Effect of garlic oil nanoemulsion against *Corynebacterium pseudotuberculosis*.

1Aya M soliman, Awaad, Ashraf. Abd El-Tawab, and 2Amany, Omar. Selim2

1 Bacteriology, Immunology and Mycology Dep., Fac. Vet. Med. Benha Univ. Benha, Egypt

2 Bacteriology Dept., Benha Provincial Laboratory,Animal Health Research Institute, Agriculture Research Center, Cairo,Egypt

1 Aya.Suleiman20@fvtm.bu.edu.eg, ORCID identifier is 0009-0004-2833-2389, 1 ashraf.awad@fvtm.bu.edu.eg

2 Amany.omar@ahri.gov.eg

Abstract

Background: - Caseous lymphadenitis is caused *by C. pseudotuberculosis*, which has the peptidoglycan cell wall lead to strong impermeable barrier for the Corynebacterium genus so the traditional treatment was effectiveness so using of nanoemulsions, Garlic essential oil (GO) which has been researched widely for its effective antibacterial activity against various micro-organisms as treatment of Caseous lymphadenitis

Objective the study aimed to detect the effect of garlic oil nanoemulsion on *Corynebacterium (C.) pseudotuberculosis*.

Methods 8 positive isolates of *C. pseudotuberculosis* were grown on Baird parker agar followed by extraction of the bacterial DNA for detection of *rpo*B, *PLD, sig*E genes. A PCR product was purified and sequenced to establish sequence identity to Gene Bank accessions then Phylogenetic analyses were done. Garlic oil nanoemulsion was prepared from garlic extract, Tween 80, and deionized water then tested for the physicochemical characteristics and cytotoxicity assay.

The antibacterial activity was observed then detects the detrimental effects by the transmission electron microscope.

Results :-All isolates were positive for *rpo*B gene, only 2 were positive for *PLD* gene, while *sig*E gene was absent. The sequence of *rpo*B gene has accession number beta (*rpo*B) gene, partial CDs, accession ON993360 with 351 PB for nucleated. Garlic oil nanoemulsion was characterized by TEM as 103-143 nm size with a narrow size distribution The zeta potential had a 13.2 mV, negatively charged with automatic polarity and conductivity of 89 us/cm. This oil was safe to be used and the fatty acids, especially oleic acid, were the major constituents of it. It has detrimental effects as cell lysis, cytoplasmic leakage, and nanoparticle internalization to the bacterial cell.

Conclusion, Garlic oil nanoemulsion has been detected to have an anti-corynebacterial effect, resulting in changes to the outer surfaces, internal properties as well as the biological activity of the cells.

Keywords :-*C. pseudotuberculosis* - *rpo*B gene sequence- Garlic oil nanoemulsion – TEM.

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1. Introduction:-

Caseous lymphadenitis (CLA) is a contagious and chronic bacterial disease that threatened livestock in many countries. That's because it's annoying clinical signs on the herd range from superficial and internal abscesses all over the body, weight loss, abortion, recurrent infection,

and occasionally death (Oreiby et al. 2014; Burmayan and Brundage, 2021). Once the animal becomes infected, it survives and replicates within the immune cells to the point that they become like its carrier inside the body. Then once the disease is established, CLA is laborious to be eradicated (Mohamed et al. 2017). It is caused *by C. pseudotuberculosis*, Gram-positive, short coryneform, highly resistant to antimicrobials, attributing its virulence from an exotoxin, phospholipase D (PLD) with the peptidoglycan cell wall (Markey et al. 2013). Most of these lipids are the mannose-associated phosphatidyl myoinositol anchors, lipomannan, lipo glycans, and lipoarabinomannan (Mishra et al. 2008) The combination of these lipids within the cell wall results in a characteristic strong impermeable barrier for the Corynebacterium genus then illustrates the induction of chronic granulomatous disease (Rebouças et al. 2020). Late onset of symptoms makes the traditional treatment effectiveness (Alcantara et al. 2021)

Regarding the guidance in (Almeida et al. 2021), our concern detoured a modern technique against CLA. Nearly decades ago, Numerous studies were published dealing with natural compounds in all branches of medicine. On account of the pivotal role that natural compounds often cover in the finding of novel drugs, their in-depth analysis could streamline the invention of new antimicrobial agents (Guglielmi et al. 2020).

