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Zinc Oxide nanoparticles and curcumin affect some virulence genes of *Staphylococcus aureus* and *Candida albicans,* the cause of sub-clinical mastitis in buffalo

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ABSTRACT

Nowadays, there are progressive advancements in the biomedical employment of nanotechnology in human and animal health. Today, the dangers of infectious animal diseases and the continuous application of traditional antibiotics resulted in the elevation of the activities of the drugs resistant genes and the failure of their control. Hence, it is necessary to prevent fungal and bacterial pathogens pollution and their toxicity in dairy animal products. Sixty samples were collected from dairy buffalo farms suffering from subclinical mastitis (20 Samples of each from subclinical mastitis milk, animal feeds, and bedding). The species of *Staphylococcus* is considered the most predominant species, and *Staphylococcus aureus* was the most prevalent bacterial isolate. While, yeast, especially *Candida albicans,* was the most dominant fungal isolate from different samples of dairy buffalo farms. Our antibiogram of *S. aureus* and *Candida albicans* showed a high resistance rate against several traditional antimicrobial agents. Therefore, searching for effective drug materials to overcome drug resistance is needed. Herein, ZnONPs and Curcumin are evaluated singly and, in a composite, to inhibit the growth of *C. albicans* and *S. aureus*. The inhibitory dose of ZnONPs for *C. albicans* and *S. aureus* were (600 and 600 µg /ml) and in Curcumin were (2% and 3%), respectively. However, the hybrid nanocomposite of ZnONPs (100 µg/ml) with Curcumin (0.25% and 1%) caused suppression of the growth of these pathogens. The nanocomposite of low levels of ZnONPs (100 µg /ml) with (0.25% curcumin) significantly increased the inhibitory effects against *C. albicans* and *S. aureus*. The molecular study to detect the efficiency of the treatment doses indicated that the exposure of *C. albicans* and *S. aureus* to a low dose of ZnONPs (100 µg/ml) did not affect the DNA expression of virulent genes. While the high dose (600 µg/ml) prevented the DNA expression. However, in the Curcumin treatments of isolates with low doses (0.25), the genes' DNA expression was not affected. While, the high dose (1%), removed the DNA band. All inhibition and removal of virulent genes' DNA of pathogens can be obtained by the combination of low levels of ZnONPs and Curcumin. Hence, it is concluded that the nanocomposites of ZnONPs and Curcumin caused significant effects against pathogens.

Keywords: Zinc Oxide nanoparticles, Curcumin, Buffaloes, Mastitis, *S. aureus, C .albicans.*

INTRODUCTION

Worldwide progressive attention is made to food safety and human and animal health; hence, animal products are of massive economic significance for human use as milk, meat, and others (Hassan *et al.,* 2020a & b, 2021, 2022). Several animal diseases are adversely suppressing their health and productivity, particularly dairy animal mastitis, which suppresses milk and meat yields (Hassan *et al.,* 2021). These diseases have resulted from immune suppression of animals, microbial agents, and adverse climatic factors (Hassan *et al.,* 2020 a & b). The majority of studies illustrated that the most predominant pathogens that cause subclinical mastitis were *Staphylococcus, Streptococcus,* and *Escherichia coli* species (62.9%, 15.5%, and 12.4**%),** respectively (Sikarwa and Batra, 2011 and Shon *et al.,* 2013, Hassan *et al.,* 2014, 2020, 2022). Therefore, the problem of sub-clinical mastitis in bovines has an enormous significance and health hazard for human consumers (Bhattarai *et al*., 2020; Brennecke *et al.,* 2021; Damian *et al.,* 2021). Whereas different fungal pathogens, especially Candida albicans, were isolated from cases of bovine mastitis as it was detected in the milk of mastitis buffaloes with an incidence of 10.59% (Mosherf, 2005), and 32% and 24% in affected sheep and goats, respectively (Hassan *et al.,* 2012). Moreover, subclinical mastitis has

several adverse problems in animals and their productivity, in addition to its zoonotic ability via the consumption of infected milk and other products (Khazaie and Ahmadi, 2021). Hence, more attention was undertaken to control the growth of pathogenic causes by conventional drugs, but frequent uses of this antimicrobial agent initiated the drug resistance of these pathogens (El-Hamaky *et al.*, 2022). So, there are urgent requirements for novel and effective agents to eliminate the viability and growth of animal pathogens to overcome drug resistance (Fahmy *et al.,* 2020).

Nowadays, the progressive advancements in the application of nanotechnology in veterinary medicine potentiate the production of new agents of antifungals and antimicrobials (Weiss *et al.,* 2006, Hassan *et al.,* 2020 a-c, 2021; 2022 a, and El-Hamaky *et al.,* 2022). They detected the multi drugs resistant genes in some bacterial and fungal strains and the elimination of their DNA signals expression by copper and zinc nanoparticles and a hybrid of Copper-Chitosan nanocomposites, which in successful antifungals and antibacterial potentials.

