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# An investigation of the anti-hypertensive effect of mad honey and *Rhododendron luteum* sweet extract induced by N- ω -Nitro L-Arginine Methyl Ester (L-NAME) in rats

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The aim of this study was to investigate the anti-hypertensive effect of mad honey and *Rhododendron luteum* sweet extracts containing grayanotoxin (GTX)-III in a rat model of hypertension induced by N-ω-nitro L-arginine methyl ester (L-NAME). Thirty Sprague Dawley rats were divided into five groups - control (0.9% NaCl, 1 mL for 30 days, oral gavage [o.g.]), hypertensive (40 mg L-NAME /kg, bw for 30 days, intraperitoneal [i.p.]), standard (40 mg L-NAME /kg, bw for 30 days, i.p. + 20 mg Captopril/kg, bw for the last 15 days, o.g.), treatment I (40 mg L-NAME /kg, bw for 30 days, i.p. + mad honey, 12.5 mg GTX-III /kg, bw for the last 15 days, o.g.), and treatment II ( 40 mg L-NAME /kg, bw for 30 days, i.p.+ *R. luteum* blossom extract, 155.8 mg GTX-III /kg, bw for the last 15 days, o.g.). In addition to evaluating blood pressure using the tail-cuff method, some biochemical parameters were also measured in serum samples. Moreover, nitric oxide (NO) and glutathione (GSH) concentrations were also analyzed in heart, liver and kidney tissues to measure tissue damage caused by hypertension. The chromatographic analyses revealed GTX-III levels in mad honey and *R. luteum* of 24.94±0.10 mg/kg and 155.80±0.10 mg/kg, respectively. Both extracts used for animal application had a significant anti-hypertensive effect compared to the control and captopril groups. The systolic and diastolic values of the mad honey and blossom extract groups were 157.97-164.16 and 119.92-120.47, respectively.

**Keywords**: Grayanotoxin, Hypertensive, Mad honey, *Rhododendron*

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*Rhododendron* species are short shrub plants that grow in different geographical regions. *Rhododendron ponticum* L. (purple flower, also known as 'kumar' in this region [Anatolia]), *Rhododendron luteum* Sweet (yellow flower, also known as 'zifin'), and *Rhododendron caucasicum* Pall. (white flower, also known as 'komar') are three endemic species found in the coastal areas of the Black Sea region[1,2]. All three plant species have the potential to produce nectar and are the main source of *Rhododendron* honey, known as 'mad honey'. This honey may be toxic because it contains grayanotoxins (GTXs) derived from *Rhododendron* plants[3]. Although GTXs have several isomers, mad honey contains generally GTX I-III isomers[3].

It is thought that the general mechanism of GTXs toxicity can be described with some biochemical processes based on the cellular level that are detailed with the inotropy forming by blocking the sodium ion channels. GTXs bind to sodium channels and change plasma membrane permeability[4]. To explain in more detail in terms of molecular level, phenylalanine (Phe) and tyrosine (Tyr) residues play an important role in binding GTX to receptors, thus increasing the affinity of grayanotoxin to the sodium channels[5,6]. After attaching GTXs to sodium channels, they block their inactivation; consequently, their membrane permeability shows a change in the direction of increase[5]. Following the increase, GTXs inhibit their repolarization, cause the formation of the hyperpolarization direction in membrane potential, and extend depolarization. In short, at the end of this whole biological process, GTXs act as a cholinergic agent associated with bradycardia, depression of respiratory rate, and hypotension[5].

*Rhododendron* honey is a well-known natural compound used in complementary medicine and especially consumed in the Black Sea region of

Türkiye due to its potential medicinal effects. It is especially believed that this honey has a blood pressure-lowering and sexual enhancement effect[7,8].

To the best of our knowledge, there have been no controlled clinical studies of the anti-hypertensive effects of mad honey, although such effects have been mentioned in case reports. The purpose of this study was therefore to investigate the anti-hypertensive effects of mad honey and *Rhododendron* blossom extract in a rat model.In this study, a well-established method based on the induction of hypertension by the NOS blocker N(G)-nitro-L-arginine methyl ester (L-NAME)[9,10] was applied to obtain positive responses.

