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Evaluation the antibacterial effect of quercetin nanoparticles (QUENPS) on drug-resistant *E. coli* strains in rabbits

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ABSTRACT
The present study aimed to evaluate the antibacterial effect and the antioxidant potential of quercetin nanoparticles on drug-resistant *E. coli* O157 in New Zealand White Rabbits. Initial surveillance lasting two years (2018-2019) was performed to isolate and molecular detection of some virulence genes of some drug-resistant *E.coli* strains circulating in NZW rabbits in Egypt.The selected *E.coli* O157 strain in our experimental work produced strong biofilm and sxt1 (MN902223), sxt2 (MT832770) and eaeA (MN813493) (similarities were 99-100% compared to other *E. coli* O157 strains in gene bank). The most important results of experimental work in the present study showed that rabbits treated with quercetin nanoparticles showed neither clinical signs nor post mortem lesions. *E. coli* O157 was re-isolated only at 3 dPI in comparison to that in other experimental infected groups. The groups of rabbits treated with quercetin and nano-quercetin showed a significant increase in antioxidant enzymes before and after infection and in comparison with quercetin, the highest activity in GPX and SOD level appeared in nano-quercetin group at 10 dPI, while at 3 dPI the lowest activity in MDA appeared in NQ group compared to other groups. There were no significant changes in biochemical parameters except nano-quercetin group showed a significant decrease in total cholesterol at 3,10 dPI. In conclusion**,** Quercetin nanoparticles could be used as a preventive therapeutic agent and a powerful antioxidant drug for protection against *E. coli* O157.
Keywords: Drug-resistant *Escherichia coli, E.coli* O157, Quercetin nanoparticles, Antioxidant enzymes, Rabbits.

INTRODUCTION

Rabbit breeding is one of lucrative productive projects that gained the interest of the breeders. The most common problem facing the breeders is the digestive disorders that are dominantly seen in suckling and weaned rabbits causing weight losses, severe diarrhea and high mortalities and subsequently leading to sever economic losses (Milon *et al.,* 1999). Different serotypes of *E. coli* were isolated from rabbits (Swennes *et al.,* 2013, Khafagy *et al.,* 2015). Among the different serotypes of *E. coli* isolated from rabbits, *E. coli* O157:H7 represents the most important and dangerous one as it is incriminated in causing food and waterborne outbreaks leading to serious hemorrhagic diarrhea and hemorrhagic colitis (Rangel *et al.,* 2005). Previous study indicated that infant rabbits that clinically exhibited hemorrhagic diarrhea and hemorrhagic colitis were infected with EHEC strains (Ritchie et al., 2003). Virulence associated genes, Shiga toxin and intimin, are the key workers in detection the pathogenesis of EHEC strains including *E.coli* O157 (Gyles, 2007). *Escherichia coli* O157:H7 produces Intimin that facilitates its intimate adherence to the intestinal epithelium (Wang *et al.,* 2014) .

Scientific community have concerned about the rapid evolving of resistance of pathogenic bacteria to antibiotic (Viswanathan, 2014). The development of new antibiotic to the rising crisis of antibiotic resistance become urgent needs (Rossiter *et al.,* 2017). Antibacterial of plant-origin is one of the alternative choices of new therapeutics (Hossion *et al.,* 2011). Quercetin (3, 5, 7, 3', 4'-pentahydroxyflavone) is one of the flavonoids that found in many plants like vegetables, fruits and herbal plants (Wiczkowski *et al.,* 2008, Nishimuro *et al.,* 2015). The unparalleled mechanism of quercetin in scavenge and neutralize free radicals, inhibit lipid peroxidation and increase glutathione levels candidate it to be one of the most powerful natural antioxidant (Bentz, 2009). Specifically it was found that quercetin could successfully reduce oxidative stress in the body and safeguard it from different illnesses like cardiovascular disorders, chronic inflammation, neurodegenerative diseases, retinal degeneration and atherosclerosis in rabbits (JuŸwiak *et al.,* 2005 and Truong, 2017). Quercetin exerted potent antibacterial effect against *Escherichia coli* (Al-Saif *et al.,* 2014). Specifically quercetin showed anti-inflammatory and anti-oxidative activities that successfully prevent *E. coli* O157:H7 induced inflammasome activation (Xue *et al.,* 2017). Quercetin was also found to combat *E. coli* O157:H7 infection through inhibiting integrin β1 expression and FA formation and subsequently interferes with the attachment of *E. coli* O157:H7 to host cell (Xue *et al.,* 2019). However quercetin is considered as an antioxidant (Robaszkiewicz et al., 2007) and exerted an antibacterial effect (Li and Xu, 2008), its low water solubility and poor bioavailability hinder its use in medical field (Cai *et al.,* 2013). Nanomedicine offers an alternative solution with sustaining advantages compared to the conventional medicines such as improvement of solubility and enhancement of the bioavailability of poor soluble drugs as well as targeted delivery of

chemotherapeutic drugs (Aithal *et al.,* 2011). The unique character of quercetin nanoparticles candidate it to be more efficient in combating the multidrug resistant pathogenic bacteria especially with their zoonotic importance that threatens the public health. Therefore, the present study is a contribution to share in solving this problem through the evaluation of antibacterial effect and antioxidant potential of quercetin nanoparticles on drug- resistant *E. coli* O157 strain in NZW rabbits. The aim of the present study: The present work aimed to study the antibacterial effect and the antioxidant potential of quercetin nanoparticles on drug-resistant *E. coli* O157 in NZW Rabbits.

MATERIAL AND METHODS

Sample collection:

Different organs including intestine, liver, lung, kidney, spleen and heart were collected randomly from 150 freshly dead suckling and weaned New Zealand White Rabbits ranged from 2 weeks to 2 months of age. The samples collected from 5 different farms (30 rabbit/farm) located in Qalyoubia governorate with case history of diarrhea and high mortalities. The collection period lasted 2 years from 2018 to 2019.

Isolation and biochemical identification of *E. Coli:*

E.coli were isolated from the examined organs on MacConkey agar (LAMB, UK) and XLD agar (Himedia L.B.S Marg, Mumbia) according to method described by Markey et al. (2013) and were identified biochemically by using MicrogenTM GnA+B-ID System (Microgen Bioproducts Ltd., Admiralty Way, Camberley surrey GU153OT,UK),

In-Vitro anti-microbial sensitivity test for antibiotics:

The susceptibility of ninety *E.coli* isolates recovered from the 150 examined rabbits were tested to five different anti-microbial agents that commonly used in the treatment of rabbits including, Gentamicin (10µg), Oxytetracycline (10µg), florfenicol (30µg), sulphaTrimethoprim (25µg), and azithromycin (15 µg), using Disc Diffusion Method as described by Markey et al. (2013).

