

# EFFECT OF DIETARY INCLUSION OF *SILYBUM MARIANUM* OIL EXTRACTION BYPRODUCT ON GROWTH PERFORMANCE, IMMUNE RESPONSE AND CECAL MICROBIAL POPULATION OF BROILER CHICKEN

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**Abstract:** The aim of this study was to evaluate the effects of *Silybum marianum* oil extraction byproduct (SMOEB) supplementation on performance and some physiological parameters of broiler chickens. Treatments were consisted of different levels of SMOEB (0%, 3%, 6%, 9% and 12% of the diet). From day 8-21, dietary inclusion of 3%, 9% and 12% of SMOEB into the diet increased ( $P<0.05$ ) feed intake and body weight gain of broilers. From day 22-42, increasing SMOEB level increased feed intake linearly. Increasing SMOEB level increased FI linearly and quadratically from day 8-42. On day 16, a higher value was recorded for wing-web thickness in birds that were received 9% of SMOEB in the diet only at 24h following the PHA-P injection ( $P<0.05$ ). The higher value was observed for CBH response at 12 and 24h post-injection on day 21 and day 35 in broilers were fed by 9% of SMOEB supplemented into the diet for toe web thickness. It can be concluded that SMOEB could be added to the diet of broilers without any adverse effect on performance parameters and also, it can improve immune parameters of birds at levels up to 12%.

**Key words:** Body weight, Broiler, By-product, Immunology, Microbiology

## Introduction

Due to COVID-19 crisis and drastic changes in feed chain and feed supply, search for alternative feed supplies becomes urgently apparent and needs further research (Hafez and Attia, 2020). Also, poultry production has become more important for developing countries following increasing human population and

demand for animal protein. According to Longe (1986), poultry production represents the fastest means for compensating the shortage of animal protein availability due to their fast rate of production and quick return on investment. Unfortunately, severe drought and shortage of water resources has increased the costs of conventional feedstuff production for animals in many developing countries including Iran. Therefore, low-cost by-product feeds are often used to decrease feed costs and improve farm profitability (Rogers and Poore, 1994). Chemical and physical properties of By-product can influence the production efficiency. For instance, the use of Agro-industrial by-products (AIBs) in animal feed can dramatically reduce the cost of feed (Longe, 1986). Considerable efforts have been made to improve the utilization of these AIBs in practical monogastric nutrition.

Plants have been a constant source of drugs and recently, much emphasis has been placed on finding novel therapeutic agents from medicinal plants. Today many people prefer to use medicinal plants rather than chemical drugs (Fatehi et al., 2004). The by-products provided through oil extraction process, which is a part of the medicinal plants production, can be used for animal nutrition.

Milk thistle (*Silybum marianum* L. Gaernt.), sometimes called wild artichoke, is a medicinal plant that has been used for thousands of years as a remedy for a variety of ailments (Rainone, 2005). *Silybum marianum* is used presently to treat alcoholic hepatitis, liver cirrhosis, liver poisoning, and viral hepatitis, and to protect the liver from side effects of medications (Luper, 1998). Standardized *Silybum marianum* extract is known as silymarin that is a combination of at least seven chemicals (Muriel, 1992) and it is a naturally occurring polyphenolic flavonoid (Atanasoff et al., 2015). Silymarin and its major constituent, silybin, have been reported to work as antioxidants scavenging free radicals and inhibiting lipid peroxidation. Some studies also suggest that they can protect against genomic injury, increase hepatocyte protein synthesis, decrease the activity of tumor promoters, stabilize mast cells, chelate iron, and slow down calcium metabolism (Flora et al., 1998). Flavonoids are a group of natural compounds known to have various pharmacological actions such as antioxydative, anti-inflammatory and diuretic (Havesteen, 2002). It has been reported that extracts of plants that are rich in flavonoids possess antimicrobial activity (Tim and Andrew, 2005). Abed et al. (2015) showed that the extracted flavonoids of *Silybum marianum* can act against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*. In short, no study has been conducted in Iran to determine the optimal dietary inclusion level and the mode of action for these plant by-products in order to enhance the growth performance in poultry production. Therefore, the present study is aimed at investigating the efficacy of an oil extraction by-product of *Silybum marianum* on the performance, and some physiological parameters of broiler chickens.

## Materials and Methods

All the procedures under taken in our study were approved by the Animal Ethics Committee at agricultural sciences and natural resources university of Khuzestan, Ahvaz, Iran.

### *Preparation and analysis of Silybum marianum oil extraction by-product*

*Silybum marianum* oil extraction by-product (SMOEB) was prepared from the Barij Essence Pharmaceutical Company, Kashan, Iran. Four samples of SMOEB were prepared and were analyzed for dry matter (DM), crude protein (CP, crude fiber (CF), ether extract (EE), and ash according to AOAC (2002). Total phenolic and flavonoid content was analyzed in the study by *Senguttuvan et al.*, (2014). The results are shown in Table 1.

**Table 1. Chemical composition of *Silybum marianum* oil extraction byproduct (%)**

| DM | CP | EE | CF | Ash  | Lignin | Total of Phenol* | Total of Flavonoid* | Tannin   | ME <sup>1</sup> | NFE <sup>2</sup> |
|----|----|----|----|------|--------|------------------|---------------------|----------|-----------------|------------------|
| 93 | 20 | 10 | 30 | 3.78 | 13.75  | 206.18           | 571.62              | 232.12** | 1522.98         | 29.22            |

<sup>1</sup>Metabolizable energy value was calculated using an equation from NRC (cottonseed meal, expeller or solvent) (Janssen, 1989) (kcal/kg).

