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Effect of *Oreganum onites* L. Aromatic Water Supplemented to Whole Milk on Performance, Blood Metabolites and Oxidative Status of Holstein Calves During the Pre-Weaning Period

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SO designed and executed the experiment and wrote the first draft of the manuscript. OO collected blood from calves and analyzed. SE obtained aromatic water from Oregano leaves. IGY evaluated blood results. DP and WN analyzed the data and revised the manuscript.

Key words
Oxidative status, Antioxidative status, *Oreganum onites* L., Aromatic water, Pre-weaning period, Holstein calves

ABSTRACT

In intensive growing systems, the growth and yield of livestock are suppressed due to the oxidative stress to which they are also exposed. In the present study, it was investigated whether *Oreganum onites* L. aromatic water (OW) could serve as an agent of reducing the effects of oxidative stress while improving the growth performance of suckling Holstein calves. Twenty-eight newborn calves (n=7, in each group) were randomly selected and assigned to the following four treatments: Control, 40, 60 and 80 ml OW supplemented whole milk (WM) per day. The applied 60 and 80 ml OW tended to improve the antioxidative stress mechanism of calves while supporting their growth (e.g., total and daily weight gain, body measurements). The findings of the present study showed that *Oreganum onites* L. aromatic water, a by-product, can be used safely in alleviating the problems such as oxidative stress that may occur in intensive growing systems.

INTRODUCTION

Calf rearing requires a sensitive and important management system in cattle farms. Thus, feed additives to protect the health of newborns and improve their performance are very important in terms of farming (Seifzadeh *et al*., 2017). Livestock is exposed to oxidative stress which frequently causes protein, lipid and DNA damage due to intensive farming conditions (McCall and Frei, 1999). Feeding cattle with medicinal plants is beneficial for their welfare and reduces their stress (Hosoda *et al*., 2006). Medicinal plants have started to be used in recent years due to the ban of antibiotics use as a feed additive (Anadon, 2006). Many herbal products are based mainly on oregano that possesses intense antimicrobial and antioxidant properties (Pizzale *et al*., 2002; Ozkaya *et al*., 2018). The leafy parts of oregano and its essential oil are used as a preserver as well as flavor and aroma additive in foods and at the same time oregano is used as a medication in traditional treatment methods (El-Nekeety *et al*., 2011). Herbs and spices can prevent lipid peroxidation through their antioxidant compounds. The antioxidative properties of plants and spices depend on the content of phenolic substances (flavonoids, hydrolysable tannins, proanthocyanidins, phenolic acids, phenolic terpenes) and some vitamins (E, C and A). Herbs such as rosemary, thyme, oregano, sage, green tea, chamomile, ginko, dandelion and marigold are rich in these phenolics compounds (Pizzale *et al*., 2002; Ozkaya *et al*., 2018; Halliwell *et al*., 1995; Craig, 2001; Baydar *et al*., 2004;

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Cetkovic *et al*., 2004; Skerget *et al*., 2005; Bakirel *et al*., 2008; Fasseas *et al*., 2008; Ozkan *et al*., 2010).