The ancients used to utilize garlic plants in food and medicine as it has been cultivated everywhere. Not only used as a traditional ingredient in numerous palatable food but also had a vital role in dealing with

intestinal worms, hypertension, healing wounds, headaches, bites, atherosclerosis, tumors, and other diseases (Liu et al. 2022). For the time being, the garlic plant is considered a global significant Allium species regarding its medical and nutritional benefits (Mossa et al. 2018). It has been registered that garlic and its derived products have acaricidal (Mossa et al. 2018), antimicrobial (Daka, 2011; Liu et al. 2022) insecticidal (Yang et al. 2009). and antioxidant activities (Mnayer et al. 2014). A recent study noticed that garlic essential oil can bind with different sorts of protein, inducing good inhibition for the host receptor. (Bizzoca et al. 2022).

Nanotechnology has attracted global attention because nano-particles have novel and unique properties from their bulk equivalents. Nanotechnology is summed up as the usage of any molecule at sizes in the nanoscale range, approximately between 1 and 100 nm (European Food Safety Authority, 2009) (Handford et al. 2015). Among the nano-encapsulation systems, Nanoemulsions are especially applicable for essential oil encapsulation regarding their low turbidity and good stability (Moghimi et al. 2016) The nano-emulsions are distributions of nanodroplet size ranging from 20 to 200 nm (Mossa et al. 2018) It has successfully produced nanoemulsions with a small particle size from various essential oil, still, the format of garlic essential oil nanoemulsion through high-power ultrasound with low surfactant content is quiet in its babyhood (Liu et al. 2022). Moreover, the antibacterial activities of essential oil have been changed variously when they are converted to nanoemulsions, Garlic essential oil (GO) has been researched widely for its effective

antibacterial activity against various micro-organisms (Zhang et al. 2019) It is a promising utilization of nano-sized nanoemulsion as it has a raised active surface area compared to pure essential oil, which may lead to altered antibacterial activity and physical stability (Liu et al. 2022).

Furthermore, this paper aims to detect the effect of GO nano-emulsion on C. *pseudotuberculosis* isolated and record the reaction with a High-resolution transmission electron microscope (HRTEM).

2. Materials & Methods

Ethical Approval:-

All samples were taken from many ranchers within Gharbiya & Menoufia Governorates during a survey for the veterinary directorate. The ranchers were welcome to take samples for the research completion. The entire samples were taken from animals in the rest stage without stress or any sedations.

the National Institutes of Health's Guidelines for the use and care of animals and authorized by the Ethical Committee of Research at the Benha University of Egypt, Faculty of Veterinary Medicine (approval no.: BUFVTM121022).

Isolation & Identification of Bacteria:

22 positive isolates of *C. Pseudotuberculosis* were taken from 215 total samples (collected from swollen affected lymph nodes). Specimens were inoculated duplicate into Baird parker agar with tween 80 and Brain Heart Infusion agar (HIMEDIA) supplemented with 5% fresh sheep blood. After inoculation, the plates were incubated for 48 h at 37◦C, then the isolated colonies were

macroscopically characterized, stained using the Gram stain, and subjected to biochemical tests. Colonies that were immotile, Gram-positive, and exhibited positive reactions to catalase and urease tests, were identified as *C. Pseudotuberculosis*. All tested isolates were compared for similarity by the reference strain of *C. pseudotuberculosis* (ATCC® 19410TM).

