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Effect of Essential Oils on Biological Criteria of Gram-Negative Bacterial Pathogens Isolated from Diseased Broiler Chickens

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ABSTRACT

The present study aimed to investigate Gram-negative bacterial pathogens isolated from liver affections in broiler chickens. The antibacterial effect of some essential oils (EOs) and their effects on isolated bacteria's biological and phenotypic criteria were also investigated. One hundred and twenty liver samples were subjected to bacterial isolation, biochemical and molecular identification of the isolated bacteria. The antibacterial effect of cinnamon, oregano, clove, turmeric, and eucalyptus EOs at different concentrations was evaluated using the agar dilution method. Pre-and Post EOs treatment biological activity (motility, biofilm formation, antimicrobial resistance, and virulence gene detection or expression) were evaluated in selected bacteria. Results revealed 85% prevalence of Gram-negative bacteria, including *Escherichia coli (E. coli)*, *Pseudomonas aeruginosa (P. aeruginosa)*, *Mannheimia haemolytica* (*M. haemolytica)*, *Aeromonas hydrophila (A. hydrophila)* and *Salmonella enteritidis* (*S. enteritidis*). Most isolates were multiple antibiotic resistant, including quinolones and fosfomycin antimicrobials. A 0.1% concentration of cinnamon and oregano EOs effectively inhibited the growth of 80-100% and 50-100% of the tested bacterial spp, respectively; however, clove, turmeric, and eucalyptus EOs had no effect. Interestingly, after treatment with oregano or cinnamon oils, few of the resistant *S. enteritidis*, *E. coli*, and *P. aeruginosa* isolates were susceptible or intermediately sensitive to some antimicrobials. Similarly, the motility of *S. enteritidis* and, to a more extent *P. aeruginosa* was significantly reduced. Both EOs had no significant effect on *S. enteritidis* biofilm-forming ability; however, they inhibited the biofilm-forming ability of 10% and 100% of the tested *E. coli* and *P. aeruginosa* isolates, respectively. Standard PCR and real-time quantitative PCR did not reveal any significant change of either detection or differential expression of selected genes in the treated isolates. In conclusion, EOs are promising alternatives to minimize synthetic antimicrobials against multiple antibiotic-resistant bacteria. Finally, the antibacterial activity of EOs are mainly attributed to the induced structural disturbances in the treated bacteria.

Key words: Broiler chickens, *E. coli*, *Salmonella*, Essential oils, Cinnamon, Oregano.

INTRODUCTION

Poultry is considered the most appropriate protein supply source of high nutritional value for humans with a relatively lower production cost and short life cycle. However, infectious diseases impede the poultry industry due to increased mortality, growth retardation, and the curative and preventive use of chemotherapeutics (Daehre et al. 2018). Several bacteria such as *E. coli*, *Salmonella* spp, *Proteus* spp, *Enterobacter* spp. *Pseudomonas* spp, *Klebsiella* spp, *Staphylococcus* spp, and *Streptococcus* spp. were found prevalent in diseased poultry (Ali et al. 2019; Abd El-Mawgoud et al. 2021). For instance, *E. coli* is the most prevalent bacteria causing respiratory infection in poultry (Roth et al. 2019; Mehmood et al. 2020). Also, *Salmonella* is responsible for substantial economic losses to the poultry industry (Lee et al. 2013). Importantly, *Salmonella*-infected poultry meat and contaminated eggs are primary sources of human salmonellosis and food-borne illness worldwide (Renu et al. 2018; Hussein et al. 2019).

Though effective antibiotic therapy usually eliminates the infecting organism, the continuous use of antibiotics

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leads to drug-resistant pathogens. Additionally, drug residues in poultry products represent a potential health hazard for humans (Michael and Schwarz 2016). Increasing antimicrobial resistance is a critical public health concern, and the emergence and spread of antimicrobial resistance is a complex problem driven by numerous interconnected factors (Radwan et al. 2021). *In-vitro* antimicrobial sensitivity testing of veterinary pathogens provides valuable guidance to the veterinarian in the choice of appropriate drug treatment (Radwan et al. 2016). Moreover, it is very useful to detect multidrug-resistant (MDR) isolates. Therefore, the appropriate antibiotic should be selected on the basis of its sensitivity that could be detected by laboratory examination.

In recent decades, great attention was paid to essential oils (EOs) as antimicrobial agents. The EOs are volatile, natural, complex compounds having antiseptic, antibacterial, antiviral, antifungal, and other medicinal properties (Bakkali et al. 2008). EOs have several properties to support their use in poultry, including antioxidant, antiseptic, and insect-repellent activities. The antibacterial properties of EOs and their components have been reviewed in the past (Ebadollahi et al. 2021; Kaur et al. 2021). Due to the large numbers of different chemical compounds in the EOs, it is most likely that their antibacterial activity is not considered one specific mechanism (Lopez-Romero et al. 2015).

The proposed modes of action include penetration of the bacterial cell wall lipids due to their hydrophobicity and causing structural and permeability disturbances. Additionally, the inactivation of extracellular enzymes, reduced intracellular ATP, coagulation of cytoplasm, and interruption of electron flow and active transport were proposed (Ultee et al. 2002). Indirect effects against some intestinal pathogens, such as decreasing digesta viscosity and improving nutrient digestion via stimulation of pancreatic enzyme activities, have been suggested for some EOs (O'Bryan et al. 2008; Toosi et al. 2016; Zhai et al. 2018). The current study was designed to investigate the antibacterial effect of some EOs on Gram-negative bacteria isolated from diseased broiler chickens. Additionally, the effects of selected EOs on the isolated bacteria's biological and phenotypic criteria (motility, biofilm formation, and virulence gene expression) were studied.