Furthermore, several metal nanoparticles, particularly those of metal oxides such as ZnO and Selenium Oxid, have significant antimicrobial potentials (Sawai *et al.*, 2000; Hassan *et al.,* 2020a-c; 2021**).** These metal nanomaterials enable the toxic effect against microbial pathogens without affecting human or animal cells **(**De Romana *et al*., 2002; Reddy*,* 2007**)**. For example, ZnONPs is used as a food supplement to treat zinc deficiency and are effective against a wide range of strains such as *E. coli*, *Salmonella, and S. aureus* **(**Jones *et al.,* 2008 and Liu *et al.,* 2009; Hassan *et al.,* 2020 a&b; 2021**).** In addition, several medicinal plants and their oils have significant abilities for eliminating and suppressing the growth of pathogens causing bovine mastitis (Lopes *et al.,* 2020; El-Hamaky *et al.,* 2022). The most famous plant powder of wide use is Curcumin, which has an active principal chemical compound (Tumeric acid) that enables therapeutic effects against several disorders such as inflammations, alimentary tract affections, and tumors **(**Goel *et al.,* 2008**).** Furthermore, it has the potential to be an antioxidant through the prevention of lipid peroxidation and DNA oxidative damage (Jayaprakasha *et al.,* 2005; Hassan et al., 2020 d).

Therefore, the present study was undertaken for the detection of the fungal and bacterial pollution causes of dairy buffalo's subclinical mastitis. The drug resistance of predominant isolates was determined. In addition, the application of use ZnONPs and curcumin and their hybrid material as novel antimicrobial agents were evaluated. In addition, the availability of these treatments to detect the influences in virulent genes' expression after exposure to the present nanomaterials and composite agents was subjected to molecular biology technique.

MATERIALS AND METHODS

Samples:

Sixty samples (20 each of apparently normal buffalo's milk samples from normal udder after giving positive reaction for White Side Test (WST) to detect subclinical mastitis according to APHA (1978), bedding and animal feeds samples) were obtained from diseased buffalo farm. They were collected under sterile conditions and transferred directly to the laboratory for both bacteriological examination and mycological examination as described by Koneman *et al.* (1997).

Zinc Oxide Nanoparticles and Curcumin:

Synthesis and identification of zinc oxide nanoparticles have been done by Sigma chemical company, and it was in amorphous form with 50 nm. The Curcumin was obtained from Al Gomhorya company, Egypt.

Isolation and identification of bacteria and fungi:

The samples were poured into the nutrient broth and incubated overnight at 37ºC_Then, they were transferred MacConkey agar, Bairda parker, EMB and 7% sheep blood agar media plates and the appeared colonies characterized as a method as Quinn *et al*. (2002); Chitra *et al.* (2015). On the other hand, the samples were subjected to mycological examination. The recovered moulds and Yeast colonies were classified and characterized as illustrated by identified by the authors according to the technique recommended by Refai *et al.* (2012) and ISO (2008) and Pitt and Hocking (2009).

Antimicrobial susceptibility testing:

The bacterial isolates suspension was inoculated onto Muller-Hinton agar palates. it was kept under aerobic condition at 37°C for 24h using different antimicrobial disks: gentamycin (CN10), ampicillin(AM10), flumequine(UB30), Ceftiofur (EFT30) imipenem(IPM10), amoxicillin(AML10), cefotaxim(CTX30), Ciprofloxacin(CIP5),

and ofloxacin(OFX5). The minimal inhibitory concentration of disks was determined as to the nearest millimetre using calibrated rulers according to CLSI (2015).

Standards of antifungal agents:
The in vitro susceptibility testing of most recovered fungi (*C.albicans*) to commercial antifungals was applied as method as National Committee for Clinical Laboratory (NCCLS, 2002). Briefly, an inoculum of *C. albicans* culture was streaked over SDA plate, then antifungal discs (Fluconazole, Clotrimazole, Ketoconazole, Amphotericin B and Nystatin) were spread on the surface plate and incubated at 37°C for 24hrs. The width of inhibition zone was determined. Evaluation of the activities of ZnONPs and Curcumin against pathogens of mastitis (Jin *et al.,* 2009; Jeff-Agboola *et al.*, 2012):

Preparation of spore suspension of isolates:
The isolated *C. albicans and S.aureus* from the present study were inoculated on respective agar (SDA and MacConkey agar) and kept (24-48 hours at 37°C), respectively, in parallel with positive control **(**Gupta and Kohli, 2003). At the end of incubation period, filtration to discard the mycelia and the spores determined by haemocytometer slide to (10^4spores /ml).

Measurement of MIC of prepared ZnONPs singly and in combination with curcumin against fungi and bacteria isolated from diseased cases of buffalos (CLSI 2008):
It was measured as recommended by (Balachandran *et al*., 2015); (NCCLS, 2002). Briefly, In sterile tubes, 900 µl of broth SD broth medium or nutrient broth and 100 µl of spores were poured (*S. aureus,* 2.5 x 10^3cells/ml) and *Candida albicans to* (5 x 10^4 cells/ml). Then, 100 µl of Zinc nanoparticles concentrations (50, 100, 200, 400, 600 µg/mL) and/or 100 µl of Curcumin (0, 0.25%, 0.5%, 1%, 2%, 3%) for bacteria and fungi, were added and incubated for 1-2 days at 37 °C. Combination effects of ZnONPs and Curcumin. The minimum inhibitory concentration was the lowest level of ZnONPs inhibits viability and growth of bacteria or yeast. The absorbance, transmittance % and optical density of the tests tubes content were observed every 24 hrs. These items were detected spectrophotometrically at 405 nm wavelength.