There are studies on herbs and spices which illustrate their effects on health and performance of livestock. Herbal feed additives increase the daily weight gain by improving the dry matter intake of calves, however, did not affect the feed conversion ratio. In addition to all these findings, herbal additives positively affected the health status of calves (Seifzadeh *et al*., 2017). Herbal feed additives (mint, clove, lemongrass) added to the diets of Holstein steers positively affected nutrient metabolism, antioxidant activity, immune system and ruminal fermentation (Hosoda *et al*., 2006), it was reported that rumen fermentation is manipulated, however, this modification had no effect. The supplementation of oregano aromatic water to calf milk replacer has no effect on live weight and body measurements, however, it tends to improve the immune system of calves and therefore has a positive effect on general health profile of calves (Ozkaya *et al*., 2018). It has been reported that oregano essential oil does not affect the weight gain and feed intake of male rats, however, it has a protective effect against aflatoxin toxicity and thus is a potential antioxidant (El-Nekeety *et al*., 2011). In a study conducted with oregano essential oil, it was reported that the oil could be an appropriate to manipulate rumen fermentation, however, it had no effect on daily weight gain, dry matter intake and feed conversion ratio of calves (Vakili *et al*., 2013). Cumin and garlic, which are used as herbal feed additives, improved the general health status, metabolic functions, performance and nutrient digestibility of buffalo calves (Hassan and Abdel-Rahem, 2013). Oregano essential oil can be used as an ideal liquid feed additive to improve feed efficiency and growth performance and also reduce the incidence of diarrhea cases and facilitate early weaning of calves (Tapki *et al*., 2020). Plant extracts (carvacrol, capsicum oleoresin and cinnamaldehyde) added to young pig rations exposed to oxidative stress conditions showed antioxidative properties (Frankic *et al*., 2010). In our previous study, we investigated the effect of *Oreganum onites* L. aromatic water supplemented to milk replacer on performance, immunity and general health profiles of calves. However, in current study, we hypothesize that supplementation of *Oreganum onites* L. aromatic water to milk would support its antioxidative defense mechanism of calves. As far as we know, no studies have been conducted to investigate the effects of *Oreganum onites* L. aromatic water supplemented to milk on nutritional oxidative stress in calves (It is an imbalance between pro-oxidant load and antioxidant defense as a result of excessive oxidative load or of inadequate supply of the organism with nutrients). Therefore, the focus of our research was to investigate the effects of *Oreganum onites* L. aromatic water on oxidative stress as well as growth performance of suckling calves.

MATERIALS AND METHODS

Animals and dietary treatments

The experiment was performed in Osmanli Farm, Serik, Antalya and its protocol was reviewed by the Akdeniz University Local Committee on Animal Research Ethics (Protocol number: 2015.01.004), Antalya, Turkey. The study was carried out with calves born between October 2014 and February 2015 in the Osmanli Dairy Cattle Farm in Karincali village of Serik district of Antalya, Turkey.

Twenty-eight newborn Holstein calves were penned in individual boxes in a naturally ventilated barn. The calves were randomly selected and allocated into four groups (n=7; the power analysis method was used to determine the number of calves in the groups with the highest mean value for *Enterobacteriaceae* count in the gut was 6.31, the lowest mean value was 4.91 and the standard deviation was 1.09) under natural ventilation and lighting conditions. Four-day-old calves were included in the experiment after feeding with colostrum for 3 d in order to eliminate the effect of the colostrum. The calves had free access to a starter and clean water. A total of 4L whole milk (WM) in the morning (2L) and in the evening (2L) was provided to the calves in equal portions. Treatment included: (CNT) control diet (4L WM), (T_2) control diet (4L WM) + 40 ml aromatic water, (T_3) control diet (4L WM) + 60 ml aromatic water, (T_4) control diet (4 l WM) + 80 ml aromatic water according to Ozkaya *et al*. (2018). Chemical composition of ration used in the present study is shown in Table I. The crude protein ratio of starter was determined by using Kjeldahl method (20; method: 954.01) and ether extract was determined by using Soxhlet method (20; method 920.39). Neutral detergent fibre (NDF), Acid detergent fibre (ADF) and Acid detergent lignin (ADL) were analysed using the ANKOM220 Fibre Analyser (Ankom Technology, Macedon, NY, USA). Metabolic energy was calculated according to Turkish Standards Institution (1991). Fat% (Method: IDF 141C:2000), protein% (method: IDF 141C:2000), lactose% (Methods: IDF 141C:2000) of WM were analysed using a Bentley B150 milk analyser (Bentley Combi 150, Bentley Instruments, Inc. Minnesota, Chaska, USA).

Sample collection

Calves were weighed after fasting of 12 h and BW was recorded weekly. Daily feed intake (DFI) of calves was determined by weighing at the same time every day. Calves were weaned when they started consuming an average 800 g of starter for three days consecutively.

Daily weight gain (DWG) was calculated as

Total BW gain (kg)/total days of the study

Feed conversion ratio (FCR) was calculated as

Total dry matter intake (DMI) (kg)/total BW gain (kg).

The calves body growth was monitored weekly. Body length (BL, cm), body depth (BD, cm), withers height (WH, cm), ad hip height (HH, cm) was determined with measuring stick and chest girth (CG, cm) was determined by measuring tape (Ozkaya *et al*., 2018).

