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Antibacterial and anti-biofilm activity of *Eucalyptus* extract in-ovo inoculation on post hatch *Pseudomonas aeruginosa* infection in broiler chickens

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ABSTRACT

Pseudomonas aeruginosa (P. aeruginosa) is considered as an important pathogen that threatens poultry industry resulting in severe economic losses. The ability of this pathogen to form biofilm and resistance to the antimicrobial agents represents a very great danger that produces treatment difficulties. So that, this study was conducted to isolate *P. aeruginosa* from 100 diseased broiler chickens farms in Dakahlia Governorate and studied the in vitro and in vivo antibacterial and anti-biofilm activities of *Eucalyptus* extract on *P. aeruginosa*. *P. aeruginosa* were isolated from 100 diseased broiler chickens farms in Dakahlia Governorate with a percentage of (12%) and it showed higher resistance to amoxicillin/ clavulanic (91.7%), ampicillin/sulbactam (83.3%), doxycycline and erythromycin (75% for each). Eight *P. aeruginosa* isolates were strong biofilm producers (8/12, 66.7%) and 4 isolates (4/12, 33.3%) were moderate. The in vitro antibacterial and anti-biofilm activities of *Eucalyptus* extract on the isolated *P. aeruginosa* showed the higher activities of 50, 75 and 100 mg/ 1ml concentrations than 40 mg/ 1ml. The in vivo studies in experiment (1) showed (100%) hatchability with no embryonic mortalities in the inoculated groups with the *Eucalyptus* extract. In experiment (2), the study showed that inoculating group 5 with a 100 mg/ml concentration of Eucalyptus extract in-ovo had beneficial effects. It prevented deaths, lessened clinical symptoms, enhanced growth performance, and reduced *P. aeruginosa* colonization in internal organs compared to group 3 (inoculated with 50 mg/ml) and group 4 (inoculated with 75 mg/ml).

The expression of *psl*A gene (responsible for biofilm formation) was examined using RT- PCR and the results showed significant down regulation (decrease in the expression or activity of a gene) in the examined liver of chicks in group (5) in comparison with other groups. The obtained results suggested that application of *Eucalyptus* extract in-ovo inoculation is an alternative natural approach to control *P. aeruginosa* characterized by multidrug resistance and biofilm formation in broiler farms.

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INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen infecting human being and animals (**Badr et al. 2016& Farghaly et al. 2017& Yong et al. 2020**). It is a Gram negative, non-capsulated, non-spore forming (**Shukla and Mishra, 2015**), motile rod shaped bacterium and usually produces water soluble green pigment with a fruity smell (fluorescent and pyocyanin) (**Kebede, 2010**).

P. aeruginosa is of zoonotic importance which threatens the poultry industry resulting in economic losses **(El-demerdash, et al. 2020 & Abd El-Ghany, 2021 & Marouf et al. 2023)** due to the higher morbidity and mortality rates in chickens (**El-demerdash, et al. 2020**) in addition to the rapid spreading of infection between poultry flocks causing mortalities in all ages (**Shukla and Mishra, 2015**) and the difficulties in treatment of antibiotic resistant strains (**Abd El-Ghany, 2021**). *P. aeruginosa* is highly virulent in the young chicks (1 - 10 days) and less pathogenic at 11 - 20 days old but the older chickens (>20 days) have been found to resist the infection (**Kebede, 2010**).

The pathogenicity of *P. aeruginosa* may resulted from the existence of virulence regulators and antimicrobial resistance genes (**Farghaly et al. 2017**) and has the ability to infect the host via wound and/or respiratory route (**Kebede, 2010**) and incase of lower immunity (**Samanta et al. 2012**). Poultry litters can also play a role in spreading the antimicrobial resistant *P. aeruginosa* in the environments (**Farghaly et al. 2017**).

P. aeruginosa considered as an important bacterial disease infecting poultry particularly eggs and newly hatched chicks (**Yong et al. 2020**). Infected chicks showed symptoms of enteric and respiratory infections, septicemia and higher mortalities but the infected embryos showed death in the egg shell (**Kebede, 2010 & Abd El-Ghany, 2021**). **Shukla and Mishra, (2015**) explained the symptoms of *P. aeruginosa* infection in chicks at 4 days old in form of drooping wings, ruffled feather and diarrhea. The in vivo experimental infections conducted by **Marouf et al. (2023**) with multidrug resistant *P. aeruginosa* revealed severe pathogenicity in oral and subcutaneous challenged birds with 40% and 100% mortalities respectively.

Biofilms are communities of bacteria that attach to a surface and embed into an extracellular matrix that supports the stability, defense and nutrition of the bacteria (**Wilson et al. 2017**). One of the most common bacteria producing biofilm is *P. aeruginosa* (**Vallet et al. 2001 & Zhang et al. 2013**) which persists in some tissues and forms biofilm structure via a series of cellular interactions and adhesion to surfaces (**Vallet et al. 2001**). The extracellular matrix of biofilm is composed of exopolysaccharides, nucleic acids, proteins and lipid vesicles. The main components of this matrix are three exopolysaccharides (pel, psl and alginate) which protect the bacterial cell from the actions of the antimicrobial agents and immune responses (**AL-Sheikhly et al. 2020**). In *P. aeruginosa* infections, biofilms considered as one of the bacterial virulence factors which give protection against antibiotics and host immunity (**Al-Wrafy et al. 2017 & AL-Sheikhly et al. 2020**). **Overhage et al. (2005)** identified *psl* gene cluster (15 cotranscribed genes) which involved in the biosynthesis of the exopolysaccharide and formation of biofilm in *P. aeruginosa* isolates.

Eucalyptus has become one of the most planted species in the world. *Eucalyptus camaldulensis* (The River Red Gum) is a plantation species in many parts of the world. It has significant antimicrobial properties against many Gram negative and positive bacteria **(Sabo and Knezevic, 2019).** In Egypt, the *Eucalyptus camaldulensis* is a well-known plant tree (**Elhariri et al. 2016**) and considered as a medicinal herb with antimicrobial and antioxidant properties which improves growth performance and health of broiler chickens (**Mashayekhi et al. 2018**). Some researchers such as **Kamel et al. (2011)** & **Bachheti et al. (2011) and Pereira et al.(2014**) explained the antibacterial properties of *Eucalyptus* against *P. aeruginosa* pathogen. The *Eucalyptus* leaves extract considered as a potential alternative approach with significant ability in preventing biofilm formation in *P. aeruginosa* (**Kaur et al. 2018**).

MATERIALS and METHODS

Samples collection and bacteriological identifications

One hundred diseased broiler chickens (aged 25- 37 days) from 10 farms (10 birds/ farm) in Dakahlia Governorate, Egypt were collected in this study. The examined birds were subjected to clinical and postmortem (PM) examinations whereas the observed clinical signs and lesions were recorded. The diseased birds were suffered from ruffled feather, respiratory distress, diarrhea in some cases and mortality (30%). The PM lesions showed septicemia, congested internal organs, perihepatitis, pericarditis, pneumonia and enteritis.

Samples from internal organs such as liver, heart, lungs, spleen and intestine were collected from each bird aseptically conditions to avoid cross contamination then labeled and transported to the Reference laboratory for veterinary quality control on poultry production (RLQP) to complete further examinations.

