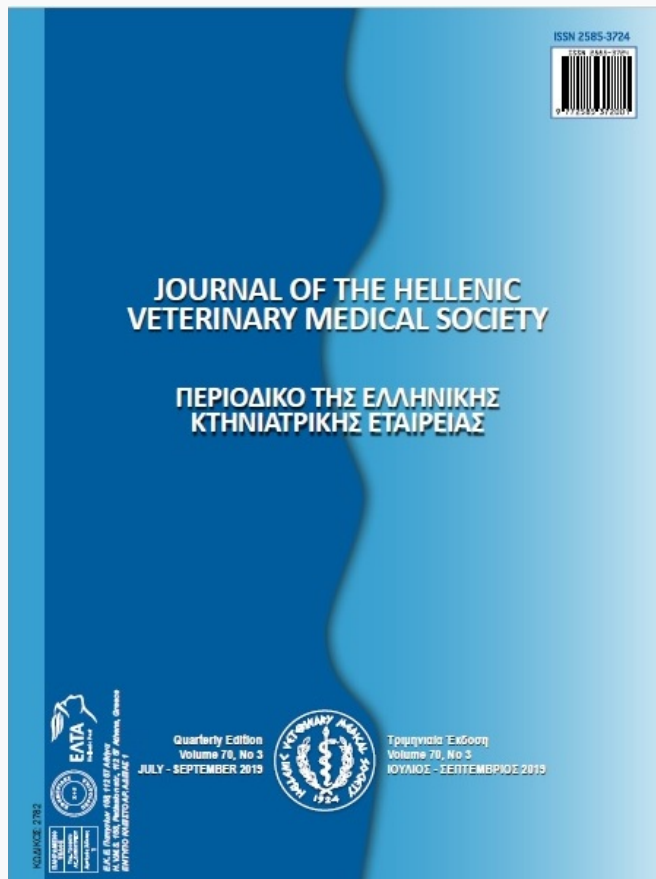


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Effect of *Ferula communis* L. on reproductive parameters in Awassi ewes

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ABSTRACT. Nutrient composition of the diet affects follicular activity, embryonic development and reproductive hormones in ruminants. The objective of the present study was to investigate the effects of *Ferula communis* L. on some reproductive parameters of Awassi sheep. The experiment was carried out on 29 (15-16 months old) Awassi ewes. All ewes were allocated to receive either a control (14% CP and 11.7 MJ ME/kg, n=9) or a diet supplemented with 5% (75g, n=10) or 10% (150g, n=10) powdered *F. communis* root, respectively for 21 days. Oestrus was synchronized using intravaginal sponges, while oestrus behaviour was observed 24, 36 and 48h after the sponge removal. Blood samples were collected for the assessment of oestradiol and LH. At the end of the 21-day period, animals were slaughtered and ovarian structures were recorded. Corpora lutea tissues were cultured *in vitro*, and progesterone production was measured. The results indicate that the treatment of animals with 5% of *F. communis* root increased the percentage of animals in heat (80%, 60% and 10% for 5C, 10C, and the control group, respectively). Furthermore, the number of small follicles (1-3 mm) in treated groups was significantly higher than those of the control group. Moreover, the number of large follicles (>4 mm) in the control group was higher than those of the treatment groups. Plasma concentration of oestrogen and LH peak were similar in the control and treatment groups. Progesterone production by luteal cells cultured *in vitro* was higher for both treatment groups compared to the control. Herewith, supplementation of the diet of Awassi Sheep with *F. communis* root during the breeding season may enhance ovulation rate and luteal activity.

