University of Szeged

Graduate School of Pharmaceutical Sciences

Ph.D programme:	Pharmacodynamics, Biopharmacy and Clinical Pharmacy
Programme director:	István Zupkó Ph.D.
Institute:	Department of Pharmacodynamics and Biopharmacy
Supervisor:	Róbert Gáspár Ph.D.

Adrienn Seres Pharm.D.

SEXUAL HORMONE EFFECTS OF HONEYBEE *(APIS MELLIFERA)* DRONE MILK IN MALE AND FEMALE RATS

Final Exam Committee:

Chairmen:	Prof. György Falkay DSc
Members:	Imre Földesi Ph.D. János Zádori Ph.D.

Ph.D. Committee:

Chairmen:	Prof. Piroska Révész DSc
Official Reviewers:	Prof. Éva Szökő DSc Pál Perjési Ph.D.
Committee Members:	István Baczkó Ph.D. Gerda Szakonyi Ph.D.

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Introduction

Numerous honeybee *(Apis mellifera)* products, such honey, propolis and bee venom, have been used in traditional medicine to prevent or treat illness and promote healing. The effects of all of these honeybee products were utilized first in folk medicine and were later proved by evidence-based medicine.

Nowadays, one of the most intensively studied beehive products is the royal jelly (RJ). RJ is the essential food for the queen bee pupa and the queen and it is secreted by the hypopharyngeal and mandibular glands of worker honeybees. It is known, that the fertility of honeybees is mainly driven by the quality and quantity of their food, accordingly when bee larvae are nourished with RJ, they differentiate into queens. The adult queens also consume a high amount of RJ, which contributes to the maintenance of their fertility. These facts and the traditional usage of RJ in the treatment of menopausal symptoms suggested that RJ may possibly have some estrogen-like effect. In fact, *in vitro* and *in vivo* estrogenic effects of raw RJ have been described and its isolated effective compounds (10-hydroxy-*trans*-2-decenoic acid and its derivatives) exhibit weak estrogen receptor (ER) binding affinities.

Drone milk (DM) is a relatively little-known honeybee product that is secreted (similarly to RJ) by the hypopharyngeal and mandibular glands of the worker honeybees. DM is main component of drone brood which also contains larvae and pupae of drones in the comb. DM is a light yellow, slightly sweet, thick liquid which is separated from drone brood by extraction to eliminate larvae and pupae during the harvest. DM is an essential food of the drone larvae and drone honeybees, and its consumption is presumed to be related to the fertility of drones. Although our knowledge of the effects of DM is very limited, a drone brood preparation is traditionally used in Romania for the rehabilitation and activation of aged people and to treat neurovegetative and sexual problems. Data have been reported on the hormone-like strengthening effects of the drone larvae and brood in Eastern European and Asian folk medicine and both androgenic and anabolic effects of Apilarnil (drone bee larvae) on male broilers have been described. The folk medicine experience and the previously described data suggest the putative hormone-like activity of DM.

Aims

The purpose of this work was to investigate the sexual hormone-like effects of DM on rats and to confirm the folk medicine experience. Therefore the following aims were set:

1. The primary aim of this work was to investigate the androgenic effect of raw DM in castrated rats using Hershberger assay. To verifying of the *in vivo* results we wanted to examine the prostatic mRNA and protein expression of the androgen-dependent Spot14-like androgen-inducible protein (SLAP) with real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot techniques. After the androgenic effect of crude DM was proven, we wanted to identify the compounds responsible for its hormonal effect, therefore gas chromatography–high-resolution mass spectrometry (GC-HRMS) and nuclear magnetic resonance spectroscopy (NMR) investigations were performed to identify the active component(s) gained by bioactivity-guided fractionation.

2. We set to determine the estrogenic effect of crude DM in female rats using uterotrophic assay. After the *in vivo* results, we wanted to test the expression of estrogen-dependent Complement component 3 (C3) in the rats uteri with real-time RT-PCR and Western blot techniques. Finally, similarly to the androgenic effect of DM, we wanted to identify the effective compound(s) using bioactivity-guided fractionation, high-resolution electrospray ionization–mass spectrometry (HR-ESI-MS) and NMR.

3. Our third aim was to investigate the gestagenic effect of DM with pregnancy maintenance assay. Real-time RT-PCR and Western blot techniques were used to determine the expression of the gestagen-sensitive Calcitonin receptor-like receptor (CRLR) in rats uteri.

Materials and methods

Animals

Animal investigations were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission numbers: IV/01758-0/2008 and IV./198/2013.). The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII. tv. 32.§). For all experiments, Sprague-Dawley rats were housed under controlled conditions and were maintained on tap water and rodent pellet diet *ad libitum*.

Collection of drone milk

DM was prepared during rape blooming and harvested by the separation from drone larvae and pupae in the late spring. DM is extracted from the comb using centrifugal force. The raw liquid material was stored at -20 °C until the beginning of the investigation and it was diluted with distilled water in all animal experiments.

Investigation of *in vivo* hormonal effects

Hershberger assay

Androgenic activity was investigated by a simplified Hershberger assay. Mature male Sprague-Dawley rats (230-240 g) were castrated. After 10 days, healthy rats were randomized and assigned to groups. Body weights were recorded on days 1, 4, 7 and 10 of the study. The animals were treated with the investigated compounds once daily for 10 consecutive days. One day after the final treatment, the rats were sacrificed. Androgen-dependent organs (the glans penis, seminal vesicle, ventral prostate and levator ani muscle) were removed and weighed. Organ weights were expressed as relative weights (organ weight mg/100 g body weight). Before the exsanguinations, 1 mL of blood was taken from each of 5 animals in each group by cardiac puncture. Plasma samples were stored at -70 °C until assay. Plasma testosterone levels were determined with a testosterone EIA kit. Absorbancies were measured with a SPECTROstar Nano microplate reader.

