Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 55 (1):152-161. Oct. 2017
DOI: 10.5455/ajvs.275350

Effect of Rosemary (*Rosmarinus Officinalis*) Dietary Supplementation in Broiler Chickens Concerning immunity, Antioxidant Status, and Performance

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Key words:
Broilers, Immunity, Antioxidant, Performance, Feed additives, Rosemary

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ABSTRACT

This study was conducted to evaluate the effect of rosemary leaves on the growth performance, blood parameters, and immune response of broiler chickens by determination of the serum immunoglobulins (IgA, IgM, and IgG), interferon-γ (INF-γ) and interleukin-10 (IL-10). Also, malondialdehyde (MDA), total superoxide dismutase (T.SOD), glutathione S-transferase (GST), and glutathione reduced (GSH) levels in the thigh and breast muscles were determined to evaluate the effect of rosemary in the broiler chicken's muscles. To achieve this aim, 120 Cobb of one-day-old chicks were allocated into four equal groups as a control group that supplemented by the basal diet, while the other three groups were fed basal diet supplemented with 0.5, 1.0, and 1.5% of rosemary. The data of growth performance indicated that supplementation of broiler with rosemary had no growth-promoting effects. Feeding diet with rosemary leaves meal significantly increased the serum total protein and globulin, while significantly decreased total cholesterol and triacylglycerol levels. Rosemary significantly increased the IgG, IgM, INF-γ, IL-10, and muscle GSH levels and T. SOD and GST activities. Whereas, muscle MDA levels were significantly decreased, so rosemary could be considered as a natural antioxidant in broiler diet. Concomitantly, provide a healthy broiler's meat with less MDA that favorable to human consumption

1. INTRODUCTION

The European Commission banned antibiotic growth promoters in broiler nutrition. Therefore, many research studies have been conducted to explore the use of possible effective substitutes. One possibility is the application of herbs or their essential oils (Sarica *et al.*, 2007). Numerous *in vitro* studies have already confirmed the antibacterial actions of these feed additives. Consequently, several *in vivo* studies were performed to confirm their beneficial qualities. Medicinal plants are resources of new drugs and many of the modern medicines that improve the health status of animals (El-Far *et al.*, 2016b; El-Far *et al.*, 2017). The active principles of essential oils act as a digestibility enhancer, balancing the gut microbiota and stimulating the secretion of endogenous digestive enzymes and thus improving the growth performance in poultry (Cross *et al.*, 2007; Ayoub *et al.*, 2011; Barakat *et al.*, 2016; El-Far *et al.*, 2016a).

Rosemary (*Rosmarinus officinalis*) has been used as a medicinal and aromatic herb since ancient Greek and Roman (al-Sereiti *et al.*, 1999). In folk

medicine, rosemary extract is a treatment for urinary ailments, chronic weakness, nervous disorders, hair loss, and peripheral vascular diseases. In addition, rosemary is a traditional astringent, carminative, tonic, rubefacient, antispasmodic, anti-inflammatory, expectorant, emmenagogue, digestive, and diaphoretic (Haloui *et al.*, 2000). Rosemary is broadly used in the food industry, and it is highly appreciated for its several functional properties, such as aromatic properties, antioxidant, and antimicrobial (Afonso *et al.*, 2013). Therefore, the current study was conducted to evaluate the impact of rosemary feed supplementation on broiler chicken's health status targeting the immunity, antioxidant potential, and performance.

2. MATERIAL AND METHODS

2.1. Birds, accommodation, and management

The present study is affirmed by the Ethics of Animal Experiments Committee, Damanhour University, Egypt. Whereas, one hundred and twenty Cobb of one-day-old broiler chicks were incubated and randomly allocated into four equal groups at the first week of age. Each one was subdivided into three replicates (10 birds per replicate). The housing of chicks was done in a clean well-ventilated room. The room temperature was adjusted according to age by electric heaters. Furthermore, the birds were vaccinated by Hitchner IB (7^{th} day), Gumboro (14^{th} day), and Gumboro and Clone (21^{st} day) by eye drop.

