and wavenumber values were calculated from 75% of height. For visual representation, the averaged spectra were baseline corrected and normalized. The second derivative vector normalization method was applied to the 1700-1600 cm-1 region, which consists of unresolved bands, to determine the protein secondary structure changes (11).

Statistical Analysis: The statistical analyses were conducted using GraphPad Prism (Version 9.3.1) software. Data were subjected to the Shapiro-Wilk test to characterize their normality. Since the data did not show a normal distribution, the ATR-FTIR results for each group were statistically analyzed by employing non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. The values are expressed as "mean ± standard deviation". The calculated p values less than 0.05 were considered as statistically significant.

RESULTS

Figures 1A-C depict the average ATR-FTIR spectra of rat livers of experimental groups in the wavenumber ranges of 3700-3025 cm^{-1}, 3025-2800 cm^{-1} and 1800-950 cm^{-1}. In these figures, the essential bands are numbered and assignment of each band according to the literature is given in Table 1.

Table 1. Major bands in the ATR-FTIR spectrum of the rat liver and their assignments (11, 12).

Band No	Wavenumber (cm^{-1})	Band Assignment
1	**3313**	**Amide A:** Proteins (N-H stretching), O-H stretching of polysaccharides, water
2	**3012**	**Olefinic HC=CH stretching:** Unsaturated lipids
3	**2965**	**CH_3 antisymmetric stretching:** Lipids and protein side chains
4	**2929**	**CH_2 antisymmetric stretching:** Mainly lipids
5	**2874**	**CH_3 symmetric stretching:** Mainly proteins
6	**2856**	**CH_2 symmetric stretching:** Mainly lipids
7	**1743**	**Carbonyl (C=O) ester stretching:** Triglycerides, cholesterol esters and phospholipids
8	**1640**	**Amide I:** Proteins (80 % C=O stretching)
9	**1547**	**Amide II:** Proteins (40% C-N stretching, 60% N-H bending)
10	**1456**	**CH_2 bending:** Mainly lipids
11	**1400**	**COO^- symmetric stretching:** Lipids and proteins
12	**1308**	**Amide III:** Proteins
13	**1238**	**PO_2^- antisymmetric stretching:** Phospholipids and nucleic acids
14	**1153**	**CO-O-C antisymmetric stretching:** Phospholipids, cholesteryl esters and nucleic acids
15	**1120**	**Ribose ring vibrations:** RNA
16	**1082**	**PO_2^- symmetric stretching:** Phospholipids and nucleic acids **C–O stretching:** Polysaccharides, glycolipids
17	**1033**	**C–O stretching:** Glycogen
18	**1025**	**DNA vibrations**
19	**972**	**C-N^+-C stretching:** Nucleic acids, ribose-phosphate main chain vibrations of RNA

