# Determination of Grayanotoxin-III from in Rhodendron Ponticum And Mad Honey Samples by Liquid Chromatography−Mass Spectrometry

Talha Sıddık Akkaya & Perihan Unak

Institute of Nuclear Science, Department of Nuclear Applications, Ege University, 35100, Bornova, Izmir, Turkey

**Correspondence**

Prof. Dr. Perihan UNAK, Institute of Nuclear Science, Department of Nuclear Applications, Ege University, 35100, Bornova, Izmir, Turkey
E-mail: perihan.unak@gmail.com; Fax: 00 90 232 388 64 66

## Abstract

Grayanotoxin-III and its glycoside derivatives were isolated from their natural sources. Grayanotoxin-III was extracted from Rhododendron Ponticum L. by liquid-liquid extraction and was isolated by prep-HPLC. Grayanotoxin-III glycoside was extracted from mad honey by SPE method. For isolation of grayanotoxin-III, a reverse phase C-18 column has been used in gradient conditions starting with 40/60 % (Methanol (A) /Water (B)) following 0 min. 34/66 (A/B), 11 min. 53/47 (A/B), 19 min. 70/30 (A/B), 22 min 100/0 (A/B) at 25 oC. Isolated fragments were examined with LC-MS analysis. The main ion of grayanotoxin-III was observed at m/z 370 with 100 % abundance with some of its fragments. Grayanotoxin-III was detected at 204 nm with Diode Array Detector at 7.5 min. with the above-mentioned conditions. Grayanotoxin-III glycoside from mad honey was identified by LC-MS and NMR analysis.

GTX-III can be transferred to honey from Rhododendron Ponticum plant according to obtained results. As a conclusion, because of the toxicity of the GTX-III compound the honey from these regions should be carefully consumed.

**Keywords:** Grayanotoxin-III, extraction, LC-MS, Mad honey, Rhododendron Ponticum L.

## 1. Introduction

Grayanotoxins (GTXs) are natural toxic diterpenoids, which have been isolated from species of Ericaceae family (Zhou et al., 2012; Booth et al., 2012; Zhang et al., 2015; Niu et al., 2016). Rhodendron Ponticum (RP) is one of the member of the Ericaceae, which contain GTXs, spread in Spain, Portugal, Turkey (Black Sea Region), United Kingdom, and Bulgaria (Milne & Abbott, 2000; Avci, 2004). Because of its use in folk medicine, these species were attractive for scientific studies. Various studies have been carried out on pharmacological and biological activity of RP (Popescu, 2013). Some other studies focused on the content of RP. Although it has temporary toxicological effects (Silici S. and Atayoglu, 2015, Popescu et al., 2009, Kim et al., 2010, Cucer and Eroz, 2010, Gunduz et al. 2014), mad honey is known as GTX contaminated honey from RP (Kurtoglu et al., 2014, Kaplan et al., 2014). Due to GTXs, mad honey can cause intoxication in human. Among many intoxication symptoms, especially dizziness, blurred vision, bradycardia, vomiting, nausea and presyncope can be seen (Silici and Atayoglu, 2015). The main structure of GTXs consists of a tetracyclic diterpenoid carbon skeleton with hydroxyl groups (Terai et al., 2000). GTX-III is the most toxic and the most similar compound to the main structure of GTXs (Wong et al., 2002). Several extraction methods like soxhlet, liquid-liquid extraction, column chromatography, prep-HPLC, have been used for isolation of the GTX-III from plants (Zhou et al., 2012, Terai et al., 2000, Wong et al., 2002, Sakata et al., 1977, Tiedeken et al., 2014). However, honey sample extraction has been used only for detection of the GTX-III with solid phase extraction (SPE) and direct dilution (Kurtoglu et al., 2014; Kaplan et al., 2014; Silici et al., 2014; Sahin et al., 2015). Detection and identification of the GTX-III was done by various methods, for example, TLC, GC, HPLC (RID), LC-MS, GC-MS, NMR (Terai et al., 2000; Wong et al., 2002; Holstege et al., 2000; Meguri et al., 1993; Nishida et al., 1990; Holstege et al., 2001; Masutani et al., 1979). GTX-III has been examined quantitatively with LC-MS analysis in rumen content, feces, urine, RP plant, ‘‘mad honey’’, and blood sample (Kurtoglu et al., 2014; Tiedeken et al., 2014; Holstege et

al., 2001; Hough et al., 2010; Cho et al., 2014).

GTX-III and GTX-III glycosides were extracted from RP and mad honey samples and GTX-III was detected with Diode Array Detector (DAD) at 204 nm wavelength in this work. Isolation of GTX-III was done with prep-HPLC from RP extract and identification was done by using DAD coupled HPLC, NMR and LC-MS analysis.

## 2. Materials and Methods

### *2.1 Reagents and solutions*

HPLC grade water and methanol (Sigma Aldrich) were used. Ethanol, chloroform, ethyl acetate, and acetone chemicals were obtained from Merck Chemical Co.

### *2.2 Apparatus*

Vacuum filtration apparatus (Sartorius), SPE cartridge (Sep-Pak), 0,22 μm filter (Ministar), 0,45 μm filter (Millex), centrifuge (Hettich 31, Rotina 38 R), rotary evaporator (Heidolph, Hei- VAP) were used.

### *2.3 Plant materials and honey samples*

The flowers and leaves of the forest roses (Family: Ericaceae, Species: Rhododendron ponticum L.) were picked up at 1300 m height of Topuk plateau of the Bolu mountain in the city of Düzce, in Turkey. Mad honey samples were gathered in the same area as forest rose samples were received.

### *2.4 Extraction of plant and mad honey*

#### *2.4.1 Extraction of the leaves and flowers of the forest rose*

The leaves and flower samples were dried at room conditions for three or four weeks, then they were milled to size of 1 mm. The extraction processes both for leaves and flowers were carried out according to Terai's report (Terai et al., 2000). Separately, 28 g of the leaves and flower powder was mixed with 280 mL of methanol for 30 min then centrifuged

at 2000 rpm for 5 min. Activated carbon was added into the supernatant at 50 ˚C then the supernatant was filtered. The liquid part of filtrate was evaporated at 1000 rpm, at 40 ˚C and then the residue was extracted with 150 mL of chloroform. The chloroform layer was evaporated at 200 rpm, at 40 ˚C with a rotary evaporator. The red colored residue was dissolved in 100 mL of ethyl acetate and applied activated carbon while boiling. The solution was filtered and evaporated at the room conditions for crystallization.

