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RESEARCH ARTICLE

EFFECTS OF DIETARY SUNFLOWER SEEDS ON BLOOD ANTIOXIDANTS STATUS OF LAYERS

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ABSTRACT

The current trial was performed to investigate the influence of sunflower seeds on the oxidative status of laying hens. Fifty (Hisex) bird, 20 week old, were obtained from Animal Production Research Center (kuku), were divided into two groups (n=25) for each one. The control group (A) was under control diet based on corn, maintains the (NRC, 1994) requirements for laying hens, the experimental group, supplemented by 10% sunflower seeds added to the diet. The trial run for eight weeks, blood samples were collected once per month (week 4 and 8), in EDTA coated vials, immediately placed into iced-container, centrifuged at 3000rpm/20 min, samples were separated in aliquot, and stored at -20°C and -80°C until analysis. Plasma analysis for enzymatic and non-enzymatic antioxidants, revealed no significant different level of uric acid, malondialdehyde (MDA) and vitamin C, between the control group and the treated one, while catalase (CAT), superoxide dismutase (SOD), vitamin E, and vitamin A, levels were significantly enhanced by the addition of 10% sunflower seeds.

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INTRODUCTION

Natural dietary antioxidants are currently receiving considerable attention in animal nutrition fields due to their association with feed high-quality characteristics and their contribution to the protection against oxidant stress. Peroxide is reactive oxygen species (ROS) that causes oxidative damage, a consequence of insufficient antioxidant potential; excessive oxidative stress is the other possible consequence in animals (Safary and Daneshyar, 2012). Under normal circumstances, the animal could remove excessive ROS using a nonenzymatic antioxidant system and a series of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) (Seven *et al*., 2010; Safary and Daneshyar, 2012). A high ant oxidative status has been regarded as one of the major factors positively affecting the production performance in the intensive poultry industry (Lin *et al*., 2006; Mujahid *et al*., 2007). In addition, feedstuff can be easily oxidized during processing and storage by a series of oxidative reactions, which would also negatively affect birds' performance (Mujahid *et al*., 2007). Normally, dietary oxidation stability is one with major factors influencing the shelf life of feed. Therefore, supplementation of the antioxidants to mitigate the oxidative stress has become a commercial practice by the industry in regard to poultry feeding. Today's poultry producers are confronted with numerous challenges to prevent diseases and maintain health without the use of sub-therapeutic antibiotics. As food safety and animal welfare concerns continue to increase, researchers will continue to seek better alternatives to current methods applied to molting laying hens (Lin *et al*., 2006; Liu *et al*., 2014).

Studies have suggested that PUFAs not only reduce the levels of serum lipids but also alter membrane FA composition in humans and animals (Camara *et al*., 1996; Demirel *et al*., 2004; de Smet *et al*., 2004; Mach *et al*., 2006; Haak *et al*., 2008). However, a high concentration of PUFA makes membranes sensitive to peroxidative degradation. PUFAs become preferential targets for the action of free radicals that induce an oxidative stress. This type of stress is facilitated if an imbalance occurs between the respective amounts of PUFA and antioxidant systems. Recent studies have established that dietary lipids and nutrients play important roles in determining the strength of cellular antioxidative defense mechanisms (Reddy and Lokesh, 1994; Scislowski *et al*., 2005). Sunflower seeds (*Helianthus annuus* L) is a coarse, stout and erect annual plant 1-3 meters high. It produces greyish green or black seeds encased in tear-dropped shaped grey or black shells that often times feature black and white strips. Seeds encased in plant

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contains monoterpenes (α-pinene, Sabinene) [(Ceccarinia, Macchia, Flamini, Cioni, Caponi and Morelli, 2004), (Verma and Singh, 2008)], diterpenes (Helikauranoside) (Francisco, Ascensión, José, Molinllo, Rosa and Diego, 1996), oleic acid, triacyl glycerol, alkaloids, cyanogenic glycosides, saponins, cardiac glycosides, tannins, fixed oils, flavanoids (Bohm, Bruce and Stuessy, 2001), sesquiterpenes lactones (Francisco, Ascensión, José, Molinllo, Rosa and Diego, 1996), alkaloids (Javed, 2001). Flowers contain quercimeritrin, anthocyanin, abundant amount of cholin and betain, triterpene (Ukaya, Akihisa, Yasukawa, Koike, Takahashi, Suzuki and Kimura, 2007), saponins (Chirva, Chirva, Cheban andLazur`evskii, 1968). Seeds contain 45 to 48 percent fixed oil, tannins (Catherine, Imungi, Okoth, Momanyi, Biesalski and Vadive, 2011), polyphenols (Kenneth, Cecil Randolph and Iuan, 1970). *Helianthus annuus* L. is a folk remedy for bronchiectasis, bronchitis, carbuncles, catarrh, cold, colic, cough, diarrhoea, dysentery, dysuria, epistaxis, eyes, fever, flu, fractures, inflammations, laryngitis, lungs, malaria, menorrhagia, pleuritis, rheumatism, scorpion stings, snakebite, splenitis, urogenital ailments, whitlow, and wounds (Duke and Wain, 1981).

