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Lippia citriodora (verbascoside) extract supplementation: Effect on rabbit semen quality *in vivo* and *in vitro*

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Abstract: Verbascoside, the main component of *Lippia citriodora* extract, is one of the most powerful free radical scavengers exhibiting a wide biological activity. In *in vivo* study 20 adult New Zealand white rabbit bucks were divided into two homogeneous groups, one control (CON) and one verbascoside-supplemented (0.1%) in feed mixture (EXP) and later *in vitro* effects of verbascoside on the motility aspects of rabbit spermatozoa were analysed. The spermatozoa concentration, ejaculate volume, spermatozoa motility, progressive motility, distance parameters, velocity parameters and type of spermatozoa movement were negatively affected by *Lippia citriodora* leaves extract after the first 4 weeks of dietary treatment, till the end of experiment (8 weeks). Four weeks after the suspension of feed additive supplementation, all spermatozoa traits values returned to the normality, and in line with CON group. For *in vitro* findings, ejaculates from 10 male New Zealand white bucks were collected using an artificial vagina. Then it was diluted in physiological saline solution containing different concentrations of verbascoside at the concentration of 0, 0.0024, 0.0219, 0.157, 120.0 mg/ml (Ctrl, VB1, VB2, VB3, VB4 groups, respectively), using a dilution ratio of 1 : 4. The obtained data proved that verbascoside at the concentration of 0.0024 and 0.0219 mg/ml had no adverse effect on spermatozoa. Additionally, we found that verbascoside at higher concentrations (0.157 and 120.0 mg/ml) significantly altered all the motility parameters analysed in the experiment. In conclusion a possible negative effect of verbascoside supplementation into feed mixture (0.1%) on semen quality parameters in rabbit bucks as well as *in vitro* can be stated, obviously considering that target organs of antioxidant activities of phenylpropanoid glycosides are various. In addition it has to be emphasized that the extract showed a reversible action, since the semen traits of treated animals returned to the normality after the dietary administration period.

Keywords: feed additive; *Verbenaceae*; rabbit; spermatozoa; motility

Male rabbits are one of the founding blocks of reproductive success in intensive rabbit farming, especially when we consider that one single male can influence the fertility and prolificacy of approximately one hundred females since artificial insemination (AI) is routinely performed in the

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rabbit farms (Eid 2008). The largely expanding rabbit production is mainly attributable to rabbit's high rate of reproduction, high potential of genetic selection, rapid growth rate, early maturation, efficient feed utilization and high quality of meat. The reproductive performance of male livestock is of economic importance, and improving semen quantity and quality, especially for artificial insemination, additionally helps to avoid the loss of valuable genotypes.

There is an international interest concerning application of natural plant sources in animal production fields. The search for improvements in semen quality and, thereby, pregnancy rates using feed supplementation has obtained much less interest in application to male livestock than to female ones. This probably has economic reasons since a far higher proportion of the reproducing population is female, especially since the establishment of artificial insemination (Clement et al. 2012). The generation of reactive oxygen species (ROS) is a normal physiological process in both animal and human tissue and organs including the testis. However, the over generation of ROS may be detrimental to spermatozoa, and has even been associated with male infertility. The spermatozoa of vertebrates including rabbits display high rates of metabolic activity and are also rich in polyunsaturated fatty acids (Castellini et al. 2006). This makes them particularly susceptible to oxidation by ROS, especially under stress conditions in domestic birds (Eid et al. 2006) and also rabbits (Castellini et al. 2000). ROS can modify the spermatozoa cytoskeleton and axoneme resulting in a reduction of spermatozoa motility and the inhibition of sperm–oocyte fusion that in turn leads to reduced fertility (de Lamirande and Gagnon 1992). ROS can also attack the DNA within the spermatozoa nucleus. Such damage to the genome may be responsible for infertility resulting in reduced reproductive performance and hence, significant financial losses. In a normal situation, the antioxidant mechanisms present in the reproductive tissues and their secretions are likely quench these ROS and protect against oxidative damage to gonadal cells and mature spermatozoa. Within the endogenous antioxidant system of spermatozoa, many substances extracted from plants are able to improve plasma stability of lipid profile and to monitor ROS production. They play a crucial role in breaking the chain reaction of peroxidation, initiated by ROS. Low amount of ROS are necessary to start capacitation and acrosome reaction; when they exceed, cell damage or inactivation of critical enzyme pathways occur (Castellini et al. 2003).

In rabbit, many studies were carried out to evaluate the effect of different antioxidants derived from grape polyphenols (Sgorlon et al. 2005), grape pomace (Eid 2008), and green tea (Eid et al. 2011), since researches attribute increasing importance to the dietary strategy in order to improve the productive performance in intensively reared animals.

