

# Immunomodulatory effects of Oregano and Artichoke based pharmaceuticals on Barki ewes' immune response to sheep pox vaccine

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## ABSTRACT

Lately, there's been a growing recognition of the therapeutic potential of natural plant extracts. These extracts have shown promise in treating a range of diseases and in partially mitigating the oxidative stress often linked with illnesses or the use of antibiotics. This work studied the effect of two pharmaceutical products based on plant extracts on the humoral and innate immune response of Barki ewes to the Sheep pox Vaccine. For this purpose, 15 Barki ewes were injected with the life-attenuated pox vaccine only (Pox group), 15 Barki ewes were injected with the same vaccine and orally treated with 50ml/head of Immune Care® (based oregano extract) for 5 consecutive days, (Immune Care Group (IG)), 15 Barki ewes were injected with the same vaccine and orally treated with 50ml/head of Bovi Care® (based on artichoke) for 5 consecutive days. Blood samples were collected from them, NI and innate immunity, and clinicopathological parameters were estimated and statistically analyzed. Immune Care® and Bovi Care® didn't improve the humoral immune responses of vaccinated ewes to the vaccine. However, they maintained the vaccinated ewes' total antioxidant capacity, erythrocyte counts, and total protein levels within normal ranges. Furthermore, they exhibited better values in serum renal and hepatic function tests, neutrophil phagocytic index, albumin, and globulin. Conclusion: Immune Care® and Bovi Care® did not have any effect on the humoral immune response of the Barki ewes to the pox vaccine but they effectively mitigated some of its side effects.

## Introduction

Sheep pox poses a significant threat to the sheep industry in Egypt due to its high contagious nature, resulting in substantial economic losses. The disease presents various detrimental effects, such as high morbidity and mortality rates among infected sheep, decreased milk and meat production, increased abortion rates, inferior wool quality, and compromised skin conditions. Additionally, it imposes limitations on international trade in animals and animal products. Sheep pox tends to be more fatal in newly introduced animals, while local inbred animals from endemic regions may experience milder symptoms (Fares *et al.*, 2019; Hamdi *et al.*, 2021).

Vaccination stands as the primary method to prevent contagious disease outbreaks like sheep pox (Eldakrouy *et al.*, 2021; Darwish *et al.*, 2023). However, outbreaks may occur in both vaccinated and unvaccinated animals due to vaccine failure, rapid vaccine deterioration, and inadequate immune response in animals. Consequently, there's a critical need to boost sheep's immune response to vaccination. One strategy involves administering an immunostimulant alongside the vaccine (Dabbir and Nanjundaiah, 2020; Doğan, 2022). Additionally, vaccination in sheep triggers an innate immune response, leading to unwanted clinicopathological changes such as anemia, elevated hepatic and renal function values, and oxidative stress (Darwish *et al.*, 2023).

In recent times, natural plant extracts like those from the oregano plant have demonstrated their effectiveness in treating various diseases and partially alleviating oxidative stress associated with illnesses or antibiotic use. Oregano (*Oregano vulgare*), a perennial shrub belonging to the mint family, predominantly thrives in the Mediterranean region. Its extract has been utilized in traditional medicine to address a multitude of ailments including cutaneous sores, muscle pain, asthma, cramps, diarrhea, indigestion, and common colds, while also promoting overall health. Moreover, recent research indicates its potential as a food preservative due to its appealing flavor and its ability to combat a broad spectrum of food spoilage bacteria (Cattelan *et al.*, 2013; Coccimiglio *et*

*al.*, 2016; Cattelan *et al.*, 2018; Khoshbakht *et al.*, 2020).

In veterinary medicine, oregano plant extracts have good antibacterial properties against enteric bacteria *in vitro* and *in vivo* and markedly improved some clinicopathological alterations in lamb bacterial enteritis treatment. It also controlled the *Clostridium perfringens* diarrhea in boilers and enhanced intestinal mucosa healing. Furthermore, oregano plant extracts exhibited promising results against certain antibiotic-resistant bacterial strains. These therapeutic benefits are primarily attributed to the abundant presence of carvacrol and thymol volatile oils in oregano plant extract (Du *et al.*, 2015; Darwish *et al.*, 2021).