MATERIALS AND METHODS

Broiler Chicken Samples

One hundred and twenty liver samples were collected from diseased broiler chickens of different ages (2-3 weeks) from different farms in El-Fayoum Governorate in Egypt from January 2020 to March 2020. The diseased chickens suffered from septicemia, peri-hepatitis, air saculitis, pneumonia, peritonitis, enteritis, yolk sac inflammation, or typhlitis. Birds were subjected to clinical, postmortem, and bacteriological examinations.

Bacteriological Examination of the Collected Liver Samples

Bacterial isolation was conducted as described previously (Collee et al. 1996). Briefly, about 10gm of liver tissues were inoculated into tryptone Soya broth and MacConkey's broth (Oxoid™ Thermo Fisher Scientific Inc., USA) and incubated at 37°C for18-24hr. One loopful was taken from the broth cultures and cultivated on tryptone soya agar, eosin methylene blue (EMB), MacConkey's agar, Pseudomonas cetrimide agar, or 5% sheep blood agar (Oxoid™ Thermo Fisher Scientific Inc., USA) and incubated at 37°C for 24hrs. For *Salmonellae* isolation, the specimens were inoculated into Selenite-F broth and incubated at 37°C for 18-24hrs. Then, a loopful of this culture was streaked out onto MacConkey's agar, Xylose Lysine Deoxycholate (XLD), and Salmonella-Shigella (SS) agar media and incubated at 37°C for 18-24hrs. The colonies were examined for their cultural characters and morphological appearance. The isolated bacteria were subjected to Gram's staining, biochemical identification (Collee et al. 1996; Quinn et al. 2011). According to the manufacturer's instructions, the API 20E and API 20NE identification systems (BioMérieux, France) were used for the biochemical identification of Gram-negative bacterial isolates.

Molecular Serotyping of *Salmonella* Isolates

The bacterial DNA was extracted from the identified *Salmonella* isolates using the QIAamp DNA mini kit according to the manufacturer's instructions. *Salmonella* isolates were subjected to multiplex PCR for intra-serotyping using *inv*A, IE1 and *flic*C primers as previously described (Paiao et al. 2013). Briefly, the mPCR was conducted in a 25µL reaction consisted of 10× DreamTaq Buffer 12.5µL, 1µL of each forward and reverse primer, 4µL template DNA, and 2.5µL nuclease-free water. The m-PCR protocol included an initial denaturation step for 5min at 95°C was followed by 30 cycles of 1min at 95°C, 1min at 58°C, and 30s at 72°C and by a final extension step for 7min at 72°C. The PCR products were analyzed by electrophoresis using 1.5% agarose gels with TBE (45 mmol L-1 Tris, pH 8.3, 45mmol L-1 borate, and 2mmol L-1 EDTA) as the running buffer. The amplicon sizes were 796, 316, and 432bp *inv*A, IE-1 and *flic*C genes, respectively.

Antimicrobial Susceptibility Profiles of Bacterial Isolates

Bacterial isolates were tested for their antimicrobial susceptibility to 14 different antimicrobial discs (Oxoid, Basing Stoke, UK) according to the previously described method (Collee et al. 1996). Briefly, standard inoculums visually equivalent to the 0.5 McFarland standards (1.5×10^8CFU/mL) were prepared. The test was performed by streaking a swab from the standard inoculum onto Muller Hinton agar in one direction over the entire surface then rotating the plate about 60°. The step was repeated three times to ensure equal distribution of the inoculum. After plate dryness, the selected antimicrobial discs were placed onto the inoculated agar plates' surfaces, and then the plates were inverted and incubated for 18-24hrs at 37°C. The complete inhibition zone diameters were measured, and the result was interpreted as sensitive, intermediate, or resistant (CLSI 2017). The multiple antibiotic resistance (MAR) index for bacterial isolates was calculated using the formula:

$$MAR\ Index = \frac{Number\ of\ antibiotics\ resisted}{\text{Total number of antibiotics Tested}} \times 100$$

Isolates with MAR index values of more than 0.2 or 20% were considered highly resistant (Krumperman 1983).

Detection of the Antibacterial Effect of the Essential Oils on the Bacterial Isolates

The agar dilution method was used to evaluate the antibacterial activities of EOs (Mekonnen et al. 2016). Briefly, cinnamon, oregano, clove, turmeric, and eucalyptus EOs were purchased from Maven Experts USA Inc. The EOs were filtered through 0.45µm cellulose filter membrane, and 0.1, 0.01, and 0.005% concentrations were added separately to sterilized cooled tryptone soya agar (~55°C) then poured in Petri dishes and left to solidify. The standard inoculums visually equivalent to the 0.5 McFarland standards (1.5×10^8CFU/mL) were prepared. A sterile cotton swab was dipped into the adjusted suspension tubes. The swab was firmly pressed and rotated against the tubes' wall several times to remove excess inoculum. Then, the inoculum was streaked onto the entire surface of oil agar plates with different concentrations for each EO and incubated for 18-24hr at 37°C. The incubated plates were examined for growth or inhibition of the bacterial growth.