Lippia citriodora, a plant species in the *Verbenaceae* family, is characterized by the presence of several phenolic compounds, including flavonoids, phenolic acids, luteolin derivatives and phenylpropanoids (Valentao et al. 2002). Phenylpropanoid, particularly verbascoside [2-(3',4'-dihydroxyphenyl) ethyl-O-α-L-rhamnopyranosyl-(l-3)-β-D-(4-O-caffeoyl)-glucopyranoside] is the most abundant compounds in *Lippia* extract (Funes et al. 2009). Verbascoside contains a rhamnose unit bound to glucose, which acts as a bridge, and it also belongs to the extensive family of phenylpropanoids and exhibits a number of biological activities including anti-inflammatory (Deepak and Handa 2000) and antioxidant (Pastorelli et al. 2012). The protective activity may be attributed either to the caffeoyl residue in the molecule or to the phenylethyl moiety (Xiong et al. 1998). Previous studies (Palazzo et al. 2011; Casamassima et al. 2017; D'Alessandro et al. 2017) showed that dietary supplementation with *Lippia* extracts in different species, hares, rabbit does and donkeys, showed an improvement in biochemical parameters, such as blood lipid profile and plasma antioxidant markers. In *in vitro* experiments, verbascoside has been shown to modulate nitric oxide production and the expression of inducible nitric oxide synthase in activated macrophages (Lee et al. 2005). It also inhibits histamine, arachidonic acid release and prostaglandin E2 production in RBL-2H3 mast cells suggesting a possible application of the compound as anti-inflammatory remedy (Lee et al. 2006). More recently it has been reported that verbascoside showed a pro-oxidant effects on the developmental potential of fresh and vitrified ovine prepubertal oocytes (Dell'Aquila et al. 2014). To the date, only few findings on verbascoside application on *in vitro* spermatozoa cell model have been concluded.

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In this preliminary research in New Zealand White rabbits, the dietary effects of *Lippia citriodora* natural extract, with verbascoside as main component, on rabbit buck semen quality was examined, and in addition the *in vitro* effect of verbascoside on the motility aspects of rabbit spermatozoa was investigated, using the computer-assisted semen analysis (CASA).

MATERIAL AND METHODS

In vivo experiment, animal and experimental design. All the experimental procedures and management of animals were conducted in accordance with European Council Directive 86/609/EEC regarding the protection of animals for experimental purpose. The choice of rabbit is due to their being a good model for assessing the effects of toxic agents on semen quality, fertility and developmental toxicity. Rabbit semen is collected, evaluated and the fertility is tested under controlled conditions through artificial insemination (Foote and Carney 2000), thereby providing a direct comparison with human semen analysis. The trial lasted 90 days and was conducted in the National Agricultural and Food Centre – Research Institute for Animal Production Nitra (Slovak Republic) on 20 adult New Zealand White rabbit bucks. Animals were individually housed in wire cages arranged in flat-decks on one level. Cages were equipped with a hopper for feed and an automatic watering system. A cycle of 16 h of light and 8 h of darkness was used throughout the trial. Temperature and humidity in the rabbitry were recorded continuously by a digital thermograph positioned at the same level as the cages. Heating and forced ventilation systems allowed the building temperature to be maintained within 18 ± 4°C throughout the trial. Relative humidity was ~70 ± 5%. The tested animals (aged 20.5 ± 1.5 months; 5.30 ± 0.27 kg body weight) were divided into two homogeneous groups, one control (CON; n = 10) fed with commercial feed, one experimental (EXP; n = 10) which received in feed an extract from *Lippia citriodora* in the amount of 10 mg of verbascoside (main active compound) per kg of feed (Table 1). The natural extract was produced and provided by Monteloeder (PLX®23; Monteloeder Ltd, Spain). The composition and ingredients of diets are reported in Table 1.

Table 1. Composition of control (CON) and experimental (EXP) diets

Ingredients	Diet	
	CON	EXP
Yellow corn	6.9	6.9
Wheat bran	20.1	20.1
Barley grain	11.1	10.4
Soybean meal	20.4	20.1
Berseem hay	35.0	35.0
Lippia extract	–	1.0
Molasses	3.0	3.0
Salt	1.0	1.0
Limestone	2.0	2.0
Premix[1]	0.5	0.5
Total	100	100

[1]1 kg of premix contains: vitamin A 12 000 000 IU, vitamin D3 220 000 IU, vitamin E 1000 mg, vitamin B1 1000 mg, vitamin B2 4000 mg, vitamin B6 100 mg, vitamin B12 10 mg, pantothenic acid 3.33 mg, biotin 33 mg, folic acid 0.83 g, Zn 11.79 g, Mn 5 g, Fe 12.5 g, Cu 0.5 g, Se 16.6 mg, Mg 66.7g

The semen samples were collected at days 0, 30 and 60 (basal, 4 weeks and 8 weeks, respectively) of feeding period with the help of artificial vagina. The obtained semen samples were diluted with physiological solution in the ratio 1 : 5. After processing, the samples were stored in the heat stage at the temperature 37°C and were analyzed immediately.

