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RESEARCH ARTICLE

Modulating Effect of Peppermint and Eucalyptus Essential Oils on vVND Infected Chickens

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A B S T R A C T

Treatment of broiler chickens with the peppermint and eucalyptus essential oils blend improved protection and productive performance as compared with the infected positive control birds. It decreased total mortality, increased final body weight, and decreased final feed conversion ratio (FCR) ($P \leq 0.05$). The peppermint and eucalyptus essential oils blend had a positive effect on macroscopic and microscopic lesion scoring and enhanced HI and ELISA titers against Newcastle disease virus vaccine, as compared to their untreated control groups ($P \leq 0.05$). In conclusion; the peppermint and eucalyptus essential oils blend used in the present investigation significantly controlled vVND infection where it has immuno-modulatory effect and positively evoked the immune response against this very hot challenging virus which is of great devastating economic impact on poultry industry.

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INTRODUCTION

Herbal products and their oil extracts have been used for natural therapy as pharmaceuticals; however, only in recent years; aromatic plants and their extracts were introduced to the animal feeding (Awaad *et al*., 2014). Further complications arise because herbal products and their oil extracts feed additives may vary widely with respect to botanical origin, processing, and composition. Most studies investigated blends of various active compounds and reported their effects on production performance rather than the physiological impacts (Renata *et al*., 2012; Lee *et al*., 2013). Eventually; immuno-modulated components strengthen the defense and immune mechanisms of the body and currently usable for stimulating the non-specific immune responsiveness in both the human and Veterinary Medical practice (Awaad *et al*., 1999). Peppermint and eucalyptus essential oils blend proved to have an immunostimulant effect on the humoral and cell mediated immune response against Newcastle disease in chickens (Awaad *et al.*, 2009a). Their utility in modulating the immune response of immunocompromised birds after infection with infectious bursal disease virus (IBDV) and/or vaccination against IBDV as compared with untreated control groups was also evident (Awaad *et al.*, 2009b).

The present trial was dedicated to determine the possible modulating effects of usage of peppermint and eucalyptus essential oils in emulsifiers on broiler chickens infected with velogenic Newcastle disease virus (vVND).

MATERIALS AND METHODS

Essential volatile oils blend: Peppermint and eucalyptus essential volatile oils in emulsifiers blend produced commercially under the trade name "Mentofin®" by EWABO Co., Germany was used. Mentofin consists of Cineol (42.2%), limonene (3.5%), L-menthol (48.7%), phellandrene (0.5%), a-pinene (1.0%), b-pinene (0.3%), and terpineol (0.3%).

Challenging Newcastle disease virus strain (vVNDV): The virus was isolated in 10 day-old chicken embryos and identified as NDV using PCR (Viljoen *et al*. 2005) (Fig.1). Sequence of PCR product proved to be similar to genotype VII, Chicken/China/SDWF07/2011-acc.no.: JQ015296 (Fig.2). The velogenicity of vVNDV was monitored by intramuscular inoculation of 10 birds (28 day-old broiler chickens free from antibodies to ND) with a dose of $10^{6.8}$ EID_{50}/ml/bird which resulted in 100% mortality.

Experimental design: One day-old Arbor Acres broiler chickens (n=300) were used in this study. They were allotted into 5 equal groups (1-5) consisting of 60 each. Chickens of all groups have been assigned into 3 equal

replicates (20 each) which kept in isolators with strict isolation measures. The birds had free access to water and fed on a commercial ration *ad libitum*. Experimented birds of groups 2, 3 and 4were vaccinated against different diseases according to the vaccination programs usually adopted in Egyptian chicken broiler farms. Chickens of groups 1 and 5 were kept as a control groups without ND vaccination.

