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Effect of clove buds powder supplementation on hematological profile, biochemical parameters, lymphoid organs, and cell-mediated immunity of broilers

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Abstract

Background: Phytogenic feed additives are products derived from plants used to improve the performance and health of animals. Nowadays, this type of phytogenic feed additive is widely used as an alternative to antibiotic growth promoters in poultry feed, and clove bud is one of the most effective medicinal herbs that has caught the attention of researchers.

Aim: This experiment was conducted to evaluate the effects of adding clove bud powder to the broiler chicken's feed on the hematological profile, biochemical parameters, lymphoid organs, and cell-mediated immunity.

Method: A total of 360 unsexed chicks (Ross 308) were allotted to three groups. All chicks were raised under the same normal management conditions from 1 day to 6 weeks of age. The first group was fed a basal diet and the other two groups were assigned to add 0.5% and 1.0% of clove bud powder to the basal diet. The studied traits were total leukocyte counts (TLC), differential leukocyte counts (DLC), blood cholesterol level, blood liver enzymes [aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase (ALP) enzymes], the weight of lymphoid organs (bursa of Fabricius and spleen), and cell-mediated immunity.

Results: Despite the significant and varied changes ($p \leq 0.05$) that resulted from adding clove buds powder to the broiler feed in the second and third weeks, the results at the end of the experiment indicated that there was no negative effect of adding clove powder on the TLC and DLC, as well as the heterophils/lymphocytes ratio. In addition, feeding on a diet containing clove buds powder had no significant effect on the level of cholesterol and liver enzymes in the blood, except for (ALP), which showed a significant increase ($p \leq 0.01$) in comparison to the control group. Moreover, the results showed in the second and fifth weeks a significant increase ($p \leq 0.05$) in the relative weight of the spleen, but, in general, there were no significant effects at the end of the experiment on the relative weight of the lymphoid organs. Furthermore, the broiler chickens that consumed clove bud powder at a rate of 0.5% showed a highly significant ($p \leq 0.01$) cellular immune response.

Conclusion: This study concluded that the addition of clove bud powder had no negative effect on leukocyte counts or differentiated leukocyte counts. The addition also raised the spleen weight and improved the level of blood alkane phosphatase activity and cellular immune response in broiler chickens during the growth stages.

Keywords: Broiler, Clove, Hematological profile, Biochemical parameters, Immunity.

Introduction

Antibiotics have been used in animal production as non-nutritive additives because of their positive effect on nutritional efficiency and to control infectious diseases. The use of antibiotics in the poultry industry as growth promoters has been banned in many countries around the world due to increasing levels of microbial resistance to drugs (Castanon, 2007). Currently, research is directed toward natural and healthy alternatives to improve the productivity and health of poultry. Ever since the day medicinal plants are considered a natural and safe alternative to improve the performance and health of broilers (Amad *et al.*, 2013), they have been attracting a great deal of research in the field of developing poultry feed. Cloves (*Syzygium aromaticum*) have several therapeutic properties and contain many biologically active compounds such as eugenol, eugenyl acetate, and beta-caryophyllene (Alma *et al.*, 2007). The major component in clove is eugenol (Barceloux, 2008). Clove also contains hydrolyzable tannins (Dibazar *et al.*, 2014), which have antimicrobial activity (Leite *et al.*, 2007; Shrivastava *et al.*, 2014), antiviral

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(Hussein *et al.*, 2000), antifungal (Campaniello *et al.*, 2010), anti-inflammatory, and antioxidant properties (Wati *et al.*, 2015). Cloves have also been used as a natural growth enhancer (Sjafani *et al.*, 2022), and this may be due to the significant improvement of the morphological condition of the intestine and increased intestinal absorption surface (Agostini *et al.*, 2012; Othman *et al.*, 2022). Also, dietary supplementation with clove stimulates the immune response (Gandomani *et al.*, 2014). This experiment aimed to investigate the effect of adding different levels of clove bud powder to broiler chicken feed on the hematological profile, biochemical parameters, lymphoid organs, and cell-mediated immunity.

Materials and Methods

The experiment was conducted at the Poultry Research Station, Faculty of Agriculture, University of Tripoli, Libya. A total of 360 1-day-old- unsexed chicks (ROSS 308) were distributed to three treatment groups. Every group was replicated 6 times with 20 chicks/replicate, one chick was randomly taken from each replicate at the end of each week of the experiment to measure traits. The treatments included the control group that fed on the basal diet (Table 1) and the second group that fed on (the basal diet + 0.5% clove bud powder) and the third group that fed on (the basal diet + 1.0% clove bud powder).

