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RESEARCH ARTICLE

Determination of the Genotoxic Potential of Annatto Seed by Single Cell Gel Electrophoresis in Layer- Hen

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Abstract

The aimed of this study is to investigation the toxicity of annatto seeds added to the diet of layer hens for different level. A total of 240 Lohmann hens 67 week of ages for 12 weeks were distributed randomized blocks into four treatments (60 hens) with three replicates 20 hens of each. The following treatments were applied: T1- control feed on based die; T2- feed on Annatto seed at 75gm/100kg, T3- feed on Annatto seed at 150gm/100kg, T4- feed on Annatto seed at 225gm/100gm. In the end of study blood samples were collected from 9 birds from each group from the wing vein in a test tube with coagulant for cytogenetic parameters. The result demonstrated that 2nd group recorded maximum significant ($p \leq 0.05$) differences compared to other nourished groups in all comet assay parameters(%DNA in head, Tail length,% DNA in tail, Tail moment and Olive tail moment) then followed by T3 regressed maximum significant ($p \leq 0.05$) differences of comet length. T1, T4 recorded a significant ($p \leq 0.05$) decrement in comet assay parameter respectively compared with other studied groups. The addition of Annatto seed at 225gm / 100kg to the diet of layer hens has an adverse effect on blood lymphocyte and cases highly damage of DNA, while added at level 75gm/kg(T2) and 150 gm/kg(T3)which protect lymphocyte from damage as shown in comet assay parameters.

Keywords: *Annatto seed, Single Cell Gel Electrophoresis (comet assay), Layer.*

Introduction

Some of feed additive adding to the diet of the farm animal to enhance growth and improve health statues by balanced GIT ecosystem or strength immune system, like antioxidant ,antibiotics, amino acid , spice ,etc, on the other hands some of these additive added to improve customer demand because visual appearance, especially color, is one of the most important characteristic of foods and examination the acceptance or refuse of the product by the consumer, This statement is also true for poultry products, in which the color of skin, meat and egg yolk plays a fundamental role to some ethnic and regional consumers [1, 4].

Poultry producers add colorants to broiler and layer diets as a means of improving the attractiveness of these products [5, 6]. Pigments are a feed additive that can increase the color of broiler skin and egg yolk. They cannot be synthesized in animal bodies, but they can be transformed and metabolized, so they must be ingested from feed [7]. There is growing interest and demand for natural colors and dyes in place of the synthetic ones [8]. This is justified by the low toxicity of the natural colorants compared with the poor biodegradability and risks of cancer and skin disorders associated with the synthetic dyes [9] also [10] indicate that synthetic pigments have the disadvantage of being more expensive and lacking safety .In contrast, natural colorant products are inexpensive, are of high quality and may be beneficial to human health. Among the natural products, carotenoids have been the most widely accepted for commercial use in poultry feeds [11].

In birds, carotenoids function as color in feathers and skin, antioxidants, precursors of vitamin A and play various roles in the endocrine and immune systems [12]. Animals, including poultry, absorb carotenoids from their diets and store them

after having modified their structure by oxidative metabolism [13]. Annatto seed is a yellow-orange-red colorant, a natural pigment derived from a non-carcinogenic and non-toxic native plant (*Bixa orellana* L.) found in Central and South America, India, and East Africa. Annatto seeds present high levels of the carotenoids. The predominant coloring or pigment principles of annatto are bixin (the oil-soluble form) and norbixin (water-soluble form). Bixin has the chemical formula C25H30O4, is non-toxic and can be extracted from the seed pulp [14], having several applications in human foods and animal feeds [15], while norbixin has the chemical formula C24H28O4 in small amount in seed and their ratio varies according to cultivar, with a general predominance of bixin [16]. Used natural dye derived from plant than synthetic making people increasingly choose those of natural origin, believing that they are devoid of toxic effects.

This is not completly true, because even a medication from a natural source can be a poison or toxic, depending on the dose that is administered and period of administration. The failure to require in-depth data related to toxicological and chemical analyses for the registration of food additives derived from natural sources [17, 18]. Among the twenty-one activities researches, those with the numerous of studies performed were antifungal activity [12], antibacterial activity [12], anti-malarial activity [6], and mutagenic activity [3]. Cytotoxic activity and toxicity have been little studied, with three and two studies, respectively [19]. So this research essentially focuses on the determination of the optimal levels of annatto seed added to the diet of layer hens for a long period which may be causes damage to DNA of blood lymphocyte by application Single Cell Gel Electrophoresis (comet assay) technique.