Chickens of groups 1, 3 and 5 were treated with peppermint and eucalyptus essential volatile oils in emulsifiers blend via both oral and spray routes. Oral treatment via drinking water was carried out at several intervals: 4-7, 16-18 and 25-39 days of age at a dose level of 0.25 ml/l for 12 hours/day. While Spray treatment carried out at 3, 7, 12, 13,15,16,19, 23, 27, 31, 35 and 39 days of age at a dose level of 0.1ml/20 ml water/10 birds using fine spray (< 3 mu diameter) as recommended by the producer. The birds of groups 2 and 4 were kept without treatment. At 28th day of age; 2 birds were randomly chosen for challenge from each replicate (6 birds/group) of groups 1-4 and infected intramuscularly with a dose of $10^{6.8}$ EID_{50}/ml/bird of vVND virus and kept with other pen mates for contact infection. The birds of group 5 were kept without infection as negative controls. Infected bird groups kept under close observation for further 14 days post infection (PI) for survival rate and macroscopic lesion scoring of dead as well as sacrificed birds at the end of observation period. At 1st day of age blood samples were collected from randomly selected 12 birds for determining mean maternal antibody titer to NDV. In addition, 36 birds/group (12/replicate) were collected at 7, 14, 21 and 28 days of age and tested using ELISA (Idexx, ME, USA) and hemagglutination inhibition test (HI) (Swayne *et al*., 1998). At 35 days of age; 3 randomly chosen contact infected chickens/group of all infected experimented groups were sacrificed and samples of cecal tonsils, lungs and trachea were collected and subjected to histopathological examination for microscopic lesion scoring. The remaining chickens were subjected to chicken performance response variables.

Statistical analyses: One-way analysis of variance was adopted using SAS software general liner models procedure (SAS Institute, 2004). The main factor was Mentofin supplementation as a mean effect. Mean values assessed for significance using Duncan's multiple range tests. The following model was used for data analysis: $Y_{ij} = \mu + \alpha_i + e_{ij}$ Where; Y_{ij}: The Jth observation of the ith dose of Mentofin.μ: Overall mean.α_i: Effect of dose of Mentofin (I=1, 2).e_{ij}: Unexplained error. Statements of statistical significance based upon P≤0.05.

RESULTS

High survival rate (100%) was observed in the negative control group (Treated/Vaccinated/Non-challenged), while 100% mortality was recorded in the positive control group (Untreated/Non-vaccinated/Challenged). Concomitant use of volatile oils with ND vaccination in group 3 (Treated/Vaccinated/Challenged) gave the superior results in protection against vVND challenge (80%) (Table 1). The survival rate against vVNDV challenge reached 18.33% and 61.67% in group 1 (Treated/Non-vaccinated/ Challenged) and group 2 (Untreated/Vaccinated/Challenged) respectively. The postmortem lesion scoring varied between groups and was higher in group 4 (Untreated/Non-vaccinated/Challenged). Essential oils supplementation significantly improved performance parameters (body weight and FCR) of the treated groups (Table 2).

Mean ELISA titers were significantly higher at all studied intervals in ND vaccinated/volatile oils treated groups 3 and 5 as well as non-vaccinated/volatile oils treated group 1 vs. positive control group 4 (P≤0.05) (Table 3). HI titers of ND vaccinated/essential oils treated groups (3 and 5) gave the higher significant increase over those of groups 1 and 4 at all studied intervals (P≤0.05) (Table 4).

Table 1: Results of vVNDV challenge of different broiler chicken groups.

Groups	Treatment	No. of survived birds/ Total No. of birds*	Mortality rate	Protection %	** Mean macroscopic lesion scoring in dead and sacrificed survivors (at 42days of age)					
					Septc***	L.	Pr	PP	CT	Sum
1	Treated/Non-vaccinated/Challenged	11	81.67	18.33	3	2	3	1	3	12
2	Untreated/Vaccinated/Challenged	37	38.33	61.67	2	1	2	2	2	9
3	Treated/Vaccinated/Challenged	48	20	80.00	1	0	1	1	2	5
4	Untreated/Non-vaccinated/Challenged	0	100	0.00	4	3	3	2	2	14
5	Treated/Vaccinated/Non-Challenged	60	0	100.00	0	0	0	0	0	0

*Total No. of birds/group = 60 ** Lesion score:(0) Absent, (1) Minimal, (2) Slight, (3) Moderate and (4) Severe lesions. *** Septc.=Septicaemia. L.=Liver hemorrhage (Hg). Pr= Hemorrhage of Proventriculus. PP = Peyer's patches ulceration. CT=Haemorrhage and Hyperplasia of Caecal tonsils.