A starter feed was provided from 1 to 21 days and a finisher feed from 22 to 42 days of the experiment (Table 1), and water was available *ad libitum*. The chicks were raised under normal management conditions on floor pens with a wood-shaving floor (1.5 × 1.3 m), and the temperature was gradually decreased from 35°C to 21°C till the end of the experiment (42 days). All chicks were vaccinated according to the vaccination program implemented by National Center for Animal Health, Libya.

Table 1. Nutrient content of basal diet over different periods of production.

Nutrient	Starter diet	Finisher diet
Dry matter (%)	90.11	89.86
Metabolizable energy (Kcal/kg)	3004.94	3042.16
Protein (%)	21.19	18.75
Ether extract (%)	4.20	3.75
Linoleic acid (%)	1.95	1.73
Crude fiber (%)	2.21	2.20
Calcium (%)	1.00	0.90
Avail. phosphorus (%)	0.482	0.434
ARG (%)	1.449	1.238
LYS (%)	1.289	1.077
MET (%)	0.555	0.509
THR (%)	0.847	0.735

The experiment was designed according to a completely randomized design. Analysis of variance was implemented by using Statistical Analysis System (SAS, 2002), and Duncan's multiple range tests were used to determine significant differences between treatment means (Duncan, 1955).

The studied traits included blood constituents, biochemical parameters, the determination of immune organ masses, and cell-mediated immunity. The blood constituent traits were total leukocyte counts (TLC), differential leukocyte counts (DLC), and heterophil/lymphocyte ratio (H/L). The biochemical parameters were serum cholesterol level (mg/dl) (Allain *et al.*, 1974) and Liver enzymes in the blood: aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Schumann *et al.*, 2002), and alkaline phosphatase (ALP) (IU/l) (Wenger *et al.*, 1984). The determination of immune organ masses was the spleen/body weight ratio and the bursa of Fabricius/body weight ratio (Heckert *et al.*, 2002). The effect of clove in chicken broiler feed on cell-mediated immunity was assessed by the hypersensitivity response of the chicken's skin to dinitrochlorobenzene (DNCB) as per the method described by Tiwary and Goel (1985). On day 28, six chickens were randomly selected from each group, marked by different colors and sensitized by applying 250 µl of 1% DNCB dissolved in acetone on the side of the right thigh. On day 42 (after 14 days), the same chickens were challenged by topical application of 0.5% DNCB dissolved in a 4:1 mixture of acetone and olive oil on the same sensitized site (sike of the right thigh) and calculated as per the method of Chowdhury *et al.* (2005).

Ethical approval

This study was approved by the Graduate School of the University of Tripoli, Faculty of Agriculture, Department of Animal Production. All animal welfare protocols were followed.

Results

The results in Figure 1A show that adding cloves at a rate of 0.5% and 1% to the broiler diet had no significant effect on the TLC compared with the control group at the end of the experiment (6 weeks of age). Nevertheless, the statistical analysis of the second week of the experiment showed a significant decrease ($p \leq 0.05$) in the TLC (716.7/mm^3 and 833.3/mm^3) in the two groups that fed on a diet supplemented with clove powder at a rate of 0.5% and 1%, respectively, compared to the control (1,250/mm^3). The results of the statistical analysis of DLC (heterophil, eosinophil, basophil, lymphocyte, and monocyte) in Figure 1B–F show that there was no significant effect of adding clove powder to the broiler diet in the end of the experiment among the experimental groups. While the weekly statistical analysis of the experimental data showed the presence

Fig. 1. Effect of adding clove buds powder to broiler feed in different levels on leukocyte counts and DLC. (*): Clove Buds Powder. Different letters within a treatment indicate a significant difference between the means at the level of probability differences between the means at the level of probability ($p < 0.05$).

of some significant changes, the results showed a highly negative effect ($p \leq 0.01$) on the percentage of heterophil in the third week in the broilers of the third group, which was (11%) compared to the control group (37%). Similarly, there was a significant decrease ($p \leq 0.05$) in the second week in the percentage of eosinophils in the second and third groups (2.83% and 1.83%, respectively) compared with the control group (3.50%). On the contrary, the addition of clove powder to the diet of broilers had a highly positive significant ($p \leq 0.001$) effect in the second and third weeks on the percentage of lymphocytes, and the increase was the

highest in the third group (85.17%) compared with the broilers of the control group (59.83%) in the third week of the experiment. In addition to the above, the results in Figure 1G indicated that supplementation with clove powder caused a significant increase in the H/L-ratio in the third week, while there was no significant effect in the sixth week of the experiment.