Keywords: Awassi, *Ferula communis*, reproductive hormones, luteal tissue, ferutinin

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INTRODUCTION

The genus *Ferula* belongs to the family *Apiaceae* which consists of 170 species distributed to Mediterranean area and Central Asia (Canogullari et al., 2009; Rahali et al., 2018). *Ferula communis* L. subsp. *communis* (giant fennel) is a perennial phytoestrogenic plant with dense roots, found across the Mediterranean coast. Sub-varieties with poisonous and non-poisonous properties also exist (Appendino et al., 2001; Akaberi et al., 2015). Giant fennel has been used as a traditional medicine. *In vitro* and *in vivo* pharmacological properties have been reported recently (Akaberi et al., 2015). Phytoestrogens are oestrogen-like substances with similar molecular composition to plasma oestrogen. Non-poisonous chemotype of *F. communis* contains ferutinin, an aromatic ester of daucane alcohol, reported to contain phytoestrogen of the daucane type (Sacchetti et al., 2003; Akaberi et al., 2015, Rahai et al., 2018) which acts through stimulating the receptors in the hypothalamus to release luteinizing hormone (Canogullari et al., 2009). Ferutinin may interact with oestrogen receptors especially with oestrogen receptor (ER) α (Zanoli et al., 2009). Roots of *F. communis* especially rich in daucane esters.

Beside its phytoestrogenic effect, *F. communis* can prevent lipid peroxidation through its anti-oxidant properties. Both synthetic and natural anti-oxidants counteract detrimental effects of oxidative stress on reproduction. Synthetic anti-oxidants may have possible side effects in addition to their toxicity (Rahali et al., 2018). Local farmers in Mediterranean coast of Turkey traditionally feed livestock with *F. communis* root for its uterine stimulant activities to improve fertility rates. The present study was designed to further investigate the effects of *F. communis* on ovulation rates, ovarian structures and reproductive hormones of Awassi sheep.

MATERIALS AND METHODS

This experiment was carried out during the breeding season, on 29 sexually mature and cyclic Awassi ewes from the same farm, in Hatay province of Turkey (35° 52' - 37° 40' N and 35° 40' - 36° 35' E). Sheep were separated into groups of Control (C, n=9), 5% *Ferula communis* (5C, n=10) and 10% *F. communis* (10C, n=10) considering live weights (40.4, 40.3 and 40.2 kg, respectively) and body condition scores (2.40, 2.35 and 2.35, respectively). Feed consumptions were recorded daily to calculate subsequent feed. Animals were fed 1.5 \times maintenance ME

requirement (Maintenance=0.42 MJ/kg^{0.75}) for 21 days with a concentrated diet containing 18% CP and 13.5 MJ ME/kg. Animals in treatment groups received 75 g (5C) or 150 g (10C) *F. communis* (diluted in 0.5 L water) orally, with the aid of a cannula.

Oestrous cycles were synchronized using intravaginal progestogen sponges (an impregnated sponge containing progestogen; 0.3g cronolone, Intervet) for 14 days. Teaser ram were introduced 24h, 36h and 48h following progestogen withdrawal to find ewes in heat and hand mating was carried out.

Blood samplings were performed on 5 ewes in each group on days 12, 14, 16 and 17 after sponge insertion, once daily, for oestradiol assessment; on days 16 and 17 after sponge removal, in 2-hour intervals, for LH assessment. All samples were centrifuged at 2060 \times g for 10 min to separate the plasma and stored at -20°C until the analyses.

At the end of feeding period of 21-days, all animals were slaughtered and reproductive tracts were taken immediately in a flask to the laboratory. The number of corpora lutea (CLs) and follicles were recorded. Mature CLs, identified on the basis of their gross appearance (Oldham and Lindsay, 1980) were isolated from ovarian tissues. Excisions were performed on a sterile warm stage (35°C) and each CL was then bisected and sliced by scalpel to provide a pair of luteal tissue sections (approx. diameter = 0.3 mm). These sections were weighed and cultured individually in 3 mL of Medium 199 with Earle's salts (Sigma) containing 0.68 mM L-glutamine, 26.19 mM sodium bicarbonate, 50 IU/mL penicillin and 50 mg/mL streptomycin sulphate in 30 mm culture dishes for 4h at 38.5°C in a humidified atmosphere of 5% CO₂ in the air. For each pair of sliced CL tissue sections, one section was incubated in the absence and one in the presence of 100 μ IU equine LH (Sigma; 1 μ IU \equiv 1 mg NIH-LH-S1). At the end of the culture period, culture medium was collected and stored at -20°C for further analysis.