Each of thus prepared samples were evaluated using a Computer Assisted Semen Analyzer (CASA) system – Sperm Vision (Minitub, Germany) equipped with a microscope (Olympus BX 51, Japan) to assess the spermatozoa motility (Massanyi et al. 2008). Each sample was placed into Makler Counting Chamber (depth 10 μm; Sefi-Medical Instruments Ltd, Israel). Using the rabbit specific set up, the following parameters were evaluated : spermatozoa concentration (CONC, 10^9/ml), volume of ejaculate (ml), total motile spermatozoa (%, motility > 5 μm/s) and progressive motile spermatozoa (%, motility > 20 μm/s), DAP (distance of average path, μm), DCL (distance of curved line, μm), DSL (distance of straight line, μm), VAP (velocity of average path, μm/s), VCL (velocity of curved line, μm/s), VSL (velocity of straight line, μm/s), STR (straightness, VSL : VAP ratio), LIN (linearity, VSL : VCL ratio), and WOB (wobble, VAP : VCL ratio).

In vitro experiment, sample collection and processing. Ten male New Zealand white rabbit

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bucks (age of 18 ± 1 months) were used. Animals were individually housed in stainless steel cages at room temperature (18 ± 4°C) with a relative humidity of 50–60% and on a 16 h light and 8 h darkness cycle. The animals had free access to commercial pellet diet (dry matter (DM) 88.5%, crude proteins 16.5%, ether extract 3.2%, neutral detergent fibre 36.9%, acid detergent fibre 20.6%, digestible energy 11.71 MJ/kg DM) and water *ad libitum*. Rabbits and diet were obtained from National Agricultural and Food Centre – Research Institute for Animal Production Nitra (Slovak Republic). Ejaculates were collected using an artificial vagina and a teaser doe. Aliquots from each ejaculate were initially evaluated under a phase contrast microscopy (200×) for concentration and motility. The ejaculates with the highest sperm motility (> 70%) were applied. Samples from rabbits with nearly the same percentage of motility were pooled. Rabbit heterosperm sample was diluted in physiological saline solution (PS; sodium chloride 0.9% w/v; Bieffe Medital, Italia) containing different concentrations of the verbascoside (Carbosynth Ltd, UK) at the concentration of 0, 0.0024, 0.0219, 0.157, 120.0 mg/ml (Ctrl, VB1, VB2, VB3, VB4 groups, respectively), using a dilution ratio of 1 : 4. The samples were processed at laboratory temperature (22–25°C). The Ctrl group (medium without verbascoside supplementation, containing normal PS) was compared with the experimental groups.

Spermatozoa motion characteristics were assessed using CASA system (equipment described in *in vivo* experiment) and 10 µl of each sample were placed into a Makler counting chamber (depth 10 µm, 37°C; Sefi-Medical Instruments Ltd) and assessed at 0 time, after 1 h and after 2 h of incubation at 37°C (Paal et al. 2017). Seven microscopic fields were subjected to each analysis in order to ensure capture of at least 300 cells. The following traits and motility features were recorded: concentration (10^9/ml, CONC), percentage of motile spermatozoa (%, MOT), percentage of progressive motile spermatozoa (%, PROG), distance average path (mm, DAP), velocity average path (µm/s, VAP) and amplitude of lateral head displacement (µm, ALH).

Statistical analyses. Obtained data were statistically analyzed with the help of the PC program Excel and a commercially available statistical package SAS (Statistical Analysis System, Version 8.0, 2003) using Student's *t*-test and Scheffe's test. In *in vivo* experiment the single rabbit was the experimental unit, whereas in *in vitro* test the group was considered the experimental unit. Statistical significance was indicated by *P*-values of less than 0.05, 0.01 and 0.001.

RESULTS AND DISCUSSION

In vivo experiment. The dietary supplementation with verbascoside extract from *Lippia citriodora* did not cause any changes in the animal body weights, and did not induce any evident clinical signs in rabbits over the 12-week experimental period. But the experimental treatment significantly affected almost all of the tested parameters (Tables 2–4).