Artichoke (*Cynara scolymus* L.) is a perennial herbaceous plant cultivated worldwide and celebrated for its integral role in the Mediterranean diet. Its historical application as a health tonic spans centuries. In recent decades, numerous studies have shed light on its remarkable health benefits. Notably, it significantly bolsters cardiovascular and gastrointestinal health, while exhibiting properties that combat cancer, metabolic disorders, obesity, and diabetes. Furthermore, it functions as a prebiotic and probiotic, offering renal protection and demonstrating efficacy against immune-related ailments, arthritis, photoaging, reproductive issues, nervous system disorders, fungal infections, and periodontal diseases. These multifaceted benefits are primarily attributed to artichoke's rich content of phenolic compounds such as cynarin. These compounds have potent anti-inflammatory antioxidant activities and a pronounced influence on liver function and lipid profiles (Afifi *et al.*, 2014; Osama *et al.*, 2014; Ben Salem *et al.*, 2017; Kaymaz *et al.*, 2017; Mahboubi, 2018; Salekzamani *et al.*, 2019; Ghasemian *et al.*, 2022; Napoli *et al.*, 2023).

In the veterinary practice, dietary supplementation of artichoke improved the performance, shell weight, breaking strength, thickness of shell, and egg yolk color and reduced serum triglyceride, LDL, and VLDL concentration, liver fat ratio, and increased the serum antioxidants enzymes in laying hens (Ürüšan, 2023). It also enhanced carcass traits and humoral immune response of boilers to Newcastle disease and bronchitis infections (Dhiab and Ali, 2022). Hassan *et al.* (2023) noticed that the dietary supplementation of 10mg of Artichoke Aqueous extract/kg diet,

resulted in considerable improvement in the growth performance and feed efficiency without adverse effects on carcass characteristics or blood or serum constituents of treated rabbits. Demirci *et al.* 2023 referred to the value of artichoke residue (leaves and stems) remaining after flower harvesting in ruminant feeding as silage.

This research aimed to study and compare how two pharmaceutical products derived from oregano and artichoke plant extracts affect the immune response of Barki ewes to the Sheep pox Vaccine. Specifically, the research focused on examining their impact on some hematological and biochemical alterations in vaccinated ewes.

## Materials and methods

After the ethical approval of the Animal and Poultry Health Department, Desert Research Center (DRC), Mataria, Cairo, Egypt. Forty-five apparently healthy Barki ewes aged (3-5 years), weight 50-55 Kg, were kept under adequate nutrition system and housing conditions, in closed pens at sustainable development Centre of Matrouh resources (SDCMR).

All animals were injected with sheep pox vaccine (Live attenuated sheep pox virus (SPV) vaccine (Rominan strain) which supplied from Pox Research Department, Veterinary Serum and Vaccine Research Institute, Egypt (VSVRI) (batch no. 8). The vaccination of animals was performed with 0.5 ml of field dose containing  $10^3$  TCID<sub>50</sub> of tissue culture Rominan SPVV, inoculated intradermal in the tail (OIE, 2018).

The vaccinated animals were divided into 3 groups:

Pox group: 15 ewes didn't receive any treatment with the pox vaccine.

Immune Care Group (IG): 15 ewes were orally treated with 50ml/head of Immune Care® for 5 consecutive days, starting immediately before vaccination. Supplied by New Vet Care company®. Each liter of Immune Care® contains 30g of carvacol and 2000 mg of thymol.

Bovi Care Group (BG): 15 ewes were orally treated with 50ml/head of Bovi Care® for 5 consecutive days, starting immediately before vaccination. Supplied by New Vet Care company®. Each liter contains 7500 mg of cynara. All doses and routes of administration are recommended by the manufacturing company.

