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The Impact of Anthocyanin supplementation on Oxidative Stress Reduction and Improve Milk Production in Friesian Crossbred Cows

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Abstract:

This study was conducted for eighty days at the Dalmaj cattle station in Wasit Governorate. The study included the use of different levels of anthocyanins to determine their effect on some productive and physiological parameters of dairy cows. Twelve crossbred Friesian dairy cattle (average body weight 370 ± 23 kg) were allotted to feed in a completely randomized design (CRD) with three levels of anthocyanin supplementation (0, 200, and 400 mg/kg). additionally. The cows were split into three groups of four for each of the three experimental treatments. The results showed the possibility of extracting anthocyanins from Solanum melongena, which reached an average concentration of 673.056 mg/L. In addition to the ability of the extracted anthocyanins to scavenge free radicals, the antioxidant activity reached 83.12%. Compared to the synthetic antioxidant compound BHT (96.25%), The addition of anthocyanins (400 mg/kg of feed) increased milk production (19.22 kg/d), improved milk components, and reduced oxidation indicators in the blood, as the MDA value reached 1.05 mmol/L.

Keywords: anthocyanins, antioxidant activity, dairy cow, milk production and Oxidative stress

اهمية الاضافات الغذائية الانثوسيانيدات على تقليل الإجهاد التأكسدي وتحسين انتاج الحليب لأبقار الفريزيان المهجنة.

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الخلاصة

أجريت هذه الدراسة لمدة ثمانين يوماً في محطة أبقار الدلمج في محافظة واسط. تضمنت الدراسة استخدام مستويات مختلفة من الانثوسيانيدات لتحديد تأثيرها في بعض المعايير الإنتاجية والفسيولوجية لأبقار الحليب. تم تخصيص اثنا عشر بقرة فريزيان مهجنة لإنتاج الحليب (متوسط وزن الجسم 370 ± 23 كغم) للتجربة في تصميم عشوائي تام (CRD) مع ثلاثة مستويات من مكملات الانثوسيانيدات (0، 200، و400 ملغم / كغم). بالإضافة الى ذلك تم تقسيم الابقار إلى ثلاث مجموعات ولكل مجموعة أربعة حيوانات. أظهرت النتائج إمكانية استخلاص الانثوسيانيدات من نبات *Solanum melongena* حيث بلغ متوسط تركيزه 673.056 ملغم/ لتر. بالإضافة إلى قدرة الانثوسيانيد المستخرج على كسح الجذور الحرة، فقد بلغ النشاط المضاد للأكسدة 83.12% بالمقارنة مع مركب مضاد الأكسدة الاصطناعي BHT (96.25%)، فإن إضافة الانثوسيانيد (400 ملغم/كغم من العلف) أدى إلى زيادة إنتاج الحليب (19.22 كغم/يوم)، وتحسين مكونات الحليب، وانخفاض مؤشرات الأكسدة في الدم، حيث بلغت قيمة MDA وصلت إلى 1.05 مليمول / لتر.

الكلمات المفتاحية: **الأنثوسيانين، نشاط مضادات الأكسدة، بقرة الألبان، إنتاج الحليب والإجهاد التأكسدي**

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Introduction

Oxidative stress occurs when the production of oxidants and free radicals overtakes the ability of ruminants to neutralize and eliminate these reactive forms(Filomeni *et al*., 2015). Dairy cows, due to their high metabolic demands for maintenance and production, are particularly susceptible to oxidative stress, which can lead to various health complications such as ketosis, and pneumonia (Sundrum, 2015). Micronutrients (active compounds) have an important role in improving the performance of high ruminants in milk production by supplementing their diet (Tian *et al.*, 2022 a). Currently, new natural antioxidants are gaining popularity due to their safety for consumers (Tian *et at.*, 2022 b). These bioactive compounds have the ability to delay aging in organisms by scavenging free radicals, playing a crucial role in preventing oxidative damage (De Beer *et al.*, 2002). Xiao *et al.* (2021) suggested that supplementing dairy cow diets with vitamin E and selenium could enhance antioxidant status and immune responses, thereby improving overall health. Numerous studies have shown that anthocyanins and other flavonoid compounds can reduce oxidative stress in ruminants (Stoldt *et al.*, 2016: Suman *et al.*, 2015). Anthocyanins are large flavonoid compounds that serve as potent natural antioxidants and are commonly found in food and natural plant products (Tian *et al.*,2021: Luo *et al.*, 2022). They have significant antioxidant potential and can donate extra electrons to free radicals, thus improving animals' oxidative stress status and reducing oxidative damage to cells, tissues, proteins, cellular membranes, and mitochondria (Canuto *et al.*, 2016: Tian *et al.*, 2018). Moreover, anthocyanins are a potent reservoir of antioxidants and could potentially boost ruminant production by altering intracellular oxidative stress and ruminal fermentation (Leatherwood, 2013). This study will explore the possibility of extracting anthocyanins from the peels of (*Solanum melongena*) and examine their value as antioxidants through which anthocyanins work to amplify the antioxidant potential and reduce oxidative stress by eliminating free radicals. The effect of anthocyanins on milk production and components were also included.