Materials and Methods

This study was set in the poultry farm in the Agricultural ministry\ circle Agricultural Research / Baghdad. The experiment was carry on 240 laying hens (Lohmann ,67 week of ages, for 12 week), weighed and at random distributed into wood shavings covered floor pen then divided into 4 experimental sub groups 60 layers in each. Each subgroup composed of three replicate pens with 20 layers. The hens were randomly allocateto a treatment and were kept in an environment similarity to that of most modern industry laying facilities. The rate temperature was 25 °C and the average relative humidity was 40%. The lighting was on for 16 h and off the remaining 8 h of the day.

Dietary Treatment

The base diet met the [20] nutrient recommendations. mix the supplements into the base diet, the appropriate amount of annatto was weighed and then was mixed into a small amount of the base diet to ensure it was well incorporated. This mixture was then added to more feed and mixed well then added to the dietary treatments as: T1-control(0) ,T2- Annatto seed 75 gm/100 kg, T3- Annatto seed 150gm\ 100kg, T4- Annatto seed 225gm\ 100kg. The formulas and calculated nutrient of the basal diet are presented in Table 1.

Table 1: The ingredients and chemical composition of diet used in experiment

Ingredients	**%**	**Ton**
corn	40.1	403
wheat	30	300
soya	14	140
premix	5	50
Di calcium	0.9	9
limestone	9.5	95
salt	0.3	3
	1000 kg	1000 kg

Ingredient %	Finisher
Total crude protein (%)	15.6
Fiber (%)	2. 4
Fat (%)	2.55
Methionine + cyctine	0.62
caicium	14.4
phosphors	0.53
methionine	0.35
argeneine	0.54
lysine	0.80
Total metabolizable energy (kcal / kg)	2734

Blood Collection

At the 72 weeks of age, blood samples were collected from 9 birds from each group from the wing vein in a test tube with coagulant for cytogenetic parameters according [21]. Cytogenetic parameters/ Evaluation of DNA damage using single-cell gel electrophoresis (comet assay) Protocol of SCGE, involving gathering samples, blood lymphocyte separation, slide preparation, cell lysis, electrophoresis and neutralization is conducted on Day I then fixation, staining and microscopy on Day II. The comet assay was performed under alkaline condition. Essentially according to the procedure described by [22] with a slight modification [23].The slides stained with ethidium bromide are observed under a bright-field light microscope and captured using CCD camera.

Thus captured images can be analyzed using commercially available software. Images of 100 randomly selected cells (50 cells from each of two replicated slides) were analyzed. Measurements of DNA density were performed using image analysis (comet score™). for slide detection used virtual lens(x100) and (x40), zoom lens(x1.9) to detected comet length (px),%DNA in head, Tail length (px),%DNA in tail, Tail moment and Olive moment. Statistical Analysis / Data gained were evolution by using analysis of variation ANOVA. Least significant difference (LSD) among different groups at 5% level was applied [24].

Result

The means of comet assay parameters were listed in Table 2 and Fig 1. There were significant ($p \leq 0.05$) differences among groups in layer hens feed on Annatto seed for different level among three month. The 4th group recorded increase of significant differences ($P \leq 0.05$) compared with other groups on comet length, DNA% in Tail, Tail Length, Tail moment, Olive tail moment as(68.50 ±0.68, 71.91 ±2.84, 38.25 ±1.49, 41.42 ±4.98, 20.35 ± 0.34) respectively, while recorded lowest value in DNA% in Head (68.50 ±0.68).

The second and third groups recorded greatest value in DNA% in Head (87.29 ±1.82, 82.30 ± 1.64) respectively then followed by first group which recorded (54.22± 0.97). T2 recorded lowest value in DNA% in Tail (12.30 ±1.84) then followed by T3, T1 (20.00 ±2.39, 33.85 ±1.23) respectively. The result denoted no significant differences between T2, T3 in Tail Length, Tail moment and Olive tail moment. Fig(1,2,3(A,B), 4,5(A,B) represented image of blood lymphocyte of layer hen nourished of Annatto seed for different level. This image explains different level of migration of DNA in field of gel electrophoresis.

