

# Investigation of the Toxic Effects of Rhododendron Honey on Mouse Cardiac Muscle Tissue Lipids at Molecular Level<sup>[1][2]</sup>

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## Abstract

The purpose of this study is to investigate the effects of different concentrations of Rhododendron honey (RH) on mouse cardiac muscle lipids by Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy at molecular level. For this purpose, a total of eighteen male *Mus musculus* mice were divided into three groups of six animals each, one being the control group and the others being the 25 and 50 mg/kg of RH-treated groups. RH was given via gavage and the cardiac muscles of these mice were investigated 24 h after the administration. The results revealed that 25 mg/kg of RH did not cause any significant effect except lipid peroxidation. However, 50 mg/kg RH caused increases in the amounts of saturated and unsaturated lipids, in the ratios of lipid/protein and CH<sub>2</sub>/CH<sub>3</sub> and a decrease in the CH<sub>3</sub>/lipid which all indicate a change in the lipid metabolism of the tissue. Moreover, the treatment with 50 mg/kg of RH caused lipid peroxidation, a decrease in lipid order and an increase in membrane dynamic. These results revealed that RH causes significant toxic effects on cardiac muscle tissue lipids and these effects are dose-dependent.

**Keywords:** Rhododendron honey, Mad honey, ATR-FTIR spectroscopy, Heart, Cardiac muscle, Lipid

## Ormangülü Balının Fare Kalp Kas Dokusu Lipitleri Üzerindeki Toksik Etkilerinin Moleküler Düzeyde İncelenmesi

### Öz

Bu çalışmanın amacı, farklı konsantrasyonlardaki ormangülü balının (OB) fare kalp kası lipitleri üzerindeki etkilerinin Azaltılmış Toplam Yansıma-Fourier Dönüşüm Kızılötesi (ATR-FTIR) spektroskopisi ile moleküler düzeyde incelenmesidir. Bu amaç doğrultusunda, toplam on sekiz adet *Mus musculus* erkek fare, her biri altı hayvan içeren, birisi kontrol ve diğerleri 25 ve 50 mg/kg OB uygulanmış gruplar olmak üzere üç gruba ayrıldı. OB gavaj yoluyla verildi ve bu farelerin kalp kasları uygulamadan 24 saat sonra incelendi. Sonuçlar 25 mg/kg OB'nin kalp kası lipitleri üzerinde lipit peroksidasyonu dışında herhangi anlamlı bir değişikliğe sebep olmadığını ortaya çıkarmıştır. Ancak 50 mg/kg OB doymuş ve doymamış lipitlerin miktarlarında, lipit/protein oranında ve CH<sub>2</sub>/CH<sub>3</sub> oranında artışa ve CH<sub>3</sub>/lipit oranında azalmaya sebep olmuştur. Tüm bunlar dokunun lipit metabolizmasında bir değişiklik olduğunu göstermektedir. Ayrıca 50 mg/kg OB lipit peroksidasyonuna, lipit düzeninde bir azalmaya ve membran düzeninde bir artışa sebep olmuştur. Bu sonuçlar, OB'nin kalp kası lipitleri üzerinde önemli toksik etkiler meydana getirdiğini ve bu etkilerin doza bağlı olduğunu ortaya çıkarmıştır.

**Anahtar sözcükler:** Ormangülü balı, Deli bal, ATR-FTIR spektroskopisi, Kalp, Kalp kası, Lipit

## INTRODUCTION

Rhododendron honey (RH), also known as mad honey, is obtained from plants belonging to the genus Rhododendron in the Ericaceae family. The rhododendrons growing

extensively in the Black Sea region of Turkey show also distribution in China, Tibet, Nepal, Tropical Asia, Europe and North America<sup>[1]</sup>. RH causes poisoning in humans and animals due to the toxic compound grayanotoxin (GTX) found in some species of the Rhododendron genus. However,



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in different regions of the world, this honey is widely used in the treatment of various disorders such as hypertension, gastrointestinal complaints, sexual dysfunction toothache, colds, etc.<sup>[2]</sup>.

