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Antimicrobial activity of Liposomal colistin against resistant *E. coli* in vitro and in vivo

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ABSTRACT

This study was performed to investigate the liposome coated colistin for enhancing the oral pharmacokinetics, bioavailability, delivery and efficacy of the drug against resistant *E. coli* strain. Minimum inhibitory concentration (MIC) of colistin (C) and liposomal colistin (L) was 1.56 µg/ml and 0.00156 µg/ml for O125 sensitive colistin strain (CS and LS), while 100µg/ml and 0.025 µg/ml for O125 resistant strain (CR and LR). In vitro, time kill kinetics of liposomal colistin against sensitive and resistant O125 recorded 100% and 82.8% as a reduction% at 1 MIC, while ≥99.9% at 2 MIC for both strains after 1hr incubation time. The pharmacokinetic/ pharmacodynamics profiles were studied by single oral dose of colistin and liposomal colistin at 100000 IU/kg b.wt in healthy and diseased chicken. The pharmacokinetic parameters; Cmax/MIC ratio for CS and CR were 3.5 and 0.06. While, liposomal colistin recorded $3.9x10^3$ and 247.2 for LS and LR, respectively. AUC/MIC ratios were 13.4, 0.248, $40.9x10^3$ and $2.6x10^3$ for CS, CR, LS and LR, respectively; proving the high efficacy of liposomal colistin with less significant activity of colistin. There was significant increment of $t_{1/2}$ Beta and MRT of liposomal colistin groups in comparison with colistin groups. Contraries, clearance Time (CL/F) was significantly decreased in liposomal colistin than colistin groups. Liposomal colistin enhanced the bioavailability% from 5.2% to 49.2%. Liver *E. coli* count revealed highly significant decrease of LC for both strains nearly similar to negative control group after repeated treatment for 5 consecutive days; indicating the great effect by liposomal colistin on both *E. coli* strains especially for colistin resistant strain. This study recommends the liposomal colistin formulation against multidrug resistant *E. coli* infections.

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INTRODUCTION

Avian Pathogenic *E. coli* (APEC) is considered as one of pathogenic and lethal bacteria in most poultry product (broilers, layers, breeding flocks, ducks, and geese). The APEC showed colibacillosis and colisepticemia signs such as air sacculitis, cellulitis, omphailitis, pericarditis, perihepatitis, swollen head syndrome, and other colibacillosis manifestations. On the other side, they threat public health via harboring and transferring antibiotic resistance genes around the world **(Nolan et al. 2013).** Furthermore, the outcomes attest to the relationship between colistin usage and the acquisition of antimicrobial-resistant bacteria from food-producing animals with transmission to human being **(Mezhoud et al. 2016).**

Colistin, polymyxin E (PME), has been used in veterinary medicine since 1959. It is isolated from the bacterium *Bacillus polymyxa colistinus.* It has a strong effect against Gram negative bacteria as *Escherichia coli, Salmonlla, Bacillus, Hemophilus* and *Pseudomonas aeruginosa* **Soliman et al. (2016)**. PME has two particular forms, colistin sulphate (for oral and topical use). The other form is negatively-charged methane sulfonate (MSA) salt of colistin, known as colistin methane sulfonate (CMS), or sodium colistimethate (SCM) in aerosol and inject able forms. CMS is a poly methanosulfonylated inactive prodrug of colistin and is microbiologically inactive; it is hydrolysed spontaneously to release active PME **Pacheco et al. (2019)**. Colistin's mechanism of action depends on binding to the bacterial outer membrane lipopolysaccharide (LPS) and bacterial endotoxins leading to deactivation and neutralization of bacterial endotoxins by dis-

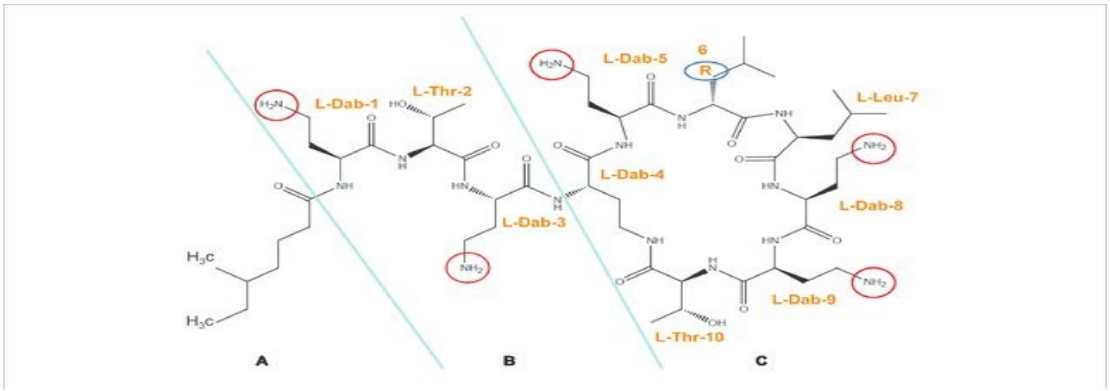

Fig (1): Chemical structure of colistin. All reactive amino acids groups are encircled except R6 (polymyxin B active site).

The extensive use of antibiotics for treatment of multidrug resistant bacteria especially *Enterobacteriacea* affect the strength of colistin efficacy. Also, it was leading to the development of colistin resistant bacteria as occupied mostly in different strains of *E. coli* **Mead et al. (2021)**. On the other hand, the low absorption of colistin through the gastro-intestinal tract even in infected animals prove that oral colistin administration is increasing colistin resistance by exerting selection pressure (due to antibiotic) on the intestinal flora of animals **Kumar et al. (2020).**

Liposomes are one of the oldest delivery system to transport the bioactive agents to cells and tissues while, protecting the drug from physiological barriers **(Kashapov et al. 2021).** Liposomes structure mainly consists of enclosed vesicles of concentric self-assembling lipid bilayers mainly composed of phospholipids and cholesterols (**Allen 1997).** There are different administrative routes of liposomes as drug delivery carriers such as oral, parenteral, nasal, ocular, transdermal, and pulmonary routes (**Liu et al. 2022).** The main advantages of liposomes are their safety and biocompatibility due to their similarity to natural membranes. Consequently, liposome surfaces can be easily modified by coupling to ligands and/or polymers to target delivery **(Bozzuto and Molinari 2015).** Moreover, liposomal antibiotics have an advantage of prolonged release and so longer duration of action with reduced frequency of administration **(Allen and Cullis**

2013). Colistin is effective when loaded in liposomes due to the electrostatic interaction with the anionic lipid **(Li et al. 2016).**

Here, we highlighted the effect of liposomal colistin versus (vs) commercial colistin against different strains of *E. coli* either sensitive or resistant strain to colistin by studying the pharmacokinetics/pharmacodynamics model in broilers in addition, effective bioassay experimentally.