The concentration of spermatozoa was significantly different ($P < 0.001$) between experimental group and control group after 4 weeks until the end of the feeding trial (8 weeks). The experimental value came back to the normality, and in line with the control group, 4 weeks after the dietary treatment. In other words, a possible negative effect on this parameter was recorded. Whereas at the end of the feeding period in the EXP group a significantly and lower value ($P < 0.05$) of spermatozoa volume, compared to the CON group, was observed. Also Okab et al. (2013), feeding rabbit bucks with dried seaweed (2%), showed a significant decrease in spermatozoa concentration, percentage of live spermatozoa and ejaculate volume.

The spermatozoa motility parameters (motility and progressive motility) (Table 2) were significantly different ($P < 0.001$) between control group and experimental group with verbascoside supplementation. In fact after 4 and 8 weeks of dietary treatment, the EXP-group values were significantly lower compared to CON-group, reaching almost the same value one month after the treatment.

Even though the mechanism of action of the substances contained in these plants has not been clearly demonstrated, it could be explained because high doses of antioxidant additives can be expected to have destroyed the functional integrity of the axosome and mitochondria of the spermatozoa, which are associated with motility. There may have been the inhibition of the proliferation of spermatogenic cells within the epithelium of the seminiferous tubules, thus reducing spermatozoa production (Herbert et al. 2005).

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Table 2. Semen characteristics of control (CON, n = 10) and experimental (EXP, n = 10) buck rabbits

Semen traits	Treatment		P-value
	CON	EXP	
Spermatozoa concentration (10^9/ml)			
Basal	0.99 ± 0.03	1.07 ± 0.09	ns
After 4 weeks	0.85 ± 0.05	0.57 ± 0.02	***
After 8 weeks	0.66 ± 0.04	0.37 ± 0.01	***
4 weeks after treatment	0.62 ± 0.07	0.63 ± 0.04	ns
Ejaculate volume (ml)			
Basal	0.78 ± 0.05	0.79 ± 0.03	ns
After 4 weeks	0.79 ± 0.01	0.77 ± 0.02	ns
After 8 weeks	0.81 ± 0.03	0.74 ± 0.01	*
4 weeks after treatment	0.71 ± 0.01	0.69 ± 0.04	ns
Spermatozoa motility (%)			
Basal	81.97 ± 1.80	83.77 ± 1.43	ns
After 4 weeks	90.68 ± 2.69	72.51 ± 2.13	***
After 8 weeks	75.08 ± 2.78	57.93 ± 2.61	***
4 weeks after treatment	75.77 ± 1.23	71.62 ± 1.74	ns
Progressive spermatozoa motility (%)			
Basal	68.49 ± 1.94	72.17 ± 2.19	ns
After 4 weeks	81.82 ± 1.01	51.84 ± 3.07	***
After 8 weeks	60.13 ± 3.81	36.79 ± 3.19	***
4 weeks after treatment	63.33 ± 1.36	58.21 ± 1.62	ns

*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant

Table 3. Semen characteristics of control (CON, n = 10) and experimental (EXP, n = 10) buck rabbits – distance parameters

Semen traits	Treatment		P-value
	CON	EXP	
DAP (μm)			
Basal	24.92 ± 1.03	24.59 ± 0.84	ns
After 4 weeks	26.28 ± 0.47	19.69 ± 1.01	***
After 8 weeks	29.45 ± 0.49	21.50 ± 0.77	***
4 weeks after treatment	26.73 ± 0.62	26.51 ± 0.43	ns
DCL (μm)			
Basal	47.72 ± 1.71	49.95 ± 1.16	ns
After 4 weeks	52.62 ± 0.80	40.08 ± 1.59	***
After 8 weeks	48.82 ± 1.76	37.41 ± 1.17	***
4 weeks after treatment	57.63 ± 1.23	53.30 ± 1.45	ns
DSL (μm)			
Basal	18.06 ± 0.30	17.79 ± 0.28	ns
After 4 weeks	19.86 ± 0.94	15.09 ± 0.65	***
After 8 weeks	23.94 ± 0.87	18.44 ± 0.69	***
4 weeks after treatment	19.34 ± 0.56	20.96 ± 0.25	ns

DAP = distance of average path, DCL = distance of curved line, DSL = distance of straight line
*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant

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Table 4. Semen characteristics of control (CON, n = 10) and experimental (EXP, n = 10) buck rabbits – velocity and other parameters

