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Research paper

Analysis of grayanatoxin in Rhododendron honey and effect on antioxidant parameters in rats

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ABSTRACT

Ethnopharmacological relevance: Rhododendron honey, locally known as "mad honey", contains grayanotoksin (GTX) and thus induces toxic effects when consumed in large amounts. But, it is still popularly used for treating medical conditions such as high blood pressure or gastro-intestinal disorders. The aim of this study was to evaluate the effect of GTX on antioxidant parameters measured from rats fed with Rhododendron honey.

Material and methods: A total of sixty Sprague-Dawley female rats were divided into five groups of 12 rats each, one being the control group (Group 1) and the others being the experimental groups (Groups 2 to 5). Group 2 was treated with 0.015 mg/kg/bw of Grayanotoxin-III (GTX-III) standard preparation via intraperitoneal injection. Groups 3, 4 and 5 were respectively given Rhododendron honey (RH) at doses of 0.1, 0.5, and 2.5 g/kg/bw via oral gavage. After one hour, blood samples were collected from the rats. Glutathione peroxidase (GSh-Px), superoxide dismutase (SOD), catalase (CAT) activities and malondialdehyde (MDA) contents were examined in blood, heart, lungs, liver, kidney, testicles, epididiymis, spleen and brain specimens.

Results: The data from the rats in Groups 2 (GTX) and 5 (RH at 2.5 g/kg/bw) showed negative effect on the antioxidants parameters in blood and all tissue samples examined at the specified doses and time period. Administration of GTX to rats at dose of 0.015 mg/kg/bw resulted in lipid peroxidation. (This part needs to be enhanced more).

Conclusion: It has been observed that both Grayanotoxin and high dose Rhododendron honey treatments showed oxidant effect on blood plasma and organ tissues investigated.

1. Introduction

The genus *Rhododendron L.* (Ericaeceae) comprises 8 subgenera with over 800 species (Bhattacharyya, 2011). There are five species of *Rhododendron* in Turkey, the most widespread ones being *Rhododendron ponticum* and *Rhododendron flavum*. Rhododendron honey (RH) is produced by honeybees, which collect nectar from flowers of plant *Rhododendron* species. Beekeepers in Turkey purposely produce RH for its supposed therapeutic effects on a range of health problems (Sogut et al., 2009). RH is also known as "*Deli Bal*" in the region, literally meaning "Mad Honey".

Grayanotoxins which are also known as rhodotoxins are a group of closely related toxins found in Rhododendron's, and consumption of Grayanotoxin (GTX)-containing Rhododendron honey may result in intoxication. Grayanotoxins are toxic polyhydroxylated diterpenes and about 25 grayanone-type diterpenoids have been isolated from *Rhododendron* species (Qiang et al., 2011). Kim et al. (2010) reported that among the 18 isoforms of GTX found in mad honey, GTX I-IV are unique classes of toxic diterpenoids; GTX-III is the principal toxic isomer. In addition, Scott et al. (1971) detected intraperitoneal LD_{50} value for Grayanotoxin-III as 0.908 mg/kg in mice.

A number of honey intoxication cases, or "mad honey" cases have been documented in Turkey (Onat et al., 1991; Ozhan et al., 2004; Gunduz et al., 2006; Yilmaz et al., 2006; Okuyan et al., 2010). Symptoms of mad honey intoxication are dose-related and the most common symptoms include dizziness, hypotension and

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bradycardia, while rare symptoms such as excessive salivation and perspiration, impaired consciousness and seizures, atrioventricular block (AVB) have also been reported at high doses (Jansen et al., 2012; Gunduz et al., 2006).

Despite their toxicity, *Rhododendron's* have been used in ancient medical systems such as traditional Chinese and Indian medicines and also in European and American folk medicines. In traditional medicine, they have been used mainly against inflammation, pain, skin ailments, common cold and gastrointestinal disorders (Popescu and Kapp, 2013). In several studies, tests on plant extracts and isolated compounds of *Rhododendron* produced diverse biological activities including anti-inflammatory, analgesic, antioxidant, anti-microbial, anti-diabetic, insecticidal and cytostatic activity (Oztasan et al., 2005; Erdemoglu et al., 2008; Prakash et al., 2008; Jing et al., 2009; Silici et al., 2010; Yarlioglues et al., 2011). Research on the medicinal use of the Rhododendron honey, while still in its early stages, has revealed initial results confirming, to a limited extent, some of the claims made by indigenous medicine.

Up to now, there has been no research on antioxidant parameters in rats treated with GTX or Rhododendron honey. To the best of our knowledge, our research is the first investigation aimed at determining the effects of Grayanotoxin and Rhododendron honey on antioxidant parameters when administered at different doses. In the following, we describe experimental protocols and data gathering procedures employed in our investigation to achieve the stated objective.