MATERIALS and METHODS

Drugs

Colistin sulphate®: is manufactured by Vetwic, Egypt. It is water soluble powder. Each one gm contains colistin sulphate 5,000,000 IU.

Chemical and reagents

Colistin sulphate standard, span 60, tween 65, tween 80, cholesterol, soyalecithin, ammonium hydroxide, ethanol (95%) and chemicals of analytical grade were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Milli-Q system (Millipore, Bedford, MA, USA) was used for deionized water purification. Acetonitrile and Methanol of HPLC grade were obtained from Merck (Darmstadt, Germany).

Preparation and characterization of liposomal colistin:

Preparation of Liposomal colistin was done according to **(Aboumanei et al. 2021)**. Nano liposome was prepared by thin film hydration method. Briefly, cholesterol: surfactant Tween (65 and 80): soyalecithin in a ratio (1:3:1) were dissolved in 10 ml of chloroform. Colistin (25 mg) was dissolved in 15 ml of chloroform and methanol mixture. The mixture was sonicated for 30 minutes then evaporated at 50°C. The traces were reconstituted in 10ml phosphate buffer at pH 7.4. The compound was stabilized by keeping it at 4°C for 24hrs to have unilamellar liposomal structure with nanometer size range from 5-10nm **(Chandrawati et al. 2010).** The compound (Liposomal colistin) was characterized by TEM (Transamination electron microscopy) Model JEOL JSM-6400, UK **(Hirschle et al. 2016).** The polydispersity index (PdI) was calculated by dividing the square of the standard deviation over the mean particle diameter (**Tekade 2018)**

Biological characterization of *E. coli* isolates and serotyping:

Pathogenic *E. coli* isolates were collected previously from internal organs (liver, heart and lung) of the diseased chicken and identified as described by **(Nolan et al. 2013).** Stored isolates (n= 24) were incubated aerobically into buffer peptone water at 37°C for 24 h. A loopful from each incubated isolate was streaked onto MacConkey agar (Oxoid, UK) and Eosin Methylene Blue agar (Lioflichem, Italy) plates were then incubated at 37° C for 24 h. The suspected colonies appeared as a pink color colony on MacConkey and green metallic sheen colonies on Eosin Methylene Blue agar. Suspected *E. coli* colonies were subjected for further biochemical examination (indole test, methyl red, voges Proskauer "VP", citrate utilization, oxidase test, and Triple Sugar Iron "TSI"). Furthermore, serotyping of *E. coli* isolates were performed using Somatic (O) antiserum according to the kit instruction of (DENKA SEIKEN Co., Tokyo, Japan).

Antimicrobial Sensitivity Test (AST)

Applied antibiotic sensitivity test for identified *E. coli* isolates using Colistin sulphate (CT) 10µg disc (Oxoid, Basingstoke, UK) on Mueller-Hinton agar as previously described **(WHO, CDC, 2003)**, and inhibition zones were interpreted following the guidelines of Clinical and Laboratory Standards Institute **(CLSI, 2021).**

Quantitative determination of Colistin:

Determinations of Minimum Inhibitory Concentration (MIC) by (E- Test):

On examination of some *E. coli* isolates using Colistin Ezy MIC strip (CL) (0.016- 256 µg/ml). The sensitive strain shows ≤ 2 µg/ml, but the resistant strain shows ≥4 µg/ml **(CLSI, 2021).**

Estimation of MIC and MBC for colistin and liposomal colistin:

Preparation of Inoculums standardized to give density 10^6 colony-forming units (CFU/ml) to put 100 µl of prepared inoculum according to **(Elisha et al. 2017)** for determination of MIC of selected isolates (colistin sensitive and colistin resistance *E. coli* isolates) against col-

istin sulphate and Liposomal colistin in addition, Minimum Bactericidal Concentration (MBC) using broth micro-dilution method according to **Yu et al. (2004).** MIC was determined using microplate dilution method on 96 well plates (U- shaped). Concentrations range of colistin sulphate was 0.39-1000 µg/ml, while liposomal colistin range was 0.0078-100 µg/ml. Briefly 100 µl of 10^6 CFU/ml of each of the tested bacterium was inoculated in wells (10 well) with equal volumes of tested colistin sulphate and liposomal colistin in different concentrations. The microplate was incubated aerobically at 37 °C for 24 h. The last two wells contain controls (organism control and material under test control). The lowest concentration (highest dilution) of the tested material that produced no visible growth (no turbidity) after 24 h when compared with the control well was considered as initial MIC which confirmed after plating of all concentrations on MacConkey or TBX agar media. Also, MBC value was determined after sub culturing the test dilutions which showed no visible turbidity on to freshly prepared MacConkey or TBX agar media. The agar plates were incubated further for 18-24 h at 37°C. The highest dilution that yielded no single bacterial colony on the agar plates was taken as MBC while, the previous concentration with lowest bacterial growth considered MIC.

Assessment of antimicrobial activity
Invitro time-kill test:

The suspension based in vitro time-kill test has been standardized by ASTM International **ASTM E2315 (2003)** which is the Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure. It measured the change in *E. coli* count within a specified sampling time after exposure to 1 and 2 MIC of colistin and liposomal colistin in vitro. These concentrations were tested against sensitive and resistant strains (O125) on Mueller-Hinton broth and incubated at 37°C for 24hr.

Ex vivo Time-Killing Curve:

Serum samples collected from healthy chicks which had been given colistin and liposomal colistin orally at 100000 IU/kg b.wt. were used for the time killing experiments. Samples were collected at the following time points: 0, 0.5, 1, 2, 4, 8, 12, and 24 h after colistin and Liposomal colistin administration. Counting of *E. coli* O125 for sensitive and resistant strain was done using the aforementioned method at the in vitro time killing studies. Ex-vivo time-killing curve was calculated by the mean log10 (CFU/ml) values (n = 5) vs. time (h) with different serum samples concentrations at the above mentioned time points.

In Vivo assay by chick's challenge:
Preparation of *E. coli* inoculum for Oral Challenge:

The selected *E. coli* strains were inoculated in buffered peptone water broth aerobically for 24 hr at 37°C. Broth was diluted with sterile buffer saline and adjusted using spectrophotometer (OD.600 wave length) to be 10^8 CFU/ml according to (**Wang et al. 2017).** Chicks were orally challenged with 0.1 ml of prepared *E. coli* suspension by a sterile automatic pipette for 6 groups.