Plasma concentration of LH was measured using a sensitive competitive enzyme immunoassay method (Yildiz et al., 2002). Briefly, D-Biotinoyl- ϵ -aminocaproic acid-N-hydroxysuccinimide ester (Biotin-X-NHS; Sigma-Aldrich, Taufkirchen, Germany) was used for labeling oLH [NIDDK-oLH-I-4 (AFP-8614B)]. Affinity purified goat IgG antirabbit IgG was attached to the solid phase and labelled and non-labeled (sample) oLH were competed against the

anti-oLH raised in rabbit [NIDDK-anti-oLH-1 (AFP-192279)]. Optimum dilutions of biotinyl-LH and oLH antiserum were found to be 1:5000 and 1:3200000, respectively. Standards used in the current study were between 0.39 and 50 ng oLH/mL. The minimum detection limit of the assay was 0.70 ng oLH/mL. Intra and interassay coefficients of variation were calculated at two levels of control samples and as quadruplicates in two different locations of the plate. At 12.2 ng/mL level, the intra- and inter-assay coefficients of variation were 10.8 and 11.5%, respectively.

The estradiol and progesterone concentrations were measured according to ELISA procedure using two commercially available assay kits (DRG EIA-2693 and DRG EIA-1561, DRG International Inc., Mountainside, New Jersey 07092, USA). The procedures were based on competition principle and microplates used as solid phase.

A sample containing unknown amount of progesterone and estradiol present in the sample and a fixed amount of estradiol or progesterone conjugated with horse-radish peroxidase compete for the binding sites of a polyclonal estradiol or progesterone antiserum coated onto the wells. After 120 min for estradiol and 60 min for progesterone incubation, the microtiter plates were washed three times in order to stop competition reaction. The substrate solution was added into each well for 15 min to stop competition reaction. The optical density was measured at 450 nm, and the concentrations of estradiol and progesterone are inversely proportional to the optical density measured.

It has been suggested that intra- and inter-assay coefficient of variation should be <20–25% (Findlay et al., 2000; Shah, 2007; Valentin et al., 2011; Birdwhistell et al., 2017). The values in the current study are much lower than that of the reported criteria and therefore tests are quite reliable (Table 1).

Table 1. Intra- and inter-assay coefficients of variations (CV) at different quality control (QC) levels

Analyte	Intra-assay variation		Inter-assay variation	
	QC	CV (%)	QC	CV (%)
Progesterone (ng/mL)	1.21	8.3	1.31	9.9
	2.62	4.6	2.71	4.8
	12.3	5.2	11.9	6.5
Estradiol (pg/mL)	478	6.3	91.89	2.0
	1167	5.0	276.1	3.8
			562.5	2.6

The IBM-SPSS (v23 for Windows) program was used to analyse all data. Data were analysed using ANOVA (GLM procedure) for evaluation of differences between treatments. Data were presented as mean±standard error (SE). The p value used to determine significance in all tests was 0.05.

RESULTS

The number of ewes in heat following sponge removal was increased ($P<0.05$) due to supplementation

of *F. communis*, while no differences were observed 36h later. Feeding animals with *F. communis* root decreased the number of large follicles (≥ 4 mm) and an increase for 5C group was observed in terms of small to large follicles ($P<0.05$). *F. communis* root induced formation of CL ($P<0.05$) but the fact remains that CL weights remained similar for each group (Table 2).