The effect of adding clove powder to broiler feed on the level of cholesterol and liver enzymes in the blood serum was shown in Figure 2A–D. It is clear from the statistical analysis that adding cloves at a rate of 0.5% and 1% did not have a significant effect on the level of cholesterol in the blood compared with the control group. Similarly, the addition of clove powder had no significant effects on the enzymes ALT and AST in the blood serum compared with the control group. On the other hand, the results indicated a highly significant increase ($p \leq 0.01$) in ALP enzyme with an average of (366.85 and 450.63 U/l) when fed with 0.5% and 1%, respectively, clove powder compared to the control group, whose average enzyme level was (224.58 U/l).

Figure 3 show the effect of the inclusion of clove powder in broiler feed on the weight of lymphoid organs. Through the experimental data analysis, Figure 3A showed that adding clove powder had no significant effect on the relative weight of the spleen at the end of the experiment (6 weeks of age). It can also be noticed that the high relative weight of the spleen was observed in the second week ($p \leq 0.001$) and the fifth week ($p \leq 0.05$) of the experiment (0.096% and 0.180%, respectively) compared to the control group, in which the mean relative weight of the spleen was (0.066%) in the second week and (0.143%) in the fifth week of the experiment. The results of the experiment in Figure 3B also indicated that the inclusion of clove powder in broiler feed had no significant effect on the bursa of Fabricius/body weight ratio either at the end of the experiment or through the weekly analysis of the experimental data.

The effect of adding clove powder to broiler feed on the cellular immune response is illustrated in Figure 4A and B. The results showed that the cellular immune response did not change significantly on hours 24, and 48-post challenge with DNCB in the second and third experimental groups compared with the control group. But on hours 72-post challenge with DNCB, a significant response ($p \leq 0.05$) was recorded in the broilers of the second group that were fed a diet containing 0.5% clove powder, the percentage of skin thickness was 275% compared with the broilers of the control group 166.67%. On the other hand, there was no significant cellular immune response in the birds in the third group after 72 hours of the injection. The results of the statistical analysis were supported by the