Animals and Experimental design:

One hundred and five clinically normal male Cobb one day old chicks and forty clinically normal chickens were selected from commercial private farm, Egypt. They were kept in separated cages at biosecurity level- two (BSL-2) animal facilities at Animal Health Research Institute (AHRI), Dokki, Egypt. They fed on a standard commercial ration free from any antibiotics before starting till the end of the experiment and water *ad Libitum*. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Animal Health Research Institute.

Efficacy of colistin sulphate and liposomal colistin study:

The experiment was designed to investigate the efficacy of liposomal colistin and colistin in 3 days old chicks infected with two strains of *E. coli* O125 (sensitive and resist to colistin). Birds were kept off feed for 12 hrs in order to reduce crop bulk, thus expediting flushing of the inoculating organism. Chicks were divided equally into 7 groups as follow: **Gr N** kept as negative control (non-infected non treated), **Gr PS** was experimentally infected with *E. coli* sensitive strain (S) (O125) to

colistin sulphate as positive control, **Gr PR** was experimentally infected with *E. coli* resistant strain (R) (O125) to colistin sulphate as positive control, **Gr CS** was experimentally infected with *E. coli* sensitive strain (S) to colistin sulphate and treated orally in a dose of 100,000 IU colistin base/kg. b. wt. once daily for 5 consecutive days, **Gr CR** was experimentally infected with *E. coli* resistant strain (R) to colistin sulphate and treated orally in a dose of 100,000 IU colistin base/kg. b. wt. once daily for 5 consecutive days, **Gr LS** was experimentally infected with *E. coli* sensitive strain (S) to colistin sulphate and treated orally in a dose of 100,000 IU Liposomal colistin/kg. b. wt once daily for 5 consecutive days and **Gr LR** was experimentally infected with *E. coli* resistant strain (R) to colistin sulphate and treated orally in a dose of 100,000 IU Liposomal colistin /kg. b. wt once daily for 5 consecutive days. The chicks were observed periodically till the end of the experiment (2 weeks). Clinical signs were noticed periodically and subjected to PM examination for any dead chicks and one euthanized chicks daily from each group for *E. coli* count.

Pharmacokinetics/pharmacodynamics modeling study:

Forty clinically normal chickens were divided equally into 6 groups. Four groups (Gr CS, Gr CR, Gr LS and Gr LR) were designed as the efficacy experiment but treated orally as a single dose of colistin and liposomal colistin. The other two groups were **GrCH** which treated with colistin orally in a single dose of 100,000 IU colistin base/kg. b. wt (colistin treated non infected) and **Group LH** that treated with liposomal colistin orally in a single dose of 100,000 IU/kg. b. wt (liposomal colistin treated non infected).

The pharmacokinetic parameters were calculated according to equations integrated by **(Baggot 1977), (Baggot 1978 a)** and **(Baggot 1978 b).** AUC/MIC and the Cmax/MIC ratios were determined to detect the efficacy of colistin and liposomal colstin.

Sample collection

Samples for estimation of efficacy on *E. coli* count: Three chicks were euthanized daily from each group till complete the treatment application (for 5 consecutive days). Liver samples were collected from each chick individually and subjected for *E. coli* count according to **(ISO/BS 16649-2:2001)** then the suspected colonies (blue colonies on TBX medium) were counted and calculated.

Samples for pharmacokinetic parameters:

Blood samples were collected from the right jugular vein of all treated groups at 0.25, 0.5, 1, 2, 4, 6, 8,10, 12 and 24 hours after oral administration of drug in clean tubes without anticoagulant. The serum was separated by centrifugation at 2000 rpm /10 min) and stored at -20°C until colistin and liposomal colistin estimation by HPLC assay.

Colistin and Liposomal colistin HPLC assay:

HPLC system and chromatographic conditions:

HPLC [Dionex-UltiMate® 3000, autosampler, column compartment, Ultimate 3000 pump, Diode array detector), The samples were analyzed on reversed phase (RP) Thermoscientific C18 column (4.6 mm i.d., 250 mm, 5 µm). The isocratic elution mobile phase was acetonitrile: 2% acetic acid: methanol in a ratio (65: 30:5 v/v) the flow rate of 1.0 ml/min. The detection wavelength was at 280nm with injection volume (20µl) and the column temperature was set at 25°C.

Standard preparation:

Stock standard solution of colistin and liposomal colistin were prepared by dissolving 10 mg in 10 ml of 20% acetonitrile to have 1 mg/ml. The fortification solution was diluted to a concentration of 10 µg/ml. Fortification solution was freshly prepared daily. Calibration curve of serum was prepared by spiking blank serum with various volumes of fortification solution to a concentration range of 0.25, 0.5, 1.0, 2.5 and 5µg/ml and spike blank serum to prepare quality control (QC) samples at 0.3, 0.6 and 0.9 µg/ml. The prepared liposomal colistin was calibrated on HPLC by centrifugation at 9000 xg for 15 minutes at 4°C (S-16 KL, Sigma). The supernatant was eluted in methanol to release the entrapped colistin (**Aboumanei et al. 2021).** The QC samples

were used for achieving the validation requirements in terms of linearity and range, precision, recovery Limits of detection and quantification (LOD & LOQ), specificity, robustness and system suitability test (SST) according to **USP, 2019**. The extraction of serum samples was performed according to **Matar and Al-Refai (2020).**

Statistical analysis

The results were calculated as mean ± standard error (SE). Statistical investigation was determined by Statistical Package for Social Science (SPSS), version 20 (SPSS Inc., Chicago, IL, USA) for windows. The data between various groups was compared by one-way analysis of variance (ANOVA) for all tests with t-test for pharmacokinetic parameters; values with $P < 0.05$ were considered as statistically significant **(Kim, 2014).** The pharmacokinetic variables were determined using PK Solver: An add-in program for Microsoft Excel, version 2 (**Zhang et al. 2010**).

RESULTS

Characterization of liposomal colistin by Transmission electron microscope (TEM):

TEM was used for determining the size and morphology of liposomal colistin nanoparticles. TEM showed sphere shape, no aggregation, and narrow size distribution 4.92±0.83nm with a polydispersity index (PdI) 0.14±0.01 indicating the higher stability, and uniformity of the vesicle size as shown in Fig. (2).