*2.4.2 Extraction of mad honey*

Extraction of honey samples was carried out according to Kurtoglu's method (Kurtoglu et al., 2014). 100 gr of mad honey was dissolved in 100 mL of water and 500 mL of ethanol was added. The solution was centrifuged at 3000 rpm for 5 min. The supernatant was filtered through 0.22 um filter with vacuum filtration (1st filtrate). The first filtrate was mixed with 500 mL of ethanol and was centrifuged at 3000 rpm for 5 min., then was filtered through 0.22 um filter with vacuum filtration (2nd filtrate). The second filtrate was mixed with 500 mL of ethanol and was centrifuged at 3000 rpm for 5 min., then was filtered through 0.22 um filter with vacuum filtration (3th filtrate). The last filtrate was passed through the SPE cartridge that was conditioned with 2x5 mL ethanol and 2x5 mL water. The SPE cartridge was washed with 5 mL of acetone/water (20:80). Finally, the compounds that trapped in the cartridge were removed with 6 mL of methanol. The filtrate was dried at 40 ±5 $^{o}$C under argon gas. The residue was dissolved in 12 mL of ethanol. Afterwards the solution was centrifuged at 3000 rpm for 5 min and the supernatant was stored at + 4 $^{o}$C.

*2.5 High performance liquid chromatography (HPLC) analysis of forest rose and mad honey extracts*

The extraction samples of leaves and flowers of the forest rose and mad honey were analyzed by HPLC. The HPLC method described by Meguri was used with some modifications (Meguri et al., 1993). HPLC system was coupled with Shimadzu LC-10AT quaternary pump, SLC-10A VP system collector, SIL-20 HT Auto Sampler, SPD-M20A

Diode Array Detector (Shimadzu Corporation, Kyoto, Japan), RID-10A Refractive Index Detector (Shimadzu Corporation, Kyoto, Japan), CTO-10AS VP Oven, FRC-10A Fraction Collector and C18 column.

*2.5.1 HPLC analysis of leaves and flowers extract*

The conditions of HPLC analysis of leaves and flower extracts are following, Detector: DAD, Wavelength: 204 nm, Mobile phase: Methanol / Water (Gradient), Flow rate: 0.7 mL/min, Oven temperature: 25 C, gradient conditions of leaves and flower extracts were given in Table 1.

*2.5.2 HPLC analysis of mad honey extract*

The conditions of HPLC analysis of mad honey extracts are following, Detector: RID, Mobile phase: Methanol / Water (Gradient), Flow rate: 0.7 mL/min, Oven temperature: 25 C, Gradient conditions of mad honey extract analysis was given in Table 1.

*2.6 Identification of molecular structure of GTX-III by LC-MS and NMR methods*

Identification of molecular structure of GTX-III from leaf extract by LC-MS method

Flowers, leaves, mad honey extracts and one of the HPLC fractions of a leaf extract (peak 3) were analyzed by LC-MS (Thermo Scientific, TSQ Quantum Access MAX) in the FABAL laboratory at the Pharmacy Faculty of Ege University. The samples were given by direct injection and ionized by electron spray ionization. Mass analyzer was Quadrupole. Scanning mode was Q1MS and scanning interval was between 300-500 (m/z) and 300-900 (m/z) for extracts (leaves, flower and honey) and peak 3, respectively. Instrument method parameters were following; liquid flow rate: 200 uL/min., sheath gas pressure: 35 psi, auxiliary gas flow (Arbitrary units): 10, spray voltage: 3000 V, ion transfer tube temperature: 280 ºC, injection volume: 10 uL, flash volume: 400 uL, flush speed: 100 uL/s, syringe speed: 8 uL/s, Injection mode: partially loop, tray temperature: 10 C, typical nitrogen consumption: 8 L/min.

*2.7 Identification of molecular structure of GTX-III from mad honey by NMR method*

Mad honey extracts were analyzed by 13C and 1H NMR (Varian mercury 400 MHz Spectrometer) at the Chemistry Department of Science Faculty of Atatürk University in Erzurum. The samples were analyzed overnight mode in methanol. ACD software was used to obtain the theoretical NMR spectra of GTX III for comparing experimental results.

## 3. Results and Discussion

The main ion of GTX III, which corresponding to m/z 370 was observed in LC-MS spectra of the leaf extracts (Fig 1). HPLC chromatogram belongs to fractions of the leaf extracts was presented in Fig 2. Main ion of GTX III was obtained from peak 3 by 100% relative absorbance and possible fragments of GTX III compounds were also detected (Fig. 3). Comparison of theoretical fragments of GTX III with experimental results in m/z values has shown in Table 2.

In the LC-MS analysis of the flower extract, main ion of GTX III (m/z 370) was observed (Fig. 4). Similar LC-MS spectra were obtained from the leaves and flower extracts (Fig.1 and Fig.5). And also the same extraction procedure was carried out to the leaves and flower samples. Thus GTX III was isolated with HPLC analysis of flower extracts with reference to peak 3 of leaf extract by overlapping the peaks with HPLC analysis (Fig 5).

The main ion of GTX III (at m/z 370) was determined in the mad honey extract (Fig 6). Besides, HPLC analyses of mad honey exhibited single main peak with small amount of impurity in the RID detector (Fig 7). RID has been used widely for the determination of many kinds of sugars (Karkacier et al., 2003; Victorita et al., 2008; Delgado et al., 2015). Furthermore $^{13}C$ NMR and $^{1}H$ NMR spectra of mad honey extract were overlapping with the theoretical NMR spectra of GTX III (Table 3, Fig.8). Both NMR spectra and (m/z 370) peak in the LC-MS assumed that this compound may be a GTX-III-glycoside.

Because of different parameters and conditions, the main peak and fragment ions can be

varied for each LC-MS study of the GTX-III (Table 4) (Kurtoglu et al., 2014; Kaplan et al., 2014; Tiedeken et al., 2014; Silici et al., 2014; Holstege et al., 2001; Hough et al., 2010; Cho et al., 2014; Michie et al., 2011; These et al., 2015).

In the previous studies, GTX-III was determined by in the rumen contents, homemade wine, feces and urine by LC-MS/MS analysis (Hwang et al., 2018; Cho et al., 2014; Holstege et al., 2001). The main ion was determined at m/z 335 with MS and fragmentation ion at m/z 299 with MS/MS. However, fragmented ions also determined in the MS spectra. In this study a pure GTX-III standard have been used (Holstege et al., 2000). Other studies were done for determination of the GTX-III in a compost of Rhododendron ponticum. GTX-III was determined at m/z 393.22 as sodium adduct. The standard GTX-III hemi (ethyl acetate) which has 414.53 g/mol of Mw, was supplied from Sigma Aldrich Company (Hough et al., 2010; Michie et al., 2011). Tiedeken et al. has determined GTX-III in honey samples and GTX-III was determined at m/z 415.3 with negative ion mode by using a commercial standard of the GTX-III from the same company (Tiedeken et al., 2014). In another study GTX-III was determined at m/z 369 with the negative ion mode in the blood sample by using the similar commercial standard of the GTX-III (Silici et al., 2014). Cho et al. developed an LC-MS method for determination GTX-III in the blood sample. They determined at m/z 335 as base ion and fragments were at m/z 299 by using Sigma- Aldrich GTX-III standard (Cho et al., 2014). Kurtoglu et al. examined mad honey and determined the main ion of the GTX-III at m/z 335 and fragmentation at m/z 315 (Kurtoglu et al., 2014). Kaplan et al., determined GTX-III in honey samples. Main ion was at m/z 335 and fragments were at m/z 316, m/z 299 and m/z 91(Kaplan et al., 2014). Sahin et al., determined GTX-III in a honey sample by using the negative ion mode. The main ion was at m/z 369 and fragments were at m/z 315, m/z 297 and m/z 279 by using same standard (Sahin et al., 2015).

