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The Effects of Supplementing Whole Milk with Juniper (*Juniperus oxycedrus)* Aromatic Water on Growth and General Health Parameters of Holstein Calves

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Abstract

The illness of the calves during the suckling period suppresses the growth of calves and causes them to be removed from the breeders. In the present study, it was investigated whether Juniper aromatic water (JOW) would be suitable for promoting healthy growth performance of suckling Holstein calves. Twelve newborn calves (n=3, in each group) were randomly selected and assigned to the following four treatments: Control, 50, 100 and 200 ml JOW supplemented milk per day. The supplementation of JOW showed an increase in calves' live weight and body measurements. In addition, the supplementation of whole milk with JOW decreased the frequency of occurrence of calves' diarrhea and disease. The best result was observed in the supplementation of whole milk with 50 ml JOW. The findings of the study showed that Juniper aromatic water, a by-product, can be used safely in the healthy rearing of calves.

Introduction

In newborn calves, resistance to diseases is low due to insufficient immune system. Antibiotic treatments applied during this period reduce the numbers of pathogens and also non-pathogenic bacteria. This causes the growth to stop or decline in the early period and therefore it causes economic losses (Soltan, 2009). The restriction and/or prohibition of the use of antibiotics in livestock have increased the interest in medicinal aromatic plants in recent years (Anadon, 2006).

The juniper, which is the subject of our study, has an important place in medicine manufacturing industry in Europe and many countries of the world because of its pharmacological properties medicine, due to and the extractive substances it contains. The juniper has been used as wood and material for making medicine from its fruits and leaves for many years in Anatolian geography as well as over the world. Medicines have been prepared from fruits and leaves for curing pain, cough, rheumatism, tuberculosis and it has also been used as antibacterial (Tumen and Hafizoglu, 2003). It has been determined that the JOW has selective antibacterial properties, also show antioxidant and iron-reducing properties depending on the concentration used (Isik *et al.,* 2020).

There are a few studies on the use of aromatic waters, a by-product of extracting plant extracts, in animal nutrition and breeding and their health effects. In this study, it was aimed to investigate the effects of aromatic water, which is produced as a by-product and has no economic value, on the healthy growth of calves while extracting oil from juniper (*Juniperus oxycedrus*) fruits and leaves.

Materials and Methods

The study was approved by the MAKÜ animal experiments local ethics committee with decision dated 04.10.2019 and numbered 507.

Animals and Dietary Treatments

The study was carried out in Isparta University of Applied Sciences, Faculty of Agriculture, Education,

Research and Application farm and 12 holstein calves were used, whose birth weight were the closest to each other. Power analysis method were used in determining the number of calves which was found as 3 calves in each group with the highest mean value of 4.73, the lowest mean value of 4.34 and the standard deviation of 0.3 for 95% power. In order to eliminate the colostrum effect, calves were experimented at the 4-day-old after suckling with colostrum for 3 days. Calves housed in individual boxes were provided by *ad libitum* to starter and clean water. A total of 4 l whole milk (WM) in the morning (2L) and in the evening (2L) was given to the calves in equal portions. Experimental groups were formed as follows: (CNT) control diet (4 l WM), (D1) control diet (4 l WM) + 50 ml aromatic water (1.25% JOW added milk), (D2) control diet (4 l WM) + 100 ml aromatic water (2.5% JOW added milk), (D3) control diet (4 l WM) + 200 ml aromatic water (5% JOW added milk). JOW doses were determined according to Minimum Inhibition Concentration analysis results (Isik *et al.* 2020). The chemical composition of starter and whole milk used in the study is presented in Table 1. The crude protein and ether extract ratio of starter used in the study was determined according to method 954.01 and 920.39, respectively (AOAC, 1990). Metabolic energy was calculated according to Turkish Standards Institution (TSE, 1991). Fat% (Method: IDF 141C:2000), protein%) method: IDF 141C:2000), lactose% (Methods: IDF 141C:2000) of WM were analyzed using a Bentley B150 milk analyzer (Bentley Combi 150, Bentley Instruments, Inc. Minnesota, Chaska, USA).

