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Antibacterial and antibiofilm effect of nano zinc-oxide and propolis nanoemulsion against strong biofilm producer coliform species isolated from chickens

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ABSTRACT

A total of 150 samples collected from freshly dead chickens were examined for *Coliform* bacteria. The results revealed isolation of 141 isolates (84 *E. coli* (56%), 6 *Klebsiella pneumoniae* (4%), 10 *Klebsiella oxytoca* (6.6%), 17 *Klebsiella ozanae* (11.3%), and 24 *Citrobacter freundii* (16%)). Strong biofilms on Congo red agar were produced by 37 *E. coli* isolates (44%), 3 *Klebsiella pneumoniae* (50%), 5 *Klebsiella oxytoca* (50%), 7 *Klebsiella ozanae* (41%) and 10 *Citrobacter freundii* (41%). Antimicrobial susceptibility test on strong biofilm producer isolates revealed that all isolates exhibited high resistance to Amoxicillin, Oxytetracycline and Erythromycin, Trimethoprim Sulfamethoxazole, while their sensitivity to gentamicin was high. Strong biofilm *E. coli* isolates were serotyped to O91:H21, O17:H18, O78, O114:K90, O26:K60, O121:H7, O128:H2, O113:H4, O159, O44:K74 and O55:K59. *Klebsiella pneumoniae* serotypes wereK1 and K2. Zinc oxide nanoparticles were45.86 ± 45.71 nm in size, 0.223 ± 0.149 PDI and zeta potential -14.2 ± 1.68 mv, while Propolis-NPs had particle size 291.6 ± 23.32 nm, 0.351 ± 0.026 PDI and zeta potential -9.88 ± 2.53 mv. SRB **cytotoxicity** assay showed that the highest concentration of propolis-NPs (75%) caused 88.88% cell viability, while ZnO-NPs showed cell viability more than 95% untill17.5 µg/ml. ZNO-NPs MIC was 35µg/ml for all *coliform* isolates. Its antibiofilm effect was observed at concentrations 8.75 and 17.5µg/ml for both *E.coli* and *Citrobacter freundii*, while *Klebsiella* species biofilm formation was inhibited at 17.5 µg/ml ZNO-NPs. Moreover Propolis-NPs MIC against all tested isolates was 37.5% while antibiofilm activity was detected at 18.75%.

1. INTRODUCTION

Coliform bacteria are present in the environment and the digestive tracts of animals, including humans, and are found in their wastes. It has the ability to ferment lactose with production of acid and gas. Its detection in food indicates unhygienic conditions. It includes *Escherichia coli*, *Klebsiella*, *Enterobacter* and *Citrobacter (Markey et al.*, 2013*)*. The most pathogenic member of coliform is *E. coli* that causes many diseases such as colisepticemia, coligranuloma, swollen head syndrome, meningitis, air sacculitis, and pericarditis in both humans and animals (Fairbrother et al., 2005).Bacteria attached each other forming biofilm which protect it from antimicrobial agents causing high economic losses (Chakraborty et al., 2018). Genus *Klebsiella* has many species as *K. pneumoniae, K. oxytoca, K. ozanae* and *K. rhinoscleromatis.* They are opportunistic pathogens causing nosocomial infections, septicaemia, pneumonia, rhinoscleroma, ozena, chronic granulomatous disease and hemorrhagic colitis(Tantawy et al., 2018). *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigens(Tajbakhsh et al., 2016) while *Klebsiella* species are sero-grouped by their capsular (K) antigens. Citrobacter causes severe diarrhea, urinary tract infections, pneumonia, neonatal meningitis and brain abscesses (Murray et al., 2010). The main *citrobacter* species are *Citrobacter diversus* and *Citrobacter freundii*. *Citrobacter freundii* has many virulence factors as toxins, proteolysis, hemolysis and biofilm formation (Fakruddin et al., 2014). Excessive use of antimicrobial drugs leads to multi-drug resistant coliform bacteria that cause multiple untreatable diseases and high mortality. Nanotechnology has become alternative method for finding a treatment of pathogenic bacteria (Siddiqi and Rahman, 2018). Nanoparticles with small size have more antimicrobial activity than that of large size (Chwalibog et al., 2010).Zinc oxide nanoparticle is an inorganic material that acts as antimicrobial agent against wide range of pathogenic bacteria (Chitra and Annadurai,2013).There have been very few reports of the nano-propolis because of lower size of it, the body absorbs nano-propolis more readily so antibacterial activity of nano-propolis may be more effective than propolis (AbdelMageed et al., 2019). Propolis is a resinous bee product that possesses several biological properties including antiviral, antibacterial, antifungal, anti-cancer, anti-oxidant and anti-inflammatory activities (Hegazi and Hady, 2001). So this study was aimed to evaluate the antibacterial and antibiofilm effect of nano zinc oxide and propolis nanoemulsion against multi-drug resistance coliform species isolated from freshly dead chickens.