<sup>2</sup>Nitrogen free extract=100-(Moisture + CP + EE + CF + Ash)

\*Values were analyzed according to micro/g DW

\*\* Value was analysed according to mg/g DW

### *Birds, diets, and general procedures*

One-day-old male chicks (Ross 308) were obtained from a local hatchery and were housed in floor pens, and were fed according to a standard broiler diet for week 1. Feed and water were provided *ad libitum* for their consumption. On day 8, totally 275 one-day-old male broiler chicks were weighed individually and were distributed randomly into 5 treatment groups, with 5 replicates (11 chicks) for each treatment group in a completely randomized design. Treatments were consisted of different levels of SMOEB (0%, 3%, 6%, 9% and 12% of diet). The feeding regimen was consisted of a starter (for day 8-21), and grower (for day 22-42) diet. The diets were formulated to meet the nutrient requirements of broilers according to *NRC (1994)*. Mash feed and water were provided *ad libitum* throughout the experiment. The ingredients and chemical composition of the diets are shown in Tables 2 and 3. Feed was prepared weekly and was stored in airtight containers. Light intensity was kept on same level continuously for the first 3 days of post-hatching, after which a 23L:1D lighting schedule was maintained during the experiment. At day 1 of age, the temperature was set at 33°C and subsequently was reduced by 2°C/wk.

**Table 2. Composition and nutrient contents of the basal diet and diets with increasing levels of *Silybum marianum* oil extraction byproduct (SMOEB) in the starter phase (d 8 to 21 posthatch)**

| Ingredients (%)                                 | Levels of SMOEB (%) |       |       |       |       |
|-------------------------------------------------|---------------------|-------|-------|-------|-------|
|                                                 | 0                   | 3     | 6     | 9     | 12    |
| Corn                                            | 58.69               | 56.84 | 54.85 | 51.63 | 48.61 |
| Soybean meal (43% CP)                           | 32.30               | 31.50 | 30.82 | 30.00 | 29.20 |
| SMOEB                                           | 0.00                | 3.00  | 6.00  | 9.00  | 12.00 |
| Wheat bran                                      | 1.76                | 0.87  | 0.00  | 0.00  | 0.00  |
| Fish meal                                       | 3.00                | 3.00  | 3.00  | 3.00  | 3.00  |
| Soybean oil                                     | 0.70                | 1.20  | 1.70  | 2.70  | 3.52  |
| Dicalcium phosphate                             | 1.26                | 1.30  | 1.33  | 1.36  | 1.35  |
| Limestone                                       | 1.24                | 1.23  | 1.23  | 1.23  | 1.23  |
| Common salt                                     | 0.26                | 0.26  | 0.26  | 0.26  | 0.26  |
| Sodium bicarbonate                              | 0.15                | 0.15  | 0.15  | 0.15  | 0.15  |
| Vitamin and mineral premix <sup>3</sup>         | 0.50                | 0.50  | 0.50  | 0.50  | 0.50  |
| DL-Methionine                                   | 0.14                | 0.15  | 0.16  | 0.17  | 0.18  |
| Calculated analysis (% unless stated otherwise) |                     |       |       |       |       |
| ME (kcal/kg)                                    | 2959                | 2959  | 2959  | 2959  | 2959  |
| Crude protein                                   | 21.21               | 21.17 | 21.18 | 21.15 | 21.15 |
| Methionine                                      | 0.50                | 0.50  | 0.50  | 0.50  | 0.50  |
| Lysine                                          | 1.32                | 1.29  | 1.26  | 1.22  | 1.19  |
| Calcium                                         | 1.00                | 1.00  | 1.00  | 1.00  | 1.00  |
| Non-phytate P                                   | 0.45                | 0.45  | 0.45  | 0.45  | 0.45  |
| Sodium                                          | 0.20                | 0.20  | 0.20  | 0.20  | 0.20  |

<sup>1</sup> Supplied per kilogram of diet: retinyl acetate, 1.55 mg; cholecalciferol, 0.025 mg;  $\alpha$ -tocopherol acetate, 20 mg; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 10 mg; calcium pantothenate, 10 mg; choline chloride, 400 mg; nicotinamide, 50 mg; pyridoxine HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B<sub>12</sub> (cobalamin), 1.013 mg; Fe, 60 mg; Mn, 100 mg; Zn, 60 mg; Cu, 10 mg; I, 1 mg; Co, 0.2 mg; Se, 0.15 mg.

**Table 3. Composition and nutrient contents of the basal diet and diets with increasing levels of *Silybum marianum* oil extraction byproduct (SMOEB) in the grower phase (d 22 to 42 posthatch)**

| Ingredients (%)                                 | Levels of SMOEB (%) |       |       |       |       |
|-------------------------------------------------|---------------------|-------|-------|-------|-------|
|                                                 | 0                   | 3     | 6     | 9     | 12    |
| Corn                                            | 65.41               | 62.39 | 59.33 | 56.23 | 53.38 |
| Soybean meal (43% CP)                           | 27.71               | 27.00 | 26.27 | 25.62 | 24.71 |
| SMOEB                                           | 0.00                | 3.00  | 6.00  | 9.00  | 12.00 |
| Fish meal                                       | 2.00                | 2.00  | 2.00  | 2.00  | 2.00  |
| Soybean oil                                     | 1.70                | 2.41  | 3.15  | 3.88  | 4.60  |
| Dicalcium phosphate                             | 0.90                | 0.90  | 0.95  | 0.95  | 0.95  |
| Limestone                                       | 1.35                | 1.35  | 1.33  | 1.33  | 1.34  |
| Common salt                                     | 0.20                | 0.20  | 0.20  | 0.20  | 0.20  |
| Sodium bicarbonate                              | 0.17                | 0.17  | 0.17  | 0.17  | 0.17  |
| Vitamin and mineral premix <sup>3</sup>         | 0.50                | 0.50  | 0.50  | 0.50  | 0.50  |
| DL-Methionine                                   | 0.06                | 0.08  | 0.10  | 0.12  | 0.15  |
| Calculated analysis (% unless stated otherwise) |                     |       |       |       |       |
| ME (kcal/kg)                                    | 3100                | 3100  | 3100  | 3100  | 3100  |
| Crude protein                                   | 18.91               | 18.95 | 18.98 | 19.03 | 19.10 |
| Methionine                                      | 0.38                | 0.38  | 0.38  | 0.38  | 0.38  |
| Lysine                                          | 1.13                | 1.10  | 1.07  | 1.05  | 1.02  |
| Calcium                                         | 0.90                | 0.90  | 0.90  | 0.90  | 0.90  |
| Non-phytate P                                   | 0.35                | 0.35  | 0.35  | 0.35  | 0.35  |
| Sodium                                          | 0.15                | 0.15  | 0.15  | 0.15  | 0.15  |