2.2. Diet and experimental design

The chicks were fed on the two phases feeding programs from 1^{st} to 21^{st} days on the starter and from 22^{nd} to 35^{th} days on grower diets. The control diet composition was represented in Table 1 and analyzed according to AOAC (2005). The diet was formulated to meet the requirements of NRC (1994). Rosemary was obtained from a local market, washed, ground, refined, and mixed with the ration at the concentration of 0.5% in Rosemary I, 1% in Rosemary II and 1.5% in Rosemary III groups, while control one was fed a basal diet. The water was accessed *ad libitum* to all birds.

Table 1. The starter and grower diet's ingredients percentage and calculated composition (as fed basis)

Ingredients	Starter diet	Grower diet
Corn	52.87	60.47
SBM (CP 44%)	34.26	29.31
Corn gluten (CP 60%)	5.5	3.0
Corn oil	3.3	3.26
Limestone	1.35	1.53
Dicalcium phosphate	1.74	1.47
L-Lysine	0.11	0.13
Dl-methionine	0.17	0.13
Vitamins and minerals premix	0.3	0.3
NaCl	0.4	0.4
Total	100	100
Composition		
ME (Kcal/Kg diet)	3061.2	3119.35
CP %	23.0	20.0
Calorie/protein ratio	133.1	155.97
Lysine %	1.3	1.16
Methionine %	0.58	0.48
Calcium %	1.0	0.9
Av. (P) %	0.45	0.40
NaCl	0.15	0.15

SBM= Soybean meal, ME = Metabolizable Energy, CP = crude protein, Av. (P) = Available phosphorous

*L-lysine 99% feed grade

**Dl-methionine 99% feed grade China

***Vitamin and mineral premix (Hero mix) produced by Heropharm and composed (per 3 kg) of vitamin A 12000000 IU, vitamin D3 2500000 IU, vitamin E 10000 mg, vitamin K3 2000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1500 mg, vitamin B12 10 mg, niacin 30000 mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid 10000 mg, manganese 60000 mg, zinc 50000 mg, iron 30000 mg, copper 4000 mg, iodine 300 mg, selenium 100 mg, and cobalt 100 mg.

2.3. Gas chromatography–mass spectrometry (GC-MS) analysis

The fine powder of rosemary was extracted by *n*-hexane by a dilution of 1: 3 (*w*: *v*). 10 µl of thyme *n*-hexane extract was injected in Trace GC Ultra-ISQ mass spectrometer with a direct capillary column TG–5MS (30 m×0.25 mm×0.25 µm). Helium carrier gas was used with a flow rate of 1ml/min. The oven temperature program was initiated at 50°C for 2 min, the rate of 4°C/min up to 160°C for 5 min, the rate of 8°C/min up to 220°C for 2 min, the rate of 15°C/min up to 280°C for 5 min. Injector and flame ionization detector temperatures were 250°C and 290°C, respectively. 1 µl of each extract was injected with a split ratio of 1:200 (Hay et al. 2015). The mass spectra of the identified components were determined by comparison to Wiley Registry mass spectral database of 8th edition.

2.4. Serum parameters

The blood samples were collected from wing vein at 3rd and 5th weeks. Each blood sample was left to coagulate at room temperature and centrifuged at 3000 rpm for 5 min. The clear sera were subjected to determination of total protein, albumin, alanine aminotransferase (ALT, EC 2.6.1.2), creatinine, total cholesterol, and TAG following the instructions enclosed in the manufactured kits produced by Biodiagnostic Company, Egypt. Also, serum globulin levels were calculated by subtraction of albumin value from the total protein value of the same sample (Coles, 1986).

2.5. ELISA assays

The serum levels of immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), Interferon-γ (INF-γ), and interleukin-10 (IL-10) were determined by ELISA kits manufactured by Elabscience Co.