The collected internal organs from each bird individually were pooled as one sample. The collected samples were inoculated in buffered peptone water (BPW) then incubated at 37°C for 24 hours in aerobic conditions. The inoculated broth was streaked onto Pseudomonas selective agar (**HiMedia**) supplemented with glycerol agar, Trypticase soya agar and MacConkey agar and incubated at 37°C for 24-48 hours. Lactose fermentation and pigment production were checked in the agar plates. The suspected colonies were subjected to Gram staining and biochemical identifications (**Quinn et al. 2002**)

Antimicrobial sensitivity pattern

The antimicrobial sensitivity of isolated *P. aeruginosa* strains were examined using disc diffusion methods against 9 antimicrobial agents (**Oxoid**) belonged to 7 classes that commonly used in broiler farms; Aminoglycosides: streptomycin (10 µg, S), Macrolides: erythromycin (15 µg, E), Tetracyclines: doxycycline (30 µg, DO) and oxytetracycline (30 µg, OT), Amphotericols: florfenicol (30 µg, FF), Polypeptides: colistin sulphate (10 µg, CT), B-Lactam : amoxicillin/clavulanic acid (20/10 µg, AMC), ampicillin/sulbactam (SAM; 20 µg) and quinolones: norfloxacin (10 µg, NOR). The diameters of the obtained inhibition zone diameters were measured and categorized into sensitive or resistant according to Clinical and Laboratory Standards Institute **(CLSI, 2020)**. *P. aeruginosa* isolates that exhibited resistance to one antimicrobial agent in three or more classes were considered as multidrug resistant (MDR). Multidrug resistance index (MARI) was recorded (Number of antimicrobial agent showed resistance / Total number of the tested antimicrobial agents).

Detection of biofilm formation

According to **Christensen et al. (1982)**, qualitative technique (tube method) was used for the detection of biofilm formation in *P. aeruginosa* isolates (n= 12). Loops of *P. aeruginosa* culture were inoculated in sterile tubes containing 10 mL of trypticase soy broth. The inoculated tubes were incubated for 24 hours at 37 °C. The inoculated broth was decanted and the tubes were washed in buffered saline (pH 7.3) and dried. The dried tubes were stained with crystal violet (0.1%) then deionized water was used to remove any remaining stain. The tubes were dried at upside down position. Tube system scoring was performed based on the performance of *P. aeruginosa* control strains (supplied from **Tawakol et al. 2018)** the presence of transparent film on the tubes wall and bottom indicated the biofilm formation. It was rated 1, weak/none; 2, moderate, or 3, high/ strong (experiments were conducted in triplicate).

Antibacterial activity of *Eucalyptus* extract against *P. aeruginosa*

***Eucalyptus* plant extraction:** Fresh leaves of *Eucalyptus camaldulensis* plant was collected from Dakahlia Governorate in September 2023. According to **Ammer et al. (2016),** the leaves were washed under tap water and dried for 2 days at room temperature. The dried leaves were crushed into powder and 50 gram was added in flask and mixed with 200 ml methanol then heated for 1 hour on boiling water bath. The mixture was kept for 5 days at room temperature. The extract was centrifuged for 10 minutes at 5000 rpm and the clear were allowed to evaporate the solvent in hot water

bath to obtain dried methanol free extract. The extract was diluted in phosphate buffered saline (PBS) containing 2% v/v tween 80 (as a suspending agent) for yielding different concentrations of 40, 50, 75 and 100 mg / 1ml PBS (the lowest concentration 40 mg/ ml and highest concentration 100 mg / ml werer selected zccording to the study performed **by kamel et al. (2011)** on the antibacterial activities of these Eucalyptus exact concentration on *p. aeruginosa* isolated from poultry.

Antibacterial assay: The antibacterial activity of *Eucalyptus* extract was evaluated using agar disc diffusion methods against 18 *P. aeruginosa* isolates recorded in this study. Four concentrations of the prepared extract were used: 40, 50, 75 and 100 mg/ 1ml. According to **Ammer et al. (2016),** a loop-full of the tested bacterial culture was inoculated into nutrient broth and incubated for 24 hours at 37 °C. The turbidity of the bacterial suspension was adjusted at a range of $1x10^8$ bacterial cells/ml using McFarland standard and 100 µl of the prepared suspension was spread onto Mueller Hinton agar plates. Filter paper discs (diameter: 6 mm) were soaked with 15 ml of each extract concentration and evenly distributed in the Mueller Hinton agar plates. The agar plates were incubated aerobically for 16-18 hours at 37 °C. After incubation the agar plates were examined for the presence of inhibition zones (mm). The experiments were performed in triplicates**.**

In vitro anti-biofilm activity of *Eucalyptus* extract

As previously mentioned for the detection of biofilm by tube method. The presence of transparent film on the tubes wall and bottom were examined and scored as previously mentioned by **Christensen et al. (1982)**: 1, weak/ none; 2, moderate, or 3, high/ strong (experiments were conducted in triplicate).

In ovo-inoculations of Eucalyptus extract in SPF embryonated chicken eggs

The role of *Eucalyptus* extract in controlling of *P. aeruginosa* post hatching was studied. **Experiment (1) design:** 75 Specific pathogen-free (SPF) embryonated chicken eggs were obtained from a breeder (Ross broiler breeder flock, age 38 weeks) which was free from *P. aeruginosa* and not vaccinated. The eggs were weighed and divided into 5 groups (15 eggs/ group) and the eggs with similar average egg of weights were placed in the same group. All eggs were incubated under standard conditions (37.5 °C and 54% relative humidity) (**De Oliveira et al. 2014 & Nabil et al. 2023**). The eggs were candled and the viability of embryos was checked all over the incubation period. Three effective *Eucalyptus* extract concentrations (50, 75 and 100 mg/ 1ml) which produced antibacterial activities against *P. aeruginosa* were used in the inoculation process. At the 17th day of incubation, the eggs were disinfected outside the incubator with alcohol (70%) and the inoculation process was conducted as follows: control group non inoculated (1) and (2) inoculated in the yolk sac with phosphate buffered saline (PBS), group (3) was inoculated in the yolk sac with a *Eucalyptus* extract (50 mg/ 1ml for each kg of eggs), group (4) inoculated in the yolk sac with a *Eucalyptus* extract 75 mg/ 1ml for each kg of eggs) and group (5) was inoculated in the yolk sac with a *Eucalyptus* extract (100 mg/ 1ml for each kg of eggs). Each egg in groups 3, 4 and 5 was inoculated with 70µl of the extract using pipette attached to a needle (23- ga). After the inoculation process the eggs were sealed with paraffin and returned to the incubator again. The eggs were examined to determine the hatchability at 21th days (number of hatched chicks/ number of the inoculated eggs per group) and embryonic mortalities.