Sunflower oil have commonly been used as energy source in poultry diets because it contains the higher level of linoleic acid (omega 6). Linoleic acid improves the laying performance in poultry (Baiao and Lara, 2005; Özdoğan, Sarı, Kanatlı rasyonlarına and yağ katkısı, 2001).

MATERIALS AND METHODS

The experiment was held in the Veterinary Research Institute (VRI), from January to march 2014. The duration of the experiment was 8 weeks.

Four, full wire cages were made, each cage was (2X 1.5X 1 meter), and the capacity of each cage was 15 birds. The cages were placed at an open poultry house. Fifty laying hens (Hisex) breed, 20 week old, obtained from animal production research center (kuku), were utilized in this study. The birds were divided into three groups, 25 birds per group.

Experimental Diets

The diets were formulated to meet the requirements of egg production according to the directions of the national research council (1994). Two formulae of diets were prepared by inclusion of, sunflower seeds (10%).

The supplementary source was subjected to proximate analysis, to determine its content of protein, fat, fiber, N.F.E and energy.

Management

Each group received its experimental diet from day one. Drinking system contained two tanks for each cage, the tanks were cleaned, and the water was changed twice daily. Birds received 24 hour light/day throughout the experiment. Three (ml) of blood was collected from twenty bird of each group, the blood was taken using a three (ml) syringe, and received into EDTA coated vials, and immediately were kept in iced container, the samples were centrifuged at 3000rpm for 20 minutes, and plasma was aliquot transferred into plane vials. Plasma samples were stored at -20 and -70°C until analysis.

Fatty Acids Analysis

Lipids were extracted in chloroform-methanol (2:1 v/v), according to the method of (Floch; Lees, and Sloane-Stanely., 1957). Methyl esters of the lipid extract were prepared according to (Wang; Sunwoo; Cherian, and Sim, 2000).

Gas Chromatograph Analysis

Fatty acid composition was determined using (2010, Shimadzu, Japan) gas chromatograph, fitted with Flame ionization detector (FID). Separation of fatty acids was achieved using DB-WAX column, serial number (us6551263 H), of 0.25um film thickness, 30 meter length and 0.25 mm inner diameter.

Fatty acids methyl esters were identified by comparison of retention times with standards, and expressed as percentage of methyl esters.

Table 1.

	D.M%	Moisture%	Protein %	Fat %	Fiber%	Ash%	N.F.E%	Energy%
Sunflower Seeds	98	2	26.00	36.49	11	6	18.5	3054.07

Table 2-1. Diets composition

Group	A	B
Raw Materials%		
Corn %	70	59.0
Wheat hull %	0	5.4
Groundnut cake %	14.3	10
Concentrate %	5	5
CalciumCarbonate%	10	10
Salt (Nacl) %	0.125	0.125
Methionine %	0.34	0.31
Lysine %	0.15	0.11
Mycofix %	0.1	0.1
Sunflower seeds%	----	10
Premix*	0.1	0.1

**Supplied per kilogram of diets: Vitamin A, 5000 IU; Vitamin D ,500 IU; Vitmin E, 5 IU; Vitmin K, 1 IU; Vitmin B, 1.5 mg, Vitmin B, 2.5mg, 1 2 Ca-pantothenate, 2.5mg, niacin acid, 10 mg; pyridoxine,3mg; biotin, 0.1mg; folicacid, 0.25mg; Vitmin B , 0.005mg. Supplied per kilogram 12 b of diets: MnSO. 7H O100mg,; FeSO . 7H O, 220mg; ZnSO. 7H O, 150mg; CuSO. 7H O, 20mg; KI, 2mg; Na SeO, 0.4 mg.*