2.6. Preparation of muscle tissue homogenate

At the end of the experimental period, the birds of control and rosemary-treated groups (*n*= 10) were sacrificed under anesthesia with an intramuscular injection of sodium pentobarbital (50 mg/kg BW), and then muscle samples from left breast and left thigh of each bird were immediately dissected and soaked in ice-cold saline 0.9%. They were homogenized using a motor-driven Teflon and glass Potter-Elvehjem homogenizer in 0.1 M Tris-HCl buffer of pH 7.4 containing 5 mM β-mercaptoethanol (1:4 *w*/*v*). The homogenates were centrifuged at 105,000 ×g for 60 min at 4°C; the supernatants were divided into aliquots then stored at -20°C for further evaluation of oxidative stress and antioxidant parameters.

2.7. Determination of oxidative stress parameters

The frozen aliquots of muscle homogenates were utilized for the colorimetric assessment of MDA and GSH contents, as well the T.SOD and GST activities.

2.7.1. Determination of lipid peroxidation

Malondialdehyde is the main aldehyde by-product of lipid peroxidation in biological systems. It was analyzed after the incubation of supernatants with thiobarbituric acid at 95°C for 30 min (pH 3.6) to form thiobarbituric acid-reactive substances (TBARS), a pink colored compound. MDA levels were measured at 532 nm and expressed as nmol MDA /mg protein (Ohkawa *et al.*, 1979).

2.7.2. Determination of reduced glutathione levels

Reduced glutathione assay was based on the reductive cleavage of 5, 5′-dithiobis 2-nitrobenzoic acid (DTNB) by compounds containing sulfhydryl groups and development of a yellow color (Sedlak and Lindsay, 1968). The quantity of reduced chromogen is directly proportional to the GSH content. The absorbance was recorded at 412 nm and expressed as µmol GSH/mg protein.

2.7.3. Determination of the Total superoxide dismutase activity

The reduction of nitro blue tetrazolium with NADH-mediated by phenazine methosulfate (PMS) under aerobic conditions was inhibited upon addition of superoxide dismutase. This observation indicated the involvement of superoxide anion radical in the reduction of nitro blue tetrazolium, the radical being generated in the reoxidation of reduced PMS. This assay was determined at 560 nm and represented as U/mg protein (Nishikimi *et al.*, 1972).

2.7.4. Determination of the glutathione S-transferase activity

The activity of GST was measured according to the method of Vessey and Boyer (1984). This assay was based on monitoring the rate of enzyme–catalyzed conjugation of the 1-chloro-2,4-dinitrobenzene (CDNB)with GSH. GST activity was measured as the increase in absorbance at 340 nm and represented as U/mg protein.

2.7.5. Determination of tissue protein

Protein concentrations in muscle homogenates were determined using bovine serum albumin as the standard according to the method of Bradford (1976).

2.8. Performance parameters

The basal diets of both starter and grower phases were formulated according to the recommendation of National Research Council Nutrient Requirements for Broiler Chickens (NRC, 1994). Performance parameters include the final body weight, feed intake, feed conversion ratio (FCR) (Lambert *et al.*, 1936) and protein efficiency ratio (McDonald *et al.*, 1987) were determined throughout the experimental period.

2.9. Statistical Analyses

The obtained data were analyzed by one-way analysis of variance (ANOVA), with Duncan's multiple range tests for significance between means using SPSS software package v.20. The data of ELISA assays and antioxidant status were analyzed by One-way ANOVA, Tukey's multiple range tests by GraphPad Prism 5. All declarations of significance depended on $p < 0.05$.

3. RESULTS

The data illustrated in Table 2 and presented in Fig.1 revealed the chemical composition of one sample was carried out using the GC–MS analysis led to the identification of eleven different components;1,8-cineole (23.76%), camphor (3.87%), α-terpineol (1.32%) eugenol (1,59) caryophyllene oxide (0.17%) oleic acid (5,16%), and ethyl iso-allocholate (3.15%).

The data illustrated in Table 2 represented that at the 3rd and 5th weeks, the serum levels of total protein and globulin were significantly increased in Rosemary I, Rosemary II, and Rosemary III in relation to control group, while serum albumin levels were non-significantly increased**.** Also, no significant changes were recognized in serum ALT activities and creatinine levels when the rosemary-treated groups were compared with control one. Furthermore, the data shown in Table 2 revelated significant decreases in the levels of total cholesterol and TAG at the 3rd and 5th weeks in comparison to control.