Table 2: Effect of peppermint and eucalyptus essential oils on body weight and FCR of different broiler chicken groups

Group	Treatment	Experimental Days					
		1	7	14	21	28	35
Body weight (g)							
1	Treated/Non-vaccinated/Challenged	39.5±0.3	132.2±2.4	358.8±4.5^{b*}	543.5±7.8^{c}	998.5±17.9^{b}	1212.1±13.2^{d}
2	Untreated/Vaccinated/Challenged	39.9±0.3	127.5±2.6	350.5±5.8^{b}	580.9±11.3^{ab}	1117.9±47.1^{a}	1282.1±23.6^{c}
3	Treated/Vaccinated/Challenged	39.7±0.3	129.5±2.4	353.6±4.1^{b}	573.7±9.1^{b}	1123.0±41.9^{a}	1368.3±27.6^{b}
4	Untreated/Non-vaccinated/Challenged	39.9±0.2	132.7±2.2	328.3±5.9^{c}	473.8±5.3^{d}	979.4±13.7^{b}	1077.5±18.7^{e}
5	Treated/vaccinated/Non-Challenged	39.7±0.3	130.8±2.5	379.1±3.2^{a}	605.3±10.4^{a}	1099.5±15.9^{a}	1525.0±36.2^{a}
	Probability	0.7318	0.3256	0.0001	0.0001	0.0001	0.0001
Feed Conversion Ratio (FCR)							
1	Treated/Non-vaccinated/Challenged		1.259±0.018	1.325±0.019	1.829±0.032	1.829±0.027	2.255±0.031^{b*}
2	Untreated/Vaccinated/Challenged		1.311±0.029	1.338±0.019	1.807±0.029	1.772±0.024	2.382±0.027^{a}
3	Treated/Vaccinated/Challenged		1.279±0.028	1.325±0.029	1.817±0.036	1.761±0.027	2.225±0.030^{b}
4	Untreated/Non-vaccinated/Challenged		1.223±0.028	1.346±0.031	1.933±0.043	1.768±0.031	2.460±0.038^{a}
5	Treated/vaccinated/Non-Challenged		1.271±0.028	1.376±0.027	1.862±0.034	1.858±0.028	2.066±0.027^{c}
	Probability		0.0557	0.6176	0.1072	0.0732	0.0001

Mean±SD with different superscripts within column, within age, are significantly different (P≤0.05).

Table 3: Results of mean ELISA–conversion titers during first 4 weeks of age

Group	Treatment	Mean ELISA-titers				
		1day	7 days	14 days	21 days	28 days
1	Treated/Non-vaccinated/Challenged	15252±0.12	8371±0.23[b]	3549±0.43[b]	3737±0.40[b]	4590±1.06[b]
2	Untreated/Vaccinated/Challenged	15252±0.23	8342±0.39[c]	2986±0.57[c]	3494±1.14[c]	3950±0.1.14[c]
3	Treated/Vaccinated/Challenged	15252±0.23	8451±0.32[a]	3687±0.55[a]	3941±0.48[a]	4879±1.01[a]
4	Untreated/Non-vaccinated/Challenged	15252±0.23	7023±0.50	3367±0.42	1813±0.86	1170±0.91
5	Treated/vaccinated/Non-Challenged	15252±0.23	8551±0.46[a]	3693±0.41[a]	3972±1.91[a]	4955±1.78[a]

Mean±SD with different superscripts within column, within age, are significantly different (P≤0.05).

Table 4: Results of Haemagglutination-Inhibition (HI) seroconversion titres during first 4 weeks of age

Group	Treatment	HI-titers (Log_2)			
		7days	14 days	21 days	28 days
1	Treated/Non-vaccinated/Challenged	5.50±0.13[d*]	4.42±0.18[c]	3.83±0.06[d]	1.92±0.11[c]
2	Untreated/Vaccinated/Challenged	7.50±0.13[c]	7.75±0.07[b]	7.00±0.23[b]	6.67±0.21[a]
3	Treated/Vaccinated/Challenged	8.83±0.14[a]	8.00±0.14[ab]	8.08±0.19[a]	7.00±0.28[a]
4	Untreated/Non-vaccinated/Challenged	5.42±0.16[d]	4.00±0.23[c]	4.42±0.20[c]	3.50±0.11[b]
5	Treated/vaccinated/Non-Challenged	8.33±0.08[b]	8.25±0.12[a]	8.08±0.28[a]	7.00±0.25[a]
	Probability	0.0001	0.0001	0.0001	0.0001

Geometric mean maternal antibody titer at 1 day of age is 7.1. Mean±SD with different superscripts within column, within age, are significantly different (P≤0.05).