Experiment (2): After hatching at 21th days the effects of the *Eucalyptus* extract inoculation in embryonated chicken eggs were studied in the hatched chicks which challenged with *P. aeruginosa* (selected from the current study). Fifty hatched chicks were selected from the previous groups (10 chicks from each group) (groups kept their previous numbers as previously mentioned). The experiments were performed in animal facility (BSL2 +). The chicks in all groups were supplied with starter ration (protein 23%) and drinking water adlibitum. Groups (2), (3), (4) and (5) challenged by subcutaneous route at the 3rd day of age with 0.1ml the bacterial suspension containing 10^7 CFU/ml multidrug resistant *P. aeruginosa* in brain heart infusion broth (**Bakheet and**

Torra, 2020). Group (1) control negative and group (2) control positive (challenged with *P. aeruginosa*). The experimental chicks were fed with broiler starter and supplied with water ad-libitum. Clinical signs and mortalities were recorded daily and any dead chicks were subjected to post-mortem (PM) examination. At the end of the experiment (10th day of age, 7th day post *P. aeruginosa* challenge) (7th dpc) growth performance parameters; body weight (BW) feed conversion ratio (FCR), feed intake (FI), and body weight gain (BWG) were determined. Chicks were killed by cervical dislocation at the end of the experimental period. Samples from liver, lung, heart and spleen were collected aseptically from each chick and subjected to *P. aeruginosa* isolation and counting (CFU/ gm) as follow; the tissue sample was weighed and homogenized in sterile NaCl solution (0.9%). One ml from the prepared homogenate was transferred into a tube containing 9 ml of sterile BPW and tenfold serial dilution was performed. A total of 0.1 ml from each dilution was plated into Pseudomonas selective agar and incubated for 48 hours at 37° C. The obtained greenish yellow colonies were counted (**ISO, 2004**).

Gene expression assay was used on the collected liver from the experimental chicks to evaluate the activity of the *Eucalyptus* extract. The samples were subjected to RNA extraction from liver tissues using QIAampRNeasy Mini kit (Qiagen, Germany, GmbH): 30 mg of the sample was added to a volume of 600 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml. The samples homogenization was performed by placing the tubes into adaptor sets that were fixed into the clamps of the Qiagen-tissueLyser. The disruption was conducted in 2 minutes high-speed (30 Hz) shaking step. One volume of ethanol (70%) was added to the cleared lysate then the steps were completed according to the Purification of Total RNA from Animal Tissues protocol of the QIAampRNeasy Mini kit (Qiagen, Germany, GmbH). On column DNase digestion was done to remove residual DNA. The used oligonucleotide Primers used were obtained from **Metabion (Germany) (table 1)**.

SYBR green rt-PCR: the primers were used in a 25- µl reaction which contain 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl RevertAid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl from each primer of 20 pmol concentration, 8.25 µl water, and 3 µl RNA template. The reaction was conducted in a Stratagene MX3005P real time PCR instrument.

The results of SYBR green rt-PCR results were analyzed as follow: the CT values and the amplification curves were detected using stratagene MX3005P software. To detect the variation of the gene expression on RNA of the different examined samples, the CT value of each sample was compared with that of the positive control group according to the "ΔΔCt" method recorded by **Yuan et al. 2006** using the following ratio: (2^{-DDct}).

ΔΔCt = ΔCt reference – Δcttarget& Δ*Ct target* = *Ct control – Ct treatment* and Δ*Ct reference* = *Ct control- Ct treatment*

Statistical analysis

Statistical analysis was conducted using SPSS version 29. One-Way ANOVA test was used to detect the significant differences between the experimental groups ($P < 0.05$).

Table 1. SYBR green rt-PCR (primers sequences, target genes and cycling conditions)

Target gene	Primers sequences	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Reference
				Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation	
Pseudomonas 16S rRNA	GACGGGTGAG-TAATGCCTA CAC-TGGTGTTCCTTCCTATA	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	50˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	50˚C 1 min.	94˚C 1 min.	Spilker *et al.*, 2004
*psl*A	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG				60˚C 30 sec.			60˚C 1 min.		Ghadaksaz *et al.*, 2015

RESULTS

P. aeruginosa isolation and identification

Bacteriologically, the characteristic *P. aeruginosa* colonies in this study showed irregular, large, translucent, fruity smell and greenish diffusible pigment on the agar plates. Gram staining revealed a Gram negative rod shaped bacteria. Indole, MR and VP tests were negative however gelatin hydrolysis and citrate utilization produced positive results.

From the bacteriological examinations, 12 *P. aeruginosa* isolates were recovered from 12% of tested 100 diseased broiler chickens collected from farms in Dakahlia Governorate.

Antimicrobial sensitivity pattern

The results in **table, (2)** showed high resistance to amoxicillin/clavulanic (91.7%) followed by ampicillin/sulbactam (83.3%), doxycycline and erythromycin (75% for each). Lower resistances were recorded to oxytetracycline and florfenicol (58.3% for each).

The investigation of the antimicrobial resistant *P. aeruginosa* isolates showed that 11 out of 12 isolates (91.7%) showed multidrug resistant to at least one agent in three or more antimicrobial classes. Twelve antimicrobial agent pattern profiles were recorded in the examined isolates with a multidrug resistant index which ranged from 0.22 to 1 **(table 3**).

Table 2. Antimicrobial sensitivity pattern of *P. aeruginosa* (12 isolates)

	NOR	DO	OT	AMC	SAM	S	E	FFC	CT
Resistant	8 (66.7%)	9 (75%)	7 (58.3%)	11 (91.7%)	10 (83.3%)	8 (66.7%)	9 (75%)	7 (58.3%)	8 (66.7%)
Susceptible	4 (33.3%)	3 (25%)	5 (41.7%)	1 (8.3%)	2 (16.7%)	4 (33.3%)	3 (25%)	5 (41.7%)	4 (33.3%)

Norfloxacin: NOR, doxycycline: DO, oxytetracycline: OT, amoxicillin/clavulanic acid: AMC, ampicillin/ sulbactam: SAM, Streptomycin: S, eyrthromtcin; E, florfenicol: FFC and colistin sulphate: CT.

Table 3. *P. aeruginosa* antimicrobial resistant pattern profiles .

Antimicrobial agent pattern profiles	Antimicrobial agent	NO. of isolates	No. of resistance markers	MDRI
1	DO-AMC-SAM-S-E	1	5	0.56
2	NOR-DO- OT-AMC-SAM-E-FF-CT	1	8	0.89
3	NOR-DO- OT-AMC-SAM-FF-CT	1	7	0.78
4	NOR-DO-AMC-S-E-FF-CT	1	7	0.78
5	NOR-DO-AMXC-S-E-FF	1	6	0.67
6	NOR-DO-AMC-SAM-S-E-FF	1	7	0.78
7	OT-AMC-SAM-S-E-CT	1	6	0.67
8	SAM-S	1	2	0.22
9	NOR-DO- OT-AMC-SAM-S-E-FF-CT	1	9	1
10	OT-AMC-SAM-E-CT	1	5	0.56
11	NOR-DO-OT-AMC-SAM-S-E-CT	1	8	0.89
12	NOR-DO-OT-AMC-SAM-FF-CT	1	7	0.78

Biofilm formation

The obtained results showed that all isolates formed biofilm: 8 isolates (8/12, 66.7%) were strong producers, 4 isolates (4/12, 33.3%) were moderate.

Antibacterial activity of *Eucalyptus* extract against *P. aeruginosa*

The concentrations of 40 mg/ 1ml had antibacterial activity against 4 out of 12 examined isolates. However, the concentrations 50, 75 and 100 mg/ 1ml showed antibacterial activity against all *P. aeruginosa* isolates. The mean diameter of the inhibition zones were 3±4.5mm, 8.8±0.72 mm, 12.4±0.8 mm and 14.2 ± 0.72mm for the concentrations 40, 50, 75 and 100 mg/ 1ml respectively .

