Research Article

Molecular and Conventional Detection of Antimicrobial Activity of Zinc Oxide Nanoparticles and Cinnamon Oil against *Escherichia coli* and *Aspergillus flavus*

Atef A. Hassan[1], Khairy F. Abo-Zaid[2], Noha H. Oraby[1*]

[1]*Department of Mycology and Mycotoxins;* [2]*Serology Unit (Bacteriology), Animal Health Research Institute, Agriculture Research Center (ARC), Dokki, Cairo, Egypt.*

Abstract | The antimicrobial activity of zinc oxide nanoparticles (ZnONPs) and cinnamon oils (C.O.) was evaluated by conventional and molecular methods against *Aspergillus flavus* (*A.flavus*) and *Escherichia coli 0157* (*E.coli*) that recovered from cattle mastitis. In agar well diffusion method (WD), Minimum inhibitory concentration (MIC) of ZnONPs and C.O. for *A.flavus* was (100 µg/ml; 0.25%) and for *E.coli 0157* were (50 µg/ml; 0.25%), respectively. The synergistic effects of these materials caused higher significant inhibition of all microbial growth by low and high doses by agar method. But, the molecular detection of virulent genes of *E. coli* (*stx1*) *and A. flavus* (*AflR)* by polymerase chain reaction (PCR) and the real-time PCR (RT-PCR) yielded uncorrelated results with WD tests. It is concluded that no direct correlation between WD, PCR, and RT-PCR and the WD tests are still inexpensive, eco-friendly, and rapidly applicable for screening of antimicrobials activity than genetic methods.

Keywords | Antimicrobial, Nanotechnology, Real-Time PCR.

Received | May 23, 2020; **Accepted** | June 03, 2020; **Published** | July 18, 2020
***Correspondence** | Nora H Oraby, Department of Mycology and Mycotoxins; Cairo, Egypt; **Email:** nohaoraby25@gmail.com
Citation | Hassan AA, Abo-Zaid KF, Oraby NH (2020). Molecular and conventional detection of antimicrobial activity of zinc oxide nanoparticles and cinnamon oil against *Escherichia coli* and *Aspergillus flavus*. Adv. Anim. Vet. Sci. 8(8): 839-847.
DOI | http://dx.doi.org/10.17582/journal.aavs/2020/8.8.839.847
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

Copyright © 2020 Oraby *et al*. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

Nowadays, the microbial infections resulted from bacteria and fungi are common and caused a significant reduction in the productivity of animals and the occurrence of human food poisoning (Quinn et al., 2002). Mastitis is the most common disease in dairy animals and resulted in significant economic losses due to a decrease in milk yield. Several studies recovered *Staphylococcus aureus* (*S.aureus*), *E.coli*, *C. albicans*, *Aspergillus,* and *Penicillium* species from mastitic milk of sheep and cattle as (Alyssa et al.,2012; Hassan et al., 2014). Hence, the development of more effective novel antimicrobial therapies is of great importance to prevent microbial infections particularly in dairy animals. Recently, the advances in nanotechnology enable synthesis metals nanomaterial which is used as an antimicrobial against common infectious agents without driving antibiotic resistance in organisms (Beyth et al., 2015; Mohan and Renjanadevi, 2016). The metals nanomaterial particularly ZnONPs have several applications as antimicrobial potentials against several pathogens (Liu et al., 2009 ; Hassan et al., 2015a). While, feed supplement with ZnONPs improves the immunity in dairy cattle (Sahoo et al., 2014) and have antimicrobial activity (Gong et al., 2007). These activities occur due to the penetration of nanoparticles into the cell membrane of organisms, generation of oxidative stress which destroys microbial cells (Seil and Webster, 2012). ZnONPs have significant effects as growth promotion, immune-modulatory, antibacterial and elevated efficiency of the reproduction in animals (Partha et al., 2015; 2016). However, the plant extracts and oils are nontoxic compounds and used in disease control replacing synthetic preservatives (Lee et al., 2007). The correlated detection of antimicrobials activities by agar diffusion tests and the molecular biology methods was previously evaluated (Chomvarin et al., 2004). They detected that disc

diffusion (DD), microdilution tests (MD) were more applicable than genotyping RT-PCR. Therefore, this study was undertaken to evaluate the antimicrobial potentials of ZnONPs singly and/or in combination with cinnamon oil against recovered *E. coli* and *A. flavus* from dairy cattle mastitis. Moreover, the comparison between the conventional agar diffusion tests and molecular methods was investigated to evaluate the use of any of them in the rapid and effective detection of antimicrobial activities in large scales application.

MATERIAL AND METHODS

Samples

Two hundred samples (125 of mastitis milk and 25 of each of water, litter, and ration) were collected from dairy cattle farms in which animals suffered from mastitis. Milk and water samples were collected in sterile bottles, while, ration and litter samples taken in sterile polyethylene bags. All methods of collection and preparations were done as a method of (APHA, 2003). Each sample was divided into two parts and subjected for mycological and bacteriological examination.

Zinc Oxide Nanoparticles and Cinnamon Oil

ZnONPs were synthesized and characterized by the laboratory of ALDRIK Sigma chemical company, USA and it was in powder form with 50 nm particle size. While cinnamon oil was purchased in crud form from Al Gomhorya chemical company, Egypt.

Mycological Examination

Ten grams of finely grind rations and litter samples and 10 ml of milk sediment and water samples were added separately to 90 ml of 1% peptone water and stirred vigorously by electric blender for preparation of homogenate (APHA, 2003). One milliliter of homogenate was inoculated into Petri-dish plates and mixed with Sabouraud's dextrose agar (SDA) and incubated 3-5 days at 25-28°C and identification of appeared mold and yeast colonies were identified according to, Pitt and Hocking (2009).