The data illustrated in Fig. 2(A) represented the effects of rosemary dietary supplementation on serum immunoglobulins where IgA levels non-significantly differed ($p>0.05$) at the 3rd and 5th weeks compared to control. Serum IgG levels were significant increase in Rosemary I ($p<0.05$) and Rosemary III ($p<0.01$) in relation to control at the 3rd week, while at the 5th week the IgG levels in rosemary-treated groups were significantly increased in Rosemary I ($p<0.001$), Rosemary II ($p<0.01$), and Rosemary III ($p<0.01$) compared to control (Figure 2B).The data illustrated in Figure 2.C represented the effects of rosemary dietary supplementation on serum IgM and revealed that at the 3rd and 5th weeks, the IgM levels were significantly increased ($p<0.001$) in Rosemary I, Rosemary II, and Rosemary III compared to control.

The data illustrated in Fig. 3(A) represented the effects of rosemary dietary supplementation on INF-γ and revealed that at the 3rd and 5th weeks, its levels were significantly increased ($p<0.001$) in the Rosemary I, Rosemary II, and Rosemary III compared to control. Results in Fig. 3(B) represented significant increases ($p<0.05$) in the levels of IL-10 in Rosemary II and Rosemary III compared to control at the 3rd week. Similarly, at the 5th week, the IL-10 levels showed significant increases in the Rosemary I ($p<0.05$), Rosemary II ($p<0.01$), and Rosemary III ($p<0.01$) compared to control.

Table 2. GC-MS analysis of rosemary *n*-hexane extract (antioxidant constituents)

	Compound Name	RT (minutes)	Area %	Molecular Formula
1	1,8-Cineole	12.43	23.67	$C_{10}H_{18}O$
2	(-)-camphor	18.01	3.87	$C_{10}H_{16}O$
3	α-Terpineol	18.91	1.32	$C_{10}H_{18}O$
4	Eugenol	24.58	1.59	$C_{10}H_{12}O_2$
5	Caryophyllene oxide	25.35	0.17	$C_{15}H_{24}O$
6	Oleic acid	38.88	5.16	$C_{18}H_{34}O_2$
7	Ethyl iso-allocholate	39.58	3.15	$C_{26}H_{44}O_5$

Table 3. Effect of dietary Rosemary supplementation on serum total protein, albumin, globulin, ALT, creatinine, total cholesterol, and triacylglycerol

Item	Control	Rosemary supplementation		
		Rosemary I	Rosemary II	Rosemary III
3rd week				
Total protein (g/dl)	4.45±0.05[b]	5.12±0.21[a]	5.28±0.06[a]	5.25±0.05[a]
Albumin (g/dl)	3.48±0.15[a]	3.56±0.09[a]	3.61±0.08[a]	3.57±0.11[a]
Globulin (g/dl)	0.97±0.13[b]	1.56±0.18[a]	1.66±0.08[a]	1.69±0.06[a]
ALT(U/l)	17.5±0.5[a]	17.25±0.48[ab]	16±0.41[ab]	15.75±0.63[b]
Creatinine (mg/dl)	0.33±0.05[a]	0.33±0.02[a]	0.39±0.03[a]	0.39±0.02[a]
Cholesterol (mg/dl)	144.11±3.56[a]	126.76±1.59[b]	125.14±2.31[b]	127.76±4.02[b]
TAG (mg/dl)	153.37±5.69[a]	131.97±3.37[b]	127.96±3.89[b]	130.32±1.94[b]
5th week				
Total protein (g/dl)	5.14±0.05[b]	5.62±0.1[a]	5.62±0.04[a]	5.84±0.12[a]
Albumin (g/dl)	3.53±0.05[a]	3.62±0.09[a]	3.57±0.02[a]	3.45±0.09[a]
Globulin (g/dl)	1.6±0.08[c]	2±0.17[b]	2.05±0.05[ab]	2.39±0.12[a]
ALT (U/l)	16.25±0.48[a]	16.5±0.65[a]	15±1.08[a]	14.25±0.63[a]
Creatinine (mg/dl)	0.47±0.02[ab]	0.43±0.02[b]	0.53±0.03[a]	0.47±0.03[ab]
Cholesterol (mg/dl)	150.38±2.49[a]	127.59±2.63[b]	126.63±3.32[b]	126.05±3.73[b]
TAG (mg/dl)	158.53±1.98[a]	132.23±2.94[b]	122.58±0.84[c]	130.62±2.57[b]