Uterotrophic assay

To determine the estrogenic activity of DM, immature female Sprague-Dawley rats (20−21 days old) were randomly assigned to groups and were treated once daily for 4 consecutive days with the test compounds. One day after the final treatment, the rats were sacrificed, their

uteri were removed and weighed. Uterus weights were expressed as relative weights (organ weight mg/100 g body weight).

Pregnancy maintenance assay

To investigate the gestagenic activity of DM, mature female (160-180 g) and male (220-240 g) Sprague-Dawley rats were mated in a special mating cage. The presence of pregnancy was determined by the assessment of sperm in the vaginal smear (first day of pregnancy). On day 8 of the pregnancy, the animals were ovariectomized and the numbers of fetuses (implantation sites) were registered. The ovariectomized females were randomly assigned to 3 groups and were treated with the test compounds once daily from day 8 to day 14. On day 15 of pregnancy, the number of surviving fetuses was recorded and was given as a ratio of all fetuses.

Determination of hormone-dependent proteins by RT-PCR and Western blot analyses

To determine the sexual hormonal activity of DM, mRNA and protein expression of hormone-dependent proteins were investigated: the androgen-dependent Slap, the estrogen-dependent C3 and the gestagen-dependent CRLR. Ventral prostate and uterus tissues were isolated from castrated/ovarietomized Sprague-Dawley rats after the treatment with the investigated compounds.

For the RT-PCR measurements, total RNAs from tissues were extracted by using a TRIsure Kit. 1 μg of total RNA and the TaqMan RNA-to-CT 1-Step Kit or High-capacity RNA-to-cDNA Kit were used for reverse transcription and amplification. RT-PCR was performed with an ABI StepOne Real-Time cycler. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant increase in the fluorescence signal was defined as the threshold cycle (CT).

For the Western blot analyis, the tissues were homogenized in RIPA Lysis Buffer. 50 μg (from prostate tissues) and 20 μg (from uterus tissues) of protein per well was subjected to electrophoresis on 4−12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units. Proteins were transferred from gels to nitrocellulose membranes by using the iBlot Gel Transfer System. Antibody binding was detected with the WesternBreeze Chromogenic Western Blot Immundetection Kit. The optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. Optical densities were calculated as arbitrary units after local area background subtraction.

Bioactivity-guided fractionation

The crude DM was diluted with water, extracted with petroleum ether and re-extracted with water. The combined aqueous phase was applied to an octadecyl-silica column and subjected to low-pressure reversed-phase column chromatography (RPCC). Gradient elution was performed with water, aqueous methanol (MeOH), 100% MeOH and dichloromethane. The androgenic activity of the fractions was controlled by real-time RT-PCR or the fractions were examined by uterotrophic assay to determine the estrogenic activity. The active fractions were further fractionated through repeated low-pressure RPCC. Elution was carried out with different mixtures of aqueous MeOH or acetone, using stepwise gradient elution. The whole separation procedure was controlled by RP TLC and the separation was detected by the use of a vanillin-sulfuric acid spray reagent.

HRMS and NMR spectroscopy

To identify the androgenic compound(s) of raw DM, GC-HRMS analyses were performed on a Waters GCT-Premier mass spectrometer. The data acquisition software used was MassLynx V.4.1. To identify the estrogenic compound(s) of crude DM, HR-ESI-MS were performed on an LTQ FT Ultra spectrometer. Data acquisition and analysis for HRMS were performed with Xcalibur software version 2.0.

NMR spectra were recorded in MeOH-*d4* at 298 K with a Varian 800 MHz NMR spectrometer equipped with a 5 mm $^{1}H(^{13}C/^{15}N)$ Triple Resonance ^{13}C Enhanced Salt Tolerant Cold Probe, operating at 800 and 201 MHz for ^{1}H and ^{13}C nuclei, respectively. Pulse sequences in all experiments [^{1}H, ^{1}H-Presat, GHSQCAD, GHMBCAD, and one- and two-dimensional zTOCSY] were taken from the VNMRJ-3.2 software library without any modification.

Statistical analysis

All statistical analyses for biological determinations were performed with the Prism 4.0 software. All data were analyzed by one-way ANOVA followed by the Newman-Keuls and Dunnett's test and each value is reported as a mean ± SEM. Significance was accepted at $p<0.05$.

Results

Androgenic effect of drone milk

In vivo androgenic effect of crude drone milk

There was no significant difference between the increases in body weight of the crude DM-treated and non-treated (control) groups. The raw DM increased the relative organ weights of the glans penis, seminal vesicle and levator ani muscle in rats. It also increased the average prostate weight, though this change was not statistically significant. Testosterone (T) enhanced all the organ weights, and was more effective than the DM. The organ weights were not changed by flutamide (F) treatment, but flutamide inhibited the organ weight-increasing effect of the DM and blocked the effects of testosterone ***(Fig. 1)***.