Detection of virulent genes *of C. albicans and S. aureus* by PCR before and after treatments:
Preparation of treated *C. albicans and S. aureus*:
Briefly, in sterile test tubes, 20 ml of SD broth medium (for fungus) and nutrient broth (for bacteria) and 0.2 ml of 10^4 spores were poured. Each strain was subjected to 6 doses of treatments (low, 100 µg /ml ZnO NPs), (high, 600 µg/ml ZnONPs) and (low, 0.25% ~~c.~~ curcumin), (high, 1%~~C.~~ curcumin), (combination, 100 µg /ml ZnONPs+ 0.25% C.), (combination, 100 µg /ml ZnONPs+ 1% C.). The negative control was (*Fusarium)(E.coli*) and the positive was *C.albicans and S.aureus*. All the tubes were incubated at 30°C for 3 days and kept at 5-8 °C till DNA extraction.

DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer's instructions.
Oligonucleotide Primer. Primers used were supplied from **Metabion (Germany) and** are listed in Table (1).
PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), briefly, 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. For multiplex PCR, primers were utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1.5 µl of each primer of 20 pmol concentration, 9 µl of water, and 11 µl of DNA template.

Analysis of the PCR Products:
The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR products and 40 µl of the multiplex PCR products were loaded in each gel slot. A plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions for detection of the virulent factors of *S. aureus* and *C. albicans*

Agent	Target gene	Primers sequences 5` to 3`	Amplified segment (bp)	Amplification (35 cycles)					Reference
				Primary denaturation	Secondary denaturation	Annealing	Extension	Final extension	
S. aureus	*clfA*	F.GCAAAATCCAGCACAACAGGAAACGA R.CTTGATCTCCAGCCATAATTGGTGG	638	94˚C 10 min.	94˚C 1 min.	55˚C 1 min.	72˚C 1 min.	72˚C 10 min.	**Mason *et al.*, 2001**
C. albicans	*SAP*	F. CTG ATT TAT GGG TTC CTG AT R- TGGCAGCATTGGGAGAGTTG	390	94˚C 5 min. 1 cycle	94˚C 1 min. 35 cycles	55˚C 1 min. 35 cycles	72˚C 2 min. 35 cycles	72˚C 5 min. 1 cycle	**(Bautista-Munoz *et al.*, 2003)**

Statistical analysis:

The results were subjected to determine the mean and their standard deviation. Significance of the data was measured by a one-way analysis of F- test and the least significant difference as well as t-student test (SPSS 14, 2006).

Results

In the present study, sixty samples (20 each of apparently normal buffalo's milk samples from normal udder, bedding and animal feeds samples) were subjected for both bacteriological and mycological examination and the obtained results were tabulates in the following tables.

Table 2. Incidence of bacterial isolates recovered from subclinical mastitic buffalo's milk, animal feeds and bedding samples:

Bacterial isolates	Total No. of isolates	subclinical mastitic milk (20)		Animal feeds (20)		Bedding (20)	
		No	%	No	%	No	%
S. aureus	12	6	30	4	20	2	10
E. coli	11	5	25	2	10	4	20
CNS (coagulate negative Staphylococcus)	12	7	35	3	15	2	10
St. faecalis	6	2	10	1	5	3	15
Klebsiella spp.	5	-	-	-	-	5	25
Ps. Aeruginosa	5	-	-	1	5	4	20

Table 3. Incidence of fungal isolates recovered from subclinical mastitic buffalo's milk, animal feeds and bedding samples:

Fungal isolates	Total No. of isolates	Subclinical mastitic milk (20)		Animal feeds (20)		Bedding (20)	
		No	%	No	%	No	%
Yeast spp. *Candida albicans*	14	7	35	5	25	2	10
Candida parapsilosis	9	4	20	2	10	3	15
Candida krusei	7	2	10	3	15	2	10
Rhodotrula mucilaginosa	5	1	5	2	10	2	10
Trichosporon	4	2	10	2	10	-	-
Mould spp. *Asperagillus fumigatus*	5	2	10	1	5	2	10
Asperagillus niger	4	1	5	2	10	1	5
Asperagillus flavus	2	1	5	1	5	-	-

Table 4. Antimicrobial susceptibility pattern of *S. aureus* isolates (n=12):

Antibiotics	Sensitive		Resistant	
	No.	%	No.	%
Amoxicillin (AXL10 µg)	5	41.67	7	58.33
Flumequine (UB30 µg)	9	75.00	3	25.00
Ceftiofur (EFT30 µg)	4	33.33	8	66.67
Imipenem (IPM10 µg)	12	100	-	-
Cefotaxime (CTX30 µg)	4	33.33	8	66.67
Gentamycin (CN10 µg)	8	66.67	4	33.33
Ampicillin (AM10 µg)	2	16.67	10	83.33
Ciprofloxacin (CIP5 µg)	12	100	-	-
Ofloxacin (OFX5 µg)	11	91.67	1	8.33

Table 5. Inhibition zones diameter of antifungal drugs used for disc diffusion method of *C. albicans* (Rosco diagnostic, 2007-2008):

Type of isolates	Fluconazole	Clotrimazole	Ketoconazole	Nystatin	Amphotericin B
C. albicans	+++	-	+	++++	+++

Table 6. Absorbance and degree of turbidity of *C. albicans* and *S. aureus* exposed to gradual concentration of ZnONPs

Concentrations of ZnONPS (ug/ml)	*C . albicans*		*S. aureus*	
	Absorbance (OD at 405 nm w/l)	DT& GT	Absorbance (OD at 405 nm w/l)	DT& GT
0	2.40	4	2.20	4+
50	0.91	3+	1.35	3+
100	0.38	2+	0.35	2+
200	0.30	1+	0.21	2+
400	0.20	1+	0.18	1+
600	0.20	00	0.18	00

OD: Absorbance at wavelength; 405 nm. - DT: Degree of turbidity - GT: Growth.