DNA extraction and PCR amplification

Out of identified *C. pseudotuberculosis*, 8 selected isolates were grown on BHI agar (Oxoid) for 24-48 hours at 37 °C followed by extraction of the bacterial DNA using QIAamp DNA Mini Kit Catalogue no. 51304 (Qiagen Inc. Valencia CA) according to the manufacturer instructions. Using the extracted bacterial DNA, Amplification of *rpo*B was done using the primers included in the table (1) below acc. to (Sammra et al. 2014)

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Gene	Sequence	Amplified product	Reference
*rpo*B	CGWATGAACATYGGBCAGGT	406 bp	Sammra et al. 2014
	TCCATYTCRCCRAARCGCTG		

Using the extracted bacterial DNA, virulence genes *PLD* and *sig*E were amplified using the primers included in the table (2) acc. To (Pacheco et al. 2012; ILHAN 2013)

Using the extracted bacterial DNA, virulence genes *PLD* and *sig*E were amplified using the primers included in the table (2).

Gene	Sequence	Amplified product	Reference
Pld	ATA AGC GTA AGC AGG GAG CA	203 bp	ILHAN et al. 2013
	ATC AGC GGT GAT TGT CTT CCA GG		
*sig*E	GGMACCGCAGCDTT CGACGC	490 bp	Pacheco et al. 2012
	CGTCCRCGGTGRAT WCGGGA		

Amplification reactions were prepared in 25 µl containing PCR master-mix (2X premix) (Emerald Amp GT PCR master mix, Takara kit, code RR310A) 12.5 µl, 1 µl for each forward and reverse primers, 5 µl template DNA completed to 25 µl by 5.5 µl PCR grade water. PCR was performed using *C. pseudotuberculosis* (ATCC® 19410TM) as a reference strain (control positive) in a T3 Thermal cycler (Biometra). The controlling negative used was sterile DNAse/RNAse free and Diethyl Pyrocarbonate (DEPC) water instead of template DNA. The cycling conditions of the primers used were: 1- initial denaturation at 94 °C for 5 min. 2- secondary denaturation at 94 °C for 30 sec. 3-annealing at (52°C for 40-sec *rpo*B), (56 °C for 30 sec. for *PLD*, and 40 sec. at 50°C for *sig*E). 4- extension at 72 °C for (45 sec. for *rpo*B), (30 sec. for *PLD*, 45 sec. for *sig*E) 5- then 35 cycles for each gene followed by a final extension at 72 °C for 10 min. The

amplified PCR products were electrophoresed in Gel Casting Apparatus (Biometra) through 1.5 Agarose gel in TBE buffer for 1 hr. against Gene ruler 100 bp DNA ladder (cat. no. SM0243) supplied from Fermentas. The DNA bands were stained with Ethidium bromide, photographed by a gel documentation system and the data was analyzed through computer software The data were analyzed by a computer software (automatic image capture protein simple formerly cell bioscience, USA) (Sambrook et al. 1989 ;WHO 2002).

Sequencing reaction

A PCR product of sample no.1 was purified by using QIA quick Gel Extraction Kit Protocol: (Qiagen Inc. Valencia CA): and sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer *(*ABI, 3130, USA*)*. Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No. 4336817. according to the instruction of the manufacturer. A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al. 1990) was initially performed to establish sequence identity to Gene Bank accessions.

Phylogenetic analysis:

A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, which was designed by Thompson et al. (1994) then Phylogenetic analyses were done using maximum likelihood, neighbor-joining and maximum parsimony in MEGA6 (Tamura et al. 2013).

Preparation of garlic nano-emulsion

Garlic oil nanoemulsion was prepared by using garlic extract obtained from National Research Centre (NRC), Tween 80 (non-ionic surfactant) obtained from the Sigma-Aldrich Co., and deionized water (D.W). The organic phase (surfactant and oil) was prepared by mixing 2ml garlic oil with 4ml tween 80 then slowly adding 92ml D.W (aqueous phase) to the mixture. The mixture was subjected to Sonication for 30 min. using a Probe sonication. Nano garlic emulsion (2%) was prepared in the nanomaterials Research and synthesis unit (27).