In vitro anti-biofilm activity of *Eucalyptus* extract

The concentration of 40 mg/ 1ml, had the ability to inhibit the biofilm formation in 4 *P. aeruginosa* isolates while concentrations 50, 75 and 100 mg/ 1ml showed anti-biofilm activity in all isolates.

In ovo-inoculations of *Eucalyptus* extract in SPF embryonated chicken eggs

Effects of *Eucalyptus* extract in-ovo inoculation on the embryonic mortalities and egg hatchability:

On the first day of hatching, the hatchability was calculated and the results showed that the hatchability was (93.3%), (100%), (100%), (100%) and (100%) in groups (1), (2), (3), (4) and (5) respectively. The embryonic mortalities were recorded in group 1 (non- inoculated groups). The examination of dead embryo revealed the presence of sticky embryo to the eggs shell. The other groups showed no embryonic mortalities.

Evaluation of *Eucalyptus* extract in-ovo inoculation on post hatch *P. aeruginosa* infection in experimental chicks.

The health of the experimental chicks was monitored daily until the end of the 10-day experiment, which included a challenge with

Pseudomonas aeruginosa on the 7th day. The negative control group showed no clinical signs. Chicks in (positive control) displayed signs of depression, ruffled feathers, respiratory distress, and diarrhea starting from the 2nd day post-challenge (dpc). Groups 3 and 4 exhibited mild clinical signs such as depression, ruffled feathers, and respiratory distress starting from the 3rd dpc and lasting throughout the experiment. In Group 5, slight signs of depression and respiratory distress appeared at 3 dpc, gradually disappearing thereafter. By the 6th and 7th dpc, chicks in Group 5 regained their vitality. No deaths were recorded in Groups 1, 3, 4, and 5. However, in Group 2, 50% mortality occurred within 4 dpc. Postmortem examinations revealed pneumonia, septicemia, unabsorbed yolk sac, and congested internal organs in Group 2 (positive control). Groups 3 and 4 also showed pneumonia and congested internal organs during postmortem examinations. Group 5 exhibited slight congestion in the lungs and liver of some chicks. No postmortem lesions were observed in Group 1. figure 1 .

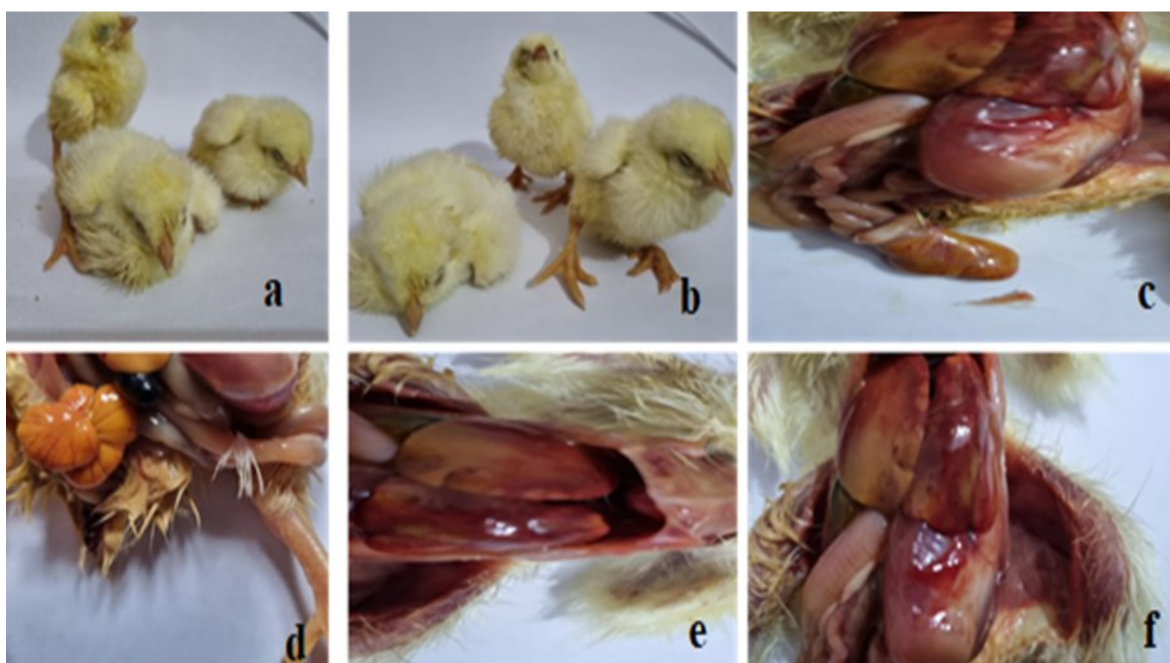

Figure (1) Clinical signs and PM lesions of the experimental chicks , Figure 1a & 1b show images of chicks displaying clinical signs such as depression, ruffled feathers, and respiratory distress. Figure c, septicemia Figure d , unabsorbed yolk sac, Figure e and f exhibited slight congestion in the lungs and liver of some chicks .

Growth performance parameters such as body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were calculated at the end of experiment at (7th dpc) (**table, 4**). Significant improvement of the growth performance parameters were recorded in group (5) when compared with other groups. No significant difference was recorded between groups (1) and (4). The parameters were significant higher in group (4) and (5) than group (3). Lower performance parameters were recorded in the positive control (group, 2) when compared with other groups.

Table (4) mean values of body weight (BW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR) of the experimental chicks

Parameters Groups No.	Body weight (BW) (g)	body weight gain (BWG) (g)	feed intake (FI) (g)	feed conversion ratio (FCR)
Group 1	291.5±0.67^{b}	250.8±0.74^{b}	286.1±0.77^{ab}	1.14±0.005^{c}
Group 2	175.1±6.1^{d}	134.5±6.13^{d}	181.8±8.9^{a}	1.36±0.045^{a}
Group 3	271±0.3^{c}	230.4±0.31^{c}	294.9±1.03^{a}	1.28±0.005^{b}
Group 4	285.7±0.33^{b}	244.9±0.41^{b}	288.4±0.4^{ab}	1.18±0.002^{c}
Group 5	306.5±0.37^{a}	265.2±0.68^{a}	281.6±0.4^{b}	1.06±0.002^{d}

* Mean values mean ± SEM (mean± standard error). The Mean values expressed by different letters (a, b, c and d) showed the significant differences between the experimental groups at $P < 0.05$.

After the PM examination, the isolation of *P. aeruginosa* from the collected internal organs (liver, lung, heart and spleen, and intestine) showed on microbiological examination a Gram staining rod shaped bacteria with irregular, large, translucent colonies in addition to fruity smell and greenish diffusible pigment on the agar plates. Indole, MR and VP tests were negative however gelatin hydrolysis and citrate utilization produced positive results. No *P. aeruginosa* was detected in negative control group (1). The mean CFU of *P. aeruginosa* ($\log_{10}$ CFU/g) was detected in the liver, lung, spleen and heart samples and the results showed that *P. aeruginosa* was localized in the examined internal organs in group (2) (positive control) with 6.95, 6.6, 5.9 and 5.3 $\log_{10}$ CFU/g respectively. Lower colonization of *P. aeruginosa* was detected in group (4) than group (3). In group (5) the recorded colonization in the examined internal organs was lower than group (3) and (4). Group (5) showed colonization in liver, lung, spleen and heart samples with 1.3, 1.2, 1 and 1 $\log_{10}$ CFU/g respectively (**table, 5**).