Semen traits	Treatment		P-value
	CON	EXP	
VAP (mm/s)			
Basal	57.75 ± 2.67	50.97 ± 2.37	ns
After 4 weeks	60.72 ± 1.40	44.89 ± 1.84	***
After 8 weeks	50.10 ± 1.75	31.80 ± 1.68	***
4 weeks after treatment	61.08 ± 2.64	59.98 ± 2.75	ns
VCL (mm/s)			
Basal	116.67 ± 3.12	105.66 ± 2.74	ns
After 4 weeks	121.10 ± 4.20	90.57 ± 3.88	***
After 8 weeks	113.90 ± 3.11	87.46 ± 2.91	***
4 weeks after treatment	130.46 ± 3.99	120.14 ± 4.35	ns
VSL (mm/s)			
Basal	42.47 ± 1.24	41.88 ± 2.08	ns
After 4 weeks	46.03 ± 1.82	34.52 ± 1.13	***
After 8 weeks	56.35 ± 2.31	43.99 ± 2.10	**
4 weeks after treatment	44.56 ± 2.54	47.72 ± 1.74	ns
STR ratio			
Basal	0.77 ± 0.01	0.74 ± 0.02	ns
After 4 weeks	0.75 ± 0.02	0.68 ± 0.01	ns
After 8 weeks	0.67 ± 0.03	0.77 ± 0.04	ns
4 weeks after treatment	0.72 ± 0.02	0.74 ± 0.01	ns
LIN ratio			
Basal	0.37 ± 0.01	0.36 ± 0.02	ns
After 4 weeks	0.38 ± 0.04	0.33 ± 0.02	ns
After 8 weeks	0.40 ± 0.02	0.38 ± 0.01	ns
4 weeks after treatment	0.39 ± 0.03	0.41 ± 0.01	ns
WOB ratio			
Basal	0.48 ± 0.01	0.47 ± 0.03	ns
After 4 weeks	0.50 ± 0.02	0.44 ± 0.01	*
After 8 weeks	0.56 ± 0.01	0.49 ± 0.02	**
4 weeks after treatment	0.46 ± 0.02	0.48 ± 0.02	ns

VAP = velocity of average path, VCL = velocity of curved line, VSL = velocity of straight line, STR = straightness, LIN = linearity, WOB = wobble

*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant

Controversially Yousef et al. (2003) observed an improvement of spermatozoa motility parameters after the dietary supplementation of ascorbic acid and vitamin E, alone and in combination, in male rabbits. Also Attia el al. (2017) showed that the dietary integration of milk thistle seeds and rosemary leaves in rabbit bucks significantly improved the semen quality and also increased the economic efficiency of farm.

The distance parameters (DAP, DSL, DCL) (Table 3) and the velocity parameters (VAP, VSL, VCL) (Table 4) of spermatozoa were significantly different (P < 0.001) between control group and experimental group with verbascoside supplementation. In fact after 4 and 8 weeks of dietary treatment, the EXP-group values were significantly lower compared to CON-group, reaching only one month after the dietary treatment the same value of CON animals.

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Also the type of spermatozoa movement (STR, LIN, WOB) (Table 4) showed a statistical effect of the dietary treatment only for the WOB ratio. In fact verbascoside supplementation decreased the value after 4 weeks of treatment till the end of the dietary period.

Motility, distance and velocity parameters and type of spermatozoa movement are important factors for the ejaculate characteristics. All these traits are involved in the ability of the spermatozoa to ascend the reproductive tract to reach the site of fertilization, as well as to act on fertilization itself. The results of the present work demonstrate that the ejaculates from EXP group would be not useful for instrument insemination, and the further prolificacy characteristics of does could be altered or compromised. Differently from our results, Okab et al. (2013) observed an improvement of the semen fertility characteristics of bucks, feeding animals with 2% dried seaweed for a period of 8 weeks.

Although in our study semen susceptibility to oxidation was not determined, a negative effect of *Lippia citriodora* extract on lipid peroxidation could be hypothesised, because peroxidation and spermatozoa viability are strictly related, and in our study this parameter was significantly lower in the treated rabbit bucks. In addition, according to Abe et al. (2002), verbascoside has been shown to exert an anti-proliferative activity, largely depending on the substituents of the phenethyl group, such as caffeic acid.