Table 2-2. Nutritional values calculated

Parameter	A	B
ME. Kcal/Kg	2729	2821
C.P%	17.25	18.24
E.E%	5	6.2
C.F%	4.2	4.1
Available phosphorus%	0.52	0.63
Calcium%	3.9	3.9

Table 3. Fatty acids profile of control and experimental diets-page (Baiao and Lara, 2005)

Fatty acid	Control group (A)	Sunflower seeds supplemented group (B)
	(g/100 g total fatty acids)	
SFA	43.5306	8.58
MFA	17.68	17.09
PUFA	38.38	74.35
C18:3 (*n*-3)	1.38	8.45
Σ*n*-3	1.38	8.45
C18:2 (*n*-6)	36.94	65.9
C20:4 (*n*-6)	0.21	1.37
Σ*n*-6	37.15	67.27
PUFA/SFA	0.88	8.7
Σ*n*-3/Σ*n*-6	0.04	0.13

SFA*= Saturated fatty acids,* ***MFA****=Mono unsaturated fatty acids,* ***PUFA****=Poly unsaturated fatty acids,* ***C18:3 (n-3)****=Linolenic acid (omega-3),* ***C18:2 (n-6)****=Linoliec acid (omega-6),* **C20:4 (*n*-6)=** Arachidonic acid (omega-6).

Determination of plasma oxidative status

Enzymatic antioxidant determination

Catalase was determined using commercial kits (Nanjing Jiancheng, China), spectrophotometric method, the instructions of manufactured was followed. Ammonium molybdate can pause H_2O_2 decomposition reaction catalyzed by catalase (CAT) immediately, residual H_2O_2 can react with ammonium molybdate to produce a yellowish complex. It enables calculate CAT activity by measuring OD value at 405 nm. Superoxide dismutase (SOD) was determined using commercial kits (Nanjing Jiancheng, China), ELISA method, the instructions of manufactured was followed. Superoxide dismutase (SOD) plays an important role in oxidation-antioxidation balance of organisms, this enzyme can remove superoxide anion radicals (O_2^-) to protect cells away from damage.

Non-Enzymatic antioxidants determination

Malondialdehyde (MDA) was determined using commercial kits (Nanjing Jiancheng, China), spectrophotometric method, the instructions of manufactured was followed. Lipid hydroperoxide decomposition products can condensate with thiobarbituric acid (TBA) to produce red compounds which has absorption peak at 532 nm. Uric acid was determined using commercial kits (Biosystem, Spain), spectrophotometric method described by, (Bablock., 1998; Colombo., 1994). Uric acid in the sample originates, by means of the coupled reactions described below, a colored complex that can be measured by spectrophotometer at 546 nm.

The level of vitamin C was estimated by spectrophotometric method described by (Omaye; Turnbull, and Sauberlich.., 1979). Ascorbic acid is oxidized by copper to form dihydroscorbic acid. The product was treated with 2, 4 dinitrophenyl hydrazine to form tris 2, 4 dinitrophenyl hydrazine which undergoes rearrangement to form a product with the absorption maximum at 520 nm in spectrophotometer.

Vitamin E content was estimated by spectrophotometric method, described by (Palan; Mikhail; Basin, and Romney., 1973). This method involves the conversion of ferric ions to ferrous ions by α-tocopherol and the formation of red coloured complex with 2, 2 dipyridyl. Absorbance of chromophore was measured at 520 nm in the spectrophotometer. Vitamin A content was estimated by spectrophotometric method, described by (Rutkowski *et al*., 2006).

The proposed method depends on (1) saponification and extraction of the vitamin A and carotene from serum on a micro scale with solvents of low volatility; (2) measurement of the light absorption of the small volumes at 328 and 460 nm; (3) destruction of the vitamin A absorption at 328 rnp without affecting the absorption of other compounds at this wave-length; and (4) remeasurement of the absorption at 328 nm.

Statistical Analysis

The data were analyzed by using Statistics-10 program designed for Windows. Differences between obtained values were carried out by analysis of variance (ANOVA) the LCD test was used for determining the significance level of at least $p<0.05$.

RESULTS

The first month revealed significant ($p<0.01$) high concentration of plasma catalase in group (B), compared to the control group (A). The control group (A), recorded significant ($p<0.01$) low concentration of plasma catalase compared to the experimental group which received 10% sunflower seeds. The first month, revealed significant ($p<0.01$) high level of SOD, recorded by group (B), compared to the control group (A).