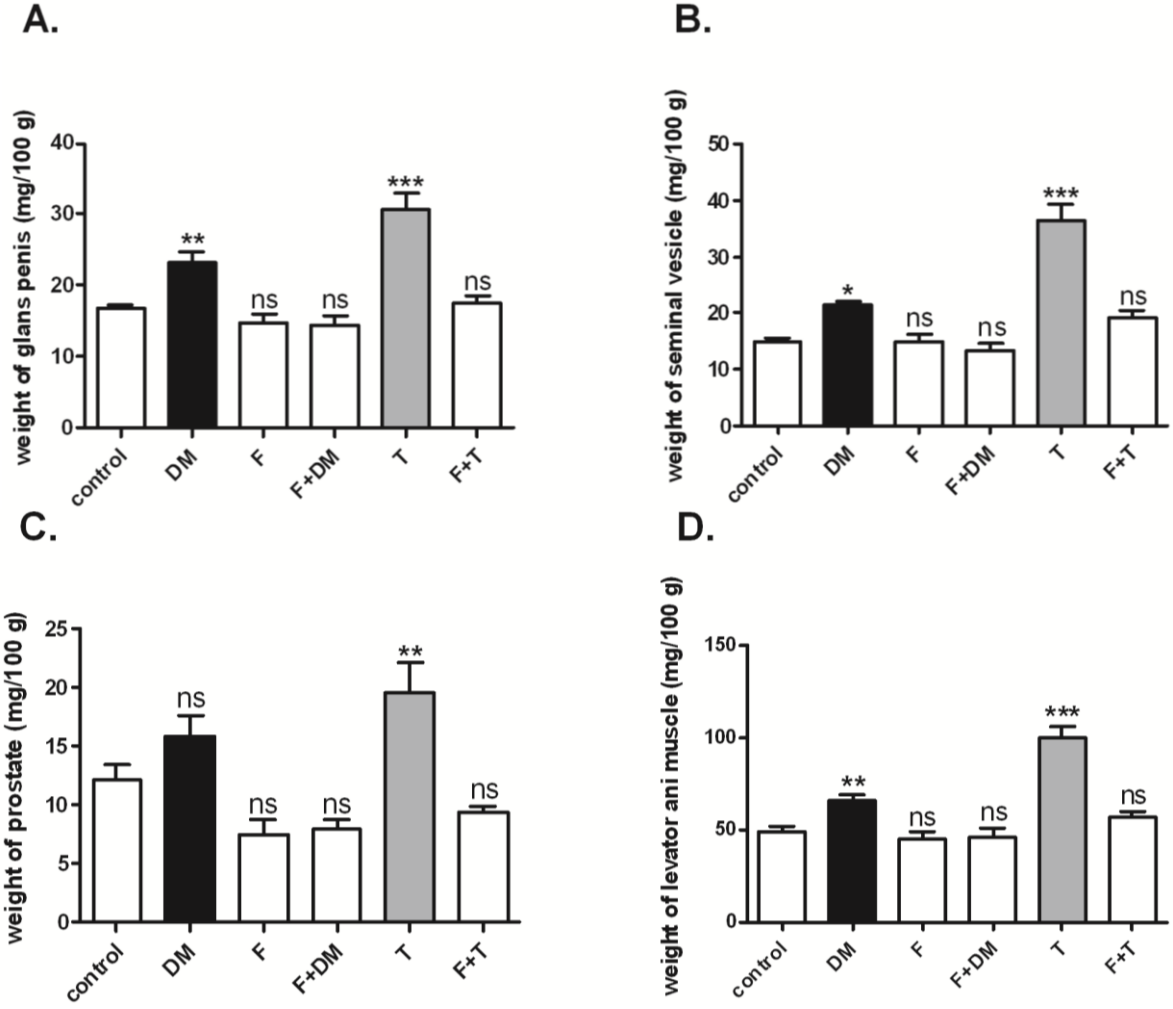

Figure 1. Changes in weight of androgen-sensitive organs following treatment with crude drone milk (DM) and testosterone (T) in castrated male rats. The drone milk and the positive control testosterone increased the relative weights (mg/100 g body weight) of the glans penis **(A)**, seminal vesicle **(B)** and levator ani muscle **(D)**. These effects were flutamide (F)-sensitive in all cases. The raw drone milk, in contrast with testosterone, did not exhibit a significant weight-increasing effect on the prostate **(C)**. *p<0.05; ** p<0.01; *** p<0.001; ns: not significant

Both the raw DM and the testosterone treatment increased the plasma testosterone level. Flutamide treatment blocked the plasma testosterone level-increasing effects of both compounds. After a single administration of flutamide, the hormone level remained unchanged.

Measurements of prostatic Slap mRNA and protein

The crude DM increased the expression of Slap mRNA in ventral prostate, but this effect was significantly lower than that of testosterone. In Western blot measurements, DM was found to increase the level of SLAP in the ventral prostate tissue ***(Fig. 2)***.

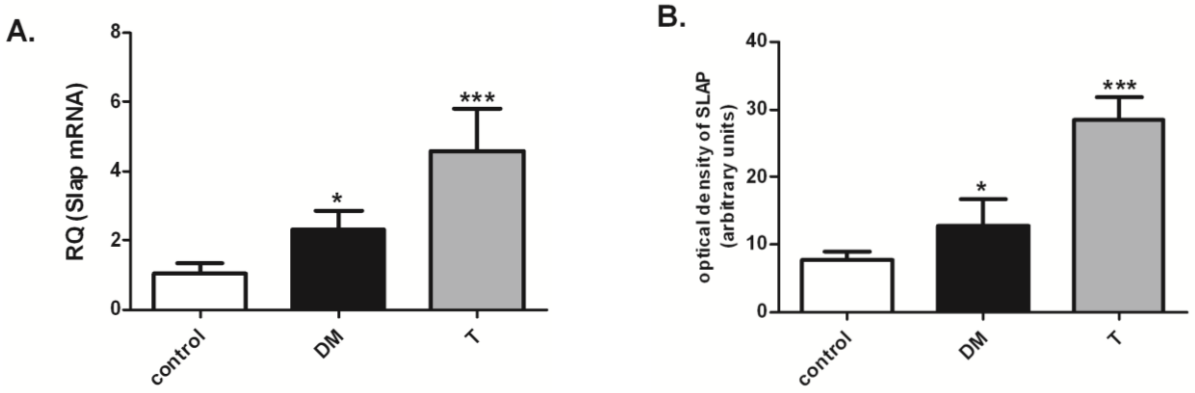

Figure 2. RT-PCR (A) and Western blot (B) analysis of Spot14-like androgen-inducible protein (SLAP) in rat prostate. The crude drone milk (DM) increased the mRNA expression of the androgen-dependent Slap in rat prostate tissue **(A)**. Western blot analysis **(B)** revealed the SLAP protein expression-increasing effect of DM in the prostate tissue. *p<0.05; *** p<0.001; RQ: relative quantity