Table 5. *In vitro* effect of verbascoside on rabbit spermatozoa quality traits

	Treatment					SEM	*P*-value
	Ctrl	VB1	VB2	VB3	VB4		
Spermatozoa concentration (10^9 /ml)							
0 h	0.54^{a}	0.47	0.55^{a}	0.43	0.33^{b}	0.016	0.001
1 h	0.62^{a}	0.59^{a}	0.37^{b}	0.33^{b}	0.28^{b}	0.039	0.001
2 h	0.49	0.44	0.49	0.36	0.47	0.020	0.128
Spermatozoa motility (%)							
0 h	79.88^{a}	78.43^{a}	82.11^{a}	58.08^{b}	15.17^{c}	5.834	0.001
1 h	84.35^{a}	81.71^{a}	42.28^{b}	11.38^{c}	15.17^{c}	6.055	0.001
2 h	65.25^{a}	58.40^{a}	42.95^{a}	6.40^{b}	6.98^{b}	4.082	0.001
Progressive spermatozoa motility (%)							
0 h	61.64^{a}	64.03^{a}	65.78^{a}	39.89^{b}	1.26^{c}	5.677	0.001
1 h	71.92^{a}	71.51^{a}	34.60^{b}	2.42^{c}	0.00^{c}	5.564	0.001
2 h	50.50^{a}	47.12^{a}	32.30^{a}	1.05^{b}	0.00^{b}	3.342	0.001
Distance of average path (μm)							
0 h	21.10^{a}	23.20^{a}	23.70^{a}	18.20^{a}	2.92^{b}	1.729	0.05
1 h	27.15^{a}	27.39^{a}	17.76^{a,b}	10.68^{b}	0.00^{c}	2.194	0.05
2 h	26.68^{a}	27.21^{a}	21.27^{a}	7.72^{b}	0.00^{b}	1.882	0.001
Velocity of average path (μm/s)							
0 h	49.04^{a}	52.98^{a}	53.77^{a}	41.46^{a}	6.57^{b}	4.071	0.05
1 h	63.43^{a}	63.77^{a}	41.42^{b}	24.59^{b}	0.00^{c}	5.169	0.05
2 h	61.50^{a}	62.30^{a}	48.18^{a}	17.19^{b}	0.00^{b}	4.227	0.001
Amplitude of lateral head displacement (μm)							
0 h	4.15^{a}	4.40^{a}	4.00^{a}	3.74^{a}	0.89^{b}	0.297	0.001
1 h	4.51^{a}	4.50^{a}	3.29^{a,b}	2.31^{b}	0.00^{c}	0.414	0.05
2 h	4.21^{a}	4.27^{a}	3.85^{a}	1.40^{b}	0.00^{b}	0.367	0.001

Ctrl = medium without verbascoside supplementation, VB1 = medium with 0.0024 mg of verbascoside, VB2 = medium with 0.0219 mg of verbascoside, VB3 = medium with 0.157 mg of verbascoside, VB4 = medium with 120 mg of verbascoside, SEM = standard error of the means

$^{a–c}$different letters within the same row indicate significant differences

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In vitro experiment. It is well known that, for the major of natural compounds, the line between benefit and toxicity is represented by the administered dose, differences in the cellular metabolism, dimension, and cultured cell lines, leading to differences in substances uptake which may result as a beneficial or toxic concentration to the cell.

Our *in vitro* experiment shows dose- and time-dependent effects of verbascoside on the spermatozoa quality parameters (Table 5).

At the end of the experiment (after 2 h of cultivation) concentrations (10^9/ml) of spermatozoa were similar between different dose-groups compared with the Ctrl group.

The spermatozoa motility (%) was significantly and negatively affected by verbascoside treatment, in particular in VB3 (after 1 h of cultivation) and VB4 doses (0 h). The Ctrl and VB1 (lowest dose) groups showed acceptable percentage of motility (around 60%) after 2 h of cultivation.

The percentage of progressive motility (%) resulted in a similar trend, as for the motility, in the experienced doses. VB3 and VB4 doses presented an altered value, compared with Ctrl group, from the beginning of the experiment, reaching the null value at the end. Ctrl value was more than 50% after 2 h of cultivation.

The distance average path (DAP, mm) analysis revealed that VB2 and VB1 doses were in line with the Ctrl group after 2 h of cultivation; whereas the others experienced doses showed a negative effect at the beginning of the experiment (for VB4 dose) and after 1 h of cultivation (for VB3 dose). Similar trend of values was observed for the velocity average path (VAP, μm/s), in which the VB1 and VB2 doses showed non-toxic effect compared with Ctrl group. VB3 and VB4 doses negatively and significantly affected the spermatozoa solution in a dose-manner way.

Measurement of the amplitude of lateral head displacement (ALH, μm) at the end of experiment was lower in the VB3 and VB4 groups compared with the Ctrl group. The other two experimental groups (VB1 and VB2) showed acceptable values, totally in line with the Ctrl group.

To the date, findings related to the application of verbascoside on spermatozoa cells have been scarce, making our results difficult to compare. In the literature, previous experience (Santoro et al. 2008) reported a genotoxic effect of verbascoside on human lymphocytes, attributing this biological property to its derivative (caffeic acid) after 72 h of cultivation. Also Dell'Aquila et al. (2014) reported a pro-oxidant effect of verbascoside on *in vitro* developmental potential of ovine prepubertal oocytes, with an increase of catalase activity and reactive oxygen species formation. Contrariwise, the research performed by Martino et al. (2016) showed protective effect of nanomolar concentration of verbascoside against reactive oxygen species affecting oocyte, resulting in enhanced embryo development.