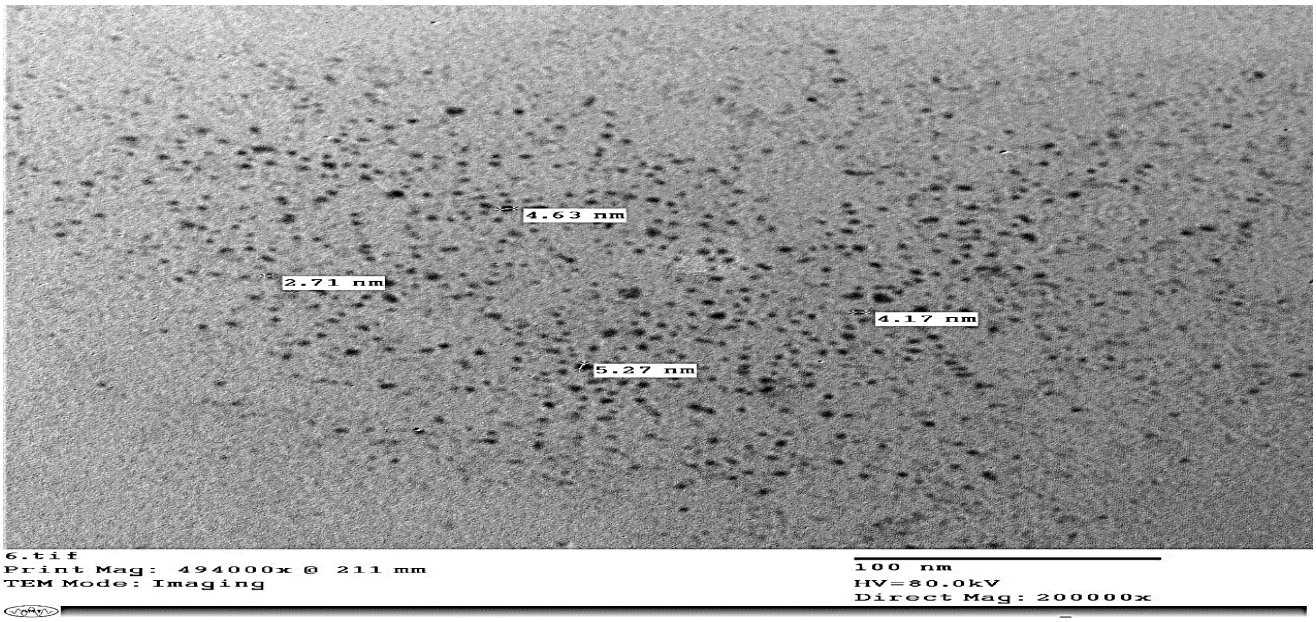

Fig (2): TEM image of liposomal colistin nanoparticles.

HPLC method validation and chromatograms:

Data of the validation procedures for HPLC method was illustrated in **Table 1**. The specificity and selectivity were clarified in **Fig. 3.**

Table (1) Mean ±SD of colistin concentrations (µg/ml) in broiler's serum:

Parameter		Serum	Acceptance Criteria
Range (µg/ml)		0.25-5	-
Retention time		2.2	-
Regression equation		y = 0.5965x -5.6652	-
Correlation coefficient(r^2)		0.9996	>0.99
Intraday precision (RSD %)		0.08	<1
Inter-day precision (RSD %)		0.3	<2
Recovery%		98.5- 100.6	85-115
Accuracy		99.7±1.1	± 2%
DL (µg/ml)		0.002	-
QL (µg/ml)		0.006	-
Pooled robustness RSD %)		1.9	<6
SST	Theoretical Plates	8199.8± 2.3	>2000
	Tailing Factor	0.96±0.001	≤2
	Symmetry Factor	1.048±0.006	<1

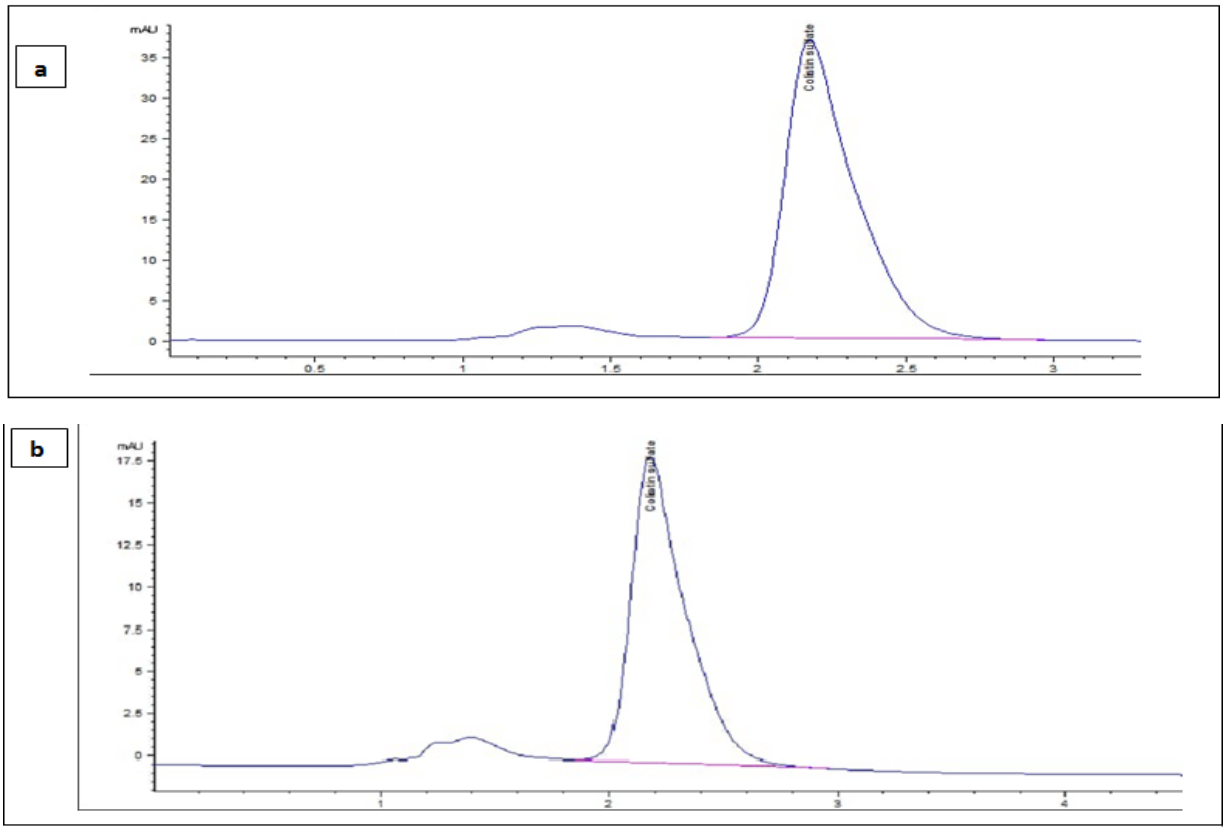

Fig (3): chromatogram of a: colistin at 1µg/ml, b: lipo-colistin compound at concentration 0.5µg/ml.