<sup>1</sup> Supplied per kilogram of diet: retinyl acetate, 1.55 mg; cholecalciferol, 0.025 mg;  $\alpha$ -tocopherol acetate, 20 mg; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 10 mg; calcium pantothenate, 10 mg; choline chloride, 400 mg; nicotinamide, 50 mg; pyridoxine HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B<sub>12</sub> (cobalamin), 1.013 mg; Fe, 60 mg; Mn, 100 mg; Zn, 60 mg; Cu, 10 mg; I, 1 mg; Co, 0.2 mg; Se, 0.15 mg.

### Performance

Weekly body weight gain (BWG) and feed intake (FI) of each pen were recorded. Feed conversion ratio was calculated by dividing FI with BWG.

### Size of different organs

At the end of the experiment, 2 birds from each replicate (which were close to the mean BW of the replicate) were selected and slaughtered to evaluate the relative weights (based on BW) of the breast, thigh, pancreas, liver, gizzard, and abdominal fat. The length of intestinal segments including duodenum, from the pylorus to the distal portion of the duodenal loop; jejunum, the segment between the point of entry of the bile ducts and Meckel's diverticulum, ileum, from Meckel's diverticulum to the ileocecal junction and cecum (left and right) were also measured separately (*Sadeghi et al., 2015*).

### **Apparent ileal digestibility of nutrients**

The chromic oxide ( $\text{Cr}_2\text{O}_3$ ) marker method reported in the study by Vries *et al.*, (2014) was used to measure ileal nutrient digestibility. Marker-containing diets (supplemented with 3 g/kg of  $\text{Cr}_2\text{O}_3$ ) were given for 7 consecutive days, from day 36 to day 42 of the experimental period. At the end of study, 2 birds per replicate were killed by cervical dislocation and ileal contents, from Meckel's diverticulum to the ileocecal junction, and all were collected in sealed bags. The pooled digesta samples (on pen basis) were kept frozen at  $-20^\circ\text{C}$  until further analysis was performed. Feed samples and digesta were ground (using a 0.5 mm screen) prior to chemical analysis. The samples were analyzed for dry matter, organic matter, crude protein, and ether extract according to the standard procedures of *Association of Official Analytical Chemists* (2002). Chromium oxide content in the experimental diets and digesta was measured according to the study conducted by Saha and Gilbreath (1991). Digestibility coefficients were calculated using the following formula (Hafeez *et al.*, 2016):

Eq. 1

### **Apparent Digestibility(%)**

$$= 100 - \left[ \left( \frac{\text{Cr2O3 diet}}{\text{Cr2O3 digesta}} \right) \times \left( \frac{\text{nutrient in digesta}}{\text{nutrient in feed}} \right) \times 100 \right]$$

### **Gut microflora**

On day 28, 2 birds per replicate and 10 birds per treatment were selected and cecal digesta (1 g) from each bird were transferred aseptically into 9 mL of sterile saline solution and were diluted serially. *Lactobacilli* was enumerated on De Man-Rogosa-Sharpe (MRS) agar, *E. coli* and *coli*- form were counted on Mac Conkey (MC) agar after incubation at  $37^\circ\text{C}$  for 48 h in an anaerobic chamber, and for 24 h in an aerobic chamber, respectively (Guban *et al.*, 2006). All samples were plated in duplicate.

### **Cellular immunology**

In order to assess the *in vivo* cell-mediate immune response, we prepared a solution containing 1 mg/ml of phytohemagglutinin-P (PHA-P; Sigma Chemical Co., St. Louis, MO) by adding 1 ml of sterile PBS (0.15 M at pH = 7.4) to 1 mg of PHA using the septum. At 16 days of age, 8 birds per treatment (2 birds of each replicate) were selected. Then we injected 0.1 ml of PHA-P solution either intra- or sub-dermally into the right wing web of the elbow joint (or interdigital skin or wattle) as well as 0.1 ml of sterile PBS (0.15 M at pH = 7.4) into the left wing web as a sham control. The thickness of the wing web was measured, using digital calipers (pressure-sensitive), to the nearest 0.05 mm immediately before and at 24 hour and 48 hours after injection. The mitogen stimulation index (SI) was calculated as follows: (Grasman 2010). Eq 2.

The mitogen stimulation index (SI) =

$$[(\text{the increased thickness of right wing web}) - (\text{the increased thickness of left wing web})]$$

### **CBH response**

The CBH response to phytohemagglutinin-P (PHA-P; Sigma Chemical Co., St. Louis, MO) was used to assess *in vivo* cell-mediated immune response. At 21, and 35 days of age, 8 birds per treatment received 100 µg of PHA-P in 0.1 mL of sterile PBS (0.15 M at pH = 7.4) which was injected intradermally in interdigital skin between the second and third digits of the right foot. 0.1 mL of PBS was injected into the left foot as a sham control. The thickness was measured, using digital calipers, to the nearest 0.05 mm immediately before and at 12, 24, and 48 hours post-injection, and the response was evaluated as follows: (Akhlaghi *et al.*, 2013). Eq 3.

“Cutaneous basophilic hypersensitivity response = [(thickness of right toe web postinjection – thickness of right toe web preinjection) – (thickness of left toe web postinjection – thickness of left toe web preinjection)]

### **Lymphoid organs**

At the end of the experiment, 2 birds from each replicate were slaughtered and the spleen and bursa of fabricius were weighed and calculated as a percentage of live BW.