The sunflower supplemented group showed significant ($p<0.01$) high concentration of plasma SOD compared to the control group (A) by the end of week (8), Table (4).

There was no significant different concentrations of plasma MDA, between the control and experimental group by the end of the trial period Table (5-1).

The first month revealed that, group (B) recorded significant ($p<0.04$) high concentration of plasma uric acid compared to the control group (A), while the second month showed that, though the plasma concentration of uric acid was higher in group (B), but it was not significantly different compared to uric acid concentration in the control group (A) Table (5-1).

The first month revealed, no significant different concentration of plasma vitamin **C**, was noticed between the experimental and the control group. The second month revealed, slightly high concentration of vitamin **C**, in group (A), compared to group (B), but the difference was not significant at ($p<0.05$),

Table (5-2). In regard to the first month, significant ($p<0.01$) high concentration of vitamin E, was recorded by group (B), compared to group (A), and the same result was obtained by the end of the second month, Table (5-2).

The sunflower supplemented group, recorded significant ($p<0.01$), high concentration of plasma vitamin A, compared to the control group, Table (5-2).

Table 4. Plasma enzymatic antioxidants concentration

Parameter	CAT (U/ml)		SOD (U/ml)	
Group	4th week	8th week	4th week	8th week
A	41.000^{B}	40.667^{B}	32.500^{B}	33.267^{B}
	±6.0431	±7.8811	±2.4710	±2.8718
B	66.667^{A}	67.333^{A}	41.333^{A}	41.267^{A}
	±6.0431	±7.881	±2.4710	±2.878

A: Control group, B: Fed 10% sunflower seeds.
CAT= Catalase, SOD=Superoxide dismutase.
Data are means ± standard error. Means in the same column followed by the same letters are not significantly different at (p < 0.05).

Table 5-1. plasma non-enzymatic antioxidants concentration

Parameter	MDA (mmol/L)		Uric Acid (mg/dl)	
Group	4th week	8th week	4th week	8th week
A	10.333^{A}±	10.867^{A}±	9.143^{B}±1.	9.143
	1.5275	1.6871	0723	^{AB}±1.0723
B	9.667^{A}±1.	9.233^{A}±1.	11.900^{A}±	11.900^{A}±1.2
	5275	6871	1.2687	687

A: Control group, B: Fed 10% sunflower seeds. MDA= Malondialdehyde
Data are means ± standard error. Means in the same column followed by the same letters are not significantly different at (p < 0.05).

Table 5-2. plasma non-enzymatic antioxidants concentration

Parameter	Vitamin C (mg/dl)		Vitamin E (mg/dl)		Vitamin A (µmol/l)	
Group	4th week	8th week	4th week	8th week	4th week	8th week
A	10.600^{A}±	16.000^{A}	2.9091^{B}±	3.1833^{B}±	0.3433^{A}±	0.5200^{B}±
	0.659	±1.116	0.277	0.43	0.1343	0.132
B	10.625^{A}±	15.000^{A}	4.0091^{A}±	5.4500^{A}±	0.3333A	1.1667^{A}
	0.736	±1.116	0.277	0.43	±0.1343	±0.132

A: Control group, B: Fed 10% sunflower seeds.
Data are means ± standard error. Means in the same column followed by the same letters are not significantly different at (p < 0.05).

DISCUSSION

Uric acid is an antioxidant, produced in vivo, primarily as product of the degradation of purine compounds, rather than consumed in diet (Becker, 1993). Uric acid reacts with the hydroxyl radicals, peroxy radicals, hypochlorous acid and hydrogen peroxide, to varying degrees, giving it potent anti-oxidant properties in vivo (Becker, 1993).

The plasma concentration of uric acid at the first month was significantly high in treated groups compared to the control group, this result could be attributed to high plasma triglycerides concentration in the treated groups at the first month, because plasma triglycerides is related to hyperuricemia (Chen; Zhu; Chen; Dai; Ren; Chen; Chen, and Fang, (2007); Abdu Allah; Hasan and Raigangar, (2009), triglycerides synthesis needs great amounts of NADPH (Clausen; Borch-Johnsen; Ibsen and Pedersen,1988), the synthesis of fatty acids in the liver is associated with the de novo synthesis fatty acids in liver is associated with the de novo synthesis of purines, thus accelerating uric acid production (Matsuura; Yamashita; Nakamura; Nishida; Nozaki; Funahashi and Matsuzawa., 1998). Also this result is in agreement with the findings of (Hazem *et al*., 2010), who reported that, adding sunflower oil and corn oil to the diets of Japanese quail, resulted in significant elevation of plasma uric acid concentration, compared to fish oil and flax seeds oil, while the concentration of uric acid between the sunflower oil group and corn oil supplemented group was not significant.