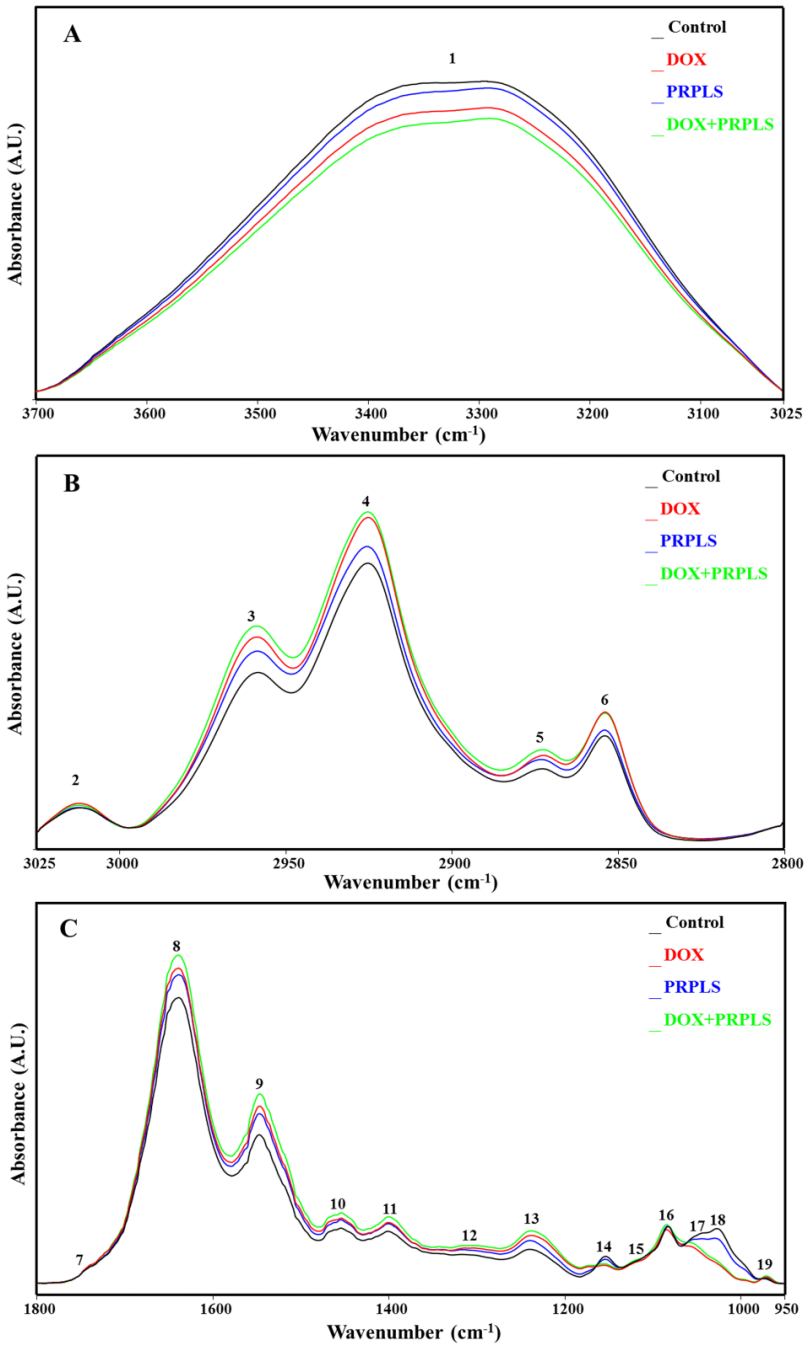

Figure 1. Average, baseline corrected and normalized ATR-FTIR spectra of control, DOX, PRPLS and DOX + PRPLS treated rat liver tissues in the A) 3700-3025, B) 3025-2800 and C) 1800-950 cm^{-1} wavenumber regions. Spectra were normalized with respect to the CH_2 antisymmetric stretching band (A) and to the Amide A band (B and C).

In the current study, the area ratios of some specific infrared bands, which provide information about the amounts of the molecules, were analyzed to eliminate errors that may result from differences in experimental conditions (11, 13). The comparisons of area ratios of infrared bands between the control and treated groups are given in Table 2. The area ratios utilized in our study, the functional groups utilized to obtain these ratios and the functions of the calculated ratios are presented as Supplementary Material (Supplementary Table 1).

Changes in the amounts of saturated lipids were examined by calculating the band area ratio of CH_2 sym./CH_2 sym. + antisym. (13). As seen from Table 2, this ratio increased significantly in the DOX and DOX + PRPLS administered groups compared to control group. The olefinic HC=CH/lipid ratio, which was used to detect the alterations in the amount of unsaturated lipids, decreased significantly in the DOX and DOX + PRPLS treated groups in comparison with control group (Table 2).

Table 2. Alterations in the band area ratios of a subset of ATR-FTIR bands obtained from the liver spectra of control, DOX, PRPLS and DOX + PRPLS treated rats.