## 4. Conclusions

GTX III compounds were isolated from the leaves and flowers of Rhodendron ponticum L., and detected with HPLC and LC-MS analysis with HPLC-DAD system. In the LC-MS studies, the main ion of the GTX-III that was determined in the leaves and flowers of Rhododendron Ponticum was also determined in honey samples, which was collected from the same region with the plants. These results indicate that GTX-III can be transferred to honey from Rhododendron Ponticum plant. As a conclusion, because of the toxicity of the GTX-III compound the honey from these regions should be carefully consumed.

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## References

Avci M. 2004. Rhododendron and their natural occurrences in Turkey. İstanbul Üniversitesi, Edebiyat Fak. Coğrafya Bölümü, Coğrafya Dergisi, 12: 13-29. (in Turkish)

Booth N.L., Kruger C.L., Hayes A.W., Clemens R. 2012. An innovative approach to the safety evaluation of natural products: Cranberry (Vaccinium macrocarpon Aiton) leaf aqueous extract as a case study. Food and Chemical Toxicology. 50: 3154-3165.

Cho H.E., Ahn S.Y., Kim D.W., Woo S.H., Park S.H., Hwang K., Moon D.C., Kim S. 2014. Development of a liquid chromatography–tandem mass spectrometry method for the determination of grayanotoxins in rat blood and its application to toxicokinetic study. Biomedical Chromatography, 28: 1624–1632.

Cucer N., Eroz R. 2010. Investigation of mutagenic effects of grayanotoxins II and III on cultured human lymphocytes. Al Ameen Journal of Medical Sciences. 4: 293-299.

Delgado D.D., Cuenca C.E.N., Sanchez L.P.R., Kushalappa A., Vasquez, M. (2015). Development and validation of a liquid chromatographic method to quantify sucrose, glucose, and fructose in tubers of Solanumtuberosum Group Phureja. The Journal of Chromatography. 975: 18-23.

Gunduz A., Eraydin I., Turkmen S., Kalkan O.F., Turedi T., Eryigit U., Ayar A. 2014. Analgesic effects of mad honey (grayanotoxin) in mice models of acute pain and painful diabetic neuropathy. Human and Experimental Toxicology. 33: 130–135.

Holstege D.M., Francis T., Puschner B., Booth M.C., Galey F.D. 2000. Multiresidue screen for cardiotoxins by two-dimensional thin-layer chromatography. Journal of Agricultural and Food Chemistry. 48: 60-64.

Holstege D.M., Pushchner B., Le T. 2001. Determination of grayanotoxins in biological samples by LC-MS/MS. Journal of Agricultural and Food Chemistry. 49: 1648-1651.

Hough R.L., Crews C., White D., Driffield M., Campbell C.D., Maltin C. 2010. Degradation of yew, ragworth and rhododendron toxins during composting. Science of the Total Environment. 408: 4128-4137.

Hwang T., Noh E., Jeong JH., Park S-K., Shin D., Kang H. 2018. Determination of Grayanotoxins from Rhododendron brachycarpum in Dietary Supplements and Homemade Wine by Liquid Chromatography-Quadrupole Time-of-Flight-Mass Spectrometry and Liquid Chromatography-Tandem Mass Spectrometry, J. Agric. Food Chem., 66: 1935-1940.

Kaplan M., Olgun E.O., Karaoglu O. 2014. Determination of grayanotoxins in honey by liquid chromatography tandem mass spectrometry using dilute-and-shoot sample preparation approach. J. Agric. Food Chem., 62: 5485-5491.

Karkacier M., Erbas M., Uslu M.K., Aksu M. 2003. Comparison of different extraction and detection methods for sugars using amino-bonded phase HPLC. The Journal of Chromatographic Science. 41: 331-333.

Kim S.E., Shin M.C., Akaike N., Kim C.J. 2010. Presynaptic effects of grayanotoxin III on excitatory and inhibitory nerve terminals in rat ventromedial hypothalamic neurons. NeuroToxicology. 31: 230–238.

Kurtoglu A.B., Yavuz R., Evrendilek G.A. 2014. Characterization and fate of grayanatoxins in mad honey produced from Rhododendron ponticum nectar. Food Chemistry. 161: 47-52.

Masutani T., Kawazu K., Uneyama K., Torii S., Iwasa J. 1979. Assignment of $^{13}$C-NMR Spectra of Grayanotoxin-I and –III. Agricultural and Biological Chemistry. 43: 631-635.

Meguri H., Katakawa J., Tetsumi T., Uchida S., Sumi M., Katai M., Terai T., Fukumato K. 1993. Simple and Rapid Determimation of Grayanotoxins by High−Performance Liquid Chrornatography. The Japanese Society of Pharmacognosy. 47: 429-433.

Michie D., Litterick A., Crews C. 2011. The influence of outdoor windrow composting on the concentration of grayanotoxins in Rhododendron leaves. Compost Science & Utilization. 19: 44-51.

Milne R., Abbott R.J. 2000. Origin and evolution of invasive naturalized material of Rhododendron ponticum L. in the British Isles. Molecular Ecology. 9: 543-556.

Nishida R., Fukami H., Iriye R., Kumazawa Z. 1990. Accumulation of highly toxic Ericaceous diterpenoids by the Geometrid Moth, Arichanna gaschkevitchii. Agricultural and Biological Chemistry, 54: 2347-2352.

Niu C.S., Li Y., Liu Y.B., Ma S.G., Li L., Qu J., Yu S.S. 2016. Analgesic diterpenoids from the twigs of Pieris Formosa. Tetrahedron, 72: 44-49.

Popescu R., Krupitza G., Kopp B. 2009. Novel insights into the mechanism of action of grayanotoxin III. Planta Medica, 75: 970-970.

Popescu R.K. 2013. The genus Rhododendron: An ethnopharmacological and toxicological review. The Journal of Ethnopharmacology. 147: 42-62.

Sahin H., Turumtay E.A., Yildiz O., Kolaylı S. 2015. Grayanotoxin-III dedection and antioxidant activity of mad honey. International Journal of Food Properties, 18: 2665-2674.