Blood samples were collected from jugular veins of calves at the weaning. Blood samples were placed into non-additive tubes to measure biochemical blood parameters (alanin aminotransferase, ALT; aspartate aminotransferase AST; alkaline phoshatase, ALP; glucose, GLU; Urea; total cholesterol, TC; creatine, CREA; uric acid, UA; triglycerides, TG and total antioxidant capacity, TAC; total oxidant capacity, TOC) and transferred to laboratory with ice packs. Serum was separated by centrifugation at 3000 rpm for 10 min. Biochemical blood parameters were analysed by Mindray BS120 Vet (Mindray Corporation, Nanshan, China).

Table I. Composition of WM supplemented with and without OW and composition of calf starter.

Ingredients, (g kg^{-1})	CNT	T_2	T_3	T_4	Calf starter*
Dry matter	128.00	127.00	126.50	126.00	882.00
Crude protein	22.0	21.0	22.0	22.0	181.0
Ether extract	3.02	3.00	3.01	3.02	59
Ash	5.50	5.45	5.49	5.46	70.80
Lactose	45.30	43.70	43.20	43.60	
P (mg/kg)	1936	2067	2324	1948	
Ca (mg/kg)	2019	2086	2173	2060	
Freezing point (-ºC)	0.57	0.58	0.55	0.52	
Acid detergent lignin					34.3
Neutral detergent fibre					298.0
Acid detergent fibre					121.0
Metabolic energy, kcal kg^{-1}					2779.5

*Components: Wheat bran, corn, sunflower seed meal, molasses, soybean meal, barley, marble powder, rye, salt, vitamin-mineral premix

TAC and TOC determination in blood serum

Rel Assay kits (Baran Medical, Turkey) were used for TAC and TOC measurements. TAC and TOC measurements were performed by an automated method developed by Erel (2004, 2005). TAC is a method that measures the total antioxidant capacity of the living systems against free radicals and TOC is a colorimetric method (Mercan, 2004; Erel, 2005). Oxidative stress index (OSI) which is an indicator of the degree of oxidative stress was calculated according to the following formula (Mercan, 2004; Kamiloglu *et al*.,2011):

OSI = TOC / (TAC x 10)

TAC and TOC parameters were analysed by T80+ UV/VIS Spectrometer (PG Instruments Ltd. Leicestershire, UK).

Production and analysis of Oreganum onites *L. aromatic water*

According to the method described in the European Pharmacopoeia (1975) a mixture of 500g dried oregano leaves collected in Isparta province and 2.5 L tap water were placed in a Clevenger hydrodistillation device and OW was separated from the oregano oil and collected in a distillation flask (750 ml).

Total phenolic compounds of OW were extracted (Tassoni *et al*., 2005) and were determined spectrophotometrically (PG T80+ UV/VIS Spectrometer, PG Instruments Ltd. Leicestershire, UK) by Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Chromatographic analysis was used to reverse-phase high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) for determination of phenolics (Capanio *et al*., 1999).

To determine the free radical activity of the OW, the method of Shimada *et al*. (1992) was used.

Statistical analysis

Statistical analysis of the collected numerical material was carried out using one-way analysis of variance. For this purpose, the GLM procedure was used (SPSS 16, SPSS Inc., Chicago, IL, USA). The differences between group means were analyzed with the Tukey test.

RESULTS

Carvacrol and thymol values of oregano aromatic water were 99.1% and 0.9%, respectively.

The OW had no significant effect on the performance of calves (Table II). Final BW was the highest (61.83 kg) for T_3 and the lowest (55.50 kg) for T_2. Total WG was the highest (18.17 kg) for T_4 and the lowest (11.67 kg) for T_2. The highest DWG (0.493 kg) was found in T_4 among others (P = 0.36). These numerical differences were not found to be significant.

DFI and FCR were not affected significantly by OW supplementation (Table II). DFI and FCR were the highest (0.425 kg and 2.22, respectively) for T_2 while they were the lowest (0.352 kg and 1.71, respectively) for CNT. DM and Crude protein intake (CP) were not affected significantly by OW supplementation (Table II). The highest DMI and CPI were found 2.70 and 0.300 kg/d for T_2 and the lowest were found 1.84 and 0.190 kg/d for CNT.