Table 5. Mean of colonization ($\log_{10}$ CFU/g) *P. aeruginosa* in the internal organs of experimental chicks

Groups	Group 1	Group 2	Group 3	Group 4	Group 5
Liver	0	6.95	3.9	2.85	1.3
Lung	0	6.6	3.6	2.3	1.2
Spleen	0	5.9	2.78	1.9	1
Heart	0	5.3	2.95	1.48	1

The effects of *Eucalyptus* extract in-ovo inoculation on *psl*A gene expression which responsible for biofilm formation in the post hatch *P. aeruginosa* infection was examined by quantitative RT-PCR (**figure, 2**). The mean values of the fold change expression were compared to Group (2) whereas the transcription of *psl*A gene was significantly down regulated in the in-ovo inoculated groups (3), (4) and (5). However the gene transcription was significantly down regulated in group (5) when compared with group (3) and (4) (p<0.05) (**table, 6).**

Table 6. Mean fold change of *psl*A gene expression

Group no.	Group 2	Group 3	Group 4	Group 5
Mean fold change	1±0.0[a]	0.76±0.01[b]	0.64±0.02[c]	0.12±0.003[d]

* Mean values expressed by mean± standard error. The Mean values expressed by different letters (a, b, c and d) showed the significant differences between the experimental groups at $P< 0.05$.

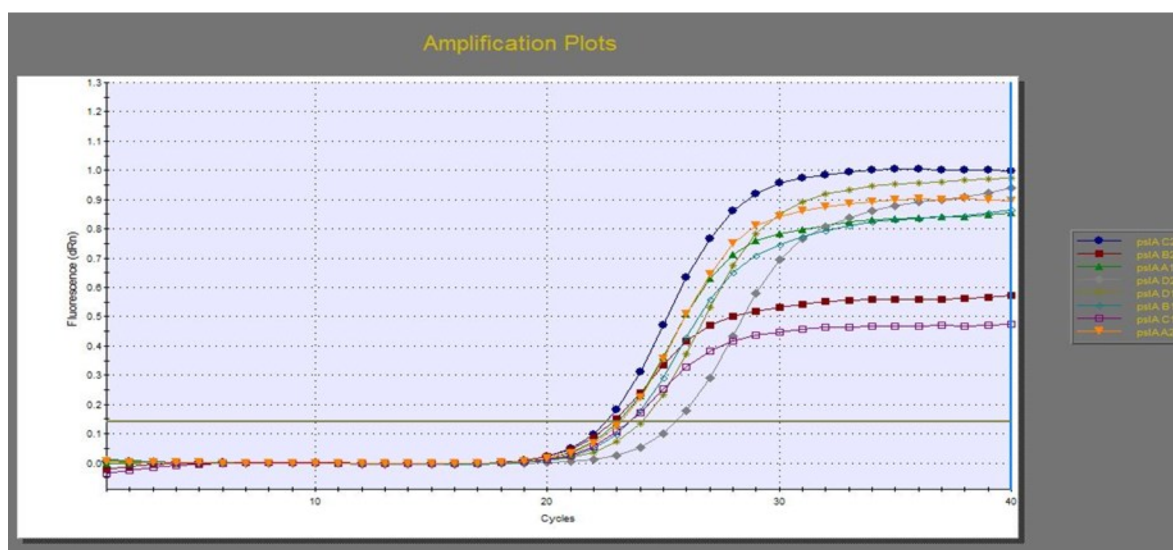

Figure 2. amplification plots of *psl*A gene expression using quantitative RT-PCR technique

DISCUSSION

P. aeruginosa is an opportunistic pathogen that causes serious problems in the chicken farms. It is one of the most important bacterial pathogens producing opportunistic infections in human. Poultry has been considered as a reservoir for antibiotic resistant bacteria, which may exacerbate the problem of pseudomonas infection (**Badr et al. 2020**). In the current study, *P. aeruginosa* was isolated from 100 diseased chickens collected from broiler farms in Dakahlia Governorate. The obtained results were nearly similar to **Radwan et al. (2018)** who isolated *P. aeruginosa* with a percentage of 10.4% from broiler chickens. Another research studies conducted by **Badr et al. (2016) and Farghaly et al. (2017)** identified lower *P. aeruginosa* incidence from chickens than recorded in the current study with percentages of 6.5% and 8.75% respectively. However higher incidences were reported by **Hassan et al. (2020)**, **El-demerdash et al. (2020), Badr et al. (2020)** and **Marouf et al. (2023)** with percentages of 18%, 20%, 39.78% and 48.1% respectively.

The antimicrobial sensitivity of the isolated *P. aeruginosa* in this study showed higher resistances to amoxicillin/clavulanic, ampicillin/sulbactam, doxycycline and erythromycin with percentages (91.7%), (83.3%), (75%) and (75%) respectively. Meanwhile, low resistance was recorded with percentage of (58.3%) for oxytetracycline and florfenicol. The isolated *P. aeruginosa* exhibited multidrug resistance in 11 out of 12 isolates (91.7%) in addition to 12 antimicrobial agent pattern profiles. Our findings nearly agreed with **El-demerdash et al. (2020)** who reported resistance of *P. aeruginosa* to amoxicillin/clavulanic acid and ampicillin. Another study conducted by **Radwan et al. (2018)** recorded resistance to amoxicillin, ampicillin, amoxicillin–clavulanic acid, doxycycline and florfenicol with percentages of

(80%), (100%), (100%), (96%), (84%) and (80%) respectively. In contrast to our study, **Ramatla et al. (2024)** recorded resistance of *P. aeruginosa* isolated from broiler chicken to ampicillin (26%) and amoxicillin-clavulanic acid (4%) with (26%) and (4%) respectively and **Abd El- Tawab et al. (2014)** who reported complete resistance (100%) to streptomycin, florphenicol, and doxycycline. **Salem et al. (2024)** found complete resistance of *P. aeruginosa* to streptomycin, amoxicillin, doxycycline and erythromycin.

The biofilm formation in the isolated *P. aeruginosa* was studied using tube method and all isolates exhibited ability to produce biofilm. The obtained results recorded that 8 isolates (8/12, 66.7%) were strong producers for biofilm and the remaining 4 isolates (4/12, 33.3%) were moderate producers. These findings were in similarity with the previous study performed by **Tawakol et al. (2018).** However **Abdelraheem et al. (2020)** recorded strong biofilm, moderate and weak biofilm formation in *P. aeruginosa* isolates with percentages of (14%), (7%) and (6%) respectively.