### **Statistical analysis**

Data were analyzed in a completely randomized design using General Linear Model procedures of SAS (SAS Institute, 2001). The following model was assumed in the analysis of all studied traits:  $Y_{ij} = \mu + T_i + \varepsilon_{ij}$ , (Eq.4)

Where  $Y_{ij}$  is the observed value for a particular character,  $\mu$  is the overall mean,  $T_i$  is the effect of the  $i^{\text{th}}$  treatment, and  $\varepsilon_{ij}$  is the random error associated with  $ij^{\text{th}}$  recording. Data were analyzed considering the pen of birds as the experiment unit regarding performance parameters, and the individual chicken was measured as the experimental unit for the rest of the parameters. The mean values were compared using Duncan's multiple range test. Statistical significance was determined at  $P < 0.05$ . Microbiological counts were subjected to base-10 logarithm transformation before analysis.

## **Results**

### **Performance**

Data representing the effect of dietary inclusion of SMOEB on performance of broilers are shown in Table 4. Mortality was lower than 3% with no differences

between the groups. From day 8 to day 21, dietary inclusion of 3%, 9% and 12% of SMOEB into the diet caused the increase ( $P < 0.05$ ) in feed intake (FI) and body weight gain (BWG) of broilers compared to those birds that were received 6% of SMOEB. From day 22 to day 42, FI was increased ( $P < 0.05$ ) by the inclusion of 12% of SMOEB into the diet compared to the other groups, while BWG was not influenced. The supplementation of SMOEB at the level of 12% into diet increased ( $P < 0.05$ ) FI compared to the birds receiving the control diet and the diets containing 3% and 6% of SMOEB from day 8 to day 42. Orthogonal contrast comparisons (Table 4) showed that FI quadratically decreased with increasing levels of SMOEB in d 22–42 ( $p = 0.022$ ) and d 8–42 ( $p = 0.021$ ). The FCR improved quadratically by SMOEB supplementation ( $p < 0.023$ ) from d 8 to 42. However, there were no significant differences in feed conversion ratio (FCR) between treatments, during experimental growth phases, and over the whole period of experiment.

**Table 4. Effect of treatments on growth performance traits of broilers at different phases**

|                                | Levels of SMOEB (%) |                      |                      |                       |                      |        |          | Contrast |           |
|--------------------------------|---------------------|----------------------|----------------------|-----------------------|----------------------|--------|----------|----------|-----------|
|                                | 0                   | 3                    | 6                    | 9                     | 12                   | SEM    | P- Value | Linear   | Quadratic |
| Starter phase (8 to 21 d)      |                     |                      |                      |                       |                      |        |          |          |           |
| Feed intake (g/b)              | 853.8 <sup>ab</sup> | 880.3 <sup>a</sup>   | 799.0 <sup>b</sup>   | 878.6 <sup>a</sup>    | 900.7 <sup>a</sup>   | 21.10  | 0.034    | 0.187    | 0.073     |
| Body weight gain (g/b)         | 430.6 <sup>ab</sup> | 445.8 <sup>a</sup>   | 400.9 <sup>b</sup>   | 440.4 <sup>a</sup>    | 454.7 <sup>a</sup>   | 11.70  | 0.046    | 0.266    | 0.078     |
| Feed conversion ratio (g:g)    | 1.98                | 1.97                 | 1.99                 | 1.99                  | 1.98                 | 0.006  | 0.200    | 1.000    | 0.453     |
| Grower phase (22 to 42 d)      |                     |                      |                      |                       |                      |        |          |          |           |
| Feed intake (g/b)              | 2773.6 <sup>b</sup> | 2748.9 <sup>b</sup>  | 2709.6 <sup>b</sup>  | 2857.5 <sup>b</sup>   | 3104.8 <sup>a</sup>  | 76.677 | 0.016    | 0.006    | 0.022     |
| Body weight gain (g/b)         | 1376.7              | 1450.1               | 1458.1               | 1471.0                | 1508.6               | 54.897 | 0.561    | 0.121    | 0.749     |
| Feed conversion ratio (g:g)    | 2.01                | 1.89                 | 1.85                 | 1.94                  | 2.05                 | 0.085  | 0.424    | 0.736    | 0.067     |
| Experiment overall (8 to 42 d) |                     |                      |                      |                       |                      |        |          |          |           |
| Feed intake (g/b)              | 3627.4 <sup>b</sup> | 3629.26 <sup>b</sup> | 3508.69 <sup>b</sup> | 3736.13 <sup>ab</sup> | 4005.57 <sup>a</sup> | 92.063 | 0.018    | 0.009    | 0.021     |
| Body weight gain (g/b)         | 1807.4              | 1895.9               | 1859.0               | 1911.4                | 1963.3               | 63.944 | 0.524    | 0.126    | 0.947     |
| Feed conversion ratio (g:g)    | 2.01                | 1.92                 | 1.88                 | 1.95                  | 2.04                 | 0.049  | 0.196    | 0.583    | 0.023     |

<sup>a-b</sup> Means with different superscripts within the same row differ significantly ( $P \leq 0.05$ )

SMOEB: *Silybum marianum* oil extraction byproduct

### **Size of different organs and length of intestine**

The size of different organs is presented in Table 5. None of the dietary treatments led to remarkable changes in any of the organs ( $P > 0.05$ ). The length of the duodenum, jejunum, and cecum was increased in broilers fed by SMOEB at the levels of 6%, 9%, and 12% supplemented into the diet compared to control ( $P < 0.05$ ). The ileum length was enlarged significantly ( $P < 0.05$ ) at the levels of 9%



and 12% of SMOEB compared to the control group. Orthogonal contrast comparisons (Table 5) showed that length of duodenum, jejunum, ileum, and cecum linearly increased with increasing levels of SMOEB in the diet ( $p=0.0005$ ,  $p=0.0006$ ,  $p=0.001$ ,  $p<0.0001$ , respectively).