## Materials and Methods

#### Chemicals and apparatus

GTX-III Hemi (ethyl acetate) standard and N- ω -nitro L-arginine methyl ester L-(NAME) and 5,5'-Dithiobis (4-nitrobenzoic acid) were supplied by Sigma-Aldrich (St. Louis, MO, USA). High-grade hydrochloric acid (HCl), glacial acetic acid, and acetonitrile were obtained from Sigma-Aldrich (St. Louis, MO, USA). Liquid Chromatography (LC) syringe filters (RC-membrane, 0.45 μm) were obtained from Sartorius Minisart RC 15, Sartorius (Darmstadt, Germany). Kapril® tablet that contains 25 mg captopril was supplied by the Mustafa Nevzat Pharmaceuticals Industry Company in Türkiye. Tail-cuff plethysmography (Biopac Systems, MP150, USA) was used to measure the rats' blood pressures.

#### Samples

The honey sample was obtained from a beekeeper in the Hopa district of Artvin, Türkiye. The botanical characteristics of the honey were defined based on its *Rhododendron* pollen content using melissopalynological analyses[11].

*Rhododendron* spp. blossoms were collected from Madur Mountain, Köprübaşı District, Sürmene, Trabzon, Türkiye in 2018 (Permit no. 72784983-488.04-177033 from the Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks, Ankara). The blossoms were dried at room temperature, ground, and stored in a freezer. Next, 3 g of dry powder sample was placed in a falcon tube, to which was added 30 mL of 98% methanol, followed by mixing on a shaker (Heidolph Promax 2020, Schwabach, Germany) for 24 h at 20°C. This mixture was evaporated by a rotary evaporator (IKA®-Werke, RV 05 Basic, Staufen, Germany), the remaining extract being used.

#### Grayanotoxin-III analyses using LC-MS/MS

GTX-III was analyzed using a LC-MS/MS device (Thermo-Scientific Instrument, San Jose, CA, USA) and a chromatographic column (15 cm×3 mm×5 μm). The procedures were based on a previous study by Sahin *et al*. (2015), including analytical determination methods such as optimization and validation for standardization and calibration[3].

#### Experimental animal study

Approval for the study was granted by the Karadeniz Technical University (Trabzon, Türkiye) animal experiments ethical committee under protocol number 2018/31. Thirty healthy female Sprague-Dawley rats (180-220 g) were housed in cages under a 12 h light/dark cycle at 22±2°C. *Ad libitum* access was allowed to standard rat chow and drinking water.

The rats were randomly divided into five groups - Control, L-NAME, Captopril, Treatment I (honey) and Treatment II (*Rhododendron*). The procedures applied in the different groups are shown below. Honey and blossom extract were administered by oral gavage (o.g.) after the first 15 days of L-NAME treatment[12].

**Group 1**: **Control**: 0.9% NaCl, 1 mL for 30 days, o.g.

**Group 2**: **L-NAME**: 40 mg L-NAME /kg, bw for 30 days, intraperitoneally (i.p.)[13]

**Group 3**: **Standard: Captopril**: 40 mg L-NAME /kg, bw for 30 days, i.p. + 20 mg captopril/kg, bw for the last 15 days, o.g.

**Group 4**: **Treatment I:** 40 mg L-NAME /kg, bw for 30 days, i.p. + 12.5 mg GTX-III in mad honey /kg, bw for the last 15 days, o.g.

**Group 5**: **Treatment II:** 40 mg L-NAME /kg, bw for 30 days, i.p. + 156 mg GTX-III in *R. luteum* blossom extract /kg GTX-III for the last 15 days, o.g.