Effect of Selected EOs on Biological Criteria of Selected Gram-negative Bacteria

Two isolates from highly resistant bacteria that could grow in the presence of cinnamon and oregano oils at 0.01% concentration were selected to investigate the potential effect of EOs on the antimicrobial resistance profile and virulence genes expression.

Effect of EOs on Antimicrobial Susceptibility Profile

The test was conducted as described above using the method already described (Collee et al. 1996; CLSI 2017).

Motility Assay

The selected bacterial isolates' motility was assessed pre-and post-exposure to EOs (Collee et al. 1996). A semisolid agar medium was prepared using tryptone soya broth and 0.4% bacteriological agar (Oxoid™ Thermo Fisher Scientific Inc., USA). For motility assay, a single colony of pure culture was stabbed to a depth of only 0.3 to 0.5 inches in the middle of the tube of semi-solid agar medium by using sterile wire. The stabbed media were incubated for 24hr at 37°C. The positive result indicted by observing diffuse, hazy growths that spread throughout the medium, while the negative result indicted by observing a growth confined to the stab-line.

Biofilm Formation

The Congo red (CR) assay was employed to assess *curli* production (Zhou et al. 2013). The pure colonies of selected bacterial isolates were streaked onto LB agar plates and incubated at 37°C for 48hr. Then, single colonies were picked and streaked onto YESCA CR agar plate and incubated for 48-72hr at 25°C. The red-colored bacterial colonies were recorded positive for biofilm formation (i.e., uptake of the stain), and the pink or white colonies were negative for biofilm formation.

Virulence Genes Detection

The bacterial DNA was extracted using the QIAamp DNA mini kit according to the manufacturer's instructions. The selected bacteria exposed to EOs were tested for virulence gene detection (pre and post-exposure) using conventional PCR in 25µl reaction volumes using specific oligonucleotide primers (Table 1). The reaction consisted of 12.5µL of Emerald Amp GT PCR master mix (Takara, Japan), 1µL of each forward and reverse primer, 6µL of template DNA, and 4.5µl of PCR grade water. The cycling conditions of the PCR were one cycle of primary denaturation at 94°C for 5min then 35 cycles of secondary denaturation at 94°C for 30s, annealing at 55-60°C (according to the primer) for 1min, and extension at 72°C for 1min followed by one cycle of final extension at 72°C for 10min. The PCR products were electrophoresed on 1.5% gel after staining with ethidium bromide and photographed by a gel documentation system.

Differential Virulence Genes Expression

For selected *P. aeruginosa* and *S. enteritidis* isolates, additional quantitative SYBER green real-time PCR was used to determine the virulence gene expression pre-and post-exposure to cinnamon oregano EOs (Tsai et al. 2020). The SYBER green real-time PCR reaction volume was 25µl consisted of; 12.5µL of Quantitect SYBR green PCR master mix (Takara, Japan), 0.25µL of RevertAid Reverse Transcriptase (Thermo Fisher), 0.5µL of each forward and reverse primers, 3µL of template DNA, and 8.25µL of PCR grade water. The cycling conditions of the PCR were one cycle of reverse transcription at 50°C for 30min, one cycle of primary denaturation at 94°C for 15min then 40 cycles of secondary denaturation at 94°C for 15s, annealing at 50-60°C (according to the primer) for 40s and extension at 72°C for 40s. The dissociation curve was generated by one cycle of secondary denaturation at 94°C for 1min, annealing at 50-60°C (according to the primer) for 1min, and final denaturation at 94°C for 1min.

Amplification curves and Ct values were determined by the Stratagene MX3005P software. To estimate the variation of gene expression of the RNA of the bacteria post-exposure to EOs, each sample's Ct was compared with that of the control group according to the "ΔΔCt" method as previously described (Yuan et al. 2006). Dissociation curves were compared between different samples to exclude false-positive results.

RESULTS

Gram-negative Bacterial Pathogens Associated with Liver Affection in Broilers

Out of 120 samples, 102 (85%) Gram-negative bacteria were recovered, including 78 oxidase negative (65%) and 24 oxidase-positive bacteria (20%). Oxidase negative bacteria included 20 *E. coli* (16.7%), 4 *Salmonella* spp (3.3%), 25 *Enterobacter cloacae* (20.8%), 17 *Citrobacter freundii* (14%), 5 *Proteus vulgaris* (4.2%), 3 *Klebsiella pneumoniae* (2.5%) as well as 2 *Shigella* spp. *and Serratia marcescens* (1.7% for each). Meanwhile, Oxidase positive bacteria included 10 *Pseudomonas aeruginosa* (8.3%), 6 *Mannheimia haemolytica* (5%) and 8 *Aeromonas hydrophila* (6.7%) (Table 2). Salmonella intra-serotyping by multiplex PCR revealed that all salmonellae isolates were *Salmonella enteritidis* (Fig. 1).