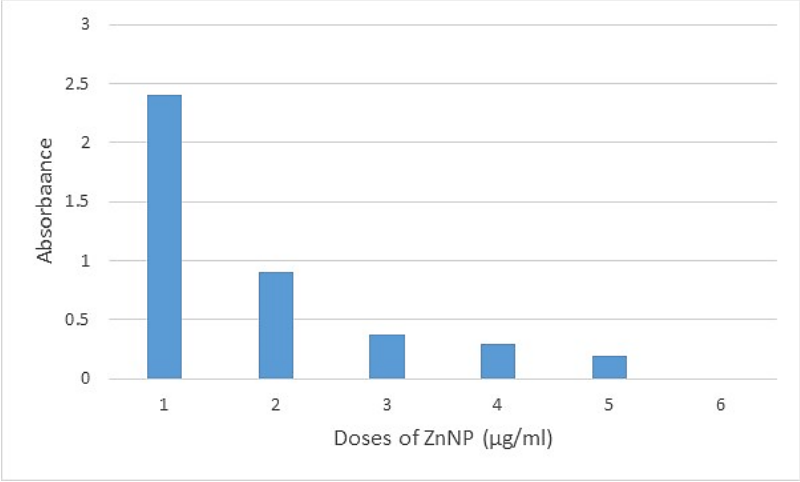

Fig. 1. Optical density of treated *C. albicans* at gradual concentration of ZnONPS
Dose1: (ZnONP 0 µg/ml), Dose2: (ZnNP 50 µg/ml), Dose3: (ZnNP 100 µg/ml). Dose4: (ZnNP 200 µg/ml), Dose5: (ZnNP 400 µg/ml), Dose6: (ZnNP 600 µg/ml)

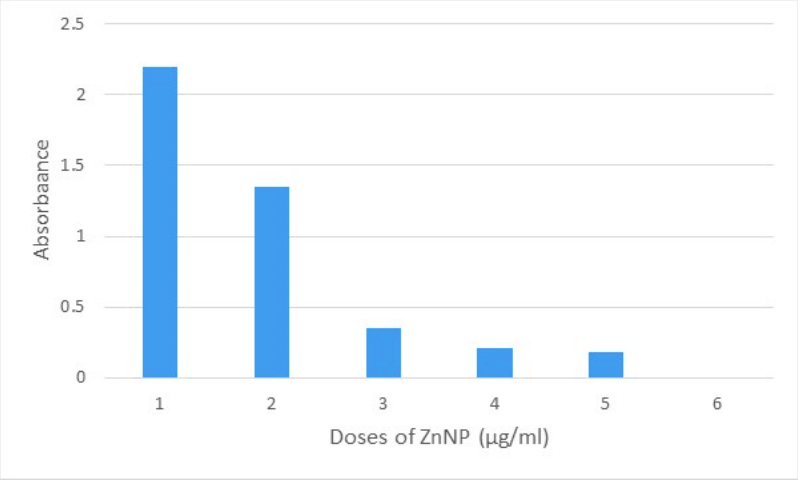

Fig. 2. Optical density of *S. aureus* at different levels of ZnONPS
Dose1: (ZnNP 0 µg/ml), Dose2: (ZnNP 50 µg/ml), Dose3: (ZnNP 100 µg/ml). Dose4: (ZnNP 200 µg/ml), Dose5: (ZnNP 400 µg/ml), Dose6: (ZnNP 600 µg/ml)

Table 7. Absorbance and degree of turbidity of *C. albicans* and *S. aureus* exposed to gradual concentration of Curcumin.

Concentrations of Curcumin %	*C . albicans*		*S. aureus*	
	Absorbance (OD at 405 nm w/l)	DT & GT	Absorbance (OD at 405 nm w/l)	DT & GT
0%	2 .07	4	2.88	4+
0.25%	0.6	3+	1.48	3+
0.50	0.4	2+	0.68	2+
1%	0.4	1+	0.55	2+
2%	0.3	00	0.46	1+
3%	0.26	00	0.39	00

OD: Absorbance at wavelength 405 nm. - DT: Degree of turbidity of treated suspension. - GT: Growth after Treatment.

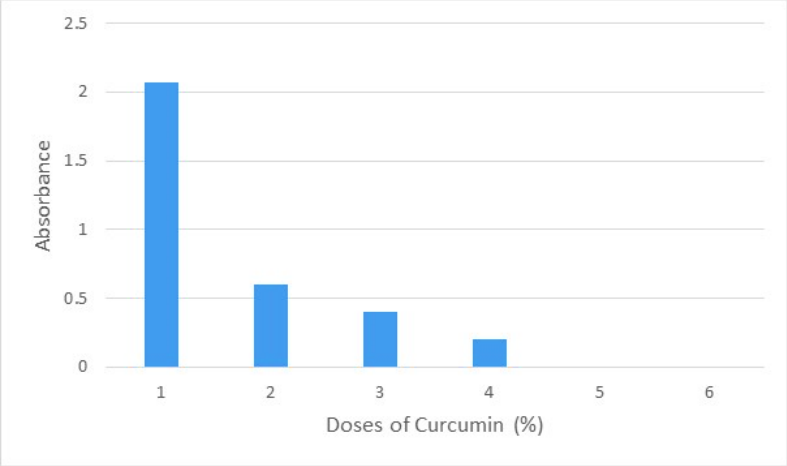

Fig. 3. Optical density of treated *C. albicans* at gradual concentration of curcumin
Dose1: (Curcumin 0 %), Dose2: (Curcumin 0.25 %), Dose3: (Curcumin 0.50 %). Dose4: (Curcumin 1 %), Dose5: (Curcumin 2 %), Dose6: (Curcumin 3 %)