Sakata K., Hattori M., Sakurai A., Hosotsuji, T. (1977). Isolation and identification of biologically active constituents of Leucothoe catesbaei A. Gray. Journal of Pesticide Science, 2: 453-456.

Silici S., Atayoglu A.T. 2015. Mad honey intoxication: A systematic review on the 1199 cases. Food and Chemical Toxicology. 86: 282–290.

Silici S., Yonar M.E., Huseyin S., Atayoğlu A.T., Ozkok D. 2014. Analysis of grayanotoxin in rhododendron honey and effect on antioxidant parameters. The Journal of Ethnopharmacology. 156: 155-161.

Terai T., Araho D., Osakabe K., Katai M., Narama I., Matsuura T., Katakawa J., Tetsumi T. Sato M. 2000. Isolation of iso-grayanotoxin II from leaves of Leucothoe grayana MAX. Its X-Ray crystallographic analysis and acute toxicity in mice. Chemical & Pharmaceutical Bulletin, 48: 142-144.

These A., Bodi D., Uecker S., Reimers K, Ronczka S., Weigert A.P., Wiederholt M.L. 2015. A case of human poisoning by grayanotoxins following honey ingestion: elucidation of the toxin profile by mass spectrometry. Food Additives & Contaminants Part A, 32: 1679-1683.

Tiedeken E.J., Stout J.C., Stevenson P.C., Wright G.A. 2014. Bumblebees are not deterred by ecologically relevant concentrations of nectar toxins. Journal of Experimental Biology. 217: 1620-1625.

Victorita B., Margihitas L.A., Stanciu O., Laslo L., Dezmiream D., Bobis O. 2008. High-performance liquid chromatographic analysis of sugars in Transylvanian honeydew honey. The Bulletin of University of Agricultural Sciences and Veterinary Medicine. 65: 229-232.

Wong J., Youde E., Dickinson B., Hale M. 2002. Report of the rhododendron feasibility

study. Prepared for the Beddgelert Rhododendron Management Group by the School of Agricultural and Forest Sciences, University of Wales, Bangor, 64-64.

Zhang M., Xie Y., Zhan Lei L., Shu P., Chen Y., Xue Y., Luo Z., Wana Q., Yao G., Zhang Y. 2015. Grayanane and leucothane diterpenoids from the leaves of Rhododendron micranthum. Phytochemistry. 117: 107–115.

Zhou C., Li X., Li H., Li R. 2012. Chemical constituents from the leaves of Craibiodendron yunnanense. Biochemical Systematics and Ecology, 45: 179–182.

Zielinski A.A.F., Braga C.M., Demiate I.M., Beltrame F.L., Nogueira A., Wosiacki G. 2014. Development and optimization of an HPLC-RI method for the determination of major sugars in apple juice and evaluation of the effect of the ripening stage. Food Science and Technology, 408: 4128-4137.

**Table legends:**

Table 1. HPLC gradient conditions of leaf, flower and mad honey extracts

Table 2. Comparison of theoretical and detected GTX III fragments for LC-MS

Table 3. Comparison of mad honey extract NMR results to theoretical NMR of GTX III

Table 4. Different LC-MS and LC-MS/MS studies of GTX-III

**Figure Legends:**

Figure 1. LC-MS spectra of the leaf extract.

Figure 2. HPLC analysis of leaf extract, 204 nm.

Figure 3. LC-MS analysis of peak 3 (Fig. 2)

Figure 4. LC-MS spectra of flower extract.

Figure 5. HPLC analysis of mad honey extract

Figure 6. HPLC analysis of flower extract and isolated peak 3

Figure 5. LC-MS spectra of flower extract.

Figure 8. Chemical structure of GTX-III (ACD software)

Table 5. HPLC gradient conditions of leaf, flower and mad honey extracts

| Gradient condition of leaf extract | | | Gradient condition of flower extract | | | Gradient condition of mad honey extract | | |
|---|---|---|---|---|---|---|---|---|
| Time program (min) | Mobile phase composition (% V-V) | | Time program (min) | Mobile phase composition (% V-V) | | Time program (min) | Mobile phase composition (% V-V) | |
| | % A (methanol) | % B (Water) | | % A (methanol) | % B (Water) | | % A (methanol) | % B (Water) |
| 0 | 34 | 66 | 0 | 34 | 66 | 0 | 34 | 66 |
| 11 | 53 | 47 | 11 | 53 | 47 | 11 | 53 | 47 |
| 19 | 70 | 30 | 14 | 100 | 0 | 13 | 100 | 0 |
| 22 | 100 | 0 | 22 | 100 | 0 | 21 | 100 | 0 |
| 33 | 100 | 0 | 24 | 40 | 60 | | | |
| 35 | 40 | 60 | | | | | | |

Table 2 Comparison of theoretical and detected GTX III fragments for LC-MS

| *Fragmentation products* | *Therotical* | *GTX-III* | *% Relative Abundance* |
|---|---|---|---|
| M | 370,48 | 370,02 | 100 |
| MH+Na | 394,48 | 394,89 | 48 |
| MH-$CH_3$ | 356,48 | 356,33 | 42 |
| M-OH | 353,48 | 352,84 | 35 |
| M-OH-$CH_3$ | 338,48 | 338,92 | 15 |
| M-2OH-$CH_3$ | 321,48 | 321,62 | 15 |

Table 3 Comparison of mad honey extract NMR results to theoretical NMR of GTX III

| ***13C-NMR*** | | | | ***1H-NMR*** | | | |
|---|---|---|---|---|---|---|---|
| ***13C No.*** | ***CHn*** | ***Experimental*** | ***Theoretical*** | ***Group*** | ***nH*** | ***Experimental*** | ***Theoretical*** |
| ***1*** | *C* | 47,5 | 51,8 | ***3*** | *1* | 3,48 | 3,48 |
| ***2*** | *C* | 84,3 | 84,6 | ***5*** | *1* | 1,70 | 1,80 |
| ***3*** | *CH* | 47,8 | 51,7 | ***7*** | *1* | 3,99 | 3,96 |
| ***4*** | *C* | 79,0 | 78,1 | ***8*** | *1* | 2,14 | 2,27 |
| ***5*** | *CH* | 57,1 | 55,2 | ***9<''>*** | *1* | 2,65 | 2,66 |
| ***6*** | *C* | 48,0 | 52,6 | ***9<'>*** | *1* | 1,69 | 1,67 |
| ***7*** | *CH* | 82,1 | 79,5 | ***10<''>*** | *1* | 1,71 | 1,72 |
| ***8*** | *CH* | 57,1 | 56,4 | ***10<'>*** | *1* | 0,94 | 0,94 |
| ***9*** | *CH2* | 27,1 | 27,0 | ***11*** | *1* | 3,82 | 3,82 |
| ***10*** | *CH2* | 17,1 | 16,0 | ***12<''>*** | *1* | 2,65 | 2,58 |
| ***11*** | *CH* | 82,1 | 82,6 | ***12<'>*** | *1* | 2,65 | 2,55 |
| ***12*** | *CH2* | 31,8 | 35,8 | ***13*** | *1* | 4,03 | 4,03 |
| ***13*** | *CH* | 73,6 | 74,2 | ***14<''>*** | *1* | 2,14 | 2,19 |
| ***14*** | *CH2* | 47,5 | 44,4 | ***14<'>*** | *1* | 1,73 | 1,73 |
| ***15*** | *CH2* | 61,5 | 60,4 | ***15<''>*** | *1* | 2,02 | 2,03 |
| ***16*** | *C* | 83,0 | 79,7 | ***15<'>*** | *1* | 1,73 | 1,77 |
| ***17*** | *CH3* | 22,5 | 21,5 | ***17*** | 3 | 1,15 | 1,11 |
| | | | | ***18*** | 3 | 1,58 | 1,56 |
| ***18*** | *CH3* | 22,5 | 21,5 | ***19,20,22, 23,24,26*** | 6 | 3,77 | 3,76 |
| ***21*** | *CH3* | 25,5 | 28,3 | ***21*** | 3 | 1,73 | 1,83 |
| ***25*** | *CH3* | 23,7 | 23,9 | ***25*** | 3 | 1,47 | 1,49 |