Table II. Effects of OW supplementation on calves' growth performance (Mean±S.E).

Growth parameters	CNT	T_2	T_3	T_4	P value
Initial body weight, (kg)	40.83±1.42	43.83±2.09	44.33±4.15	39.67±3.71	0.66
Final body weight, (kg)	55.67±0.83	55.50±1.76	61.83±1.76	57.83±2.59	0.13
Total weight gain, (kg)	14.83±1.92	11.67±0.33	17.50±3.88	18.17±1.30	0.24
Daily weight gain, (kg)	0.398±0.02	0.387±0.04	0.454±0.08	0.493±0.01	0.36
Daily feed intake, (kg)	0.352±0.02	0.425±0.03	0.406±0.03	0.405±0.06	0.59
FCR (feed intake: BW), (kg/kg)	1.71±0.06	2.22±0.36	1.74±0.37	1.78±0.06	0.51
FCR of DM	1.84±0.07	2.70±0.47	1.96±0.50	2.16±0.14	0.38
FCR of CP	0.190±0.01	0.300±0.05	0.212±0.05	0.237±0.28	0.30

FCR, Feed conversion ratio; DM, Dry matter; CP, Crude protein; CNT, control. For composition of T_2, T_3 and T_4, see Table I.

Table III. Effects of OW supplementation on calves' body measurements (Mean±S.E).

Measurements	CNT	T2	T3	T4	P value
Body length, (cm)					
Initial	69.67±0.33	69.50±1.04	70.50±2.25	67.00±2.47	0.56
Final	76.50±0.50	76.33±1,36	79.67±1.96	77.17±2.13	0.48
Difference	$6.83±0.44^{B}$	$6.83±0.73^{B}$	$9.17±0.67^{AB}$	$10.17±0.60^{A}$	0.01
Wither height, (cm)					
Initial	78.50±0.29	77.67±1.20	78.67±0.88	76.33±1.86	0.42
Final	84.00±0.58	83.83±1.17	86.17±1.42	83.67±1.20	0.53
Difference	5.50±0.87	6.17±0.73	7.50±1.44	7.33±0.88	0.49
Body depth, (cm)					
Initial	28.00±0.58	29.67±0.33	29.33±1.33	28.00±1.15	0.50
Final	32.17±0.73	33.17±0.60	34.17±0.60	33.17±0.60	0.25
Difference	4.17±0.73	3.50±0.76	4.83±1.01	5.17±0.60	0.49
Hip height, (cm)					
Initial	80.33±0.88	80.50±1.26	80.67±1.20	78.00±2.03	0.52
Final	87.00±0.58	86.50±1.32	90.00±1.26	87.17±1.36	0.23
Difference	6.67±1.45	6.00±0.58	9.33±0.83	9.17±1.48	0.49
Chest girth, (cm)					
Initial	74.00±1.53	75.00±1.00	76.00±3.06	72.67±2.19	0.72
Final	$84.00±0.01^{AB}$	$82.17±0.83^{B}$	$87.00±1.00^{A}$	$84.67±1.20^{AB}$	0.03
Difference	10.00±1.53	7.17±0.93	11.00±2.89	12.00±1.00	0.32

^{A,B} Means in a same row with different superscripts are significantly different ($P<0.05$). For composition of T_2, T_3 and T_4, see Table I.

Although the differences among groups for BL were not significant in weaning, the difference between initial and final BL was significant (Table III); it had greater values in T4 compared to CNT and T2 groups. Also, differences among groups for CG in weaning were significant ($P < 0.05$); T3 had greater values than T2 group. When body measurements were taken into consideration, an increasing tendency was observed in OW groups compared to the CNT.

Effects of OW on blood parameters are shown in Table IV. Blood parameters were not affected by OW except AST. T3 calves had significantly lower AST compared to T2 and T4 group ($P<0.01$).