**Table 5. Effect of dietary treatments on relative organ weights (% of BW) and length of the intestine (cm) at 42 d of age in broilers**

| Size of different organs | Levels of SMOEB (%) |                  |                   |                  |                 | SEM   | P-value | Contrast |           |
|--------------------------|---------------------|------------------|-------------------|------------------|-----------------|-------|---------|----------|-----------|
|                          | 0                   | 3                | 6                 | 9                | 12              |       |         | Linear   | Quadratic |
| Breast                   | 21.85               | 21.68            | 23.36             | 21.88            | 22.34           | 0.542 | 0.256   | 0.484    | 0.362     |
| Thighs                   | 19.57               | 19.63            | 19.32             | 18.97            | 19.34           | 0.272 | 0.521   | 0.198    | 0.568     |
| Pancreas                 | 0.27                | 0.27             | 0.27              | 0.25             | 0.26            | 0.008 | 0.623   | 0.175    | 0.941     |
| Liver                    | 2.23                | 2.13             | 2.18              | 2.23             | 2.17            | 0.033 | 0.178   | 0.773    | 0.628     |
| Gizzard                  | 2.49                | 2.46             | 2.48              | 2.54             | 2.57            | 0.033 | 0.183   | 0.034    | 0.215     |
| Abdominal Fat            | 1.60                | 1.50             | 1.501             | 1.82             | 1.92            | 0.147 | 0.184   | 0.052    | 0.197     |
| Length of intestine      |                     |                  |                   |                  |                 |       |         |          |           |
| Duodenum                 | 27 <sup>b</sup>     | 29 <sup>ab</sup> | 30 <sup>a</sup>   | 30 <sup>a</sup>  | 31 <sup>a</sup> | 0.828 | 0.005   | 0.0005   | 0.155     |
| Jejunum                  | 72 <sup>b</sup>     | 79 <sup>ab</sup> | 84 <sup>a</sup>   | 85 <sup>a</sup>  | 86 <sup>a</sup> | 2.681 | 0.010   | 0.0006   | 0.110     |
| Ileum                    | 78 <sup>c</sup>     | 79 <sup>bc</sup> | 89 <sup>abc</sup> | 91 <sup>ab</sup> | 94 <sup>a</sup> | 4.076 | 0.022   | 0.001    | 0.795     |
| Cecum                    | 32 <sup>b</sup>     | 33 <sup>b</sup>  | 40 <sup>a</sup>   | 41 <sup>a</sup>  | 41 <sup>a</sup> | 1.546 | <0.001  | <0.0001  | 0.145     |

<sup>a-c</sup> Means with different superscripts within the same row differ significantly ( $P \leq 0.05$ )

SMOEB: *Silybum marianum* oil extraction byproduct

### ***Apparent ileal digestibility of nutrients***

As shown in Table 6, apparent ileal digestibility of dry matter, organic matter, crude protein and ether extract were not influenced by the treatments. Orthogonal contrast comparisons (Table 6) showed that apparent ileal digestibility of organic matter increased quadratically by SMOEB supplementation ( $p=0.028$ ).

**Table 6. Effect of dietary treatments on apparent ileal digestibility of nutrients and cecal microbial population of broilers at d 42**

|                                                   | Levels of SMOEB (%) |      |      |      |      |       |         | Contrast |           |
|---------------------------------------------------|---------------------|------|------|------|------|-------|---------|----------|-----------|
| Apparent ileal digestibility (%)                  | 0                   | 3    | 6    | 9    | 12   | SEM   | P-value | Linear   | Quadratic |
| Dry Matter                                        | 77.1                | 77.3 | 77.4 | 77.1 | 76.9 | 0.240 | 0.601   | 0.422    | 0.184     |
| Organic matter                                    | 79.5                | 79.9 | 80.0 | 79.8 | 79.5 | 0.159 | 0.231   | 0.724    | 0.028     |
| Crude protein                                     | 72.8                | 72.0 | 72.5 | 72.4 | 72.6 | 0.239 | 0.337   | 0.965    | 0.189     |
| Ether extract                                     | 63.1                | 62.6 | 62.9 | 62.8 | 63.0 | 0.144 | 0.216   | 0.960    | 0.068     |
| Cecal microbial population (log CFU/g of digesta) |                     |      |      |      |      |       |         |          |           |
| Lactobacill                                       | 7.9                 | 8.0  | 8.1  | 8.1  | 8.0  | 0.070 | 0.355   | 0.237    | 0.154     |
| Coliform                                          | 7.8                 | 7.6  | 7.8  | 7.9  | 7.9  | 0.153 | 0.635   | 0.267    | 0.445     |
| E.Coli                                            | 7.8                 | 6.9  | 6.8  | 7.1  | 7.1  | 0.357 | 0.348   | 0.331    | 0.153     |
| Lactobacilli/E.coli                               | 1.01                | 1.16 | 1.19 | 1.14 | 1.12 | 0.090 | 0.127   | 0.257    | 0.098     |
| Total aerobes                                     | 7.7                 | 8.0  | 7.9  | 8.1  | 8.1  | 0.130 | 0.176   | 0.078    | 0.459     |

SMOEB: *Silybum marianum* oil extraction byproduct

### **Intestinal bacterial colonization**

The effects of SMOEB supplemented into diet on cecal microbial population of birds are shown in Table 6. Cecal population of *Lactobacillus*, *Coliform*, *E. coli*, Total aerobes and *Lactobacilli/E.coli* ratio were not influenced by the treatments ( $P>0.05$ ).

### **Immunity**

#### **CBH response**

Table 7 shows the CBH response in broiler chickens. On day 16, a higher value was recorded for wing web thickness in the birds that were received 9% of SMOEB in the diet only at 24hr following the PHA-P injection ( $P<0.05$ ). Moreover, higher value of CBH response was observed in toe web thickness at 12 and 24hr post-injection on day 21 and day 35 in broilers that were fed by 9% of SMOEB supplemented into diet. The value of CBH response for wing web thickness quadratically increased ( $p=0.048$ ) by increasing SMOEB levels in diet on day 16 only at 24hr following the PHA-P injection. On day 21, CBH response for toe web thickness at 12hr post-injection, linearly ( $p=0.016$ ) and at 24hr ( $p=0.021$ ) and 48hr ( $p=0.041$ ) post-injection, quadratically, increased by supplementation of SMOEB in the diet. The CBH response quadratically affected by supplementation of SMOEB after 12hr ( $p=0.033$ ) and 48hr ( $p=0.045$ ) post-injection on day 35. The relative weight of bursa of fabricius was not influenced by the treatments ( $P>0.05$ ; Table 7). However, higher relative weight of spleen was observed in broilers fed by 9% of SMOEB supplemented into diet compared to the other groups ( $P<0.05$ ).