Table 4 Different LC-MS and LC-MS/MS studies of GTX-III

| Reference | Analyzed Sample | MS system | Mobile phase / Conditions | Ionization Mode | MS (m/z) | MS/MS (m/z) | Rt. (min.) |
|---|---|---|---|---|---|---|---|
| Holstege et al., 2001 | Rumen content, feces and urine, | Finnigan LCQ ion trap CID | (A) % 1 acetic acid in water<br>(B) % 1 acetic acid in MeOH<br>1 min. %70 A - % 30 B<br>15 min. %10 A - % 90 B<br>3 min. hold %10 A - % 90 B | ESI (positive) | (M+Na)+ 393.4<br>MH+ 371.1<br>353.1<br>(MH-2H2O)+ 335.1<br>317.0<br>299.0<br>281.0<br>253.3 | 317.0<br>299.0<br>281.0<br>271.0 | 9.2 |
| Hough et al., 2010 | Rhododendron Ponticum (composting ) | Agilent 6200 - MSD-TOF | (A) % 1 acetic acid in water<br>(B) % 1 acetic acid in MeOH<br>1 min. %70 A - % 30 B<br>15 min. %10 A - % 90 B<br>1 min. %70 A - % 30 B<br>9 min. %70 A - % 30 B | ESI (positive) | M+Na 393,2248 | - | 10.0 |
| Michie et al., 2011 | Rhododendron Ponticum (composting of leaves and twings) | Agilent 6200, MSD-TOF | (A) % 1 acetic acid in water<br>(B) % 1 acetic acid in MeOH<br>0 min. %70 A - % 30 B<br>15 min. %10 A - % 90 B | ESI (positive) | M+Na 393,2248 | - | 11.0 |
| Tiedeken et al., 2014 | Rhododendron Ponticum nectar | ZQ | (A) water<br>(B) MeOH<br>(C) % 1 formic acid in MeCN<br>0 min. %90 A-% 0 B- % 10 C<br>20 min. %0 A-% 90 B-% 10 C<br>30 min. %0 A-% 90 B-% 10 C<br>31 min. %90 A-% 0 B-% 10 C | Negative mode | (M-H+formate)- 415.3 | - | 6.7 |
| Silici et al., 2014 | Blood sample (from rats fed RP honey and injected GTX-III STD) | Termo Scientific, TSQ Quantum Access Max. | (A) % 1 acetic acid in water<br>(B) % 1 acetic acid in MeOH<br>A/B (50:50) (isocratic cond.) | ESI (negative mode) - Selected Reaction Monitoring (SRM) | M-H 359 | 315<br>297<br>279 | - |
| Cho et al., 2014 | Blood sample (from rat) | Sciex 3200 QTRAP system | (A) % 1 acetic acid in water<br>(B) % 1 acetic acid in MeOH<br>0-9 min. % 0 - % 90 B (ramp)<br>9-18 min. % 0 - % 90 B<br>18-20 min. %90 %0 B(ramp)<br>20-30 min. %90 - % 0 B | ESI (positive) Multiple reaction monitoring (MRM) | - | (MH-2H2O)+ 335.1 (in Q1 full scan)<br>(MH+3H2O)+ 317.0<br>(MH+4H2O)+ 299.0<br>(MH+5H2O)+ 281.0<br>(MH+5H2O -CO)+ 253.3 | 10.7 |
| Kurtoglu et al., 2014 | Mad honey | 3200 Qtrap, Applied Biosystems | (A) % 0.1 acetic acid in water<br>(B) % 0.1 acetic acid in MeOH<br>0-1 min. % 10 B<br>1-10 min. %10-%80 B (Ramp)<br>10-30 min. % 80 B<br>30-31 min. % 80 - % 10 B<br>31-41 min. % 10 B | - | main ion 335 m/z | fragment 317 m/z | 5.6 |
| Kaplan et al., 2014 | Mad honey | API 4000 Q-TRAP (Applied Biosystems) | (A) % 0.1 acetic acid in water<br>(B) %0.1 acetic acid in MeOH 0-<br>10 min. % 10 -%80 B<br>10-20 min. % 80 B | ESI (positive) Selected Reaction Monitoring (SRM) | main ion 335 | fragments 316<br>299<br>91 | 6.7 |
| Sahin et al, 2015 | Mad honey | Thermo Scientific, TSQ Quantum Access Max, | (A) % 1 acetic acid in water<br>(B) % 1 acetic acid in<br>A/B (50:50) (isocratic cond.) | ESI (negative mode) - Selected Reaction Monitoring (SRM) | M-H 359 | 315<br>297<br>279 | 4.0 |
| These et al., 2015 | Mad honey | Thermo Fisher Scientific, TSQ Vantage, (HRMS Orbitrap Exactive ) | (A) % 0.2 formic acid in water<br>(B) % 0.2 formic acid in<br>ACN/WATER (95/5)<br>0-1 min. % 90 A - % 10 B<br>12 min. % 40 A - % 60 B<br>15 min. % 40 A - % 60 B<br>16 min. % 90 A - % 10 B<br>25 min. % 90 A - % 10 B | ESI (positive) | (M+Na)+ 393.2<br>M-H2O 353.1<br>(MH-2H2O)+ 335.1<br>317.0<br>299.0<br>281.0<br>253.3<br>214.1 | Main Ion (M-1H2O) 353<br>M-2H2O 335<br>M-3H2O 317<br>M-4H2O 299<br>M-5H2O 281<br>271<br>M-6H2O 263<br>241 | 4.5 |
| Present study | Rhododendron Ponticum leaves | Thermo Scientific, TSQ Quantum Access Max | (A) Water<br>(B) Methanol<br>Start : % 60 A-%40 B<br>0 min. % 66 A - % 34 B<br>11 min. % 47 A - % 53 B<br>19 min. % 30 A - % 70 B<br>22-33 min. % 0 A - % 100 B<br>35 min. % 60 A - % 40 B | ESI (positive) - Direct injection | (MH+Na)+ 394,89<br>(M) 370,02<br>(MH-CH3) 356,33<br>(M-OH) 352,84<br>(M-OH-CH3) 338,92<br>(M-2OH-CH3) 321,62 | - | 7.5 |