Table 2. The effects of powdered *F. communis* root supplementation to diet on the manifestation of estrus and on ovarian structures

	Control (n=9)	5% (n=10)	10% (n=10)
1-3 mm follicles /ovary	4.5±0.45 ^a	6.7±0.50 ^b	5.7±0.55 ^{ab}
≥ 4 mm follicles /ovary	0.89±0.20 ^a	0.05±0.05 ^b	0.06±0.05 ^b
Number of corpus luteum / ovary	0.22±0.14 ^a	0.70±0.2 ^b	0.41±0.1 ^b
Corpus luteum (weight; mg)	0.37±0.01	0.35±0.01	0.38±0.01
Ewes in oestrus % (24 h)	10 ^a	80 ^b	60 ^b
Ewes in oestrus % (36 h)	40	90	70

^{a,b} Different superscript in the same row differ at $P<0.05$. Values presented as mean±SE

Plasma peak LH (Table 3) and estradiol concentrations (Figure 1) were similar in control and *F. communis* root supplemented groups. However, plasma peak LH concentration in 5C group was tended to be higher than in control and 10C group ($P>0.05$).

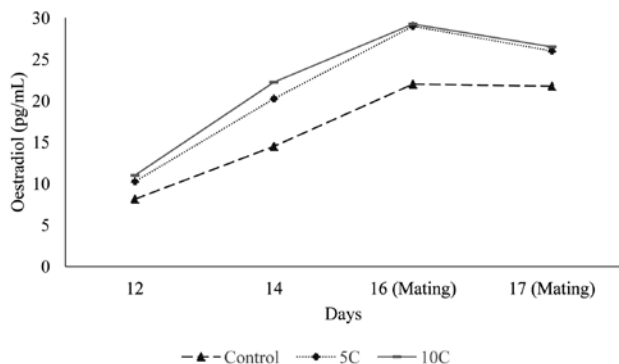


Figure 1. Plasma oestradiol concentrations of ewes fed with 0 g (control), 75 g (group 5C) and 150 g (group 10C) *F. communis* extract

Basal concentrations of LH (mean±sem; ng/mL) did not differ ($P>0.05$) between treatment groups and the control group. Similar to basal LH concentrations, neither plasma concentrations (C, 80.9±42; 5C, 123.3±13; 10C, 58.9±11) nor the time (C, 30.2±1.4; 5C, 35.2±1.8; 10C, 36.7±2.3) of LH peak were affected by supplementation of powdered *F. communis* root. Peak concentrations of LH (ng/ml) at the pre-ovulatory surge were higher ($P>0.05$) within 5% *F. communis* root supplemented group (Table 3).

Table 3. The effects of powdered *F. communis* root supplementation to diet on plasma peak LH concentration and on the time of LH peak

	Control (n=5)	5C (n=5)	10C (n=5)
Plasma peak LH (ng/ml)	80.9±42	123.3±13	58.9±11
Time of LH peak (h*)	32.0±1.4	35.2±1.8	36.7±2.3

* hours after sponge removal

Results showed that cultured luteal cells from the treatment groups produced more progesterone than those of the control ($P<0.01$). Also, progesterone production by luteal cells was stimulated by the supplementation of 100 µIU LH.

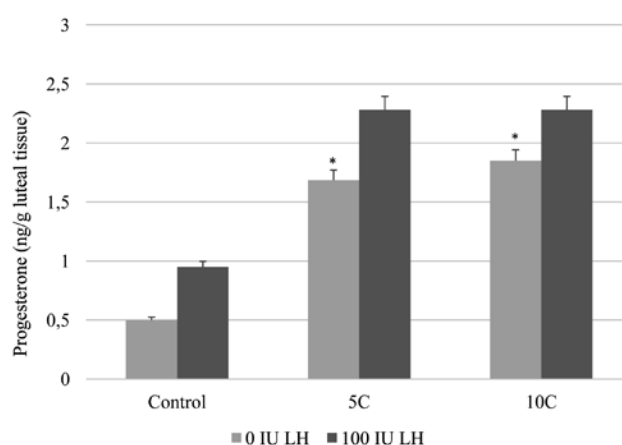


Figure 2. In vitro progesterone production by luteal cells originating from ewes fed with 0 g (control), 75 g (group 5C) and 150 g (group 10C) *F. communis* extract for 21 days. * Statistically significant at $p<0.01$

DISCUSSION

Many researchers have analysed the constituents of different organs of *F. communis*, previously (Manolakou et al., 2013; Rahali et al., 2018). Despite the fact that the chemical composition of *F. communis* root has not been analysed in the present study, mean live weights and body condition scores remained similar for each treatment group during the experimental period.