Table 2: Effect feed different level of Annatto seed on comet assay parameters (mean ± SE)

Parameters (px)	T1	T2	T3	T4
Comet length	54.22± 0.97 B	41.00 ±0.26 C	37.00 ±1.22 D	68.50 ±0.68 A
DNA% in Head	65.98 ±1.08 C	87.29 ±1.82 A	82.30 ± 1.64 B	27.80 ± 2.82 D
DNA% in Tail	33.85 ±1.23 B	12.30 ±1.84 D	20.00 ±2.39 C	71.91 ±2.84 A
Tail Length	11.32 ±0.85 B	6.33 ±1.31 C	7.00 ±0.26 C	38.25 ±1.49 A
Tail moment	3.94 ± 0.43 B	0.72 ± 0.15 B	1.28 ± 0.16 B	41.42 ±4.98 A
Olive tail moment	5.33 ± 0.28 B	2.07 ± 0.30 C	1.93 ± 0.21 C	20.35 ± 0.34 A

Different capital letter among groups denoted significant differences ($p \leq 0.05$)

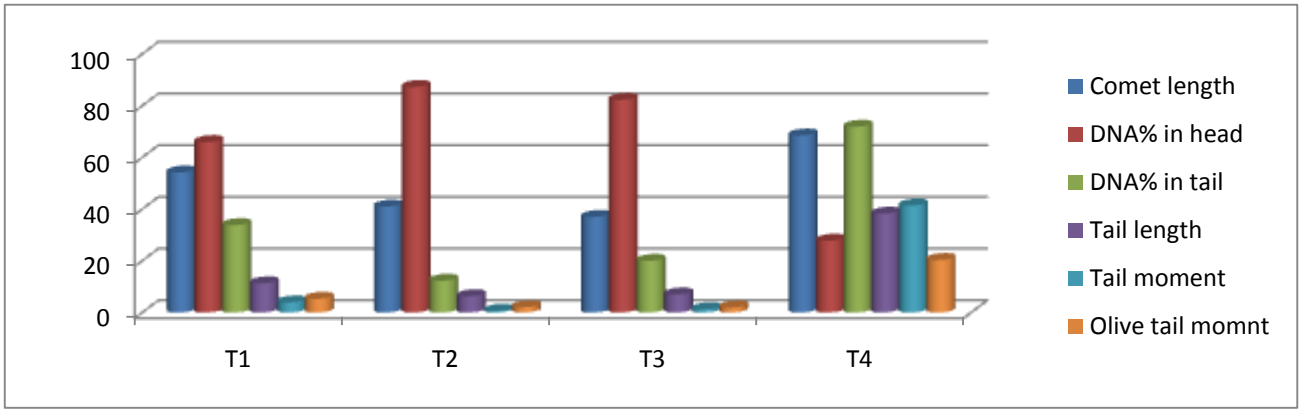


Fig. 1: Measurements DNA damage .Where (Comet length, %DNA in Head, Tail length, %DNA in Tail, Tail moment. Olive tail moments) in lymphocyte of layer hens nourished on different level of Annatto seed at 72 weeks of age.