Bioactivity-guided fractionation

After clarification of the androgen-like effect of raw DM, we launched bioactivity-guided fractionations to find the effective ingredients of this natural product. In the first RPCC separation, two of the four fractions gained (I/B and I/C) increased the mRNA level of Slap. The difference between the effects of the two fractions was non-significant. Further fractionation was performed on fractions I/B and I/C, but the further separation of fraction I/B resulted in a total loss of the androgenic effect (results not shown). In the second RPCC for fraction I/C, the ratio of load to sorbent was high (1:20) and the elution gradient steps were smaller. After each column chromatography step, the fractions with the same compositions were combined. One of the six fractions gained (II/E) increased the mRNA level of Slap ***(Fig. 3.)***.

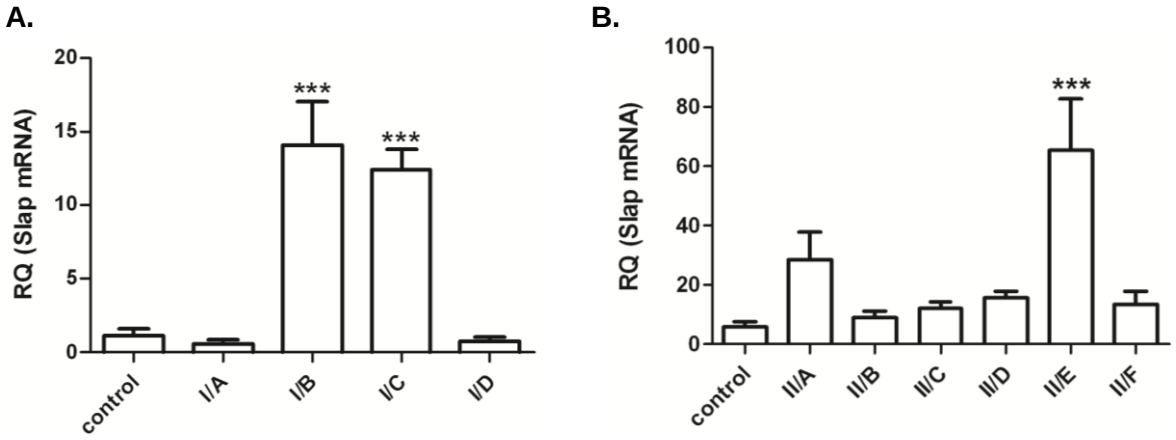

Figure 3. Bioactivity-guided fractionation. At the first step, the 40%, 60% and 80% aqueous MeOH (I/B) and 100% MeOH (I/C) fractions increased the relative quantity of Slap in the RT-PCR assay**(A**). Further separation of fraction I/B resulted in loss of the effect. From among the 4 fractions of 75% aqueous MeOH, the last fraction (II/E) increased the relative quantity of Slap in the RT-PCR assay **(B)**.

I/A: 20% aqueous MeOH fraction; **I/B:** 40%, 60% and 80% aqueous MeOH fraction; **I/C:** 100% MeOH fraction; **I/D:** dichloromethane fraction; **II/A:** 70% aqueous acetone fraction; **II/B, II/C, II/D** and **II/E:** 75% aqueous acetone fractions; **II/F:** 100% aqueous acetone fraction; *** $p<0.001$; RQ: relative quantity

Identification of the androgenic compound(s) of raw drone milk

In order to characterize the biologically active components fraction II/E was subjected to GC-HRMS and NMR spectroscopic investigation. The GC chromatogram indicated the presence of two main components in the sample (RT 9.03 and 9.73 min). The accurate mass values determined for these components were 270.0258 and 296.2707, corresponding to the elemental compositions of $C_{17}H_{34}O_2$ (methyl palmitate; MP) and $C_{19}H_{36}O_2$ (methyl oleate; MO), respectively. A database and literature search suggested that the two main components were the well-known fatty acid methyl esters, MP and MO. These structural suggestions were confirmed by comparing the ^{1}H and ^{13}C NMR data with those available in the literature, and by comparing the GC-HRMS chromatograms and spectra of the collected sample with those measured on the purchased compounds. These results unambiguously identified the two components present in the biologically active fraction II/E as MP and MO ***(Fig. 4)***.