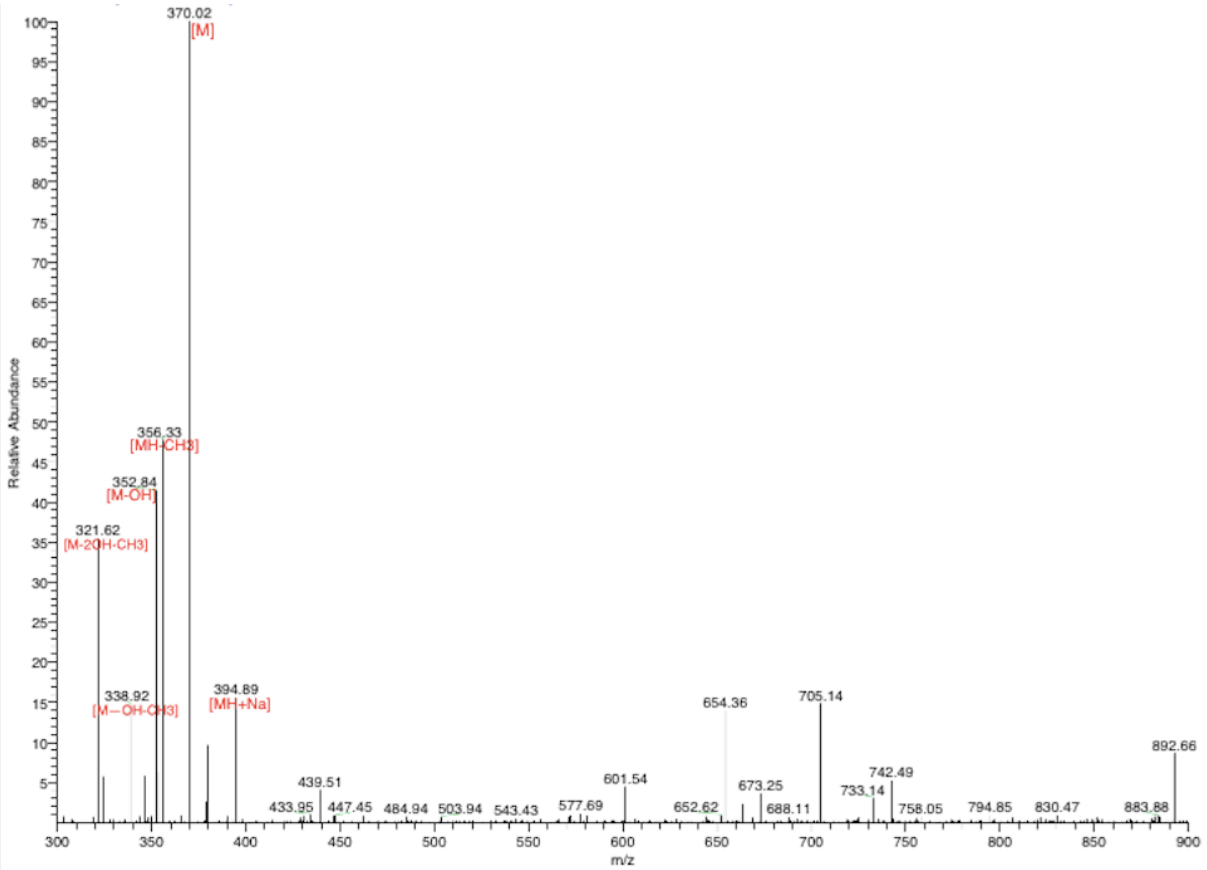


Figure 6. LC-MS spectra of the leaf extract.

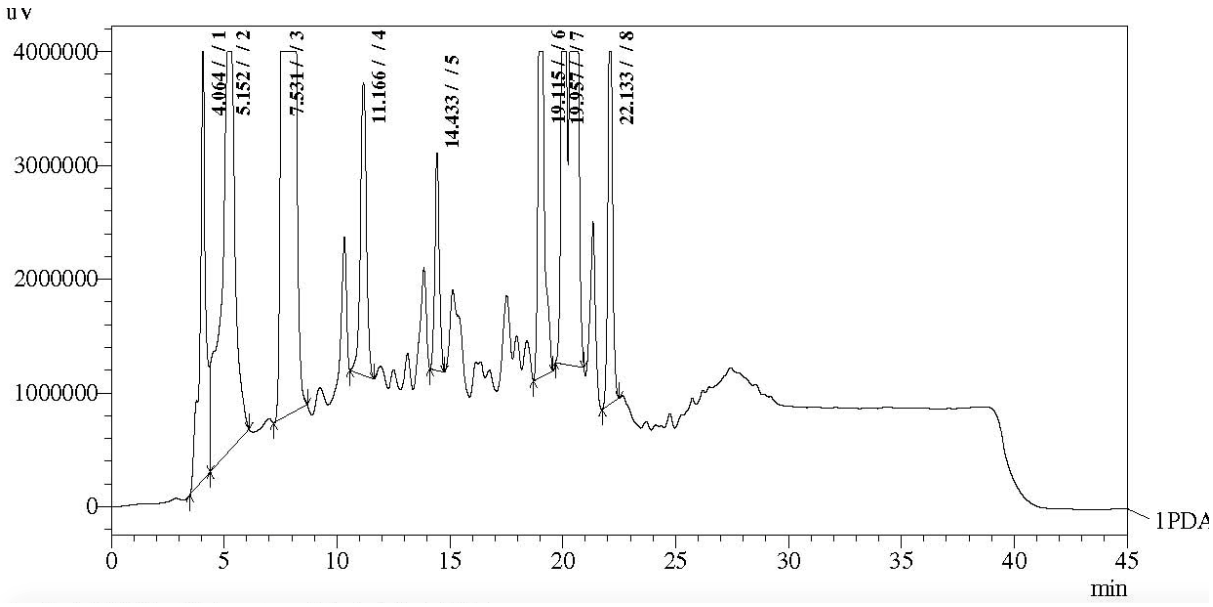


Figure 7. HPLC analysis of leaf extract, 204 nm.