The animals' blood pressures were measured from their tails on days 15 and 30. Five repetitions were performed for each rat, at three-minute intervals, and average values were calculated (Fig. 1). The blood flow through the arteries in the tail was briefly interrupted by a ring-shaped inflatable cuff (11 mm). Once the desired pressure level had been achieved, the cuff was automatically and gradually deflated by the system to resume the blood flow as slowly. The first point defined as systolic blood pressure which was recorded by the system indicated the blood

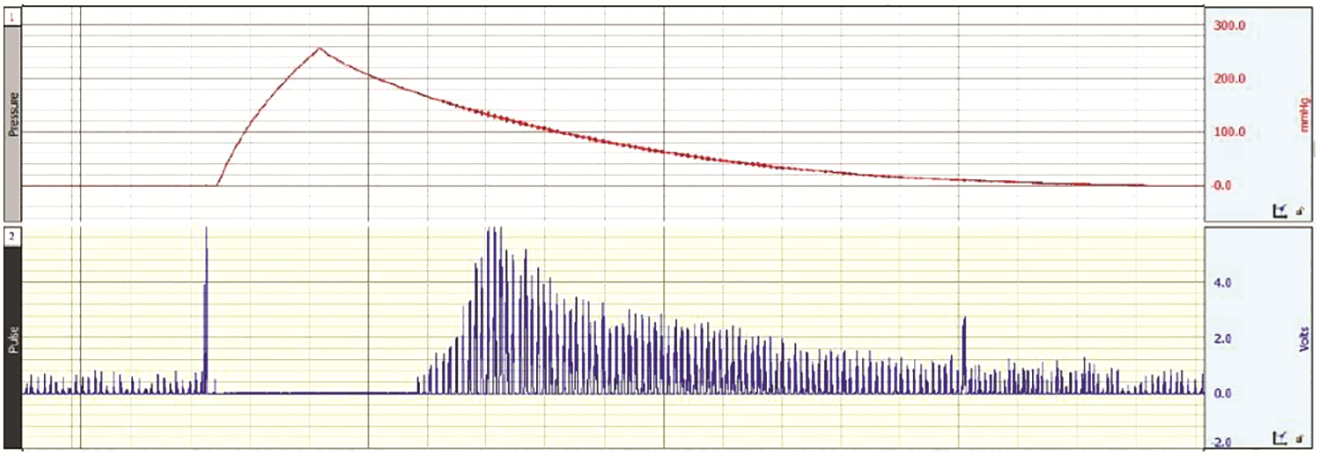


Fig. 1 — Blood pressure measurement screen

pressure exerting to artery walls in time when the first blood re-flow. And also, the second recorded point specified as diastolic blood pressure was the lowest pressure applied to the arteries in the rhythmic heartbeats with the highest beat.

At the end of the experiment all rats were decapitated. After decapitation, their blood was collected quickly into plastic tubes containing trisodium citrate dihydrate, an anti-coagulant. Liver, kidney and heart tissues were removed.

#### Blood biochemical analyses

Aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenases (LDH), creatine kinase (CK), glucose, and urea nitrogen were measured in the serum samples in the Karadeniz Technical University Medical Faculty biochemistry laboratory using commercial kits.

#### Tissue GSH concentrations

GSH concentrations of the liver and kidney tissues were calculated according to the modified method of Ellman at 412 nm, and the obtained results were given as µmol/g tissue[14].

#### Tissue nitric oxide concentrations

The measurement of the NO levels in the tissue homogenates were performed according to the Griess method[15]. The results were calculated as µmol/g tissue.

#### Analyses of the honey and blossom extract

Honey moisture, pH, and electrical conductivity analyses were performed in line with harmonized International Honey Commission methods[16]. The color characteristics of the honey were measured using a Hunter Lab device (Konica, Minolta, CM-5, Minolta, and Osaka, Japan). The Hunter Lab values represented means of (L) for darkness/lightness (0 black, 100 white), a (−a greenness, +a redness), and b (−b blueness, +b yellowness[17]. The amount of proline, expressed as mg/kg, was determined spectrophotometrically based on the color reaction of the amino acid with ninhydrin[16]. Fructose, glucose, and sucrose profiles were determined by HPLC-RID[18]. Total phenolic content (TPC) and total flavonoid content (TFC) were measured for each extract sample using Folin-Ciocalteu's method[19] and the $AlCI_3$ method[20], respectively. The total antioxidant capacities of the honey and blossom were determined in terms of the reduction capability of the iron (III) TPTZ complex, known as the FRAP method[21].