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2. MATERIAL AND METHODS

2.1. Ethical Approval

This research was approved by Institutional Animals Care and Use Committee of faculty of veterinary medicine, Benha university (approved number BUFVTM) 21-04-23)

2.2. Collection of samples

A total of 150 samples were collected from freshly dead chickens (spleen, liver, gizzard, intestine, heart and lung), from different farms and shops at Qaluobia governorate for isolation of coliform bacteria.

2.3. Isolation and Identification of coliform (Markey et al., 2013)

A loopful from each sample was inoculated in MacConkey broth (oxoid) and incubated at 37ºc for 24hrs, then subcultured on MacConkey agar. Pink colonies were picked and identified by their cultivation on differentiated media as Brilliant green media**,** Xylose-lysin-deoxycholate (XLD) agar, Eosin methylene blue agar medium *(EMB)* and biochemical tests with reference strains (*E.coli* ATTCC25922, *Citrobacter freundii* ATCC8090, *K. pneumoniae* ATCC700603).

2.4. Biofilm formation by isolated strains (Subramanian et al., 2012)

The biofilm production was detected on Congo Red Agar (CRA) medium. Black colonies with a dry crystalline consistency indicated strong biofilm production. Weak biofilm remained pink while darkening of the colonies with absence of dry crystalline colonies indicated intermediate result. The experiment was performed in triplicate. *Edwardsiella tarda* MW362141 was used as control positive.

2.5. Antibiotic sensitivity assay (Markey et al., 2013)

The strong biofilm producer isolates were subjected to the disk diffusion test against 5 antimicrobial discs (Bio analyse); Amoxicillin (25µg), Oxytetracycline (30µg), gentamicin (10µg), Erythromycin (15µg), Trimethoprim Sulfamethoxazole (25µg).The inhibition zones were interpreted according to CLSI (2008)

2.6. Serological identification (Markey et al., 2013)

Strong biofilm *E. coli i*solates were serotyped using *E. coli* polyvalent and monovalent O antisera, H and K sera (SEIKEN supplied from MAST ASSURE™). *K.pneumoniae* specific antiserum (Statens Serum Institute, Copenhagen, Denmark) was used for serotyping of *K.pneumoniae*

2.7. Characterization of ZnO-NPs and propolis-NPs

ZnO-NPs and propolis-NPs (75%) were obtained from Animal Health Research Institute in Dokki. They were characterized by determination of their particle size, size distribution and zeta potential by photon correlation spectroscopy using particle size analyzer Dynamic Light Scattering (DLS) (Zetasizer Nano ZN, Malvern Panalytical Ltd, United Kingdom). Moreover, the cytotoxicity of different concentrations of ZnO-NPs (2.18µg/ml-35µg/ml) and propolis-NPs (4.68%-75%) were determined by sulforhodamine B (SRB) assay, using Green monkey kidney cells (Nawah Scientific Inc., Mokatam, Cairo, Egypt), according to (Skehan et al., 1990)

2.8. Antibacterial and antibiofilm effect of ZNO-NPs and Propolis-NPs

Minimum inhibitory concentrations (MICs)and antibiofilm effectof ZNO-NPs and Propolis-NPs were determined against strong biofilm producer coliform isolates according to (Basumatari et al., 2021).Different concentrations of propolis-NPs(75, 37.5, 18.75, 9.3 and 4.68 %) and ZnO-NPs (35, 17.5, 8.75, 4.3 and 2.18µg/ml) were used