The effect of GTX is predominantly on the voltage-dependent sodium channel. It binds to the sodium channel in the open position and the sodium channel becomes incapable of closing. As a result, action potential enters a prolonged period of hyperpolarization process<sup>[3,4]</sup>. Through this action mechanism, GTX affects the heart directly and causes a wide range of systemic effects including hypotension, arrhythmia, nausea and a reduction in spontaneous motion by affecting the central nervous system<sup>[1]</sup>. Most of the published studies on RH have presented case reports either on patient complaints after eating the honey or cases of patients treated at emergency services<sup>[3]</sup>. In clinical studies examining the effects of RH on the cardiac system, it has been reported that hypotension and bradycardia were seen in more than 90% of the patients<sup>[5]</sup>.

In the literature, experimental studies on the effects of RH on the cardiac system are extremely limited in number, compared to the clinical trials on the subject. For example, Onat et al.<sup>[6]</sup> observed obesity-related hypotension, bradycardia and respiratory rate depression in RH-treated mice. In another study, blood pressure and heart rate were measured in rats with experimental hypertension and it was reported that RH reduced the blood pressure and heart rate in the hypertensive rats<sup>[7]</sup>. Zushi et al.<sup>[8]</sup> have reported that the effect of GTX on the neuromuscular junction was achieved by increased permeability of the membrane to sodium. In a recent experimental study investigating the dose-related cardiovascular effects of GTX-III, it was reported that it could be lethal at high doses due to cardiac arrest<sup>[5]</sup>.

Fourier transform infrared (FTIR) spectroscopy is a high-tech tool that gathers valuable information about biological tissues and membranes by measuring the vibrations of molecules<sup>[9]</sup>. By using the attenuated total reflection (ATR) unit in FTIR spectroscopy, it is possible to detect spectral changes in biological tissues faster and more accurately, regardless of sample thickness<sup>[10]</sup>. The ATR-FTIR spectroscopy is a unique technique that enables the detection of absorption bands of lipids, proteins, carbohydrates and nucleic acids in biological systems in a single spectrum and at the same time, monitoring these molecules without using any labeling technique<sup>[10]</sup>. The main advantage of this technique is that the samples can be investigated without any preparation processes by placing them directly on the ATR crystals.

Recent studies have revealed the importance of intracellular myocardial and pericardial lipid deposits, showing that even the smallest changes in these fat deposits cause significant changes in cardiac performance<sup>[11]</sup>. It is known that RH leads to cardiac side effects and functional

disorders; however, there is no study reporting the effects of RH on the structure and function of lipids in the cardiac muscle. The ability of a tissue to function properly is related to its structure and ATR-FTIR spectroscopy gives information about the structure of the tissue at molecular level. In this study, we aimed to reveal the molecular effects of two different doses of RH on the composition, structure and function of the cardiac muscle lipids by using ATR-FTIR spectroscopy. To the best of our knowledge, this is the first study to investigate the effects of RH on cardiac muscle lipids at the molecular level.

## MATERIAL and METHODS

The RH that we used in our study was obtained from Düzce beekeepers. As a result of the palynological analysis, the honey sample was confirmed to dominantly ( $\geq 45\%$ ) consist of the *Rhododendron ponticum* pollen<sup>[12]</sup>.

The amount of GTX-I and GTX-III in the RH used in this study was detected by liquid chromatography-mass/mass spectrometry (LC-MS/MS) using the method developed by Kaplan et al.<sup>[13]</sup>. The limit of detection and the limit of quantification values of the validated analysis method were 0.0033 mg/kg and 0.01 mg/kg, respectively. The GTX-I and GTX-III amounts of the samples were found to be 32 and 8  $\mu\text{g/g}$ , respectively, which were higher than the average values reported previously<sup>[13]</sup> and in parallel with the amounts of toxic substances in RH used in other studies<sup>[14-16]</sup>.

All experimental procedures were approved by the Abant İzzet Baysal University Medical Faculty Experimental Animals Ethics Committee (2015/42). 18 male *Mus musculus* mice (20-25 g), 8-12 weeks old, were used. The animals were housed in a 12 h light - 12 h dark cycle at a constant room temperature ( $22 \pm 2^\circ\text{C}$ ) and fed with mouse food and water. The mice were divided into three groups: the control group ( $n=6$ ), the 25 mg/kg RH-treated group ( $n=6$ ) and the 50 mg/kg RH-treated group ( $n=6$ ). The different concentrations of RH were prepared by dissolving in water and they were administered via gavage to the animals in 0.01 mL per gram body weight ratio. 24 h after administration, the animals were decapitated and their heart tissues were removed and stored at  $-80^\circ\text{C}$  for latter ATR-FTIR spectroscopy experimentation.