Sample Collection

Live weight and body measurements of calves were monitored weekly until weaning. Starter and water were drawn from the calves in the evening before the weighing, and the calves were weighed when their stomach were empty. The feed consumption of calves was recorded daily by using 1g sensitive electronic scales (TESS, Comak Tarti LTD., Turkey). In this way, calves consuming 800g of feed on consecutive days were determined and weaned.

Body measurements (Body length (BL), body depth (BD), withers height (WH), and hip height (HH) and chest girth (CG)) of calves were recorded weekly.

Blood samples were taken from vena jugularis of calves at the beginning of the experiment and at the weaning. Total cholesterol TC, Triglycerides TG, glucose GLU, Urea, Creatine CREA, Albumin ALB, Total protein TP, liver enzymes (Alanin aminotransferase ALT, Aspartate aminotransferase AST, Alkaline phoshatase ALP, Gamma-glutamyltransfarase GGT, Lactate dehydrogenase LDH) were determined in blood samples. Blood samples were centrifuged at 3000 rpm for 10 minutes, and the obtained blood serum was analyzed with Mindray BS120 Vet (Mindray Corporation, Nanshan, China).

The feces of calves were monitored daily and scored for consistency (Larson *et al.,* 1977): 1- normal, 2- soft, 3- fluid, 4- juicy. The number of days with diarrhea (NDD) and illness of calves (NID) has been recorded. When the feces score (FS) is 3 and above, the calf was registered with diarrhea. The respiratory rate (RR) and pulse rate (PR) of calves were recorded daily. Respiratory scores (RS) of calves were determined by Heinrichs *et al.* (2003): 1- normal, 2- mild cough, 3- moderate cough, 4- moderate to severe cough, 5- severe and chronic cough.

Production and Analysis of JOW

According to the method described in the European Pharmacopoeia (1975) a mixture of 200 g juniper fruits and leaves and 1.5 L tap water were placed in a Clevenger hydrodistillation device and JOW was separated from the juniper essential oil and collected in a distillation flask (5 L).

Total phenolic compounds of JOW were determined according to Singleton and Rossi (1965) using Folin-Ciocalteu colorimetric method and spectrophotometer (PG T80+ UV/VIS Spectrometer, PG Instruments Ltd. Leicestershire, UK) reading were made at 765 nm wavelength. Chromatographic analysis was used to reverse-phase high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) for the determination of JOW components (Caponio *et al.,* 1999).

Table 1. Chemical composition of WM which supplemented JOW and without JOW, and calf starter.

Ingredients, %	CNT	D1	D2	D3	Calf starter
DM	8.90	9.00	8.80	8.60	91.26
CP	3.20	3.20	3.20	3.10	17.55
EE	3.40	3.40	3.40	3.40	3.45
Lactose	4.90	4.90	4.90	4.70	
Freezing point(-^{o}C)	0.57	0.57	0.56	0.55	
pH	6.63	6.63	6.57	6.59	
ME, kcal kg^{-1}					2848.90

* DM: Dry matter, CP: Crude protein, EE: Ether extract, ME: Metabolic energy

Antiradical activity was determined using 1,1-diphenyl-2-picrylhydrazyil (DPPH) (Shimada *et al.,* 1992). 1 mL of 0.2 mM DPPH was added to 1 mL samples (at concentrations of 50, 100, 250 ppm) and mixed well with vortex.

The readings were made at 517 nm after 30 minutes in the dark environment and room temperature. The free radical of the samples was calculated using the formula: Antiradical activity (%): [(absorbance value of control – samples absorbance value) / (absorbance value of control)] x 100.

The iron reduction capacity was determined using the method of Oyaizu (1986). Accordingly, 2.5 mL of 200 mM sodium phosphate buffer (pH: 6.6) and 2.5 mL of 1% potassium ferricyanidine were added and mixed with a 2.5 mL sample. After the samples were kept at 50 ^{o}C for 20 minutes, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 200 rpm for 10 minutes.

A 5 mL of the upper phase was taken and 5 mL of deionized water and 1 mL of 0.1% ferric chloride were added on it. Then, the absorbance values of the samples at 700 nm wavelength were measured in the spectrophotometer. Comparison with synthetic antioxidants such as BHA (Butylated hydroxyanisole), BHT (Butylated hydroxytoluene) and Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) as high absorbance value indicates high iron reducing capacity. The iron binding strength at the samples was revealed.