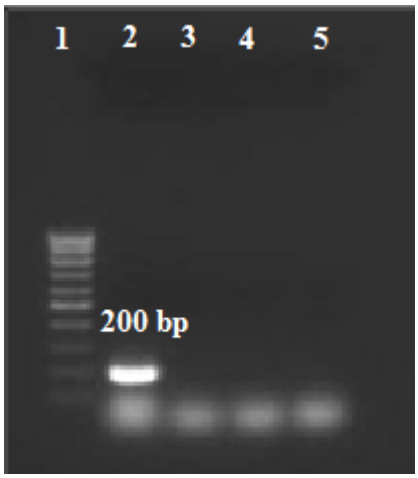

Fig. 1: PCR electrophoretic pattern of multiplied gene of fusion protein of NDV. Lane1: 100 base pairs molecular ladder. Lane2: Egg fluid sample showed +ve result at 200 bp. Lane3-5: Negative control sample for egg fluid sample.

TGCAGCAATGCTCTCTTGACGGCAGGCCCCTTGCAGTTA
GGAGAATTGTAGTAACGGGAGATAAGGCAGTCAATGTAT
ACACCTTTTTTcAAACAGGGTCAATCATAGTCAAGTCGCT
CCCGAATATGCCCAGAGATAAGGAGGCATGTTCAAGGGT
TGCAGCAATGGTCTCTTGcACGGCAGGGACCACtCAGCA
GCAGGAATTTGGTGAGTCTATCCGGAGGATACAAGGGTC
TGTATCCACGTCCGGAGGAAGGAGACAAAAACGTATTAT
AGGTGCTGTTATTGCCAGTGTAGCGTCCTACTGGTTTTA
CAAACCTGTGATGGCCTCGAAGACCCTCGAGGCTACCT

Fig. 2: Amino acid sequence of the fusion protein of NDV isolate.

No obiviuos histopathological lesions could be detected in the negative control group 5 (Treated/Vaccinated/Non-challenged). Moreover; birds of this group showed an increase in the follicular size comprizing the cecal tonsils with lymphoblastic activation of follicular elements. Tracheal microscopic lesions revealed variable histopathological alterations that were more severe with significant increase in group 4 (Untreated/Non-vaccinated/Challenged) (P≤0.05) vs. group 5 (Treated/Vaccinated/Non-challenged) represented by marked deciliation and necrosis of tracheal epithelium associated with focal hyperplastic proliferation of epithelium associated with intense lymphocytic infiltration, congestion, hemorrhage and severe edema in the lamina propria. Lesions were less severe in group 1 (Treated/Non-vaccinated/Challenged) than group 2 (Untreated/Vaccinated/Challenged). Lesions in group 3 (Treated/Vaccinated/Challenged) characterized by goblet cell hyperplasia of tracheal epithelium with lymphocytic infiltration and dilatation of blood capillaries in the lamina propria (Fig.3). Lung microscopic lesions in group 1 (Treated/Non-vaccinated/Challenged) revealed mild interlobular and interstial edema with degeneration of epithelial lining the secondry bronchi associated with edema, congestion and increased lymphocytes in lamina propria associated with edema and lymphocytes infiltating the parabronchi with congestion and perivascular and interstitial edema. More advanced lesions were observed in group 2 (Untreated/Vaccinated/Challenged) than group1 that characterized by more interstitial and perivascular edema and lymphoctic infiltration in the secondry bronchi and parabronchus extending into infundibulum and air capillaries assocaited with congestion of blood capillaries. lesions were very mild in group 3 (Treated/Vaccinated/Challenged) that was restricted to perivascular interstitial edema and no other lesion were detected in parabronchi or air capillaries. On the other hand; significant marked severe lesions were recorded in the group 4 (Untreated/Non-vaccinated/ Challenged) (P≤0.05) that characterized by severe necrotic and inflammatory reaction involving the secondry bronchi, parabronchi and air capillaries associated with lymphocytes and heterophils infiltartion and proteineuos exudated filling the lumen of air capillaries and focal hemorrhage (Fig.4).Caecal tonsils microscopic lesions characterized by lymphoid depletion and necrosis of lymphoid elements of lymphoid follicles. The lesions were significantly severe in group 4 (Untreated/Nonvaccinated/Challenged) (P≤0.05), less in group 1 (Treated/Non-vaccinated/Challenged) than group 2 (Untreated/Vaccinated/Challenged) while in group 3 (Treated/Vaccinated/Challenged), the lesions were mild lymphocytic depletion assocaited with lymphoblastic activation with increase in the follicular size (Fig.5). Group 4 (Untreated/Non-vaccinated/ Challenged) showed the worst total significant microscopic lesion score (34.25) vs. the other challenged groups which had 18.75, 20.5 and 17.25 scores in groups 1, 2 and 3 respectively (P≤0.05) (Table 5).