Detection of biofilm formation of *E. coli* isolates by using Cong red agar media:

Cong red agar Plates were inoculated with *E.coli* isolates and incubated aerobically for (24-48) hour at 37˚C. Appearance of black colonies with a dry crystalline consistency indicated positive results and pink colonies with darkening at their center indicated weak slime producers and dark colonies with lack of a dry crystalline consistency indicated an intermediate result according to method described by Pramodhini et al. (2012).

Serological identification of *E. Coli:*

A total of 20 Positive biofilm formation *E.coli* strains and showed resistant to antibiotics used in the present study were selected for serotyping by using slid e agglutination test according to method described by Markey et al. (2013). The antisera used in serotyping of *E. coli* were "SEIKEN" that supplied from MAST ASSURE™.

Molecular detection of some Virulence genes

Six *E.coli* serotypes including O125, O119, O26, O1, O143 and O157 were selected to be examined by multiplex PCR for detection of some virulence genes including stx1, stx2, eaeA and ompA according to Sambrook et al. (1989). Description of target genes, Primers sequences and amplicon sizes are listed in **Table** (**1**).

Sequencing of the sxt1, sxt2 and eae A genes of *E. coli* O157:

Sequencing of the amplified part of sxt1, sxt2 and eae A Sequencing of the PCR amplified product was conducted by sigma Company using ABI 3730xl DNA sequencer for forward primers according to Sanger et al., (1977). The traditional Sanger technology is combined with the new 454 technology for sequencing 348 bp, 584 bp and 397 bp PCR product of the sxt1, sxt2 and eae A genes, respectively. In order to establish sequence identity to GenBank accessions, a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was initially performed.

Table 1. Description targeted genes, primers sequences and amplicon sizes used in multiplex PCR reaction.

Target gene	Primer sequences ('5----------3')	Amplicon	Positive control strain	Negative control strain	Reference
***stx*1**	F:CAGTTAATGTGGTGGCGAAGG	348 bp	AJ314838	MRSA ATCC 43300	Cebula et al. (1995)
	R:CACCAGACAATGTAACCGCTG				
***stx*2**	F:ATCCTATTCCCGGGAGTTTACG	584 bp	FN252457		
	R:GCGTCATCGTATACACAGGAGC				
***eae*A**	F:ATTACCATCCACACAGACGGT	397 bp	MK761168		Fratamico and Strobaugh (1998)
	R:ACAGCGTGGTTGGATCAACCT				
***omp*A**	F:AGCTATCGCGATTGCAGTG	919 bp	AF23428.1		Ewers et al. (2007)
	R:GGTGTTGCCAGTAACCGG				

Determination of in-vitro safety of nanoquercetin on BHK using MTT assay:
Quercetin (Sigma-Aldrich, Egypt) and nanoquercetin was dissolved in dimethylsulfoxide (DMSO; Sigma, USA) to obtain 5 mg/ml stock solution for each, which then were diluted in the medium to desired concentrations. The MTT assay was used to test the effect of quercetin and nanoquercetin on BHK cell proliferation. Briefly, the cells were seeded in 96-well plates at a density of 5×103 cells/well and cultured in MEM with Hank's salt (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and antibiotics (penicillin 100 U/mL, streptomycin 100 U/mL). After being cultured for 24 hours, the cells were treated with quercetin and nanoquercetin for each at different concentrations (2500µg/ml, 1250 µg /ml, 625 µg/ml, 321.5 µg/ml, 156.25 µg/ml, 78.13 µg/ml, 39.06 µg /ml, 19.53 µg /ml, 9.77 µg/ml, 4.88 µg/ml, 2.44 µg/ml) and cultured for 24 hours and 48 hours, respectively. At each time point, the cells were washed twice with PBS, and add 50ug/ml from MTT (Amresco, USA) solution and incubated at 37°C for 3hours. Finally, the absorbance was measured at 570 nm using an ELx Ultra Microplate Reader (BioTek, USA). The test was performed three times.

Experimental protocol:

Animals :
Sixty White New Zealand rabbits of 30 days of age were purchased from a commercial rabbit breeding farm located in Qalyoubia governorate. The rabbits were randomly allocated into five groups of 12 animals per group. Rabbits were housed in wire-floored, metal cleaned cages and kept under the observation for 7 days to ensure that they free from E.coli prior to experiment initiation. The experimental rabbits fed on a commercial pellet ration ad libitum and were free access to water. The ration contains (18% crude protein and 14 % crude fiber) and it was free from antimicrobial additives.

Challenged inoculum:
The isolated enterohemorrhagic strain, E.coli O157 which produces Stx1 (MN902223), Stx2 (MT832770) and Intimin (MN813493) were chosen to be used in the experimental work. Rabbits in all groups except the control negative group no.1 were challenged by E.coli O157 on day 51 of age at rate of 1ml of 3×10^8 CFU/ml via oral route.

Treatments:

Quercetin:
Quercetin was purchased from Sigma-Aldrich, Egypt (purity ≥ 95% (HPLC))

Quercetin nanoparticles:
Quercetin nanoparticles were prepared by the anti-solvation precipitation method (Kakran et al., 2012) with some modification according to (EL-Sayed et al., 2018). Briefly, quercetin was dissolved in ethanol as an organic solvent at concentration of 5mg/ml. the syringe was filled with 10 ml of the prepared solution and was quickly injected at a fixed flow rate (10ml/min) into deionized water (antisolvent) of a definite volume at ratio 1:12 under magnetic stirring (1300 rpm). Stirring was allowed for 2 hours (EL-Sayed et al., 2018) then the formed quercetin nanoparticles were filtered and air dried. Quercetin nanoparticles morphology was observed using a SEM (Quanta 3D FEG/ FEI) with 20 kV, a collector bias of 300 V. The powder samples were spread on a SEM stub and sputtered with gold before the SEM observations. Preparation of quercetin nanoparticles was carried in Naqaa Foundation, Giza, Egypt.

FloRicol®
1ml contain 100mg florfenicol

Experimental design:
The experiment was carried out in centre of laboratory animal's research, Faculty of veterinary medicine, Benha University, Egypt. The treatments of the present study were conducted as shown in (table,2).

Sampling from experimental rabbits:
Blood samples of 3 rabbits from each group were collected at 51 days of age (prior infection) and at 3th day and 10 th day post infection for estimating serum biochemical and antioxidant parameters. The samples were immediately transferred into sterile test tubes and serum was harvested after centrifuged at 3000 rpm for 10 min. The serum was stored at −20°C for later analysis of antioxidant and biochemical parameters.