Band Area Ratio	Control	DOX	PRPLS	DOX + PRPLS
CH_2 sym./CH_2 sym + antisym.	1.306±0.022	1.366±0.025*	1.325±0.044	1.377±0.046*
Olefinic HC=CH/ Lipid	0.042±0.004	0.034±0,004*	0.040±0.004	0.032±0.004*
Amide I/Amide I + II	7.024±0.457	8.107±0.440*	7.485±0.746	8.250±0.798*+
RNA/Protein	0.055±0.008	0.043±0.007*	0.047±0.006	0.043±0.003*
RNA/Lipid	0.669±0.067	0.509±0.039**	0.612±0.077	0.532±0,059*
DNA/Protein	0.152±0.016	0.046±0.009***	0.122±0.02#	0.049±0.005**
DNA/Lipid	1.715±0.145	0.542±0.066***	1.459±0.238#	0.630±0.087**
RNA/DNA	0.375±0.024	1.235±0.088**	0.447±0.075	1.063±0.100**
C-O/Protein	0.315±0.035	0.122±0.021**	0.267±0.051#	0.126±0.018**
C-O/Lipid	3.823±0.419	1.449±0.252***	3.422±0.209#	1.556±0.174**
C-O/PO_2^-	1.409±0.170	0.657±0.090**	1.262±0.159#	0.705±0.080**
CH_2 antisym./CH_3 antisym.	2.512±0.170	2.251±0.097*	2.407±0.278	2.258±0.202*
C=O/Lipid	1.154±0.055	0.950±0.079**	1.078±0.085	0.941±0.068**
CH_3 antisym./Lipid	0.223±0.015	0.269±0.009**	0.237±0.015	0.278±0.020**
Amide I/Amide II	2.361±0.086	2.173±0.078*	2.239±0.212	2.118±0.161*
Lipid/Protein	0.082±0.002	0.085±0.001*	0.078±0.007	0.084±0.002*

Degree of significance was denoted as $^{*,\#,+}p<0.05$, $^{**,\#\#,++}p<0.01$, ***,$^{\#\#\#,+++}$ p<0.001. (*): control vs. DOX, PRPLS and DOX + PRPLS; (#): DOX vs. PRPLS and DOX + PRPLS; (+): PRPLS vs. DOX + PRPLS

Alterations in the protein concentrations were evaluated by calculating Amide I/Amide I + II ratio (13). As can be seen from Table 2, this ratio increased significantly in the DOX and DOX + PRPLS treated groups compared to control group. In addition, the DOX + PRPLS treated group presented significantly higher level of this ratio in comparison with those observed in the PRPLS group. The band at 1120 cm^{-1} arises from ribose ring vibrations in the RNA and gives information about the RNA molecules in the system. Using this band, RNA/protein and RNA/lipid ratios were calculated to attain information about the amount of RNA in the tissue (12). To obtain information about the amount of DNA, DNA/protein and DNA/lipid ratios were calculated using the 1025 cm^{-1} band, which is a special DNA band (12). All these ratios decreased significantly in the DOX and DOX + PRPLS administered groups compared to control group (Table 2). The changes in the RNA/DNA ratio were utilized to obtain information about the transcription status of the cells (12). As can be seen from Table 2, this ratio increased significantly in the DOX and DOX + PRPLS treated groups compared to control groups. As also seen from Table 2, DNA/protein and DNA/lipid ratios were found to be significantly higher in the PRPLS treated group when compared to DOX administered group.

Since the C-O stretching band, which appears at 1033 cm^{-1}, gives information about glycogen, C-O/protein and C-O/lipid ratios were calculated to analyze the alterations in the concentration of glycogen in the system (11). As seen from Table 2, these ratios significantly decreased in the DOX and DOX + PRPLS treated groups in comparison with control group. To attain information about the changes in the metabolic activity of the tissue, C-O/PO_2^- (glycogen/phosphate) ratio was used (12) and it has been observed that this ratio decreased significantly in the DOX and DOX + PRPLS treated groups compared to control group (Table 2). C-O/protein, C-O/lipid and C-O/PO_2^- ratios were also found to be significantly higher in the PRPLS treated group when compared to DOX administered group.

CH_2 antisym./CH_3 antisym. ratio was utilized to detect the changes in the chain length of phospholipids, while the C=O/lipid and CH_3 antisym./lipid ratios were utilized to detect the changes in the carbonyl status of and methyl concentration in the tissue, respectively (11, 16). As seen from Table 2, the CH_2 antisym./CH_3 antisym. and C=O/lipid ratios decreased and the CH_3 antisym./lipid ratio increased significantly in the DOX and DOX + PRPLS treated groups in comparison with control group. To obtain information about the structural alterations in the proteins, the ratio of Amide I/Amide II was calculated (11) and it has been seen that this ratio decreased significantly in the DOX and DOX + PRPLS treated groups compared to control group. In addition, to compare the relative alterations in the amounts of lipids and proteins, the lipid/protein ratio was calculated (16). This ratio increased significantly in the DOX and DOX + PRPLS treated groups in comparison with control group (Table 2).