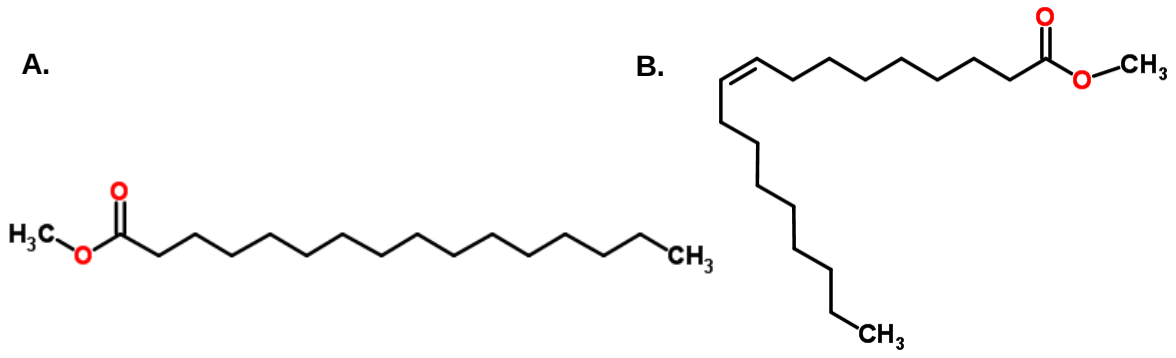

Figure 4. Structure of methyl palmitate (A) and methyl oleate (B) (ChemSpider database)

We investigated their contributions to the androgenic effect with our simplified Hershberger assay and plasma testosterone assay. MO, MP and their specific combination (molar ratio: 4:3=MO:MP) were administered in doses of 2.5, 25 and 250 μg/kg. The 25 μg/kg dose of MP and the highest dose (250 μg/kg) of the MO and MP combination increased the weights of androgen-sensitive organs (except the prostate tissue). These androgenic effects, similarly to those of raw DM, were flutamide-sensitive ***(Fig. 5)***.

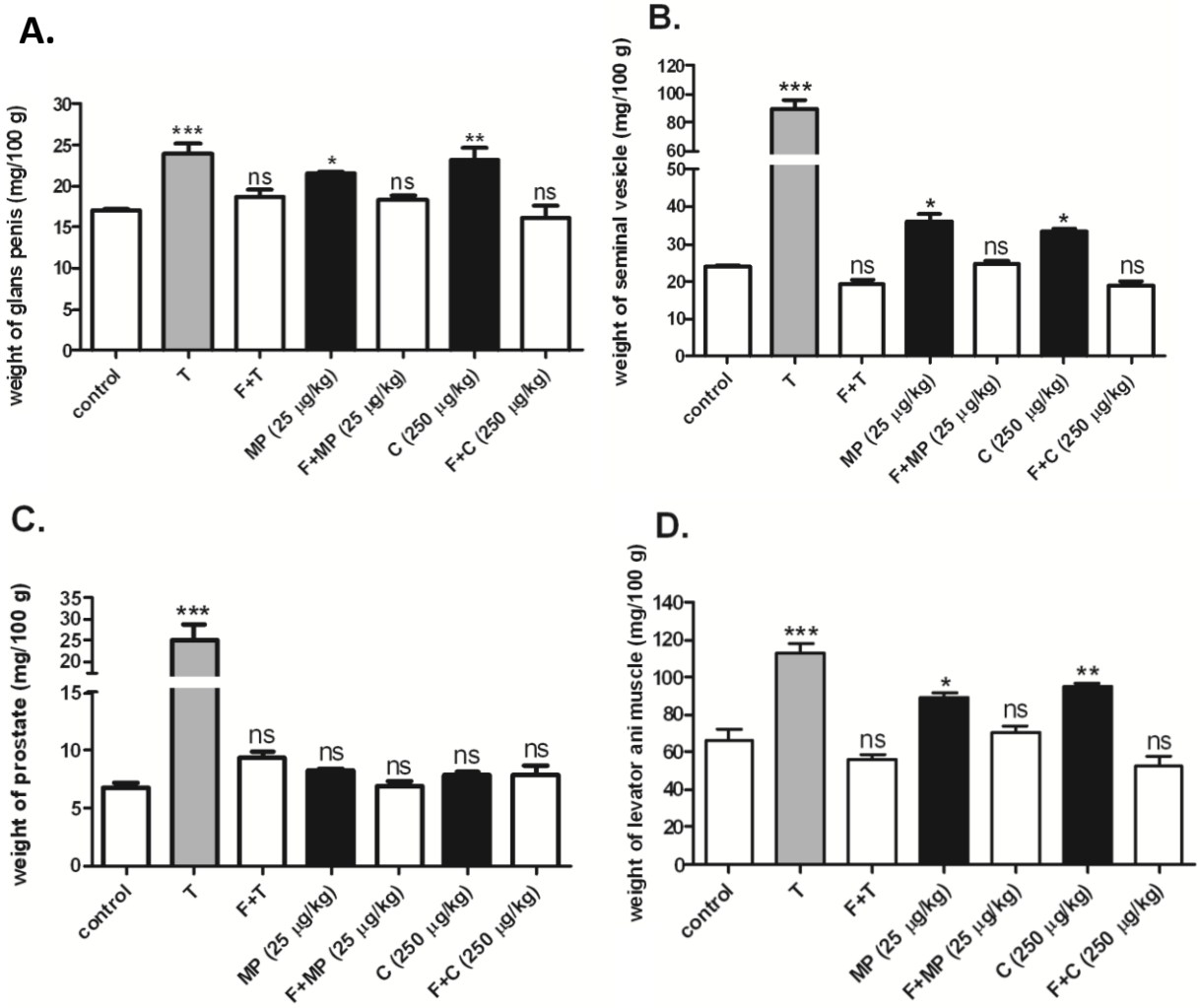

Figure 5. Changes in weights of androgen-sensitive organs following treatment with a 25 μg/kg (middle) dose of methyl palmitate (MP) and a 250 μg/kg (high) dose of the combination of methyl oleate and methyl palmitate (C) in castrated male rats. These doses increased the relative weights (mg/100 g body weight) of the glans penis (**A**), seminal vesicle (**B**) and levator ani muscle (**D**). These effects were flutamide (F)-sensitive. Similarly to the crude drone milk, the compounds had no effect on the prostate (**C**). Testosterone (T) was used as positive control. *p<0.05; ** p<0.01; *** p<0.001; ns: not significant

The plasma testosterone level was not affected by either MO or MP in any of the applied doses. In contrast, when the combination was applied in the highest dose, an increase in plasma testosterone level was detected.