Table 5: Statistical analysis of histopathological lesion scoring of vVND challenged broiler chickens

Histopathological lesions	vVND challenged groups			
	G1*	G2	G3	G4
Trachea				
Deciliation & Desquamation & necrosis	2.00±0.31^{ab}	1.75±0.47^{ab}	1.50±0.28^{b}	3.00±0.57^{a}
Inflammatory reaction	1.40±0.40^{b}	1.80±0.37^{ab}	1.25±0.25^{b}	2.75±0.74^{a}
Congestion	1.66±0.66^{a}	1.75±0.25^{a}	1.40±0.24^{a}	2.66±0.66^{a}
Edema	1.66±0.66^{a}	1.50±0.28^{a}	1.40±0.24^{a}	2.50±0.50^{a}
Lungs				
a-Secondary bronchi				
Desquamation & necrosis	1.00±0.40^{b}	2.70±0.80^{ab}	0.80±0.20^{b}	3.00±0.31^{a}
Inflammatory reaction	2.00±0.40^{a}	2.00±0.83^{a}	1.25±0.25^{a}	2.80±0.37^{a}
Congestion	1.40±0.54^{a}	1.80±0.64^{a}	1.16±0.40^{a}	2.60±1.14^{a}
Edema	1.40±0.24^{a}	1.80±0.73^{a}	1.25±0.25^{a}	2.40±0.50^{a}
b-Parabronchi & air capillaries				
Desquamation & necrosis	1.10±0.23^{ac}	1.64±0.25^{ab}	0.77±0.19^{c}	2.33±0.25^{a}
Inflammatory reaction	1.00±0.25^{b}	1.23±0.35^{b}	0.86±0.16^{b}	2.86±0.29^{a}
Congestion	1.75±0.16^{ab}	1.66±0.31^{ab}	1.13±0.16^{c}	2.82±0.26^{a}
Interstitial and perivascular edema	1.60±0.26^{ab}	1.47±0.35^{ab}	1.14±0.14^{b}	2.29±0.31^{a}
Cecal tonsils				
Lymphocytic depletion	1.25±0.25^{ab}	1.00±0.00^{b}	1.00±0.00^{b}	1.83±0.30^{a}

*G1 = Treated Non-vaccinated. G2 = Untreated Vaccinated. G3 = Treated Vaccinated. G4 = Untreated Non-vaccinated (Positive control). Mean±SD with different superscripts within row, within age, are significantly different (P≤0.05). No lesions could be detected in group 5.