#### Statistics

Statistical analyses were carried out on SPSS version 11.5 software (IBM SPSS Statistics, Armonk, New York, USA). Descriptive statistics are presented as mean±SD. Correlation analysis was performed with the Mann–Whitney U-test. Significance was set at $p<0.05$ and $p<0.001$. Statistical significance tests were performed using one-way analysis of variance (ANOVA).

## Results

Table 1 shows various analysis parameters of the *Rhododendron* honey and its blossom used in the study. Predominant and secondary pollen species were measured using melissopalynological analysis based on microscopic evaluation of the pollen morphology. The major pollen detected in the honey

Table 1 — Some characteristic features of the studied samples

| Parameters | *Rhododendron* Honey (Mad Honey) Number of Sample (n)= 1 | *Rhododendron Blossom* Number of Sample (n)= 1 |
|---|---|---|
| % Predominant pollen | *Rhododendron*; 15.50±1.50* | |
| Secondary pollens | *Castanea sativa, Tilia, Trifolium, Robinia, Lamiaceae, Rosaceae* | N.A. |
| pH | 4.20±0.00* | N.A. |
| Moisture | 19.00±0.50* | N.A. |
| Conductivity | 0.56±0.00* | N.A. |
| Optic Rotation $[\alpha]_{20}$ | -2.15±0.05* | N.A. |
| | Color (Hunter Lab) | |
| L (0-100) ( darkness/lightness) | 81.53±0.75* | N.A. |
| a (0-100) ( −a greenness +a redness) | 27.43±0.42* | N.A. |
| b (0-100) (−b blueness +b yellowness) | 72.65±1.28* | N.A. |
| GTX-III (mg/kg) | 24.94±0.10* | 155.80±0.10* |
| Proline (mg/Kg) | 853.40±2.40* | N.A. |
| Total Fructose (g/100 g) | 36.40±0.10* | N.A. |
| Total Glucose (g/100 g) | 25.60±0.24* | N.A. |
| Total Sucrose (g/100 g) | N.D. | N.A. |
| Fructose/Glucose (F/G) | 1.42±0.10* | N.A. |
| Total Polyphenol Content (TPC) (mg GAE/ g) | 16.10±0.40* | 57.40±1.80* |
| Total Flavonoids Content (TFC) (mg QUE/ g) | 1.00±0.00* | 5.30±0.20* |
| Total antioxidant Capacity (FRAP) µmol $FeSO_4.7H_2O$/g | 3.30±0.54* | 132.00±0.03* |

* In all the results given, the analyses were performed in triplicate; N.D.: Not detected; N.A: No Analysis; the current analysis was not performed for this sample.

Table 2 — Blood pressures in the studied groups

| Groups* | Systolic Blood Pressure | Diastolic Blood Pressure |
|---|---|---|
| G 1 Control | 132.33±7.63[a] | 75.48±8.44[a] |
| G 2 L-NAME | 229.36±6.37[b, c] | 136.22±7.27[b, c] |
| G 3 Standard Captopril | 140.30±6.46[a] | 93.92±6.69[a] |
| G 4 Treatment I-Honey Extract | 157.97±9.50[a] | 119.92±9.54[b] |
| G 5 Treatment II-Blossom Extract | 164.16±8.12[a] | 120.47±7.95[b] |

*The number of samples (n) for each group is 6. [a-c] Values are significantly different from each of group ($p<0.001$).