Physico-chemical characters of GO nanoemulsion

GO nanoemulsion components were measured at Nawah Scientific Inc. (AlMokattam, Cairo, Egypt) for GC-MS. Particle size analysis (Dynamic light scattering) was determined by the NANOTRAC-WAVE II Zeta-sizer (MICROTRAC, USA) for the average particle size and similarity, polydispersity indexes (PDI) (homogeneity and distribution of the particles), surface charge (zeta potential), electrical conductivity (El-Oksh et al. 2022). the morphology of the garlic nanoemulsion was investigated by transmission electron microscopy.

Cell culture

Oral epithelial cells (OEC) were obtained from Nawah Scientific Inc. (AlMokattam, Cairo, Egypt). cells were maintained in DMEM media, supplemented with 100 units/mL of penicillin, 100 mg/mL of streptomycin, and heat-inactivated fetal bovine serum (10%) in a humidified atmosphere with 5% CO2 (v/v) at 37ºC.

Cytotoxicity assay

Cell viability was assessed by sulforhodamine B (SRB) assay (Routine Analysis IC50) with five concentrations 2%, .2%, 0.02%, 0.002%, and 0.0002% for 72hr post-inoculation. (Allam et al. 2018)

Determination of the antibacterial activity by Minimum Inhibitory Concentration (MIC) (Panphut et al. 2017).

100 µl MHB broth was dispensed in each well of the 96-well plate. 100 µl of stock solution of garlic nanoemulsion and serial two-fold dilutions were done in rows on the plate with resulting concentrations ranging from 2% to 0.125%. In each well, a 100 µl of *C. pseudotuberculosis* of concentration 3×10^5 CFU/ml was added. The plates were incubated at 37 C for 24 hrs. Then 30 µl of Blue tetrazolium solution 0.015% was added to each well and the plates were re-incubated for 1-2 hours. A change in color from colorless to blue indicated the growth of bacteria and the MIC was defined as the lowest concentration of the drug that prevented this color change.

Processing of specimens for TEM

That work was done in the transmission electron microscope (TEM) laboratory at Cairo university research park faculty of agriculture (FA-CURP). TEM was carried out to detect the detrimental effects of Garlic nano-emulsion on *C. pseudotuberculosis*. Ultra-thin tissue sections were examined by TEM JEOL (J EM-1400 TEM) at the candidate magnification. Slice tissue samples into *1 mm slices. then immediately fixed in 3% glutaraldehyde molecule (v/v) in 0.1M sodium phosphate buffer (Ph.7.2-7.4) over 4˚c. Slice tissue was processed by washing in buffer, fixation in osmium tetroxide,

dehydrated in ethanol, and embedded in an epoxy resin, the embedding of the specimens in beam capsule lowed. Thin sections were stained with toluidine blue (1X). The uranyl acetate and lead citrate were used to stain ultrathin sections prepared at 75–90 µm thickness. 500nm to 1000 nm thickness sections were prepared by use of a Leica Ultra-cut UCT ultra-microtome. Images were captured by CCD Camera model AMT, an optronics Camera with 1632 * 1632 pixel format as side mount configuration (Mohamed et al. 2016; El Sayed et al. 2022).

3.Result:

As in Fig. (1): all 8 isolates of *C. pseudotuberculosis* isolates were positive to *rpo*B gene at 406 bp. And two isolate only showed positive to gene (*PLD*) as in fig (2) while sigma factor σE gene (*sig*E) was negative to all 8 *C. pseudotuberculosis* as in fig(3)