Statistical Analysis

The data obtained in the study were analyzed by analysis of variance (ANOVA), and the differences between groups were examined with Tukey test (Minitab 2017 v 18.1, Minitab Ltd. UK).

Results

Components and Total Antioxidant Capacity of JOW

The components of the JOW and their percentage values are presented in Table 2. When the phenolic components of JOW were examined, it was observed that the highest component was α-cedrol (54.43%) and the lowest component was trans-pinocarveol (3.57%).

The oxidant capacity, iron reduction capacity and total phenolic content of the JOW are shown in Table 3.

The total content of JOW was obtained as 1.84 mg GAE/g. Antioxidant capacity and iron reducing capacity increased in parallel with the increase in concentration.

Table 2. Components and percentages of JOW.

Components	Percentages
α-Cedrol	54.43
Verbenone	20.16
Verbenol	14.90
Berneol	6.04
Trans-pinocarveol	3.57

Growth Performance of Calves

The effect of JOW supplementation on the growth performance of calves is shown in Table 4. The effect of JOW supplementation on the weaning age of calves was not found to be significant.

Table 3. Phenolic content, antioxidant capacity and iron reducing power of JOW.

	TPC mg GAE/g Mean±SEM	Antiradical % (50 µl/ml) Mean±SEM	Antiradical % (100 µl/ml) Mean±SEM	Antiradical % (250 µl/ml) Mean±SEM	Iron reducing (50µl/2.5ml) Mean±SEM	Iron reducing (250µl/2.5ml) Mean±SEM
JOW	1.84±0.01	29.49±0.02	45.40±0.10	70.43±0.13	0.49±0.01	4.46±0.02
Trolox	----------	85.20±0.20	91.30±0.30	97.25±0.25	3.32±0.02	10.93±0.03
BHT	----------	94.70±0.21	94.80±0.25	97.53±0.08	6.31±0.01	23.65±0.02
BHA	----------	97.30±0.23	96.75±0.15	98.70±0.20	3.62±0.02	18.52±0.02

* TPC: Total phenolic content, BHT: Butylated hyroxytoluene, BHA: Butylated hyroxyanisole

However, the trial groups tended to be weaned early compared to be CNT.

Numerical differences between initial and final live weight (LW) of calves were found to be significant ($P < 0.05$). Similarly, no significant differences were found between daily live weight gain (DLWG) and total live weight gain (TLWG) averages.

The effect of JOW supplementation on body measurements of calves was not found to be significant. However, it tended to improve the growth of calves.

The difference between the daily feed consumption (DFC) averages of groups was found to be significant ($P < 0.05$). While the highest DFC was obtained in D1, the lowest consumption was in CNT.

Healthy Parameters of Calves

The numerical difference among the averages of all values observed as health parameters of groups (Table 5) was found significant ($P < 0.05$).

Among these parameters, the highest average values for RR, PR and RT were observed in D3, while the highest RS value was obtained in CNT.

Results revealed that illness and diarrhea were less common in the calves in the trial groups compared to the CNT, and when Table 5 is examined, it can be seen that the FS, NDD and NID values were higher in the CNT than the others.

Table 4. Effects of JOW supplementation on calves' growth performance.

	CNT	D1	D2	D3	P value
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	
Weaning age, d	44.33±2.91	36.67±1.86	43.33±2.85	42.67±2.33	0.21
Initial LW, kg	42.33±5.67	42.17±1.69	41.17±1.64	40.33±1.86	0.97
Final LW, kg	56.17±6.72	56.33±1.17	57.67±2.35	54.67±4.76	0.97
DLWG, kg	0.313±0.02	0.386±0.01	0.386±0.04	0.330±0.09	0.63
TLWG, cm	13.83±1.09	14.16±0.88	14.50±0.76	14.33±4.38	0.85
TBLG, cm	7.50±1.04	9.33±0.67	11.00±1.80	9.67±1.69	0.41
TBDG, cm	3.33±0.16	3.83±0.88	5.33±1.09	6.10±0.44	0.08
TWHG, cm	5.16±0.60	8.67±1.30	7.83±1.01	6.66±0.72	0.12
THHG, cm	5.33±0.44	7.16±0.44	7.67±1.09	6.50±0.76	0.21
TCGG, cm	8.33±0.66	8.17±0.44	11.17±0.60	11.50±1.80	0.49
DFC, kg	$0.237±0.02^{B}$	$0.327±0.03^{A}$	$0.281±0.02^{AB}$	$0.246±0.03^{B}$	0.01