Selection of *E. coli* strain:

Twenty four *E. coli* strains were identified and serogrouped to 10 isolates O125, (3) isolates for each O86a and O111 serotype, also, O18, O127 and O157 were 2 isolates for each but O18 and O166 were identified only in one isolate. Selecting two strains (sensitive and resistance to colistin) by AST test which showing sensitive for all tested isolates except 3 isolates were resist to colistin sulphate (O125 "n=2" and O127 "n=1"). They were confirmed by Colistin Ezy MIC strip (CL); the selected sensitive strain had 0.75µg/ml while, the selected resistant strain had 4µg/ml of the O125

serotyped *E. coli* strains.

Estimation of MIC for colistin and liposomal colistin:
The two *E. coli* stains (sensitive and resistant to colistin) were tested against colistin and liposomal colistin. MIC of colistin and liposomal colistin against colistin resistant *E. coli* strain was 100 µg/ml and 0.025 µg/ml, respectively. While, it was 1.56 µg/ml and 0.00156 µg/ml, respectively against colistin sensitive *E. coli* strain.

Assessment of antimicrobial activity.
The in vitro time-kill kinetics profile of resist and sensitive strains of *E. coli* (O125) either by colistin or Liposomal colistin was shown in Table 2. This study determined of the speed of bactericidal activity of colistin vs liposomal colistin. It is noticed that the increment of liposomal colistin concentration to 2 MIC has prompted the maximal killing action with reduction % ≥ 99.9 (the Log 10 decreased 3 counts than the initial count) at 1hr for sensitive strain and 2hrs for resistant strain. Unlike Gr LS and LR, bacterial re-growth of Gr CS and CR was noticed after 3hrs at both 1 MIC and 2 MIC.

Table (2) Time-kill kinetics antibacterial study of Colistin and liposomal colistin against sensitive and resistant *E. coli* (O125) strains at 1 and 2 MIC:

Time interval (hr)	**Log CFU of strains**				**Reduction% (resulted count-initial count/ initial count)x100**			
					1 MIC			
	CS	LS	CR	LR	CS	LS	CR	LR
0	4.01	3.99	4.22	4.03	5.13	2.23	1.70	11.39
1	4.00	3.95	4.20	3.99	6.04	7.12	10.97	15.38
2	3.97	3.92	4.16	3.96	10.45	11.56	14.95	19.04
3	4.05	3.79	4.16	3.85	12.20	17.84	16.50	24.19
4	4.48	3.72	5.18	3.79	6.34	22.30	16.92	39.23
6	4.60	2.48	6.28	3.08	3.75	50.05	1.99	51.94
24	4.60	0.00	6.45	1.11	3.75	100.00	0.46	82.80
					2 MIC			
0	3.79	2.00	4.22	2.62	6.97	50.97	7.21	42.27
1	3.78	0	4.20	2.00	11.27	≥99.9	10.97	57.63
2	3.72	0	4.16	0	15.96	≥99.9	14.95	≥99.9
3	3.79	0	4.24	0	17.84	≥99.9	16.50	≥99.9
4	4.55	0	4.88	0	4.95	≥99.9	21.72	≥99.9
6	4.21	0	4.58	0	15.07	≥99.9	28.59	≥99.9
24	4.02	0	4.42	0	20.81	≥99.9	31.82	≥99.9

The Ex- vivo time-kill curves were significantly elucidated at 2 hr time point for both Gr CS and Gr CR; while, 1h for Gr LS and Gr LR using samples collected after oral dosing of colistin and liposomal colistin at 100000 IU/kg **Fig 4 (a, b, c and d)**. Viable bacteria count was below 10 CFU at Gr LS after only 1 hr incubation period.