Bacteriological Examination

Samples were cultured onto MacConkey agar medium for 24 hr_s at 37°C, then a peptone water cultures were prepared from appeared colonies to inoculate biochemical tests (Quinn et al., 2002). While, serological identification for *E. coli* species was undertaken according to (Neville and Bryant, 1986).

Antimicrobial potential OF ZnONPs and C.O. (Jin et al., 2009 and Jeff-Agboola et al., 2012).

The *A.flavus* and *E.coli O157* that recovered from the present samples and the standard control of each were cultivated on (SDA and MacConkey agar) and incubated for (1-3 days at 28°C or 24 hours at 37°C), respectively. The spore suspension was prepared and counted in the hemocytometer slide. One ml of 10^5 spores of microbes was aseptically added to plates and covered with SDA medium (for fungus) and nutrient agar (for bacteria). Wells of 5 mm in Φ were made on surface of plates and add 100 µl of ZnO NPs (0, 25, 50, 100, 150, 200, 250 µg/ ml) or 100 µl of C.O (0, 0.25%, 0.5% , 1%, 2%, 3%) and incubated for 1-5 days at 28-37 °C.

Synergistic antimicrobial potential of ZnONPs with C.O. :

In a separate 4 wells in plates, we added 50 µl of ZnONPs+ 50 µl of C.O. of the following concentrations: (0.25% of C.O.+ 100 µg/ml ZnONPs), (1% C.O+ 100 µg/ml ZnONPs), (0.25% C.O.+ 200 µg/ml ZnONPs) and (1% C.O.+ 200 µg/ ml ZnONPs). Incubation of plates for 1-5 days at 28-37 °C. Then the plates were tested for the growth inhibitory zones around wells. All procedures were repeated 3 times to pooled data.

Detection virulent genes of *E. coli O157* and *A.flavus* by PCR. Preparation of treated strains of *A.flavus* and *E. coli O157:*

The *A.flavus* and *E. coli O157* that recovered from the present samples were subjected to PCR detection of virulent gene expression before and after treatments with ZnONPs and C.O. In 50 ml sterile test tubs, add 20 ml of sterilized SD broth medium (for fungus) and nutrient broth (for bacteria) and 0.2 ml of 10^5 spore suspensions of 7 days old for all used microbes was inoculated into the tubes. Each strain was subjected for 6 doses treatments (low, 100 µg /ml ZnO NPs), (high, 500 µg/ml ZnONPs) (low, 0.25% C.O.), (high, 1%C.O.), (combination, 100 µg /ml ZnONPs+ 0.25% C.O.), (combination, 100 µg /ml ZnONPs+ 1% C.O.). The negative control was (*Fusarium for A.flavus*)(*Staph aureus for E.coli O157*) and the positive were (*A,flavus and E.coli O157)*. All the tubes were incubated at 30°C for 3 days and kept at 5-8 °C till DNA extraction.

DNA extraction (Fittipaldi et al., 2012 and Hossain et al., 2015):

Genomic DNA of the strains was obtained using the genomic DNA Extraction Kit (Quick-DNA Miniprep DNA purification kit, cat. No. D3024) following the manufacturer's instructions. DNA concentration was determined spectrophotometrically at 260/230 nm using SPECTROstar Nano" BMG LABTECH" and stored at –20°C until PCR amplification.

Table 1: General primers

Genes	Primer	Primer Design5-´-3´.	Amplicon bp
Stx1	*stx*1-F	GACTTCTCGACTGCAAAGAC	306
	*stx*1-R	TGTAACCGCTGTTGTACCTG	
AflR	*Afl* R-F	AACCGCATCCACAATCTCAT	800
	Afl R-R	AGTGCAGTTCGCTCAGAACA	

Table 2: Standard cycling mode

Step	Temperature	Duration	Cycles
UDG activation	50°C	2 minutes	Hold
Dual-Lock DNA polymerase	95°C	2 minutes	Hold
Denature	95°C	15 seconds	40
Anneal/extend	60°C	1 minute	

Table 3: Prevalence of fungal species i in samples of mastitis in cattle

Fungal Species	Examined samples									
	Mastitis milk (125)		Letter (25)		Water (25)		Ration (25)		Total(200)	
	No.	%	No.	%	No.	%	No.	%	No.	%
A. flavus	20	16	9	36	19	76	8	32	56	28
A. niger	10	8	2	8	4	16	4	16	20	10
A.ochraceous	5	4	1	4	0	0	0	0	6	3
A.fumigatus	10	8	2	8	0	0	0	0	12	6
Pencillium sp.	25	20	3	12	0	0	5	20	33	15.5
Cladosporium sp.	5	4	1	4	1	4	0	0	14	7
Mucor sp.	0	0	3	12	0	0	3	12	6	6
Fusarium sp.	0	0	0	0	0	0	3	12	3	1.5
C. albicans	20	16	0	0	0	0	0	0	20	10
Rhodotrella sp.	15	12	0	0	0	0	0	0	15	7.5

Tables 4: The incidence of bacterial species in samples of mastitis in cattle

Bacterial Species	Type of examined samples									
	Mastitic milk (125)		Letter (25)		Water (25)		Ration (25)		Total(200)	
	%	No.	%	No.	%	No.	%	No.	%	No.
E. coli (total)	16	**20**	28	7	12	3	4	1	15 .5	31
***E**.coli O157*	12	**15**	4	1	4	1	4	1	9	18
E.coli O55	4	**5**	-	-	-	-	-	-	2.5	5
E.coli O111	8	**10**	8	2	4	1	4	1	7	14
E.coli O26	4	**5**	-	-	4	1	-	-	3	6
C. freundi	-	-	4	1	4	1	-	-	1	2
C.diversus	-	-	4	1	-	-	-	-	0.5	1
K.pneumonia	4	**5**	8	2	4	1	-	-	2	4
K. oxytoca		-	8	**2**	-	-	-	-	1	2
Ps.aeurginosa	4	**5**	20	5	12	3	4	1	7	14
***S**. aureus*	8	**10**	12	3	8	2	-	-	7	7