**Table 7. Effects of dietary treatments on cutaneous basophilic hypersensitivity response to phytohemagglutinin-P (PHA-P) injection as well as the relative weight of lymphoid organs in broilers**

|                                     | Levels of SMOEB <sup>1</sup> (%) |                    |                    |                   |                     |       |         | Contrast |           |
|-------------------------------------|----------------------------------|--------------------|--------------------|-------------------|---------------------|-------|---------|----------|-----------|
| Immunity                            | 0                                | 3                  | 6                  | 9                 | 12                  | SEM   | P-value | Linear   | Quadratic |
| Wing web thickness, mm (d 16)       |                                  |                    |                    |                   |                     |       |         |          |           |
| 24h                                 | 0.20 <sup>b</sup>                | 0.23 <sup>b</sup>  | 0.23 <sup>b</sup>  | 0.39 <sup>a</sup> | 0.17 <sup>b</sup>   | 0.040 | 0.028   | 0.421    | 0.048     |
| 48h                                 | 0.19                             | 0.21               | 0.21               | 0.32              | 0.16                | 0.052 | 0.343   | 0.840    | 0.225     |
| Toe web thickness, mm (d 21)        |                                  |                    |                    |                   |                     |       |         |          |           |
| 12h                                 | 0.27 <sup>b</sup>                | 0.31 <sup>b</sup>  | 0.37 <sup>ab</sup> | 0.45 <sup>a</sup> | 0.36 <sup>ab</sup>  | 0.036 | 0.037   | 0.016    | 0.087     |
| 24h                                 | 0.23 <sup>c</sup>                | 0.30 <sup>bc</sup> | 0.37 <sup>ab</sup> | 0.41 <sup>a</sup> | 0.33 <sup>abc</sup> | 0.031 | 0.032   | 0.014    | 0.021     |
| 48h                                 | 0.11                             | 0.16               | 0.22               | 0.28              | 0.12                | 0.049 | 0.208   | 0.366    | 0.041     |
| Toe web thickness, mm (d 35)        |                                  |                    |                    |                   |                     |       |         |          |           |
| 12h                                 | 0.56 <sup>b</sup>                | 0.57 <sup>b</sup>  | 0.62 <sup>ab</sup> | 0.71 <sup>a</sup> | 0.55 <sup>b</sup>   | 0.033 | 0.032   | 0.343    | 0.033     |
| 24h                                 | 0.45 <sup>b</sup>                | 0.46 <sup>b</sup>  | 0.49 <sup>b</sup>  | 0.62 <sup>a</sup> | 0.36 <sup>b</sup>   | 0.069 | 0.032   | 0.798    | 0.060     |
| 48h                                 | 0.21                             | 0.37               | 0.37               | 0.41              | 0.28                | 0.063 | 0.254   | 0.365    | 0.045     |
| Lymphoid organs <sup>2</sup> (d 42) |                                  |                    |                    |                   |                     |       |         |          |           |
| Bursa of fabricius (%)              | 0.18                             | 0.13               | 0.20               | 0.15              | 0.17                | 0.025 | 0.383   | 0.872    | 0.775     |
| Spleen (%)                          | 0.11 <sup>b</sup>                | 0.11 <sup>b</sup>  | 0.11 <sup>b</sup>  | 0.15 <sup>a</sup> | 0.10 <sup>b</sup>   | 0.012 | 0.045   | 0.866    | 0.178     |

<sup>a-c</sup> Means with different superscripts within the same row differ significantly ( $P \leq 0.05$ ).

<sup>1</sup> SMOEB: *Silybum marianum* oil extraction byproduct

<sup>2</sup> Lymphoid organs are expressed as a percentage of live body weight

## Discussion

### Growth performance

To our knowledge, our study is the first study investigating the effect of various levels of SMOEB on broiler performance. The literature also lacks reliable experimental data regarding this topic. We showed that from day 8 to 21, the groups that were fed by 6%, 9% and 12% of SMOEB exhibit the highest FI and BWG and those that were fed by the diet containing 3% of SMOEB as well as the control diet had the lowest level regarding these parameters. Meanwhile, from day 22 to 42 and day 8 to 42, the birds that were fed by SMOEB at the level of 12% had highest FI and there was no significant difference in the BWG between treatments. The reason for this observation is presumably the high palatability caused in part by the high concentration of SMOEB (6%, 9% and 12% of the diet). Another reason for high values of FI could be related to the lignin content of SMOEB. As shown in Table 1, SMOEB contains 13.75% lignin, and thus any increase in the levels of SMOEB, can increase the lignin content of diets. This is also supported by the findings of *Ricke et al. (1982)* which they have observed the increase in FI in broilers caused by the dietary supplementation of lignin in the diet. Also, there are some reports indicated that low concentrations of tannins increased feed intake and thus increased performance of monogastric animals (*Huang et., 2018*). The high feed intake might have enhanced body weight gain of