Concerning the obtained results of the in vitro antibacterial and anti-biofilm activities of different *Eucalyptus* extract (40, 50, 75 and 100 mg/ 1ml) on 12 *P. aeruginosa* isolated in this study, it was found that the concentration of 40 mg/ 1ml had antibacterial and anti-biofilm activity against 4 out of 12 examined isolates. However, the concentrations 50, 75 and 100 mg/ 1ml showed antibacterial and anti -biofilm activities against all of the *P. aeruginosa* isolates. The mean values of the inhibition zones diameters (mean with standard deviation) were 3±4.5 mm, 8.8±0.72 mm, 12.4±0.8 mm and 14.2±0.72 mm for the concentrations 40, 50, 75 and 100 mg/ 1ml respectively. These findings were supported by **Kaur et al. (2018) and Al-taai et al. (2022)** who recorded that *Eucalyptus* extract prevented biofilm formation in *P. aeruginosa* pathogen. Our results were nearly coordinated with **Kamel et al. (2011)** who recorded higher antibacterial effects of *Eucalyptus globules* at a concentration of 100 mg/ 1ml against *P. aeruginosa* isolated from poultry. The results in this study was supported by **Sabo and Knezevic, (2019)** who mentioned that *Eucalyptus camaldulensis* extract and essential oils showed activity against Gram negative and Gram positive bacteria. The *Eucalyptus camaldulensis* extracts and essential oil had the most antibacterial activity in comparison with other species of the genus *Eucalyptus.* **Bachheti et al. (2011)** reported significant antibacterial effects of essential oil extracted from different *Eucalyptus* species against *Pseudomonas, Streptococcus, Staphylococcus aureus, E.coli* and *Lactobacillus* isolates. Another study conducted by **Mota et al. (2015)** mentioned that *Eucalyptus globulus* oil exhibited antimicrobial activity against different pathogens.

With regard to the in ovo-inoculations of *Eucalyptus* extract in SPF embryonated chicken eggs (experiment 1), the effects of the extract on the embryonic mortalities and egg hatchability recorded that the inoculated groups (3), (4) and (5) showed (100%) hatchability and no embryonic mortalities. These findings reflected the safety of *Eucalyptus* extract on the embryonated chicken eggs with any negative effects on hatchability. **Fathi et al. (2020)** reported that the supplementing of quail diet with 0.1 *Eucalyptus* leaves powder as a feed additive enhanced the eggshell quality, immunocompetence and help in the reduction of broken Japanese quails eggs.

The evaluation of *Eucalyptus* extract in-ovo inoculation on post hatch *P. aeruginosa* infection in experimental chicks (experiment 2) showed no clinical signs in group 1 (negative control). Depression, ruffled feather, respiratory distress, diarrhea, (50%) mortalities, pneumonia, septicemia and congested internal organs were recorded in group (2, positive control). Some research studies identified clinical signs, gross lesion and mortalities resulted from experimental infection of chicks with *P. aeruginosa* such **Badr et al. (2016)** who indicated that the subcutaneous injection of *P. aeruginosa* in 3 days old chicks produced (100%) mortalities within 24-72 hours after injection and **Shukla and Mishra, (2015)** who reported 100% mortalities after intramuscularly and 30% after swapping of the palatine cleft with *P. aeruginosa.* The PM lesions showed congested liver, distended gall bladder, congested

internal organs (heart, lungs and kidneys) and enlarged yolk sac. **Bakheet and Torra, (2020)** recorded 70% mortalities after *P. aeruginosa* subcutaneous infection within 3 dpc and the unabsorbed yolk sac, congested heart and lung and pale liver with petechial foci.

It was observed from this study that the *Eucalyptus* extract prevented mortalities and helps the reduction of clinical signs. In the inoculated groups (3) (received 50 mg/ml) and group (4) which received 75 mg/ml, mild clinical signs (depression, ruffled feather and respiratory distress with pneumonia and congested internal organs) were recorded. However in group (5) which received the higher concentration (100mg/ml) showed slight congestion in lung and liver of some chicks and slight clinical signs (depression and respiratory distress) which disappeared gradually and the chicks regain their viability at the 6dpc and 7dpc. The obtained findings may be attributed to the findings of **Mashayekhi et al. (2018**) who stated the positive effects of the extract as antimicrobial and antioxidant in addition to improving the bird's immunity, **Kamel et al. (2011)** who reported the antibacterial effect of *Eucalyptus* against *P. aeruginosa* and **Mustafa, (2019)** who found that the dietary supplementation with *Eucalyptus* powder decreased the mortalities in broiler chicken**.**

From **table (4),** the growth performance parameters that recorded at the end of experiment at (7th dpc) showed significant improvement in group (5) when compared with other groups. No significant difference was recorded between group 1 (negative control) and 4. The parameters were significant higher in group (4) and (5) than group (3). Lower performance parameters were recorded in the positive control (group, 2) when compared with other groups. The group (5) which received the higher *Eucalyptus* extract concentration (100 mg/ 1ml) showed higher improvement than group (4) which received 75 mg/ 1ml and group (3) which received 50 mg/ 1ml and these findings may be attributed to the using of higher concentration increase the absorbance of feed nutrients in the gastrointestinal tract. These findings were supported by **Mustafa, (2019)** who found that the supplementation of broiler with *Eucalyptus* improved relative length of the small intestine and duodenum (increasing number of goblet cells, crypt depth, villus height, improved lipase, amylase, chymotrypsin and trypsin, count of *Lactobacillus*). A researchers study performed by **Mashayekhi et al., (2018)** mentioned the effectiveness of *Eucalyptus* powder when added with a concentration of 0.5% in broiler diets as an alternative for antibiotics and improvement of growth performance. **Mustafa, (2019)** recorded significant higher body weight and body weight gain in case of the supplementation of broiler diet with *Eucalyptus* powder. On the other side **Farhadi et al. (2017)** reported different results about the dietary supplementation of *Eucalyptus* leaf powder in decreasing body weight gain during 7-28 days of age.

The mean CFU of *P. aeruginosa* (log_{10} CFU/g) was detected in the liver, lung, spleen and heart samples and the results showed that *P. aeruginosa* was localized in the examined internal organs in group (2) (positive control) with 6.95, 6.6, 5.9 and 5.3 CFU/g respectively. Lower colonization of *P. aeruginosa* was detected in group (4) than group (3). Lower colonization of *P. aeruginosa* was detected in group (4) than group (3). Group (5) showed lower colonization of *P. aeruginosa* in liver, lung, spleen and heart samples with 1.3, 1.2, 1 and 1 log_{10} CFU/g respectively (**table, 5**). For chicks in group (5) *Eucalyptus* had the ability to reduce the colonization of *P. aeruginosa* than the other inoculated groups and this may explain the positive effect of the higher concentration of the extract (100mg/ml) in increasing the birds immunity, gut health and performance. These findings supported by **Mashayekhi et al. (2018**) and **Farhadi et al. (2017)** who reported the ability of *Eucalyptus* in improving immunity in broiler chickens. Some researchers such as **Kamel et al. (2011)** & **Bachheti et al. (2011) and Pereira et al. (2014**) reported the antibacterial activities of *Eucalyptus* against *P. aeruginosa* pathogen. The uses of *Eucalyptus* oil via spray or drinking water in broiler chicken can potentially help in controlling respiratory pathogens (**Petrolli et al. 2019).** The supplementation of *Eucalyptus globulus* extract in poultry feed showed antibacterial activity and help the re-

duction of *Escherichia coli*, *Salmonella* Pullorum and *C. perfringens* Type A counts in experimental birds (**Ullah et al. 2021**).

