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Effects of different levels of dietary *Citrus Limon* essential oil on some blood parameters and antioxidant status in Afshari Ewes

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Abstract: Citrus Limon Oil (CLO) is known as antioxidant resource and contains limonoids, flavonoids and phenolic compounds. This study was conducted to investigate the effects of different levels of CLO on blood parameters and antioxidant status in Afshari ewes. Six adults Afshari ewes (3-4 years old and 51±5 kg) were randomly allocated to 3×3 Latin square design with three diets in 21 days period. Dietary treatments included:1) control diet, 2) control diet with 200 mg/day CLO, and 3) control diet with 400 mg/day CLO. To evaluate the antioxidant effect of the CLO, sustainable elimination of free radicals by DPPH and ABTS methods were used. The antioxidant activity of essential oils in DPPH method at doses of 32.5, 45, 130, 260 and 520 mg/ml were 9, 16, 31, 49 and 89%, respectively. Also, antioxidant activity of essential oils in ABTS method at doses of 32.5, 45, 130, 260 and 520 mg/ml were 49, 73, 81, 89 and 95%, respectively. CLO treatments did not affect glucose, blood urea nitrogen, albumin, total protein, low density lipoprotein, while improved the concentration of high-density lipoprotein ($P>0.01$). Results showed that supplementation with CLO significantly decreased ($P<0.01$) cholesterol, triglycerides and very low density lipoprotein concentrations compared with control. There was no significant difference in analyzed blood bio-chemicals and serum enzymes level between different antioxidant activity methods and groups, suggesting general well-being of ewes. These results suggest that, CLO supplementation had a positive impact on blood traits and antioxidant status of the Afshari ewes.

Key words: Afshari ewe; Antioxidant status; Biochemical profile; Blood parameters; Citrus Limon Oil.

Introduction

Sheep are an important livestock resource and the Afshari breed is one of the most usual local sheep breed in Iran that is known to be the most appropriate for breeding in the mountain conditions. The nutrient requirements of grazing animals are often below their need, and therefore, for optimum production, the animal's diet should be fortified with some feed additives (1, 2). On the basis our knowledge, commonly used feed additives in sheep production and industry include amino acids, minerals, vitamin, and phytobiotics in Iran. Consequently, there has been a growing interest in plant food additives have received widespread attention in livestock industry in the world (3, 4).Citrus family is well known as medical plant species and antioxidant resource (5). Citrus fruits and juices are a very good source of bioactive compounds, including antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectin, and of great importance in human nutrition are important. Herbal essential oils generally contain a variety of volatile compounds, which may have medicinal properties (6, 7, 8). Numerous reports about the existence of a positive correlation between phenolic compounds and antioxidant activity of the extracts and essential oils provided, so that the antioxidant properties of fruits and vegetables significantly in the presence of high amounts of phenolic compounds increases (9,10).

According to previous findings and the importance of finding viable alternative to antibiotics and the use of natural ingredients in the food chain animals and also due to the fact that limited research on the use of essential oils of lemon on feeding small ruminants has been made, the main aim of this study was to investigate the performance of antioxidant lemon essential oil to laboratory methods and effects of essential oil of lemon on some blood parameters and antioxidant status in Afshari ewes.

Materials and Methods

Location

This study was performed at Animal Reproduction Laboratory of University of Zanjan, located in Zanjan province; Iran (36°11'N, 48°48'E) from June to December 2016.

Animal and Treatments

Six adults Afshari ewes (3-4 years old and 51±5 kg) were randomly allocated to 3×3 Latin square design with three diets in 21 days period. Dietary treatments included: Treatment 1: control diet, Treatment 2: control diet with 200 mg/day CLO, and Treatments 3: control diet with 400 mg/day CLO. Experimental diet with a ratio of 65% forage and 35% concentrate and nutritional requirements based on the recommendations of NRC (2007) was adjusting (Table 1).

Blood samples (5 mL) were blood taken after eating

Table 1. Composition of basal diet.