Bacteriological examination:
E. coli O157 was re-isolated from intestine from 3 animals from each experimental group at 3rd, 7th and 10th day post infection. Re-isolation percentages of *E. coli O157* from intestine were calculated from the experimental groups.

Table 2. The experimental design in the present study.

Experimental design				
Groups	Treaments	Period of treatments	Challenge by *E.coli* O157 at rate of 1ml of 3 × 10^8 CFU/ml via oral route	Age of challenge
1	Did not receive any treatment	-	-ve	-
2	Did not receive any treatment	-	+ve	on day 51 of age
3	Treated with 1ml of quercetin solution via oral route which contain 10 µg//kg BW/day	from 37 days of age till the end of experiment	+ve	on day 51 of age
4	Treated with 1ml of quercetin nanoparticles solution via oral route which contain 20 µg/Kg BW/day	from 37 days of age till the end of experiment	+ve	on day 51 of age
5	Treated with FloRicol® by oral route at dose 1 ml/kg BW /day (1ml contain 100mg florfenicol)	for 5 successive days starting from 1st appearance of clinical signs	+ve	on day 51 of age

Biochemical parameters analysis:
The biochemical activities such as ALT, AST enzymes, total protein, albumin, uric acid, creatinine, triglycerides and total cholesterol in tested sera were determined using the Bioanalytica test kits (Spinreact colorimetric kits, S.A./ S.A.U. Ctra.Santa Coloma, 7 E-17176 Sant Esteve De Bas (GIRONA), Spain). The quality system implemented by SPINREACT, has been certified according standards ISO 13485-2016. Serum globulin (G) was calculated as follows: G = total protein – albumin.

Determination of antioxidant indices:
The glutathione peroxidase (GPx), superoxide dismutase (SOD), and malondialdehyde (MDA) levels were determined by ELISA kits produced by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to Dalton et al. (2000).

Statistical analysis:
The data were analyzed by determine the normality via Shapiro-Willk test. The differences between groups were analyzed by One-Way ANOVA followed by Duncan's multiple comparison Post Hoc tests (Duncan, 1955). The Statistical Package for Social Science (SPSS Inc. Released, 2009) (version 20.0, SPSS Inc., Chicago IL, USA) was used for performing the statistical analyses to determine difference between groups. Significance between mean values was set a statistically at P<0.05.

RESULTS

Characterization of Quercetin nanoparticles :
Quercetin nanoparticles prepared by solvation/anti-solvation technique was characterized by using Scanning Electron Microscope (SEM). As shown in **Figure (1)**, Quercetin nanoparticles were appeared smaller particles (182-240 nm) and were uniform in their size and shape.

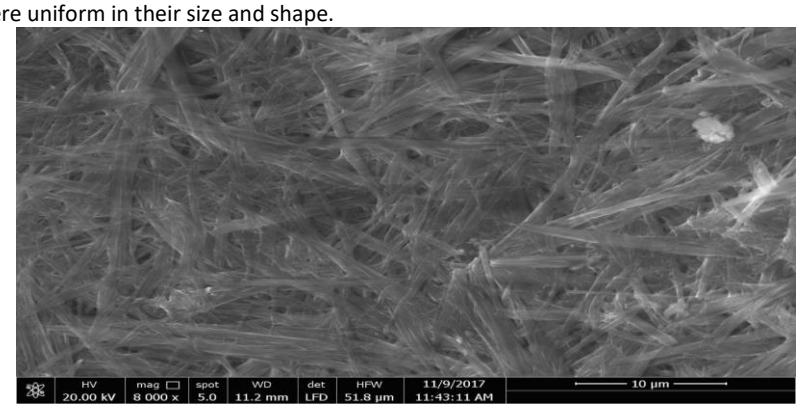

Fig. 1. Scanning electron microscope (SEM) photograph showing quercetin nanoparticles. (Cited from El-Sayed, et al., 2018).

***E. coli* Strains:**

Isolation Percentages of *E. coli* isolates recovered from different examined organs of freshly dead rabbits were shown in Table, (**3**). A total of 90 *E. coli* isolates were recovered from 150 freshly dead rabbits with percentage of 60%. The isolation percentages from examined organs were shown in **Table (3).**

Table (3) Isolation Percentages of *E.coli* isolates from different examined organs.

***E.coli* isolates (90 isolates)**	**Organs**					
	Intestine	**Liver**	**Lung**	**Kidney**	**Heart**	**Spleen**
No. of isolates	24	20	14	12	11	9
percentages	26.7%	22.2%	15.5%	13.3%	12.2%	10 %

Antibiotic sensitivity test:

In the present study as shown in Table (**4**), *E. coli* isolates recovered from rabbits showed highly sensitivity to azithromycin (84.44%) followed by Enrofloxacine (72.22) while they showed highly resistance to sulphaTrimethoprim (62.22%), Oxytetracycline (51.11%), florfenicol (46.7%) and Gentamicin (45.6%).

Table 4. In-Vitro anti-microbial Sensitivity test for 90 isolated E. coli strains.

Anti-microbial agents	***E. coli* (90 isolates)**						**AA**
	Sensitive		**Intermediate**		**Resistant**		
	No.	**%***	**No.**	**%***	**No.**	**%***	
Azithromycine (15µg)	76	84.4	4	4.4	10	11.1	S
Florfenicol (30µg)	35	38.9	23	25.6	42	46.7	R
Gentamicin (10µg)	39	43.3	10	11.1	41	45.6	R
Enrofloxacin(10µg)	65	72.2	8	8.9	17	18.9	S
Oxytetracycline (10µg)	26	28.9	18	20	46	51.1	R
Sulfa-trimetoprim (25µg)	23	25.6	13	14.4	56	62.2	R

%* in relation to total number of *E.coli* isolates (90), AA Antibiogram Activity

Biofilm formation

Biofilm formation by congred agar media was shown in **Table (5).**

Table 5. Biofilm formation for 90 isolated *E. coli* strains by congred agar media.