* LW: Live weight, DLWG: Daily weight gain, TLWG: Total live weight gain, TBLG: Total body length gain, TBDG: Total body depth gain, TWHG: Total wither height gain, THHG: Total hip height gain, TCGG: Total chest girth gain, DFC: Daily feed consumption, ^{A,B}: Shows the difference between averages on the same row

Table 5. Health parameters of calves.

	CNT	D1	D2	D3	P value
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	
RR	$79.06±0.49^{B}$	$72.89±0.63^{C}$	$70.98±1.06^{C}$	$82.03±0.32^{A}$	0.00
PR	$82.99±0.52^{B}$	$82.37±0.59^{B}$	$82.90±0.62^{B}$	$85.26±0.42^{A}$	0.00
RS	$1.15±0.03^{A}$	$1.00±0.00^{B}$	$1.05±0.02^{B}$	$1.01±0.01^{B}$	0.00
FS	$2.08±0.06^{A}$	$1.44±0.05^{B}$	$1.52±0.06^{B}$	$1.94±0.05^{A}$	0.00
RT, ℃	$38.54±0.03^{B}$	$38.39±0.03^{C}$	$38.52±0.03^{B}$	$38.82±0.03^{A}$	0.00
NDD	$10.00±2.31^{A}$	$0.33±0.33^{B}$	$3.33±3.33^{AB}$	$5.67±1.20^{AB}$	0.04
NID	$2.67±1.33^{A}$	$0.00±0.00^{B}$	$0.33±1.00^{B}$	$0.00±0.00^{B}$	0.03

* RR: Respiratory rate, PR: Pulse rate, RS: Respiratory score, FS: Feces score, RT: Rectal temperature, NDD: Number of days with diarrhea, NID: Number of illness days, $^{ABC:}$ Shows the difference between the averages in the same row.

Biochemical Blood Parameters of Calves

Blood serum biochemical values of calves are shown in Table 6. Numerical differences among observed blood serum biochemical values of all groups were not found to be significant.

Discussion

Components and Total Antioxidant Capacity of JOW

In our study, α-cedrol has the highest percentage in JOW components (Table 2) in line with Isik *et al.*'s (2020) study.

The total phenolic content of aromatic water obtained from different Juniper species shows differences. Indeed, Taviano *et al.* (2013) stated that the total phenolic contents of two different Juniper species used in their study were 5.14 and 17.89 mg GAE/g respectively, while Isik *et al.* (2020) and Miceli *et al.* (2009, 2011) reported the total phenolic contents of Juniper as 1.85 mg GAE/g and 17.64 mg GAE/g in their studies.

The impact of environmental conditions in the regions where Juniper grows may be the cause of this difference.

Many researchers reported that both essential oils and extracts obtained from Juniper species have high antioxidant properties and can be used instead of synthetic antioxidant (Elmastas *et al.,* 2006; Loizzo *et al.,* 2006; Miceli *et al.*, 2009; Lesjak 2011; Isik *et al*., 2020). Our findings revealed that JOW can be used as an alternative of synthetic antioxidants at a concentration of 250µl/ml, JOW (Table 3). Iron reduction capacity, which has an important place in determining antioxidant activity (Menconi *et al.*,

Table 6. Effects of JOW supplementation on calves blood parameters.