birds from day 8 to day 21. In our experiment, inclusion of SMOEB influenced feed consumption, but BWG and FCR were not influenced in whole period of experiment, suggesting that the primary effect of SMOEB can be on the feed intake. Similarly, Fani Makki *et al.* (2013) evaluated the ability of *Silybum marianum* seeds on performance of the broiler chickens contaminated with aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and they reported that *Silybum marianum* seeds increased FI and BWG, and improved FCR, however, AFB<sub>1</sub> had a negative effect. Gowda and Sastry (2000) confirmed the improvement of BWG caused by receiving *Silybum marianum* seeds. They also attributed these effects to antioxidant activity that stimulated protein synthesis by the bird's enzymatic system. Perhaps the improvements in BWG of birds that were fed by high levels of SMOEB (6%, 9% and 12% of diet) from day 8 to day 21 is attributed to polyphenolic flavonoid compounds (Atanasoff *et al.* 2015) such as silymarin in SMOEB that has remained after processing by antioxidant activity. It can be pointed that flavonoid compounds like silymarin could have improved the body weight gain by influencing microbial population in digestive system (Štastník *et al.* 2016). Similarly, Tedesco *et al.* (2004) reported that the addition of *Silybum marianum* seeds to the broiler diets caused an improvement in body weight gain. In contrast to our findings, Suchy *et al.* (2008) reported that supplementation of *Silybum marianum* seed cakes at the levels of 0.2% and 1%, caused a decrease in BWG of broiler chickens. In the current study, both BWG and FCR were similar among birds were fed by the basal diet and the diet supplemented by different levels of SMOEB during the whole period of experiment. FCR is a measure representing how well a flock converts feed intake into weight gain. It is also the ability of the livestock to turn feed mass into body mass. Birds that have low feed conversion ratio are referred to as efficient food consumers. Presumably, due to the similar nature of SMOEB, corn, and soybean meal, broilers treated with these ingredients exhibited similar responses of BWG and FCR. As shown in Tables 2 and 3, SMOEB was replaced with corn and soybean meal in the control diet.

### ***Size of different organs and length of intestine***

Results shown in Table 5 indicate that relative weights of the breast, thigh, pancreas, liver, gizzard, and abdominal fat of broilers at 42 days were not influenced by dietary treatments. In contrast, Zahid & Durrani (2007) fed broilers by different levels of *Silybum marianum* seed, and found significantly higher breast weights at the level of 1.5 % of feed. Fani makki *et al.* (2013) investigated the interaction between different levels of *Silybum marianum* seeds (0.5% and 1%) and aflatoxin B<sub>1</sub> (0, 25 and 50 %) in broiler diet. They also reported that relative weight of thigh was not influenced by different levels of aflatoxin B<sub>1</sub> and *Silybum marianum* seeds. However, the highest weight of breast muscle was observed in birds fed by 1% of *Silybum marianum* seeds compared to the birds that were received different levels of aflatoxin B<sub>1</sub>. Schiavone *et al.* (2007) reported that

different levels of Silymarin (0, 40 and 80 mg/kg) influenced carcass and thigh yields of broilers negatively.

The reason for the increase in length of the duodenum, jejunum, ileum, and cecum on day 42 in chickens that were received high levels of SMOEB (9% and 12%) is presumably due to the high level of crude fiber (30%) in SMOEB as shown in Table 1. This result is supported by the findings of *Sadeghi et al. (2015)*, who showed the length of the jejunum was increased in broilers fed by sugar beet pulp (3%) and sugar beet pulp/rice hull compared to those received rice hull (3%). Sugar beet pulp and sugar beet pulp/rice hull enlarged the ileum significantly compared to the other dietary treatments. The amount of crude fiber in sugar beet pulp and rice hull in that study was equal to 15.1% and 44.7%, respectively. In agreement with this, *Jiménez-Moreno et al. (2013)* reported longer intestines in chicks received sugar beet pulp compared to those fed by rice hull at 6 days and 12 days of age. In contrast, *Saki et al. (2011)* demonstrated that supplementing a greater ratio of soluble fiber decreased ileum length at 14 days of age. The measurements in the present study confirm that intake of high fiber diets causes a significant increase in length of the caecum. This observation has been confirmed in previous studies on birds (*Savory & Gentle 1976; Savory 1992*), and also on other animal species such as rat (*Hansen et al. 1992; Zhao et al. 1995*) and pig (*Jorgensen et al. 1996*). These changes can influence the energy metabolism as visceral organs have a high rate of energy expenditure relative to their size (*Pekas and Wray 1991*).

#### **Apparent ileal digestibility of nutrients**

Depending on the dietary fiber levels, the diet passes the digestive tract at different speeds. This speed is a crucial digestion parameter (*Mateos et al. 2012*). The coefficients of total tract apparent digestibility of nutrients, such as the dietary metabolizable energy content, can be increased significantly if fiber is included in the diet (*Gonzalez-Alvarado et al. 2010*). Also, several studies have reported that inclusion of moderate amounts of insoluble fiber sources in the diet can increase hydrochloric acid (*Jiménez-Moreno et al., 2010*), bile acids, and enzyme production (*Hetland et al., 2003*) and result in increased nutrient digestibility and growth performance of broilers fed by low-fiber diets (*Jiménez-Moreno et al., 2009 a, b; González-Alvarado et al., 2010*). In the current study, by increasing the level of SMOEB in the diet, crude fiber level of diet was increased, as shown in Table 1, SMOEB is contained of 30% of crude fiber. The difference in apparent ileal digestibility of nutrients was almost identical between diets that were supplemented by SMOEB and the control diet.

#### **Intestinal bacterial colonization**

It is important to consider that some feed additives originating from plant products have a profound effect on gut microflora either directly or indirectly (*Cowan 1999*). In the current study, birds fed by the diets containing different levels of SMOEB showed a similar microbial population of cecum compared to those fed by

control diet. Antimicrobial activity has been recognized as the major beneficial effect of phytochemicals on animal production, although the exact antimicrobial mechanism has not been fully understood. Some *in vitro* studies demonstrated that flavonoids of *Silybum marianum* exhibited antimicrobial activity against intestinal microbes such as *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* (Abed et al., 2015). Antimicrobial action of flavonoids is mediated by lipophilic property to perforate the bacterial membrane, which releases membrane components from the cells to the external environment (Helander et al., 1998). But in the current study, although, SMOEB was contained of flavonoid (as shown in Table 1) but there was not significant alterations between treatments in terms of various species of microorganisms. On the other hand, in an *in vivo* study, it seems that the findings regarding the effect of flavonoids including *Silybum marianum* on gastrointestinal microflora are not consistent with the present study, even though *Silybum marianum* has been recognized generally as an antimicrobial agent. Therefore, it is speculated that the *in vivo* antimicrobial property of phytochemical in birds can be influenced by basal diet and environmental conditions.