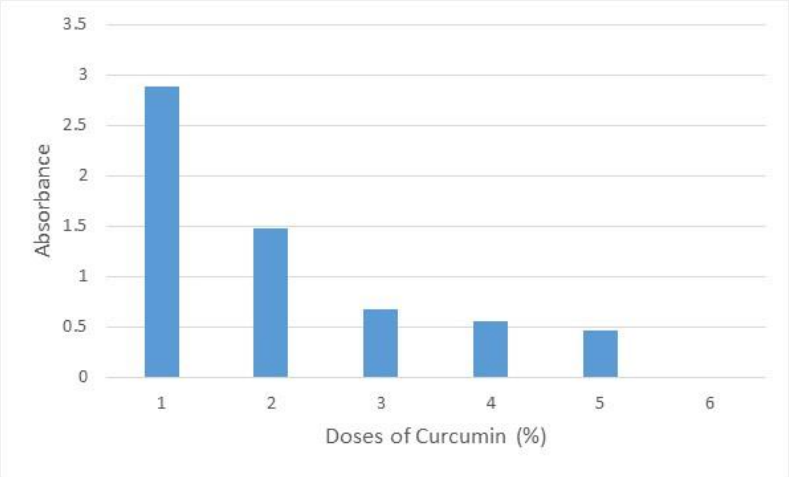

Fig. 4. Optical density of treated *S. aureus* at gradual concentration of Curcumin
Dose1: (Curcumin 0 %), Dose2: (Curcumin 0.25 %), Dose3: (Curcumin 0.50 %). Dose4: (Curcumin 1 %), Dose5: (Curcumin 2 %), Dose6: (Curcumin 3 %)

Table 8. Absorbance and degree of turbidity of treated *C. albicans* and *S. aureus* treated with hybrid ZnONPs with curcumin

Concentrations of ZnONPS hybrid (**u**g/ml) with curcumin	***C . albicans***		*S. aureus*	
	Absorbance (OD at 405 nm w/l)	DT& GT	Absorbance (OD at 405 nm w/l)	DT& GT
0	2.40	4	2.20	4+
100 ug/ml(ZnONPs)+ 0.25% Curcumin)	0.0	00	0.0	00
100 ug/ml(ZnONPs)+ 1.0% Curcumin)	0.0	00	0.0	00

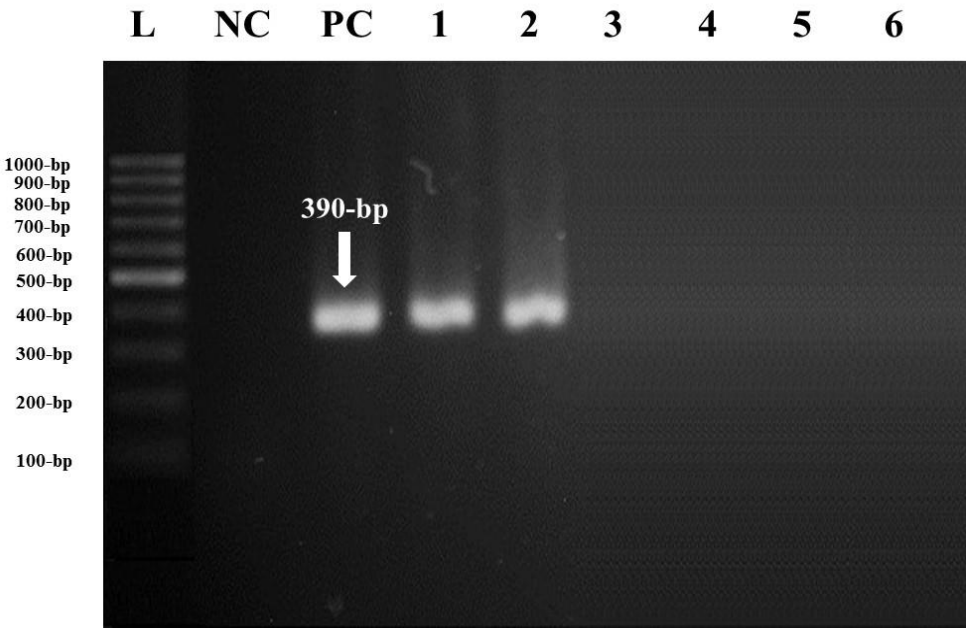

Fig. 5. The PCR gel electrophoresis for *SAP* gene *of C. albicans* (at 390 pb) Lane L: 100 bp DNA ladder standard. Lane NC: Negative control (*Fusarium spp*.), Lane PC: Positive control of *C. albicans*, Lane 1: Treated with 100 µg /ml of ZnONPs). Lane2: Treated with 0.25% of Curcumin). Lane 3: Treated with 1% Curcumin. Lane 4: Treated with 600 µg/ml of ZnONPs, Lane 5: exposure to conjugation of 100 µg /ml of ZnONPs+1% Curcumin. Lane6: Conjugation of 100 µg /ml of ZnONPs+ 0.25% Curcumin