2. Materials and methods

2.1. Animal material

A total of 60 Sprague-Dawley female rats (6–8 months old; 250–300 g; Laboratory Animal Unit, Erciyes University, Kayseri, Turkey) were used. The rats were divided into five groups of 12 rats each, one being the control group (Group1) and the remaining being the experimental groups (Groups 2 to 5). The rats were housed at a fixed temperature of 22–24 °C in standard cages and maintained in a 12:12 light/dark photoperiod and were fed a standard pelleted diet and tap water ad libitum. The chemical analysis showed that the pellets contained 23% crude protein, 7% crude cellulose, and 2600 Kcal/kg diet. The study protocol was approved by the Ethical Committee of the Veterinary Faculty of Erciyes University.

2.2. Honey samples

Grayanotoxin-III Hemi (ethyl acetate) was purchased from Sigma-Aldrich (Germany). Eleven fresh honey samples (extracted honey type) of *Rhododendron* from different parts of the Black Sea region of Turkey were collected from beekeepers in the period of June–July in 2012, when the *Rhododendron* species were flowering. The samples were stored at room temperature in jars for further analysis. Honey doses used in the study were calculated as 0.1, 0.5 and 2.5 g/kg/bw. These amounts were equivalent to the average amount of honey that a70 kg individual would consume; 7 g, (one teaspoon), 35 g (1–2 tablespoons) and overdose of 175 g (7–8 tablespoons).

2.3. Analysis of sediment for the identification of honey samples

The honey samples were classified according to their botanical origin after the pollen spectrum was obtained and melissopalynological analysis was carried out using the methods of Louveaux et al. (1978) and Von der Ohe et al., 2004. Pollen grains were microscopically observed and compared with reference slides for identification. Pollen grains were identified under the microscope and counts were expressed as percentages. Botanical classification was achieved when the pollen spectrum contained $>45\%$ of the corresponding dominant pollen. Monofloral Rhododendron honeys ($>45\%$) were used for the GTX analysis.

2.4. GTX-III analysis of Rhododendron honey samples

2.4.1. Reagents

GTX-III standard was supplied as Grayanotoxin-III Hemi (ethyl acetate) from Sigma-Aldrich (St. Louis, MO, USA). Hypergrade methanol for LC-MS, and glacial acetic acid were obtained from LiChrosolv® (Merck KGaA, Darmstadt, Germany). High quality ultra pure water was supplied by Human Zeneer Navi Power I Integrate (Human Corporation, Korea). LC syringe filters (RC-membrane, 0.2 μm) were from Sartorius Minisart RC 15, Sartorius (Darmstadt, Germany).

2.4.2. Analysis of GTX-III in honey

Monofloral Rhododendron honey samples ($n=6$) were dried at room temperature for the experiments. About 5 g of honey was extracted with 30 mL methanol in a flask attached to the condenser at 60 °C in 6 h. Extract was filtered to remove particles and final volume was determined. The remaining extract was evaporated and dried with a rotary evaporator (IKA, Werke, USA) at 40 °C. The residues were dissolved in 10 mL distilled water and transferred to C_{18} solid phase extraction (SPE) cartridge, which was initially conditioned with 5 mL methanol followed by 5 mL water. The cartridge was washed with 5 mL water to remove unbound materials. GTX-III was eluted from C_{18} SPE using 5 mL methanol. The organic solvents were evaporated in the rotary evaporator with reduced pressure at 40 °C. The residue was weighed and dissolved in methanol for LC/MS–MS analysis. 1 mg Grayanotoxin-III hemi (ethyl acetate) was dissolved with 1 mL hypergrade methanol. For calibration graphs and method validation, different dilutions ranging from 0.03125 to 2.5 μg/mL of the standard were used.

2.4.3. LC-MS/MS analysis

A Thermo-Scientific LC coupled with a TSQ Quantum Access Max triple stage quadrupole mass spectrometer (San Jose, CA USA) was used for all analyses. The analytical column was a Phenomenex C-18 (15 cm × 3 mm × 5 μm) (Torrance, California, USA). GTX-III was eluted under isocratic conditions using a mobile phase consisting 50:50 water/methanol solution containing 1% acetic acid into 8 min. Flow rate was 0.3 mL/min. MS data was acquired ESI in negative ion mode using Selected Reaction Monitoring (SRM) after described the real molecular weight of GTX-III by full scan in the range of 200–500 *m/z*. The instrument was tuned on MS/MS mode by optimizing the response of *m/z* 369 as the negative ion form of *m/z* 370 using 1 μg/mL GTX-III into the mobile phase at 10 μL/min flow rate with flash syringe. Fragmentation pattern study was applied for *m/z* 279, m/z 297, *m/z* 315 mass ions as product masses of *m/z* 369.

2.5. Experimental groups

The experimental groups received the following treatments:

Group 1 (control): 1 ml of 0.9% NaCl solution was given via intraperitoneal injection; ($n=12$)
Group 2 (GTX): 0.015 mg/kg/bw of Grayanotoxin-III was given via intraperitoneal injection; ($n=12$)