The alterations in the wavenumbers of the CH_2 antisymmetric and symmetric stretching bands and bandwidth of the CH_2 antisymmetric stretching band were analyzed to examine the order and fluidity of the biological membrane (16). As seen from Table 3, the wavenumber values of the CH_2 antisymmetric and symmetric stretching bands shifted to higher values in the DOX and DOX + PRPLS treated groups and the bandwidth of the CH_2 antisymmetric stretching band increased significantly in the same groups compared to control group.

Table 3. Alterations in the wavenumber and bandwidth values of a subset of ATR-FTIR bands obtained from the liver spectra of control, DOX, PRPLS and DOX + PRPLS treated rats.

Functional Group	Control	DOX	PRPLS	DOX+PRPLS
Peak position (Wavenumber)				
CH_2 antisym. str.	2928.031±0.628	2928.996±0.733*	2928.361±0.911	2928.646±0.342*
CH_2 sym. str.	2855.149±0.390	2855.75±0.411*	2855.421±0.342	2855.739±0.366*
Amide I	1640.674±0.324	1639.791±0.871*	1640.183±0.674	1639.964±0.201**
Bandwidth				
CH_2 antisym. str.	9.609±0.156	9.903±0.103*	9.778±0.716	9.941±0.316*
Amide I	34.556±0.221	34.116±0.291*	34.307±0.385	33.866±0.558*

Degree of significance was denoted as $^{*,\#,+}p<0.05$, $^{**,\#\#,++}p<0.01$, ***,$^{\#\#\#,+++}$ $p<0.001$. (*): control vs. DOX, PRPLS and DOX + PRPLS; (#): DOX vs. PRPLS and DOX + PRPLS; (+): PRPLS vs. DOX + PRPLS.

In order to attain information about the conformational alterations in proteins, the wavenumber and bandwidth of the Amide I band were analyzed (11). As seen from Table 3, the wavenumber of this band shifted to lower values and its bandwidth decreased in the DOX and DOX + PRPLS groups in comparison with control group. To evaluate the changes in the secondary structures of proteins, vector normalized second derivative ATR-FTIR spectra were analyzed in the Amide I band region (11). As seen from Table 4, the intensities of the turn and random coil structures increased significantly and the intensities of the alpha-helix and beta-sheet structures decreased significantly in the DOX and DOX + PRPLS treated groups. As also seen from Table 4, the intensity of alpha-helix structure decreased and the intensity of random coil structure increased significantly in the DOX + PRPLS treated group compared to the PRPLS treated group.

Table 4. Alterations in the intensity values of the principle protein secondary structures from the Amide I band region of vector normalized second derivative liver spectra of control, DOX, PRPLS and DOX + PRPLS treated rats.

Functional Group	Control	DOX	PRPLS	DOX+PRPLS
Turns (1684 cm^{-1})	0.130 ± 0.005	0.137 ± 0.005*	0.132 ± 0.007	0.139 ± 0.008*
Alpha-helix (1651 cm^{-1})	0.298 ± 0.007	0.283 ± 0.008*	0.309 ± 0.009	0.283 ± 0.011*+
Random coil (1640 cm^{-1})	0.111 ± 0.007	0.127 ± 0.009*	0.109 ± 0.010	0.129 ± 0.009*+
Beta-sheet (1633 cm^{-1})	0.111 ± 0.014	0.075 ± 0.009**	0.097 ± 0.019	0.077 ± 0.009**

Degree of significance was denoted as $^{*,\#,+}p<0.05$, $^{**,\#\#,++}p<0.01$, ***,$^{\#\#\#,+++}$ $p<0.001$. (*): control vs. DOX, PRPLS and DOX + PRPLS; (#): DOX vs. PRPLS and DOX + PRPLS; (+): PRPLS vs. DOX + PRPLS.