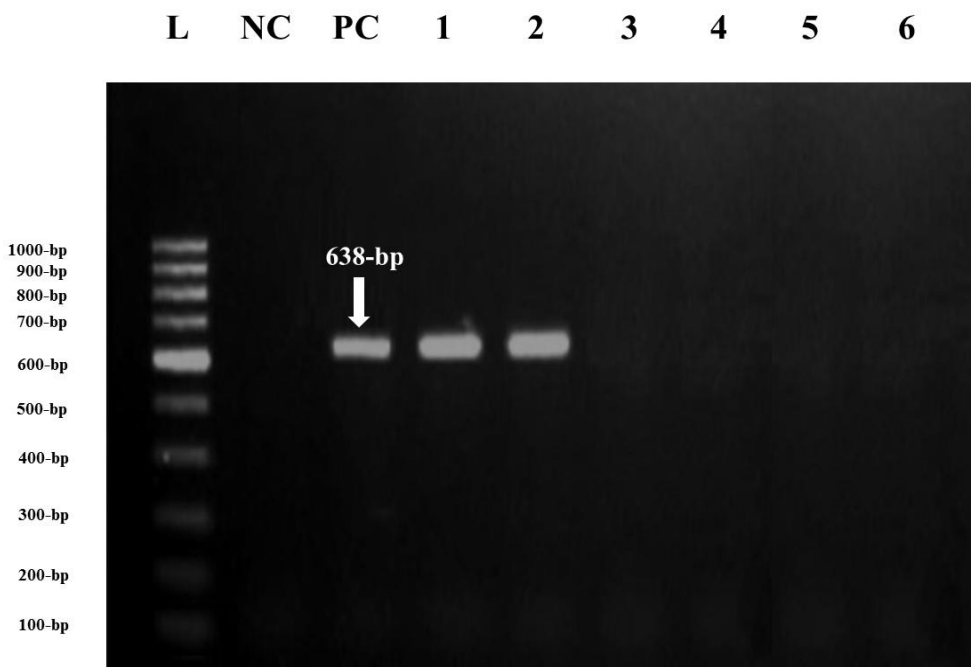

Fig. 6. The PCR amplification for *S. aureus clfA gene* (at 638 pb), Lane 1:100 bp DNA ladder standard. Lane 2: Negative control *(E.coli),* Lane 3: Positive control of *S. aureus*, Lane 4: Treated with 100 µg /ml of ZnONPs. Lane 5:Treated with 0.25% of Curcumin. Lane 6: Treated 1% Curcumin. Lane 7: Treated with 600 µg /ml of ZnONPs. Lane 8: exposure to conjugation of 100 µg /ml of ZnONPs+1% Curcumin. Lane9: Embedded ZnONPs (100 µg /ml) + (0.25%) Curcumin.

Discussion :

Nowadays, Subclinical mastitis disease is of massive economic significance in dairy animal productivity. Because of the overusing of antimicrobial compounds in dairy farms, to prevent intramammary infections, cause pathogens resistance that results in a substantial risk of disease resistance (Abed *et al.,* 2018).

In this study, *Staphylococcus spp.* is considered the most predominant isolates from different samples in dairy farms, where results illustrated in a table (2) revealed that out of 20 subclinical mastitis milk samples, 13 (65%) were found positive for *Staphylococcus* spp., followed by *E. coli* 5(25%) and *Streptococcus faecalis* 2 (10%). Our findings came in accord with the results obtained by Chhabra *et al.* (2020); Patil *et al.* (2021), where they detected that the predominant etiological agent causing subclinical mastitis in buffalo was members of *Staphylococcus* sp. and caused food poisoning in consumers. In this study, six isolates (30%) were identified as *S. aureus*, and seven isolates (35%) were *CNS.* These results nearly agreed with other studies on subclinical mastitic buffalo in Egypt recorded by Ahmed *et al.* (2020). A lower prevalence of *S. aureus* was reported by Salem-Bekhit *et al.* (2010) (16.1%) and Amin *et al.* (2011) (14.9%). On the contrary, Ali *et al.* (2015) obtained a higher incidence of *S. aureus* (60%) recovered from subclinical mastitis in buffalo in India. Moreover, the prevalence of *E. coli* in our study was nearly similar incidence (30%) observed by Kumar (2009), whereas a comparatively Low frequency of *E. coli* was documented by Vásquez-García *et al.* (2017) in dairy buffalo. Herein, *S. aureus, CNS, E. coli, St. faecalis,* and *Ps. aeruginosa,* were isolated from the 20 animal feed samples in a percentage of 20, 15, 10, 5, and 5, respectively. In addition, the microorganisms, which were isolated from 20 bedding samples, were *Klebsiella spp. E. coli, Ps. Aeruginosa,* and *St. faecalis* at an incidence of 35, 20, 20, and 15, respectively. While *S. aureus* and *CNS* were isolated in a percentage of 10 for each. Similarly, Zadoks *et al.* (2011), Elbably *et al.* (2013), Byomi *et al.* (2020), and Nesma *et al.* (2020) reported that out of 57 bedding samples, 68% tested positive for *Klebsiella* spp. in dairy farms.