3. RESULTS

3.1. Prevalence of coliform bacteria

Coliform species were isolated from 141/150 samples. They were identified as *E. coli* (84, 56%),*Klebsiella pneumoniae* (6, 4%), *Klebsiella oxytoca* (10, 6.6%), Klebsiella *ozanae* (17, 11.3%), and *Citrobacter freundii* (24, 16%), (Figure 1)

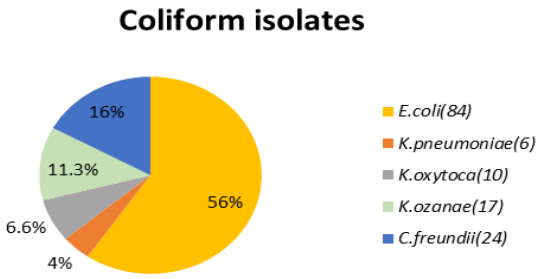

Fig1. Prevalence of different coliform isolates

3.2. Biofilm formation

Strong biofilms were produced by 37 *E. coli*isolates (44%), 3 *Klebsiella pneumoniae* (50%), 5 *Klebsiella oxytoca* (50%), 7 *Klebsiella ozanae* (41%) and 10 *Citrobacter freundii* (41%) (Table 1)

Table (1):- Biofilm formation by coliform isolates

	Strong		Intermediate		Weak	
	N	%	N	%	N	%
E.coli(84)	37	44	29	34.5	18	21.4
K. pneumoniae(6)	3	50	2	33.3	1	16.6
K.oxytoca(10)	5	50	3	30	2	20
K. ozanae(17)	7	41.2	4	23.5	6	35.3
C. freundii (24)	10	41.7	9	37.5	5	20.8

3.3. Antimicrobial sensitivity test

Coliform isolates showed resistance to more than one antibiotic. More than 81.08% of *E. coli* and 70% of *Citrobacter freundii* isolates were resistant to Amoxicillin, Oxytetracycline, Erythromycin and Trimethoprim, Sulfamethoxazole, while *Klebsiella* spp isolates showed complete resistance (100%) to them. Lowest degree of resistance was detected to Gentamicin (Table 2)

3.4. Serotyping of strong biofilm producer coliform isolates

Serotyping of *E. coli* revealed 11 *E. coli* serotypes, O114:K90 was the predominant one (Table 3), while *Klebsiella pneumaniae isolates* were serotyped into K1 (2/3) and K2 (1/3)

3.5. Characterization of ZnO-NPs

ZnO-NPs showed particle size of 45.86±45.71 nm, 0.223±0.149 PDI, Zeta potential of -14.2 ± 1.68 mv, while propolis-NPs had particle size of 291.6 ± 23.32 nm, 0.351 ± 0.026 PDI, Zeta potential of -9.88 ± 2.53 mv. SRB cytotoxicity assay proved that all propolis-NPs concentrations were safe on Vero green monkey kidney cells; their viability at highest concentration (75%) was 88.88%. Also ZnO-NPs showed cell viability more than

95% till conc.17.5 ug/ml and the Half-maximal inhibitory concentration (IC_{50}) was 31.42ug/ml (Table4)

Table (2).Antimicrobial sensitivity tests for strong biofilm coliform isolates

	Amoxicillin		*Oxytetracycline*		*Gentamicin*		*Erythromycin*		*Trimethoprim sulfamethoxazole*	
	R	S	R	S	R	S	R	S	R	S
	N %	N %	N %	N %	N %	N %	N %	N %	N %	N %
E.coli (37)	35 94	2 6	33 90	4 10	7 19	30 81	31 84	6 16	30 81	7 19
K.pneumoniae(3)	3 100	0 0	3 100	0 0	0 0	3 100	3 100	0 0	3 100	0 0
K.oxytoca (5)	5 100	0 0	5 100	0 0	1 20	4 80	5 100	0 0	5 100	0 0
K.ozanae (7)	7 100	0 0	7 100	0 0	1 14	6 86	7 100	0 0	7 100	0 0
C.freundii (10)	8 80	2 20	9 90	1 10	3 30	7 70	8 80	2 20	7 70	3 30