Antimicrobial Susceptibility Profile of the Isolated Gram-negative Bacterial Pathogens

Results of *in-vitro* susceptibility tests revealed that all *P. aeruginosa*, *M. haemolytica*, *A. hydrophila*, *E. coli*, and

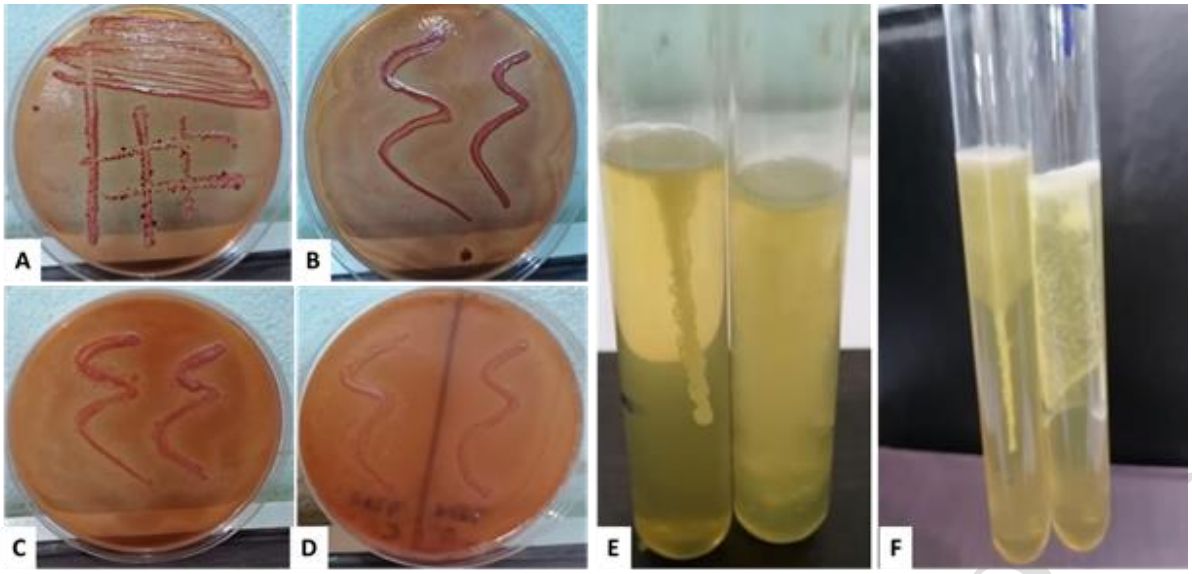


Fig. 1: Cinnamon and Oregano EOs affect the biofilm and motility of E. *coli, S. enteritidis,* and *P. aeruginosa*. Strong biofilm-producing *E. coli* pre-(A) and post-exposure to Oregano (C). Strong biofilm-producing P. aeruginosa pre-(B) and post-exposure to Oregano (D). Motility pre-(left) and post-exposure (right) to Cinnamon of *S. enteritidis* (E) and *P. aeruginosa* (F).

Table 1: Oligonucleotide primers sequences for molecular characterization, virulence gene, and SYBER green real-time PCR.

Target organism	Gene		Primer sequence	Amplicon size	References
E. coli	*iut*A		*ATGAGCATATCTCCGGACG* *CAGGTCGAAGAACATCTGG*	587	Kaczmarek et al. (2012)
	*fim*H		*GATCTTTCGACGCAAATC* *CGAGCAGAAACATCGCAG*	389	
	*fim*A		*CGGCTCTGTCCCTSAGT* *GTCGCATCCGCATTAGC*	500	
	*pap*C		*GACGGCTGTACTGCAGGGTGTGGCG* *ATATCCTTTCTGCAGGGATGCAATA*	328	Le Bouguénec et al. (2001)
	*fel*A		*GGTCAASCAGCTAAAAACGGTAAGG* *CCTTCAGAAACAGTACCGCCATTCG*	239	Moulin-Schouleur et al. (2006)
	tsh		*GGTGGTGCACTGGAGTGG* *AGTCCAGCGTGATAGTGG*	640	Dozois et al. (2000)
Salmonella spp	*adr*A		*ATGTTCCCAAAAATAATGAA* *TCATGCCGCCACTTCGGTGC*	1113	Bhowmick et al. (2011)
Pseudomonas spp	*pelA*		*CATACCTTCAGCCATCCGTTCTTC* *CGCATTCGCCGCACTCAG*	786	Ghadaksaz et al. (2015)
Salmonella typing	*inv*A gene	*Inv*A F *Inv*A R	*CGG TGG TTT TAA GCG TAC TCTT* *CGA ATA TGC TCC ACA AGG TTA*	796	Fratamico (2003)
	S. enteritidis	IE1 F IE1 R	*AGT GCC ATA CTT TTA ATG AC* *ACT ATG TCG ATA CGG TGG G*	316	Wang and Yeh (2002)
	S. typhimurium	*flic*C F *flic*C R	*CCCGCTTACAGGTGGACTAC* *AGCGGGTTTTCGGTGGTTGT*	432	Paiao et al. (2013)
SYBER green real-time PCR	*P. aeruginosa*	16S rDNA	*GGGGGATCTTCGGACCTCA* *TCCTTAGAGTGCCCACCCG*	NA	Spilker et al. (2004)
		*psl*A	*TCCCTACCTCAGCAGCAAGC* *TGTTGTAGCCGTAGCGTTTCTG*	656	Ghadaksaz et al. (2015)
	Salmonella spp.	16S rRNA	*CAGAAGAAGCACCGGCTAACTC* *GCGCTTTACGCCCAGTAATT*	NA	Yang et al. (2014)
		*csg*D	*TTACCGCCTGAGATTATCGT* *ATGTTTAATGAAGTCCATAG*	651	Bhowmick et al. (2011)