CONCLUSION

In conclusion it can be stated that this study underlines a possible negative effect of verbascoside supplementation into feed mixture (0.1%) on semen quality parameters in rabbit bucks, obviously considering that target organs of antioxidant activities of phenylpropanoid glycosides are various. In addition it has to be emphasized that the extract showed a reversible action, since the semen traits of treated animals came back to the normality after the dietary administration period. For these reasons, the *Lippia citriodora* extract, used in the experiment, appears to be promising contraceptive agents. However, more detailed and extensive *in vivo* studies would be required to find out the exact biological pathway showing the anti-fertility activity, and further studies are planned in this direction. The obtained data from the present *in vitro* study proved that verbascoside at the concentration of 0.0024 and 0.0219 mg/ml has no adverse effect on spermatozoa, reported data similar to that of control group. Additionally, we found that verbascoside at higher concentrations (0.157 and 120.0 mg/ml) significantly altered all the motility parameters analysed in the experiment. Further researches are needed to confirm the current results, in order to understand and/or clarify the involved pathway responsible for the adverse effects.

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REFERENCES

Abe F., Nagao T., Okabe H. (2002): Antiproliferative constituents in plants 9. Aerial parts of Lippia dulcis and

https://doi.org/10.17221/35/2018-CJAS

Lippia canescens. Biological and Pharmaceutical Bulletin, 25, 920–922.

Attia Y.A., Hamed R.S., Bovera F., Abd El-Hamid A.E., Al-Harthi M.A., Shahba H.A. (2017): Semen quality, antioxidant status and reproductive performance of rabbits bucks fed milk thistle seeds and rosemary leaves. Animal Reproduction Science, 184, 178–186.

Casamassima D., Palazzo M., Vizzarri F., Ondruska L., Massanyi P., Corino C. (2017): Effect of dietary Lippia citriodora extract on reproductive and productive performance and plasma biochemical parameters in rabbit does. Animal Production Science, 57, 65–73.

Castellini C., Lattaioli P., Bernardini M., Dal Bosco A. (2000): Effect of dietary α-tocopheryl acetate and ascorbic acid on rabbit semen storage at 5°C. Theriogenology, 54, 523–533.

Castellini C., Lattaioli P., Dal Bosco A., Minelli A., Mugnai C. (2003): Oxidative status and semen characteristics of rabbit buck as affected by dietary vitamin E, C and n-3 fatty acids. Reproduction Nutrition Development, 43, 91–103.

Castellini C., Cardinali R., Dal Bosco A., Minelli A., Camici O. (2006): Lipid composition of the main fractions of rabbit semen. Theriogenology, 166, 703–712.

Clement C., Witschi U., Kreuzer M. (2012): The potential influence of plant-based feed supplements on sperm quantity and quality in livestock: A review. Animal Reproduction Science, 132, 1–10.

D'Alessandro A.G., Vizzarri F., Palazzo M., Martemucci G. (2017): Dietary verbascoside supplementation in donkeys: Effects on milk fatty acid profile during lactation, and serum biochemical parameters and oxidative markers. Animal, 11, 1505–1512.

Deepak M., Handa S.S. (2000): Anti-inflammatory activity and chemical composition of extract of Verbena officinalis. Phytotherapy Research, 14, 463–465.

de Lamirande E., Gagnon C. (1992): Reactive oxygen species and human spermatozoa I. Effect on the motility of intact spermatozia and on sperm axonemes. Journal of Andrology, 13, 368–378.

Dell'Aquila M.E., Bogliolo L., Russo R., Martino N.A., Filioli Uranio M., Ariu F., Amati F., Sardanelli A.M., Linsalata V., Ferruzzi M.G., Cardinali A., Minervini F. (2014): Prooxidant effects of verbascoside, a bioactive compound from olive oil mill wastewater, on in vitro developmental potential of ovine prepubertal oocytes and bioenergetic/oxidative stress parameters of fresh and vitrified oocytes. BioMed Research International, 2014: 878062.

Eid Y.Z. (2008): Dietary grape pomace affects lipid peroxidation and antioxidative status in rabbit semen. World Rabbit Science, 16, 157–164.

Eid Y., Ebeid T., Younis H. (2006): Vitamin E supplementation reduces dexamethasone-induced oxidative stress in chicken semen. British Poultry Science, 47, 350–357.