Feed components (% Dry matter)	
Alfalfa	40
Corn silage	22
Barley	32
Wheat bran	5
Mineral-vitamin supplements	1
Dietary nutrient composition (% Dry matter)	
Metabolizable energy (Mega Calories/Kg Dry matter)	2.86
Total digestible nutrients (% Dry matter)	69.35
Neutral detergent fiber (% Dry matter)	40.12
Acid detergent fiber (% Dry matter)	25.7
Calcium (% Dry matter)	0.57
Phosphorus (% Dry matter)	0.39
Crude protein (% Dry matter)	11.2

the morning meal of treatments from the jugular vein of all ewes into evacuated tubes. Blood samples were centrifuged (×4000g for 10 minutes) to obtain serum, and serum stored at −20°C pending assayed. Samples were evaluated for glucose, urea nitrogen, albumin, total protein, high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol, triglycerides, very low density lipoprotein (VLDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were analyzed with a semi-automated analyzer (RX Monza, UK) using commercial kits (Pars Azmoon Co., Tehran, Iran). Standard commercial kits were applied to analyzing as recommended by the manufacturer of these kits.

To study the antioxidant effect of the essential oils mentioned methods 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis (-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were used. For this purpose, the stock solution of the radical, prepared by dissolving 24 mg DPPH in 100 mL methanol, was kept in a refrigerator until further use. In a test tube, 3 mL DPPH working solution was mixed with 100 µL essential oil (1mg/mL) or the standard solution. The absorbance was measured at 517 nm for a period of 30 min. The percent antioxidant or radical scavenging activity was calculated using the following formula:

%Antioxidant activity = [(Ac − As)/Ac] × 100

Where, Ac and As are the absorbance of control and sample, respectively. The triplicate samples control contained 100 µL methanol in place of essential oil (11).

The working solution of ABTS•+ radical was made by reacting ABTS (9.5 mL, 7 mM) with potassium per sulfate (245 µL, 100 mM), and raising the volume to 10 mL with distilled water. The solution was kept in the dark at room temperature for 18 h, and then diluted with potassium phosphate buffer (0.1 M, pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm. Essential oil sample were prepared in methanol with dilutions 50–1250 µg/mL. A sample (10 µL) was placed in a test tube and mixed thoroughly with 2.99 mL ABTS radical working solution. Absorbance of the resulting clear mixture was recorded at 734 nm. The percent antioxidant or radical scavenging activity was calculated using the following formula:

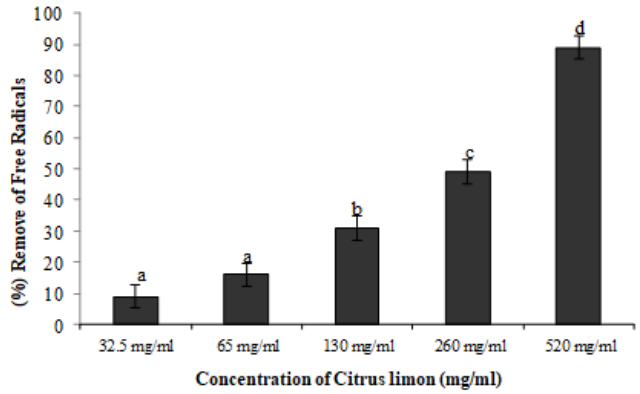

Figure 1. The antioxidant activity of different concentrations of the essential oil of lemon by DPPH method. a, b, c, d in the columns indicate significant differences (p <0.01).

%Antioxidant activity = [(Ac − As)/Ac] × 100

Where, Ac and As are the absorbance of control and sample, respectively. The triplicate samples control contained 100 µL methanol in place of essential oil (12).

Statistical analysis

Statistical analysis of the data was done with SAS software (13). The mean values (±SE) for blood parameters concentrations and antioxidant status were calculated. To see the magnitude of gestation age variation in blood parameters concentrations and various antioxidant statuses, the data were subjected to one-way analysis of variance (ANOVA). Significance between means was tested using Duncan's multiple range tests. The levels p<0.01 were considered as significant.

Results

The antioxidant activity of essential oils in DPPH method at doses of 32.5, 45, 130, 260 and 520 mg/ml were 9, 16, 31, 49 and 89%, respectively. Remove the free radicals treatments for 65, 130 and 260 mg/ml, 16, 31 and 49%, respectively. The results showed a significant relationship (p <0.01) among the different amounts of essential oils (Figure 1).