Formononetin and biochanin A are biotransferred to estrogenic isoflavone equol, a metabolite produced *in vivo*, by the action of ruminal bacteria and gut microflora. (Muthyala et al., 2004; Retana-Marquez et al., 2012). The main route of entry of phytoestrogens into body is via the diet. According to recent studies, consumption of some plants which contain phytoestrogens such as soybean may result in infertility problems, abortions and even stillbirth. Such plants exert these effects through agents like isoflavens and ferutinin (Appendino et al., 2001). Isoflavens, ferutinin and plasma oestrogen compete for binding to oestrogen receptors located in the anterior pituitary, since they have similar molecular structure (Adams, 1995; Ikeda et al., 2002). The estrogenic activity of pituitary decreases about 1/1000 to 1/10000 times once isoflavens bounded to the oestrogen receptors (Raymer et al., 1999). In line with this hypothesis, ovulation rates and the number of small follicles increased in the present study. This increase in the number of small to large sized follicles is in agreement with the assumption of Billig et al. (1993) who reported that phytoestrogens may prevent apoptosis. Increased oestrogen levels in follicular fluid is related to the number of decreased

atretic follicles. Oestrogen treatment was reported to prevent ovarian apoptotic DNA fragmentation (Billig et al., 1993). Recent studies reported that oestrogenic isoflavens resulted an increase in expression of anti-apoptotic factor while decrease in expression of pro-apoptotic factors (Kundu et al., 2018).

In the present study we demonstrated that ingestion of *F. communis* resulted enhanced earlier manifestation (24 h) of oestrus behaviour of Awassi ewes. These results seem to support the folkloric reputation of *F. communis* root as a sexual stimulant. Recent studies are available which reported both stimulating effects of the genus *Ferula* on reproductive behaviour in male rats. Hadidi et al. (2003) observed a significant increase in the copulatory behaviour of rats depending on the extract, when fed orally.

Overproduction of reactive oxygen species (ROS) in follicular microenvironment may cause meiotic arrest and impaired oocyte development (Rizzo et al., 2012). The present findings lead us to suggest that *F. communis* powder might exert its provocative action on female receptivity and proceptivity through acting like ROS scavenger and thus promoting the development of small to large sized follicles in this regard.

Plasma oestrogens and androgens have been reported to inhibit luteal function through different mechanisms. Wocławek-Potocka et al (2013) reported that high concentrations of active metabolites of phytoestrogens may disturb CL function by inhibiting progesterone secretion. However, our findings suggest that *in vitro* progesterone production by luteal tissues

has been increased after the ingestion of *F. communis*.

Similarly, higher plasma LH concentration (Bindon et al., 1982) and/or enhanced sensitivity of LH secretion induced by GnRH-A (Arispe et al., 2013) for sheep consuming phytoestrogenic plants has been reported.

Finally, Guan et al. (2006) has shown that lipid activities of soybean and kudzu improved lipid profile, including total cholesterol and certain lipoprotein ratios. The authors also pointed that research on phytoestrogens other than genistein and daidzein is limited. Ferutinin in the *F. communis* has been reported to have effects on secretory gland activities by increasing Ca²⁺ channel activity (Zamaraeva et al., 1997).

CONCLUSION

In conclusion, *F. communis* root extract should exert pharmacological role on reproductive parameters of Awassi ewes dose-dependently and can be added in the diet at 5% with no adverse effects.

Concerning the potential therapeutic role of ferutinin, it could exert some beneficial endocrinological effects to the current hormonal replacement therapy in livestock, especially for ewes. In addition, the pharmacological profile of *F. communis* as a strong antioxidant and oestrus promotive could lead to further therapeutic benefits, which will be investigated in our future research.

CONFLICT OF INTEREST

None declared.

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