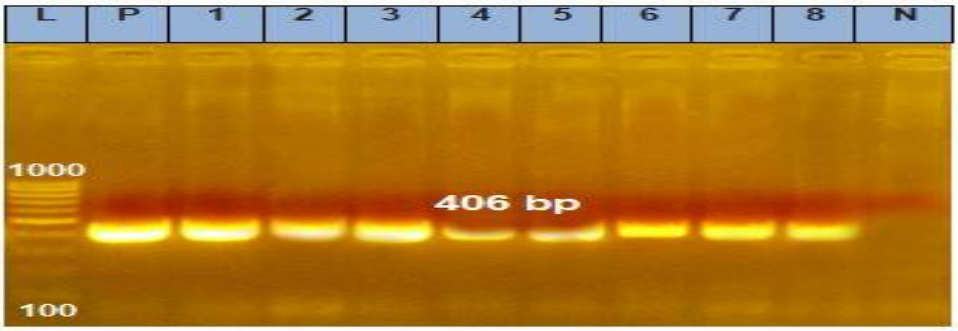


Fig (1):- Agarose gel electrophoresis of PCR for amplification products of the β subunit of bacterial RNA polymerase for 8 *C. pseudotuberculosis* isolates. Lanes (1 to8)show positive amplification of *rpo*B gene at 406 bp. Lane L: DNA ladder at 100-1000bp. N.: Negative control (sterile DNAse/ RNAse free DEPC water). P.: Positive control (*C. pseudotuberculosis* (ATCC® 19410TM). lane 1 to8 were positive to *rpo*B gene at 406 bp.

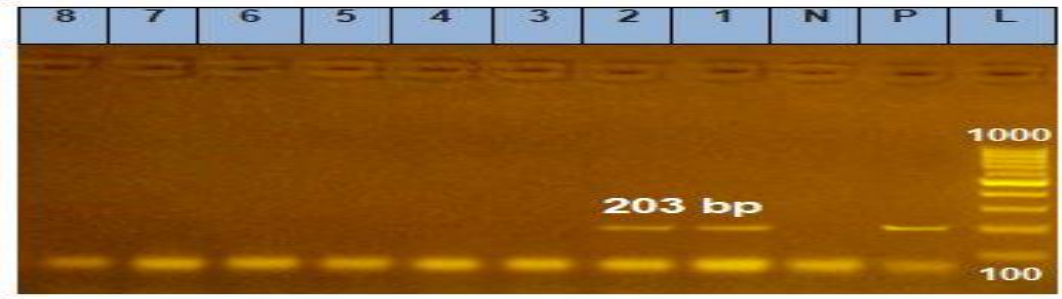


Fig. (2): Agarose gel electrophoresis of PCR for amplification products of Phospholipase D gene (*PLD*) for 8 *C. pseudotuberculosis* isolates. Lanes no 1, and 2 show positive amplification of *PLD* gene at 203 bp. Whilst Lanes no 3,4,5,6,7,8 show negative amplification. Lane L: DNA ladder at 100 1000bp. N.: Negative control (sterile DNAse/ RNAse free DEPC water). P.: Positive control (*C. pseudotuberculosis* (ATCC® 19410TM).

Fig. (3): Agarose gel electrophoresis of PCR for amplification products of the extra-cytoplasmic function (ECF) sigma factor σE gene (*sig*E) for 8 *C. pseudotuberculosis* isolates. All Lanes show negative amplification of *sig*E gene at 490 bp. Lane L: DNA ladder at 100-1000bp. N.: Negative control (sterile DNAse/ RNAse free DEPC water). P.: Positive control (*C. pseudotuberculosis* (ATCC® 19410TM).

The result of *rpo*B gene sequence of *C. pseudotuberculosis*

The accession number of beta (*rpo*B) gene, partial CDs. Was ON993360 with 351 PB for nucleated and has similarity with 99.43% with others in gene bank and the accession number of amino acid is WBD86631 with 117 amino acids the most with the similarity the phylogenetic tree for nuclide and amino acid in fig 4,5.