Mean values with different letters in the same row differ significantly at P<0.05

Table 4. Effect of dietary turmeric supplementation on growth performance of broiler chickens

	Control	Rosemary supplementation		
		Rosemary I	Rosemary II	Rosemary III
IBW (g)	160±1.54[a]	155.4±2.12[a]	157.08±2.13[a]	157.39±1.72[a]
FBW (g)	1608.25±24.59[a]	1646.2±21.56[a]	1595.21±23.68[a]	1578.04±23.05[a]
BWG (g)	1448.25±23.1[ab]	1490.8±19.48[a]	1438.13±21.66[ab]	1420.65±21.45[b]
FI (g)	2884±16.44[a]	2907.5±23.65 [a]	2916.89±30.23 [a]	2901.76±25.35 [a]
FCR	2.00±0.03[ab]	1.96±0.03[b]	2.04±0.03[ab]	2.05±0.03[a]
PER	2.58±0.04[a]	2.66±0.03[a]	2.56±0.04[a]	2.56±0.03[a]

Mean values with different letters in the same row differ significantly at P<0.05
IBW = initial body weight; FBW = final body weight; BWG = body weight gain; FI= Feed intake; FCR = feed conversion ratio; PER = protein efficiency ratio

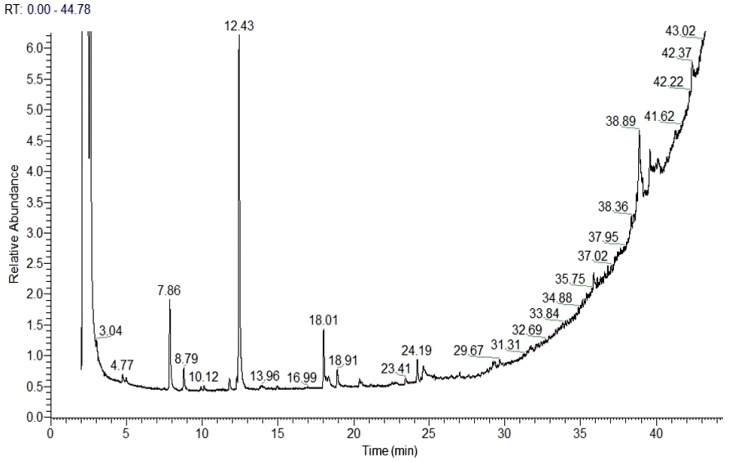

Fig. 1. GC-MS analysis chromatogram of rosemary *n*-hexane extract

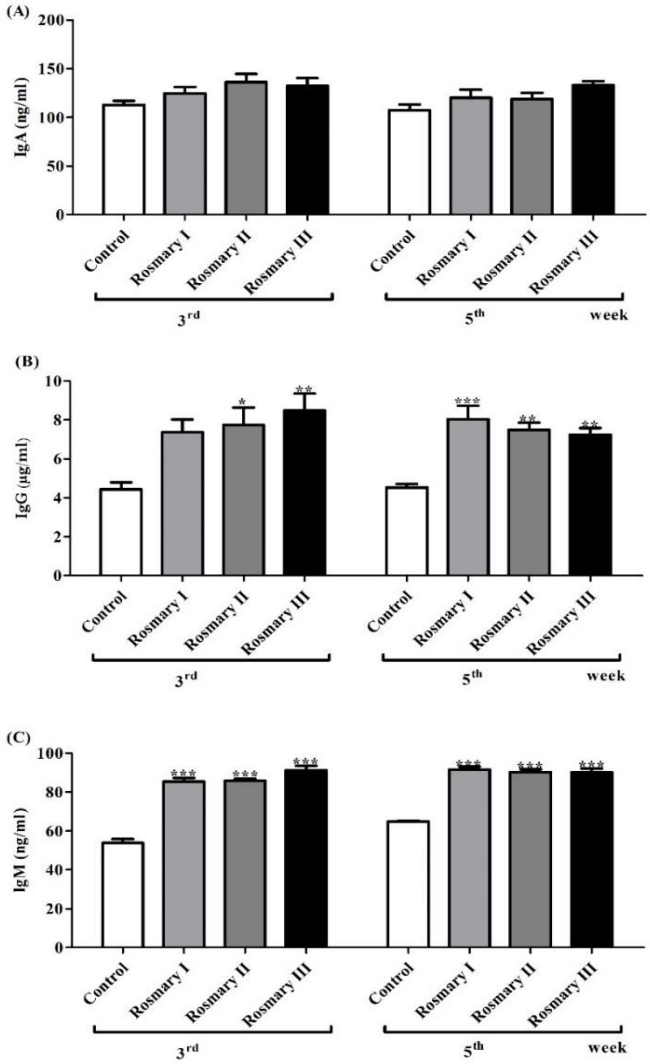

Fig. 2. Represents the effects of rosemary on serum (A) IgA, (B) IgG, and (C) IgM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control.

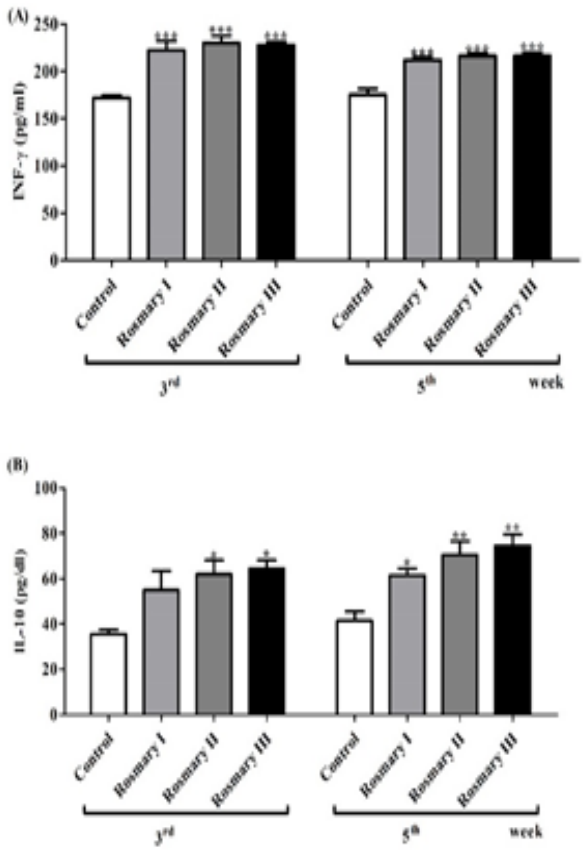