Eid Y., Zeweil H., Ahmed M., Basyony M., Farok M. (2011): Effect of plant source of omega-3 fatty acids and green tea powder on the performance and meat quality of growing rabbits. Egyptian Rabbit Science, 21, 115–134.

Foote R.H., Carney E.W. (2000): The rabbit as a model for reproductive and developmental toxicity studies. Reproductive Toxicology, 14, 477–493.

Funes L., Fernandez A.S., Laporta O., Pons A., Roche E., Segura C.A., Fernandez G.A., Micol V. (2009): Correlation between plasma antioxidant capacity and verbascoside levels in rats after oral administration of lemon verbena extract. Food Chemistry, 117, 589–598.

Herbert H., Ozoje M.O., Adejumo D.O. (2005): Effect of leucaena and gliricidia leaf meals on the seminal characteristics, testis weights and seminiferous tubule diameters of rabbits. Animal Research, 54, 173–178.

Lee J.H., Lee J.Y., Kang H.S., Jeong C.H., Moon H., Whang W.K., Kim C.J., Sim S.S. (2006): The effect of acteoside on histamine release and arachidonic acid release in RBL-2H3 mast cells. Archives of Pharmacal Research, 29, 508–513.

Lee J.Y., Woo E.R., Kang K.W. (2005): Inhibition of lipopolysaccaride-inducible nitric oxide synthase expression by acteoside through blocking of AP-1 activation. Journal of Ethnopharmacology, 97, 561–566.

Martino N.A., Ariu F., Bebbere D., Uranio M.F., Chirico A., Marzano G., Sardanelli A.M., Cardinali A., Minervini F., Bogliolo L., Dell'Aquila M.E. (2016): Supplementation with nanomolar concentrations of verbascoside during in vitro maturation improves embryo development by protecting the oocyte against oxidative stress: A large animal model study. Reproductive Toxicology, 65, 204–211.

Massanyi P., Chrenek P., Lukac N., Makarevich A.V., Ostro A., Zivcak J., Bulla J. (2008): Comparison of different evaluation chambers for analysis of rabbit spermatozoa motility parameters using CASA system. Slovak Journal of Animal Science, 41, 60–66.

Okab A.B., Samara E.M., Abdoun K.A., Rafay J., Ondruska L., Parkanyi V., Pivko J., Ayoub M.A., Al-Haidary A.A., Aljumaah R.S., Massanyi P., Lukac N. (2013): Effects of dietary seaweed (Ulva lactuca) supplementation on the reproductive performance of buck and doe rabbits. Journal of Applied Animal Research, 41, 347–355.

Paal D., Strejcek F., Tvrda E., Vasicek J., Balaz A., Chrenek P., Massanyi P. (2017): Taurine does not improve the quality of short-term stored rabbit spermatozoa in vitro. Reproduction in Domestic Animals, 52, 1046–1051.

Palazzo M., Vizzarri F., Cinone M., Corino C., Casamassima D. (2011): Assessment of a natural dietary extract, titrated

https://doi.org/10.17221/35/2018-CJAS

in phenylpropanoid glycosides, on blood parameters and plasma oxidative status in intensively reared Italian Hares (*Lepus corsicanus*). Animal, 5–6, 844–850.

Pastorelli G., Rossi R., Corino C. (2012): Influence of Lippia citriodora verbascoside on growth performance, antioxidant status, and serum immunoglobulins content in piglets. Czech Journal of Animal Science, 57, 312–322.

Santoro A., Bianco G., Picerno P., Aquino R.P., Autore G., Marzocco S., Gazzaro P., Lioi M.B., Bifulco M. (2008): Verminoside- and verbascoside-induced genotoxicity on human lynphocytes: Involment of PARP-1 and p53 proteins. Toxicology Letters, 178, 71–76.

Sgorlon S., Stradaioli G., Stefanon B., Altimer G., Della Loggia R. (2005): Dietary grape polyphenols modulate oxidative stress in ageing rabbits. Italian Journal of Animal Science, 4, 541–543.

Valentao P., Fernandes E., Carvalho F., Andrade P.B., Seabra R.M., Bastos M.L. (2002): Studies on the antioxidant activity of Lippia citriodora infusion: Scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid. Biological and Pharmaceutical Bulletin, 25, 1324–1327.

Xiong Q., Hase K., Tezuka Y., Tani T., Namba T., Kadota S. (1998): Hepatoprotective activity of phenylethanoids from Cistanche deserticola. Planta Medica, 64, 120–125.

Yousef M.I., Abdallah G.A., Kamel K.I. (2003): Effect of ascorbic acid and Vitamin E supplementation on semen quality and biochemical parameters of male rabbits. Animal Reproduction Science, 76, 99–111.

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