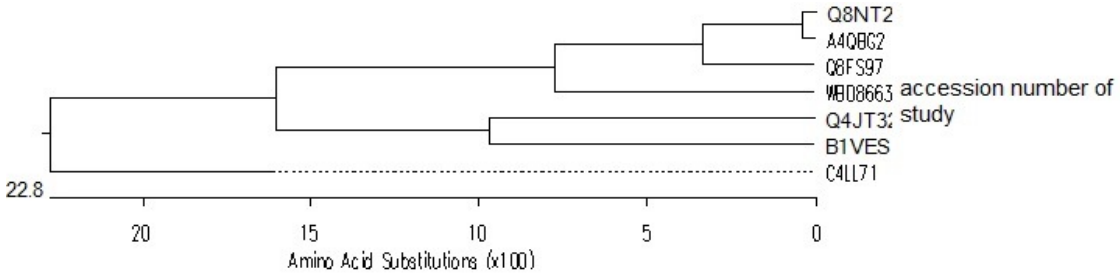


Fig. 4 of alignment of amino acid *rpo*B gene with other in gene bank

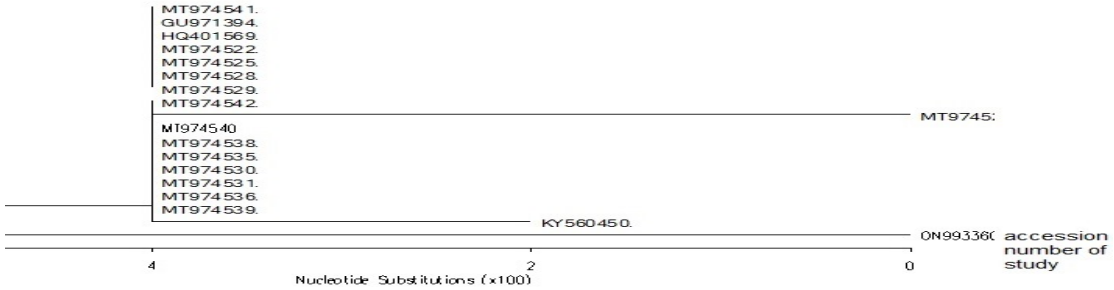


Fig.5 alignment of nucleotide of *rpo*B gene of *C. pseudotuberculosis* ON993360.

Characterization of GO nanoemulsion: GO nanoemulsion was characterized by TEM nano-emulsion size, 103-143 nm as in (Fig.6) with a narrow size distribution indicating greater homogeneity in nanodroplet size. The zeta potential indicates stable suspensions, taken by dynamic light scattering (DLS), which had a 13.2 mV, negatively charged with automatic polarity and conductivity 89 us/cm.

