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Original Research Article

EFFECT OF THE MEDICINAL PLANT (*AZADIRACHTA INDICA*) ON *Chlamydophila psittaci* INFECTION IN BROILER CHICKENS

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Abstract: *Chlamydophila psittaci* is considered one of the important bacterial agents which affecting poultry with zoonotic importance to human health. This study designed to investigate the antibacterial action of aqueous neem leaves extract (*Azadirachta indica*) against experimentally infected broiler chickens with chlamydia. Seventy, one day old Hubbard chicks were randomly selected and divided equally into seven groups, three of the infected groups were treated with neem extract at varied concentration of 4%, 6% and 8% in drinking water compared with the fourth that treated with oxytetracycline. Based on clinical and postmortem examination, growth performance, serum biochemical analysis and histopathological findings the results were evaluated. Chicks received 8% extract at 8 days old for five successive days and control showed normal level of alanine aminotransferase, aspartate aminotransferase, uric acid and creatinine 30 U/L, 26 U/L, 3.90 mg/dl and 0.67 mg/dl respectively, while treated infected groups revealed lower levels unlike infected untreated showed higher levels 68 U/L, 62 U/L, 5.20 mg/dl and 1.62 mg/dl respectively. Moreover treated groups with neem extract of 4%, 6% and 8% recorded significantly better body weights 1130.56 gm, 1135.70 gm and 1254.60 gm respectively, than infected untreated group 963.25 gm at 32 days old as well as feed conversion rate 2.26, 1.94 and 1.77 than 3.80 respectively, at 24 days old. Histopathological examination of infected group showed pulmonary inflammation, myocarditis and hepatic necrotic foci while confirmed that treatment with 8% neem extract resulted in complete recovery of lung tissue and normal myocardium. It is concluded that aqueous leaves neem extract especially 8% concertation had an excellent antichlamydial medication without side effects and recommended in the control of chicken chlamydiosis.

Key words: broilers; *Chlamydophila psittaci*; neem; oxytetracycline; enzymes

Introduction

Chlamydiosis is an infectious disease of zoonotic importance affect humans, animals and birds where it may resulted in outbreaks of in domestic avian, pet and wild birds (1,2). It is caused by obligate intracellular bacteria that responsible for wide range of earnest threats on human and birds (3). The phylogenic analysis of the 16S and 23S rRNA genes showed that order chlamydiae contained two distinguished groups at the family level, *chlamydia* and *chlamydophila* (4).

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Chlamydophila psittaci (C. psittaci) is endemic in birds causing psittacosis or avian chlamydiosis which plays a remarkable role in respiratory infections leading to severe losses in poultry industry (5). The control system of the disease depended on antibiotic treatment commonly oxytetracycline (OTC) , macrolides and doxycycline that inhibit protein synthesis of on chlamydia ribosome**,** but extended exposure of *chlamydia* to antibiotic yielded a greater chance for drug resistance development (6,7). Although the bacteria respond very well to OTC treatment in human and birds (8,9), it has many disadvantages as adverse side effects on birds health and prolonged withdrawal period with residual results (10).

Continuous search for new compounds with antibacterial influences on *chlamydia* and other bacterial species such as *staphylococci* and *lactobacilli* is important (11). Emerging usage of medicinal plants against various diseases is gradually gaining importance. *Azadirachta indica*, known as neem, belongs to the family *Meliaceae* contains various active substances as azadiractin, nimbin, nimbindin, quercetin and others. It has antioxidant, antibacterial, antifungal, antiviral, insecticidal and antiprotozoal properties beside immune-stimulatory actions (10). Therefore the study aspired to assess the therapeutic activity of aqueous extract of neem leaves at different concentrations 4, 6 and 8% against *C. psittaci* infection in chickens.