PCR amplification (Somashekar et al., 2004 and Hossain et al., 2015):

The PCR amplification used primers for the detection of aflatoxin regulatory gene *(aflR)* of *A.flavus* and *Shiga toxin* gene *(stx1)* of *E.coli O157* were prepared by Invitrogen Company (Table 1). The amplification conditions for *AflR* gene were: 5 min initial step at 95 °C followed by 35 cycles at 95 °C for 30 sec, 56 °C for 30 sec and 72 °C for 30 sec and a final extension step at 72 °C for 10 min. While, the PCR amplification of *Stx1* gene were: initial step at 95°C for 5 min, 25 cycles for 5s at 96 °C, 10 s at 54 ° C, 15 sec. at 68°C. Amplification products were electrophoresed with 1 ul of ethidium bromide per agarose gel added for visualization under UV light (1.5% w/v) (Sigma, USA), Using 100 bp DNA Ladder H3 RTU (Ready-to-Use) Cat. No. DM003-R500 from Gene Direx, Inc. Company, Litwania.

Real-Time PCR (Sharma and Nystromi, 2003 and Cruz and Buttner, 2008):

The RT-PCR was used to detect the DNA cycle threshold for *aflR* and *stx1* genes using specific oligonucleotide primers and syber green Mix. The quantities were determined by RT-PCR in 20 µL containing 1 µL of DNA template, 10 µL of syber Green (Biosystems, and Catalog number: A25741). 7 µL PCR grade water, 1 µL of primer with a level of 10 pmol/µL. cycle was done in real-time PCR machine (Chrom4-BIO-RAD, USA), amplification condition as Standard cycling mode (Table 2)

Statistical Analysis

The obtained data were computerized and analyzed for calculation mean ± standard error according to SPSS 14 (2006).

RESULTS AND DISCUSSION

In the current study, the most common recovered fungi was *A. flavus* from mastitis milk, letter, water and ration (16%, 36%, 76% and 32%), respectively. While, the yeasts spp. were detected only in mastitis milk samples as *C. albicans and Rhodotorela* sp. (16%,12%), respectively (Table 3). Whereas other genera of molds were recovered in variable frequencies. *Aspergillus flavus* constitute a public health hazard due to the production of aflatoxins which cause some degree of acute toxicity and are potential carcinogens (FDA, 2000). On the other hand, *E. coli* potentiated the occurrence of bovine clinical mastitis (Hogan and Smith,2003) and recovered from milk and its product (Quinn et al., 2002). Herein, *E. coli* was the most predominant isolates from mastitis milk, letters, water, and animals' ration (16%, 28%, 12%, and 4%), respectively. Currently, the strains of *E.coli O157 and E.coli O111* were recovered from all examined samples at the rates of (12%, 8%) in mastitic milk, (4%, 4%) in water, (4%, 4%) in the ration and (4%, 8%) in letter samples respectively (Table 4). Seyffert et al. (2012) recovered *S. aureus* and *E. coli* from mastitic milk. Moreover, Parul et al. (2014) recovered *E. coli* from dairy animal litters, water and ration samples (8.33%, 3, 0%) and (3%, 11.11%, 0%) by Vanitha et al. (2018), respectively. The significant disease of *E.coli* O157: H7 which produced Shiga toxins (stx1 and stx2) is the hazard of food poisoning (Vali et al., 2007) and dairy cattle is the primary reservoir of infection (Perera et al., 2015). Hence, the discovery of the novel effective antimicrobial agents is required to overcome the microbial infections and resistance to commercial antibiotics (Whitesides, 2003). Today, ZnONPs have significant antimicrobial potentials and friendly safe to the environment (Violeta et al., 2011). Herein, A.*flavus* and *E.coli O157* were the most prevalent isolates in samples of mastitis cattle and were subjected for investigation of the antimicrobial potentials of ZnONPs and C.O. by WD test. The (MIC) of ZnO NPs against *A.flavus and E.coli O157* were (100, 50 µg /ml) and inhibition zones were (10±1.0, 13±0.7 mm), respectively (Table 5). In *A. flavus*, when ZnONPs levels increased from (100-900 µg/ml), the zones of inhibition also increased (10±1.0-30±3.0 mm).While, in *E.coli O157*, the inhibition zones elevated (13±0.7 to 29±2.0 mm), as levels of ZnONPs increased from (50- 900 µg/ml). The antimicrobial potential of ZnO NPs was detected against bacteria and fungi (Raghupathi et al., 2011; Hassan et al., 2017) that caused skin infection in buffaloes (Hassan et al., 2015b) and mastitis in cattle (*E.coli* and *A. flavus*) (Sabir et al., 2014). This is due to the penetration of ZnO-NPs the microbial cell wall, destruction and death of cells (Brayner, et al., 2006). Currently, MIC of C.O. against *A.flavus and E.coli O157* were (0.25% for each) and inhibition zones increased as concentration levels increased (Table 6). El-Baroty et al. (2010) detected the significant antimicrobial activity of C.O. and the MIC values were ranged from (20-120 µg/ml). This activity due to C.O. rich with eugenol and cinnamaldehyde which enable them to penetrate the bacterial or fungal cell membrane and mitochondria cause cell death (Anwer et al, 2009) and impairment of cell enzyme system and cause gene toxicity (Abd El-Baky and El-Baroty, 2008). Recently, the awareness about the toxicity of nanoparticle applications resulted in significant attention for its conjugation with natural materials to avoid the toxic doses in animals. Currently, the synergistic effects of ZnONPs (100, 200 µg/ml) with C.O. (0.25% and 1%), resulted in significant growth inhibition of *A.flavus* and *E.coli O157* (Table 7). The combination of low level of ZnONPs (100 µg /ml) with (0.25% C.O) caused increase in inhibitory zone (10±1.0 to15±2.0) and (17±0.2 to 20±0.8), respectively. While, elevation of C.O. concentration to (1%) at a low level of ZnONPs, increased significantly the inhibitory zones to (20±1.5 to 25±2.0), respectively. These results increase the availability of the application of nanomaterial in biomedicine by decreasing the used doses via conjuga