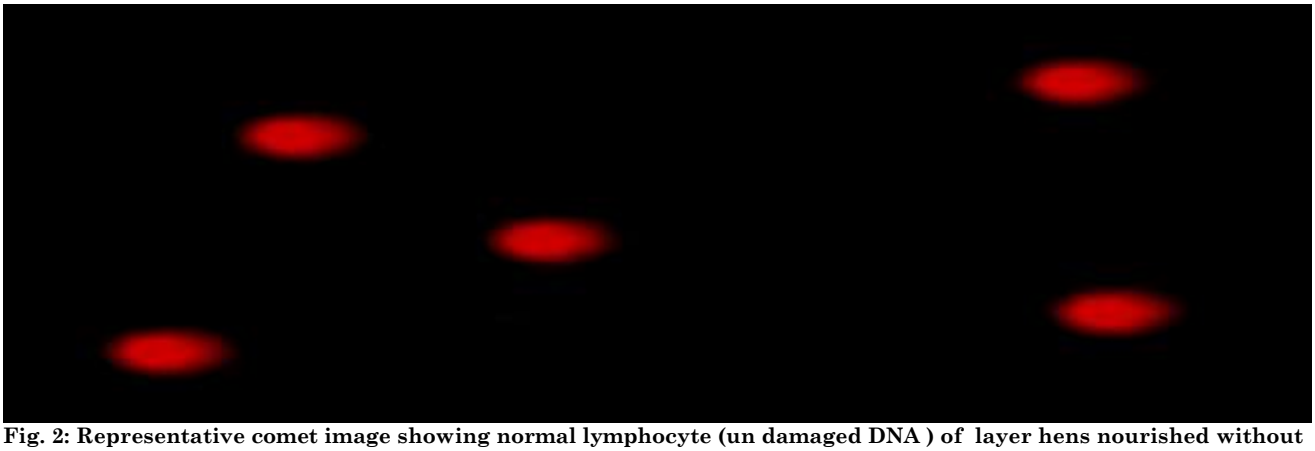
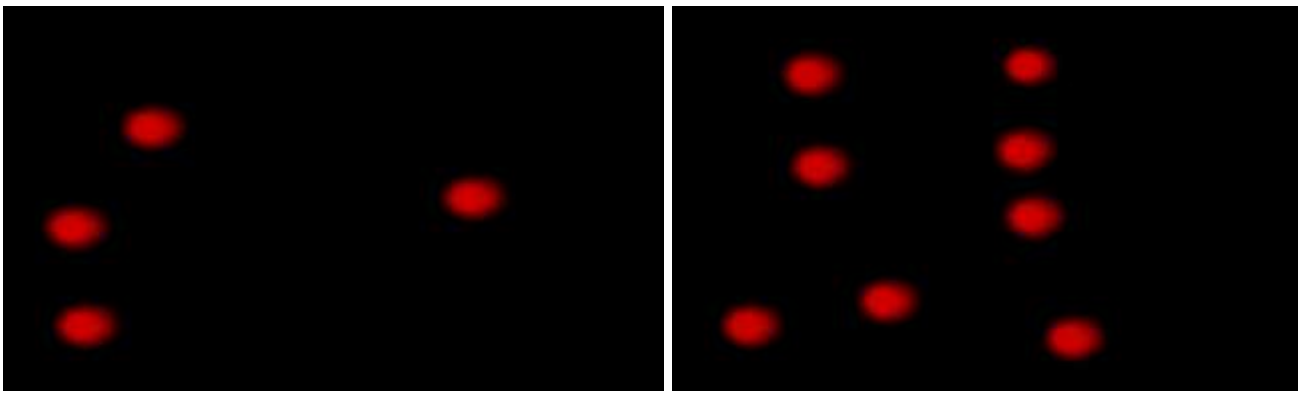


Fig. 2: Representative comet image showing normal lymphocyte (un damaged DNA) of layer hens nourished without any additives control treatment (T1) in 72 weeks of age.x190



A B

Fig. 3(A, B): Representative comet image showing blood lymphocyte of layer hen nourished on Annatto seed at75gm/ 100kg (T2) in 72 weeks of age.x190