Material and methods

Experimental birds

Seventy, one day-old Hubbard chicks obtained from EL-Dakahlia poultry Company, were reared in a floor based system at experimental units in Faculty of Veterinary Medicine, Zagazig University. Birds were fed on commercially prepared diet sourced from El-Eman Feed Millers formulated to contain 21% protein and yield 3100 Kcal/kg. Approval of the study was obtained from the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Aqueous neem extract

Leaves were collected from middle aged green trees at agricultural orchards of Faculty of Agriculture, Zagazig University, dried in oven at 37°C for 24 hours and grounded in metallic grinder. Weigh of 40, 60 and 80g of the grind separately soaked, in three nonmetallic jars contain 1 liter of hot boiled distilled water each, for 5-8 hours at room temperature preparing 4%, 6% and 8% neem extract (12).

Oxytetracycline (OTC)

Commercial product contains 200 mg of oxytetracycline HCL (OXYVET-PHARMA) used for treatment of infected chicks with *C. psittaci* in a dose 20 mg/ kg life body weight in drinking water for 5 days (8).

C. psittaci strain

The bacteria, used for challenge was isolated and supplied by Hala (13), prepared in embryonated chicken eggs after Alethea et al. (14) and stored at -80°C. According to Enany et al. (15), briefly 20% suspension of the yolk sac harvest was inoculated into Vero cell, two passages were performed. The SPG buffer (10% fetal calf serum, 10 Ug gentamicin/ml, 100 Ug vancomycin/ml, 2 Ug amphotrecin/ml) was added to the tissue culture flask (1medium:1SPG buffer) undergo subsequent freezing and thawing to release the individual chlamydia from the cells. The harvest was sonicated (1 min) and centrifuged to remove cell fragments (2790 rpm for 10 min). The inoculum was titrated in Vero cell to calculate $TCID_{50}$ with Reed and Muench guidelines (16).

Experimental design

Birds were randomly divided into seven equal groups, 10 birds each, five groups were challenged intranasal with 0.2 ml of 10^6 TCID *C. psittaci* at 7days old (17) while one group received the higher concentration of neem 8% for investigation of possible side effect on liver and kidney function by monitoring (AST, ALT, creatinine, uric acid) and other one left as uninfected not treated control. Four of the infected groups were treated orally with OTC and aqueous neem leaves extract at 4, 6 and 8%

concentration respectively for five successive days started at second day post inoculation.

Samples

Blood and tissues **(**lung – heart - liver) were collected from 2 euthenazed birds from all infected, treated and uninfected not treated control groups at 14 days old, to perform biochemical tests and histopathological examination.

Biochemical tests

Alanine aminotransferase (ALT), aspartate aminotranseferase (AST), albumen, uric acid and creatinine levels in serum were analyzed at Animal Health Research Institute (Zagazig) for detection of liver and kidney function.

Cytological examination

Impression smears from the cut surfaces of the internal organs were fixed in methanol for 5 minutes and dipped in sealed staining jar containing diluted freshly prepared Giemsa stain (1 volume of stock stain + 49 volume of neutral distilled water) and incubated at 37°C overnight. The slides washed by distilled water and alcohol successively then examined microscopically by oil immersion lens for detection of *chlamydia* inclusion bodies (15).

Histopathological evaluation

Collected specimens of lung, liver and heart were fixed in 10% formalin buffered solution, dehydrated in a graded ethanol series, cleared in xylene and finally embedded in paraffin wax. Paraffin sections of 5 µm thickness were stained by hematoxylin and eosin (H&E) and examined microscopically (18).

Performance parameters

Body weight (BW) and feed conversion rate (FCR) for each group were recorded on weekly basis.

Statistical analysis

Data were compiled and analyzed using one-way analysis of variance (ANOVA) with the statistical Package for Social Science version 20.0 (SPSS for windows 20.0 Inc., Chicago, IL, USA) and Duncan multiple range test used to separate means at $P<0.05$ (19).

Results

Clinical and post mortem examination

Signs onset 3 days post infection with depression and anorexia then respiratory signs, rhinitis, sneezing and coughing were developed 10 days post infection with no mortalities. Grossly the scarified birds presented congestion in parenchymatous organs with diffuse thickening of the air sac, fibrinous pericarditis and nephritis. Recorded lesions were milder in birds of treated groups with neem 4%, 6%, 8% and OTC as compared with infected untreated birds.