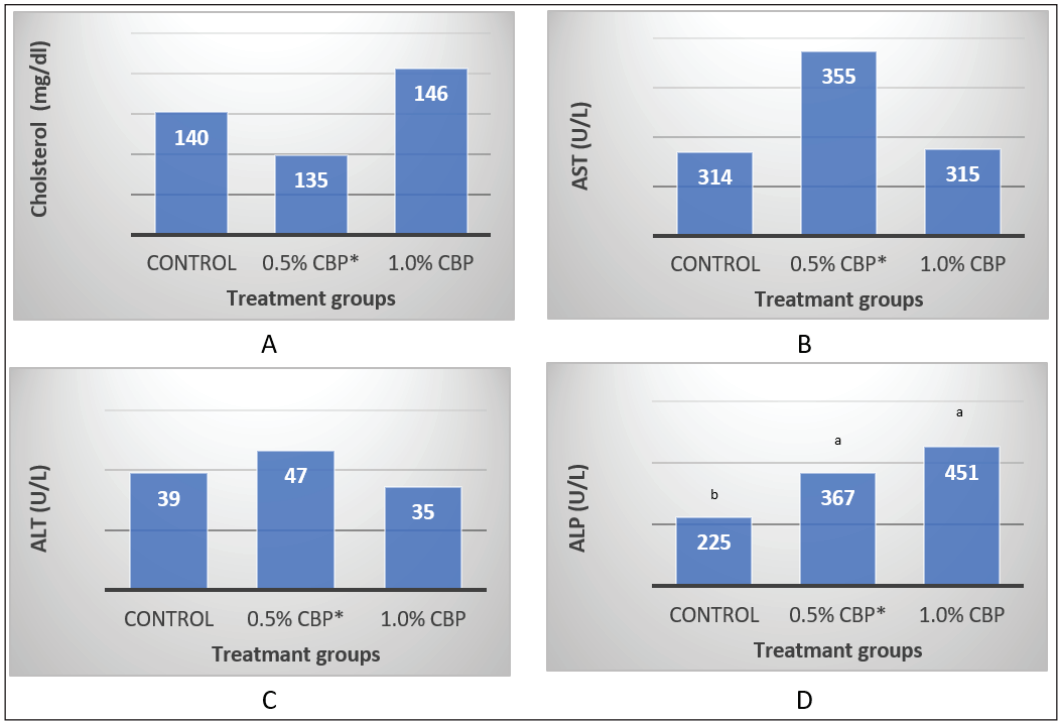

Fig. 2. Effect of adding clove buds powder to broiler feed in different levels on Serum cholesterol level and Liver enzymes at 42 days of the experiment. (*): Clove Buds Powder. Different letters within a treatment indicate a significant difference between the means at the level of probability differences between the means at the level of probability ($p < 0.01$).

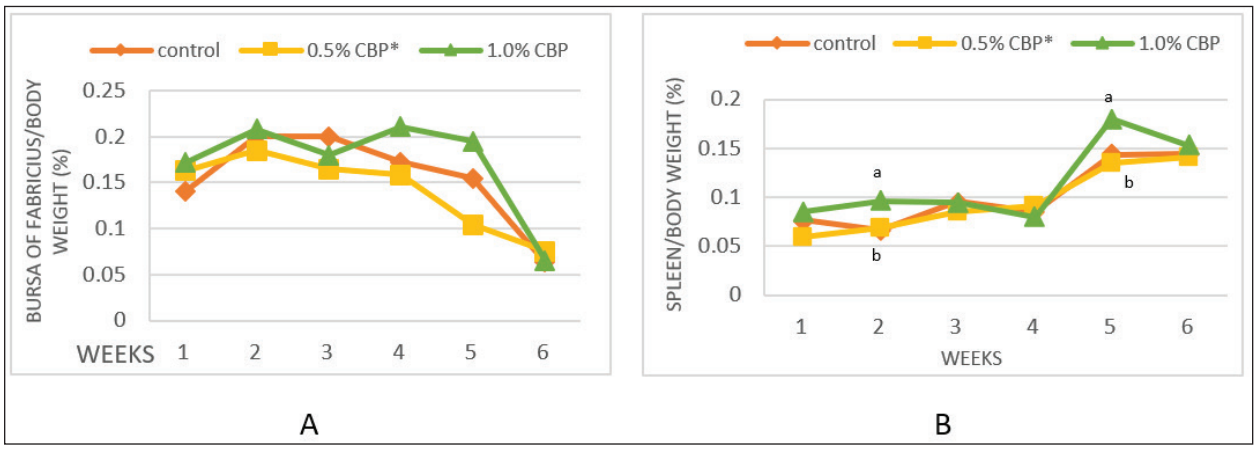

Fig. 3. Effect of adding clove buds powder to broiler feed in different levels on lymphoid organs weight in broiler chickens. (*): Clove Buds Powder. Different letters within a treatment indicate a significant difference between the means at the level of probability differences between the means at the level of probability ($p < 0.05$).