R: resistant S: sensitive

Table (3): Serotyping of *E.coli* isolates (n=37)

Serotype	No	%
O91:H21	3	8.1
O17:H18	1	2.7
O78	4	10.8
O121:H7	1	2.7
O128:H2	3	8.1
O113:H4	1	2.7
O159	1	2.7
O44:K74	3	8.1
O55:K59	3	8.1
O114:K90	10	27
O26:K60	7	18.9

%= According to number of *E.coli* isolates

Table (4): Viability of cells to Propolis-NPs and ZNO-NPs

Propolis-NPs		ZNO-NPs	
Conc	Viability	Conc	Viability
75 %	88.8845%	35µg/ml	4.6205 %
37.5 %	90.205%	17.5µg/ml	95.3191 %
18.75 %	93.92%	8.75µg/ml	95.3594 %
9.3 %	95.7233%	4.3µg/ml	96.064 %
4.68 %	96.0583%	2.18µg/ml	96.5875 %

Table (5): Antibacterial and antibiofilm effect of nanozinc oxide on isolated coliform

Nano zinc conc	*E.coli* O114:K90 and O26:K60	*Klebsiella Pneumoniae* (K1 and K2)	*Klebsiella oxytoca*	*Klebsiella ozanae*	*Citrobacter freundii*
35 µg/ml	-ve (MIC)	-ve (MIC)	-ve (MIC)	-ve (MIC)	-ve (MIC)
17.5 µg/ml	Very low(Antibiofilm)	Very Low (Antibiofilm)	Low (Antibiofilm)	Low (Antibiofilm)	Low (Antibiofilm)
8.75 µg/ml	Low (Antibiofilm)	High	High	High	Low (Antibiofilm)
4.3 µg/ml	High	High	High	High	High
2.18 µg/ml	High	High	High	High	High

High, low and –ve refer to bacterial growth

Table (6): Antibacterial and antibiofilm effect of propolis-NPs on isolated coliform

propolis-NPs conc	*E.coli* O114:K90 and O26:K60	*Klebsiella Pneumoniae* (K1 and K2)	*Klebsiella oxytoca*	*Klebsiella ozanae*	*Citrobacter freundii*
75 %	-ve	-ve	-ve	-ve	-ve
37.5 %	-ve (MIC)	-ve (MIC)	-ve (MIC)	-ve (MIC)	-ve (MIC)
18.75 %	Low (Antibiofilm)	Low (Antibiofilm)	Low (Antibiofilm)	Low (Antibiofilm)	Low (Antibiofilm)
9.3 %	High	High	High	High	High
4.68 %	High	High	High	High	High

High, low and –ve refer to bacterial growth.

3.6. Antibacterial and antibiofilm effect of ZNO-NPs and Propolis-NPs on strong biofilm producer coliform isolates

The MIC of nano zinc-oxide against *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella ozanae* and *Citrobacter freundii* was 35ug/ml. Antibiofilm was observed at ZNO-NPs concentrations 8.75 and 17.5 µg/ml for both *E.coli* and *Citrobacter freundii*, while *Klebsiella* species biofilm was inhibited at 17.5 µg/ml (Table 5). Propolis-NPs MIC was 37.5 %for *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Klebsiella ozanae* and *Citrobacter freundii*, while it inhibited biofilm formation by all of them at concentration 18.75 %(Table 6)