Table 5: Antimicrobial activity of Zinc oxide nanoparticles against *A.flavus* and *E.coli 0157* recovered from cattle mastitis

Examind isolates	Zones of inhibition (mm) at different concentration of Zinc oxide NPs (µg/ml)									
	50	100	200	300	400	500	600	700	800	900
A.flavus	ND	10±1.0	15±0.5	15±1.0	17±1.5	20±0.8	22±2.0	27±2.5	30±1.0	**30±3.0**
E.coli 0157	13±0.7	17±0.2	20±1.5	20±0.8	24±1.2	24±2.5	25±2.0	25±1.0	27±0.8	**29±2.0**

Table 6: Antimicrobial activity of cinnamon oil against *A.flavus* and *E.coli 0157* recovered from cattle mastitis

Tested isolates	Diameters of inhibitory zones(mm)/ gradual concentrations of cinnamon oil (%)					
	0%	0.25%	0.5%	1.0%	2%	3%
A.flavus	ND	5±0.5	10±1.5	11±0.5	15±0.8	18±1.0
E.coli O157	ND	7±1.5	12±2.5	15±1.0	17±2.0	20±2.5

Table 7: Synergistic Antimicrobial activity of Cinnamon oil with ZnONPs against *A.flavus* and *E.coli 0157* recovered from cattle mastitis

Examined isolates	Diameters of inhibitory zones(mm) / combination of cinnamon oil and ZnONPs.			
	100 µg/ml of ZnO NPs+ 0.25% of cinnamon oil.	100 µg/ml of ZnO NPs+ 1.0% of cinnamon oil.	200 µg/ml of ZnO NPs+ 0.25% of cinnamon oil.	200 µg/ml of ZnO NPs + 1.0% of cinnamon oil.
A.flavus	15±2.0	20±1.5	27±3.0	35±3.0
E.coli O157	20±0.8	25±2.0	25±2.5	35±2,0

Table 8: Comparison between agar diffusion tests, PCR amplification and RT-PCR for detection *AflaR* -gene of *A.flavus and stx1-gene* of *E.coli O157* that treated with ZnONPs singly or in combination with C.O.

Treatments Trials	Agar Diffusion Test	*AflaR -gene of A.flavus*				*stx1*-gene of *E.coli O157*			
		PCR		C.T. (RT-PCR)		PCR		C.T. (RT-PCR)	
		L	H	L	H	L	H	L	H
Control (Untreated)	G	P		26.33		P		19.46	
ZnONPs	NG	N	N	26.62	23.26	P	P	24.01	23.99
C.O.	NG	P	P	23.41	22.57	N	P	14.72	20.01
Combination of C.O.+ ZnONPs	NG	N	P	28.34	28.39	P	P	20.43	24.07

- G: growth of microbial cells -NG: no growth -P: positive DNA band -N: no DNA band - C.T.: Cycle Threshold of DNA - ZnO NPs: 100 µg/ml (Low dose), 500 µg/ml (High dose). - C.O.: 0.25% (low dose), 1% (high dose). -.Combination : 100 µg/ml of ZnONPs+ 1% C.O(high dose), 100 µg/ml of ZnONPs+ 0.25% C.O. (low dose).

tion with natural materials. Similarly, Hassan et al. (2019) detected the MIC of ZnONPs against *Fusarium sp*. was (500 µg/ml) and significantly decreased to (100 µg /ml) when combined with curcumin or probiotic (0.25% for each). This enables to prevent drug resistance and resulted in significant antimicrobial efficacy (Chow and Yu, 1999).

In the present study, PCR detection of the virulent genes in isolated *A. flavus (aflR)* and *E. coli O157(stx1)* from cattle mastitis (4 representative isolates). The DNA bands of 2 isolates of *A.flavus* were similar to the standard strain, while others showed no bands for *aflR* gene (Figure 1). The expression of the *stx1 gene* of *E.coli O157,* 2 isolates not showed any DNA fragment and other isolates were positive for *the stx1 gene* similar to the standard strain (Figure 2). Cruz and Buttner (2008) detected *the alfR* gene in *A.flavus* by PCR and different results of DNA bands occurred. While, Scherm et al. (2005), detected *alfR* and *alfQ* in *A.flavus* isolated from animal feeds. PCR detection of virulent genes *stx1* and *stx2* in *E. coli* isolated food and beef samples (Godambe et al., 2017) that cause food-born infection (Ferens and Hovde, 2011). Currently, the positive isolates of virulent genes were used for PCR detection of antimicrobial potentials of ZnONPs and C.O. (Figures, 1, 2). The PCR amplification of DNA bands of control for each used isolate was similar to the general characters of a standard reference untreated species of *A.flavus and E.coli O157* (Figures 3, 4). Whereas, treating *A.flavus* by low (100 g/ml) and high (500 g/ml) doses of ZnO NPs eliminated the signals of DNA bands (Figure 3). But DNA bands were observed in the case of *E. coli O157* with low or high doses of ZnO NPs (Figure 4). The C.O. effects on genes of *A.flavus,* either at low and