	CNT	D1	D2	D3	P value
	Mean ±SEM	Mean ±SEM	Mean ±SEM	Mean ±SEM	
Initial ALT	7.67±0.88	13.67±2.40	15.33±7.42	16.33±7.13	0.67
Final ALT	8.00±2.08	8.00±3.00	14.00±2.89	10.67±2.03	0.35
Initial AST	51.30±27.50	56.00±14.60	30.30±24.10	141.00±103.00	0.53
Final AST	75.00±16.80	43.70±22.40	175.00±100.00	49.70±22.20	0.33
Initial GGT	641.00±298.00	766.00±272.00	728.00±163.00	1414.00±247.00	0.19
Final GGT	35.00±8.14	56.00±18.50	38.00±4.04	44.33±4.10	0.53
Initial ALP	216.70±63.00	340.30±50.00	235.70±25.00	171.30±7.70	0.24
Final ALP	150.00±12.50	277.30±67.50	206.70±12.50	117.70±27.50	0.07
Initial GLU	125.70±11.20	113.70±16.60	116.70±16.20	115.00±3.06	0.91
Final GLU	99.00±7.00	98.00±7.64	96.62±6.36	92.33±2.33	0.87
Initial TC	60.30±28.70	59.30±39.40	41.30±23.60	83.67±2.60	0.75
Final TC	151.70±20.30	107.70±49.40	138.30±56.60	137.00±58.20	0.92
Initial CREA	1.38±0.22	1.32±0.08	1.32±0.06	1.54±0.34	0.86
Final CREA	1.20±0.08	1.40±0.06	1.50±0.10	1.22±0.15	0.24
Initial UREA	52.10±16.80	29.30±15.40	26.30±21.00	72.40±26.60	0.47
Final UREA	47.30±23.40	19.30±10.80	53.40±24.50	50.40±14.00	0.59
Initial TP	8.36±0.49	7.60±0.38	7.59±0.57	7.62±0.24	0.56
Final TP	7.72±0.33	7.45±0.07	6.89±0.26	6.96±0.35	0.18
Initial ALB	2.84±0.19	2.76±0.09	2.97±0.17	2.73±0.02	0.62
Final ALB	3.39±0.07	3.47±0.07	3.26±0.21	3.34±0.15	0.77
Initial LDH	648.00±35.70	651.30±79.70	763.00±152.00	821.00±161.00	0.68
Final LDH	579.00±275.00	713.70±57.30	943.70±88.00	822.30±19.30	0.40
Initial TG	35.00±12.70	60.00±18.20	47.67±9.74	46.70±11.70	0.64
Final TG	27.33±7.31	37.33±8.45	34.67±5.17	22.67±7.84	0.50

1995), increased in parallel with the increase in concentration of JOW (Table 3). Similarly, many researchers determined that the iron reduction capacity increases with an increase in the concentration (Elmastas *et al.*, 2006; Djeridane *et al*., 2006; Miceli *et al*., 2009; Isik *et al.,* 2020).

Growth Performance of Calves

The appetizing properties of medicinal herbs and their extracts stimulate digestion and increase feed consumption by promoting gastric and intestinal motility by increasing the release of enzymes.

Increasing feed consumption allows calves to be weaned at an early age (Tekeli *et al.*, 2006; Kehoe *et al.*, 2007; Ozkaya *et al.*, 2018).

It has been reported that the reason that aromatic waters obtained from medical herbs improve the live weight of calves may be due to the positive effect of aromatic waters on the intestinal flora (Tiihonen *et al.,* 2010; Sharma *et al.,* 2013; Ozkaya *et al.,* 2018). Chaves *et al.* (2008) have been stated that medicinal aromatic waters increase the digestibility of foods by increasing the total essential fatty acids concentration in the rumen and thus have positive effect on live weight gain. Herbal oils and extracts significantly increased the LW of calves (Ahmed *et al.*, 2009; Soltan, 2009; Ghosh *et al.*, 2010; 2011). However, in the study conducted with oregano aromatic water (OAW), Ozkaya *et al.* (2018) reported that the effect of OAW is not significant on the increase in LW of calves.

It was reported by Ozkaya *et al.* (2018) that the supplementation of OAW as a milk replacer tends to improve the body measurements of calves, but the numerical differences are not significant and supplementing JOW to milk showed similar results. Similarly, Unlu and Erkek (2013) reported that effect of oregano oil supplementation on the body measurements of calves is not significant.