Antioxidant activity of essential oils in ABTS method at doses of 32.5, 45, 130, 260 and 520 mg/ml were 49, 73, 81, 89 and 95%, respectively. Remove the

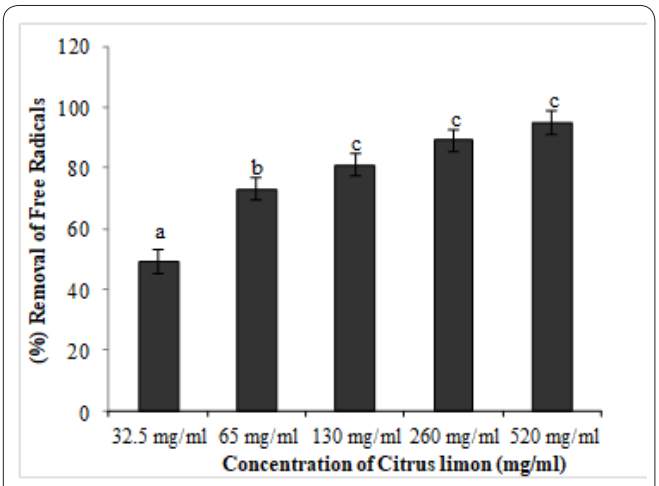

Figure 2. The antioxidant activity of different concentrations of the essential oil of lemon by ABTS method. a, b, c, in the columns indicate significant differences (p <0.01).

Table 2. Effects of Citrus Limon oil on the serum concentration of enzymatic variables in Afshari ewes (n = 6).

Enzyme (U/L)	1	2	3	P-value
ALT	19.21	21.01	26.3	0.31
AST	57.01[b]	64.22[a]	69.01[a]	0.01
ALP	199.05	212.02	223.19	0.44

ALT Alanine aminotransferase, AST aspartate aminotransferase, ALP alkaline phosphatase. * Dietary treatments included: 1: control diet, 2: control diet with 200 mg/day of oil and 3: control diet with 400 mg/day of oil. a, b in each row indicate significant differences ($p<0.01$).

free radicals treatments for 65, 130 and 260 mg/ml, 73, 81 and 89 percent, respectively. The results showed a significant relationship ($p<0.05$) among the different amounts of essential oils (Figure 2).

Table 2 presents that supplementing Afshari ewes with CLO resulted in higher dose (400 mg/day CLO), total antioxidant capacity ($p<0.01$) and activity of AST ($p<0.01$) compared with control group. Table 3 presents that the effect of CLO on some blood profile concentration in the Afshari ewes that treatment with different levels CLO. Results show that CLO have not significant effect on serum glucose, urea nitrogen, albumin, total protein, LDL ($p>0.01$). CLO was significantly increased ($p<0.01$) the concentrations of HDL, but the levels cholesterol, triglycerides and VLDL were significantly decreased ($p<0.01$) when compared with control group.

Discussion

On the basis our knowledge, this study for first time was conducted to investigate of the effects of different levels of CLO on blood parameters and biochemical profile in Afshari ewes in Iran.

Based on the results of the present study was to evaluate the antioxidant activity of essential oil of lemon by using of DPPH and ABTS methods showed that strong antioxidant activity of essential oil of lemon is the removal of free radicals, the property may be related to phenolic compounds and flavonoids found in lemons (14). The antioxidant properties of essential oil of lemon have been confirmed in other studies (15). A study shows the power of lemon approximately lipid peroxidation inhibition by synthetic antioxidants BHA and BHT or more of power (16). In another study of lemon extract on lipid peroxidation and activity of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase has been studied in mice (17).