OW affected TAC vaue not significantly ($P = 0.74$) compared to the CNT (Table III). A non-significant difference between TOC and OSI values was obtained. When the results were evaluated in general, a non-significant decrease in TOC level and an increase in TAC level were observed in the OW groups compared to the control group (Table III).

Table IV. Effects of OW supplementation on calves blood parameters and nutritional oxidative stress (Mean±S.E).

Biochemical components	CNT	T_2	T_3	T_4n±S.E.	P
Alanine aminotransferase (µ/L)	11.33±3.28	13.67±0.67	10.33±3.18	27.7±1.19	0.26
Aspartate aminotransferase (µ/L)	67.83±4.98^{AB}	71.67±2.91^{A}	56.00±0.58^{B}	79.00±2.31^{A}	0.01
Alkaline phosphatase (µ/L)	441.0±51.7	435.0±41.4	389.7±47.3	424.7±58.3	0.89
Glucose (mg/dL)	104.0±9.45	107.0±9.35	103.0±2.33	90.0±1.53	0.35
Urea (mg/dL)	24.70±2.76	25.97±9.53	19.57±6.73	23.53±6.37	0.92
Total cholesterol (mg/dL)	129.4±12.3	130.3±10.8	156.64±6.78	144.16±4.45	0.19
Creatine (mg/dL)	1.92±0.12	1.69±0.27	1.74±0.24	1.70±0.06	0.82
Uric acid (mg/dL)	1.00±0.00	1.00±0.00	0.67±0.33	1.00±0.00	0.44
Triglyceride (g/mL)	70.86±7.39	48.40±20.80	64.50±17.80	54.82±8.17	0.72
Total oxidant capacity (µmol H_2O_2/L)	3.43±1.89	2.53±0.69	2.11±1.60	2.29±1.00	0.92
Total antioxidant capacity (mmol Trolox Equivalent/L)	2.81±0.28	3.29±0.96	4.82±2.29	3.82±0.88	0.74
Oxidative stress index (TOC/TAC*10)	0.12±0.08	0.11±0.69	0.09±0.09	0.06±0.04	0.95

^{A,B}Means in a same row with different superscripts are significantly different (P<0.05). For composition of T_2, T_3 and T_4, see Table I.

DISCUSSION

Carvacrol and thymol values of oregano aromatic water were 99.1% and 0.9%, respectively.

No significant increase in BW of calves was observed at final weight (Table II). These results are similar with the studies reporting that the supplementation of various plants and extracts has no effects on the BW of calves (Hassan *et al*., 2013; Vakili *et al*., 2013; Ozkaya *et al*., 2018; Tapki *et al*., 2020). Seifzadeh *et al*. (2017) indicated that the medical plant mixtures increased BW. Total WG was the highest (18.17 kg) for T_4 and the lowest (11.67 kg) for T_2. The highest DWG (0.493 kg) was found in T_4 among others. Seifzadeh *et al*. (2017) reported that medical plant mixtures increased DWG while Hassan and Abdel-Rahem (2013), Vakili *et al*. (2013) and Ozkaya *et al*. (2018) reported that the increase was not found statistically significant. Tapki *et al*. (2020) reported that oregano essential oil significantly increased total weight gain and DWG of calves. In some studies, conducted with broilers, it was reported that oregano essential oil had no effect on live weight gain (Alp *et al*., 2012; Bozkurt *et al*., 2016) while some reported an improved live weight gain (Mohiti-Asli and Ghanaatparast-Rashi, 2015).

DFI and FCR were not affected significantly by OW among groups (Table II). DM and CP were not affected significantly by OW among groups (Table II). Hosoda *et al*. (2006) reported that the supplementation of clove, peppermint and lemongrass increased DMI ($P < 0.05$). Similarly, Seifzadeh *et al*. (2017) reported that medical plant mixtures increased DMI. In contrast, Hassan and Abdel-Rahem (2013) and Vakili *et al*. (2013) reported that DMI increase was not significant a finding that is similar to our study. Seifzadeh *et al*. (2017), Hassan and Abdel-Rahem (2013), Vakili *et al*. (2013) and Frankic *et al*. (2010) reported that the increase in FCR was not significant, as indicated in our study. Tapki *et al*. (2020) reported that the supplementation of oregano essential oil significantly reduced DFI, DMI and FCR of calves. The supplementation of oregano essential oil improved FCR in broilers (Alp *et al*., 2012; Bozkurt *et al*., 2014; Mohiti-Asli and Ghanaatparast-Rashi, 2015).