was *Rhododendron* (15.50±1.50%). Other, secondary, pollens in the sample were *Castanea sativa*, *Tilia*, *Trifolium*, *Robinia*, *Lamiaceae*, and *Rosaceae*. The honey moisture was determined as 19.00±0.50%, with a pH value of 4.20. The electrical conductivity of the honey was 0.56±0.00 mS/cm, and the optical rotation value was negative. The color of honey was measured using the Hunter method, with an L value, indicating the lightness or darkness of the honey, of 81.53±0.75. This parameter identified the honey as light blossom honey. The Hunter a* value of the honey (27.43±0.42) indicates that was greenish, and that b* value (72.65±1.28) it was yellowish. The concentration of GTX-III isomer, the toxic compound, measured by LC/MS-MS was 24.94±0.10 mg/kg in *Rhododendron* honey and 155.80±0.10 mg/kg in the blossom. The proline content, the major free amino acid, in the honey was 853.40±2.40 mg/kg. The amount of fructose and glucose, some of the main sugar components of honey, was 36.40% and 25.60%, respectively, although sucrose remained below detectable limits. The total phenolic and flavonoid contents responsible for the biological activity were 16.10±0.40 mg Gallic Acid Equivalents (GAE)/ g and 1.00±0.00 mg Quercetin Equivalents (QUE)/ g sample in the honey extract and 57.40±1.80 mg GAE/g and 5.30±0.20 mg QUE/g in the blossom extract, respectively. The total antioxidant value of each sample measured using the FRAP method was 3.30±0.54 µmol $FeSO_4.7H_2O$/g in the honey extract and 132.00±0.03 µmol $FeSO_4.7H_2O$/g in the blossom extract.

Table 2 shows the systolic and diastolic blood pressures of the five experimental groups. Using the tail cuff method, the mean systolic and diastolic vessel pressures measured from the tail were 132.33±7.63 and 75.48±8.44 in the control group. Both blood pressures increased significantly in the

rats with hypertension induced by L-NAME (229.36-136.22, $p<0.001$), while both blood pressures decreased significantly in the rats given the blood pressure-lowering agent captopril (140.30-93.92, $p<0.001$). Blood pressure also decreased significantly in the mad honey and blossom extract treatment groups compared to the L-NAME group ($p<0.001$).

The blood serum biochemistry parameters of the study groups are summarized in Table 3. One noteworthy result was that blood sugar was significantly lower in the treatment groups compared to the L-NAME group ($p<0.05$). BUN, which indicates the amount of urea in the blood, decreased significantly only in the honey and blossom extract groups compared to the L-NAME group ($p<0.05$). Compared with the control group, Aspartate transaminase (AST) activity increased significantly in the L-NAME and captopril groups compared with the control group ($p<0.05$), although a slight increase was observed in the treatment groups. A significant decrease was also observed in the L-NAME group compared to the treatment groups ($p<0.05$). Alkaline phosphatase (ALP) activity was significantly lower in the treatment groups compared to the control and L-NAME groups ($p<0.05$). Intragroup analysis in terms of creatine kinase activity also revealed a significant difference, the lowest activity being found in the captopril and extract groups ($p<0.05$). While an increase in lactate dehydrogenases (LDH) activity was observed in the treatment groups compared to the control group ($p<0.05$), the activity was highest in the honey group. The GSH concentrations in the liver and kidney tissues shown in Table 4 revealed an increase in the L-NAME and the treatment groups compared to the control group ($p<0.05$). NO levels in the heart, liver, and kidney tissues are also summarized in Table 4. NO levels were lowest in the captopril and blossom extract groups (especially in heart tissue) and highest in liver tissue in the honey group. A statistically significant increase was observed in liver tissue between the L-NAME group and the honey group, together with a significant decrease in kidney tissue between the L-NAME group and the blossom extract group ($p<0.05$).

Table 3 — The results of biochemical parameters measured in the rat serum

| Groups* | Glucose (mg/100 mL) | BUN (mg/100 mL) | AST (U/L) | ALP (U/L) | CK (U/L) | LDH (U/L) |
|---|---|---|---|---|---|---|
| G 1 Control | 171.00±3.29$^{d}$ | 20.00±2.37$^{b}$ | 155.17±5.12$^{a}$ | 136.00±1.26$^{e}$ | 5554.00±15.14$^{b}$ | 537.67±9.63$^{a}$ |
| G 2 L-NAME | 165.00±3.35$^{c}$ | 20.17±0.75$^{b}$ | 220.17±7.55$^{c}$ | 131.67±2.88$^{d}$ | 8167.50±68.61$^{c}$ | 655.00±9.25$^{b}$ |
| G 3 Standard Captopril | 169.83±4.58$^{d}$ | 19.00±1.10$^{b}$ | 233.00±5.40$^{d}$ | 113.00±4.00$^{c}$ | 12135.50±89.58$^{e}$ | 535.67±3.01$^{a}$ |
| G 4 Treatment I-Honey Extract | 43.17±3.87$^{a}$ | 14.83±1.47$^{a}$ | 164.17±1.33$^{b}$ | 79.67±1.63$^{a}$ | 8835.83±32.69$^{d}$ | 758.17±1.72$^{c}$ |
| G 5 Treatment II-Blossom Extract | 153.00±2.90$^{b}$ | 14.83±0.75$^{a}$ | 170.17±4.12$^{b}$ | 95.50±2.26$^{b}$ | 3158.17±22.86$^{a}$ | 648.33±9.75$^{b}$ |