Catalase is the first enzyme, to show alteration following induction of oxidative stress (Stara; Machova and Velisek., (2012); Jin; Zhang; Shu; Chen; Sun; Qian; Liu and Fu., (2010). The catalase activity is related to the SOD activity, because SOD, converts superoxide radicals to hydrogen peroxide (substrate of catalase), more production of superoxide radicals, more will be the activity of SOD, and hence more activity of catalase (Rodriguez-Martinez and Ruiz-Torres., 2001), the result of the current study agrees with this scientific truth.

Significant high level of plasma catalase, was observed in the treated groups compared to the control group. The alteration of catalase concentration was fluctuated, this agrees with what was reported by (Ilknur., 2013), when he obtained the same result in a study performed to evaluate the antioxidant enzymes activities of (Cyprinus carpio), fed diet containing moderate level of sunflower seeds meal, the justification of this result could be attributed to the intracellular localization of this enzyme which is responsible of different responses to oxidative stress, because catalase activity found mainly in peroximes (Trenzado; Hidalgo; García-Gallego; Morales; Furné; Domezain; Domezain; and Sanz, 2006). The values can be reflected also an adaption to diet composition (Bastrop; Spangenberg and Jurss, 1991).

In the present study, the sunflower diet enhanced the activity of the antioxidant enzyme SOD compared to the control diet, which is consistent with the previous reports (Prasad, 1997; Kratz *et al*., 2002; Liu *et al.,* 2011). Liu, (2009), suggested that, polyunsaturated fatty acids (PUFA), can scavenge free radicals, improve the activity of SOD and other antioxidant enzymes, and may exert a preventive antioxidant role against free radicals action, the fatty acids profile and the percentage of plasma polyunsaturated fatty acids in the treated groups, is agree with the suggestions mentioned above.

MDA levels in serum did not change significantly after dietary treatments in layers, which indicated that PUFAs induced an increase in the activities of antioxidant enzymes and a decrease in lipid peroxidation (Yang *et al*., 2008). The biosynthesis of ascorbic acid in mammals and birds takes place in the liver/kidney or both. In the chickens the synthesis occurs primarily in the kidneys as reported by (Roy, and Guha., 1958), usually, sugars such as glucose, fructose and mannose, serve as precursors for vitamin C synthesis. There was no significant different levels of plasma vitamin C concentration between the control group and sunflower treated group, this may be due to the capability of hens to produce the vitamin de novo at the kidney and liver, and as time passing by the concentration of vitamin C in plasma elevated, regarding individual variation of ascorbic acid synthesis.Vitamin E, is the primary lipid soluble anti-oxidant, found in food and human blood and tissues, its

well-known that vitamin E, inhibits the process of lipid peroxidation in oils and in the biological lipid-protein complexes, such as biological membranes or circulating lipoproteins (Fellenberg, and Speisky, 2006). Group B, which received 10% sunflower seeds, showed significant high concentration of plasma vitamin E, compared to the control group, this result is in agreement with what was reported by (Dorrell, and Vick., 1997), that sunflower seeds oil considered as a rich source of vitamin E. Vitamin A was measured as biomarkers for oxidative stress that could be associated with consumption of diets rich in PUFA, in the present study the plasma concentration of vitamin A was significantly increased in response to sunflower seeds addition compared to the control group, this result does not agree with (Tülay, Fatma and Mustafa, 2014), who reported significant decrease in plasma vitamin A level when Japanese quail fed diet containing sunflower oi. According to (USAD, 2012), sunflower seeds contains 50 IU/100 g of vitamin A, so the contrary result in the present study could be attributed to the oxidation of vitamin A in sunflower oil, since its more exposed to environmental condition, while the whole sunflower seed is naturally protected from the oxidation factors.

Conclusion

The results obtained from the current study appointed that, feeding laying hens 10% sunflower seeds for 8 weeks, enhance the oxidative status, without any elevation of plasma MDA, though the high content of PUFA in the experimental diet. The improvement in oxidative status in return will be reflected in improvement of laying hen health and production.

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