Material and methods

Extraction of anthocyanin

Anthocyanins were obtained from Solanum melongena waste according to the method of Al Hussainy (2010). Anthocyanins were extracted by weighing 30 g of peels and adding 100 ml of acidified methanol (85 ml methanol + 15 ml 1% acetic acid solution) and then placing it on a magnetic stirrer for 24 hours, away from light. The mixture was filtered using filter paper under a vacuum. Then the precipitate was washed with a sufficient amount of acidified methanol until the color became pale. The extract was then concentrated under vacuum pressure using a vacuum rotary evaporator at a temperature of 40 degrees Celsius and stored in the refrigerator in tightly sealed, opaque glass containers until use.

The concentration of anthocyanin:

Anthocyanins were estimated using the differential pH method according to the method of Al Hussainy (2010). Using the pH differential method, a structured solution (KCI, 0.025M, pH = 1.0) was prepared to dissolve 1.86 g KCI in 980 ml of distilled water, then the pH was adjusted to 1.0 using HCI concentrated acid, and then the volume was completed by distilled water to 1 l. The regulated solution (CH3COONa, 0.4M, pH=4.5) was prepared by melting 54.43 g of CH3COONA in 960 ml of distilled water, and the pH was adjusted to 4.5 using HCI acid. The volume was then reduced to 1 l using distilled water. Melting 1 mg of Anthocyanin extracts in 10 millimeters of acid methanol (85 methane + 15 ml HCI) drew 0.8 ml of this solution and completed the volume to 3 ml of regulated potassium chloride solution (KCI, 0.025M, pH=1.0) and repeated the same step by completing the volume to 3 ml of CH3COONa, 0.4M, pH=4.5) left 15 minutes after which the absorption of the samples was measured at 520 and 700 nm wavelengths. The same steps were made using methanol and acidic water as Blank. Anthocyanin mg/l is estimated using the following equations:

A = (A 520 nm – A 700 nm) pH 1 – (A 520 nm – A 700 nm) pH 4.5

content mg/ L Anthocyanin = A. MW. DF .1000/ £ ×L

Antioxidant activity of Anthocyanin

According to the method of Du et al. (2008), the antioxidant activity of anthocyanin was measured. Through the DPPH free radical scavenging test, take 30 mg of anthocyanins and dissolve 30 ml of methanol, then take 60 microliters of the extracted sample and add 3 ml of DPPH methanol solution (0.05 g/l). The sample is placed in a water bath for 20 minutes at a temperature of 25°C. The absorbance was measured at a wavelength of 517 nm, and TBA was used as a control sample.

$$\%\textbf{Antioxidant activity} = 100\ (\frac{1 - AC}{AD})$$

Design of the Experiments

Twelve Friesian crossbred cows were used. Live weights before the experiment were 370 ± 23 kg, and average milk production was 18.3 ± 1.5 kg/d. The study lasted for 80 days, with the first 10 days of adaptation

Dietary treatments

The animals were divided into three dietary groups (as shown in Table 1).

Table (1): Component of different concentrated ration

Nutrients	Percentage of feed materials included in experimental diets %		
	Control	T1	T2
Barley	84.5	84.5	84.5
Soy bean meal	14.5	14.5	14.5
Salt	0.4	0.4	0.4
Limestone	0.5	0.5	0.5
Mineral premix1	0.1	0.1	0.1
Anthocyanin (mg/kg)	-	200	400
Chemical composition of the feed based on dry matter (%)			
Dry matter	93.44	93.44	93.44
Organic matter	96.90	96.90	96.90
Crude protein	17.25	17.25	17.25
Ether extract	1.5	1.5	1.5
Ash	3.1	3.1	3.1

T1: given a control diet+ 200 mg/kg feed of anthocyanin.

T2: given a control diet+ 400 mg/kg feed of anthocyanin.

Sample collecting and chemical evaluations

Using a Milko Scan FT1 instrument, analyses of milk samples were performed to ascertain the contents of protein, fat, solids-not-fat, total solids, lactose, galactose, glucose, casein, and milk urea nitrogen.