*The number of samples (n) for each group is 6. $^{a-e}$Values are significantly different from each of group ($p<0.05$).

Table 4 — The results of GSH and NO levels of heart, liver, and kidney tissue

| Groups* | Heart | Liver | Kidney |
|---|---|---|---|
| | | GSH levels (μmol/g tissue) | |
| G 1 Control | - | 0.70±0.11$^{a}$ | 0.70±0.05$^{a}$ |
| G 2 L-NAME | - | 0.98±0.14$^{b}$ | 0.84±0.07$^{b}$ |
| G 3 Standard Captopril | - | 0.86±0.06$^{a}$ | 0.70±0.05$^{a}$ |
| G 4 Treatment I-Honey Extract | - | 1.71±0.32$^{c}$ | 0.96±0.01$^{c}$ |
| G 5 Treatment II-Blossom Extract | - | 1.04±0.07$^{b}$ | 1.10±0.19$^{d}$ |
| | Heart | Liver | Kidney |
| | | NOx levels (μmol/g tissue) | |
| G 1 Control | 0.22±0.03$^{c}$ | 0.24±0.06$^{b}$ | 0.22±0.01$^{a, b}$ |
| G 2 L-NAME | 0.17±0.02$^{a, b}$ | 0.23±0.05$^{a, b}$ | 0.27±0.03$^{c}$ |
| G 3 Standard Captopril | 0.19±0.01$^{b, c}$ | 0.18±0.02$^{a}$ | 0.20±0.04$^{a}$ |
| G 4 Treatment I-Honey Extract | 0.17±0.02$^{a, b}$ | 0.49±0.05$^{c}$ | 0.24±0.03$^{b}$ |
| G 5 Treatment II-Blossom Extract | 0.15±0.01$^{a}$ | 0.19±0.04$^{a, b}$ | 0.19±0.03$^{a}$ |

* The number of samples (n) for each group is 6.
$^{a-d}$ GSH values are significantly different from each of group ($p< 0.05$)
$^{a-d}$ NO values are significantly different from each of group ($p< 0.05$)

## Discussion

The honey sample in this study meets the definition of multifloral honey since the proportion of major pollen exceeds 45%[11]. However, since the major pollen was *Rhododendron*, it can also be considered *Rhododendron* honey. Additionally, it can be referred to as 'mad honey' because contains GTX-III isomer. Our previous study showed that the amount of GTX-III in *Rhododendron* honey collected in the Black Sea region (the same area as in the current study) varied between 0.70 and 68 μg/g[3]. The highest level of GTX-III was detected in the same region (Artvin-Hopa). The amount of GTX-III compound was approximately five times higher in the blossom extract than in the honey (Table 1). A negative optical rotation value ([α]-2.159), one of the physicochemical analysis parameters of honey, indicates that honey is blossom honey, while positive optic rotation indicates dew honey, such as pine, oak, and fir honeys[18,22]. The color of honey is an important physical criterion in distinguishing its botanical properties, a higher L* value identifying light-colored honey and a higher b* value identifying yellow-colored honey. The appearance of the *Rhododendron* honey in the present study was similar to that in previous studies[18]. The major sugar components of honey are fructose and glucose, followed by specific sugars such as sucrose, maltose, trehalose, melibiose, melezitose, etc.[23]. The absence of sucrose in a honey sample shows that the honey is a high-quality product and has not been adulterated with sugar syrups[24]. The honey standards specify a maximum level of 5% sucrose in honey, but the absence of sucrose indicates high-quality honey[25-27]. The honey in the present study was sucrose-free, thus confirming that the sample was a natural compound.