Estrogenic effect of drone milk

In vivo estrogenic effect of crude drone milk

E_2 increased the relative weight of the uteri and this activity was diminished by the antiestrogenic ICI 182.780 (ICI). The raw DM was able to increase the relative weight of uteri and similarly to that of E_2 the estrogenic effect of DM was blocked in the presence of ICI. The soy extract also increased the weight of the uteri ***(Fig. 6)***.

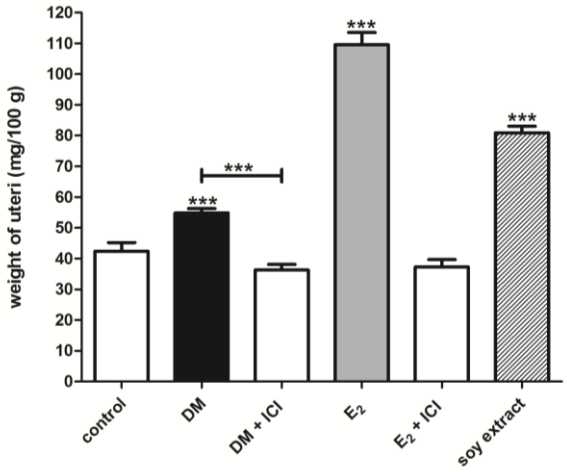

Figure 6. Changes in relative weight of the uteri in immature rats following treatment with crude drone milk (DM), 17β-estradiol valerate (E_2) or soy extract. DM, E_2 and the soy extract increased the weight of the uteri. These effects were ICI 182.780 (ICI)-sensitive. *** $p<0.001$; ns: not significant

Measurements of C3 mRNA and protein in rat uterus

By means of a real-time PCR technique, we found that DM almost doubled the relative C3 mRNA expression in the uteri. The effect of positive control E_2 was also significant. In Western blot measurement DM enhanced the level of C3 in the uterine tissue, and E_2 was also effective ***(Fig. 7)***.

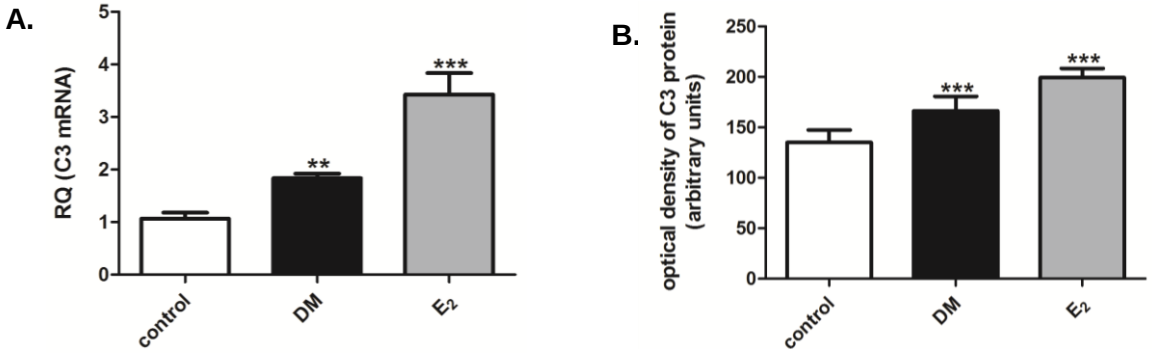

Figure 7. RT-PCR analysis and Western blot analysis of complement component C3 (C3) mRNA and protein expression in rat uteri. The crude drone milk (DM) increased the mRNA expression of the estrogen-dependent C3 in rat uterus tissue **(A)**. The crude drone milk (DM) increased the expression of the estrogen-dependent C3 protein in rat uterus tissue **(B)**. ** $p<0.01$; *** $p<0.001$

Bioactivity-guided fractionation

After the first separation, the fractions with the same compositions were combined and tested by uterotrophic assay. Further separations were done with fraction I/C, but new fractions were ineffective, therefore we did not continue the investigation of this product. On the basis of the results, fractions I/A and I/B were chosen for further separation. As the main constituent of the more active fraction (I/A) was also present in I/B, although the material content of the latter was much lower, these two fractions were combined and purified together by column cromatography (CC). The second CC separation, with the stationary phase in a high (e.g. 20-fold) excess, and elution of the constituents with a multistep gradient, proved to permit rather effective purification. ***(Fig. 8)***.

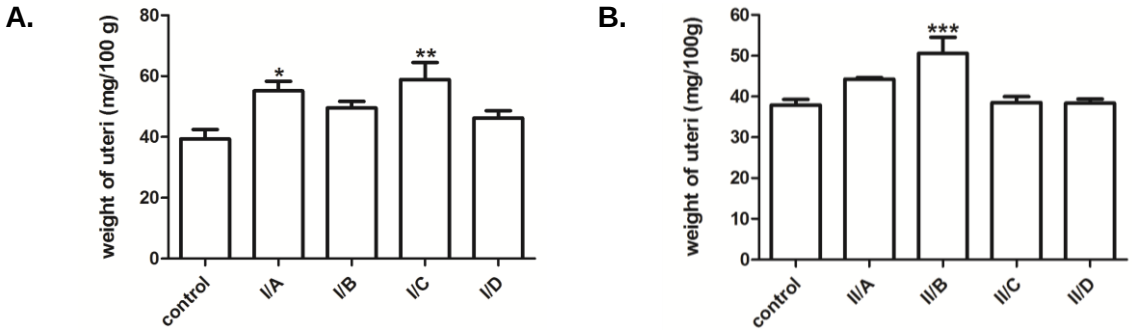

Figure 8. Bioactivity-guided fractionation. I/A and I/C fractions increased the relative weight of the uteri **(A)**. At the second step, only fraction II/B increased the relative organ weight of the uteri **(B)**.