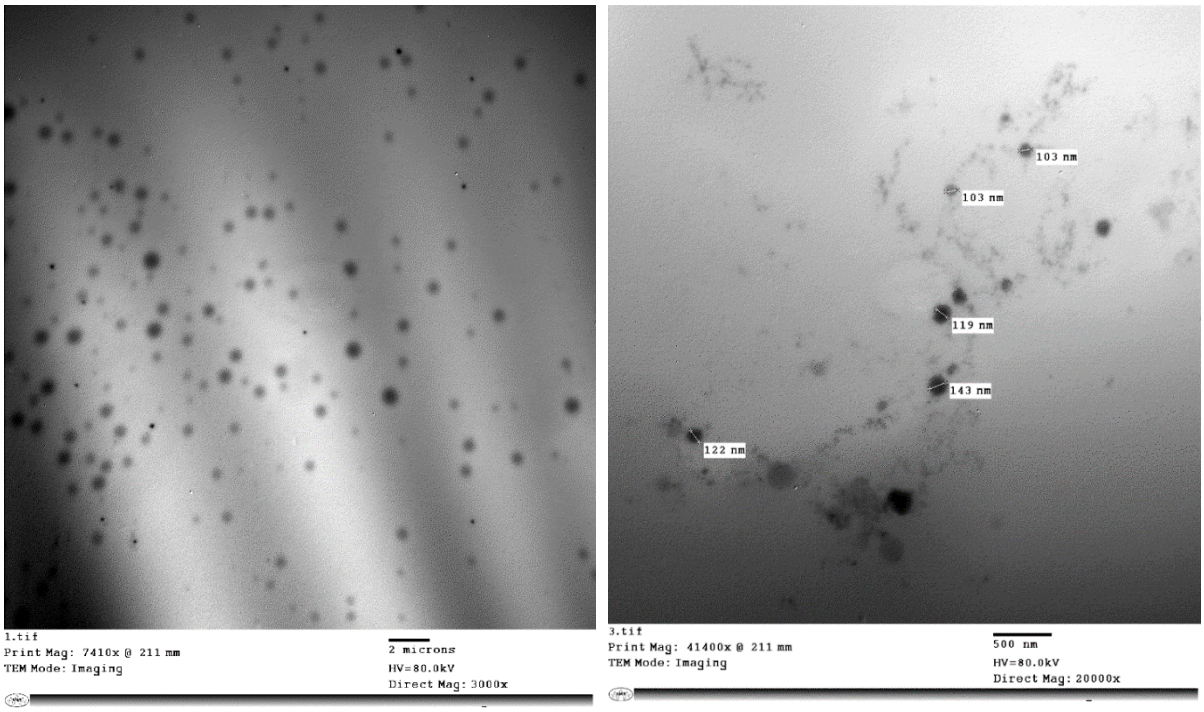


Fig.6: GO nanoemulsion under HRTEM shown nano-droplet size from 103-143 nm (average 118 nm) with a greater homogeneity

When GC-Mass was analyzed in the GO nanoemulsion as in fig. 7, 5 compounds were identified in the emulsion making up 48.84% of garlic essential oil. They were 21.08% 9-Octadecenoic acid (Z)-, methyl ester (oleic acid), 12.7% 10-octadecenoic acid, methyl ester (stearic acid), 6.62% Pentadecanoic acid, 14-methyl-, methyl ester (Myristic acid), 4.40% 1h-purine-6-amine, [(2-fluorophenyl) methyl]- 4.04% Cyclononasiloxane, octadecamethyl-. While it afforded high oil

yields as many components were found relative to Silicone oil by 3.63%, 3.58%,3.51%, 3.12% and others found relative to Benzene by 3.51%, 3.24%, 2.40%, 1.48%. Other active compounds were also found as Cyclooctasiloxane, hexadecamethyl- 2.85%, Dotriacontane 2.04%, 9,12 octadecadienoic acid (z,z)-, 2,3 bis[(trimethylsilyl)oxy]propyl ester 2.10%, 4h-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy- 1.22%.