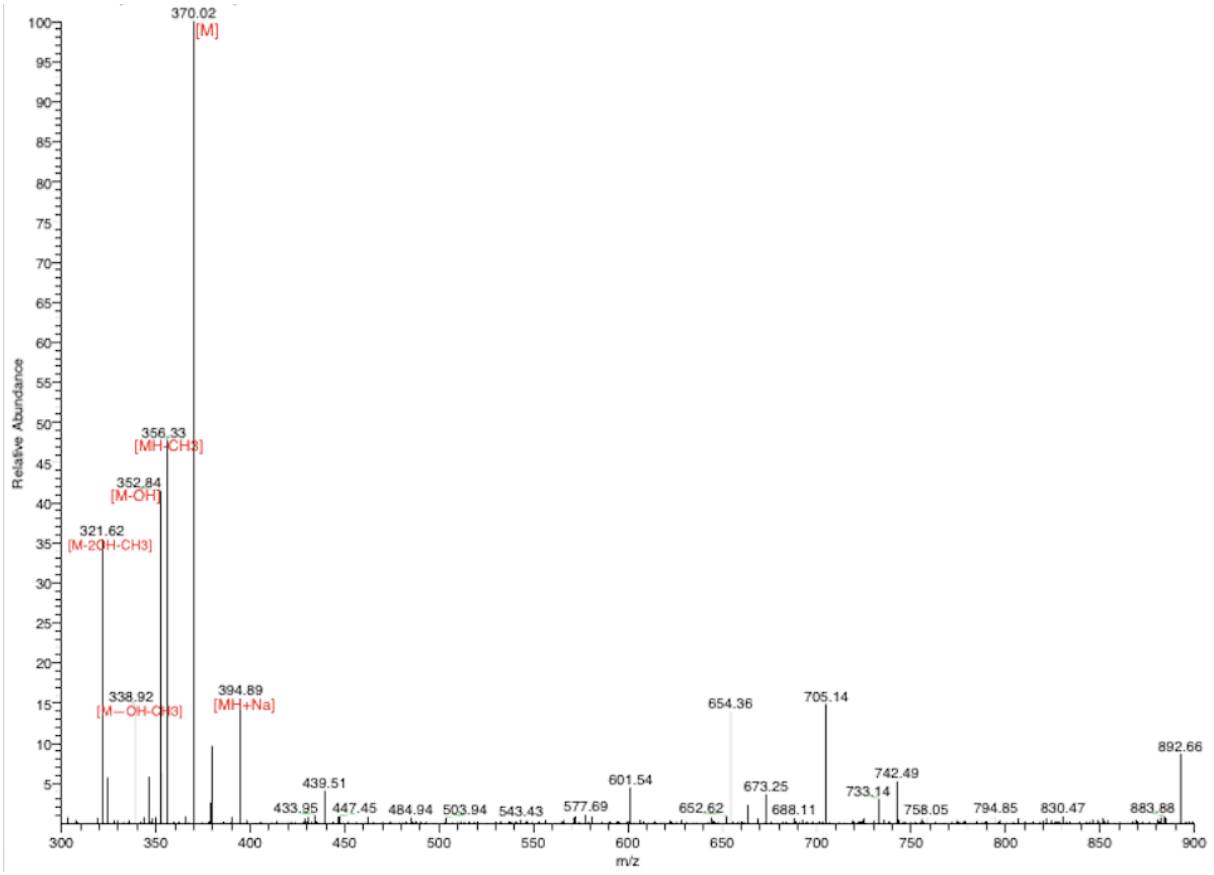


Figure 8. LC-MS analysis of peak 3 (Fig. 2)

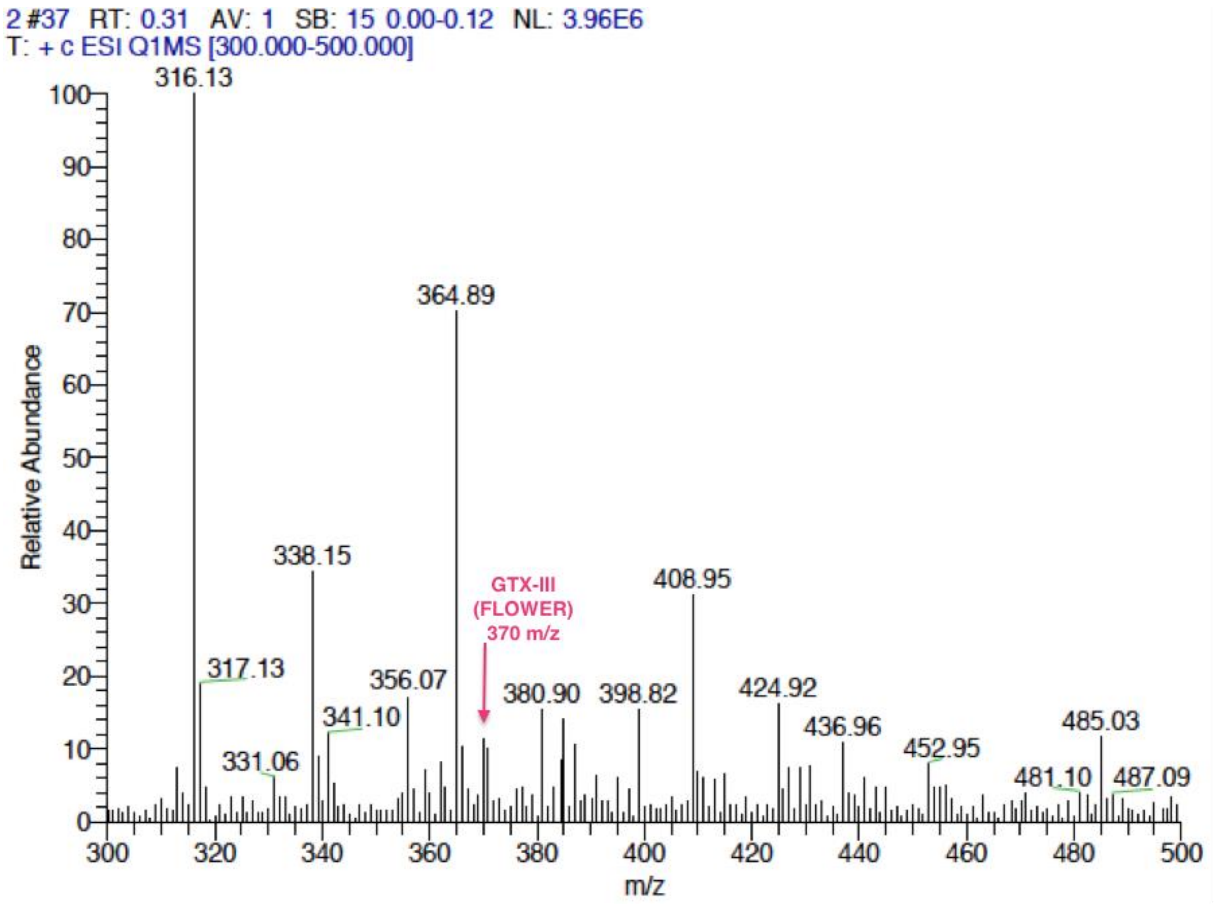


Figure 9. LC-MS spectra of flower extract.