Performance parameters

Statistical analysis of growth performance results were summarized in two tables. Table (1) clarified that group received neem 8% without infection had significantly ultimate mean BW 177.8±2.70 gm, 1018.60±42.16 gm and 1505.50±59.05 gm than other groups at 8, 24 and 32 days old respectively, whereas the lowest 654.14±26.74 gm and 963.25±33.0 gm noted at 24 and 32 days old respectively, in infected group only. Treated groups with OTC, neem 4%, and 6% exhibited no significant difference in recorded BW 522.5±13.37 gm, 495±9.35 gm and 503.7±12.17 gm, respectively at 16 days old while at 24 days old it was 857.60±25.70 gm, 841.80±14.03 gm and 856.70±17.98 gm in treated groups with OTC, neem 6% and 8%, respectively. At 32 days old control group (1313.73±25.76 gm) had no significant different mean BW than treated groups with neem 8% (1254.60±26.51 gm) whereas significantly different than groups treated with OTC (1198.10±26.19 gm), neem 4% (1130.56±27.19 gm) and neem 6% (1135.70±10.93 gm).

Table 1: Effect of Neem on body weight post *Chlamydia* infection

Age \ group	Control**	8%neem only	Infect+OTC	Infect+4%neem	Infect+6%neem	Infect+8%neem	Infected only
8days	156.8±2.05^{b}	177.8±2.70^{a}	164.6±5.4^{b}	153.9±6.60^{b}	158.7±5.78^{b}	164.6±2.75^{b}	164.4±2.47^{b}
16days	471.9±7.02^{bc}	497.5±5.87^{ab}	522.5±13.37^{a}	495±9.35^{ab}	503.7±12.17^{a}	466.8±6.56^{c}	495.4±10.5^{ab}
24days	806.73±11.87^{bc}	1018.60±42.16^{a}	857.60±25.70^{b}	771.44±16.66^{c}	841.80±14.03^{bc}	856.70±17.98^{b}	654.13±26.74^{d}
32days	1313.73±25.76^{b}	1505.50±59.05^{a}	1198.10±26.19^{cd}	1130.56±27.19^{d}	1135.70±10.93^{d}	1254.60±26.51^{bc}	963.25±33.0^{e}

*Means ± standard error within the same row carrying different superscript were significantly different at P value<0.05, ** Uninfected not treated group

Data shown in Table (2) expressed that groups received neem 8%, control and treated with neem 8% post infection had statistically the same FCR 1.24±0.032^{b}, 1.19±0.025^{b} and 1.16±0.022^{b} respectively, at 16 days old while treated group with neem 6% had significantly higher FCR 1.91±0.077^{a}. Groups infected with *C. psittaci* and treated with OTC, neem 4%, 6% and 8% statistically had parallel FCR 2.04±0.131^{b}, 2.26±0.117^{b}, 1.94±0.102^{bc} and 1.77±0.055^{bc} respectively, at 24 days old, the poorer FCR noted in infected group without treatment 3.80±0.436^{a} while the best recorded in group received neem 8% 1.44±0.091^{c}.

Table 2: Effect of Neem on feed conversion rate post *Chlamydia* infection

Age \ group	Control**	8%neem only	Infect+OTC	Infect+4%neem	Infect+6%neem	Infect+8%neem	Infected only
8days	1.54±0.027^{a}	1.36±0.068^{b}	1.39±0.047^{b}	1.57±0.042^{a}	1.46±0.058 ^{ab}	1.36±0.030^{b}	1.44±0.011^{ab}
16days	1.19±0.025^{b}	1.24±0.032^{b}	1.14±0.032^{bc}	1.05±0.017^{cd}	1.91±0.077^{a}	1.16±0.022^{b}	1.02±0.007^{d}
24days	2.09±0.105^{b}	1.44±0.091^{c}	2.04±0.131^{b}	2.26±0.117^{b}	1.94±0.102^{bc}	1.77±0.055^{bc}	3.80±0.436^{a}
32days	2.09±0.110^{d}	2.47±0.294^{cd}	3.23±0.224^{a}	2.88±0.135^{abc}	3.20±0.108^{a}	2.59±0.090^{bcd}	3.12±0.244^{ab}