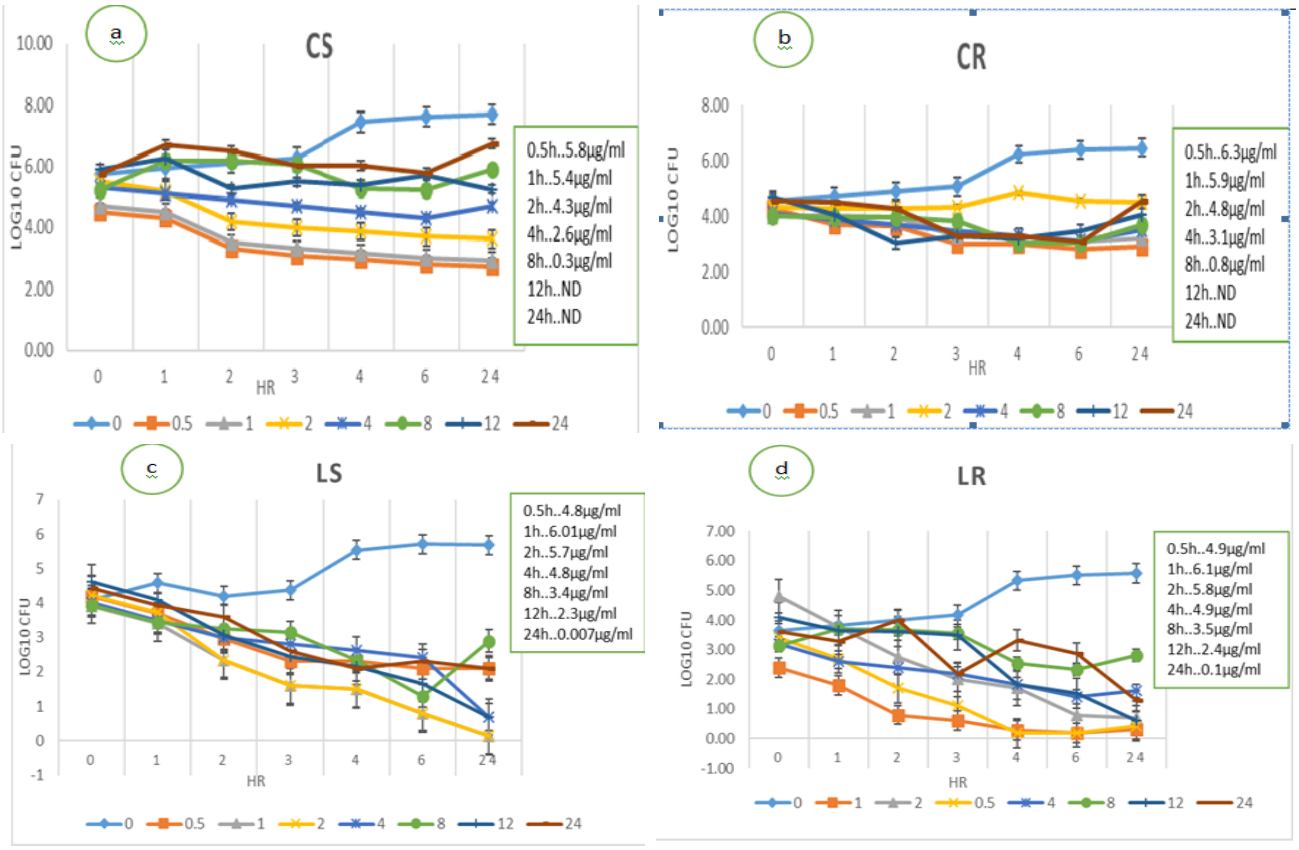

Fig 4: Ex vivo inhibition of *E. coli* in serum after oral administration of colistin and liposomal colistin in different groups CS, CR, LS and LR (sampling times of 0, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h). Values are expressed as mean ± SE. (n = 5).

Pharmacokinetics/pharmacodynamics Modeling:

The MIC of colistin and liposomal colistin for *E. coli* O125 sensitive and resistant strains were determined as 1.56, 100, 0.00156 and 0.025 µg/mL for Gr CS, Gr CR, Gr LS and Gr LR, respectively. Following oral administration of colistin and liposomal colistin 100000 IU/kg b.wt.,Cmax/MIC ratio was 3.5, 0.06, $3.9x10^3$and 247.2 for Gr CS, Gr CR, Gr LS and Gr LR, respectively. Moreover, AUC/MIC ratio was 13.4, 0.248, $40.9x10^3$ and $2.6x10^3$ for Gr CS, Gr CR, Gr LS and Gr LR, respectively. These results proved the high efficacy of liposomal colistin with less activity of colistin against the tested strains with easier development of *E. coli* resistance to colistin.

The pharmacokinetic parameters of colistin and liposomal colistin in broilers after single oral dose were illustrated in Table 3 and Fig 5. There were significant increment of $t_{1/2}$ Beta and MRT in Gr LH, LS and LR in comparison with Gr CH, CS and CR, respectively. Contraries, there was a significant decrease in CL/F (clearance Time) in Gr LH, LS and LR in comparison with Gr CH, CS and CR, respectively. These data indicated a good distribution and antimicrobial activity of liposomal colistin than colistin.

The estimated bioavailability% (F) was illustrated in Table 3. It was recorded in all groups with remarkable increase in all liposomal colistin treated groups (Gr LH, Gr LS and Gr LR) than colistin treated (Gr CH, Gr CS and Gr CR). The highest bioavailability % was detected at Gr LH (10.5%).

Table (3): Kinetic Parameters of colistin and liposomal colistin in broilers after single oral dose at 100000 IU/kg b.wt.

Kinetic parameters	**Gr CH**	**Gr CS**	**Gr CR**	**Gr LH**	**Gr LS**	**Gr LR**
$t_{1/2ka}$ (h)	0.55±0.001	0.57±0.02	0.53±0.06	0.49±0.5*	0.57±0.2	0.42±0.7***
$t_{1/2Beta}$ (h)	2.4± 0.002	2.24± 0.1	2.9±0.002	8.2±0.1*	7.86±0.3**	6.6±0.01***
V/F (mg) (µg/ml)	0.45±0.05	0.46±0.06	0.43±0.02	0.58±0.6*	0.54±0.2**	0.69± 0.024***
CL/F(mg) (µg/ml)/hr	0.21± 0.02	0.23±0.01	0.18±0.02	0.066±0.3*	0.071±0.6**	0.074±0.08***
T_{max} (h)	1.14± 0.03	1.15±0.04	1.12±0.05	1.66±0.8*	1.68±0.4**	1.75± 0.07***
C_{max} (µg/ml)	5.7± 0.24	5.48± 0.1	6.03± 0.2	6.34±0.5*	6.16±0.1**	6.18± 0.09
AUC_{0-24}(µg h/ml)	22.4± 0.6	20.9± 0.7	24.8± 0.8	68.27±0.2*	63.9±0.7**	64.2± 0.5***
MRT (h)	3.8± 0.25	3.5± 0.04	4.33±0.08	12.02±0.9*	11.5±0.1**	10.07±0.09***
F%	5.75±0.5	5.2±0.7	6.67±0.3	49.2±0.4*	45.5±0.2**	43.9±0.09***

Values are the mean ±SD (n = 5).
$t_{1/2ka}$: absorption half-life, $t_{1/2Beta}$:elimination half-life,V/F: apparent volume of distribution, CL/F: apparent total clearance of the drug from serum, T_{max}: time to reach maximum serum concentration, C_{max}: maximum serum drug concentration,AUC_{0-24}: area under the serum concentration-time curve from time zero to time 24 hs, MRT: mean residence time, F%: oral bioavailability %.
*: Significant change at $p<0.05$ with respect to Gr CH using t-test.
**: Significant change at $p<0.05$ with respect to Gr CS using t-test.
***: Significant change at $p<0.05$ with respect to Gr CR using t-test.