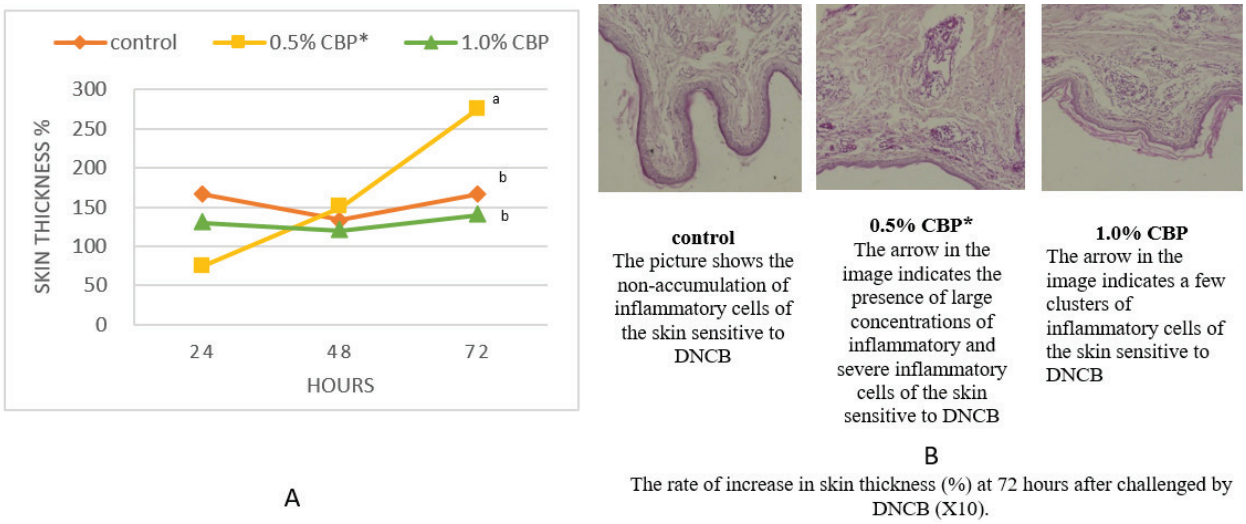

Fig. 4. The effect of adding clove buds powder to the broiler's diet in different levels clove buds powder on skin thickness in broiler chickens on hours 24, 48, and 72-post challenge with DNCB. (*): Clove Buds Powder. Different letters within a treatment indicate a significant difference between the means at the level of probability differences between the means at the level of probability ($p < 0.05$).

microscopic images of the skin tissue sections, which showed a large concentration of inflammatory cells as a result of the injections in the broilers of the second group that were fed 0.5% clove powder.

Discussion

Eugenol is the main component of clove extract (70%–90%) (Barceloux, 2008), and it is found in many other plants such as basil, cinnamon, nutmeg, and bay leaf. Eugenol is a volatile phenolic constituent of clove characterized by many beneficial effects, whether on the animal's productivity or health performance. Therefore, the measurement of blood parameters is one of the most efficient screening assessments to evaluate their effect on the immune system. The results obtained from our data (Fig. 1A) showed a limited effect of adding clove bud powder on the TCL, as well as the DCL. The non-appearance of adverse effects as a result of adding clove bud powder to TCL is considered an encouraging matter if compared with the improvement of other productive traits or physiological parameters. The same conclusion was observed by Toghyani *et al.* (2010) who mentioned that feeding broilers thyme supplementation had no adverse effect on their blood profile. Additionally, supplementation of some Libyan medicinal plants in the broiler's diet had no significant effect on TCL

(Asheg *et al.*, 2015). On the contrary, Abdel Fatah *et al.* (2020) reported a decrease in WBC in broilers fed clove powder (2%) in the diet. It can be noticed that the high concentration of clove may cause undesirable effects. It was mentioned that herbal medicines may have side effects or be ineffective (George, 2011), or be toxic (Nasri and Shirzad, 2013). Some previous studies reported that the inclusion of phytogenic feed additives increased WBC counts (Amad *et al.*, 2013; Wati *et al.*, 2015). Moreover, adding medicinal plants or their oils, such as thyme and cinnamon (Al-Kassie, 2009), thyme oil (Adam *et al.*, 2020), or bay leaf (Mohammed *et al.*, 2020) increased the WBC significantly. It is clear that adding cloves or other medicinal plants or their extracts had a positive effect on WBC counts. The superior effect of medicinal herbs on WBC may be due to the phytochemicals ability to stimulate immune systems and lymphoid organs and reduce metabolic stress in the body, which enhances better utilization of nutrients in the body to build up blood cells.