high doses (0.25%, 1%), not cause any changes in DNA bands signals. On the contrary, the treatment of *E.coli O157* with a high dose of C.O.(1%) resulted in the absence of DNA band, but a low dose (0.25%) not cause any changes. Whereas, the combination of ZnO NPs and C.O. presence of DNA band in *E. coli*. While, there was low faint DNA band in treatment of *A.flavus* with (100 μg / ml of ZnONPs+ 1% C.O.). Recently, the RT-PCR help in the generation of a specific fluorescent signal in real-time analysis and quantitation of DNA targets (Schena et al., 2004) and allow rapid, sensitive, specific, and high accurate activity than traditional DNA-PCR method (Bilodeau, 2011). Herein, the RT-PCR system directed against DNA extracted from isolates of *A.flavus* and *E.coli 0157* was done (Figures 1-4 and Table 8). The treatment doses of ZnONPs alone or in combination with C.O. increased the DNA cycle threshold (C.T). The treatments of *A.flavus* with ZnO NPs (100g/ml) resulted in a significant increase in DNA C.T. values (26.62, 28.34) higher than that DNA of non-treated isolates (26.33).

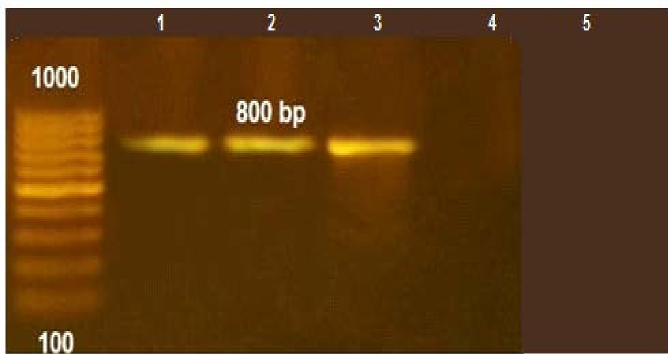

Figure 1: PCR amplification for *aflaR* gene of *A.flavus*(at 800pb) Lane L:100 bp DNA ladder standard. Lane 1: Positive control of *A.flavus*. Lane 2-5 *A.flavus isolated from mastitis*

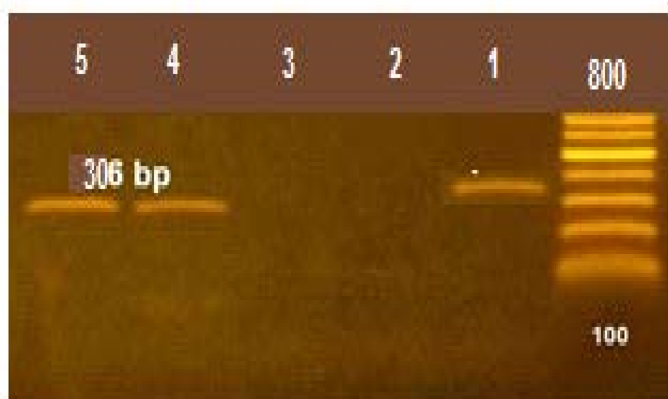

Figure 2: The PCR amplification *for stx*1gene of *E.coli O175*(at 306pb).Lane R: 100bp DNA ladder standard; Lane 1: Positive control of *E.coli 0157*., Lane 2-5: the isolates from mastitis

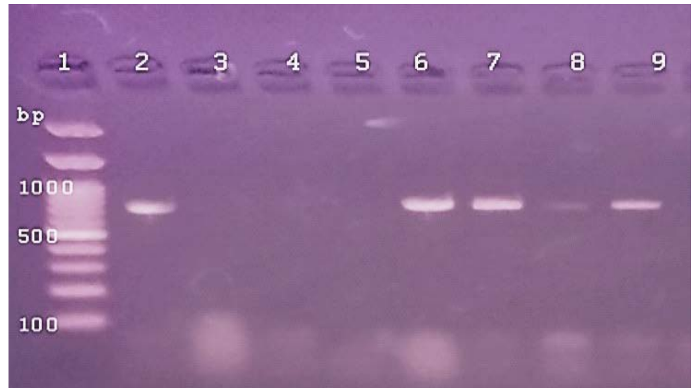

Figure 3: The PCR amplification for *aflaR* gene *of A.flavus*(at 800pb) Lane 1:100 bp DNA ladder standard. Lane 2: Positive control of *A.flavus*.Lane 3: Negative control (*Fusarium sp.*) Lane 4: Treated 100 μg /ml of ZnONPs).Lane5: Treated 500 μg /ml of ZnONPs). Lane 6: Treated 0.25% C.O. Lane 7: Treated 1% C.O. Lane 8: Combination treat. of 100 μg /ml of ZnONPs+1%C.O. Lane9: Combination treat. of 100 μg /ml of ZnONPs+ 0.25% C.O.

Figure 4: The PCR amplification *for stx*1gene of *E.coli O175*(at 306pb).Lane1: 100bp DNA ladder standard; Lane 2: Positive control of *E.coli 0157*.Lane 3: Negative control (*S.aureas)* Lane 4: Treated 100 μg /ml of ZnONPs). Lane 5: Treated 500 μg /ml of ZnONPs). Lane 6: Treated 0.25% C.O. Lane 7: Treated 1% C.O.Lane 8: Combination treat. of 100 μg /ml of ZnONPs+ 1% C.O. Lane 9: Combination treat. of 100 μg /ml of ZnONPs+ 0.25% C.O.