4. DISCUSSION

The results of the current study revealed isolation of 141/ 150 coliform bacterial isolates (94%), from visceral organs of freshly dead chickens of different ages and sexes. *E.coli* was isolated from 84 samples (56%), (figure 1). which nearly agreed with Hussein *et al*., (2022) who recorded 59.3% *E.coli* recovery from septicemic broiler chickens and Gharieb *et al*., (2023) who stated 55% *E. coli* isolation rate from visceral organs. On the other hand, higher isolation rate (86%) and lower one (30 %) were detected by Halfaoui *et al*., (2017) and Abd EL-Tawab *et al.,* (2017), respectively. Moreover, *3 Klebsiella species were isolated; K. Pneumoniae* (4%), *K. oxytoca* (6.6%), *K. ozanae* (11.3%), (Figure 1). Those species were also isolated by previous authors; Li *et al*., (2022) isolated *K. Pneumoniae* (4.6%), El-Tawab *et al.,* (2022) isolated *K. Pneumoniae* (75%) and *K. oxytoca (25%*) , El-Tawab *et al.*,(2018) isolated *K. oxytoca* (1.9%) , Fielding *et al.*,(2012) isolated *K.ozanae* 94.1% , Younis *et al*.,(2016) isolated *K. pneumoniae* (73.33 %) and *K. oxytoca*(26.67 %), Mi *et al.*,(2019) isolated *K.pneumoniae* (14.4%). Among the isolated coliforms spp., there were 24*Citrobacter freundii* isolates (16%), (Figure 1). That was slightly consistent with El-Tawab *et al*.,(2018) who isolated *Citrobacter freundii* (9.6%) from visceral organs of dead chickens. Biofilms are micro-communities formed by combination of bacterial cells together within a matrix of extracellular polymeric substances. They are important virulence factor which comes up with the protection of the microorganism not only from altered pH, osmolarity, nutrients scarcity, mechanical and shear forces