Fig. 3. Represents the effects of rosemary on (A) INF-γ and (B) IL-10. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control.

The data illustrated in Fig. 4 represented the effects of rosemary dietary supplementation on MDA and antioxidant status in breast and thigh muscles. In Fig. 4(A) the breast muscle MDA levels were significantly decreased ($p<0.05$) in rosemary-treated groups compared to control. Also, the thigh muscles MDA levels were significantly decreased ($p<0.01$) in rosemary-treated groups. The data illustrated in Fig. 4(B) revealed that the breast muscle levels of GSH were a significant increase ($p<0.001$) in rosemary-treated groups compared to control. In comparison to Rosemary I the GSH levels were significantly increased ($p<0.01$) in Rosemary II and Rosemary III. The GSH levels in thigh muscles were significantly increased ($p<0.05$) in Rosemary II and Rosemary III compared to control. Also, it showed significant increases ($p<0.05$) in Rosemary III compared to Rosemary I.

The data illustrated in Fig. 4(C) represented that the T. SOD activities in breast muscles were significantly increased ($p<0.01$) in rosemary-treated groups compared to control, while in thigh muscles its activities were significant increase in Rosemary I ($p<0.05$), Rosemary II ($p<0.05$), and Rosemary III ($p<0.01$) compared to control.