*Means ±standard error within the same row carrying different superscript are significantly different at P value<0.05
**Uninfected not treated group

Serum biochemical parameters

Infection with *chlamydia* resulted in optimum levels of ALT, AST, albumin, creatinine and uric acid 68 U/L, 62 U/L, 5.20 mg/dl, 1.62 mg/dl and 5.20 mg/dl respectively, while treated groups with OTC recorded lower levels 22 U/L, 20 U/L, 3.52 mg/dl, 0.52 mg/dl and 2.01 mg/dl respectively, compared with control group 32 U/L, 27 U/L, 3.80 mg/dl, 0.82 mg/dl and 3.40 mg/dl respectively, almost similar to unchallenged chicken which received neem 8% 30 U/L, 26 U/L, 3.20 mg/dl, 0.67 mg/dl and 3.90 mg/dl respectively. Neem treatment efficiently reduced enzyme marker where 4% recorded 20 U/L, 24 U/L, 3.70 mg/dl, 0.40 mg/dl and 3.30 mg/dl respectively, 6% logged 25 U/L, 23 U/L, 3.60 mg/dl, 0.24 mg/dl and 3.20 mg/dl respectively and 8% documented 29 U/L, 23 U/L, 3.40 mg/dl, 0.12 mg/dl and 3.07 mg/dl.

Giemsa stained impression smears

Reflected the presence of elementary bodies within the examined organs mainly lung of challenged birds, which appeared as dense basophilic extracellular bodies (Figure 1A) but not detected in treated groups.

Pathological examination

C. psittaci infection exhibited fibrinous air saculitis and pneumonia with thickened pulmonary septa through intense inflammatory exudate mainly fibrin and numerous leukocytes varied from lymphocyte, plasma cells and heterophiles (Figure 1B and C). Also multiple necrotic areas which margined and infiltrated by numerous leukocytes in the hepatic paren-

chyma (Figure 1D and E) were seen with portal bile duct proliferation or leukocytic aggregations. Intense necrotic myocarditis accompanied with focal hemorrhage and edema was prevalent (Figure 1F).