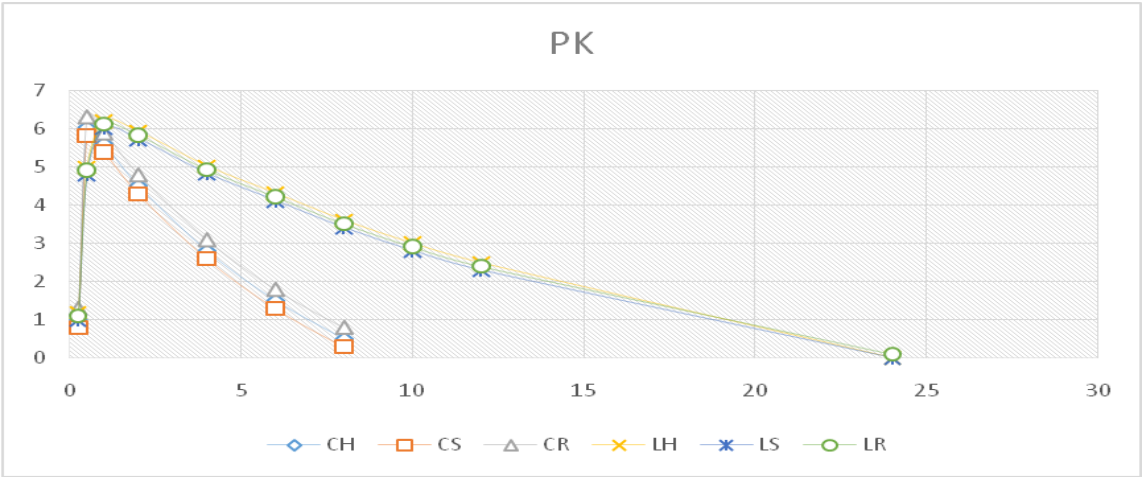

Figure (5): Mean serum levels of colistin and liposomal colistin versus the time-course after a single oral dose at 100000 IU/kg b.wt.in broilers.

In vivo assay of colistin and liposomal colistin effect in experimentally infected chicks: Mortality%, clinical signs and PM examination:

The experiment design showed no mortality rate in groups (LS and LR) while, Gr CS and Gr CR recorded 20% mortality. All infected groups showed depression and brown diarrhea with pasty vent which recovered with liposomal colistin more than colistin. On the other hand, the necropsy of periodic euthanized chicks detected that the all internal organs especially intestine of liposomal colistin treated groups (LS and LR) appeared in normal condition. Meanwhile, the treated group with colistin (CS and CR) showed nephritis and general congestion of muscle, liver, kidney and intestine as shown in Fig (6). Moreover, con-

gested retained yolk sac which normally absorbed during the first week of chick age **(Murakami et al. 1992)**. Postmortum (PM) of colistin treated groups indicated its failure for treatment of *E. coli* infection.

Enumeration of *E. coli* for experimental group:

The mean of *E. coli* count for positive control groups (PS and PR) were the highest count. Then, the count of Gr CS was lower than Gr CR. These data proved the efficacy of colistin on *E. coli* sensitive strain (O125) with great less activity on resistant colistin strain. On the other hands, the counts of Liposomal colistin treated groups (LS and LR) were slightly similar to negative group (N). This indicated the increased liposomal colistin efficacy on both sensitive and resistant strains as shown in Fig. (7). Finally, there was a highly significant difference between groups and within groups using one-way analysis of variance (ANOVA). Gr CS and Gr LS recorded high significant difference with Gr PS, while no significant difference between Gr CS and LS. Also, Gr LR showed a highly significant decreased count when compared with Gr PR and Gr CR. While no significant difference was noticed between Gr PR and Gr CR. In Conclusion, liposomal colistin is a potentially an effective antibiotic especially for treatment of colistin resistant *E. Coli* strains.

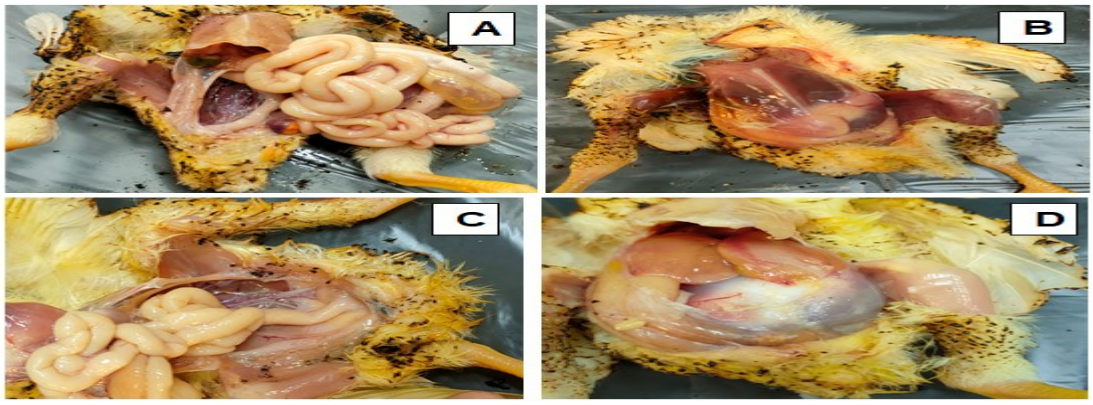

Fig (6): Post mortem (P.M) lesions of treated groups; A and B is treated group with colistin showed general congestion and nephritis with congested, retained yolk sacs, C and D is treated groups with liposomal colistin which apparently normal with very bright appearance of intestinal wall.

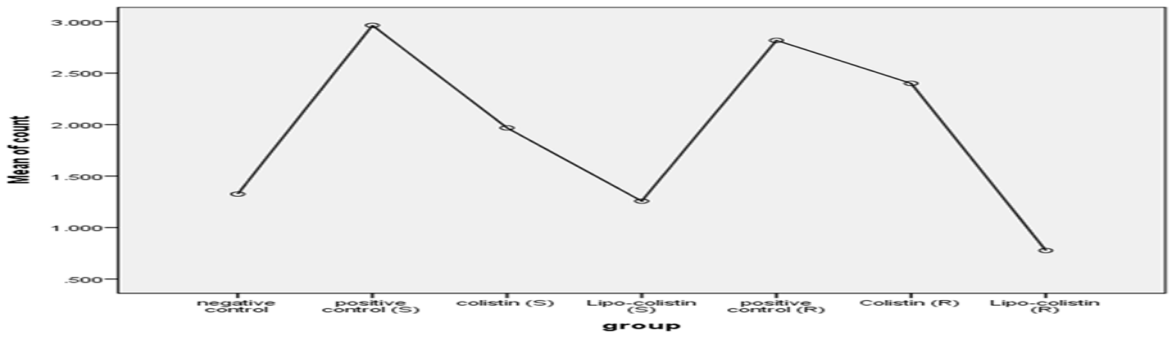

Fig (7): Mean *E. coli* counts for each experimental groups

DISCUSSION

Antibiotic-loaded liposomes can exhibit synergistic activity against bacteria beyond the activity of each antibiotic alone **(Schiffelers et al. 2002).** This help in resolving the antimicrobial resistance to colistin which rises mainly by gram negative bacteria through modifications of the negatively charged phosphate groups of lipid A or by loss of LPS in its cell wall **(Tran et al. 2016)**. The liposomes adopted the anticipated charge from the cationic colistin, indicating direct association of the amphiphilic lipopeptide with the liposome structure destructing its resistance (**Wallace et al. 2012).** In our result, the liposomal colistin is more effective than colistin especially for treatment of colistin resistance strains. However, Gr CS has no significant effect with Gr LS, but liposomal colistin avoids the side effects of colistin such as high mortality rate and internal organs lesions. These were confirmed by **(Demetriou et al. 2019)** who found new theoretical and experimental benefit in applications of diamagnetic liposomes to improve biological processes. Also, **(Salem et al. 2005)** and **(Bapolisi et al. 2020)** confirmed that encapsulation of certain antibiotics in liposomes could enhance their effect against microorganisms invading cultured cells and in animal models. As our result, **(Bapolisi et al., 2020)** represents an illustrative example of co-loading of antibiotics into liposomes, which could lead to the development of novel therapeutic tools for tackling antimicrobial resistance.