Results in Fig. 4(D) revealed significant increases ($p<0.01$) in the breast muscles GST activities in the rosemary-treated groups compared to control.

But in the thigh muscle, its activities revealed non-significant differences in rosemary-treated groups.

The data presented in Table 4 showed non-significant improvement ($p \geq 0.05$) in growth performance measurements in 0.5% rosemary-treated group in comparison to control while inclusion of high levels of rosemary deteriorates the growth performance parameters.

4. DISCUSSION

Continuous use of antibiotics in poultry diets has evoked numerous problems including the cross-resistance and environmental pollution. So that the search for alternative substances to replace classical antibiotics should be continued (Dickens et al. 2000). Therefore, vegetables, herbs, spices and edible plants were suggested as feed additives in animal nutrition (Abaza 2001).

The GC-MS analysis of rosemary stated the cineole content of rosemary was 23,67% area in the chromatogram. The essential oil isolated from rosemary was characterized by its greater content of 1,8-cineole as stated by Mathlouthi *et al.* (2011). The main active components were camphor (11-16%), alpha-pinene (15-20%) and cineole (30-35%) which has a high degree of inhibition against many bacteria and fungi (Ali and Ghazalah, 2008). The same compounds have the antioxidant potential as studied by Rašković *et al.* (2014).

The obtained data showed an immunostimulant effect of rosemary through significant increases in the serum levels of IgM, IgG, INF-γ, and IL-10 in rosemary-treated groups at the 3rd and 5th weeks, while the serum IgA level was significantly increased in the Rosemary II at the 3rd week and was significantly increased in the Rosemary III at the 5th week in comparison to control. These results come in accordance with that obtained in the study of ELnaggar *et al.* (2016) who studied the addition of rosemary to the basal diet at the concentrations of 0.25, 0.5, 0.75, and 1.0 % had significantly improved the serum immunoglobulins (IgY, IgM, and IgA), IFN-γ, and IL-10.

Lipid peroxidation leads to the formation of various products as the MDA. Therefore, blood MDA level is often determined in some studies as an indicator of lipid peroxidation in the body (Droge, 2002). In a biological system, many oxidation reactions are essential for our survival. Sometimes, inside the normal cells, oxidation reactions release uncontrolled reactions and produce unstable oxygen molecules “free radicals”. These produced compounds will react with many different important molecules in vital organs like lipid, protein, and DNA forming a new compound that damage DNA (Ercegovac *et al.*, 2010). Antioxidants are the first line of defense against free radical damage and are critical for maintaining optimum health (Lobo *et al.*, 2010). The data of the present study revealed that the supplementation of rosemary to the diet of broiler chickens reduced the muscle MDA levels, which indicates a decreased lipid peroxidation. In contrast, T.SOD and GST activities in them were significantly increased in comparison to control, while GSH was significantly increased in Rosemary III and non-significantly increased in Rosemary I and Rosemary II. These results came in accordance with that obtained in the study of Polat *et al.* (2011) where they recognized an enhancement in serum SOD activity in rosemary fed birds. The meat from broilers fed on the diet containing 500 mg/kg rosemary and sage extracts had smaller concentrations of total cholesterol oxidation products than meat from the control group (Lopez-Bote *et al.*, 1998).

Inspection of the blood data in Table 2 showed that values of serum total protein and globulin were significantly increased with the addition of rosemary compared to control, while no significant differences were observed in blood albumin. This reflects the ability of chicks to store reserve protein even after the body has reached its maximum capacity for depositing protein to tissues. In addition, the increase in the globulin indicates the effective role of rosemary in increasing immunity due to its role in developing and protecting cells and inhibiting non-enzymatic oxidation (Houghton *et al.*, 2007). These results came in accordance with that obtained in the study of Ali and Ghazalah (2008). The non-significant alterations in ALT activities and creatinine levels indicated the safe use of rosemary as a feed additive in broiler chickens diet on liver and kidney functions. Moreover, the serum total cholesterol and triacylglycerol levels were significantly reduced due to rosemary feed supplementation compared to control.