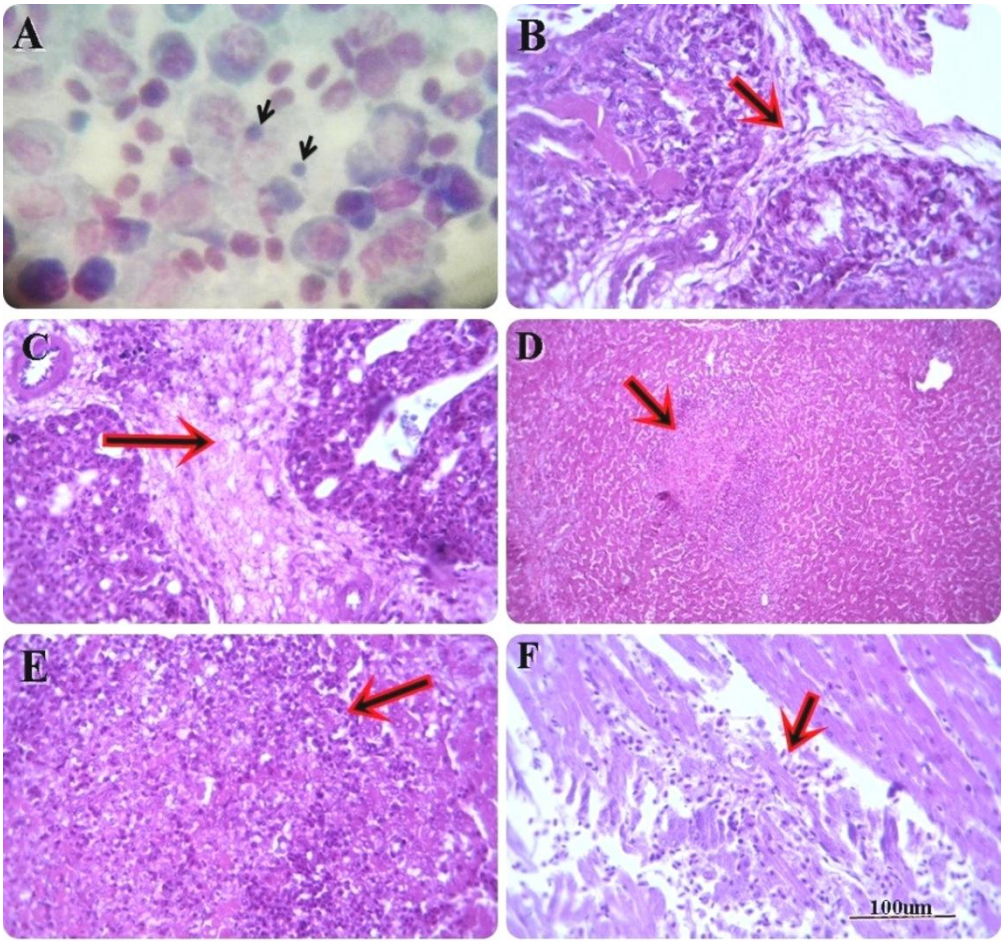

Figure 1: Photomicrograph of infected chickens with *Chlamydia* at 14 days old (A to F). A: Lung impression smear showing elementary bodies stained blue (arrows) Gimesa stain (X1000), B: Fibrinous air saculitis and interstitial pneumonia (arrow) H&E (X100). C: Thickened septa by fibrinous exudate (arrow) H&E (X100). D: Necrotic foci in the hepatic parenchyma (arrow) H&E (X100). E: Lymphocytes, plasma cells and heterophils infiltrate the hepatic necrotic area (arrow) H&E (X100). F: Intense necrotic myocarditis (arrow). H&E (X100)

Treatment with OTC reduced the intensity of the previous lesions in the examined organs, where moderate air saculitis and interstitial pneumonia together with bronchitis and hyperplastic para-bronchial lymphoid aggregation were common (Figure 2A and B). The majority of the hepatic parenchyma was apparently normal meanwhile a few interstitial lymphocytic aggregations could be seen (Figure 2C). Mild myocarditis and edema were encountered (Figure 2D).

Neem 4 or 6 % treatments showed milder lesions of the examined organs meanwhile 8% resulted in complete recovery of lesions