The most important components of honey in terms of responsibility for its biological activity are polyphenols. The amount of polyphenols in honey varies depending on its botanical characteristics. Dark-colored honey types such as chestnut, pine, and oak are particularly rich in polyphenols. The TPC value in the present study (16.10±0.40 mg GAE/ g honey sample) was associated with mostly light-colored blossom honeys, such as acacia, Anzer, and *Astragalus*[18,28].

Comparison of the TPCs of honey and blossom extract revealed a much higher value in the blossom extract. A previous study of *R. ponticum* species reported a total polyphenol amount value of 349 mg GAE/100 g, and a total flavonoid value of 311 mg QUE/100 g, the major phenolic components being ferulic acid and catechin[1]. The honey in the present study was identified as a high-quality product due to its high proline content and non-sucrose nature, together with other physicochemical parameters[18].

Systolic and diastolic blood pressures exhibited a wide range of distribution among the experimental groups (Table 2). In our knowledge, the model of L-NAME-induced hypertension, besides decreasing NO production and increased oxidative stress[29], results in increased blood pressure. According to our data, a significant decrease in blood pressure in both the treatment-I (G4) and treatment-II (G5) groups, in addition to the captopril group (G3), indicates that mad honey and blossom extract may be effective against hypertension. Detailed comparison of the G4 and G5 groups revealed no statistical difference in blood pressure parameters between these treatment groups (G4 and G5) ($p>0.001$). This outcome was also regarded as promising because the effects in the G4 and G5 groups were very similar to that of captopril, a standard drug. Fundamentally, captopril works as an inhibitor of Angiotensin-converting enzyme (ACE) and prevents the development of hypertension[29]. This enzyme catalyzes the conversion of angiotensin I to angiotensin II known as a potent vasoconstrictor[30]. Moreover, angiotensin II is also the precursor for the secretion regulation of aldosterone[31]. This hormone has a significant role in the homeostasis of blood pressure and intravascular volume[32]. As a result, if ACE inhibition occurs, the simultaneous decrease effect could be seen in both vasopressor activity and aldosterone secretion resulting anti-hypertension effect. In line with this reality, the fall in blood pressure values in G4 and G5 may be due to the presence of GTXs in the honey and blossom extracts.

Except for basing the ACE inhibition effect of the positive group-captopril and comparing each other, the well-known effect of GTX-III which was found in the treatment material on metabolism was also directly considered. For example, some researchers have also suggested that different mechanisms are also involved in the toxicity of GTX has been attributed to its ability to bind to the group II receptor site in voltage-gated sodium channels (Nav1 x) inside the cell[33,34]. It has also been suggested that GTX increases membrane permeability by affecting the Na-K ATPase gate, thus causing depolarization[34]. Besides bradycardia, hypotension is the general symptom of

mad honey poisoning caused grayanotoxins[35]. According to Gami and Dhakal (2017), the stimulation of the afferent cardiac branches of the vagus nerve take a significant role because of leading a tonic inhibition of central vasomotor centers. This inhibition results in hypotension as well as bradycardia and peripheral vasodilation[35]. In the treatment groups which was detected GTX-III, the occurrence of hypotension which was exhibited with analysis of levels of systolic and diastolic could be based on this reality.

Another fact that may be related to the current situation is the inhibition of carbonic anhydrase (CA) by CA inhibitors; essentially, they are associated with numerous physiological processes and diseases. Vasodilation known as the widening of blood vessels can also be seen as one of these processes. Right here, some studies focused on the vasodilation by CA inhibitors, affecting vascular smooth muscle, endothelium, heart, blood cells, and nervous systems[36,37]. Those studies reported that some potent CA inhibitors known such as sulfonamides have potential vasodilating action[36,37]. As a result, the CA inhibition related to vasodilation may develop a different point of view to our study because GTX-III has been previously proven to be a CA inhibitor. Here, an *in silico* docking study showed that the GTX-III isomer causes inhibition of human carbonic anhydrase I and XIV enzymes[38]. Briefly, GTXs in the honey and extract may also have caused vasodilatation in the vascular endothelium through the CA inhibition mechanism. Another *in vitro* study supporting the present study reported that mad honey inhibited CA[39].