The mouse cardiac muscle spectra were collected with a Spectrum Two FTIR spectrometer equipped with an ATR accessory (Perkin-Elmer Ltd., UK).  $0.5 \times 0.5 \times 0.1$  cm sized sections were cut from the myocardial layer of the heart for the ATR-FTIR studies and placed directly on the diamond/zinc-selenide crystals of the ATR unit. For each sample, spectra were obtained at  $4 \text{ cm}^{-1}$  resolution and 100 force gauge with 64 scans between the 4000-900  $\text{cm}^{-1}$  wavenumbers. The analyses of spectral bands were performed using Perkin Elmer software programme. Bandwidth and band wavenumber values were calculated at 75% of the band height<sup>[9,10]</sup>.

For quantitative comparison between control and treated samples, the areas under the lipid bands and the area ratios of some specific infrared bands were calculated. To examine the level of the lipid peroxidation of the system, the area under the olefinic=CH band and the unsaturated/saturated lipid ratio (the area of the olefinic=CH stretching band/the sum of the areas of the saturated lipid bands), which are the parameters used as an index for the determination of lipid peroxidation in FTIR studies, were used [9,17]. To find out the changes in the amount of saturated lipids, the areas under the CH<sub>3</sub> antisymmetric (antisym) stretching, CH<sub>2</sub> antisym stretching and CH<sub>2</sub> symmetric (sym) stretching bands and the ratio of CH<sub>2</sub> sym/CH<sub>2</sub> sym + CH<sub>2</sub> antisym stretching vibrations were evaluated [9,18,19]. To have information about the amount of triglycerides and cholesterol in the system, the area under the C=O ester stretching band was analyzed [20]. To determine the changes in the chain length of the membrane phospholipids, the CH<sub>2</sub>/CH<sub>3</sub> ratio (the area of the CH<sub>2</sub> antisym stretching/the area of the CH<sub>3</sub> antisym stretching) [21] and in the methyl concentration, CH<sub>3</sub>/lipid (the area of the CH<sub>3</sub> antisym stretching band/the sum of the areas of saturated lipid bands) ratio [20] were calculated. To compare the relative changes in lipid and protein concentrations in the system, the area ratios of the sum of the saturated lipid bands (CH<sub>3</sub> antisym, CH<sub>2</sub> antisym and CH<sub>2</sub> sym stretching bands) and proteins (Amide II band) were obtained [21]. To have information about membrane order and membrane fluidity, the wavenumber and bandwidth of the CH<sub>2</sub> antisym stretching band were analyzed, respectively [9].

Power analysis was performed to estimate the test power considering the sample size of each experimental group (n=6) for a power of 80% to achieve significant statistical differences at the 5% significance level and the calculated effect size value was 1.55. Mann-Whitney U test, which is a non-parametric test used to compare two independent groups that do not require large normally distributed samples, was performed to test the significance of the differences between the control and RH-treated groups two by two; that is, between the control and 25 mg/kg RH-treated groups and the control and 50 mg/kg RH-treated

groups. The P values less than 0.05 were considered statistically significant.

## RESULTS

Fig. 1 shows the ATR-FTIR spectrum of a control mouse cardiac muscle in the wavenumber range of 4000-900 cm<sup>-1</sup>. The main bands on the figure are numbered and their definitions are given in Table 1 according to the literature.

The cardiac muscle spectrum shown in Fig. 1 can be examined in three different regions: The large band located at 3700-3030 cm<sup>-1</sup> (band No. 1) mainly receives signals from the N-H groups of proteins with the little contribution from O-H stretching of polysaccharides, carbohydrates and water [20]. The 3025-2800 cm<sup>-1</sup> region (band No's. 2-6) is called the C-H stretching region and generally provides information about lipids [18]. The 1800-800 cm<sup>-1</sup> region (band No's. 7-17), which is called the fingerprint region, receives signals mostly from proteins and nucleic acids and in small quantities from lipids and carbohydrates [22]. In this study, since we aimed to collect information about the effects of different concentrations of RH on cardiac muscle lipids, the detailed analyses were performed mainly in the region of 3025-2800 cm<sup>-1</sup>. Fig. 2 shows the average ATR-FTIR spectra in the 3025-2800 cm<sup>-1</sup> region of the cardiac muscle of control and 25 and 50 mg/kg RH-treated mice.