	Biofilm forming		
	Strong	intermediate	weak
No. of colonies	20	23	47

Serotyping of *E. Coli:*

A total of 20 positive biofilm forming *E.coli* isolates were subjected to serotyping identification as shown in **Table (6).**

Table 6. Serological identification of 20 *E. coli* isolates

Polyvalent antisera	**Monovalent antisera**	**No. of isolates**
1	(O86a,O1, O119, O128, and O26)	12
2	(O125)	2
3	(O157,O158)	5
8	(O143)	1

Molecular detection of virulence-associated genes (sxt1, sxt2, eaeA and ompA genes):

Molecular detection of virulence-associated genes (sxt1, sxt2, eaeA and ompA genes) in the selected six *E.coli* isolates (O125,O119,O26,O1 , O157 and O143) revealed that, sxt1 and eaeA were detected in 2 isolates (serotypes O143 and O157) while sxt2 were detected only in one isolate (O157). All isolates were negative for ompA gene as shown in **Fig. (2).**

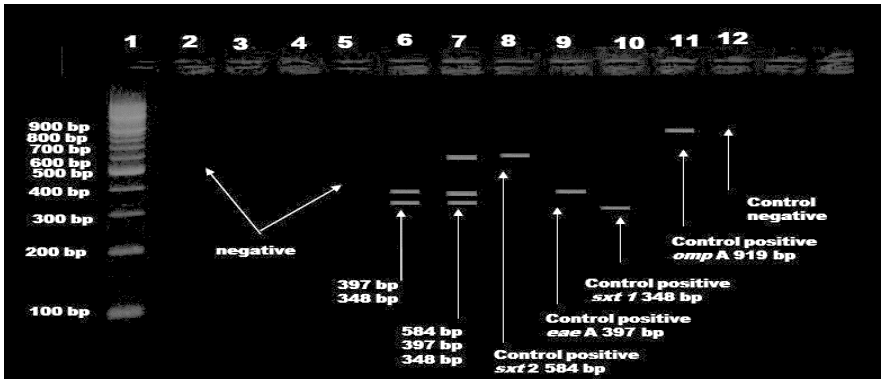

Fig. 2. Agarose gel electrophoresis reveals the PCR products of six *E.coli* isolates (O 125,O119,O26,O1,O143 and O157).

Lane 1: 100 bp DNA ladder
Lane 2 - 5: Negative (serotypes O125,O119,O26,O1).
Lane 6: show positive amplification of sxt1 and eaeA genes at 348 bp and 397bp respectively (serotype O143).
Lane 7: show positive amplification of sxt1,eaeA and sxt2gene at 348 bp, 397bp and 584 bp respectively (serotype O157).
Lane 8: Control positive shows positive amplification of sxt 2 at 584 bp.
Lane 9: Control positive shows positive amplification of eae A at 397 bp.
Lane 10: Control positive shows positive amplification of sxt1 at 348 bp.
Lane 11: Control positive shows positive amplification of omp A at 919 bp.
Lane 12: Control negative MRSA ATCC 43300.
Accession number of detected genes of *E. coli* O157 was described in **Table 7**.

Table (7) : Accession number of detected genes of *E. coli* O157

Isolate	Gene	Accession number
***E. coli* O157**	Shiga toxin 1	MN902223
***E. coli* O157**	Shiga toxin 2	MT832770
***E. coli* O157**	Intimin gamma	MN813493

In-vitro safety determination of nanoquercetin on BHK using MTT assay:
The cytotoxicity of quercetin nanoparticles were studied in comparison to quercetin using MTT assay. Microscopic features of BHK cells treated with different concentrations of (2500µg /ml to 2.44 µg/ml) of quercetin and quercetin nanoparticles for 24h and 48h were reported. At 48 hours, the IC_{50} of quercetin nanoparticles and quercetin was found to be 78.5 and 70 µg/ml, respectively **(Figure 3)**.

A) Control BHK/48h

B) BHK treated with concentration of 4.88 µg /ml of quercetin /48h

C) BHK treated with concentration of 4.88 µg/ml of quercetin nanoparticles /48h

Fig. 3. Microscopic features of control BHK cells (A) and BHK cells treated with concentrations of 4.88µg/ml of quercetin (B) and quercetin nanoparticles (C) for 48h (100 X).

Clinical signs and post-mortem lesion of the experimentally infected rabbits with *E. coli* O157:
All experimental animals were monitored daily during experimental period. After experimental infection, developments of clinical signs were recorded. Control negative rabbits (group 1) had no clinical signs, whereas infected rabbits infected with *E. coli* O157 that did not receive treatment (group 2) developed severe diarrhea and weight loss. Rabbits manifest mild and moderate diarrhea in group (3) (infected with *E. coli* O157 and treated with quercetin) and group (5) (infected with *E. coli* O157 and treated with florfenicol), respectively. None of rabbits manifested diarrhea in group (4) (infected with *E. coli* O157 and treated with quercetin nanoparticles). Mortality rate was 25%, 17% in groups (2) and (5), respectively while other groups showed no mortalities. (**Table, 8**). Enteritis was the prominent lesion that seen in the experimental animals. The degree of enteritis varied in its severity among the experimental groups (**Table, 8**).

Table 8. Clinical signs and post-mortem lesions observed in the experimental groups during experimental period.

Groups	Infection	Treatments	Clinical signs			Post-mortem lesions
			Weight loss	Diarrhea	Mortalities	Enteritis
1	Not infected	Not treated	-ve	-ve	0%	-ve
2	Infected with *E. coli* O157	Not treated	+ve	Severe	25% (3/12)	Severe
3	Infected with *E. coli* O157	Treated with quercetin	-ve	Mild	0% (0/12)	Mild
4	Infected with *E. coli* O157	Treated with quercetin NPs	-ve	-ve	0% (0/12)	-ve
5	Infected with *E. coli* O157	Treated with Florfenicol	+ve	Moderate	17% (2/12)	Moderate

-ve: Negative , +ve : Positive, NPs : Nanoparticles

Re-isolation of *E.coli* O157 from intestine from experimentally infected rabbits at three interval periods at 3rd and 7th and 10th day post infection:
Except the control negative group (1), at 3rd day post infection *E.coli* O157 was recovered from intestine from all experimental groups. At 7th day post infection *E.coli* O157 was recovered from intestine from groups (2),(3) and (5) and at 10th day post infection *E. coli* O157 was recovered from intestine from group (2) only (**Table 9**).
Table 9. Re-isolation of *E. coli* O157 from intestine from experimentally infected rabbits at 3rd and 7th and 10th day post infection.