I/A: 20% aqueous MeOH fraction; **I/B:** 40%, 60% and 80% aqueous MeOH fraction; **I/C:** 100% MeOH fraction; **I/D:** dichloromethane fraction; **II/A:** 20% aqueous MeOH fraction; **II/B:** 35% aqueous MeOH fraction; **II/C:** 70% aqueous MeOH fraction; **II/D:** 75% aqueous MeOH fraction; * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Identification of the estrogenic compound(s) of raw drone milk

The main components of fraction II/B could be identified by HR-ESI-MS and NMR data. Analysis of the ^{1}H NMR spectrum indicated the presence of a *trans* double bond, as evidenced by two doublets of triplets at 5.80 (H-2, J=15.5 and 1.5 Hz) and 6.92 ppm (H-3, J=15.5 and 6.9 Hz). The correlations of the methylene and olefinic protons observed in the one- and two-dimensional ZTOCSY spectra permitted the complete ^{1}H NMR assignment of the identified compound. The HSQC and HMBC spectra suggested the presence of two carboxylic groups, bound to C-2 and C-9. Structure of this compound, the E-dec-2-enedioic acid accords with the determined elemental composition of the main component ***(Fig. 9)***.

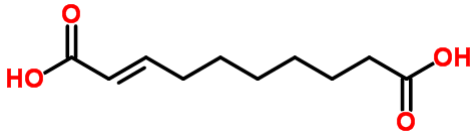

Figure 9. Structure and carbon atom numbering of E-dec-2-enedioic acid (ChemSpider database).

Gestagenic effect of drone milk

In vivo gestagenic effect of crude drone milk

The gestagenic activity of crude DM was investigated by means of pregnancy maintenance assays. The E_2 treatment (as negative control) led to the complete loss of pregnancy in ovariectomized pregnant rats, but the combination of E_2 and DM resulted in the survival of 17.4% of the fetuses. Although the number of surviving fetuses was increased, this effect was not statistically significant. The combination of E_2 and P (as positive control) displayed a well-defined pregnancy-maintaining effect, with a fetus survival rate of 98.8% ***(Fig. 10)***.

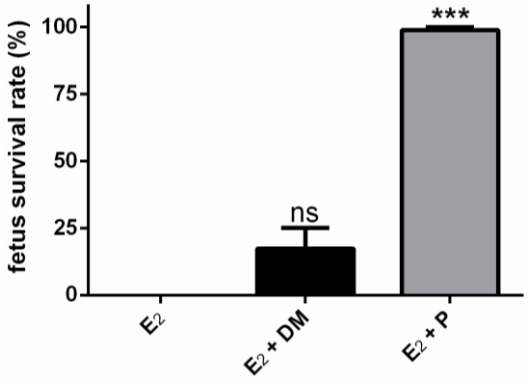

Figure 10. Changes in fetus survival following treatment with 17β-estradiol valerate (E_2), with a combination of E_2 and drone milk (DM), or with a combination of E_2 and progesterone (P). The DM treatment kept 17.4% of the fetuses alive. *** p<0.001; ns: not significant

Measurements of CRLR mRNA and protein in rat uterus

The crude DM increased the expression of CRLR mRNA in rat uterus, similarly to progesterone. In Western blot measurements we got similar results, DM treatment increased the level of CRLR protein. ***(Fig. 11)***.

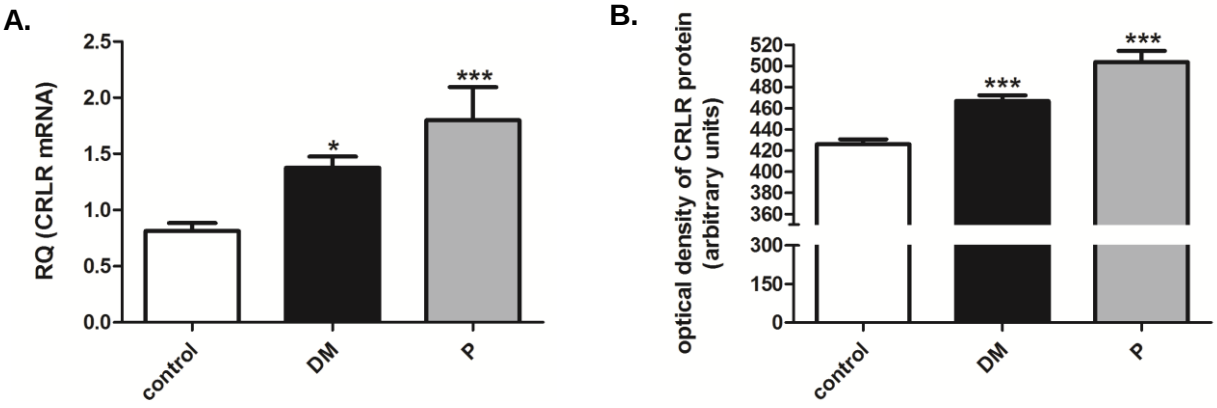

Figure 11. RT-PCR and Western blot analysis of CRLR mRNA and protein. Raw drone milk (DM) increased the mRNA **(A)** and protein **(B)** expression of the gestagen-dependent CRLR in rat uterus tissue. *p<0.05; *** p<0.001; RQ: relative quantity

Measurement of the gestagenic efficacy of drone milk

In our next experiment, we made use of the weak gestagenic compound spironolactone (SP). Spironolactone was used alone and in combination with DM. Spironolactone alone did not increase the expression of CRLR mRNA, but its combination with DM caused a marked increase in CRLR mRNA. The combination of DM and spironolactone was able to keep the 21.4% of the fetuses alive, and - unlike the action of DM alone - this effect was already significant ***(Fig. 12)***.