On the other hand, the fungal pollution of the present samples of subclinical mastitis was illustrated in Table (3). The table showed that 5 species of yeast were recovered such as *Candida albicans, Candida parapsilosis, Candida krusei, Rhodotrula mucilaginosa, and Trichosporon* from different dairy farm samples, as well as 3 genera of molds: *Aspergillus fumigates, Aspergillus niger* and *Aspergillus flavus*. The most predominant isolate of *yeast spp.* was *Candida albicans* (14) which recovered from subclinical mastitic milk, animal feeds, and bedding at an

incidence of (35%, 25%, and 10%), respectively. However, *Candida parapsilosis, Candida krusei, Rhodotrula mucilaginosa, and Trichosporon* (20%, 10%, 5%, and 10%) obtained from subclinical mastitis milk samples (10%, 15%, 10%, and 10%) from animal feeds and (15%, 10%, 10%, and 0%) in bedding samples, respectively. While mold spp. (*Aspergillus fumigates, Aspergillus niger,* and *Aspergillus flavus)* were isolated from samples at an incidence of (10%, 5% and 5%) in subclinical mastitic milk samples, (5%, 10%, and 5%) in animal feeds and (10%, 5%, and 0%) in bedding samples, respectively. These results were coincident with that recorded by Mosherf (2005), various Candida spp. in subclinical mastitic milk. Nearly similar incidences were obtained by Hassan *et al.* (2012), Abd El-Razik *et al.* (2011) recorded the prevalence of *C. albicans* and *A. fumigatus were* (23.07%) and (3.84%), respectively in mastitic buffaloes. While higher results of *C. albicans* (51.7%) were obtained by Al-Abedi *et al.* (2020) from mastitis in Basrah province.

Herein, the commercial antibiotics were tested against *S.aureus* that predominantly recovered in the present samples observed a significant drug resistance rate against Ampicillin (83.33%) followed by Ceftiofur (66.67%), Cefotaxime (66.67%) and Amoxicillin (58.33%). Currently, our results showed that Imipenem was effective against all tested isolates (100%) and Ciprofloxacin (100%), and most isolates were sensitive to Ofloxacin (91.67%), Flumequine (75%), and Gentamycin (66.67%). Similar sensitive rates were observed by Sahebekhtiari *et al.* (2011). He found that all *S. aureus* isolated from subclinical mastitic milk from dairy farms were susceptible to ciprofloxacin, imipenem, and gentamicin, while they were resistant to ampicillin (64%). It was also determined by Kenar *et al.* (2017) that *S. aureus* was isolated from subclinical mastitic milk, and it was resistant to ampicillin at a high rate (48.2%), but Imipenem inhibited their viability. While the susceptibility rate to gentamicin and ciprofloxacin was 84.3% and 68.6%, respectively.

Thus, our study reveals the predominance of *Staphylococcus spp*. as a contagious form of subclinical mastitis at the farm, which needs to be controlled with appropriate measures to prevent the further spread of infection. While the higher percentage of environmental pathogens in bedding materials may lead to udder infection due to contaminated beddings and suggested improved sanitation and hygienic practices at the farm.

The results of antifungal susceptibility in table (5) revealed that *C. albicans* is highly susceptible to Nystatin, followed by Amphotericin B and Fluconazole, while it showed resistance to Clotrimazole. Nearly similar findings were obtained by Mohamadi *et al.* (2014), who proved that *C. albicans* is susceptible to Nystatin and Amphotericin B, while the results recorded by Mohammed *et al.* (2020) indicated that the Ketoconazole, Nystatin, and Clotrimazole antifungals showed more inhibition on the *C. albicans*. The random and repeated use of this antagonist leads to the emergence of resistant species of Candida spp., therefore, it is natural that they differ from one species to another. Repeated use of antifungals leads to mutations that increase the resistance of Candida spp. and increase the factors of their virulence, and thus their resistance to fungicides (AL-Maliki and AL-Ani, 2011).

Therefore, the exploration of new anti-pathogenic drugs to compete with the drug resistance to conventional agents is urgently needed (Whitesides, 2003). Current strategy protocols for eliminating microbial infections by nanomaterials gained significant interest as ZnONPs and natural herbs (Hassan *et al.,* 2020, and 2022).

In present work, the pathogens of *C. albicans* and *S. aureus* that were predominantly recovered from cases buffaloes' mastitis and their growth inhibition by ZnONPs and Curcumin were evaluated.

The (MIC) of ZnONPs against *C. albicans* and *S. aureus* were (600 and 600 µg /ml), respectively Table (6), Fig. (1, 2). The absorbance of treated spores decreased until it reached zero and transmittance 100%. The antimicrobial potential of ZnONPs detected against bacteria and fungi (Hassan *et al.,* 2017; 2020b & C; 2021) that caused skin infection in buffaloes (Hassan *et al.,* 2015) and mastitis in cattle (*C. albicans* and *S. aureus*) (Sabir et al., 2014). The antimicrobial potentials of ZnONPs are related to their ability to cause the destruction and death of pathogens cells (Vasilache *et al.,* 2011; Hassan *et al.,* 2022).

Herein, the inhibitory concentration of Curcumin against the growth of *C. albicans* and *S. aureus* were (2% and 3%), respectively Table (7) Fig. (3, 4). The antimicrobial potential of Curcumin due to its ability to inhibit the viability of pathogen cells, such as depressing the protein and DNA synthesis (Anwar *et al.,* 2009; El-Baroty *et al.*, 2010; Hassan *et al.,* 2022) and resulting in genotoxicity (Abd El-Baky and El-Baroty, 2008; and Hassan *et al.,* 2021).