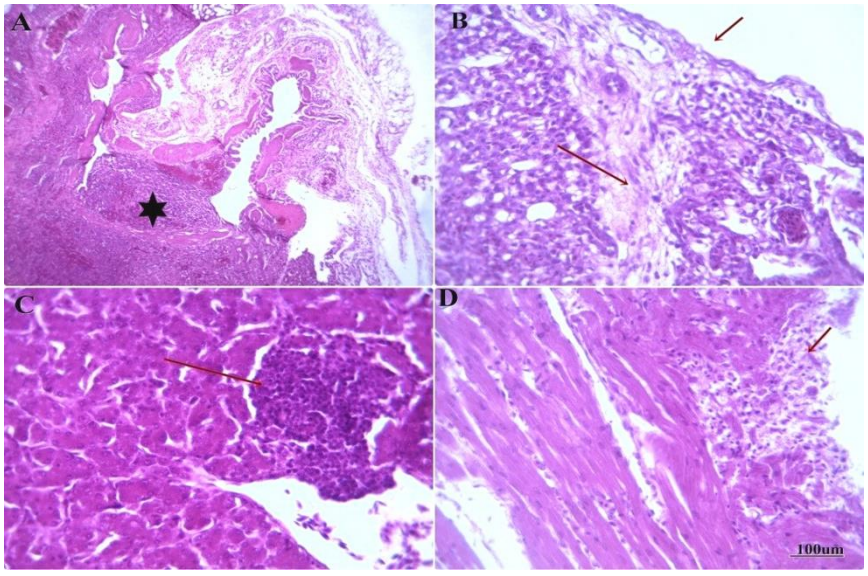

Figure 2: Photomicrograph of challenged chickens with chlamydia and treated with oxytetracycline at 14days old (A to D). A: Bronchitis (star), H&E (X400), B: Moderate air saculitis and interstitial pneumonia (arrow), H&E (X400), C: Focal interstitial lymphocytic aggregation replaced normal hepatic cells (arrow) H&E (X400), D: Focal myocarditis (arrow). H&E (X400)

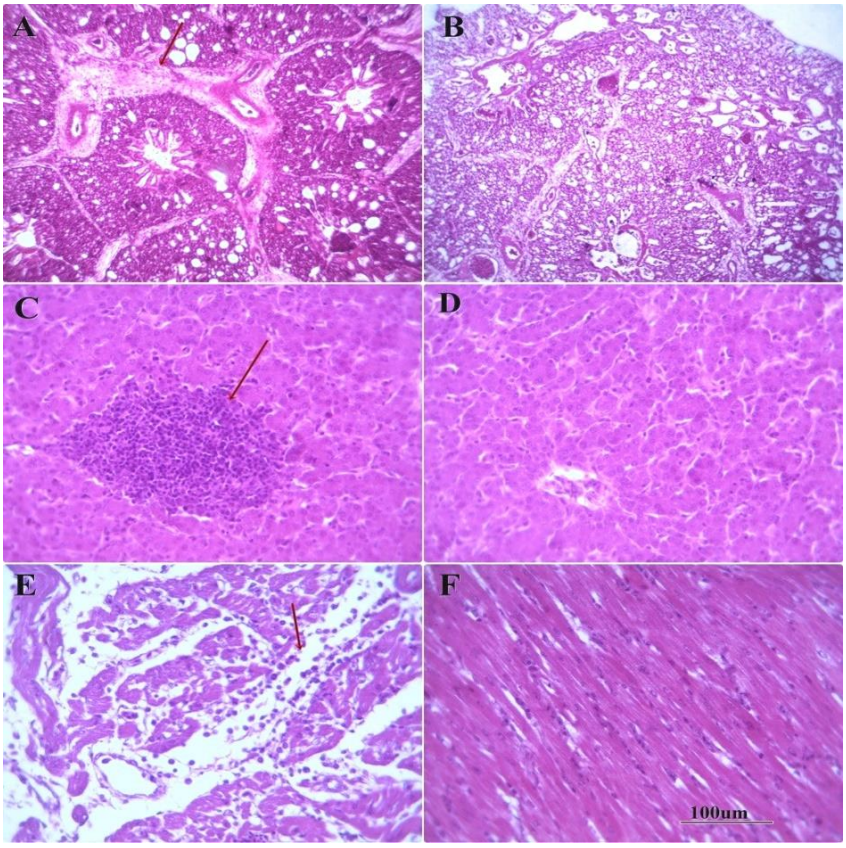

Figure 3: Photomicrograph of treated chickens with different neem concentration post *Chlamydia* challenge at 14days old (A to F), A: Mild interstitial pneumonia (arrow) (6%), H&E (X400). B: Complete recovery of lung tissue with mild congested blood vessels (8%), H&E (X400). C: Focal portal lymphocytic aggregation (arrow) (6%), H&E (X400). D: Apparently hepatic parenchyma with slightly congested hepatic sinusoid (8%). H&E (X400). E: Mild myocarditis and edema (arrow) (6%) H&E (X400). F: Normal myocardium (8%), H&E (X400)

Discussion

Challenged broilers with *C. psittaci* expressed general signs of illness, respiratory symptoms (rhinitis, sneezing, coughing) and greenish diarrhea, meanwhile the recorded gross lesions were septicemia, nephritis and thickening of the air sac which was in accordance with Yin et al, and Zhou et al, (17,20) respectively. Impression smears from liver, lung, heart and spleen that stained with Giemsa elaborate the elementary bodies as explained by Enany et al. (15).