Groups	Infection	Treatments	Re-isolation of *E. coli* O157 from intestine		
			3rd day PI	**7th day PI**	**10th day PI**
1	Not infected	Not treated	-ve	-ve	-ve
2	Infected with *E. coli* O157	Not treated	+ve	+ve	+ve
3	Infected with *E. coli* O157	Treated with quercetin	+ve	+ve	-ve
4	Infected with *E. coli* O157	Treated with quercetin NPs	+ve	-ve	-ve
5	Infected with *E. coli* O157	Treated with Flofenicol	+ve	+ve	-ve

-ve:Negative , +ve : Positive , PI: Post Infection, NPs : Nanoparticles

Effect of quercetin and quercetin nanoparticles on glutathione peroxidase (GPx), malondialdehyde (MDA) and superoxide dismutase (SOD) activity in rabbits after 2 weeks of treatments intake and before initiation of infection:
Figure (4) showed that the obtained data of antioxidant enzymes in the groups of rabbits treated with quercetin and nano-quercetin showed significant increased ($P < 0.05$) in serum GPX and SOD level and decreased in MDA values compared to control group. Nano-quercetin group showed the significantly highest level ($P < 0.05$) in serum GPX compared to quercetin and control group.

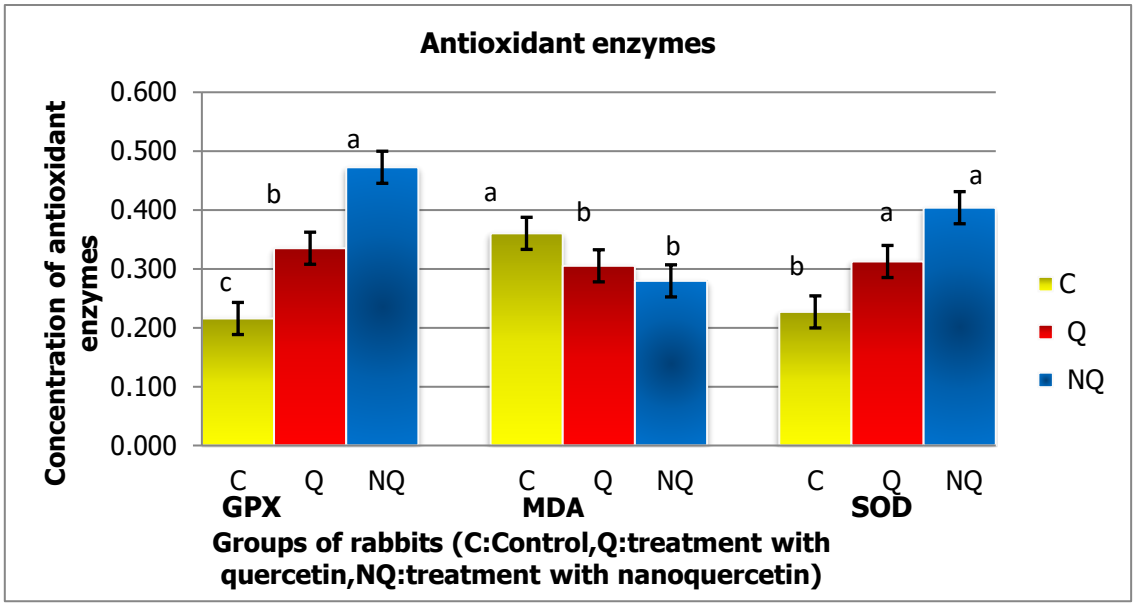

Figure 4. Effect of quercetin and quercetin nanoparticles on glutathione peroxidase (GPx), malondialdehyde (MDA) and superoxide dismutase (SOD) activity in rabbits after 2 weeks of treatments intake and before initiation of infection.
a,b,c mean values with different superscripts are statistically different at (p≤ 0.05) , *mean of 3 rabbits.

Effect of quercetin and quercetin nanoparticles on blood biochemical parameters in rabbits after 2 weeks of treatments intake and before initiation of infection:

Table (10) appeared that there were no significant changes in biochemical parameters but the differences occur in the groups of rabbits treated with quercetin and nano-quercetin showed the lowest level of Uric acid and lipid profile compared to control group.

Table 10. Effect of quercetin and quercetin nanoparticles on blood biochemical parameters in rabbits after 2 weeks of treatments intake and before initiation of infection (Mean*±SE).

groups	Blood biochemical parameters after 2 weeks of treatments intake and before initiation of infection								
	ALT(U/L)	AST(U/L)	Total protein (g/dl)	Albumin (g/dl)	globulin (g/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	Total Cholesterol	Triglycerides (mg/dl)
Control group	29.10± 0.87^{a}	126.13± 4.74^{a}	7.19± 0.338^{a}	4.59± 0.304^{a}	2.60± 0.641^{a}	5.99± 0.188^{a}	0.38± 0.006^{a}	169.21± 4.62^{a}	130.47± 2.95^{a}
group (treatment with quercetin no infection)	28.76± 0.499^{a}	125.28± 4.07^{a}	7.11± 0.251^{a}	4.48± 0.336^{a}	2.63± 0.408^{a}	4.48± 0.275^{b}	0.38± 0.42^{a}	147.36± 4.29^{b}	115.98± 4.11^{b}
group (treatment with Nano-quercetin no infection)	28.66± 0.76^{a}	124.41± 4.48^{a}	7.18± 0.315^{a}	4.57± 0.547^{a}	2.61± 0.702^{a}	4.52± 0.258^{b}	0.37± 0.009^{a}	125.37± 6.36^{c}	117.84± 3.30^{b}

a,b,c mean values with different superscripts in a column are statistically different at (p≤ 0.05) , *mean of 3 rabbits.

Effect of quercetin and quercetin nanoparticles in experimentally infected rabbits with *E.coli* O157 on glutathione peroxidase (GPX), malondialdehyde and superoxide dismutase (SOD) activity 3 and 10 days post infection:

The results of the effect of antioxidant enzyme assays are shown in Table (11). There was a significant increase in GPX and SOD with significant difference in MDA level in *E. coli* experimentally infected rabbits groups treated with quercetin (Q) and nano-quercetin (NQ) compared to all groups. At 3, 10 days post infection the group of NQ showed the significantly highest level ($P < 0.05$) in serum GPX level compared to all groups. Related to SOD activity, where both Q and NQ groups showed a significantly higher activity compared to the other groups at 3 days post infection, while the highest activity appeared in NQ with significant difference compared to other groups at 10 days post infection. The lowest activity in MDA level appeared in NQ compared to other groups at 3 days post infection. The obtained data revealed that, The GPX and SOD were efficient and increased in response to treatment with 1 ml of quercetin and nano- quercetin solution which contain 10 ug/kg B.W./day and 20 ug/kg B.W./day, respectively compared to group treated with florfenicol (FL) in *E. coli* experimentally infected rabbits.