### Immunity

The PHA-P skin response test is an *in vivo* method for measuring T lymphocyte-mediated immunity. It measures the swelling caused by inflammatory leukocyte and fluid infiltration after an intradermal injection of PHA-P. Mitogens such as PHA-P are derived from lectins, which are considered as plant or bacterial proteins that bind to specific sugar components of glycoproteins attached to the surface of cells. PHA-P specifically binds to the surface of T lymphocytes. In the skin response test, PHA-P stimulates T lymphocytes to release lymphokines, resulting in an increase in vascular permeability and the influx in a variety of leukocytes. A large increase in skin thickness indicates a strong T cell-mediated immune response (Grasman, 2010). Ezekowitz and Hoffman (1998) demonstrated that spleen cells are consisted of T cells, B cells, and macrophages. When antigens intrude into the body, the spleen notifies T and B cells, thus stimulating cell-mediated immunity. In the current study, the levels of 6% and 9% of SMOEB increased the influx in a variety of leukocytes to injection site of PHA-P. In addition, it had been shown that certain non-genetic factors such as dietary nutrient concentrations can alter to some extent the expression of the genes responsible for immune responsiveness (Rama Rao et al., 2003). Also, cell-mediated immunity system of birds is consisted of cells with the ability of quick cleavage, which requires adequate nutrient material (Vegad, 2002). Wang et al. (2008) and Brenes et al. (2008) reported that plant extracts rich in polyphenols, as natural antioxidants are good candidates (with safety and toxicity effects), because they are obtained easily from natural sources and they can prevent lipid oxidation in food products efficiently. In the current study, the improvement in cellular immunity caused by supplementing high levels of SMOEB (especially 9% of the diet), can be partly due to polyphenolic flavonoid compounds such as silymarin in SMOEB which have remained after processing. As reported by Basaga et al. (1997) milk thistle

can support the immune system through its powerful antioxidant, as well as by its direct effects on immune cells. *Chand et al. (2011)* showed that Silymarin, as antioxidant, has protective action against the oxidative damages on the immune organs (bursa of fabricius, spleen and thymus). Moreover, *Mitaru et al. (1984)* reported that the lignin content of the diets appears to have some adverse effects on protein and can bind to some of amino acids such as methionine. Also, *Rama Rao et al. (2003)* reported that methionine levels lower than 0.50 % in broiler diet would generate a poorer immune cell response compared to higher concentrations. In general, the level of requirement of methionine for increased immunity is higher rather than the one for growth. Therefore, the decline in cellular immunity in birds that were fed by 12% of SMOEB can be related to the lignin content of diets. This means the lignin content of diets, only in high levels of SMOEB (12%), could suppress the cellular immunity. Similarly, as shown in Table 1, in our experiment, SMOEB is contained of 13.75 % of lignin.

*Al-Khalifa et al. (2012)* reported that the thymus, spleen, and bursa of fabricius are the main immune organs in poultry. During an immune response, mature lymphocytes and other immune cells interact with antigens in these tissues. Consequently, in some cases immune tissue development can indicate immune system response. *Cazaban and Gardin (2012)* showed that in young birds the bursa of fabricius plays an important role in boosting immunity. In the current study, SMOEB did not influence the growth of the bursa of fabricius. But chickens fed by diets containing 9% of SMOEB had significantly greater spleen weights compared to chickens fed by 0, 3%, 6% and 12% of SMOEB. It is not clear that why the increased spleen weight was not maintained after feeding chickens by 9% of SMOEB supplemented in diet. *Fani Makki et al. (2013)* investigated the interaction between different levels of *Silybum marianum* seeds (0.5% and 1%) and aflatoxin B<sub>1</sub> (0, 250 and 500 mg/kg) in broilers diet, and found out that in diets containing AFB<sub>1</sub>, bursa of fabricius weight was decreased significantly, whereas spleen weight did not change significantly by the treatments.

According to our results, SMOEB can be used in broiler chick diets at levels up to 12%. Also, not only the use of SMOEB as by-product in the diet had not negative effect in broiler performance, but also it improved immune parameters of birds.

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## Uticaj ukljućivanja u ishranu nusproizvoda ekstrakcije ulja *Silybum Marianum* na porast, imunološki odgovor i mikrobiološku populaciju develop creva brojlerskih pilića

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## Rezime

Cilj ovog istraživanja je bio da se procene efekti dodavanja u ishranu nusproizvoda ekstrakcije ulja *Silybum marianum* (SMOEB) na performanse i neke fiziološke parametre pilića brojlera. Tretmani su se sastojali od različitih nivoa SMOEB (0%, 3%, 6%, 9% i 12% obroka). Od 8. do 21. dana, uključivanje 3%, 9% i 12% SMOEB u ishranu povećalo je ( $P < 0,05$ ) unos hrane i prirast telesne mase brojlera. Od 22. do 42. dana, povećanjem nivoa SMOEB linearno se povećavao unos hrane. Povećavanjem nivoa SMOEB povećavao se FI linearno i kvadratno od 8. do 42. dana. Veća vrednost za debljinu mrežice krila zabeležena je 16. dana kod brojlera koji su primile 9% SMOEB u ishrani samo 24 sata nakon injekcije PHA-P ( $P < 0,05$ ). Viša vrednost zabeležena je za CBH odgovor 12 i 24 sata nakon injekcije, 21. i 35. dana kod brojlera koji su hranjeni obrokom sa 9% SMOEB-a za debljinu mrežice prstiju. Može se zaključiti da bi SMOEB mogao da se doda u ishranu brojlera bez ikakvog negativnog uticaja na parametre performansi, a takođe može da poboljša imunološke parametre brojlera na nivoima do 12%.

**Ključne reči:** telesna masa, brojler, nusproizvod, imunologija, mikrobiologija

### Disclosure of interest

The authors report no conflict of interest.

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