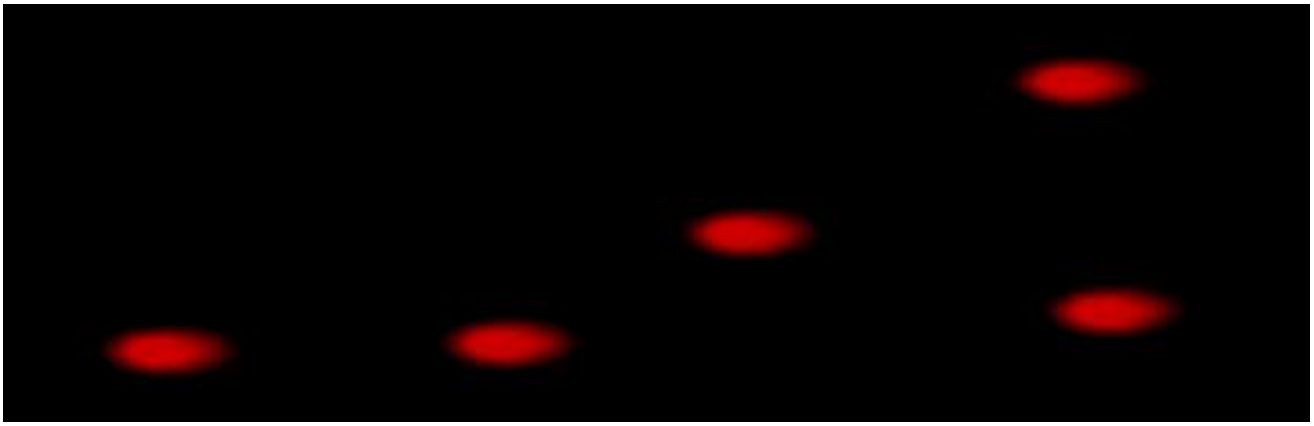
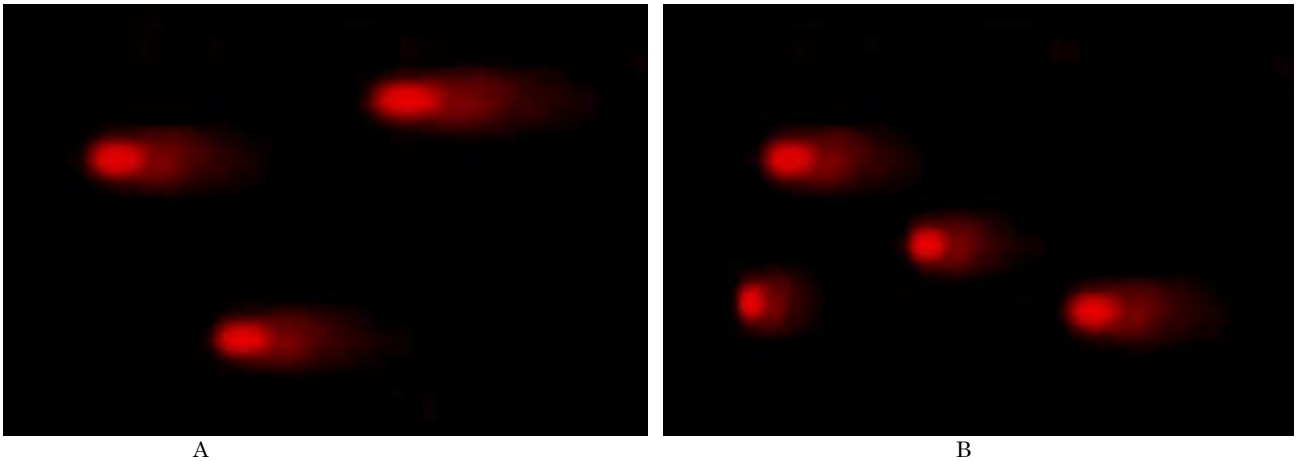
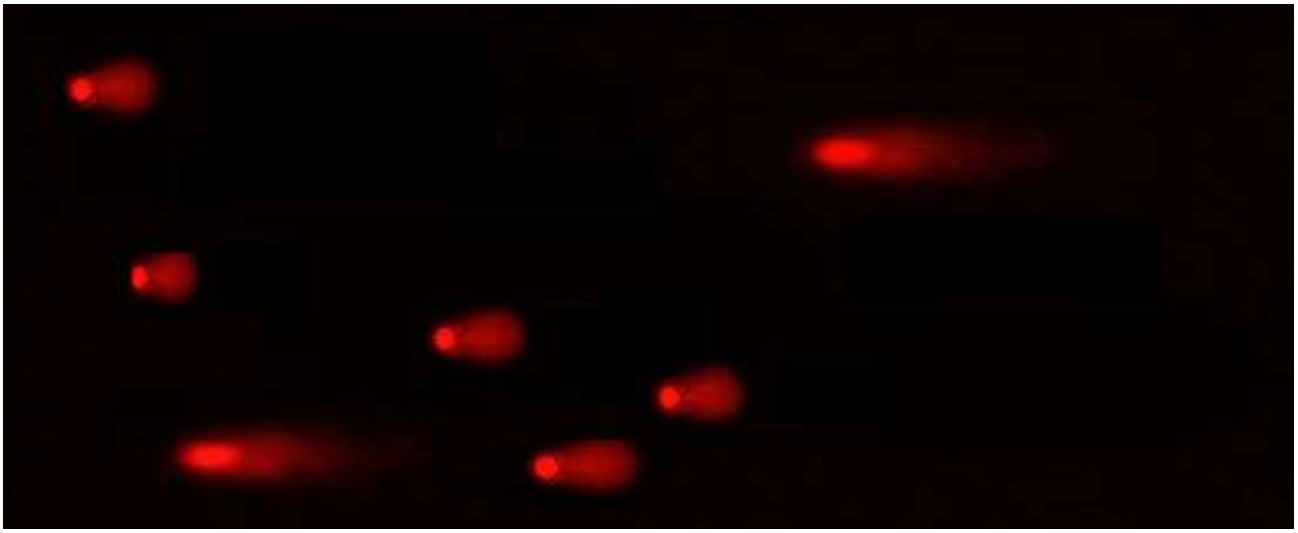