Table 11. Effect of quercetin and quercetin nanoparticles in experimentally infected rabbits with *E.coli* O157 on glutathione peroxidase (GPx), malondialdehyde and superoxide dismutase (SOD) activity 3 and 10 days post infection. (Mean*±SE).

	Antioxidant parameters					
	GPX (ng/ml)		MDA (nmol/ml)		SOD (U/L)	
	3 day	10 day	3 day	10 day	3 day	10 day
Control negative group	0.210± 0.014^{c}	0.231± 0.018^{c}	0.327± 0.017^{b}	0.374± 0.014^{a}	0.199± 0.010^{b}	0.259± 0.016^{cd}
Infected group (no treatment)	0.140± 0.010^{d}	0.183± 0.011^{c}	0.421± 0.016^{a}	0.393± 0.011^{a}	0.143± 0.007^{c}	0.206± 0.013^{d}
Infected group (Q-treatment with quercetin)	0.303± 0.027^{b}	0.372± 0.031^{b}	0.306± 0.011^{b}	0.285± 0.010^{b}	0.270± 0.017^{a}	0.323± 0.022^{ab}
Infected group (NQ-treatment with Nano-quercetin)	0.358± 0.013^{a}	0.449± 0.026^{a}	0.245± 0.008^{c}	0.311± 0.023^{b}	0.296± 0.014^{a}	0.357± 0.023^{a}
Infected group (FL-treatment with florfenicol)	0.212± 0.011c	0.205± 0.016bc	0.340± 0.013b	0.368± 0.18a	0.192± 0.013b	0.279± 0.019bc

Q, group treated with quercetin, NQ, group treated with Nano-quercetin, FL, group treated with florfenicol. a,b,c, mean values with different superscripts in a column are statistically different at (p≤ 0.05) , *mean of 3 rabbits.

Effect of quercetin and quercetin nanoparticles in experimentally infected rabbits with *E. coli* O157 on blood biochemical parameters at 3 and 10 days post infection:

Tables (**12, 13**) showed that the differences were significant (P < 0.05) among the treatments in serum AST, ALT, Uric acid, Total Cholesterol, Triglycerides and Creatinine levels. The levels of AST and ALT enzymes in serum were decreased in *E. coli* experimentally infected rabbits treated with quercetin and nano- quercetin compared to infected group only with no significant difference compared to florfenicol treatment (FL) and control negative group at 3 days post infection. The lowest level of Uric acid showed in NQ group at 3 days post infection, also decreased in Q and NQ group at 10 days post infection. The results revealed that the lowest level of total cholesterol showed in NQ group compared to the other groups.

Table 12. Effect of quercetin and quercetin nanoparticles in *Escherichia coli* experimentally infected rabbits on blood biochemical parameters at 3 days post infection (Mean*±SE).

Groups	**Blood biochemical parameters at 3 days post infection**								
	ALT(U/L)	**AST(U/L)**	**Total protein (g/dl)**	**Albumin (g/dl)**	**Globulin (g/dl)**	**Uric acid (mg/dl)**	**Creatinine (mg/dl)**	**Total Cholesterol (mg/dl)**	**Triglycerides (mg/dl)**
Control negative group	27.87± 1.22^{ab}	114.16± 3.67^{bc}	6.76± 0.46^{b}	3.42± 0.23^{b}	3.35± 0.68^{a}	5.06± 0.42^{bc}	0.40± 0.012^{b}	174.10± 3.27^{a}	119.97± 2.70^{ab}
Infected group	31.95± 1.89^{a}	144.70± 2.64^{a}	8.73± 0.36^{a}	4.96± 0.09^{a}	3.77± 0.41^{a}	8.14± 0.47^{a}	0.47± 0.018^{a}	179.01± 4.66^{a}	126.76± 2.91^{a}
Infected group (Q-treatment)	26.96± 1.18^{b}	112.39± 5.09^{c}	6.71± 0.37^{b}	3.41± 0.22^{b}	3.30± 0.21^{a}	5.74± 0.65^{bc}	0.39± 0.010^{b}	158.07± 4.95^{b}	113.86± 3.64^{bc}
Infected group (NQ- treatment)	27.30± 0.94^{b}	108.17± 2.88^{c}	6.68± 0.29^{b}	3.33± 0.30^{b}	3.34± 0.02^{a}	4.50± 0.33^{c}	0.38± 0.012^{b}	144.02± 3.61^{c}	108.49± 3.03^{c}
Infected group (FL treatment)	30.09± 0.95^{ab}	126.19± 5.53^{b}	7.57± 0.48^{ab}	3.99± 0.06^{b}	3.68± 0.45^{a}	6.58± 0.58^{ab}	0.44± 0.005^{a}	175.81± 4.81^{a}	122.18± 2.39^{ab}

Table 13. Effect of quercetin and quercetin nanoparticles in *Escherichia coli* experimentally infected rabbits on blood biochemical parameters at 10 days post infection (Mean*±SE).

Groups	Blood biochemical parameters at 10 days post infection								
	ALT(U/L)	AST(U/L)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	Total Cholesterol(mg /dl)	Triglycerides (mg/dl)
Control negative	25.89± 0.742^{bc}	182.75± 3.24^{b}	6.69± 0.325^{ab}	2.96± 0.096^{b}	3.73± 0.250^{a}	5.48± 0.335^{b}	0.38± 0.012^{b}	193.96± 4.19^{b}	148.92± 1.61^{b}
Infected group	29.74± 1.216^{a}	217.42± 1.95^{a}	7.71± 0.406^{a}	3.81± 0.174^{a}	3.90± 0.58^{a}	7.12± 0.290^{a}	0.43± 0.014^{a}	206.49± 4.03^{a}	162.86± 2.76^{a}
Infected group (Q-treatment)	23.36± 0.632^{c}	159.73± 4.57^{d}	6.55± 0.356^{ab}	2.97± 0.106^{b}	3.58± 0.37^{a}	5.18± 0.145^{b}	0.36± 0.010^{b}	164.09± 3.70^{c}	118.50± 3.47^{c}
Infected group (NQ-treatment)	24.24± 0.426^{c}	171.44± 1.62^{c}	6.34± 0.301^{b}	3.01± 0.078^{b}	3.33± 0.243^{a}	5.29± 0.468^{b}	0.37± 0.009^{b}	145.30± 4.36^{d}	124.15± 3.23^{c}
Infected group (FL-treatment)	27.88± 0.656^{ab}	189.22± 3.38^{b}	7.04± 0.38^{ab}	3.37± 0.156^{b}	3.67± 0.23^{a}	6.76± 0.445^{a}	0.39± 0.017^{b}	187.11± 3.73^{b}	156.43± 3.51^{ab}

Q, group treated with quercetin, NQ, group treated with Nano-quercetin, FL, group treated with florfenicol. a,b,c, mean values with different superscripts in a column are statistically different at (p≤ 0.05) , *mean of 3 rabbits.