DISCUSSION

The high survival rate (100%) in the negative control group (Treated/Vaccinated/Non-challenged) indicated the safety of the used ND vaccines and the administered essential oils blend. On the other hand; a 0% survival rate recorded in positive control group (Untreated/Non-vaccinated/Challenged) indicated the velogenicity of the used vVND virus. In challenged bird groups; the effect of volatile oils blend obtained in the present investigation might be due to their antiviral activity (Sökmen *et al*., 2004). This result confirms the findings of Awaad *et al* (2002) on studying the effect of peppermint and eucalyptus essential oils on concomitant infection of "ORT" and "vVND" in broiler chickens. Our obtained findings are also in agreement with those reported by Barbour *et al.* (2013) who evaluated the impact of eucalyptus and peppermint essential oils on immune modulation and production of broiler chickens challenged with a molecularly characterized velogenic Newcastle disease virus. Findings of group 1 could be explained as this blend has a potent immunomodulatory effect that confirm findings of Awaad *et al.* (2009a) who stated that eucalyptus and peppermint essential oils blend implement both innate-cell mediated and humoral immune response. Similar findings has been reported by Barbour and Danker (2005) who mentioned that essential oils of eucalyptus and peppermint improved the homogeneity of immune responses and performance in Mycoplasma gallisepticum/H9N2 virus-infected broilers.

Essential oils supplementation significantly improved performance parameters (body weight and FCR) of the treated groups. These variables correlated positively with the survival percentage. The worst parameters has been recorded in positive control group 4 (Untreated/Non-vaccinated/Challenged). The improving in performance by essential oils supplementation has been described by other workers (Cross *et al*. 2007; Windisch *et al*., 2008; Barbur *et al*., 2013). The proposed mode of action of herbal products might be attributed to their antimicrobial properties (Kollanoor-Johny *et al*., 2012; Lee *et al*., 2013), oxidative-resistant activity (Dundar *et al*., 2008; Sarac *et al*., 2009) or anti-inflammatory effect (Lee *et al*., 2009; Yanhong, 2011). The significantly higher immune status in ND vaccinated/volatile oils treated groups 3 and 5 as well as non-vaccinated/volatile oils treated group 1 vs. positive control group 4 could be attributed to the fact that peppermint oil maintains the structural integrity of immune cells due to its strong antioxidant action which protects cell membrane from free radical oxidants, thereby resulting in an improved immune response (Nickels, 1996). Awaad *et al*. (2009a) concluded that administration of eucalyptus and peppermint oils blend evoked the immune response in chickens. Enhancement of the immune system by herbal products and oils has been reported by many investigators (Lee *et al*., 2010; Lillehoj *et al*., 2010).

The improvement in vVND pathological picture (macroscopic and microscopic lesion scores) recorded in volatile oils supplemented groups (with or without vaccination) is supposed to their possible antiviral (virucidal) action that might influenced the virulence of the challenging agent (Mekay and Blumberg, 2006; Ocak *et al.*, 2008; Barbour *et al.*, 2010). Moreover; this perhaps attributed also to their effect on cell mediated and local immunity (Cserep, 2008) which has been shown to play a part in clearing virus from the tissues (Awaad *et al*., 2009a). The recorded increase in the follicular size comprizing the cecal tonsils with lymphoblastic activation of follicular elements in groups 1, 3 and 5 completely accords with those reported by Awaad *et al.* (2009a) who mentioned that treated birds with peppermint and eucalyptus essential oils resulted in lymphocytic hyperplasia and activation in Bursa of Fabricious, thymus, spleen and caecal tonsils.

Conclusions: The used peppermint and eucalyptus essential oils blend proved its immunomodulatory effects and positively evoked the immune response against vVND virus that have a great devastating economic impact on poultry industry. It also improved clinical, pathological and performance parameters.