Regarding the differential WBC count findings in Figure 1B and C, our results agreed with what was concluded by Al-Asheq *et al.* (2015). They indicated that supplementation with aromatic plants in broilers' diet significantly decreased the heterophils percentage and increased the lymphocytes percentage in the third week. While there was no significant effect at the end of the experiment (the sixth week) on the percentage of heterophils or lymphocytes. In addition, our results indicate that there was no significant change in the H/L ratio at the end of the experiment, which is in agreement with the inference of Toghyani *et al.* (2010). Furthermore, Najafi and Torki (2010) found that adding cloves did not have a significant effect on the lymphocyte percentage or heterophil percentage. In general, most studies reported that aromatic plants had a beneficial effect on differentiated WBC, and this agreed with the results of the third week of our study as shown in Figure 1G. Along the same lines, Gandomani *et al.* (2014) pointed out that the dietary inclusion of clove bud powder decreased the heterophil percentage and increased the lymphocyte percentage, which in turn reduced the H/L ratio. Additionally, supplementation with phytogenic feed additives (fennel, melissa balm, peppermint, anise, oak, clove, and thyme) significantly contributed to increasing the lymphocyte percentage and decreasing the heterophil percentage and the H/L ratio (Wati *et al.*, 2015). The decrease in Heterophil percentage and increase in lymphocytes percentage is attributed to the antioxidant and anti-inflammatory effects of clove buds (Wati *et al.*, 2015). Likewise, Gandomani *et al.* (2014) stated that clove bud had a strong antioxidant effect, which reduced oxidative stress in immune cells and increased the number of lymphocytes. The H/L ratio is a good signal of the health status of birds, as a higher ratio indicates that the birds are under severe stress (Al-Darraji, 1995). Therefore, aromatic herbs as antioxidants play an important biological role in improving immunity by lowering the H/L ratio in the blood of birds (Ojala *et al.*, 2000), and this was approved by Naderi *et al.* (2014) when feeding broilers on cinnamon and turmeric , as well as when feeding on laurel leaves (Mohammed *et al.*, 2020).

The result of our study related to the serum total cholesterol in Figure 2A was in agreement with some other studies. Broiler chicks fed on a basal diet containing clove essential oils did not show a significant change in plasma cholesterol (Najafi and Torki, 2010; Azadegan *et al.*, 2014). Other studies conducted on dietary supplementation with anise seed (Soltan *et al.*, 2008), thyme powder (Toghyani *et al.*, 2010), or basil essential oil (Riyazi *et al.*, 2015) indicated that there is no significant effect on plasma cholesterol. In addition to the above, feed additives may have hypolipidemic activity. Venkadeswaran *et al.* (2014) found that eugenol inclusion reduced total cholesterol, LDL-cholesterol, and triglyceride levels. Moreover, dietary clove powder in broilers (Mahrous *et al.*, 2017) and clove oil in laying hen diets (Şehitoğlu and Kaya, 2021) significantly lowered serum total cholesterol. Besides, supplementation with cinnamon, thyme, and *Alchornea cordifolia* leaf meal significantly decreased total cholesterol concentration in serum (Najafi and Taherpour, 2014; Oloruntola *et al.*, 2016).

Serum AST and ALT concentrations are valuable parameters to evaluate liver status. Studies conducted by Abdel Fatah *et al.* (2020) and Şehitoğlu and Kaya (2021) agreed with our results in Figure 2B and C whereas they noted that supplemental clove in broiler diets did not affect the levels of AST and ALT in the blood serum. In the same line, findings by Soltan *et al.* (2008); Gowda *et al.* (2009); and Mustafa (2016) indicated that the addition of other phytogenic feed additives had no significant effect on the level of serum liver enzymes. Moreover, some studies showed the hepatoprotective action of medicinal and aromatic plants. Soltan *et al.* (2008) indicated that the addition of anise seeds to broiler feed reduced ALT levels in the blood. Also, Oladokun *et al.* (2021) stated that chicks that received clove essential oil recorded the highest significant reduction in plasma AST level. In general, increasing AST and ALT are associated with liver damage (hepatocellular degeneration). Thus, the reduction of AST and/or ALT may have been considered evidence of the hepatoprotective activity of phytogenic feed additives (Langhout, 2000). In addition, Gowda *et al.* (2009) showed that active compounds in aromatic plants possessing antioxidant properties have a role in protecting liver cells from oxidative damage. From the previous results, it could be concluded that clove and other aromatic plants are safe for chickens and do not cause any adverse effects on the liver. In opposition, Al-Mufarrej *et al.* (2019) listed that clove powder supplementation at rates of 5% and 6% significantly increased AST, which means that high levels of clove may adversely affect liver function. Our study recorded

a significant increase in serum ALP concentration compared to the control group (Fig. 2D). Consistent with our results, Wati *et al.* (2015) reported that serum ALP was higher in the broiler group fed with plant feed additives and showed that a higher rate of ALP might be an indicator of osteoclast activity. Additionally, Şehitoğlu and Kaya (2021) revealed that supplemental oil clove in laying hens' diets significantly increased serum ALP. Otherwise, the Inclusion of clove powder (Al-Mufarrej *et al.*, 2019), anise seed (Soltan *et al.*, 2008), clove essential oil (Oladokun *et al.*, 2021), and the mixture of essential oils (Mustafa, 2016) did not affect the plasma ALP concentration. On the contrary, clove oil supplementation caused a reduction in liver AST, ALT, and ALP (Asimi and Sahu, 2016; Gashlan and Al-Beladi, 2017). These variations in the literature's results may be due to the different levels, sources, and types of phytogenic feed additives and different animal species that were used in each study.