While, the treatment of *E.coli* with low and high doses of ZnONPs caused a treatment with C.O. caused a decrease in DNA C.T. values (Table 8). It is suggested that the higher DNA C.T. due to the lower number of DNA copies of the genes in treated isolates with ZnONPs than that of untreated ones and contrary to this were reported in C.O. (Table 8). Several studies used RT-PCR for rapid detection of genes pathogens as Sharma and Nystromi (2003) and Hu et al. (2020) for *stx1* and *stx2* in *E.coli* O157: H7 in food, Scherm et al. (2005), for detecting aflatoxin regulatory genes. Copping et al. (2005) found that the inhibitory concentration of antifungals against

C.albicans elevated the activity of secreted proteinase (Sap) and SAP genes detection by RT– PCR. Labeed et al. (2016) and Hassan et al. (2017) detected the absence of DNA band in PCR of *Afla* gene after bio-control of mycotoxigenic *A. flavus* and no levels observed in chemical detection of AFB1. Rathore et al. (2018) found that variety in the correlation between disc diffusion and genotypic PCR antibiotic sensitivity pattern against virulent genes in *S. aureus* that ranged from (58.3%-100%). While, Zheng et al. (2015) resulted in significant antimicrobial activity of *Enterococcus faecium* against gram-positive and gram-negative bacteria using agar well diffusion method as a simple method and PCR amplification not detected any tested virulence genes. Furthermore, Chomvarin et al. (2004) detected that disc diffusion and agglutination tests are of the highest sensitivity and specificity and both assays are technically simple and can be easier to perform in routine laboratories than PCR in the evaluation of drugs against bacterial and fungal pathogens. Hence, the evaluation of antimicrobials by covenantal method as agar WD yielded significant specific results and despite advances in PCR and RT-PCR we detected variable in accord findings.

CONCLUSION

From the forgoing results it is concluded that the antimicrobial potential of ZnONPs was more effective than traditional antibacterials as oil and resulted in decreased and eliminated the targeted DNA gene expression of aflatoxigenic *A.flavus and E.coli*. In addition the RT-PCR confirmed these changes by increase in DNA cycle threshold . The synergistic action of ZnONPs with natural oil caused significant antibacterial potential and resulted in decrease the used doses of nanomaterial , hence, we can overcome nanomaterial toxicity for future application in veterinary medicine. The conventional laboratory diffusion tests are still most satisfactory, simple and inexpensive in comparison with genotyping methods as PCR and RT-PCR. Hence, nanotechnology has huge significant progressive advancement in biotechnology and biomedicine related to human and animal science as increase the safety of their health, production and hence elevation of national income.

CONFLICT OF INTEREST

All authors declare that there no conflict of interest.

ACKNOWLEDGMENT

The authors are gratefully acknowledged to Prof. Dr. Hazem Hassan Mahmoud, for his kind assistance and fund in identification and characterization of the prepared and used zinc nanoparticles.

AUTHORS CONTRIBUTION

Atef Hassan conceived and designed the experiments; Noha Oraby and K.Abo-zaid did the methodology, Atef Hassan and Noha Oraby did manuscript preparation, analyzed the data and wrote this manuscript; Corresponding author N.Oraby. All authors read and approved the final manuscript.

REFERENCES

- Abd El-Baky HH, El-Baroty GS (2008). Chemical and Biological Evaluation of the Essential oil of Egyptian Moldavian Balm. Int. J. Essen. Oil Therap. 2:76-81.
- Alyssa SS, Rajinder PSB, Thomas A (2012). “Hyper virulent (hypermucoviscous) *Kl.pneumoniae.* A new and dangerous breed genosis infection”, Veterans Administration Healthcare System, Buffalo, New York, USA.
- Anwar F, Ali M, Hussain AI, Shahid M (2009). Antioxidant and antimicrobial activities of essential oil and extracts of fennel *(Foeniculum vulgare Mill.)* seeds from Pakistan. Flav. Frag. J. 24: 170-176. https://doi.org/10.1002/ffj.1929
- APHA (American Public Health association) (2003). Compendium of methods for the microbiological examination of foods. 3rd ed. (USA), 675- 800.
- Beyth N, Haddad Y, Domb A, Khan W, Hazan R (2015). Alternative antibacterial approach: Nano-antibacterial materials. J. Evid. Based Complem. Altern. Med. 1-16. https://doi.org/10.1155/2015/246012
- Bilodeau GJ (2011). Quantitative Polymerase Chain Reaction for the detection of organisms in soil, Perspective in Agriculture, Vet. Sci. Nutr. Natur. Res. 6: 1-14. https://doi.org/10.1079/PAVSNNR20116014
- Brayner R, Ferrari-Iliou R, Brivois N, Djediat S, Benedetti MF, Fievet F (2006). Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Lett. 6(4): 866–870. https://doi.org/10.1021/nl052326h
- Chomvarin C, Siripornmongcolchai T, Chaicumpar K, Limpaiboon T, Wongkham C, Yutanawiboonchai W (2004). Evaluation of polymerase chain reaction, conventional and MRSA screen latex agglutination methods for detection of methicillin-resistant,-borderline and susceptible *S.aureus.* South East As. J. Tr. Med. Pub. Heal. 35(4):879-885.
- Chow JW, Yu VL (1999). Combination antibiotic therapy versus monotherapy for gram-negative bacteremia: a commentary. Int. J. Antimicrob. Agents. 11:7-12. https://doi.org/10.1016/S0924-8579(98)00060-0
- Copping VMS, Barelle CJ, Hube B, Gow NAR.,Brown AJP, Odds FC (2005). Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted proteinase genes. J. Anti. Chem. 55: 645–654. https://doi.org/10.1093/jac/dki088
- Cruz P, Buttner MP (2008). Development and evaluation of a real-time quantitative PCR assay for *Aspergillus flavus*. Mycologia. 100: 683-690. https://doi.org/10.3852/08-022
- El-Baroty G, Abd El-Baky HH, Farag RS, Saleh MA (2010). Characterization of antioxidant and antimicrobial compounds of cinnamon and ginger essential oils. Afric.J. Biochem. Res. 4(6): 167-174.
- FDA (Food and Drug Administration) (2000): Conference

on mycotoxins in animal feeds,grains and food related to human and animal health. Rockville, Maryland.