DISCUSSION

Alterations in the structure and composition of lipids, which are the main components of cell membranes and important storage molecules in liver cells, may cause important dysfunctions in this tissue. In this study, the increase detected in the ratio of CH_2 sym./CH_2 sym.+antisym. after DOX administration showed that DOX induced an increase in the concentration of saturated lipids in rat liver (13). This result suggested that DOX administration caused alterations in lipid metabolism, resulting in lipid accumulation in the liver. It has been reported that DOX increased the production of ceramides, dihydro-ceramides, sphingosine and dihydrosphingosine, which is an indicator of a disorder in the sphingolipid metabolism in the liver (17). In addition, it has been reported that DOX caused liver steatosis (fatty liver) in different experimental animals (1, 18). It has been known that liver steatosis also causes a decrease in glycogen concentration (19). In a previous study, an increase in the amount of lipid together with a decrease in the amount of glycogen during liver steatosis has been demonstrated via FTIR spectroscopy (20). Indeed, when the C-O/protein and C-O/lipid ratios were analyzed to determine the alterations in glycogen amount in the liver tissue, a decrease upon DOX administration was observed. This result indicated that DOX led to a decrease in glycogen amount in rat liver tissue (11). In consistence with our result, it has been reported that DOX reduced insulin-induced glucose uptake and glycogen synthesis in liver cells and glycogen stores in the heart tissue of rats (17, 21). Thus, the decrease observed in the amount of glycogen might have arisen from the significant changes induced by DOX in glycogen and lipid metabolism in the tissue. Changes in the amount of lipid and glycogen, which are the main energy storage materials of the liver, affect the energy metabolism of the system. Since excessive amounts of carbohydrates and proteins are converted to lipids and stored in the liver, our results indicated that the energy metabolism of the liver was significantly affected by DOX. The decrease observed in the glycogen/phosphate (C-O/PO_2^-) ratio after DOX administration, also confirmed that DOX induced significant changes in the metabolic activity of the liver tissue (12).

The double bonds in unsaturated fatty acids are extremely vulnerable to lipid peroxidation, which is a molecular injury mechanism initiated by ROS and an indicator of high oxidative stress in

tissues. If unsaturated fatty acids in lipids are attacked by ROS, a lipid peroxidation chain reaction starts and these harmful reactions lead to the breakdown of lipids and ultimately a decrease in unsaturated fatty acid concentration, namely olefinic bonds (22). For this reason, in biological FTIR spectroscopy studies the olefinic HC=CH/lipid ratio (unsaturated/saturated ratio) is utilized as lipid peroxidation index (11). In this study, the decrease noticed in the olefinic HC=CH/lipid ratio indicated that DOX caused a decrease in the concentration of unsaturated lipids in liver tissue. It has been known that semiquinone electron fragments formed in the liver after DOX administration produce ROS by reducing oxygen and these oxidative free radicals participate in the DOX-induced lipid peroxidation (14). Consistent with the literature, our findings also showed that DOX caused an increase in the level of lipid peroxidation possibly by inducing oxidative stress in liver tissue. In a previous FTIR spectroscopy study related to lipid peroxidation, it has been found that there is also a decrease in the CH_2 and carbonyl (C=O) groups and an increase in the CH_3 groups in the tissue of interest (11). When these parameters were examined in this study, it has been observed that CH_2 antisym./CH_3 antisym. and C=O/lipid ratios decreased and CH_3 antisym./lipid ratio increased significantly after DOX administration. These changes showed that lipids were broken down by ROS into smaller fragments, which have less CH_2 and C=O groups and more CH_3 groups, and could be attributed to lipid peroxidation in the DOX-treated tissue. These findings demonstrated that the action mechanism of DOX is associated with its potential for induction of oxidative stress and lipid peroxidation. In earlier studies, it has been indicated that DOX caused lipid peroxidation in the liver and other tissues. For example, Afsar et al. (23) have shown that hepatotoxins, which were formed as a result of the generation of ROS after DOX administration in the liver, caused a disruption in lipid profile and an increase in lipid peroxidation by reacting with polyunsaturated fatty acids. Ozdoğan et al. (24) have shown that elevated ROS production after DOX administration was directly related to the damage occurring in cardiac myocyte membranes via lipid peroxidation. In addition, it has been shown that DOX led to alterations in the level of endogenous antioxidant enzyme activities and formation of lipid peroxidation products (malondialdehyde) by causing oxidative stress in various tissues (4, 18). Thus, the decreases observed in the olefinic HC=CH/lipid, CH_2 antisym./CH_3 antisym. and C=O/lipid ratios and the increase in the CH_3 antisym./lipid ratio indicated that DOX caused lipid peroxidation in rat liver tissue.