Effects of OW on body measurements of calves are shown in Table III. The difference between initial and final BL and also final CG was found significant. Ozkaya *et al*. (2018) reported that the supplementation of OW to milk replacer had no significant effect on body measurements of calves, but they observed a numerical increase compared to the control group.

Effects of OW on blood parameters are shown in Table IV. Blood parameters were not affected by OW except AST. Calves which were provided with diet T_3 have significantly the lowest (P=0.01) AST in serum among others. In a study, which cloves, peppermint and lemaongrass were supplemented, cholesterol value increased by the supplementation of clove, however, the blood metabolites were not affected by the peppermint, lemongrass and the mixture of these three (Hosoda *et al*., 2006). In addition, thyme and cinnamon supplement did not affect blood metabolites (Vakili *et al*., 2013). Seifzadeh *et al*. (2017) observed that the supplementation of 3% of medical plant mixture increased the TP value but did not affect the glucose, cholesterol, triglyceride and albumin values. Hassan and Abdel-Rahem (2013) reported that cumin and garlic increased TP ($P < 0.05$) value but decreased cholesterol ($P < 0.05$), although they

observed that ALT and AST values decreased, which was not significant. Ozkaya *et al*. (2018) observed that the supplementation of OW to milk replacer had no effect on the biochemical blood parameters of calves.

When the results are evaluated in general, a non-significant decrease in TOC level and a non-significant increase in TAC level are observed in OW groups compared to control (Table III). TAC included enzymes such as superoxide dismutase, catalase and glutathione peroxidase and macromolecules such as albumin, ceruloplasmin and ferritin. TAC provides more convenient biological information compared to that obtained by measuring individual components because it considers the cumulative effect of all antioxidants present in plasma and body fluids (El-Nekeety *et al*., 2011). The decrease in TAC level indirectly indicates an increase in damage due to oxidative stress (Abdel-Wahhab *et al*., 2010; Choi *et al*., 2010). In this context, current study showed that OW increased TAC levels of calves and could be used as a protection against oxidative stress. It has been reported that rats fed by aflatoxin-contaminated diet decreased TAC levels in liver damage caused by aflatoxin, but applied *Thymus vulgaris* essential oils increases TAC levels and that this essential oil is a potential antioxidant and it protects against aflatoxin toxicity (El-Nekeety *et al*., 2011). Frankic *et al*. (2010) observed that the mixtures did not affect the TAC value and the results were close to each other.

The high the OSI level in CNT may be due to the high oxidative capacity and the low of antioxidant capacity (Ogut *et al*., 2013). It was reported that the OSI value decreased or increased parallel with the TOC value (Daggulli *et al*., 2014; Marcil *et al*., 2013; Yucel *et al*., 2015).

As far as we observed, there have been no studies published on the effects of OW supplementation on oxidative stress of calves. While we have used *Oreganum onites* L. aromatic water in our study, some studies have used oregano and thyme essential oils and some have used essential oils of other herbs and leaves. Therefore, it is difficult to compare *Oreganum onites* L. aromatic water to others.

CONCLUSIONS

Applied doses 60 and 80ml OW showed tendency to increase calves' growth performance. The results of this work indicate that supplementation of OW reduced oxidative stress. OW has a potential antioxidant activity and a protective effect on the oxidative stress and this protection was dose-dependent. It can be concluded that, better dose to feed calves is WM, which contains 60-80 ml OW, which is better to prevent oxidative stress. The use of *Oreganum onites* L. aromatic water can support the antioxidative defense mechanism and reduce the effect of oxidative stress that often occurs in intensive livestock production, thus enabling healthier calves to be raised, as well as providing economic husbandry by reducing health expenses of the enterprise. In addition to all these, the use of *Oreganum onites* L. aromatic water, which is a by-product, in animal husbandry will increase its added value and contribute to the national economy.

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Statement of conflict of interest

No potential conflict of interest was reported by the authors.

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