### Blood samples

Blood samples were collected from the jugular vein of the three groups' members using clean sterile vacutainer tubes 2 h before receiving vaccination and treatment (0 day) and 7, 14, 21, and 28, days post-vaccination, and split into three parts, 1st part was placed in a tube containing EDTA and was used instantly for evaluation of different hematological parameters [red blood cells count (RBCs), hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocytic count (TLC) and differential leukocytic count, (DLC) manually according to the method described by Feldman *et al.* (2000). Whereas the 2<sup>nd</sup> part was placed in a tube containing heparin and was used immediately for estimation of neutrophils phagocytic activity and index according to the method described by Wilkinson (1981), using *Candida albicans*. The 3<sup>rd</sup> part was placed in a clean, plain tube and was left to coagulate then was centrifuged at 3000 r.p.m for 20 minutes and serum was separated into clean Eppendorf tubes that were stored at -80°C. Later, serum was used for determination of the studied humoral and innate immunological parameters and biochemical parameters.

### Humoral immunity

#### Virus Neutralization Test (VNT)

All collected sera were screened against SPV according to the method described by OIE (2018) using Cell culture African green monkey Kidney cell line (Vero), kindly supplied from Pox Research Department,

Veterinary Serum and Vaccine Research Institute, Egypt, and Eagl's minimum essential medium (MEM) with neonatal calf serum (10% for growth medium and 2% for maintenance medium).

The neutralization index (NI) was calculated according to Reed and Muench (1938):  $NI = VT - SVT$  (virus titer) - SVT (serum virus titer).

### The innate immunological parameters and biochemical parameters

Serum cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ) levels were measured in serum by ELISA kits of Ray Biotech company following the manufacturer's instruction. Serum caeruloplasmin (Cp) concentrations were estimated by a turbidimetric method using Elabscience USA® kits. Serum concentrations of total antioxidant capacity (TAC), serum liver enzymatic activity (ALT, AST), kidney function test (BUN, creatinine (Cr)), total protein (TP), and albumin (Alb) levels were detected spectrophotometrically using commercial kits of Biodiagnostics following the manual instructions.

### Statistical analysis

Means of different statistical parameters between the different animal groups were compared by two-way ANOVA test by SPSS version 24 at 0.05 level of probability.

## Results

### The immunological parameters

#### Humoral immunity

NI of the three vaccinated groups significantly ( $P < 0.05$ ) increased from the 7<sup>th</sup> day and reached its highest value on the 28<sup>th</sup> day. Pox group had the highest NI value as 2.75 among the studied groups followed by IG with NI value as 2.41, then BG with NI value as 2.25.

#### Innate immunity

IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and Cp displayed significant increases ( $P < 0.05$ ) in the three groups, peaking on the 14<sup>th</sup> day, then significantly approaching their baseline values on the 28<sup>th</sup> day in the three groups. In contrast, TAC values significantly ( $P < 0.05$ ) decreased in the Pox, and BG groups reaching their lowest point on the 14<sup>th</sup> day in the pox group) and on the 7<sup>th</sup> in BG, then significantly ( $P < 0.05$ ) increased scoring values near their baseline values on the 28<sup>th</sup> day in the pox groups and 14<sup>th</sup> day in BG. Interestingly, TAC non-significantly ( $P \geq 0.05$ ) in the IG group changed throughout the experiment.

### Hematological parameters

Red blood cell parameters and indices (RBCs, Hb, PCV, MCH, MCV, and MCHC) significantly ( $P < 0.05$ ) decreased in the Pox group till the 14<sup>th</sup> day (except RBCs reached their bottom on the 7<sup>th</sup> day) then gradually increased approaching their initial values on the 28<sup>th</sup> day. While the BG and IG hemogram non-significantly ( $P \geq 0.05$ ) changed.

On the other hand, the leukogram of the three groups revealed a significant ( $P < 0.05$ ) leukocytosis with neutrophilia, lymphocytosis, and increased neutrophils phagocytic activity and index, began from the 7<sup>th</sup> day till the research ended in the Pox group and till the 21<sup>st</sup> day in the IG and BG.