Nowadays, there is a continuous awareness of the suspected toxicity of nanotechnology applications in biomedicine. This enforced significant investigations into the use of hybrid nanocomposites, with beneficial materials to overcome toxicity in animals. Currently, the hybrid synergism of ZnONPs (100 µg/ml) with Curcumin (0.25% and 1%) resulted in significant growth inhibition of *C. albicans* and *S. aureus* Table (8). The nanocomposite

of low level of ZnONPs (100 µg /ml) with (0.25% Curcumin), and even the elevation of Curcumin concentration to (1%) at the low level of ZnONPs, significantly increased the inhibitory effects against *C. albicans* and *S. aureus*, respectively. Similarly, Hassan *et al.* (2019) detected the MIC of ZnONPs against *Fusarium spp*. was (500 µg/ml) and significantly decreased to (100 µg /ml) when combined with Curcumin or probiotics (0.25% for each). These findings enabled the prevention of drug resistance and thus resulted in significant antimicrobial efficacy **(**Chow and Yu, 1999; Hassan et al., 2021 and 2022**)**. Moreover, Hassan et al. (2020) found the MIC of ZnO NPs and cinnamon oil against *A. flavus* and *E. coli* were (100 and 50 µg/mL for NPs), respectively, and (0.25% for each for cinnamon oil). The conjugated ZnONPs with cinnamon oil caused significant inhibition of *A. flavus* and *E. coli* growth at a lower dose.

In the present study, the molecular detection of the virulent genes in isolated *C. albicans* and *S. aureus* from buffaloes' mastitis has been performed. The DNA band of the virulence gene (SAP gene) of 2 isolates of *C. albicans* were like the standard strain, while others showed no bands for SAP gene (Figure 5). Similarly, in S. aureus, 2 isolates showed positive DNA fragments for the CFL gene like the standard strain (Figure 6). Moreover, the exposure of *C. albicans* and *S. aureus* to a low dose of ZnONPs (100 µg/ml) did not affect the genes' DNA expression and while the use of a high dose (600 µ g/ml) prevented the DNA expression of virulent genes (Figure 5, 6). On the other hand, the exposure of S. aureus and *C. albicans* to a low dose of Curcumin (0.25), the genes' DNA expression. Whereas the treatment of these isolates with a high dose of Curcumin (1%) removed the DNA expression band. Herein, the combination of low levels of ZnONPs and Curcumin resulted in the complete absence of the DNA band of virulent genes of used pathogens.

Hence, it is interesting to report here that the hybrid nanocomposite of low doses of ZnONPs with Curcumin resulted in significant prevention of the virulent genes DNA expression of dominant pathogens of bovine mastitis. This allows us to overcome the use of high doses of nanomaterials, otherwise low safe doses in conjugation with natural benefit materials as the combination of ZnONPs with Curcumin and enable to inhibit the viability of virulent genes expression as detected here by PCR. Similarly, the PCR enables the rapid detection of pathogens' genes as recommended by **Hu** *et al.* (2020), Hassan *et al.* (2021, 2022) who found that the use of nanocomposites with natural plant materials caused significant changes in aflatoxin regulatory genes. Other studies illustrated that the traditional antifungals and herbs against *C. albicans* increased the activity of secreted proteinase (Sap) and SAP genes, which can be observed by PCR (Copping *et al.* 2005; Saleh 2011). However, Al-Saad *et al.* (2016) and Hassan *et al.* (2017; 2019, 2020, 2022) observed the prevention of DNA expression of the Afla R gene after control of mycotoxigenic *A. flavus* by probiotic preparation, cinnamon, and olive oils. Moreover, Rathore *et al.* (2018) and Zheng *et al.* (2015) detected that there is a strong relation similarity between traditional diffusion tests and gene expression of bacteria such as *S. aureus* and *Enterococcus faecium* treated by traditional drugs (58.3%-100). In addition, the hybrid Nanocomposite with benefit molecules of microbial cells resulted in their damage and death (Xiao *et al.,* 2016; Meena *et al.* (2018). In another study, it was detected that nanocomposites potentiated the growth suppression of many microbial agents by the destruction of the cytoplasmic contents and hence loss of cell viability and function (Bai *et al.*, 2018).
Hence, the conjugation of nanomaterials and benefit molecules diminished the dose of nanosized metals, which significantly avoids the hazards of toxicity (Contera *et al.,* 2020; Hassan *et al.,* 2021, 2022).

CONCLUSIONS

From the foregoing results, we concluded that the pathogens of *C. albicans* and *S. aureus* were at the top of all causes of mastitis in buffaloes. They showed resistance to traditional antibiotics and hence novel agents that can remove this resistance are required. Over the past decades, there was huge and extensive use of nanomaterials, particularly ZnONPs, and medicinal herbs as antimicrobial agents. These cause the production of nanocomposites of ZnONPs with curcumin herbs, which showed successful antimicrobial potentials. These actions are due to their ability to enter the pathogen's cells and prevention their activities and final death. In addition, molecular studies by PCR to detect the influences in virulent genes DNA, which is responsible for drug resistance activities, are investigated before and after exposure to low and high doses of ZnONPs and/or Curcumin. Where the conjugation of low safe doses of ZnONPs with low and high doses of Curcumin herbs showed significant removal of the virulent genes DNA of pathogens similar in action to the exposure to high doses of ZnONPs that are suspected to be toxic in prolonged use. Hence, it is interesting to report here that the nanocomposites decreased the used dose of nanomaterial, and the toxic data of nanomaterials must be available before their use in human and animal science to avoid their toxicity risk.

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