To obtain information about the changes in the concentrations of lipid molecules after RH treatment, analyses of the areas under the lipid bands in the FTIR spectra were performed [18]. The changes in the band area values of the major functional groups are given in Table 2. As seen in Table 2, the area under the olefinic=CH stretching band (band No. 2) increased significantly in the 50 mg/kg RH-treated group indicating an increase in the amount of unsaturated lipids. As also seen in Fig. 2 and Table 2, the region under the saturated lipid bands (band No's. 3, 4, 6) increased significantly in the 50 mg/kg RH group. This result suggested that the 50 mg/kg RH administration caused an increase in the amount of saturated lipid in the cardiac muscle. The result derived from the analysis of the lipid bands in the C-H region was supported by the

**Fig 1.** ATR-FTIR spectrum of control mouse cardiac muscle in the 4000-900 cm<sup>-1</sup> wavenumber region

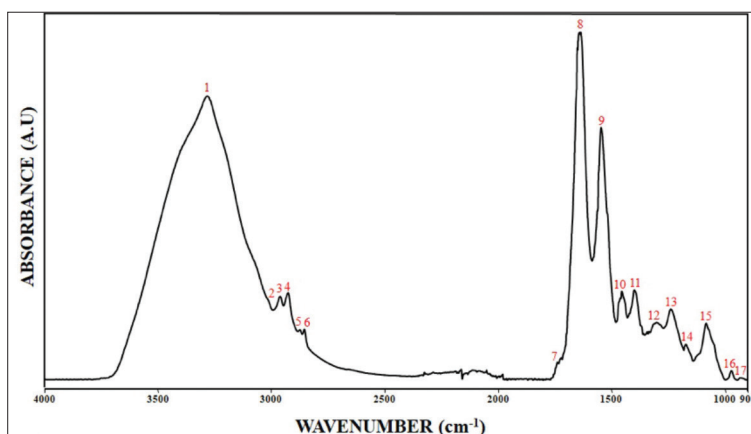
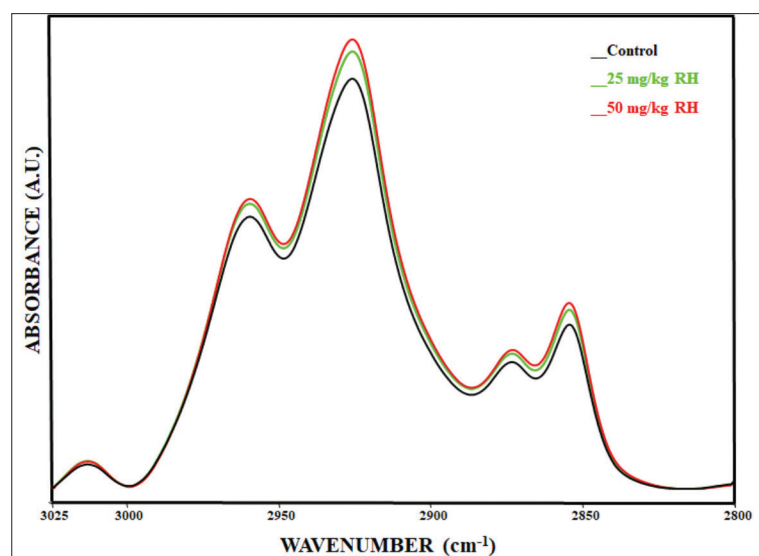