Our findings linked to the lymphoid organs (spleen and bursa of Fabricius) in Figure 3A and B correspond to many investigations conducted on clove and other aromatic plants. Some scientists found that the relative weight of the spleen, bursa of Fabricius, and *thymus* were not influenced by clove supplementation (Chowdhury *et al.*, 2005; Najafi and Torki, 2010; Al-Mufarrej *et al.*, 2019). Besides, Sadeghi *et al.* (2012) found that cinnamon, thyme, and turmeric supplementation had no significant effect on the relative weights of the spleen and bursa. Differently, Gandomani *et al.* (2014) exhibited that the addition of clove bud powder to layer diets caused an increase in the relative weight of the spleen. This inconsistency may be attributed to the differences in the sources of the clove, the levels of clove, diet composition or environmental conditions. Also, Gandomani *et al.* (2014) suggested that the finding might be associated with the likely positive interaction effects of bioactive compounds of clove bud and fatty acids on cell proliferation in such peripheral lymphoid organs as the spleen.

Eugenol was assured as an efficient antimicrobial compound, and earlier studies confirmed the bioactive effects of eugenol as antibacterial (Leite *et al.*, 2007), antifungal (Campaniello *et al.*, 2010), and antiviral (Hussein *et al.*, 2000). Moreover, the anti-inflammatory activity of eugenol has been proven (Magalhaes *et al.*, 2010). Therefore, all these beneficial activities can enhance the immune system, which supports our results that clove buds (that are rich in eugenol) can improve the cellular immune response. In the same vein, Wang *et al.* (1998) mentioned that as a result of the antibacterial action of eugenol, clove essential oils improved immunity. Additionally, herbal plants and their essential oils may have antioxidant activity (Gumus *et al.*, 2017; Elwan *et al.*, 2019) and have a positive effect on immunity (Ghanima *et al.*, 2020). Our previous result in Figure 1C showed an increase in lymphocytes in the second and third weeks, which is an indicator of improvement in the cellular immune system. This improvement could result in a reduction of oxidative stress in immune cells due to the antioxidant activity of the clove plant. Likewise, the antioxidant properties of medicinal plants might be responsible for increased antibody production (Najafi and Torki, 2010). In line with our findings in Figure 4A and B, Farhath *et al.* (2013) determined that the essential oils (geranial, geranial acetate, gingerol, and eugenol) significantly improved the specific and non-specific immunity and enhanced the proliferation of lymphocytes. Their data showed an increase in foot pat thickness due to the high production of antibodies and phagocytic functions of macrophage cells and non-specific immunity. Correspondingly, an earlier study by Carrasco *et al.* (2009) showed that clove essential oil augmented leukocyte counts and enriched delayed-type hypersensitivity responses while restoring cellular and humoral immune responses. In addition, Mahrous *et al.* (2017) stated that clove significantly enhanced the immune response in broiler chickens. Other studies reported different outcomes, Kammon *et al.* (2010) reported that there was no significant effect on the thickness of the skin's sensitivity to DNCB in chickens given chlorpyrifos. Mehr *et al.* (2014) stated that clove powder did not affect cutaneous basophil hypersensitivity. Halder *et al.* (2011) showed that the administration of clove oil decreased cell-mediated immunity. Dibazar *et al.* (2014) suggested that clove extracts could suppress T-cell cellular immunity.

Conclusion

This study concluded that the addition of clove bud powder had no negative effect on leukocyte counts or differentiated leukocyte counts. The addition of clove bud powder also raised the spleen weight, stimulated the blood ALP activity, and improved the cellular immune response in broiler chickens during the growth stages.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest.

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Data availability

All data supporting the findings of this study are available within the manuscript. Any other data are available from the corresponding author upon reasonable request.

Authors contributions

Khaled M. Ben Naser: Conception, design of the study and statistical data analysis. Bashir M. Sherif: Wrote

the manuscript and design figures. Siham M. Othman: Corresponding author and funded the research. Abdulatif A. Asheg: Supervision and discussion of blood parameters results.

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