### Biochemical parameters

#### Liver and kidney function tests

ALT, AST, blood urea, and Cr significantly ( $P < 0.05$ ) increased in the

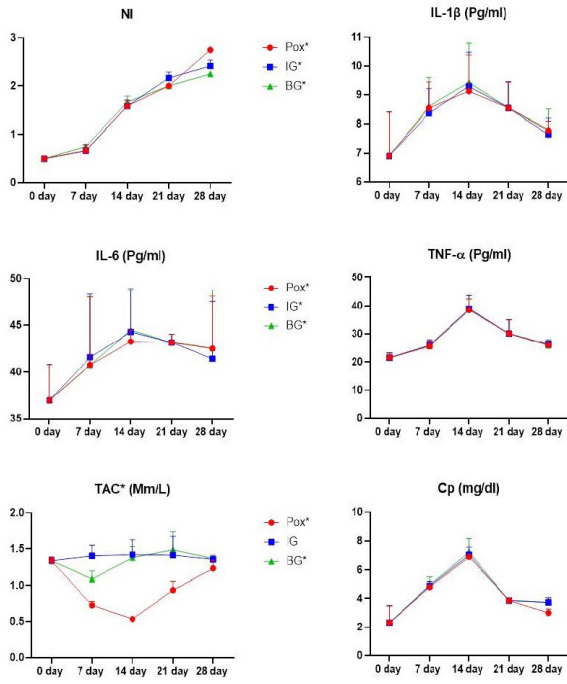


Fig. 1. Comparison between the humoral and innate immune response of the three studied groups throughout the research, (\*) on the parameter means significant between the groups, (\*) on the group means the vaccine or vaccine+immunstimulant effect is significant throughout the research. Considered significant when (P<0.05).

Pox group reaching their peaks on the 14<sup>th</sup> day and then they were significantly (P<0.05) decreased approaching their initial values on the 21<sup>st</sup> day for urea, Cr, AST and at 28<sup>th</sup> for ALT. Conversely, IG and BG groups showed a significant (P≥0.05) decline in liver and kidney function tests starting from the 7<sup>th</sup> and extended till the research end.

Protein profile

A significant (P<0.05) hypoproteinemia and hypoalbuminemia and decreased A/G were observed in the Pox group till the 14<sup>th</sup> day, then significantly (P<0.05) increased, approaching the 0-day value on the 28<sup>th</sup> day for TP and Alb. A/G decreased till the 7<sup>th</sup> day then increased and

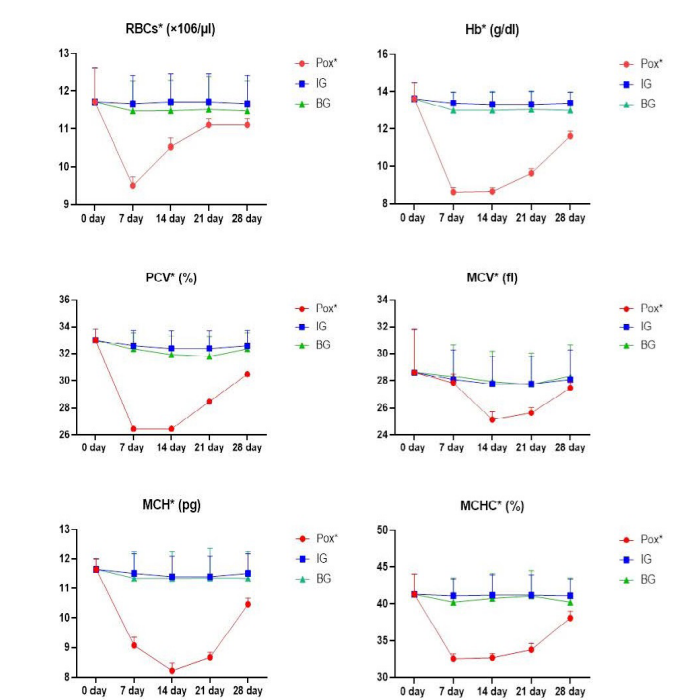


Fig. 2. Comparison between the erythrogram of the three studied groups throughout the research, (\*) on the parameter means significant between the groups, (\*) on the group means the vaccine or vaccine+immunstimulant effect is significant throughout the research. Considered significant when (P<0.05).

exceeded their original values on the 28<sup>th</sup>. Globulin significantly (P<0.05) increased, achieving two peaks on the 7<sup>th</sup> and the 21<sup>st</sup> days then scoring values close to its initial values on the 28<sup>th</sup> day.