Use a centrifuge to separate the blood serum (500 rpm at 4 C for 10 minutes) according to Croker (1967). In order to quantify the blood urea nitrogen, the plasma was then maintained at -20°C. Plasma lipid oxidation was also assessed using thiobarbituric acid reactive substances (Toaldo *et al.,* 2015). Supernatants were evaluated with absorbance at 532 nm via UV absorption spectrophotometry. The results were calculated as thiobarbituric acid reactive substances concentration expressed in μmol/L of malondialdehyde. The antioxidant capacity was determined by spectrophotometric according to the method of Martinez et al. (2006), using a stable free radical DPPH the absorbance was determined at 516 nm using a Spectra Max M3 Multi-Mode Microplate Reader, Molecular Devices, San Jose, CA, USA).

Statistical analyses

A completely randomized design was used to examine the data. One-way ANOVA approach was used to determine the effects of treatments on the traits under investigation (SPSS, 2018). Least significant Design test (L. S. D.) within the same statistical packages was used to identify the significant differences between treatment means.

Results

Concentration and antioxidant activity of anthocyanin

The results of Table 2 showed that the amount of anthocyanin extracted from *Solanum melongena* peels (673.056 mg/L). The table also showed the ability of anthocyanins to scavenge free radicals, as the antioxidant activity of anthocyanins reached 83.12% compared to the synthetic compound BHT, which reached 96.25%.

Table (2): Concentration and antioxidant activity of anthocyanin (mean ± SD)

Concentration of antioxidants (mg/L)	antioxidant activity (%)		
	Anthocyanin	BHT	P-value
673.056 ± 18.94	83.12 ± 1.6^{b}	96.25 ± 1. 4^{a}	0.005

Milk production

the anthocyanins supplementation at 400 mg/kg significantly (P<0.05) exceeded the amount of milk production of other experimental treatment (table, 3). It recorded 19.22 kg/d compared to the control (18.65 kg/d) and adding 200 mg/kg of anthocyanins (18.94 kg/d).

In addition, the table showed that there was a significant (P<0.05) improvement in the chemical composition of milk when adding 400 mg/kg of anthocyanins (fat 3.9%, protein 3.57%, solids-not-fat 9.33%, total solids 12.51%, lactose 5.67%, galactose 0.34%, glucose 0.32%, casein 2.77%, and milk urea nitrogen (13.28 mg). However, control group revealed fat (3.5%), protein (3.48%), solids-not-fat (8.23%), total solids (11.05%), lactose (4.27%), galactose (0.28%), glucose (0.25%), casein (2.59%), and milk urea nitrogen (12.81mg).

Table (3): The effect of anthocyanins in milk production and composition in Friesian cows (mean ± SD)

Item	control	T1	T2	P-value
Milk production (kg/d)				
Total milk yield	18.65 ± 1.10^{b}	18.94 ± 1.06^{b}	19.22 ± 0.35^{a}	0.021
Milk composition (%)				
Fat	3.5 ± 0.46^{b}	3.7 ± 0.70^{ab}	3.9 ± 0.19^{a}	0.001
Protein	3.48 ± 0.83^{b}	3.48 ± 0.65^{b}	3.57 ± 0.75^{a}	0.037
Solids-not-fat	8.23 ± 0.51^{b}	8.31 ± 0.36^{b}	9.33 ± 0.27^{a}	0.012
Total solids	11.05 ± 2.20^{b}	11.17 ± 1.33^{b}	12.51 ± 2.39^{a}	0.022
Lactose	4.27 ± 0.10^{b}	4.48 ± 0.26^{b}	5.67 ± 0.25^{a}	0.016
Galactose	0.28 ± 0.07^{b}	0.29 ± 0.02^{b}	0.34 ± 0.03^{a}	0.002
Glucose	0.25 ± 0.02^{b}	0.27 ± 0.05^{b}	0.32 ± 0.01^{a}	0.012

Casein	2.59 ± 0.01^{b}	2.63 ± 0.06^{b}	2.77 ± 0.03^{a}	0.032
milk urea nitrogen (mg/d)	12.81 ± 0.22^{b}	12.96 ± 0.05^{b}	13.28 ± 0.09^{a}	0.004

T1: given a control diet+ 200 mg/kg feed of anthocyanin.
T2: given a control diet+ 400 mg/kg feed of anthocyanin.

Plasma antioxidant activity and oxidative stress

The anthocyanin supplementation resulted in enhancements in the DPPH scavenging activity (table, 4). DPPH scavenging activity of anthocyanin at 400 mg/kg group (22.61%) was higher ($P \leq 0.05$) than that of group fed anthocyanin at 200 mg/kg feed per day (18.19%) and control (16.41%).

In addition, the results showed a significant ($P = 0.003$) decrease in malondialdehyde when adding anthocyanins at levels of 400 and 200 mg/kg feed, as the MDA concentration reached 1.05 and 1.13 mmol/L, respectively. Compared with the control treatment, 1.28 mmol/L.