but also deceases their susceptibility to the antibiotics and host's immune cells (Sharma et al., 2019). The ability of the isolated coliform spp. to form biofilms was evaluated by Congo red agar method (qualitative assay). The results conveyed that all of them were biofilm producer but showed varying degrees (Table 1). *E.coli* exhibited strong biofilm (44%), intermediate (34.5%) and weak biofilm (21.4%) and also Tajbakhsh *et al*., (2016) found that 80 *E. coli* isolates were able to produce biofilm; strong biofilm (18.75%), intermediate biofilm (25%) and weak biofilm (56.25 %). Within the isolated *Klebsiella spp.,* strong biofilms were formed by 50 % *of K. pneumoniae* and *K. oxytoca* and 41.2% of *K. ozanae.* Unlike, Abebe *et al*., (2020) who illustrated biofilm formation by *Klebsiella oxytoca* isolates; 74%, 16%and 5% were strong, moderate, weak biofilm producers, respectively. In addition to *Klebsiella pneumoniae* isolates that were strong (60%) and moderate (40%) biofilm producers, respectively. While Lamey *et al.,* (2023) revealed that 92.5%of *E. coli* isolates were strong biofilm producers and 7.4% were medium while 23 *Klebsiella* spp isolates showed strong biofilm (91.3%) and medium biofilm (8.6%). *Citrobacter freundii* isolates in the recent study also formed strong (41.7), intermediate (37.5%) and weak biofilm (20.8%). while other study was conducted by Bunyan *et al*., (2020) who revealed strong biofilm (71.4%), moderate biofilm (14.3%), and weak biofilm (14.3%) isolates from patients. Another study detected formation of biofilm in clinical samples from patients (Zogaj*et al*., 2003). Strong biofilm producer Coliform isolates were tested to their antibiotic susceptibility against 5 antibiotics using disc diffusion method. They were resistance to more than one antibiotic (Table 2)**.** More than 81.08% of *E. coli* and 70% of *Citrobacter freundii* isolates were resistant to Amoxicillin, Oxytetracycline, Erythromycin and Trimethoprim Sulfamethoxazole, while *Klebsiella* spp isolates showed complete resistance (100%) to them. All tested isolates exhibited lowest degree of resistance to Gentamicin (14-30%).Similary, Gharieb et al., (2023) reported that *E. coli* showed resistance to Oxytetracycline (74.54%) and sensitive to Genatamicin (56.36%), in contrast, sensitive to Trimethoprim Sulfamethoxazole (60%). Another study conducted by Abd El-Dayem *et al.,* (2020) reported that *E.coli* was resistant to Amoxicillin, Sulfamethoxazole 75%, 100%, respectively. Additionally Abd El-Tawab *et al*., (2016) that found *E. coli* had resistance to Erythromycin (100%), Trimethoprim Sulfamethoxazole (93%) and Gentamycin (40%). Regarding *Klebsiella* spp, it has been reported by El-Tawab *et al.,*(2022) that they showed 100% resistant to Oxytetracycline and Trimethoprim Sulfamethoxazole.Furthermore, previously *Klebsiella* spp showed resistance to Oxytetracycline (100%), Erythromycin (90%), and Gentamycin (65%) in study carried by MI *et al.,* (2019). Additionally, Younis *et al*., (2016) demonstrated 100% resistance to Amoxicillin by *Klebsiella* spp. Comparably *Citrobacter freundii* revealed high sensitivity to Gentamicin, and resistant to Oxytetracycline, Trimethoprime Sulfamethoxazole by El-Tawab *et al.,* (2018). Thirty-seven strong biofilm producer *E.coli* isolates were identified serologically. The results identified 11 serotypes (Table 3). The most common serotype was O114:K90 (27%), followed by O26:K60 (18.9%), O78 (10.8), O91:H21, O128:H2, O55:K59 (8.1%, each) and O17:H18, O121:H7, O113:H4, O159 (2.7%, each). The obtained results came close to findings of Eid and Merfan *et al*., (2013) who serotyped *E.coli* isolates into O114:K90 (17.86%), O26:K60 (10.71%), O44:K74 (3.57%), O55:K59 (14.29%), O125:K70 (14.29%) and O111:K58 (10.71%). Also Hussein et al., (2022) identified *E. coli* O55 (4.5%) and O44 (16.6%), Rosario *et al.,* (2004) found *E. coli* O78 (5%) and O91, While Abd El- Tawab et al., (2016) revealed identification of *E. coli*O44, O78, O114, O26 and O91. Three strong biofilm *K.pneumoniae* were identified serologically using hyper muco-viscosity capsular antigen intoK2, K1 that agreed with Jian-*Li et al*., (2017) and Lamey *et al*., (2023). Emergency of antibiotics resistance phenomena was the motive for finding antibiotics alternatives. One of them is application of Nanotechnology (Siddiqi and Rahman, 2018). In the current study, antibacterial and antibiofilm effect of Zinc oxide NPs and Propolis nanoemulsion were estimated against strong biofilm of *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella ozanae* and *Citrobacter freundii*. MIC of Zinc oxide NPs was 35 ug/ml against*Escherichia coli, Klebsiella spp* and *Citrobacter freundii* (table 5), which was close to previously determined MIC (31.25 µg/ml) against *E. coli* by Alekish et al., (2018). Propolis NPs MIC which determined by ten-fold dilution in-vitro was found to be 37.5%for *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella ozanae* and *Citrobacter freundii* growth. For the authors own sake, there have been very few reports of the nano propolis but the antibacteial effect of propolis had been reported by many authors such as (Hegazi and Hady, 2001) which determined MIC ranged from 1800 to 3200 |ig /ml for *Escherichia coli*.In contrast, *E. coli* showed resistance against propolis (Hegazi et al., 2014).

The biofilm formation was inhibited at concentrations 8.75 and 17.5µg/ml Zinc oxide NPs by both *E. coli* and *Citrobacter freundii*, while at 17.5 µg/ml for *Klebsiella* species (Table 5), While, Propolis nanoemulsion inhibited biofilm formation by all of them at concentration 18.75 % (Table 6). In our experience, it was the first report evaluated the antibiofilm effect of Zinc oxide NPs and Propolis nano emulsion against coliform spp.

5. CONCULOSIONS

From the obtained results it could be concluded that coliform spp isolated from visceral organs of freshly dead chickens showed antibiotic resistance to commonly used antibiotics; Amoxicillin, Oxytetracycline, Erythromycin and Trimethoprim Sulfamethoxazole. Zinc oxide NPs yielded a safe antibacterial effect which may provide good antibiotics alternatives and using of natural nanoemulsion like propolis as antibiofilm and antibacterial has no residue and safe in cell till high concentration.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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