A significant (P<0.05) hypoalbuminemia and hyperglobulinemia with decreased A/G were noticed in IG and BG. Then the albumin values significantly (P<0.05) increased, achieving their baseline values on the 21<sup>st</sup> day while the hyperglobulinemia as well as decreased A/G continued till the search ended in both groups. TP was non-significantly (P≥0.05) altered in IG and BG throughout the experiment.

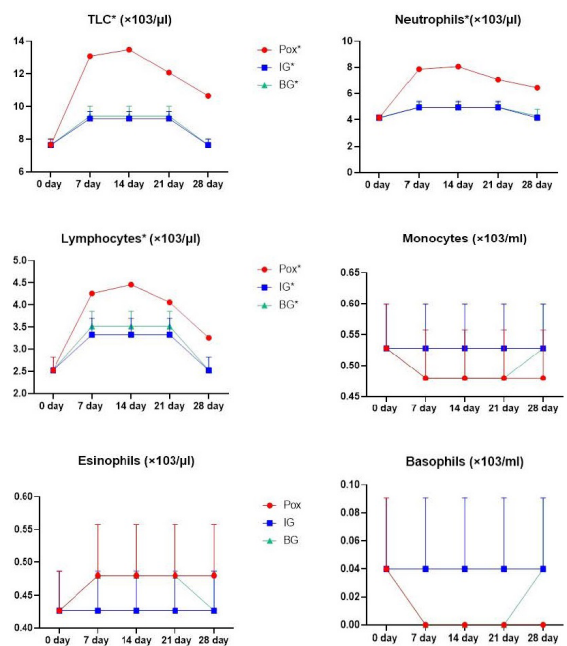


Fig. 3. Comparison between the leukogram of the three studied groups throughout the research, (\*) on the parameter means significant between the groups, (\*) on the group means the vaccine or vaccine+immunstimulant effect is significant throughout the research. Considered significant when (P<0.05).

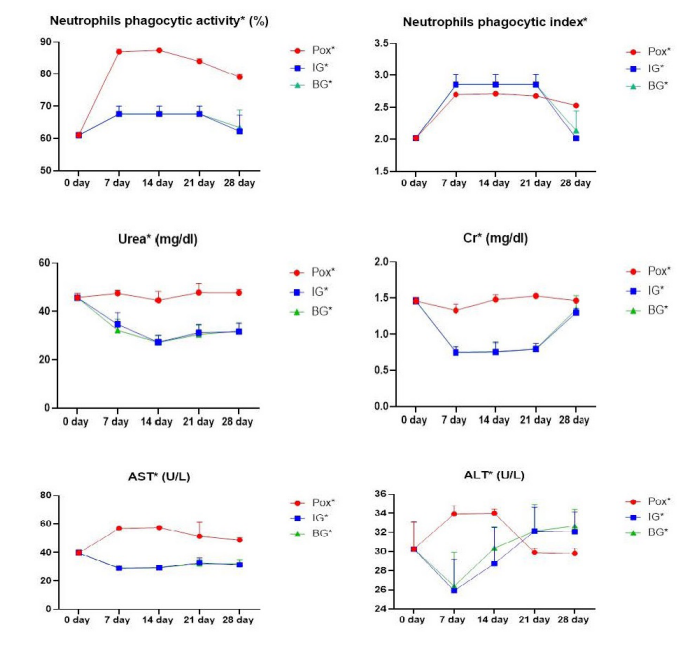


Fig. 4. Comparison between the neutrophils phagocytic activity and index and the kidney and liver function tests of the three studied groups throughout the research, (\*) on the parameter means significant between the groups, (\*) on the group means the vaccine or vaccine+immunstimulant effect is significant throughout the research. Considered significant when (P<0.05).