P. aeruginosa is one of the most common bacteria producing biofilm (**Vallet et al. 2001 & Zhang et al. 2013, Tawakol et al. 2018 and Abdelraheem et al. 2020**). It has the ability to persist in some tissues producing biofilm structure via a series of cellular interactions and adhesion to surfaces (**Vallet et al. 2001**). psl exopolysaccharide is one the main components of biofilm matrix which help the protection of bacteria from the effects of the antimicrobial agents and immune responses (**AL-Sheikhly et al. 2020**). **Overhage et al. (2005)** suggested the important role of *psl*A in the biofilm differentiation. For this reason, the inhibition of biofilm formation in *P. aeruginosa* is an important issue to control its adverse effects such as the protection of bacterial cell from the antimicrobial agents and immune responses. So that the effects of *Eucalyptus* extract in-ovo inoculation to control *psl*A gene expression was examined using quantitative RT-PCR technique. The obtained results showed lower mean values of the fold change expression in group (3), (4) and (5) in comparison to positive control group (2). However the mean fold change was down regulated in group (5) than group (3) and (5). These findings reflected the significant down regulation of *psl*A gene expression in group (5) which in-ovo inoculated with the higher *Eucalyptus* extract concentration (100mg/ml) in this study. The obtained results in this study showed the positive effects of *Eucalyptus* extract in-ovo inoculation as an alternative natural anti-biofilm substance.

CONCLUSION

It was concluded from this study that *Eucalyptus* extract showed in vitro antibacterial and anti-biofilm activities against *P. aeruginosa*. The *Eucalyptus* extract in-ovo inoculation with concentration of 100 mg/ml in embryonated chicken eggs showed no adverse effect on hatchability in addition to its ability to prevent mortalities, reduce clinical signs, improve growth performance parameters and reduce the colonization of *P. aeruginosa* in the internal organs of post hatched chicks. Furthermore the expression of *psl*A gene (responsible for biofilm formation) was significantly down regulated. These findings suggested the application of *Eucalyptus* extract in-ovo inoculation as an alternative natural approach to control *P. aeruginosa* in broiler farms. Further studies should be applied to determine the immune response and pathological changes in the experimental chicks.

REFERENCES

Abd El- Tawab AA, El-Hofy FI, Khater DF, Al-Adl, MM. 2014. PCR detection and gene sequence of *Pseudomonas aeruginosa* isolated from broiler chickens. Benha Veterinary Medical Journal, 27 (2):449-455.

Abd El-Ghany WA. 2021. *Pseudomonas aeruginosa* infection of avian origin: Zoonosis and one health implications, Veterinary World, 14(8): 2155-2159

Abdelraheem WM, Abdelkader AE, Mohamed ES, Mohammed MS .2020. Detection of biofilm formation and assessment of biofilm genes expression in different *Pseudomonas aeruginosa* clinical isolates. Meta Gene 23 (2020) 100646.

AL-Sheikhly MAH, Musleh LN, Al Mathkhury HJF. 2020. Gene Expression of *pel*A and *psl*A in *Pseudomonas aeruginosa* under Gentamicin Stress. Iraqi Journal of Science, 61 (2): 295-305.

Al-taai NA, Al-Gburi NM, Khalil NK. 2022. Antibacterial and Anti Biofilm activity of *Eucalyptus* Plant Extract Spp. REDVET - Revista electrónica de Veterinaria, 23 (3): 130- 148.

Al-Wrafy F, Brzozowska E, Górska S, Gamian A. 2017. Pathogenic factors of *Pseudomonas aeruginosa* – the role of biofilm in pathogenicity and as a target for phage therapy. Postepy Hig Med Dosw (online), 2017; (71): 78-91

Ammer MR, Zaman S, Khalid M, Bilal M, Erum S, Huang D, Che S. 2016. Optimization of antibacterial activity of *Eucalyptus tereticornis* leaf extracts against *Escherichia coli* through response surface methodology. Journal of Radiation Research and Applied Sciences 9 (2016) :376- 385.

Bachheti RK, Joshi A, Singh A. 2011. Oil Content variation and Antimicrobial activity of *Eucalyptus* leaves oils of three different Species of Dehradun, Uttarakhand, India. Int.J. ChemTech Res. 2011,3 (2): 625-628.

Badr H, Roshdy H, Abd El-Hafez AS, Farghaly E. 2016. Prevalence, pathogenicity and antibiogram sensitivity of *Pseudomonas aeruginosa* isolated from diseased chickens. Assiut Vet. Med. J. (62): 119-126.

Badr JM, El Saidy FR, Abdelfattah AA. 2020. Emergence of Multi-Drug Resistant *Pseudomonas aeruginosa* in Broiler Chicks. International Journal of Microbiology and Biotechnology, 5 (2): 41-47.

Bakheet AA, Torra DE. 2020. Detection of *Pseudomonas aeruginosa* in Dead Chicken Embryo with Reference to Pathological Changes and Virulence Genes. AJVS. Vol. 65 (1): 81-89.

Christensen GD, Simpson WA, Bisno AL, Beachey EH. 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infection and Immunity, 37(1), 318-326.

CLSI. 2020. Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

De Oliveira JE, van der Hoeven-Hangoor E, van de Linde IB, Montijn RC, van der Vossen J M B M. 2014. In ovo inoculation of chicken embryos with probiotic bacteria and its effect on post hatch Salmonella susceptibility. Poult. Sci. (93): 818–829 (2014).

El-demerdash GO, EL-shafei AA, Mahmoud AEM, Roshdy, H. 2020. Potential risk of *pseudomonas* infection in broiler chickens with detection of the antibiotic resistant genes. Egypt Poult. Sci. Vol. (40) (IV): 783-792.

Elhariri M, Hamza D, Elhelw R, Refai M. 2016. *Eucalyptus* Tree: A Potential Source of *Cryptococcus neoformans* in Egyptian Environment. International Journal of Microbiology, 4080725, 1- 5.

Farghaly EM, Roshdy H, Bakheet AA, Abd El-Hafez AS, Badr H. 2017. Advanced studies on *Pseudomonas aeruginosa* infection in chicken. Animal Health Research Journal, 5, 4 (A): 207-217.

Farhadi D, Karimi A, Sadeghi G, Sheikhahmadi A, Habibian M, Raei A, Sobhani K. 2017. Effects of using *eucalyptus* (*Eucalyptus globulus L.*) leaf powder and its essential oil on growth performance and immune response of broiler chickens. Iranian Journal of Veterinary Research, vol 18, No. 1, Ser. No. 58, Pages 60-62.

Fathi M M, Al-Homidan I, Ebeid TA, Abou-Emera OK, Mostafa MM. 2020 Dietary supplementation of *Eucalyptus* leaves enhances eggshell quality and immune response in two varieties of Japanese quails under tropical condition. Poultry Science (99):879–885.

Ghadaksaz A, Fooladi AAA, Hosseini HH, Amin M. 2015. The prevalence of some *Pseudomonas* virulence genes related to biofilm formation and alginate production among clinical isolates. Journal of Applied Biomedicine, 13 (1): 61-68.

Hassan WH, Ibrahim AMK, Shany SAS, Salam HSH. 2020. Virulence and resistance determinants in *Pseudomonas aeruginosa* isolated from pericarditis in diseased broiler chickens in Egypt. J Adv Vet Anim Res 2020; 7(3):452–463.