Through determination of in vitro and ex vivo time kill kinetic, colistin is a concentration dependent antibiotic **(Luo et al. 2019)** and kill resistant *E. coli* strains. This results were convenient with **(Cui et al. 2016)** who reported that colistin kill persisted *E. coli* in concentration dependent manner. Moreover, via pharmacokinetics/pharmacodynamics model, the liposomal colistin recorded AUC/MIC ratio higher than 100–125 with clinical success rate over 80%. The Cmax/MIC ratio was above 8–10 indicating the better clinical results and less bacterial resistance **(Levison and Levison, 2009).**

By studying the pharmacokinetic parameters, we found that the highest serum concentration was detected within 1 hour after colistin and liposomal colistin administration. In colistin treated groups (Gr CS and CR), it was in detectable limits just for 8 hours of administration. These data was in the same line with **(EMEA 2002)** who proved that colistin sulphate was poorly absorbed after its oral administration in the drinking water and no longer detection after 6 h in different species, so it has to be prescribed for gastrointestinal infection. Unlikely, liposomal colistin treated groups (Gr LS and Gr LR) achieved good detectable serum concentrations over 24hr after the initial administration. These indicated good absorption of liposomal colistin and considered as a good choice of systemic infection.

Colistin's absorption half life time ($t_{1/2}$ ka) was slight short in Gr LR expressed as 0.42± 0.2 hr than the other groups. This was explained by **(Ledwaba et al. 2020)** who reported that enteropathogenic *E. coli* (EPEC) alters the intestinal permeability due to its toxins while colistin absorbed by passive diffusion. This might explain the higher absorption rate of liposomal colistin treated groups rather than colistin group with respect of liposomal colistin slow release manner and high stability within tissues **(Wallace et al. 2012)**.

The elimination half-life ($t_{1/2\,\beta}$) of liposomal colistin was longer (6.6±0.01 to 8.2±0.1 h) than colistin (2.24±0.1 to 2.9±0.02 h). It means that liposomal colistin takes longer time to act but on the positive side its clearance is faster than colistin (**Martinez et al. 2012)**. Generally, drugs with very short half-lives can lead to dependency if taken over a long period of time **(Toutain and Bousquet-mélou 2004).** The value of Mean Residence Time (MRT) is the average time that molecules of a dosed drug spend in the body. Liposomal colistin groups recorded more MRT (10.07±0.09 to 12.02±0.9 h) than colistin groups (3.5±0.04 to 4.33±0.08 h). This time elongation means more drug absorption **(Jackson et al. 2012)** and explained elongation of $t_{1/2}\,\beta$ values.

The volume of distribution (V/F) was generally increased with infection which reflected more localization of drug at the site of infection **Matson and Fallon (2009)**. The marked concentration level was recorded at Gr LR

(0.69±0.02) µg/ml. This indicated good distribution which explained by increasing capillary permeability with the critical infection **van den Broek et al. (2021)** and the lipophilic nature of liposomal colistin **Xing et al. (2016).**

Oral Colistin mainly eliminated through liver and found in feces **EMEA, (2002).** In this study, total body clearance of liposoml colistin (CL/F) was higher (0.066±0.3 to 0.074±0.08 µg/ml/h than colistin groups (0.21±0.02 to 0.23±0.1 µg/ml/h). It is explained by the high entrapment affinity of liposome and its delivery to the hepatocytes **Baratta et al. (2009)**.

The maximum serum colistin concentrations (C_{max}) were 6.34±0.5 and 6.16±0.2 µg/ml in Gr LH and Gr LS, respectively and attained at 1.66±0.8 and 1.68±0.4 h. These results were slightly significantly higher than Gr CH and Gr CS. These data might be explained by **(Aboumanei et al. 2021)** who recorded higher Cmax in rates (16.4±0.23 µg/ml) treated with chitosan coated colistin nanoliposomes than colistin concentration (2.86±0.1 µg/ml). This proved the sustained release of liposomal colistin. The difference in values might be attributed to difference in species and addition of chitosan for nanoliposome preparation.

Groups (LH, LS and LR) attained higher area under curve (AUC) ranged from 64.2±0.5 to 68.9±0.2µg/ml/h than Gr CH, CS and CR. This indicated high antibacterial activity of liposomal colistin than commercial colistin **(Firsov and Mattie 1997)**.

Oral bioavailability % is one of the most important parameters to the drug design development. It is the fraction of an oral administered drug that reaches systemic circulation and the point of pharmacological effect **(Rhouma et al. 2015).** In this study, the bioavailability% had significant increase in liposomal colistin treated groups than colistin treated ones. Liposomal colistin groups (LH, LS and LR) were bioavailable 8 times than colistin groups (CH, CS and CR). Whereas, **(Béïque and Zvonar, 2015)** reported that the bioavailability of some antibiotics increased directly with the dose level which could reflect oral liposomal colistin bioavailability. Besides, the acquired bioavailability of liposomal colistin was in the same level of some widely used antibiotics for broiler chickens and laying hens as erythromycin which attains less than 50% bioavailability **(EMEA 2002)** with respect of less affectivity of erythromycin against *E. coli* strains **(Leclercq et al. 2013).** This indicated a clearer tendency of liposomal colistin use for *E. coli* treatment.

CONCLUSION

Liposomes coated colistin proved a synergistic activity against sensitive and resistant *E. coli* above the activity of colistin alone. Liposomal colistin overcomes the side effects of colistin when used for poultry industry. This study recommends the use of liposomal colistin in chickens because of its good pharmacokinetic and pharmacodynamics profile and overcome antimicrobial resistance.

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