Table 1. General band assignment of ATR-FTIR spectrum of cardiac muscle tissue based on literature <sup>(18,20,22)</sup>		
Band No	Wavenumber (cm <sup>-1</sup> )	Definition of the Assignment
1	3283	Amide A: Mainly N-H stretching of hydrogen-bonded amide groups of proteins with the little contribution from O-H stretching of polysaccharides, carbohydrates and water
2	3011	Olefinic=CH stretching: Unsaturated lipids
3	2959	CH <sub>3</sub> antisymmetric stretching: Mainly lipids with little contribution from proteins, carbohydrates and nucleic acids
4	2924	CH <sub>2</sub> antisymmetric stretching: Mainly lipids with little contribution from proteins, carbohydrates and nucleic acids
5	2874	CH <sub>3</sub> symmetric stretching: Mainly proteins with little contribution from lipids, carbohydrates and nucleic acids
6	2855	CH <sub>2</sub> symmetric stretching: Mainly lipids with little contribution from proteins, carbohydrates and nucleic acids
7	1738	C=O (carbonyl) ester stretching: Ester functional groups in phospholipids, triglycerides and cholesterol esters
8	1641	Amide I: C=O stretching in proteins (80%)
9	1545	Amide II: Proteins (60% N-H bending, 40% C-N stretching)
10	1454	CH <sub>2</sub> bending: Mainly lipids with little contribution from proteins
11	1396	COO <sup>-</sup> symmetric stretching: Fatty acids and amino acid side groups
12	1302	Amide III: Proteins (40% C-N stretching, 30% N-H bending, 20% C-C stretching)
13	1238	PO <sub>2</sub> antisymmetric stretching: Mainly nucleic acids with some contribution from phospholipids
14	1172	CO-O-C antisymmetric stretching: Phospholipids, cholesteryl ester and nucleic acids
15	1080	PO <sub>2</sub> symmetric stretching: Nucleic acids and phospholipids; C-O stretching: glycogen, polysaccharides and glycolipids
16	972	C-N <sup>+</sup> -C stretching: Nucleic acids, ribose-phosphate main chain vibrations of RNA
17	931	z-type DNA



**Fig 2.** Average infrared spectra in the 3025-2800 cm<sup>-1</sup> spectral region of cardiac muscle of control, 25 mg/kg and 50 mg/kg RH-treated mice (The spectra were normalized with regard to the amide I band)

increase in the value of the C=O ester stretching band located at 1738 cm<sup>-1</sup> (band No. 7). As shown in Table 2, a significant increase was observed in the area value of this band in the 50 mg/kg RH-treated group.

In order to evaluate the effects of RH on the structure and composition of the cardiac muscle lipids, the area ratios of some specific lipid functional groups were also evaluated <sup>(21)</sup>. The band area ratios calculated for this purpose are given in Table 3. As shown in Table 3, the ratio of CH<sub>2</sub> sym/CH<sub>2</sub> sym + CH<sub>2</sub> antisym stretching vibrations increased significantly in the 50 mg/kg RH-treated group. This increase confirmed the increase in the amount of saturated lipids in the system

obtained from the analysis of the lipid band areas. The unsaturated/saturated lipid ratio and the CH<sub>2</sub>/CH<sub>3</sub> ratio increased significantly in 50 mg/kg RH-treated groups compared to the control group. A significant decrease in CH<sub>3</sub>/lipid ratio suggested a decrease in the amount of methyl groups in the 50 mg/kg RH-treated group. The lipid/protein ratio increased significantly in the 50 mg/kg RH-treated group.

The changes in the wavenumber and bandwidth values of the CH<sub>2</sub> antisym stretching band are given in Table 3. As seen from this table, the wavenumbers of the CH<sub>2</sub> antisym stretching band shifted towards higher values in the 50

**Table 2.** Changes in the band area values of the major lipid bands of the ATR-FTIR spectra of the cardiac muscle of control, 25 mg/kg and 50 mg/kg RH-treated mice

Band No	Functional Group	Wavenumber (cm <sup>-1</sup> )	Control	25 mg/kg RH	P Value <sup>a</sup>	50 mg/kg RH	P Value <sup>b</sup>
2	Olefinic=CH Stretch.	3011	0.021±0.004	0.030±0.006	0.100	0.037±0.005	0.034 <sup>*</sup>
3	CH <sub>3</sub> Antisym. Stretch.	2959	0.433±0.018	0.448±0.038	0.807	0.461±0.018	0.034 <sup>*</sup>
4	CH <sub>2</sub> Antisym. Stretch.	2924	0.815±0.025	0.855±0.072	0.376	0.896±0.063	0.043 <sup>*</sup>
6	CH <sub>2</sub> Sym. Stretch.	2855	0.125±0.008	0.136±0.015	0.167	0.143±0.010	0.015 <sup>*</sup>
7	Carbonyl Ester Stretch.	1738	0.623±0.012	0.655±0.037	0.165	0.666±0.036	0.006 <sup>**</sup>