Table 4: The effect of anthocyanins in Plasma antioxidant activity and oxidative stress in Friesian cows (mean ± SD)

Item	**control**	**T1**	**T2**	**P-value**
Plasma antioxidant activity (%)				
DPPH scavenging activity	16.41 ± 1.81^{c}	18.19 ± 2.32^{b}	22.61 ± 2.07^{a}	0.003
Oxidative stress (mmol/L)				
Malondialdehyde	1.28 ± 0.01^{c}	1.13 ± 0.02^{b}	1.05 ± 0.01^{a}	0.000

T1: given a control diet+ 200 mg/kg feed of anthocyanin.
T2: given a control diet+ 400 mg/kg feed of anthocyanin.

Discussion

Anthocyanins Effect on the Activity of Antioxidants:

Living organisms, including dairy cows, possess a redox system that maintains a healthy balance (Surai *et al.,* 2019). Chemical species known as free radicals (FRs), which have unpaired electrons, are extremely reactive and unstable. Their reactivity results from their capacity to either take or give electrons to establish stability (Halliwell and Gutteridge, 2015). The interaction between a radical and a non-radical compound often results in the propagation of the radical chain reaction, leading to the generation of new free radicals (Halliwell, 2006). Under normal circumstances, free radical hydrogen peroxide is converted by the enzyme superoxide dismutase into H_2O_2 and then converted to H_2O through the activity of the enzyme glutathione peroxidase and catalase in ruminants. (Majlesi *et al.,* 2021).

Anthocyanin, which has a polyphenol-like active phenolic hydroxyl structure, may enhance antioxidant activity in one of two ways. First off, the phenolic hydroxyl group can directly scavenge oxygen-free radicals due to its own structure, increasing the body's capacity for antioxidants and the activity of enzymes associated with them. Second, by encouraging the restoration of intestinal macrobiotics, excluding pathogens, reducing intestinal barrier permeability, and boosting immune response and antioxidant activity, anthocyanin plants can decrease inflammatory processes in the intestinal mucosa. (Pieszka *et al.,* 2015: Sakano *et al.,*2005).

The decrease in peroxides is accompanied by the oxidation of reduced glutathione, which can be regenerated from glutathione disulfide through reducing equivalents from NADPH, generated by the pentose monophosphate shunt. This process results in the depletion of reduced glutathione, leading to an increased consumption of reducing equivalents. This diverts glucose away from vital physiological pathways and competes with NADPH-dependent metabolic pathways in animals, including energy metabolism, immune functions, antioxidation capacity, and calcium homeostasis (Miller *et al.,* 1993).

Anthocyanins Effect on Milk Production

Milk, being a rich source of protein, vitamins, minerals, and both enzymatic and nonenzymatic antioxidant components, is considered one of the most suitable sources of nutrition for humans (Khan *et al.,* 2019: Paraskevakis, 2015). However, the oxidation of unsaturated fatty acids can

negatively impact the antioxidant enzymes in milk (Lindmark-Månsson and Åkesson, 2000). This oxidative deterioration is a significant issue in food chemistry as it can lead to undesirable flavours and a reduction in the safety of dairy and nutritional quality foods containing lipids (Gad and Sayd, 2015).

The process of lipid peroxidation involves three stages: initiation, propagation, and termination (Lindmark-Månsson and Åkesson, 2000). Different substances, including radicals like hydroxyl, alkoxyl, peroxyl, superoxide, and peroxynitrite, can start this chain reaction. As a result, a fatty acyl side chain carbon loses a proton to a free radical, making the remaining carbon radical available to molecule oxygen to form a lipid peroxyl radical. Due to its high reactivity, this radical intensifies the chain reaction. (Catalá, 2006). As a result, conjugated dienes, peroxy radicals, and hydroperoxides are created from polyunsaturated fatty acid molecules. Over 20 lipoperoxidation end-products have been found after this cleavage, which mostly produces aldehydes. (Niki, 2009). among these, acrolein, malondialdehyde, 4-hydroxyalkenals, and isoprostanes are most frequently mentioned (Leopld and Loscalzo, 2009). malondialdehyde is a product of the lipid peroxidation of polyunsaturated fatty acid and can be used as a marker of oxidative stress as the concentration of malondialdehyde is an indicator in estimating the degree of fat peroxidation in milk (Davey *et al.*, 2005).

Conclusions

Anthocyanins have the potential to be used as natural antioxidants in dairy cows by neutralizing and reducing the production of free radicals and reactive oxygen species, thus enhancing the body's natural antioxidant activity. In addition, plants rich in anthocyanins have the ability to transfer these compounds to milk, which improves its quality and prevents fat oxidation, which in turn benefits the health of the consumer.

However, absorption and metabolism of anthocyanins in ruminants are still unclear and require further research. In addition, the raw materials examined in this study are anthocyanin-rich plants, which contain not only these compounds but also other natural antioxidants. Therefore, further in vivo studies are needed to fully understand their effects.

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