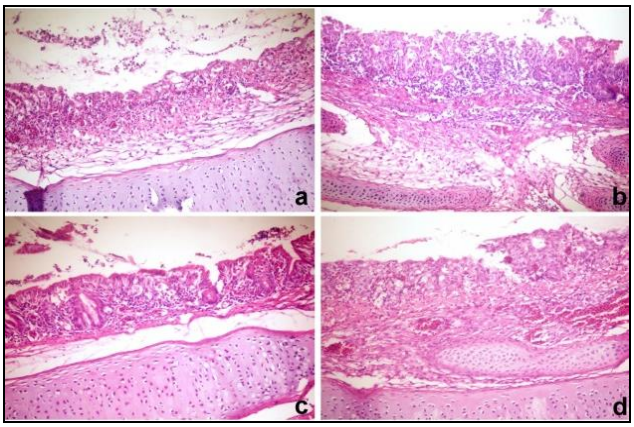

Fig. 3: Microscopic lesions in trachea. H & E X200. **a)** Mentofin treated group (group 1) showing deciliation and hyperplasia of tracheal mucosal epithelium asssociated with lymphocytic infiltration and congestion with mild edema of lamina propria with few RBCs exudation (score 1). **b)** Vaccinated group (group 2) showing hypertrophy and hyperplasia of tracheal mucosa with extensive deciliation and lymphocytic infiltration with moderete congestion and edema. **c)** Mentofin treated vaccinated group (group 3) showing focal mild deciliation of tracheal mucosa with goblet cell hyperplasia and mild inflammatory reaction involving the mucosa. **d)** Untreated non-vaccinated (positive control) group (group 4) showing extensive necrosis with hyperplasia of tracheal mucosa with severe edema, congetion and hemorrhages in lamina propria.

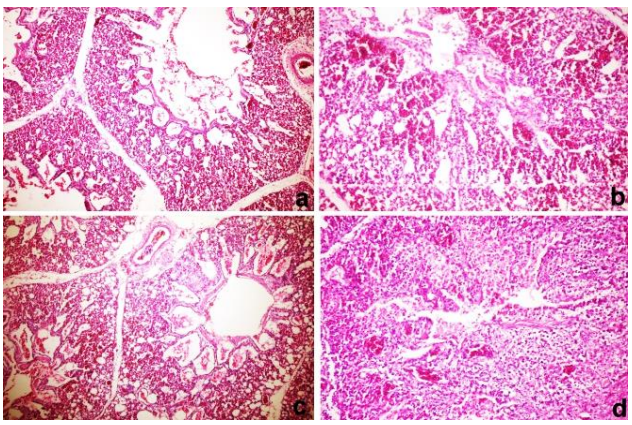

Fig. 4: Microscopic lesions in lungs. H & E. **a)** Mentofin treated group (group 1) showing no inflammatory reaction involving the parabronchus or the air capillaries except for mild congestion of blood capillaries. **b)** Vaccinated group (group 2) showing sloughing epithelial lining the parabronchus with congestion of blood capillaries and edema X400. **c)** Mentofin treated vaccinated group (group 3) showing mild congestion of blood capillaries comprising the lung lobules associated with mild perivascular edema. **d)** Untreated non-vaccinated (positive control) group (group 4) showing necrosis of epithelial lining the parabronchus and air capillaries with congestion of blood capillaries note the extensive destruction of parabronchus with severe edema of air capillaries and extensive oblitration of air capillaries X400.

Author's contribution: MHHA and HMH planed the investigation. MAA and SAZ applied the immune status assessment and recorded the mortality and post mortem lesions. FFM carried out the histopathological examination. MAE applied the performance variable values and the statistical analysis. All authors participated in draft and revision of the manuscript. All authors read and approved the final manuscript.

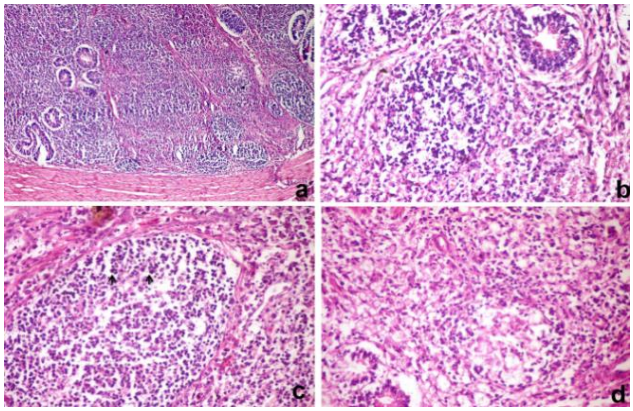

Fig. 5: Microscopic lesions of caecal tonsils. H & E. **a)** Vaccinated group (group 2) showing extensive hypeplasia of the lymphoid follicles (X100). **b)** Mentofin treated group (group 1) showing mild depletion of lymphoid elements (X400). **c)** Mentofin treated vaccinated group (group 3) showing mild lymphocytic depletion of lymphoid follicle with lymphoblastic activation (arrow) (X400). **d)** Untreated non-vaccinated (positive control) group (group 4) showing severe lymphocytolysis and necrosis of majority of lymphoid elements comprising the follicle (X400).

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