Values are given as "mean ± standard deviation" for each group. Degree of significance was denoted as \*P<0.05, \*\*P<0.01. P value<sup>a</sup>: P values are from the comparison of the control and 25 mg/kg RH-treated mice; P value<sup>b</sup>: P values are from the comparison of the control and 50 mg/kg RH-treated mice

**Table 3.** Changes in the band area ratio, wavenumber and bandwidth values of various functional groups of the ATR-FTIR spectra of the cardiac muscle of control, 25 mg/kg and 50 mg/kg RH-treated mice

Functional Group	Control	25 mg/kg RH	P Value <sup>a</sup>	50 mg/kg RH	P Value <sup>b</sup>	
Band Area Ratio	CH <sub>2</sub> sym/CH <sub>2</sub> sym + CH <sub>2</sub> antisym	0.132±0.002	0.134±0.004	0.574	0.137±0.003	0.045 <sup>*</sup>
	Unsaturated/Saturated lipid	0.019±0.0008	0.021±0.001	0.030 <sup>*</sup>	0.022±0.001	0.024 <sup>*</sup>
	CH <sub>2</sub> /CH <sub>3</sub>	1.876±0.030	1.904±0.05	0.298	1.939±0.040	0.008 <sup>**</sup>
	CH <sub>3</sub> /Lipid	0.312±0.003	0.310±0.006	0.688	0.308±0.002	0.013 <sup>*</sup>
	Lipid/Protein	0.105±0.003	0.108±0.008	0.872	0.113±0.004	0.030 <sup>*</sup>
Wavenumber	CH <sub>2</sub> antisym. str.	2927.613±0.198	2927.751±0.393	0.521	2927.851±0.076	0.045 <sup>*</sup>
Bandwidth	CH <sub>2</sub> antisym. str.	11.355±0.142	11.380±0.136	0.872	11.525±0.094	0.045 <sup>*</sup>

Values are given as "mean ± standard deviation" for each group. Degree of significance was denoted as \*P<0.05, \*\*P<0.01. P value<sup>a</sup>: P values are from the comparison of the control and 25 mg/kg RH-treated mice; P value<sup>b</sup>: P values are from the comparison of the control and 50 mg/kg RH-treated mice

mg/kg RH group compared to the control group. As seen from the same table, the bandwidth of this band increased significantly in the 50 mg/kg RH-treated group.

## DISCUSSION

It has been reported that consuming more than 50 mg/kg (approximately 1 teaspoon for humans) of RH may cause serious poisoning [23], with symptoms appearing within 20 min to 3 h and lasting for 1-2 days [24]. For this reason, in this study, doses of 50 mg/kg, which is accepted as the toxic dose limit, and 25 mg/kg, which is ½ of the toxic dose, were selected for administration to the animals. Doses similar to those used in this study have also been used in previous studies [7,25,26].

Since unsaturated fatty acids are highly sensitive to lipid peroxidation, the area under the olefinic=CH band and the unsaturated/saturated lipid ratio in FTIR spectroscopy studies are used as an index for the determination of lipid peroxidation [9,17]. In this study, significant increases were observed in the olefinic=CH band area in the 50 mg/kg RH-treated group and unsaturated/saturated lipid ratio in the 25 and 50 mg/kg RH-treated groups. This increase may have been due to an increase in lipid peroxidation end products in the cardiac tissue as a result of an elevated level of lipid peroxidation in the cardiac muscle of the 50 mg/kg RH-treated group [21]. It has been known that lipid peroxidation occurs in the double bonds in the acyl chains of polyunsaturated fatty acids [27] and it results in the loss

of olefinic bonds [28]. However, according to the results of our experiments, instead of a decrease, an increase was observed in the olefinic groups. This result indicated that the amount of unsaturated fatty acid lost during lipid peroxidation reactions was compensated by the double bonds present in the lipid peroxidation end products, as reported in some previous FTIR spectroscopy studies [17,21]. In addition, in the present study, it was determined that the area under the C=O ester stretching band increased in the spectrum of the 50 mg/kg RH-treated group. Since there are abundant C=O (carbonyl) groups in lipid peroxidation end products, such as malondialdehyde (MDA), an increase in the area value of this band also indicates an increase in lipid peroxidation end products in the system [29].