### Comparison between the immunostimulants groups and the Pox group

There were non-significant ( $P \geq 0.05$ ) changes between the three groups in NI, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and Cp levels during the research. The Pox group displayed a significant ( $P < 0.05$ ) decreased TAC, microcytic hypochromic anemia, elevated liver and kidney function tests, and hypoproteinemia, while IG and BG showed non-significant changes in TAC (except on the 7th day in BG, TAC slightly decreased and rapidly returned to its normal value on the 14th day), red blood cell parameters and indices and TP. While the liver and kidney function tests significantly ( $P < 0.05$ ) decreased in IG and BG. Both groups revealed a lower degree of hypoalbuminemia, decreased A/G, leukocytosis, neutrophilia, lymphocytosis, and the neutrophils' phagocytic activity than the Pox group. Contrariwise, IG, and BG demonstrated a higher degree of neutrophils phagocytic index and globulin, and A/G (compared to the pox group). Notably, there were non-considerable differences between IG and BG along the research.

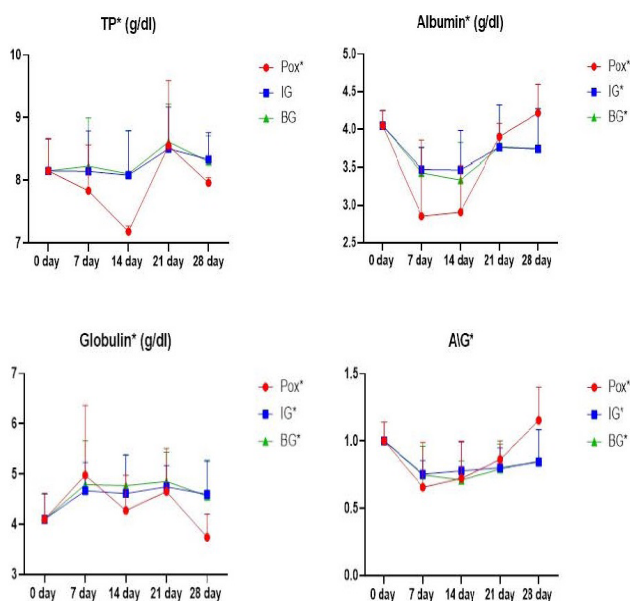


Fig. 5. Comparison between the protein profile of the three studied groups throughout the research, (\*) on the parameter means significant between the groups, (\*) on the group means the vaccine or vaccine+immunostimulant effect is significant throughout the research. Considered significant when ( $P < 0.05$ ).

### Discussion

SPPV infection continues to pose a notable socio-economic challenge for sheep farming in Egypt, characterized by its elevated levels of illness and death among the livestock. This disease represents a considerable obstacle to successful sheep production on a global scale. Vaccination emerges as the most simple and efficient means of prevention against infectious diseases, acknowledged for its humane approach and cost-effectiveness (Fares *et al.*, 2019; Eldakrouy *et al.*, 2021; Hamdi *et al.*, 2021).

In the current study, the sheep pox vaccine effectively elicited a humoral immune response, as indicated by the gradual increase in NI observed in the Pox group. This response began on the 7th day and steadily rose to the research end at the 28th day with a mean of 2.75. These findings align with Kostinov *et al.* (2020), who cleared that the vaccine's efficacy depends on its ability to mimic the natural infection to a moderate degree. Previous authors confirmed the efficacy of live attenuated sheep pox vaccine (Romanian strain) in stimulation of the humeral immune response of sheep against sheep pox virus (Oreiby *et al.*, 2022; Elsayed *et al.*, 2023). They recommended it as prophylactic measure against sheep pox infection in susceptible animals (Oreiby *et al.*, 2022; Elsayed *et al.*, 2023).