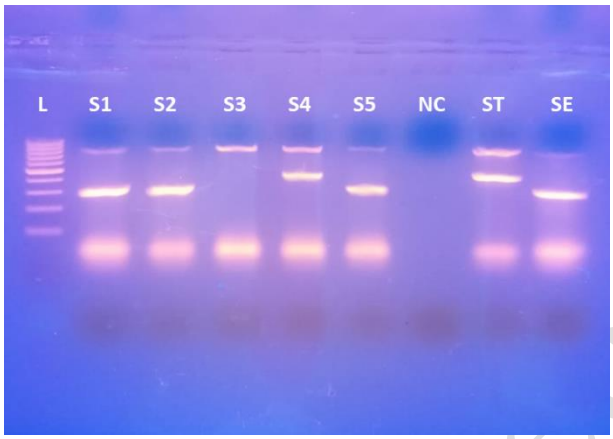
S. enteritidis isolates were multiple antibiotic-resistant. Interestingly, the isolates were highly resistant to quinolones and fosfomycin. As expected, almost all isolates were highly resistant to β-lactams and potentiated sulfa antimicrobials (Supplementary Table 1).

Inhibitory Effect of EOs Gram-Negative Bacterial Isolates

Bacterial isolates with multiple antibiotic resistance (*E. coli*; n=10, *S. enteritidis*; n=4, *P. aeruginosa*; n=4, and *M. haemolytica*; n=5) were selected to investigate the *in-vitro* efficacy of EOs. The cinnamon and oregano EOs effectively inhibited the growth of 80-100% and 50-100% of the tested bacterial spp. at 0.1% concentration, respectively. Meanwhile, cinnamon inhibited the growth of 25% of the tested *S. enteritidis* at 0.01% concentration. However, clove, turmeric and eucalyptus EOs did not affect all tested bacteria (Table 3).

Supplementary Table 1: The antimicrobial susceptibility profile of the prevalent Gram-negative bacteria isolated from broiler chicken livers (R; resistant, I; intermediate susceptible, S; susceptible).

Antimicrobial Class		Disc (µg)	*P. aeruginosa* (n=10)			*M. haemolytica* (n= 6)			*A. hydrophila* (n=8)			*E. coli* (n=20)			*S. enteritidis* (n= 4)		
			R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
β-lactams	Penicillins Amoxicillin	25 µg	100	0	0	100	0	0	75	0	25	100	0	0	100	0	0
	Amoxicillin-Clavulanic acid	30 µg	100	0	0	100	0	0	75	0	25	90	0	10	100	0	0
	Cephalosporins Cefalexin	10µg	100	0	0	100	0	0	62.5	0	37.5	100	0	0	100	0	0
	Ceftriaxone	30µg	30	50	20	66.6	33.3	0	62.5	0	37.5	100	0	0	100	0	0
	Cefotaxime	30µg	100	0	0	100	0	0	50	0	50	95	0	5	100	0	0
Amino glycosides	Amikacin	30 µg	30	0	70	33.3	0	66.6	87.5	0	12.5	25	25	50	25	0	75
	Apramycin	15 µg	100	0	0	100	0	0	25	0	75	75	15	10	75	25	0
	Gentamicin	10µg	0	0	100	66.6	33.3	0	62.5	0	37.5	70	0	30	100	0	0
	Kanamycin	30µg	100	0	0	100	0	0	62.5	0	37.5	85	0	15	75	25	0
	Streptomycin	10µg	80	0	20	100	0	0	87.5	0	12.5	100	0	0	100	0	0
Quinolones	Ciprofloxacin	5 µg	80	0	20	100	0	0	87.5	0	12.5	85	5	10	100	0	0
Tetracyclines	Doxycycline	30µg	100	0	0	100	0	0	87.5	0	12.5	95	0	5	100	0	0
Fosfomycin	Fosfomycin	200µg	100	0	0	66.6	0	33.3	50	0	50	100	0	0	75	0	25
Potentiated sulfa	Sulfamethoxazole - Trimethoprim	25 µg	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0



Supplementary Fig. 1: Salmonella isolates intra-serotyping by multiplex PCR. L; 100bp ladder, S1-S5; *Salmonella* isolates, NC; negative control, ST and SE; *Salmonella typhimurium* and *Salmonella enteritidis* positive controls, respectively.

Table 2: Recovery rates of Gram-negative bacilli from the liver of diseased broiler chickens

Bacterial isolate	No.	%
Oxidase negative		
E. coli	20	16.7
Salmonella enteritidis	4	3.3
Enterobacter cloacae	25	20.8
Citrobacter freundii	17	14.2
Proteus vulgaris	5	4.2
Klebseilla pneumoniae	3	2.5
Shigella spp.	2	1.7
Serratia marcescens	2	1.7
Total Oxidase negative	78	65
Oxidase positive		
Pseudomonas aeruginosa	10	8.3
Mannheimia haemolytica	6	5
Aeromonas hydrophila	8	6.7
Total oxidase-positive	24	20
Total Gram-negative	102	85
Gram-positive bacteria	18	15
Total No. of samples	120	100

% was calculated according to the total number (No.) of samples (n=120).