It has been known that a large part of the heart energy requirement is provided by the oxidation of free fatty acids and fatty acids are extremely sensitive to the harmful effects of free radicals [11]. Under normal conditions, free radicals resulting from oxidative stress are produced in small amounts in all body cells. However, these radicals are inactivated by antioxidant enzymes and thus prevented from attacking other molecules in the cell [30]. If the production of free radicals increases rapidly for any reason, the mechanism of inactivation cannot compensate and these radicals react with other molecules in the cell and disrupt their structure. For example, the hydroxyl radical, one of the oxidative stress-producing free radicals, reacts with long chain fatty acids and causes lipid peroxidation [30]. As mentioned above, GTX increases the sodium permeability

of the membrane<sup>[4]</sup>. The increase in intracellular sodium concentration may affect the intracellular and extracellular sodium/calcium exchange mechanisms<sup>[31]</sup>. In this way, the intracellular calcium concentration increases, while the sodium level decreases on the other side. The increase in intracellular calcium concentration is known to be one of the main mechanisms triggering the formation of free radicals<sup>[32]</sup>. It is also known that exposure to GTX can lead to dysfunction in organs such as the liver and kidneys and functional defects in organs and impaired balance in the biological system may lead to free radical formation<sup>[16]</sup>. On the other hand, it is known that GTX plays an important role in cardiotoxicity by binding to the muscarinic M2 receptors<sup>[33]</sup>. The increased vagal tone of GTX leads to the condensation of the cholinergic effect, especially in the cardiovascular system and to the impairment of the physiological balance of other systems<sup>[33]</sup>. These may indirectly lead to the degradation of the oxidant/antioxidant balance at the cellular level, the weakening of the cellular antioxidant defense system and the formation of free radicals. As a result, the increase in the area values of olefinic=CH and carbonyl ester stretching bands and the ratio of unsaturated/saturated lipids observed in this study could be attributed to lipid peroxidation as a consequence of the attack of free radicals to the fatty acids in the system. Our results showed that the mechanism of action of GTX was related to the potential for oxidative stress formation. In a study conducted by Eraslan et al.<sup>[34]</sup>, in accordance with our results, oxidative stress induced by different doses of RH caused increases in the oxidative stress markers such as MDA, NO (nitric oxide) and HNE (4-hydroxynonenal) along with changes in enzyme levels. Similarly, lipid peroxidation induced by GTX in various cells and tissues determined via increase in MDA level was reported by Silici et al.<sup>[35]</sup> In the same study, an increase in antioxidant enzyme levels in the plasma and in various tissues was also observed. Since antioxidant enzymes play an active role in converting harmful free radicals into less harmful or harmless compounds, this increase can be regarded as an indication of lipid peroxidation due to oxidative stress.

According to the results of the present study, the amount of saturated lipids increased significantly in the 50 mg/kg RH-treated group. This result was supported by the increase observed in the chain length, which is determined by the increase in the CH<sub>2</sub>/CH<sub>3</sub> ratio, in 50 mg/kg RH-treated group<sup>[20]</sup>. The increase in lipid chain length may have been due to an increase in lipid content in the cardiac muscle tissue after 50 mg/kg RH administration. As systems with longer chained lipids contain relatively fewer methyl groups, the CH<sub>3</sub>/lipid ratio decreased when the lipid chain length increased<sup>[21]</sup>. Thus, the observed reduction in the CH<sub>3</sub>/lipid ratio following the administration of 50 mg/kg RH confirmed the conclusion that the lipid chain length had increased. These results suggested that the dose of 50 mg/kg RH caused changes in lipid metabolism in the cardiac muscle, resulting in the accumulation of lipids.