In this study, the increase observed in the ratio of Amide I/Amide I+II showed that there was an increase in the concentration of proteins in the liver after DOX administration (13). This increment in the amount of proteins might be due to an increase in protein synthesis in this tissue. In previous studies, it has been shown that liver enzymes, such as alanine transaminase and aspartate transaminase increased after DOX administration (14). In addition, it has been reported that the amount of cysteine, an amino acid in thiol structure, increased in the liver after DOX administration (25). Therefore, this increase in the amount of protein might be due to the increase in the concentration of some enzymes and some amino acids after DOX administration in the liver.

The decreases detected in the wavenumber and bandwidth values of the Amide I band after DOX administration indicated a change in protein conformation (13). This finding was also supported by the decrease observed in the Amide I/Amide II ratio after DOX administration. It has been known that any pathological condition in the liver tissue causes a decrease in the Amide I/Amide II ratio and thus various alterations in the protein structure (11). The findings of the vector normalized second derivative spectra analyses revealed more detailed information about the changes in the secondary structure of proteins. The increase observed in the random coil structure showed that DOX caused protein denaturation in the liver cells (11). The protein denaturation observed in the liver might have resulted from the increase in the amount of ROS induced by DOX. The appropriate functioning of proteins is dependent on their three-dimensional structure and errors that may occur during protein folding can lead to differences in protein secondary structure and thus functional damage. The increase in DOX-induced ROS formation might have caused changes in the protein secondary structure by changing the redox potential of the cell (26). Our results are consistent with previous studies that have reported that ROS formed in the tissue after DOX administration caused deterioration in protein structure. Previously, Oz et al. (21), have shown that myofibril proteins in heart cells had more disordered structures after DOX administration. Yagmurca et al. (3) have shown that DOX caused protein oxidation together with lipid peroxidation in rat liver tissue. Oxidation of proteins (e.g. enzymes) causes significant alterations in their structure and function that are important for cells.

The alterations in proteins observed in DOX-treated groups might have also been the result of changes in the levels of gene expression or protein synthesis. In this study, decreases observed in the RNA/lipid, RNA/protein, DNA/lipid and DNA/protein ratios showed that the concentrations of nucleic acids in liver tissue decreased after DOX administration. Since DOX is a moderately lipophilic drug, it has a high binding capacity to the cellular and nuclear membranes and nucleic acids. Therefore, it may accumulate in the nuclei of liver

cells and cause DNA damage. It has been known that one of the mechanisms of action of DOX is to block DNA and RNA synthesis by locating between adjacent base pairs (27). DOX contains an amino sugar and an anthracycline ring. The aglycone part of the drug enters between DNA and RNA, binds ionically to these molecules to stabilize the intercalation of the ionic sugar structure, and as a result, DNA and RNA are deformed during synthesis (4). Thus, the decrease in the amount of nucleic acids in the tissue observed by analysing the DNA and RNA bands might have resulted from the inhibition of the synthesis of these molecules by DOX. In addition, it has been known that ROS formed in the liver after DOX administration oxidizes DNA and RNA and causes deterioration in their structures (27). The decrease noticed in the RNA/lipid ratio also implied some alterations in the proliferation state or growth capacity of cells after DOX administration (12). Regarding the nucleic acids, the RNA/DNA ratio was also calculated and an increase in this ratio was observed after DOX administration. This increase showed that the transcription rate of some genes was higher in DOX-treated groups than in the control group (12). It has been known that the transcription level of the genes responsible for the synthesis of proteins; that is the amount of mRNA; increases after DOX administration. For example, in previous studies it has been reported that hepatic injury induced by DOX begins with the expression of genes responsible for drug delivery, cell cycle progression, oxidative stress response, mitochondrial disorders, apoptosis, DNA damage and DNA repair (28).

In the current study, to determine the effect of DOX on the relative amounts of lipids and proteins in the system, the lipid/protein ratio was evaluated. This ratio also enables the analysis of changes in lipid and protein asymmetry, which are important indicators for cell functions. In addition, a change in this ratio indicates a modification in the structure, arrangement and fluidity of the membrane (11). A significant increase in the lipid/protein ratio was detected after DOX administration in the liver tissue. In our study, since an increase in both lipid and protein amount after DOX administration has been detected, the increase in this ratio indicated that the increase in lipid amount was higher. This result showed that DOX affected lipid metabolism more than protein metabolism in the liver (11, 12). This increase also showed that DOX caused alterations in lipid and protein asymmetry in the liver cell membranes and supported the result that it caused alterations in lipid and protein metabolism. Since the alterations in lipid and protein asymmetry significantly affect membrane function, they may cause significant changes in the concentrations of ions inside and outside the cells.