Nobakhat and Amiri (2014) showed that the use of dried lemon pulp decreased low-density lipoprotein cholesterol in the turkey meat, also in this study; it was shown that the use of this waste will be significantly reduced abdominal fat turkeys (18). In another study it was shown that the addition of citrus juice (orange and lemon) broiler rations reduced total cholesterol in the blood (19). In the present study, similar results were obtained in total cholesterol in the blood of Afshari ewes. In one study, the effects of essential oils of lemon in male Wistar rats suggests that low levels of essential oils on cholesterol and low-density lipoproteins, triglycerides lowering effect of weak, but the amount is not affected. Moderate and high levels of essential oils also significantly decreased total cholesterol, triglycerides and low-density lipoprotein (20). In another side, adding antioxidants to the diet of an animal can be considered as human dietary source of antioxidants and provide beneficial effects in gastrointestinal tissues and other tissues in the human body. It has been shown that plant extracts with antioxidant properties can prevent the oxidation of low-density lipoprotein (21, 22). Based on the results of research studies, plants and antioxidants reduces the absorption of fats, stimulates the secretion of cholesterol via the bile and excretion of cholesterol through the feces. Also, some plants inhibit lipoproteins, enzymes and proteins that are involved in the metabolism of lipids and lipoproteins, and thereby reduce the lipid profile (23). In a study in which the effect of lemon juice on blood plasma and liver fat in mice, it was shown that the high fat and lemon polyphenols significantly increased the receptor activator of peroxisome fat diet high and Low fat diet (that had no polyphenols lemon), in the liver. These receptors induce the expression of enzymes involved in the oxidation of fatty acids. The results showed that levels of triglycerides in mice fed a diet containing high fat and polyphenols lemon fed to mice fed diets high in fat and low fat diet (that had no polyphenols lemon) significantly decreased. The level of serum phospholipids with high fat mice that were fed a lemon and polyphenols, compared to mice fed a high fat diet was significantly less. The researchers concluded that lemon polyphenols can prevent weight gain and accumulation of fat in the body

Table 3. Effect of Citrus Limon oil on some blood profile concentration in Afshari ewes (n=6).

Blood profile (mg/dL)	Mean			SEM	P-value
	1	2	3		
Cholesterol	67.54[a]	57.38[b]	49.12[c]	0.63	0.011
Triglycerides	27.63[a]	22.41[b]	18.87[c]	0.88	0.025
High-density lipoprotein	25.48[a]	29.64[b]	31.23[c]	1.35	0.033
Low density lipoprotein	39.61	37.71	36.33	3.64	0.35
Very low density lipoprotein	6.45[a]	4.48[b]	3.79[c]	0.51	0.016
Glucose	58.13	56.73	56.48	1.79	0.21
Urea nitrogen	27.75	26.32	26.51	3.38	0.29
Albumin	4.0	3.91	3.57	1.1	0.19
Total protein	7.33	7.17	6.42	1.3	0.25

* Dietary treatments included: 1: control diet, 2: control diet with 200 mg/day of oil and 3: control diet with 400 mg/day of oil. a, b, c in each row indicate significant differences ($p<0.01$).

by increasing the effects of beta oxidation of fats zoom proxy (24).

In general, the results of this study showed that lemon essential oil has strong antioxidant properties and the effect of lowering the blood plasma lipids. Probably the essential oil of lemon with a decrease in plasma lipids and their antioxidant properties could play an important role in strengthening the antioxidant defense system of ewe. On the other hand, based on the results present study farmer can extract oil or lemon or lime and other citrus pulp as an alternative to antibiotics used in ruminant nutrition. Because of lack of information and limited data on the use of herbal extracts and essential oils in ruminants to clarify the mechanism of action and mode of action and their applications in improving the performance of ruminants, further research seems necessary. Results presents that supplementing Afshari ewes with CLO resulted in higher dose (400 mg/day CLO), total antioxidant capacity ($p<0.01$) and activity of AST ($p<0.01$) compared with control group. Hence, dietary CLO supplementation may improve the antioxidant status, indicating that it can constitute a useful additive in sheep feeding. Thus, supplying CLO in diets may help to improve relative immune response in sheep breed. Based on the results of the present study was to evaluate the antioxidant activity of lemon essential oil showed that strong antioxidant activity with removal of free radicals by using of DPPH and ABTS methods. However, the essential lipid lowering effects on blood plasma. According to these results, this oil can be used to reduce the effects of oxidative stress induced by various stresses, strengthening the immune system and improve the performance used in ruminants. Thus, a moderate level (400 mg/day) of dietary lemon essential oil may help to enhance the relative antioxidant capacity and immune response in sheep.

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