The physiological balance between lipid uptake and oxidation prevents the accumulation of excess lipids. If this balance is impaired for any reason, accumulation of myocardial lipid is observed and this situation leads to various pathological responses<sup>[36]</sup>. Fatty acids significantly affect crucial membrane functions like membrane fluidity and membrane structure stability, membranous ion and substance transport and cardiac electrophysiology, which is essential for cardiac function and excitability. In addition, they play a role as regulatory molecules in the formation of oxidative and ischemic damage, as a secondary messenger in cell signaling and transduction and as an effector in apoptosis<sup>[37]</sup>. For this reason, abnormalities in fatty acid metabolism affect the structure and function of the cardiac system adversely. In a study conducted by Oztasan et al.<sup>[25]</sup>, it has been demonstrated that RH caused changes in the lipid metabolism. In that study, diabetic rats were found to have reduced blood lipid levels after RH administration<sup>[25]</sup>. RH may cause a decrease in the lipid content in the blood due to this effect, which may in turn lead to an increase in the amount of lipids in tissues such as cardiac muscle. Most of these lipids that accumulate in the cardiac muscle tissue affect cardiac function in the worst way and cause myocardial structural damage, such as cardiac fibrosis, myocyte apoptosis and decreased contraction thought to be caused by frequent mitochondrial disorders<sup>[36]</sup>. Since consumption of RH in high amounts is known to cause cardiac side effects and functional disorders<sup>[38]</sup>, the lipids accumulated in the tissue may contribute to all these functional disorders.

In the current study, the lipid/protein ratio in the 50 mg/kg RH-treated group was significantly increased. This finding pointed out a change in the lipid asymmetry in the cardiac cell membranes and supported the conclusion that there has been a change in lipid metabolism in the system after the 50 mg/kg RH administration. It is known that changes in lipid asymmetry cause significant alterations in intracellular and intercellular ion concentrations and ultimately in membrane function<sup>[39]</sup>. The wavenumber of the CH<sub>2</sub> antisym stretching band in the FTIR spectrum gives information about the order and disorder status of the lipids in the membrane. In the present study, the wavenumber of the CH<sub>2</sub> antisym stretching band of the 50 mg/kg RH-treated group showed a significant shift towards higher values. This finding suggested that the 50 mg/kg RH administration caused a reduction in the membrane order<sup>[9]</sup>. The reduction in membrane order may have been related to lipid peroxidation caused by oxidative stress induced by RH<sup>[40]</sup>. In addition, the bandwidth of the CH<sub>2</sub> antisym stretching band was significantly increased in the 50 mg/kg RH group. This increase in bandwidth indicated an increase in membrane fluidity<sup>[9]</sup>. The observed changes in lipid fluidity may be due to the changes in the lipid composition, lipid concentrations and their changes relative to each other and to a change in the lipid/protein ratio<sup>[9]</sup>. It is known that the effects of GTX on the

skeleton and cardiac muscle are all due to changes in the cell membrane [41]. As GTX is a fat-soluble toxin, the order and fluidity of the cell membrane is very important for permeability to GTX. As a result of the action mechanism of GTX, some changes may occur in the cytosolic calcium concentration which plays important roles in events such as muscle contraction, cell division, apoptosis, and neurotransmitter release [4,42]. In our study, the decrease in the membrane order and the increase in the membrane fluidity were factors that increased the membrane permeability. Therefore, our results are consistent with the findings that RH affects the cell membrane, resulting in changes in intracellular ion concentration. Our findings showed that the 50 mg/kg RH dose disrupted the normal functioning of the cell membrane due to significant changes in membrane order and fluidity. These adverse effects on the order and fluidity of the cell membrane could be one of the toxic mechanisms of RH on the cardiac muscle.

Our results revealed that administration of 25 mg/kg of RH did not cause any significant change in the mouse cardiac muscle lipids except for an increase in the unsaturated/saturated fatty acid ratio, which is an indication of lipid peroxidation. However, 50 mg/kg RH induced significant changes on the tissue lipids at molecular level together with lipid peroxidation. The results of our study demonstrating the toxic effects 50 mg/kg RH on the structure, composition and dynamics of cardiac muscle lipids are crucial to reveal the action mechanism of RH on the functions of the cardiac muscle and have been reported for the first time. The global changes in lipids and membranes observed in this study may reflect one of the main mechanisms of the toxicity of GTX in RH. In addition, the results of this study show that the amount of RH is important and the induced effects depend on the dose consumed.

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