Fig. 4: Representative comet image showing blood lymphocyte of layer hen nourished on Annatto seed at150 gm/100kg (T3) in 72 weeks of age.x190



A B



C

Fig. 5(A, B,C) Representative comet image showing blood lymphocyte (damaged DNA) of layer hen nourished on Annatto seed at 225gm/100kg (T4) in 72 weeks of age.x190

Discussion

The result of this study explain added Annatto seed at 225gm / 100 Kg (T4) for a long period cases DNA damage as we shown in comet assay parameters, while added at level 75 – 150 gm /100 Kg (T2,T3) which enhance cell statues and recorded best value even compared with control group. Control group (T1) recorded mild damage of DNA this may be due to laying egg for along period act as stress factor on body physiology of the hen this stress generate free radical, reactive oxygen species attack the bio molecule like DNA, protein, RNA and other macromolecules [25] then cases damage. While T2 and T3 recorded lowest value of DNA damage this may be due to Annatto seed have biological compound act as anti oxidants.

This finding is similar to the study by [26] which recordedthe ethanolic extract of Bixa orellana have antiradical properties, bactericidal and fungicidal activities this due to increases the potential application of as a natural food preserver offered. In vitro assay of Bixa orellana seed extract using DPPH and Ferric iron reducing power models also showed antioxidant activity at low concentrations [27].

The Aqueous extracts of Annatto seed have Flavonoids, Steroids, Cardiac Glycosides and Terpenoids [28]. Anthraquinone[29]. Flavonoids and many other phenolic compounds of plant origin have been reported as scavengers of reactive oxygen species (ROS) [30, 13]. Researchers have shown that the more active agents in the herbs have a strong capability for scavenging superoxide radicals, hydrogen peroxide and nitric oxide from activated macrophages, reducing iron complex and inhibiting lipid peroxidation [32,34].Stress conditions can be generally divided into three main categories [35]. The most important part is nutritional stress conditions. A second group of stress factors includes environmental conditions: increased temperature or humidity, hyper oxia, radiation etc. Internal stress factors include various bacterial or viral diseases as well as allergy [36]. So may be DNA damage occurs in present study (T4) when feed hens by a large dose of Annatto seed for a long period and this may be cases allergy [37]. Improved that Annatto have many side effect like cases lower of blood pressure, urticaria and angioedema, alter glycemic and insulinlevels.

Annatto has been improved as a cause of allergic reflexe, these reactions generally taking the form of angioedema, urticaria or eczema, although one case of anaphylaxis. The Genetic potential can be influenced by increased production of free radicals is more relevant to disease and frequently the attempted target of supplementation intervention [38, 39].

Conclusion

Added Annatto seed at 225gm / 100kg (T4) to layer hen diet for 3 months has genotoxic affect on blood lymphocyte as shown in comet assay parameters. While added at level 75gm/100kg (T2), 150 gm/kg (T3) have no adverse affect on blood lymphocyte by decrease level of Comet Assay parameters especially T2 followed byT3.

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