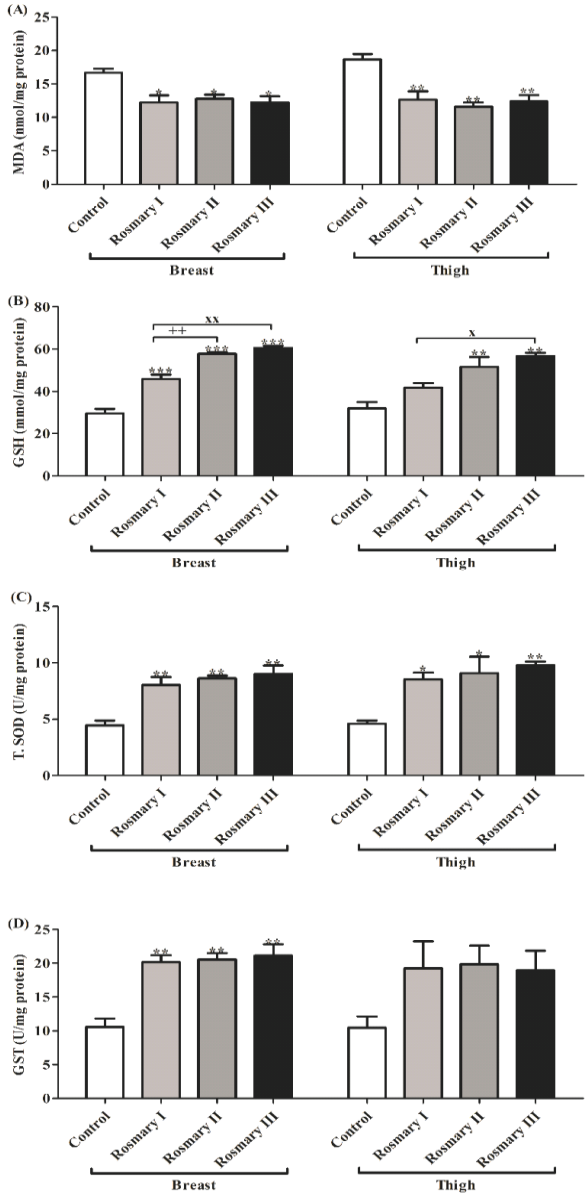

Fig. 4. Represents the effects of rosemary on (A) MDA, (B) GSH, (C) T. SOD, and (D) GST. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control. $^{++}p< 0.01$ vs. Rosemary II; $^{x}p < 0.05$ and $^{xx}p < 0.01$ vs Rosemary III.

The average body weight, average body weight gain, feed conversion, and average protein efficiency were statistically non-significantly differed in the rosemary-treated groups compared to control. These findings are confirmed by the study done by Abd El-Latif *et al.* (2013) who investigated the effects of dietary inclusion of different levels of rosemary and garlic essential oils on broilers performance and concluded that the data of growth performance indicated that supplementation of broiler diets with rosemary oil had no growth-promoting effects. Similarly, Hernandez *et al.* (2004) found no significant differences in body weight gain of broilers when a blend of extracts of sage, thyme, and rosemary was added to diets. Also, dietary rosemary and yarrow herb powders had no significant differences in the final body weight (Norouzi *et al.*, 2015). Also, these results were supported by ELnaggar *et al.* (2016) who found that low levels (0.25%) of rosemary give better growth performance compared to higher one. On the other hand, Al-Kassie *et al.* (2008) stated that 0.5 and 1.0% rosemary herb supplementation in the diet clearly improved broiler growth performance at the 42nd days of age, compared with the control treatment (Ali and Ghazalah, 2008) also found that 0.5% rosemary herb supplementation in the diet gave better results than the control treatment at the 49th days of age.

5. CONCLUSION

From the obtained results, it could be concluded that supplementing rosemary into broiler diet show no positive effect on growth performance especially higher levels. Remarkably, it, in a concentration-dependent manner, increased immunity and antioxidant activity in the broiler chickens producing a meat with fewer quantities of MDA.

6. COMPETING INTERESTS

The authors have no conflict of interest.

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