Changes in the parameters associated with the order and fluidity of the phospholipids, the main components of the cellular membrane, affect the normal functioning of membranes. The shifts towards higher values detected in the wavenumbers of the CH_2 antisymmetric and symmetric stretching bands after DOX administration indicated a decrease in the order and an increase in the acyl chain flexibility of membrane lipids (11, 16). A significant increase observed in the bandwidth of the CH_2 antisymmetric stretching band indicated that DOX caused an increase in the fluidity of membrane. These alterations might be due to the differences in the ratios of different lipid species and the lipid/protein ratio as a result of the oxidative stress induced by DOX. In addition, increased lipid peroxidation due to DOX administration might have caused some changes in membrane functions, loss of membrane structure integrity and inactivation of many membrane-bound protein receptors and enzymes (5, 10). In a previous study using electron paramagnetic spectroscopy, it has been shown that oxidative damage in the liver caused a decrease in membrane order (29). Biological membranes control a number of important functions such as signal transmission, material transfer and the activity of membrane-bound enzymes. In addition, the changes in membrane order and dynamics, secondary structures of proteins in membranes and molecular content of membranes cause many diseases by damaging the integrity and function of ion channels (11). Therefore, the order and fluidity of membrane should be at the optimum level in cells and this is very important for the degree of permeability to DOX. In consistence with previous studies, our results indicated that DOX disrupted the normal function of the cell membrane by changing its order and fluidity. These harmful effects on cellular membranes might be one of the toxic action mechanisms of DOX in biological tissues.

In order to obtain information about the effects of PRPLS on the molecules in the liver tissue, a group of animals was given PRPLS only. Our results indicated that PRPLS did not induce any significant change on the composition and structure of biomolecules in the liver when given alone to animals compared to the control group. This finding showed that PRPLS is not toxic to the body and it can be used for various purposes in medicine.

The results of the group that was given PRPLS before DOX administration showed that there were significant changes in the analyzed FTIR parameters compared to the control group and all these changes were in parallel with those observed in the DOX-treated group. This finding demonstrated that PRPLS was inadequate to prevent the harmful effects of DOX on molecules in liver tissue. It has been known that DOX is predominantly metabolized in the liver and the liver is one of the organs where DOX is mostly accumulated (28). For this reason, DOX caused

significant harmful effects on the molecules in the liver at the dose and time administered in this study. In the literature, there are a few studies showing the protective effects of PRPLS collected from different geographical regions of the world against DOX toxicity in various organs by using different methods (4, 5, 10). However, in those studies, generally a single dose of acute DOX was administered to the animals and the PRPLS doses and application times used were different from the current study's. Therefore, the reason why PRPLS could not show any protective effect against the toxicity of DOX might be that DOX at the dose and application times used in this study caused major damage on the molecules in the liver tissue and PRPLS at the dose and application times used in the current study was not sufficient to eliminate these significant toxic effects of DOX. A higher dose and longer application time of PRPLS may be required to protect the liver tissue from damaging effect of DOX. Since we believe that non-significant results are also an important building block for scientific studies, we think that our finding about the lack of protective effect of PRPLS at the dose and application times used in this study will shed light on future studies on this important bee product.

CONCLUSION

The findings of the current study showed that DOX caused significant deleterious effects on the composition, structure and function of the biomolecules in rat liver. DOX administration induced a significant decrease in the amount of glycogen and nucleic acids and a significant increase in the amount of lipid and protein in the liver. The decrease in the olefinic HC=CH/lipid ratio observed after DOX administration indicated an increase in oxidative stress and as a result lipid peroxidation occured in the liver. In addition, the decreases observed in the CH_2 antisym./CH_3 antisym. and C=O/lipid ratios and the increase in the CH_3 antisym./lipid ratios indicated that lipids were fragmented and the amount of short-chained lipids increased as a result of lipid peroxidation in the liver tissue after DOX administration. The increase observed in the amount of random coil structures showed that protein denaturation occured in the tissue due to DOX. The decrease in the C-O/PO_2^- ratio and the increase in the lipid/protein ratio indicated that DOX caused significant alterations in carbohydrate, lipid and protein metabolism in the liver tissue. In addition, DOX administration induced a decrease in the order of the phospholipid chains of the liver cell membranes and an increase in membrane fluidity. It has been known that one of the mechanisms of the toxic effect of DOX in the cell is to attach to the cell membrane and damage cellular functions. Therefore, this finding regarding the cell membrane is very important as it has revealed another toxic action mechanism of DOX for the first time – DOX damages the cell by changing the membrane order and fluidity.