Another possible mechanism is that L-NAME may similarly lead to inhibition of the NOS enzyme, which catalyzes NO formation. Tissue NO levels were measured in the present study (Table 4). NO levels were present in similar amounts in all three tissues in the control group, while a significant increase was observed in the liver in the honey group and a decrease in the extract group. While NO levels increased significantly in the kidney in the L-NAME group, a significant decrease was detected in heart tissue. A significant decrease in heart tissue was detected in both the honey and the extract groups. The decrease in heart tissue in the treated groups (including GTX-III) indicates that the compound may cause inhibition of the NOS enzyme. The detection of different NO levels in three tissues can only be explained by the presence of NOS isoenzymes[40].

Hypertension is also a stress factor associated with the production of various free radicals and oxidative stress. GSH concentrations in tissue and the oxidation speed of the sulfhydryl group to disulfide in cellular glutathione provide useful information about the state of oxidative stress. A significant difference was observed in both liver and kidney tissues following treatment with *Rhododendron* honey ($p<0.05$).

To the best of our knowledge, studies examining blood biochemistry parameters related to *Rhododendron* honey and/or GTXs in the literature are limited, and unfortunately, those we did find were not directly compatible with our data. Only some parameters, such as levels of glucose, creatine kinase (CK), and LDL, are therefore discussed here. CK and lactate dehydrogenase (LDH) are both markers of cardiac damage[41]. An increase in the levels of CK and LDH was observed in the L-NAME group during the period of application compared to the control group in the present study, and this was evaluated as normal due to L-NAME's potential effect. A decrease was determined in CK levels, especially following treatment with *Rhododendron* flower extract. A statistically significant difference was observed in blood glucose concentrations between the L-NAME and the treatment groups (honey and blossom extract) ($p<0.05$). Although decreasing trends were observed in both treatment groups, the rate of decrease was sharper in the honey group. Blood sugar is an important source of energy for cells, and adequate concentrations are essential to the physiology of brain tissue[42]. Silici *et al.* (2016) measured the blood glucose levels of rats fed with *Rhododendron* honey and observed a downtrend compared to a GTX group[43]. Salman & Akbaş (2010) reported that GTX is capable of causing hypoglycemia[44]. In addition, Uzun *et al.*[45] reported below-normal blood glucose levels in a case showing signs of poisoning with *Rhododendron* honey.

## Conclusion

The present study confirmed the important medicinal potential of mad honey and Rhododendron blossom containing GTX-III. Especially, the current results showed that mad honey and Rhododendron blossom significantly reduced blood pressure in rats with hypertension-induced L-NAME. As mentioned in the discussion section, the obtained results were discussed by focusing on four main potential reasons. The first of these was the inhibition of the formation

of angiotensin II which was the mechanism of the standard drug, Captopril; the second was the toxicity effect of GTX-III, the main component in the treatment groups, after binding to the group II receptor site in voltage-gated sodium channels (Nav1 x) inside the cell; the third was the potential inhibitory effect of the CA enzyme because there are strong acceptations about the enzyme related to having the vasodilator effect in the literature; finally, the fourth is the imbalance level of NO, it is known that NO effects on vasodilation through a cascade of biological progress. However, this study was conducted at the preliminary level, and further, more detailed animal research is now needed to testify to the mechanism and the reason for hypotension.

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**Conflict of Interest**

The authors declare no conflict of interest

**Authors' Contributions**

HE Ç: Investigation, formal analysis, methodology, validation; M M: Conceptualization, methodology, resources; H S: Supervision, formal analysis, visualization, and writing-review & editing; Y K: Investigation, methodology; S K: Supervision, project administration, funding acquisition, writing-reviewing, and editing.

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