- Ferens WA, Hovde CJ (2011). *Escherichia coli* O157:H7: animal reservoir and sources of human infection. Foodborne Pathog. Dis. 8:465–487. https://doi.org/10.1089/fpd.2010.0673
- Fittipaldi M, Nocker A, Codon F (2012). Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J. Microb. Meth. 91(2): 276-289. https://doi.org/10.1016/j.mimet.2012.08.007
- Food and Drug Administration (FDA) (2000). Conference on mycotoxins in animal feeds, grains and food related to human and animal health. Rockville, Maryland.
- Godambe LP, Bandekar J, Shashidhar R (2017). Species specific PCR based detection of *Escherichia coli* from Indian foods. Biotech. 7(130):1-5. https://doi.org/10.1007/s13205-017-0784-8
- Gong P, Li H, He X, Wang K, Hu J, Tan W, Zhang S, Yang X (2007). Preparation and antibacterial activity of Fe3O4@ Ag nanoparticles. Nanotechnology. 18(28):285604. https://doi.org/10.1088/0957-4484/18/28/285604
- Hassan AA, Oraby NA, Mohamed AA, Mahmoud HH (2014). The possibility of using Zinc Oxide nanoparticles in controlling some fungal and bacterial strains isolated from buffaloes. Egypt. J. Appl. Sci. 29(3):58-83.
- Hassan M. N., El-Sayed A. S.A., Nada H. M.S. (2015a). Detection of aflatoxins by HPLC and the expression of biosynthetic *nor-1* gene of aflatoxin and *ocrA* gene of ochratoxin. Benha Vet. Med. J. 29(2): 1-10. https://doi.org/10.21608/bvmj.2015.31538
- Hassan AA, Mahmoud HK, Taha H, Sayed El-Ahl RH, Mahmoud HH (2015b). Herbal biosynthesis of zinc nanoparticles and evaluation of their antifungal and antibacterial effect for buffaloes skin affections. Int. J. Curr. Res. 7(12): 24338–24349.
- Hassan AA, El-Shafei HM, Sayed El-Ahl RMH, El-Hamaky AM (2017). Molecular detection the influence of aflatoxin biosynthetic genes by *Aspergillus flavus* before and after *bacillus subtlis* and *candida albicans* biocontrol. 9th Scient. Congr. Egypt. J. Anim. Manag. 1-19.
- Hassan AA, Oraby NH, El-Mosalamy MM (2019). Detection of mycotoxigenic *fusarium* species in poultry ration and their growth control by zinc nanoparticle. Anim. Health Res. J.7(4):1075-1091.
- Hossain AM, Al-Arfaj AA, Zakri AM, El-Jakee JK, Al-Zogibi OG, Hemeg HA, Ibrahim IM (2015). Molecular characterization of *Escherichia coli O157:H7* recovered from meat and meat products relevant to human health in Riyadh, Saudi Arabia. Saudi J. Biol. Sci. 22(6): 725-729. https://doi.org/10.1016/j.sjbs.2015.06.009
- Hogan J, Smith KL (2003). Coliform mastitis. Vet. Res. 34: 507-519. https://doi.org/10.1051/vetres:2003022
- Hu L, Han B, Tong Q, Xiao H, Cao D (2020**)**. Detection of Eight Respiratory Bacterial Pathogens Based on Multiplex Real-Time PCR with Fluorescence Melting Curve Analysis. Canad. J. Infec. Dis. Med. Microl. 4056. https://doi.org/10.1155/2020/2697230
- Jiff-Agbola YA, Onifade AK, Osho IB (2012). In vitro antifungal activated of essential oil from Nigerian medical plants toxicogenic Aspergillus flavus. J. Med. Plants. 6(23): 4048-4056
- Jin T, Sun D, Su JY, Zhang H, Sue HJ (2009). Antimicrobial efficacy of zinc oxide quantum dots against *Listeria monocytogenes, Salmonella enteritidis*, and *Escherichia coli O157:H7*. J. Food Sci. 74: 46–52. https://doi.org/10.1111/j.1750-3841.2008.01013.x
- Labeed AA, Adnan IA, Sami AA, Naresh M, Alicia R (2016). Impact of bacterial biocontrol agents on aflatoxin biosynthetic genes, *aflD* and *aflR* expression, and phenotypic aflatoxin B1 production by *Aspergillus flavus* under different environmental and nutritional regimes. Int. J. Food Micro. 217: 123–129. https://doi.org/10.1016/j.ijfoodmicro.2015.10.016
- Lee SO, Choi GJ, Jang KS, Kim JC (2007). Antifungal activity of five plant essential oils as fumigant against postharvest and soil borne plant pathogenic fungi. Plant Pathol. J. 23(2): 97-102. https://doi.org/10.5423/PPJ.2007.23.2.097
- Liu S, Wei L, Hao L, Fang N, Chang MW, Xu R, Yang Y, Chen Y (2009). Sharper and faster nano darts kill more bacteria: a study of antibacterial activity of individually dispersed pristine single-walled carbon nanotube. ACS Nano. 3 (12): 3891–3902. https://doi.org/10.1021/nn901252r
- Mohan AC, Renjanadevi B (2016). Preparation of zinc oxide nanoparticles and its characterization using scanning electron microscopy (SEM) and x-ray diffraction (XRD). Proc. Technol. 24: 761-766. https://doi.org/10.1016/j.protcy.2016.05.078
- Neville J, Bryant AF (1986). Laboratory Immunology and Serology. 2nd ED. ,Saundei Company Copyright, Toronto, Canada.
- Partha SS, Rajendran D, Rao SBN, Dominic G (2015). Preparation and effects of nano mineral particle feeding in livestock: a review. Vet. World. 8 (7): 888-891. https://doi.org/10.14202/vetworld.2015.888-891
- Partha SS, Rao BN, Rajendran D, Dominic G, Selvaraju S (2016). Nano zinc, an alternative to conventional zinc as animal feed supplement: a review, Anim. Nutr. 2: 134-141. https://doi.org/10.1016/j.aninu.2016.06.003
- Parul SB, Sharma BB, Jain U (2014). Virulence-associated factors and antibiotic sensitivity pattern of E.coli isolated from cattle and soil. Vet. World. 7(5): 369-372. https://doi.org/10.14202/vetworld.2014.369-372
- Perera A, Clarke CM, Dykes GA, Fegan N (2015). Characterization of Shiga toxigenic *Escherichia coli O157* and non O157 isolates from ruminant feces in Malaysia, Biomed. Res. Intern. 3:1-8. https://doi.org/10.1155/2015/382403
- Pitt JI, Hocking AD (2009). "Fungi and Food Spoilage"- "Methods for Isolation, Enumeration and Identification". Chapter 4:19-55. 3rd Ed. Springer Science Intern. Publishing AG. https://doi.org/10.1007/978-0-387-92207-2_4
- Quinn PJ, Carter ME, Markey BK, Donnelly WJC, Leonard FC (2002). Veterinary Microbiology and Microbial Disease. Great Britain by MPG, Book, Ltd, Bodmin.
- Raghupathi KR, Koodali RT, Manna AC (2011). Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles. Langmuir. 27(7): 4020–4028. https://doi.org/10.1021/la104825u
- Rathore K, Joseph B, Sharma DK, Gaurav A, Sharma SK, Milind M, Patel P, Prakash C, Singh L (2018). Evaluation of multiplex polymerase chain reaction as an alternative to conventional antibiotic sensitivity test, Vet. World. 11(4): 474-479. https://doi.org/10.14202/vetworld.2018.474-479
- Sabir S, Arshad M, Chaudhari SKl (2014). *Review Article*: Zinc Oxide Nanoparticles for Revolutionizing Agriculture: Synthesis and Applications. Scient. World J. Article ID 925494, 1-8. https://doi.org/10.1155/2014/925494