Effect of EOs on Biological Criteria of Treated Gram-negative Bacteria

Post EOs treatment antimicrobial susceptibility

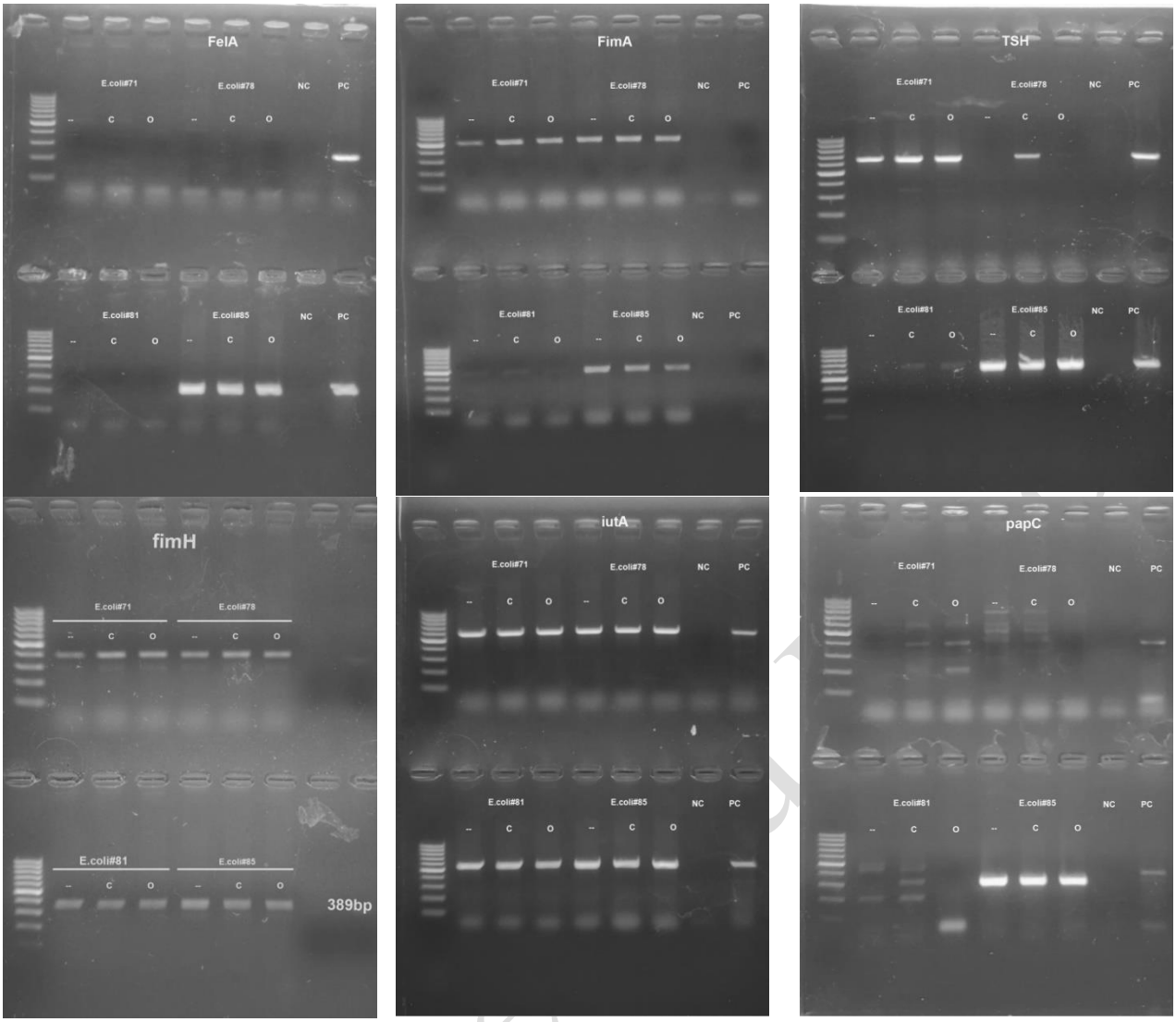
One *S. enteritidis* isolate changed to intermediate to amikacin and gentamicin; one *E. coli* isolate changed to susceptible to amikacin. One *P. aeruginosa* isolate changed to susceptible to amikacin, ceftriaxone (the other isolate changed to intermediate) and streptomycin. On the other hand, cinnamon EO treatment of resistant isolates resulted in the change of one *S. enteritidis* to be susceptible to amikacin, and one *E. coli* isolate changed to intermediate susceptible to fosfomycin and gentamicin. In contrast, no *P. aeruginosa* isolates were affected (Table 4).

Post-exposure Gram-negative Bacteria Motility and Biofilm Formation

Motile *E. coli*, *S. enteritidis*, and *P. aeruginosa* that grow in the presence of cinnamon and oregano at 0.01% concentration were evaluated for their motility. Oregano inhibited 1 out of 2 *S. enteritidis* isolates motility (50%), which were not affected by cinnamon. However, none of them affected *E. coli* isolates motility. On the other hand, both EOs inhibited the motility of 40-50% of tested *P. aeruginosa* isolates. However, both EOs have no significant effect on *S. enteritidis* biofilm-forming ability; however, they inhibited the biofilm-forming ability of 10 and 100% of the tested *E. coli* and *P. aeruginosa* isolates, respectively (Table 5; Fig.1).