Treatment with aqueous Neem leaves extract showed milder clinical sings and postmortem lesions with no inclusion bodies detection which may be explained by antimicrobial activity of azadirachtin, quercetin and B-sistosterol been in neem leaves with high concentration as reported by Awasthy et al. (21) and Siddiqui et al. (22) who noted that aqueous neem leaves extract suppress many pathogenic bacteria as *staph, salmonella* and *klebsiella*.

Antichlamydial properties of neem was verified where at 16 days old infected groups recorded nearly similar BW after treatment with 6% neem extract and OTC for five successive days furthermore at 24 days old no significant difference of BW for control and treated groups with OTC, neem 6% and neem 8%. At 32 days old, mean BW of the control group were insignificantly different with 8% neem treated group but differently with groups treated with OTC, neem 4% and neem 6%. Parallel to BW, FCR was improved significantly in treated group with OTC and neem extract along the experiment as well as chickens received 8% neem extract without infection. The results were coincided with Chakravarty and Prasad (23) who reported that the highest BW gain and best FCR were achieved in broilers which exposed to neem leave extract when compared with control. The increase of weight gain probably justified by presence of macrominerals as Potassium, Magnesium and phosphorous in addition to microminerals as Iron, copper, Manganese and zinc in *Azadirachta indica* (24), whereas deficiency of these minerals resulted in anorexia and retarded growth (25) or due to diversified effect of Neem therapy on intestinal microflora avoiding stressful conditions (26)

AST also known as serum glutamic oxaloacetic transaminase (SGOT), an enzyme which released in the blood, as a result of injury of certain organs or tissues particularly liver and heart, so its activity varies with age and productive function (27) Hitherto, AST test is more effective than ALT test for detecting liver damage where its level rises faster in hepato-cellular disorders indicating liver damage tumor, kidney or lung damage (28). The presented results showed that the levels of ALT and AST decreased with the higher concentration of aqueous neem leaf extract, proofing no toxic effect of neem within the birds' liver parenchyma and justified by receiving of 8% neem only does not alternate ALT and AST level. It was in agreement with Dkhil et al. (29) who elaborated that hepatoprotective activity of aqueous neem leaves extract lowered ALT, AST. Also serum creatinine and uric acid levels are used mainly as markers of renal function detecting the levels of kidney histological structure damage (30). Over 80% of nitrogen excreated by birds is in the form of uric acid through tubular secretions (31), elevated serum levels of uric acid and creatinine could be considered as indicators of kidney damage (32). Histobiochemical findings highlighted that *C. psittaci* caused high increase of these markers demonstrating severe damage where hyperuricemia associated with injury of tubular endothelial cells. On the other hand, treated groups with neem concentrations showed lower values which indicated by low uric acid and creatinine levels. The hepato-renal protection of neem extract could be due to stabilizing serum level of marker enzymes responsible for this damage or may be due to antioxidant effect of the plant (33). Additionally *Chlamydia* infection revealed fibrinous airsaculitis and pneumonia represented by thickened pulmonary septa with intense inflammatory exudate mainly fibrin and numerous leukocytes varied from lymphocyte, plasma cells and heterophiles clockwise with Martinov and Popov (34) who recorded multiple necrotic areas bordered and infiltrated

by numerous leukocytes allocated in the hepatic parenchyma. Although OTC treatment minimized the intensity of the previous lesions, neem 4 or 6% exhibited milder lesions meanwhile 8% neem resulted in complete recovery of these lesions.

Conclusion

It is concluded that aqueous neem extract has potent herbal anti-bacterial effect against *C. pcittacae* and enhance broiler performance due to posing many minerals that improve digestibility and body weights of broilers. Unlike most of antibiotics it does not affect hepatic or renal tissue; neem could be an efficient substitution of OTC for treatment of *Chlamydia*.

Conflict of interest

The authors declare no conflict of interest.

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