Herbal extracts improve the digestion of feeds by increasing saliva, gall and enzyme activities. However, it increases the digestion and absorption capacity of the intestine by increasing the ability of epithelium cells to regenerate villi as a result of suppression pathogenic bacteria in the intestine (Maurao *et al.,* 2006). The findings obtained in the study are line with Ghosh *et al*. (2010; 2011) who reported that herbal extracts significantly increased feed consumption. However, there are studies reporting that herbal extracts and aromatic waters do not effect feed consumption (Soltan, 2009; Unlu and Erkek, 2013; Ozkaya *et al*., 2018). The difference between these results may cause systemic losses due to mucosal secretion of herbal extracts (Jamroz *et al.,* 2006).

Healthy Parameters of Calves

Supplementation oregano aromatic water (OAW) has no effect on the RR of calves (Ozkaya *et al.,* 2018). Calves have higher RRs than adults (Plumb, 2005). However, 200 ml JOW supplementation significantly increased the RR of calves (Table 5). The fact that the NID was low in the trial groups compared to the CNT indicated that the high RR was not associated with the disease. It is thought that calves being more active than adults increase RR.

It has been reported that OAW supplementation significantly increases PR of calves (Ozkaya *et al.,* 2018). Just as increase in activity increases the RR, it also increases the PR (Akgun, 1989). Calves have higher PRs than adults, just like their RRs (Plumb, 2005). For this reason, it is thought that the reason why PRs higher in trial groups compared to CNT is due to the active and excited calves rather than the disease.

Although the difference among the RT of the groups was found to be significant (Table 5), the average values remained within the normal limits (38.6-39.4 ^{o}C) for calves (Latimer *et al*., 2003). It has been reported by many researchers RT rises above 39.5 ^{o}C in both respiratory and digestive system disease (Griffin, 1997; Smith, 2000; Gunes, 2018).

Due to the antibacterial properties and iron reduction capacity of oils and extracts of aromatic plants, they are positive effects on intestinal bacterial flora and suppress pathogenic bacteria. Therefore, plant oil and extracts significantly reduce the FS (Ishihara *et al*., 2001; Goetz *et al*., 2002; Lewis *et al*., 2003; Ghosh *et al*., 2010; 2011; Ozkaya *et al*., 2018). This explains the low FS in the trial groups (Table 5). However, there are studies indicating that oil and extracts obtained from plants have no effect on the FS (Greathead *et al*., 2000; Bampidis *et al*., 2005; Unlu ve Erkek, 2013).

Digestive system diseases suppress the growth of calves and cause death (Davis and Drackley, 1998; NAHMS, 2007). The antibacterial properties of herbal extracts suppress the growth of pathogenic bacteria such as *E. coli, Coliforms* and *Enterobacteriaceae* in the intestinal flora, thereby improving the immune system and reducing the incidence of diarrhea (Ahmed *et al*., 2009; Ozkaya *et al.*, 2018). The reason for the low NDD

is thought to be the suppression of the growth of pathogenic bacteria due to the antibacterial effects of JOW (Table 5).

Oils and their extracts obtained from medicinal aromatic plants support the immune system and reduce the incidence of disease cases in calves (El-Ashry *et al.*, 2006; Ahmed *et al*., 2009; Franz *et al*., 2010; Li *et al.,* 2012a; 2012b; Wang *et al*., 2012; Sajjadi *et al*., 2014, Zeng *et al*., 2015; Ozkaya *et al*., 2018). The reason for the low NID in the trial groups may be associated with an immune system supporting effect of JOW (Table 5).

Conclusion

The supplementation of JOW tended to improve the growth performance, increased feed consumption, reduced the incidence of diarrhea and disease of calves and enabled healthy calves rearing. The fact that no negative effects were observed in blood serum biochemical variables which is an indicator of the healthy growth and good animal welfare for calves. It was concluded that applied JOW doses could be used for rearing healthy calves, but calves reared with 50 ml JOW supplemented milk (1.25% JOW supplemented milk) showed healthier growth calves compared to other groups.

It is recommended to examine the effects of JOW supplementation on fecal bacteria count, immune system and antioxidative defense parameters in order to better reveal the effects of JOW on both the growth and health of calves.

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