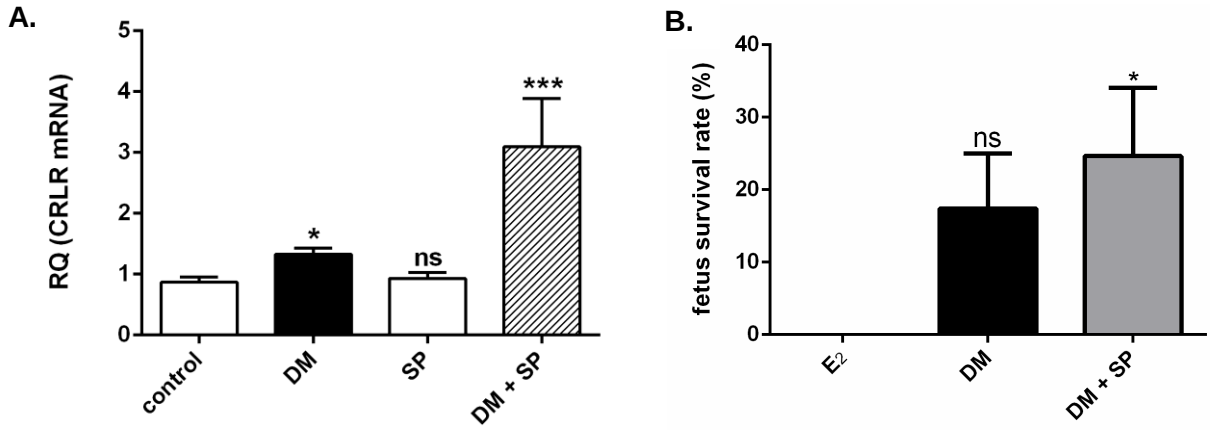

Figure 12. Effects of spironolactone (SP) and its combination with crude drone milk (DM) on Calcitonin receptor-like receptor (CRLR) mRNA expression and on fetal survival rate. Spironolactone itself did not show any changes in CRLR mRNA expression, but the combination of spironolactone and DM was more effective than DM itself **(A)**. The combination kept 21.4% of the fetuses alive **(B)**.*p<0.05; *** p<0.001; ns: not significant; RQ: relative quantity

Discussion and conclusion

DM is a lesser-known honeybee product; it is a purified extract of drone brood without larvae and pupae and the main food of drone bees and larvae. DM and frozen drone larvae are used in folk medicine and are available as a dietary supplement and an aphrodisiac (Frozen drone larvae royal jelly®, Apidom Ltd, Russian Federation; Apilarnil Potent®, S.C. Biofarm S.A., Bucuresti, Romania). Since the folk medicine uses drone brood to treat sexual problems in both genders, we hypothesized that DM may have some hormonal effects, too. Therefore we searched for both male and female hormonal effect and the active components of DM.

We found that the hitherto unknown DM exhibits significant sexual hormonal effects both in male and female rats. DM has remarkable androgenic activity by its action to increase weight of the androgen-sensitive organs, except the prostate, in castrated rats. We set out to prove the androgenic effect of DM at a molecular level, targeting mainly the androgen-sensitive prostatic proteins in castrated rats. Both the mRNA and protein expression of SLAP proved to be increased in the rat prostate. After a bioactivity-guided fractionation, we identified the compounds with NMR and MS measurements, MP and MO, which are responsible for its androgenic effect. The exact mechanism of action of MP and MO is still unknown, but according to the scientific literature, they are supposedly involved in steroidogenesis. The fact that crude DM or MP or/and MO treatments are able to increase the weights of androgen-sensitive organs without exerting an accompanying anabolic effect may project a new, natural mode for the therapy of fertility problems in men.

DM clearly shows estrogenic effect in female rats, because the crude DM was able to increase the relative organ weights of uteri in immature female rats. The changes in the uterine level of C3 protein are other evidences for the estrogenic effect of raw DM. E-dec-2-enedioic acid, isolated from crude DM as an estrogenic compounds, exhibits structural similarity to the estrogenic compounds of royal jelly, which are able to bind to ER. Because of the structural similarity, the identified E-dec-2-enedioic acid presumably binds to the ER, too. The estrogenic activity of DM justifies its use in folk medicine and may lead to new possibilities for its use in estrogen-deficient conditions in women, similarly to RJ.

Raw DM elicits not only androgenic and estrogenic activities, but also a weak gestagenic effect, which becomes relevant in combination with another weak gestagen. The mechanism of this action and the gestagenic compound(s) of DM are still unknown, but the identified androgenic or estrogenic compounds of DM may be responsible for this weak gestagenic activity.

Appendix

Publications related to the Ph.D. thesis:

I **Seres AB**, Ducza E, Bathori M, Hunyadi A, Beni Z, Dekany M, Gaspar R: Raw drone milk of honeybee elicits uterotrophic effect in rat: evidence for estrogenic activity
Journal of Medicinal Food 16:(5) pp. 404-409. (2013) *IF: 1.642*

II **Seres Adrienn**: A heretej ösztrogénhatásának vizsgálata patkányban
„Együtt a biztosabb tudományos karrierért, a jövőtervezésért" VII. Ph.D. Conference, Budapest, Hungary
Professzorok az Európai Magyarországért Egyesület, pp. 348-355. (2013)

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