This is the first report revealing the structural and related functional effects of DOX on the liver tissue at the molecular level by utilizing ATR-FTIR spectroscopy. Understanding the toxic action mechanisms of DOX in the liver is very important to increase the efficacy of this drug in malignant cells and reduce its toxic effects on healthy tissues. The fact that PRPLS did not cause any changes in the liver tissue when given alone could be used in the process of integrating this precious bee product into medicine, especially pharmacology. In addition, our study has shown that ATR-FTIR spectroscopy is a practical technique for examining the effects of a chemotherapeutic agent in a biological tissue.

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Supplementary Table S1. Band area ratios, the respective functional groups used to calculate those ratios and their assignments.

Band Area Ratio	Functional Groups Utilized	Function
CH_2 sym./CH_2 sym + antisym.	CH_2 symmetric stretching (# 6)/CH_2 symmetric stretching (# 6) + CH_2 antisymmetric stretching (# 4)	Saturated lipid concentration of the system
Olefinic CH=CH /Lipid	Olefinic=CH stretching (# 2)/CH_3 antisymmetric stretching (# 3) + CH_2 antisymmetric stretching (# 4) + CH_2 symmetric stretching (# 6)	Unsaturation level of the system (lipid peroxidation)
Amid I/Amid I + II	Amide I (# 8)/Amide I (# 8) + Amide II (# 9)	Protein concentration of the system
RNA/Protein	Ribose ring vibrations (# 15)/Amide II (# 9)	RNA concentration of the system
RNA/Lipid	Ribose ring vibrations (# 15)/ CH_3 antisymmetric stretching (# 3) + CH_2 antisymmetric stretching (# 4) + CH_2 symmetric stretching (# 6)	
DNA/Protein	DNA vibrations (# 18)/Amide II (# 9)	DNA concentration of the system
DNA/ Lipid	DNA vibrations (# 18)/ CH_3 antisymmetric stretching (# 3) + CH_2 antisymmetric stretching (# 4) + CH_2 symmetric stretching (# 6)	
RNA/DNA	DNA vibrations (# 18)/ Ribose ring vibrations (# 15)	Transcription status of the system
C-O/Protein	C-O stretching (# 17)/Amide II (# 9)	Glycogen concentration of the system
C-O/Lipid	C-O stretching (# 17)/CH_3 antisymmetric stretching (# 3) + CH_2 antisymmetric stretching (# 4) + CH_2 symmetric stretching (# 6)	
C-O/PO_2^- sym.	C-O stretching (# 17)/ PO_2^- symmetric stretching (# 16)	Metabolic activity of the system
CH_2 antisym./CH_3 antisym.	CH_2 antisymmetric stretching (# 4)/ CH_3 antisymmetric stretching (# 3)	Chain length of the lipids
C=O/Lipid	Carbonyl ester stretching (# 7)/ CH_3 antisymmetric stretching (# 3) + CH_2 antisymmetric stretching (# 4) + CH_2 symmetric stretching (# 6)	Carbonyl status of the system
CH_3 antisym./Lipid	CH_3 antisymmetric stretching (# 3) / CH_3 antisymmetric stretching (# 3) + CH_2 antisymmetric stretching (# 4) + CH_2 symmetric stretching (# 6)	Methyl concentration of the system
Amid I/Amid II	Amide I (# 8)/ Amide II (# 9)	Changes in protein structure and conformation
Lipid/Protein	CH_3 antisymmetric stretching (# 3) + CH_2 antisymmetric stretching (# 4) + CH_2 symmetric stretching (# 6)/ Amide II (# 9)	Comparison of the relative changes in the concentrations of the lipids and proteins

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