DISCUSSION

Emergence of multi-drug resistant bacteria represent stumbling block and real threaten to public health in recent years. Conventional treatments became weak to control illness caused by that dangerous type of bacteria and active search from scientists to find new alternative solutions become an imperative necessity. Here, we study the antibacterial effect of quercetin nanoparticles on drug-resistant *E. coli* O157 strain in NZW rabbits. To achieve this goal firstly we perform initial survey to isolate the currently drug resistant *E. coli* strains circulating in rabbits. The surveillance lasting two years from 2018 to 2019 from 5 different rabbit farms (30 NZW rabbits/farm) located in Qalyoubia Governorate with case history of high mortalities in young rabbits accompanied with severe diarrheic symptoms.

A total of ninety *E. coli* isolates were recovered from 150 freshly dead rabbits with percentage of 60%. similar result were recorded by Swennes et al., (2013) who obtained *E. coli* isolates from 61% of the rabbits samples. Of the ninety *E.coli* isolates in the present study, 62.22% were resistant to sulphaTrimethoprim, 51.1% to Oxytetracycline, 46.7% to florfenicol and 45.6% to Gentamicin. On the other hand 84.44% of *E.coli* isolates were sensitive to azithromycin. Different scientific studies carried out the in-vitro anti-microbial sensitivity test on *E.coli* isolates recovered from rabbits and showed variability in their results, among these studies, our results showed lower resistant rate in compared to that reported by Swennes et al. (2013) who showed that 59% of *E.coli* isolated from New Zealand white rabbits were resistant to gentamicin and differ completely with same author who showed that 100% of isolates were sensitive to trimethoprim sulfamethoxazole. Also our results showed lower resistant rate in compared to that obtained by Zhao et al. (2018) who showed that *Escherichia coli* isolated from rabbit farms in Tai'an, China showed highest resistance rate against tetracycline (78.2%). In the previous study, different serotypes of *E.coli* strains were isolated from rabbits suffering from diarrhea (Swennes *et al.,* 2013). Here, a total of twenty isolates were showed strong positive biofilm forming. These isolates were serotyped as 12 isolates (O86a, O1, O119, O128 and O26), 5 isolates (O157 and O158), 2 isolates (O125) and one isolate (O143). The Intimin (eae) gene is one of the genetic markers used to identify strains of REPEC that responsible for adherence mechanism to host epithelial cells (Badagliacca *et al.,* 2018). The eaeA gene of Enteropathogenic *Escherichia coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) strains play an essential role in intimate attachment and microvillus effacement in vitro and in animal models (Donnenberg *et al.,* 1993). Shiga toxin (Stx) is known as a lethal biological poisons and is incriminated in causing renal damage and accumulation of fluid in ileal loops in rabbits (Angela and Melton, 2014). Scientific study that carried out on rabbits in mid-1980s proved the role of Shiga toxin-producing *E. coli* (STEC) in induction of apoptosis of the cells (Keenan *et al.,* 1986). Molecular data in the present study to monitor the virulence genes of the recovered *E. coli* isolates from weaned rabbits associated with diarrheic symptoms revealed that the selected drug resistant *E. coli* serotype O157 harbor the both virulence-associated genes stx1 and stx2 and eaeA while *E. coli* serotype O143 harbor stx1 and eaeA.

Quercetin exerted potent antibacterial effect against *Escherichia coli* (Al-Saif *et al.,* 2014) and in recent study showed that, quercetin had the ability to combat *E. coli* O157:H7 infection through prevent *E. coli* O157:H7 adhesion to epithelial cells via suppressing focal adhesions (Xue *et al.,* 2019). These valuable data in the previous studies nominate quercetin as a potential therapeutic agent used in prevention of enteric pathogenic infection. Low water solubility and poor bioavailability of quercetin restricted its use in pharmaceutical field (Cai *et al.,* 2013). Nanotechnology provides good solution

to improve the solubility and enhancement of the bioavailability of quercetin (Al-Jameel and Abd ElRahman, 2017). Here, a trail was conducted to study the antibacterial effect of quercetin nanoparticles on drug- resistant *E.coli* O157 (harbor Stx1, Stx2 and eae) in NZW rabbits. The rabbits in control positive group (2) developed weight loss, severe diarrhea and enteritis. Similar clinical signs and pathological features were recorded in previous study (Panda *et al.,* 1998) and were attributed to virulence associated genes, Shiga toxin and intimin that play an important role in the pathogenesis of EHEC strains including *E. coli* O157 (Gyles, 2007). Rabbits in groups 3 treated with quercetin showed mild degree of clinical signs and pathological features in comparison to control positive group (2) and group (5) that treated with anitibiotic. These results strengthen the finding of Xue et al. (2019) who showed that "quercetin inhibit *E. coli* O157:H7 via attenuated the association between integrin β1and FAK. Given that antibiotic is not applicable for O157:H7 infection". Rabbits in group (4) that treated with quercetin nanoparticles showed no clinical signs nor pathological lesions and *E.coli* O157 was re-isolated only at 3 PI in comparison to other experimental groups (2),(3) and (5) . These results revealed the superiority of quercetin nanoparticles in prevention of *E. coli* O157 infection in rabbits. This may be attributed to the improvement of the solubility and the bioavailability as well as the unique characters of nanoparticles (Al-Jameel and Abd ElRahman, 2017).

Quercetin is effective in treatment and prevention of human diseases through it elevates glutathione enzymes activity and affect ROS production so, it is used in the medicinal field due to its antioxidant activity in vivo (Xu *et al.,* 2019). Eftekhari et al. (2018) confirmed that nano-antioxidants as quercetin nanoparticles are safe with no toxicity, biocompatible and biodegradable, they also have strong intrinsic antioxidant properties in attenuating of free radical induced oxidative damage. In the present study, after administering quercetin and nano-quercetin to the experimental groups of rabbits at concentrations of (10 and 20 µg, respectively) noticed the significantly highest level in serum GPX and SOD activities compared to all groups before and after infection and the oral administration of nano-quercetin in normal and experimentally rabbits have higher potent antioxidant property which increased GPX activities more than quercetin and this was in agreement with Eftekhari et al. (2018) who mentioned that oral utilization of nano-quercetin in rats was significantly increased in GPx level greater than free quercetin. In addition, Ghaffari et al. (2018) mentioned that oral administration of nano-quercetin in experimentally rats have powerful antioxidant property which increased antioxidant enzyme activities and lead to decrease the intracellular H2O2 production with a simultaneous increase of glutathione level and this could decrease the lipid peroxidation. On the contrary, Ghosh et al. (2013) reported that SOD and catalase levels were decreased by a single dose oral treatment of nano-encapsulated quercetin (2.7 mg/kg b. wt.) in young and aged Albino rats. Iskender et al. (2016) observed that quercetin supplementation (0.5 g/kg) in laying chickens increased the activities of GPx, GST and SOD level more significantly than hesperidin and naringin.