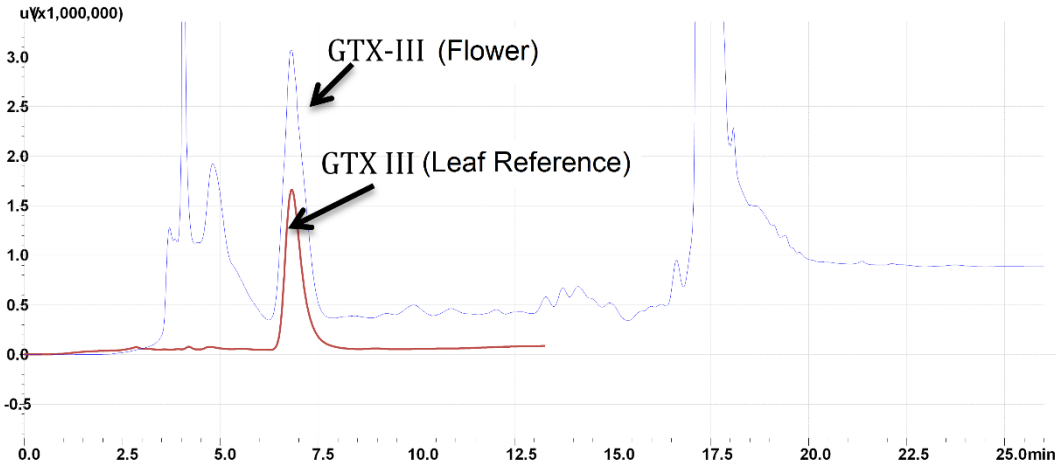


Figure 5. HPLC analysis of mad honey extract

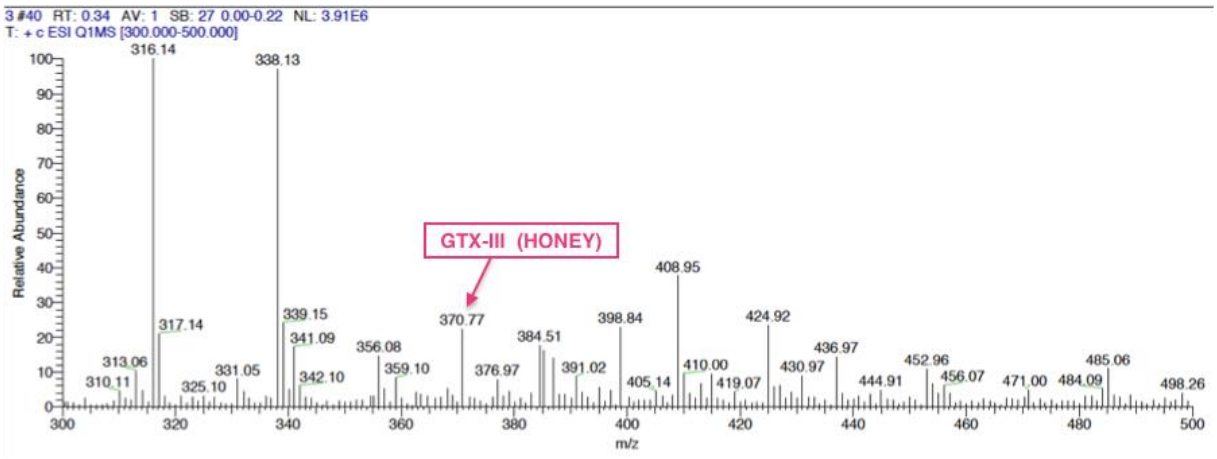


Figure 6. HPLC analysis of flower extract and isolated peak 3

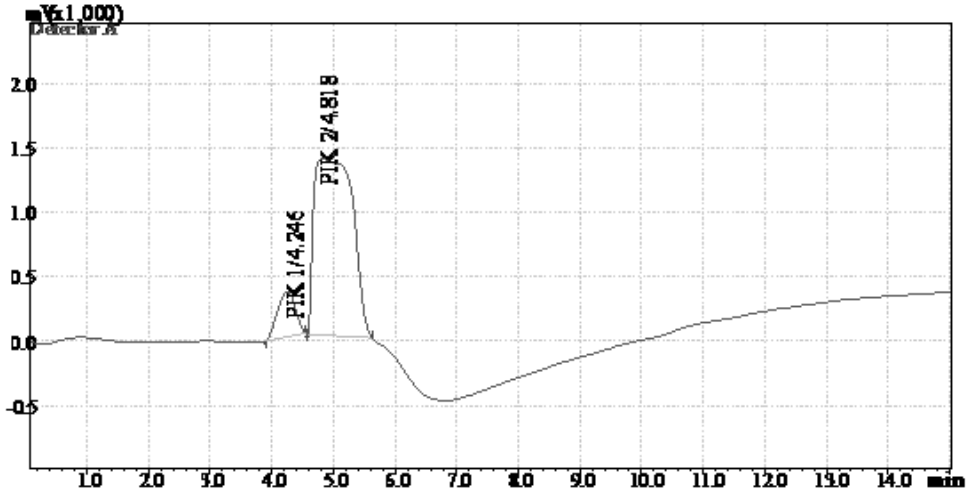


Figure 10. LC-MS spectra of flower extract.

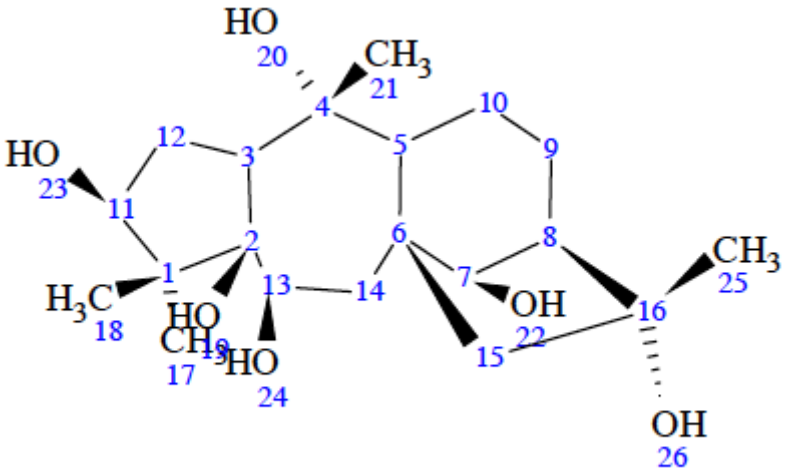


Figure 8. Chemical structure of GTX-III (ACD software)