This successful humoral immune response started with a strong cy-

tokines activation noticed in the Pox group, up to the 14th day following vaccination. Similar patterns have been observed previously different types of vaccines (El-Din *et al.*, 2014; Simon *et al.*, 2014; Christian *et al.*, 2015; Mawa *et al.*, 2017; Jarikre *et al.*, 2019; Darwish *et al.*, 2023). Cytokines are immunoregulatory proteins, that usually up-regulate post-infection or vaccination. They orchestrate the immune response, leading to the immunoglobulins formation. Early cytokine increment following vaccination improves the body's reaction to the vaccine and supposes higher levels of immunoglobulins (Bergamaschi *et al.*, 2021). Moreover, their activity post-vaccination is associated with local and systemic reactions, such as fever and fatigue, commonly observed after vaccination (Christian *et al.*, 2015; Burny *et al.*, 2019).

These pro-inflammatory cytokines evoke APPs production by hepatocytes, evidenced by the marked increase in Cp levels observed in the Pox group here up to the 14th day. Cp is a copper-containing protein and a tissue damage marker. It usually increases in different infectious diseases. Post-vaccine acute phase response was documented in different animal species (Andersen *et al.*, 2012; Arthington *et al.*, 2013; Gundasheva and Tsachev, 2015; Shawky *et al.*, 2016; Darwish *et al.*, 2023).

The oxidative stress depicted in the pox group here is another outcome of the activation of the aforementioned pro-inflammatory cytokines. Under the pro-inflammatory cytokines stimuli, different immune cells release free radicals. Free radicals are an essential part of the innate immune response, they oxidize pathogens' vital components, causing their destruction. They maximize the immune response effectiveness in both infection and vaccination. Antioxidants are responsible for body cell protection from free radicals' harm and damage. In the present work, the excess production of free radicals exceeds the neutralizing capacity of antioxidants resulting in significant oxidative stress. This is evidenced by the observed decrease in TAC up to the 14th day in the pox group. The occurrence of oxidative stress following vaccination has been consistently documented in numerous studies by Phillips *et al.* (2010); Kumar *et al.* (2019); Jarikre *et al.* (2019); Yadav *et al.* (2020); Dursun *et al.* (2022) and Darwish *et al.* (2023).

The oxidative stress experienced by the Pox group played a pivotal role in the noticeable hematological and biochemical alterations observed post-vaccination. Free radicals, primarily targeting hepatocytes, renal cells, and erythrocytes, instigate damage and subsequent destruction. Consequently, a marginal increase in liver and kidney function tests, coupled with decreased levels of RBCs, hemoglobin, and packed cell volume, were observed in the Pox group up to the 14th day, consistent with findings from previous studies (Phillips *et al.*, 2010; Kumar *et al.*, 2019; Yadav *et al.*, 2020; Dursun *et al.*, 2022; Darwish *et al.*, 2023).

Furthermore, the inhibitory effect of pro-inflammatory cytokines on bone marrow erythropoiesis via disrupting erythropoietin synthesis, intestinal iron absorption, and iron macrophage recycling, exacerbates the microcytic hypochromic anemia observed in the Pox group up to the 14th day (Paulson *et al.*, 2020). This contrasts with previous findings, where vaccinated animals exhibited a normal erythrogram (Shawky *et al.*, 2016; Jarikre *et al.*, 2019; Arfuso *et al.*, 2021). However, it is consistent with the results of Prentice *et al.* (2015) and Darwish *et al.* (2023), who observed diminished red cell parameters in vaccinated infants and sheep.

On the contrary, pro-inflammatory cytokines play a crucial role in promoting the maturation, proliferation, and release of neutrophils and lymphocytes from the bone marrow and lymph nodes into circulation. This process explains the observed increase in neutrophil count, heightened neutrophilic phagocytic activity and index, and lymphocytosis, resulting in overall leukocytosis seen in the Pox group throughout the study. Neutrophils are vital components of innate immunity, producing reactive oxygen species and aiding in the Toll-like receptor 4 (TLR4)-dependent innate clearance of pathogens, particularly bacteria, due to their abundance of digestive and hydrolytic enzymes (Kumar *et al.*, 2019). Furthermore, viral proteins may contribute to lymphocytosis in the Pox group by stimulating certain lymphocyte subpopulations such as mitogens or



superantigens (Arfuso et al., 2021). Leukocytosis, with or without neutrophilia, and increased phagocytic activity, as well as lymphocytosis, have been observed following vaccination in various animal species (Andersen et al., 2012; Shawky et al., 2016; Arfuso et al., 2021; Darwish et al., 2023).