- Sahoo AK, Sahoo D, Sahu NC (2014). Mining export, industrial production and economic growth: A cointegration and causality analysis for India. Resources Policy, Elsevier. 42(C): 27-34. https://doi.org/10.1016/j.resourpol.2014.09.001
- Schena L, Nigro F, Ippolito A, Gallitelli D (2004): Real Time quantitative PCR: a new technology to detect and study phyto-pathogenic and antagonistic fungi. Europ. J. Plant Pathol. 110(9):893-904. https://doi.org/10.1007/s10658-004-4842-9
- Scherm B, Palomba M, Serra DM, Migheli Q (2005). Detection of transcripts of the aflatoxin genes *aflD*, *aflO*, and *aflP* by reverse transcription– polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. Int. J. Food Microbiol. 98: 201-210. https://doi.org/10.1016/j.ijfoodmicro.2004.06.004
- Seil JT, Webster TJ (2012). Antimicrobial applications of nanotechnology: methods and literature. Int. J. Nanomed. 7: 2767– 2781. https://doi.org/10.2147/IJN.S24805
- Seyffert N, Le Maréchal C, Jardin J, McCulloch JA, Rosado FR, Miyoshi A, Even S, Jan G, Berkova N, Vautor E, Thiéry R (2012). *Staphylococcus aureus* proteins differentially recognized by the ovine immune response in mastitis or nasal carriage. Vet. Microbiol. 157(3-4):439-47. https://doi.org/10.1016/j.vetmic.2012.01.016
- Sharma VK, Nystromi EA (2003). Detection of enterohemorrhagic *Escherichia coli* O157:H7 by using a multiplex real-time PCR assay for genes encoding *Intimin* and *Shiga* toxins. Vet. Microbiol. 93: 247–260. https://doi.org/10.1016/S0378-1135(03)00039-7
- Somashakar D, Rati ER, Chandrashekar A (2004). PCR-restriction fragment length analysis of *aflR* gene for differentiation and detection of *A.flavus and A.parasiticus* in maiz. Int. J. Foods Microb. 93(1):101-107. https://doi.org/10.1016/j.ijfoodmicro.2003.10.011
- SPSS 14 (2006). "Statistical Package for Social Science, SPSS for windows Release 14.0.0, and 12 June, 2006. Standard Version, Copyright ® SPSS Inc. USA.
- Vali L, Hamouda A, Hoyle DV (2007). Antibiotic resistance and molecular epidemiology of *E.coli 026, 0103 and 0145* shed by two coharts of Scottish beef cattle. J. Antimic. Chemotherap. 59:403-410. https://doi.org/10.1093/jac/dkl491
- Vanitha HD, Sethulekshmi C, Latha C (2018). An epidemiological investigation on occurrence of enterohemorrhagic *Escherichia coli* in raw milk. Vet. World. 11(8):1164. https://doi.org/10.14202/vetworld.2018.1164-1170
- Violeta V, Catalin P, Constantin F, Monica A, Marius B (2011). Nanoparticles applications for improving the food safety and food processing. 7 th Int. Conf. on Mat. Sci. and Engin. Bramat. Brasov. 24-26.
- Whitesides GM (2003). The 'Right' Size in Nanobiotechnology. Nature Biotech. 21(10): 1161-5. https://doi.org/10.1038/nbt872
- Zheng W, Zhang Y, Lu H, Li D, Zhang Z, Tang Z, Shi L (2015). Antimicrobial activity and safety evaluation of *Enterococcus faecium* KQ 2.6 isolated from peacock feces. Biotechn. 15(1):30. https:/doi.org/10.1186/s12896-015-0151-y.