Virulence Genes Profiles after EOs Treatment

No significant effect of either cinnamon or oregano was observed in the virulence gene detection by PCR of isolated *E. coli* (Supplementary Fig. 2), *S. enteritidis* or *P. aeruginosa*. Therefore, we selected two isolates of *S.* Enteritidis and three isolates of *P. aeruginosa* to investigate specific virulence genes expression (*csg*D and *psl*A, respectively) by qPCR assay. The results demonstrated that there was no significant effect following both EOs treatments. The expression of the tested genes was nearly comparable to the untreated bacteria. *S. enteritidis csg*D gene expression decreased only 0.2-0.3 and 0.3-0.4 folds after treatment with



Supplementary Fig. 2: Virulence gene profiles in the pre-and-post EOs treated *E. coli* isolates (no.4). -; non treated, C; cinnamon treated, O; oregano treated.

Table 3: Antibacterial effect of tested essential oils on Gram-negative bacilli

Tested Isolates	Cinnamon			Oregano			Clove		Turmeric		Eucalyptus	
	0.005	0.01	0.1	0.005	0.01	0.1	0.01	0.1	0.01	0.1	0.01	0.1
E. coli (n=10)	0/10	0/10	10/10(100%)	0/10	0/10	10/10(100%)	0/10	0/10	0/10	0/10	0/10	0/10
S. Entertidis (n=4)	0/4	1/4(25%)	4/4(100%)	0/4	0/4	3/4(75%)	0/4	0/4	0/4	0/4	0/4	0/4
P. aeruginosa (n=4)	0/4	0/4	4/4(100%)	0/4	0/4	2/4(50%)	0/4	0/4	0/4	0/4	0/4	0/4
M. haemolytica (n=5)	0/5	0/5	4/5(80%)	0/5	0/5	4/5(80%)	0/5	0/5	0/5	0/5	0/5	0/5

Table 4: Antimicrobial susceptibility of selected Gram-negative bacteria post-exposure to the essential oils

EOs Treatment	Antimicrobial Agents													
	AMC	AML	APR	AK	CL	CIP	CTX	CTR	DO	FO	GEN	K	SF	SXT
S. enteritidis isolates (n=2)														
Cinnamon	R	R	R	100%(S)	R	R	R	R	R	R	R	R	R	R
Oregano	R	R	R	50%(R) 50%(I)	R	R	R	R	R	R	50% R) 50%(I)	R	R	R
E. coli (n=2)														
Cinnamon	R	R	R	R	R	R	R	R	R	50%(R) 50% (I)	50%(R) 50%(I)	R	R	R
Oregano	R	R	R	50%(R) 50%(S)	R	R	R	R	R	R	R	R	R	R
P. aeruginosa (n=2)														
Cinnamon	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Oregano	R	R	R	50%(S) 50%(R)	R	R	R	50%(S) 50%(I)	R	R	R	R	50%(S) 50%(R)	R

Abbreviations: AMC; amoxicillin/clavulanic acid- AML; amoxycillin- APR; apramycin- AK; amikacin- CL; colistin-CIP; ciprofloxacin-CTX; cefotaxime- CTR; Cceftriaxone- DO; doxycycline- FO; fosfomycin- GEN; gentamycin- K; kanamycin- SF; Sulfisoxazole- SXT; trimethoprim-sulphamethoxazole- R; resistant- I; intermediate susceptible- S; susceptible.

Table 5: Effect of cinnamon and oregano EOs treatment on the biological activities of selected Gram-negative bacteria

Tested isolates	Biological activities lost post-exposure to EOs									
	Motility					Biofilm formation				
	No. of treated isolates	Cinnamon		Oregano		No. of treated isolates	Cinnamon		Oregano	
		No. of affected isolates	%	No. of affected isolates	%		No. of affected isolates	%	No. of affected isolates	%
E. coli	10	0	0	0	0	10	1	10	1	10
S. enteritidis	2	0	0	1	50	2	0	0	0	0
P. aeruginosas	10	5	50	4	40	4	4	100	4	100

% was calculated according to the corresponding number (No.) of tested isolates.

Table 6: Effect of EOs cinnamon and oregano on *S. enteritidis* and *P. aeruginosa* gene expression

Sample ID	Treatment	*S. enteritidis*			Sample ID		*P. aeruginosa*		
		16S rRNA Ct	csgD				16S rDNA Ct	pslA	
			Ct	Fold change				Ct	Fold change
S1	None	21.19	22.83	-	P-1	None	18.53	19.31	-
	Cinnamon	22.31	26.07	0.2300		Cinnamon	19.62	23.45	0.1207
	Oregano	21.64	24.93	0.3186		Oregano	20.47	23.74	0.1780
S-2	None	22.05	22.64	-	P-2	None	19.14	19.88	-
	Cinnamon	22.91	25.15	0.3186		Cinnamon	20.61	24.93	0.0836
	Oregano	22.28	24.19	0.4005		Oregano	19.70	23.63	0.1103
					P-3	None	18.27	20.06	-
						Cinnamon	19.89	25.11	0.0928
						Oregano	19.91	24.48	0.1456

Abbreviations: csgD; curli activator CsgD protein- psIA; psl cluster responsible for biofilm formation- Ct; real-time PCR cycle threshold.

cinnamon or oregano, respectively, while *P. aeruginosa psl*A gene expression decreased only 0.1 and 0.1-0.2 folds after EOs treatment, respectively (Table 6).