The protein profile of the Pox group mirrored the immunological shifts post-vaccination. Elevated levels of cytokines and acute phase proteins (APPs), including  $\alpha$  and  $\beta$  globulins, directly led to the observed hyperglobulinemia in the vaccinated groups up to day 14. Subsequently, as cytokine and APP levels decreased after day 14, this hyperglobulinemia persisted due to antibody production against the vaccine ( $\gamma$  globulin) (Shawky et al., 2016; Arfuso et al., 2021). Similarly, the acute phase response and oxidative stress mentioned earlier account for the significant hypoalbuminemia noted in the Pox group. Albumin, as a negative acute phase reactant, experiences reduced synthesis by the liver due to the influence of pro-inflammatory cytokines, which prioritize the production of positive acute phase proteins instead (Shawky et al., 2016; Arfuso et al., 2021). Moreover, its antioxidant properties make it susceptible to damage by circulating free radicals. The total hypoproteinemia and decreased A/G ratio, observed in the Pox group until the 14<sup>th</sup> day and 7<sup>th</sup> day, respectively, stemmed from alterations in albumin and globulin levels, as total protein and A/G ratios predominantly depend on the collective levels of albumin and globulins, as well as the difference between them (Shawky et al., 2016; Arfuso et al., 2021).

Regarding the effect of the studied immunostimulants on the ewes' immune response to the sheep pox vaccine, both failed to enhance the vaccinated ewes' humoral immune responses to the vaccine. This result was disappointing as many researchers confirmed the immunopotential action of oregano and artichoke extracts and approved their ability to boost the immune response against different infections (Du et al., 2015; Darwish et al., 2021; Dhiab and Ali, 2022; Ürüshan, 2023). This may be because of the difference in the antigen type, active principal concentrations, and animal species.

On the other hand, they kept the vaccinated ewes' TAC (only a slight decline in BG on the 7<sup>th</sup> day and rapidly disappeared on the 14<sup>th</sup> day), and erythrogram and TP in normal ranges. In addition, they showed better values of serum renal and hepatic function tests, neutrophils phagocytic index, albumin, globulin, and A/G (compared to the pox group). While leukocytosis, neutrophilia, lymphocytosis, and the neutrophils' phagocytic activity were less prominent than Pox group. These results were mainly attributed in IG to the antioxidant characteristics of the oregano essential oils' (carvacrol and thymol) which countered the oxidative stress associated with the vaccination process (Du et al., 2015; Darwish et al., 2021). Besides their anti-inflammatory properties which restrain the pro-inflammatory cytokines activity (Du et al., 2015; Darwish et al., 2021). Similarly, Cynara (the main phenolic compound of artichoke) in BG has potent anti-inflammatory antioxidant properties with its specific influence on liver functions and lipid profile (Affi et al., 2014; Osama et al., 2014; Ben Salem et al., 2017; Kaymaz et al., 2017; Mahboubi, 2018; Salekzamani et al., 2019; Ghasemian et al., 2022; Napoli et al., 2023). Hence, both extracts effectively manage the key factors involved in the above-described clinicopathological changes (oxidative stress and pro-inflammatory cytokine activity) and safeguard the body from adverse effects associated with vaccination.

## Conclusion

Immune Care® and Bovi Care® succeeded in avoiding oxidative stress, anemia, and hypoalbuminemia usually accompanied the pox vaccination in sheep. They also improved the liver and kidney function tests, globulin and neutrophils phagocytic index but they did not affect the humoral immune response.

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## Conflict of interest

The authors have no conflict of interest to declare.

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