International Standards Organization (ISO). 2004. Microbiology of food and animal feeding stuffs. Horizontal method for detection and enumeration of *Enterobacteriaceae*, Part 2: colony count method. International Standards Organization, Geneva.

Kamel GM, Ezz eldeen NA, El-Mishad MY, Ezzat RF. 2011. Susceptibility Pattern of *Pseudomonas aeruginosa* Against Antimicrobial Agents and Some Plant Extracts with Focus on its Prevalence in Different Sources. Global Veterinaria 6 (1): 61-72, 2011.

Kaur S, Sharma N, Aanchal, Ghambir A, Shar-

ma A, Sharma A, Sharma V. 2018. Antibiofilm potential of aqueous *Eucalyptus* leaf extract against nosocomial pathogens: *Staphylococcus* and *Pseudomonas aeruginosa*. The Pharma Innovation Journal 2018; 7(11): 425-432.

Kebede F. 2010. *Pseudomonas* infection in chickens. Journal of Veterinary Medicine and Animal Health Vol. 2(4): 55-58.

Marouf S, Li X, Salem HM, Ahmed ZS, Nader SM, Shaalan M, Awad FH, Zhou H, Cheang T. 2023. Molecular detection of multidrug-resistant Pseudomonas aeruginosa of different avian sources with pathogenicity testing and in vitro evaluation of antibacterial efficacy of silver nanoparticles against multidrug-resistant *P. aeruginosa*. Poultry Science 102:102995.

Mashayekhi H, Mazhari M, Esmaeilipour O. 2018. *Eucalyptus* leaves powder, antibiotic and probiotic addition to broiler diets: effect on growth performance, immune response, blood components and carcass traits. Animal, 12:(10):2049–2055.

Mota V, Turrini RNT, Poveda V. 2015. Antimicrobial activity of *Eucalyptus globulus* oil, xylitol and papain: a pilot study. Rev Esc Enferm USP · 2015; 49(2): 215-219.

Mustafa MA. 2019. Effect of *Eucalyptus* leaves and its supplementation with diet on broiler performance, microbial and physiological statues to alleviate cold stress. Iraqi Journal of Agricultural Sciences –1029:50(1):953-963.

Nabil NM, Tawakol MM, Samir A, Hassan HM, Yonis AE, Reda RM, Elsayed MM. 2023. Synergistic infuence of probiotic and forfenicol on embryonic viability, performance, and multidrug-resistant *Salmonella* Enteritidis in broiler chickens. Scientific Reports, (13):9644.

Overhage J, Schemionek M, Webb J S, Rehm B H A. 2005. Expression of the psl Operon in *Pseudomonas aeruginosa* PAO1 Biofilms: PslA Performs an Essential Function in Biofilm Formation. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 71 (8): 4407–4413.

Pereira V, Diasb C, Vasconcelos MC, Rosa, E, Saavedra M J. 2014. Antibacterial activity and synergistic effects between *Eucalyptus globulus* leaf residues (essential oils and extracts) and antibiotics against several isolates of respiratory tract infections (*Pseudomonas aeruginosa*). Industrial Crops and Products 52 (2014) 1– 7.

Petrolli TG, Sutille MA, Petrolli O J, Stefani L M, Simionatto AT, Tavernari FC, Zotti CA, Girardini LK. 2019. *Eucalyptus* oil to mitigate heat stress in broilers. Revista Brasileira de Zootecnia 48:e20160306.

Quinn PJ, Markey BK, Carter ME, Donnelly WJ, Leonard FC. 2002. Veterinary Microbiology and Microbial Disease. (1st ed., pp: 43-122). Cornwall, Great Britain. Blackwell Science Ltd.

Radwan IA, Shehata AAE, Abd Elwahab SH. 2018. Phenotypic and Genotypic Characterization of *Pseudomonas aeruginosa* Recovered from Kidney Lesions of Broiler Chickens. Assiut Vet. Med. J., 64 (156) 2018, 110-116.

Ramatla T, Mokgokong P, Lekota K, Thekisoe O. 2024. Antimicrobial resistance profiles of *Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumoniae* strains isolated from broiler chickens. Food Microbiology 120 (2024) 104476.

Sabo VA, Knezevic P. 2019. Antimicrobial activity of *Eucalyptus camaldulensis* Dehn. plant extracts and essential oils: A review. Ind Crops Prod, (132):413-429.

Salem M, Younis G, Sadat A, Nouh NAT, Binjawhar DN, AbdelDaim MM, Elbadawy M, Awad, A. 2024. Dissemination of mcr1 and β-lactamase genes among *Pseudomonas aeruginosa*: molecular characterization of MDR strains in broiler chicks and dead-in-shell chicks infections. Ann Clin Microbiol Antimicrob (2024) 23:9.

Samanta I, Joardar SN, Palas Das, Ghosh, D, Sar TK, Taraphder S. 2012. Multi Drug Resistant Pseudomonas aeruginosa From Wild Hanuman Langur in India. Journal of Biomedical Sciences, 1 No. 2:1 doi: 10.3823/1005.

Shukla S, Mishra, P. 2015. *Pseudomonas aeruginosa* Infection in Broiler Chicks in Jabalpur. International J Ext Res. 6:37-39.

Spilker T, Coenye T, Vandamme P, LiPuma J J. 2004. PCR-Based Assay for Differentiation of *Pseudomonas aeruginosa* from Other *Pseudomonas* Species Recovered from Cystic Fibrosis Patients. Journal Of Clinical Microbiology, May 2004, p. 2074–2079.

Tawakol MM, Nabil NM, Reda RM. 2018. Molecular studies on some virulence factors of *Pseudomonas aeruginosa* isolated from chickens as a biofilm forming bacteria. Assiut Vet. Med. J, 64 (159): 43-51.

Ullah A, Anjum AA, Rabbani M, Ijaz M, Nawaz M, Ashraf M, Ali A, Rashid A, Najeeb I, Hussain A. 2021. Activity of ethanolic extract of *Eucalyptus globulus* leaves against multi drug resistant poultry pathogens in broiler chicks. Cell Mol Biol (Noisy le Grand), 67 (1): 153-158.

Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A. 2001. The chaperoneyusher pathways of *Pseudomonas aeruginosa*: Identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. PNAS, 2001, 98 (12): 6911–6916

Wilson C, Lukowicz R, Merchant S, Valquier-Flynn H, Caballero J, Sandoval J, Okuom M, Huber C, Brooks TD, Wilson E, Clement B, Wentworth C D, Holmes A E. 2017. Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. Res Rev J Eng Technol. 2017 6(4): 1-25.

Yong X, Ling Y, Xiao-cui Z, Li-zhen L, Peng L, Wei-sheng C. 2020. Rapid detection of *Pseudomonas aeruginosa* by cross priming amplification. Journal of Integrative Agriculture 2020, 19(10): 2523–2529.

Yuan JS, Reed A, Chen F, Stewart CN. 2006. Statistical analysis of real-time PCR data. *BMC Bioinformatics*, 7:85.

Zhang L, Fritsch M, Hammond L, Landreville R, Slatculescu C, Colavita A, Mah T. 2013. Identification of Genes Involved in *Pseudomonas aeruginosa* Biofilm-Specific Resistance to Antibiotics. PLoS ONE 8(4): e61625.