DISCUSSION

Gram-negative bacterial pathogens are seriously affecting the poultry industry in Egypt and worldwide, causing many diseases leading to significant economic losses (Hassan et al. 2018; El Jilal et al. 2020). The present study aimed to investigate Gram-negative bacterial pathogens isolated from liver affections in broiler chickens and the application of some EOs studying their antibacterial effect and their effects on biological and phenotypic criteria (motility, biofilm formation, and virulence gene expression) on such bacterial isolates.

In the current study, out of 120 liver samples, 102 (85%) Gram-negative bacteria were recovered with a percentage of 85%. The high prevalence of gram-negative bacteria was previously reported in Egypt (Hassan et al. 2018), with up to 82.5% of isolated bacteria from internal organs. Among oxidase-negative bacterial isolates, *E. coli* and *S. enteritidis* were the most important isolated pathogens regarding the poultry industry (Radwan et al. 2021). The oxidase-positive bacterial isolates included *P. aeruginosa*, *M. haemolytica* and *A. hydrophila*, were also the most prevalent Gram-negative oxidase-positive isolates. *P. aeruginosa* causes septicemia, diarrhea, and respiratory affections and is associated with mortality in birds (Wallmann et al. 2007). *Aeromonas hydrophila* in broiler chickens suggests that chickens could be a potential source for dissemination of food-borne Aeromonas gastroenteritis (Bhowmick et al. 2011). Therefore, Aeromonads distribution in the environment and foods of animal origin needs further studies considering their public health implications.

PCR assay using the *inv*A primers specific for *Salmonella* spp is considered an international standard procedure for detecting genus *Salmonella* and mostly reduces the number of false-negative results commonly occurring in diagnostic laboratories (Malorny et al. 2003). Besides *Inv*A, the amplification of IE1 and *flic*C genes is considered a simple, accurate, and rapid method for detecting and identifying *Salmonella* at the genus level and the serovars Enteritidis and Typhimurium, respectively (Paiao et al. 2013). In the current study, *Salmonella* intra-serotyping by multiplex PCR using *inv*A, IE1 and *flic*C genes showed that all salmonellae isolates were identified as *S. enteritidis*.

The increased emergence and spread of antimicrobial resistance are significant public health concerns. In the present study, the antimicrobial susceptibility profile of the isolated *P. aeruginosa*, *M. haemolytica*, *A. hydrophila*, *E. coli* and *S. enteritidis* were studied. Results revealed that all isolates were multiple antibiotic resistant. Interestingly, they were mostly quinolones and fosfomycin resistant. As expected, all isolates were highly resistant to β-lactams and potentiated sulfa antimicrobials (Radwan et al. 2016; Hassan et al. 2018; Radwan et al. 2021).

In an effort to minimize the use of synthetic antimicrobials, we investigated the antimicrobial effect of some EOs against multiple antibiotic-resistant bacteria considering their low toxicity and high volatility compared to antimicrobials (Zhai et al. 2018). In the current study, cinnamon and oregano EOs effectively inhibited the growth of 80-100% and 50-100% of the tested bacterial spp. (*E. coli*, *S. enteritidis*, *P. aeruginosa* and *M. haemolytica*) at 0.1% concentration, respectively. Meanwhile, clove, turmeric and eucalyptus EOs had no effect. The antibacterial activity of oreganium EO is mainly attributed to its high carvacrol and thymol content that can disintegrate Gram-negative bacteria's outer membrane, releasing lipopolysaccharides increasing the permeability of the cytoplasmic membrane to ATP (Simirgiotis et al. 2020). However, cinnamon's antibacterial activity is mainly attributed to the presence of a high percent of cinnamaldehyde that exhibits antibacterial characters (El Atki et al. 2019).

Several proposed modes of action were suggested for the antimicrobial effects of EOs; however, the current study focuses on the effect of selected EOs on biological criteria of multiple antibiotic-resistant gram-negative bacteria. Interestingly, some phenotypic changes were observed upon treatment with either oregano or cinnamon oils. Few of the treated resistant *S. enteritidis*, *E. coli*, and *P. aeruginosa* isolates were changed to either susceptible or intermediately susceptible to some antimicrobials than the parental bacterial isolates (Table 4). Additionally, the motility of *S. enteritidis* and, to more extent *P. aeruginosa* that grow in the presence of cinnamon and oregano at 0.01% concentration were greatly affected. Both EOs have no significant effect on *S. enteritidis* biofilm-forming ability; however, they inhibited the biofilm-forming ability of 10 and 100% of the tested *E. coli*, and *P. aeruginosa* isolates, respectively.

To investigate whether these phenotypic changes are due to a defect in structure or a functional disturbance of some genes (Morimoto et al. 2010), we employed standard PCR and real-time quantitative PCR to evaluate the detection or differential expression of selected genes. No significant effect on either the detection or the expression of selected genes was observed. These results support the previous studies that have demonstrated that structural disturbances are mainly associated with reduced motility and phenotypic changes (Carson et al. 2002; Ultee et al. 2002; Bakkali et al. 2008; Lopez-Romero et al. 2015).

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Author's contributions

IAR, MMM, and AA: Conceptualization- IAR, MMM, SHA, AA, and AHA: Data curation, MMM, SHA, AA, and AHA; Formal analysis- IAR, MMM, AA, and AHA; Methodology- IAR, AA, and AHA; Supervision- IAR, MMM, SHA, AA, and AHA; Writing the original draft- IAR, MMM, SHA, AA, and AHA; Writing, review & editing. All authors have read and agreed to the published version of the manuscript.

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