MDA is considered one of the end products of lipid peroxidation that reflects the degree of cellular damage and changes in the polymerization of cell membrane components (Kamboh *et al.,* 2015). At 3 days post infection, the significantly lowest level ($P<0.05$) in serum malondialdehyde activities was noticed in nanoquercetin group compared to the other groups. These results with agreement of Iskender et al. (2016) who showed that quercetin supplementation (0.5 g/kg) was significantly decreased MDA level in laying chickens than the other flavonoids. The experiment study showed that with the use of quercetin and nano-quercetin as a preventive in the groups of rabbits, the biochemical parameters were near to normal values, but showed significant reduction in lipid profile (total cholesterol and triglycerides) and uric acid compared to control negative group. The obtained results revealed no changes in protein parameters and these results in agreement with Iskender et al. (2016) who stated that all flavonoids as quercetin supplementations haven't any effect on serum protein levels in laying hens. Furthermore, similar results of quercetin in the lowering of serum or hepatic lipids were previously reported in rabbits (Kamada *et al.,* 2005) and chicken (Qureshi *et al.,* 2011). Also, Eman et al. (2018) showed that quercetin nanoparticles were significantly decreased total cholesterol level in rats. On the contrary Selvakumar et al. (2013) mentioned that oral quercetin have no influence on serum total cholesterol and triglyceride levels in rats, these differences might be due to animal species, quercetin level, quercetin sources, dietary lipid level and experiment interval duration.

In comparison with quercetin, quercetin nanoparticles showed greater effects in antioxidant activity by reactive oxygen species scavenging and achieved higher potency than the free drug (quercetin) and this may be due to nanoparticle properties as high solubility, a smaller size, and higher bioavailability (Al-Jameel and Abd ElRahman, 2017). We observed a significant GPx and SOD depletion that might be a mechanism for the *Escherichia coli* infection in rabbits, quercetin is able to increased GPX and SOD levels and this effect may also participate in protection against *Escherichia coli* infection, but for the first time here, we showed that antioxidant potential of quercetin nanoparticles was significantly increased in GPx than quercetin in both normal and infected rabbits. So, nano-sized quercetin particles more effective than quercetin and lead to the efficiency of antioxidant defence systems activity and this in agreement with Zhang et al. (2020) who confirmed that nanoparticles with prominent different particle diameters were solved the problem of solubility and evaluate the positive effects of quercetin in vitro.

CONCLUSION

Quercetin nanoparticles showed antibacterial activity against *E.coli O157* infection in NZW rabbits and could be used as a preventive therapeutic agent alternative to antibiotics against drug resistant enteric bacterial infection. Nano-quercetin could be a promising natural new powerful antioxidant drug for giving the most protection against *Escherichia coli* O157 with high solubility, safety and bioavailability than quercetin

Conflict of interest statement

The authors declare no conflict of interest.

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تقيم تأثير جزيئات النانوكيورستين كمضاد بكتيرى على عترات الإيشريشيا كولاى المقاومة للمضادات الحيوية فى الأرانب

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الملخص العربي

الهدف من الدراسة الحالية هو تقييم التأثير المضاد للبكتيريا وايضا التاثيرات المضادة للأكسدة للنانو كيرسيتين على بكتيريا الإيشيريشيا كولاى O157 المقاومة للمضادات الحيوية في الأرانب البيضاء النيوزيلندية. تم إجراء مسح أولي لمدة عامين من 2018 إلى 2019 للعزل والكشف الجزيئي لبعض جينات الضراوة لبعض سلالات الإيشيريشيا كولاى المقاومة للمضادات الحيوية المنتشرة في الأرانب النيوزيلندية في مصر .تم اختيار سلالة الإيشيريشيا كولاىO157 في تجربتنا المعملية والتي تنتج كلا من البيوفيلم القوي و(MN902223) Stx1 و (MT832770) Stx2و Intimin(MN813493)وكانت أوجه التشابه 99-100 ٪ مقارنة بسلالات الإيشيريشيا كولاى O157القولونية الأخرى في بنك الجينات. أظهرت أهم نتائج التجربة المعملية في هذه الدراسة أن الأرانب التي عولجت بالنانو كيرسيتين لم تظهر أي أعراض ولا آفات فى الصفة التشريحية. تم إعادة عزل الإيشيريشيا كولاى O157 فقط عند 3 أيام بعد العدوي بالمقارنة مع المجموعات المعدية الأخرى .أظهرت مجموعات الأرانب التي عولجت بالكيرسيتين والنانو كيرسيتين زيادة معنوية في إنزيمات مضادات الأكسدة قبل الإصابة وبعدها ، وبالمقارنة مع الكيرسيتين ، ظهر أعلى نشاط في مستوى انزيم الجلوتاثيون بيروكسيداز و السوبراكسيد ديسميوتاز في مجموعة النانو كيرسيتين عند 10 أيام بعد العدوي ، بينما عند 3 أيام بعد العدوي كان أقل نشاط في انزيم المالونديالدهيد في مجموعة النانو كيرسيتين مقارنة بالمجموعات الأخرى . لم تكن هناك تغيرات معنوية في المعايير البيوكيميائية باستثناء مجموعة النانو كيرسيتين التي أظهرت انخفاضاً معنوياً في الكوليسترول الكلي عند 3،10 أيام بعد العدوي .كما أظهر أقل مستوى من حمض اليوريك في مجموعة النانو كيرسيتين مقارنة بالمجموعات الأخرى عند 3 أيام بعد العدوي. الخلاصة, جزيئات النانو كيرسيتين يمكن استخدامها كعامل علاجي وقائي و عقارًا قويًا مضادًا للأكسدة لإعطاء الحماية ضد الإيشيريشيا كولاى O157.

الكلمات المفتاحية: الإيشيريشيا كولاي المقاومة للمضادات الحيوية ,الإيشريشيا كولاي **O157** ,جزيئات النانوكيرسيتين , إنزيمات مضادات الأكسدة , الأرانب