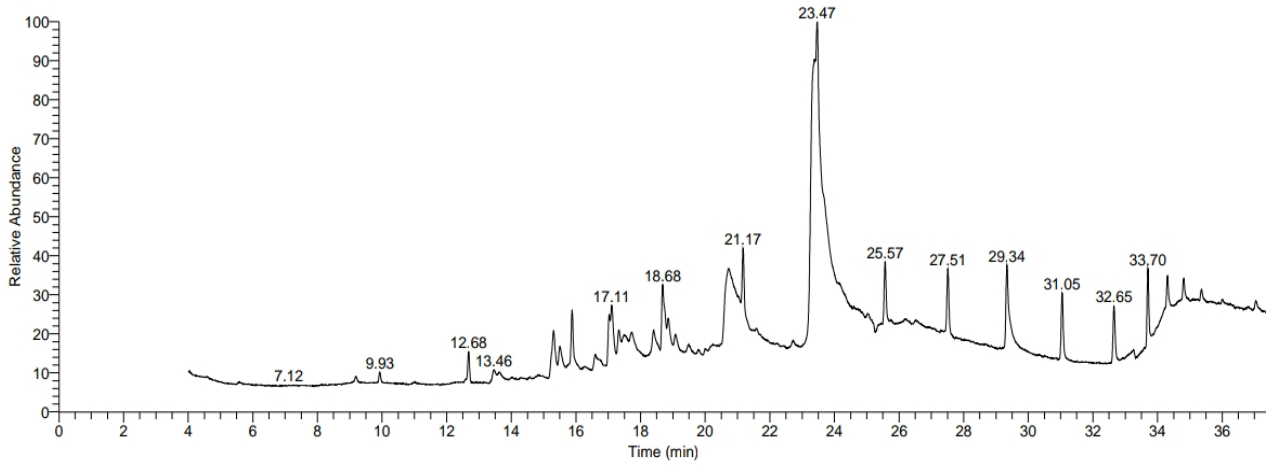


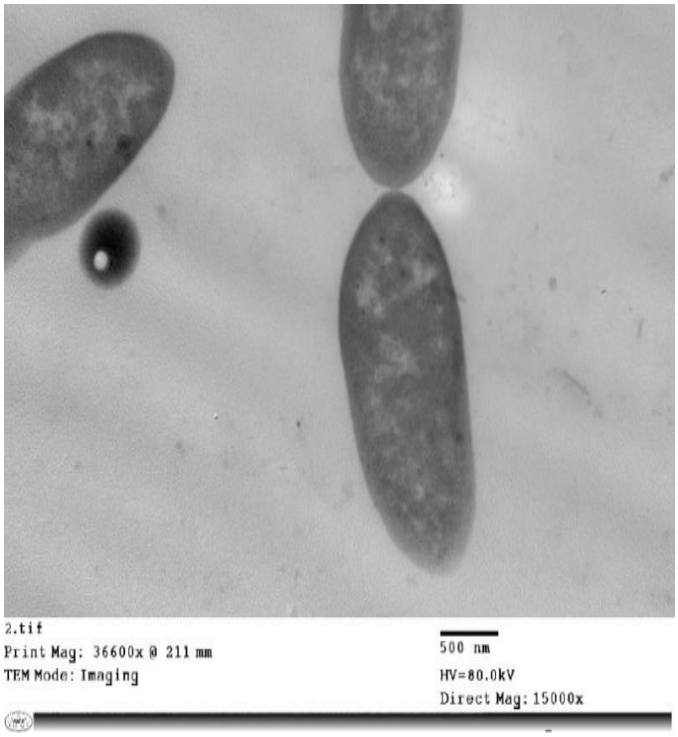
Fig. 7: Chemical components analysis of GO nanoemulsion using GC-Mass.

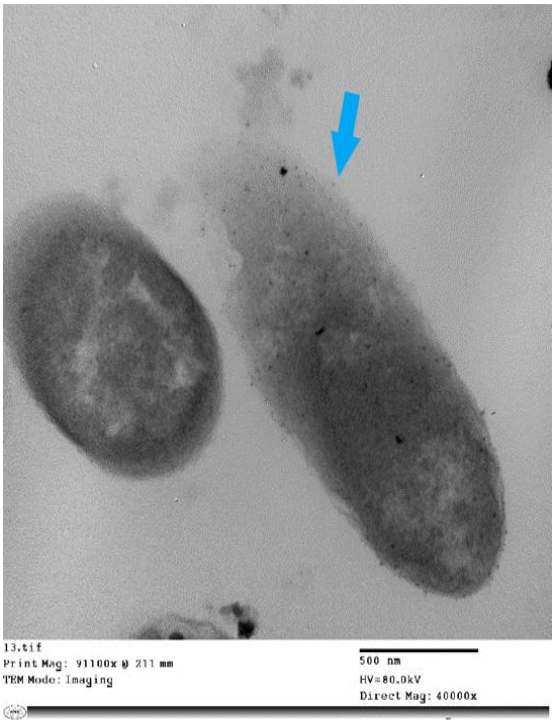
Cytotoxicity assay

On the surface of oral epithelial cells, the obtained results for GO nanoemulsion with numerous concentrations 0.0002%, 0.002%, 0.02%, 0.2%, and 2%, showed that cell viability% using SRB assay was 100.48, 100.43, 62.73, 14.60, and 0.17 %, respectively whilst IC50= .02% GO nanoemulsion.

MIC result for GO nanoemulsion against *C. pseudotuberculosis* isolates

The antimicrobial activity and micro-dilution susceptibility test of GO nanoemulsion was determined using MIC test the lowest concentration that prevented the change in color and kill the ***C. pseudotuberculosis*** was 0.125% with diameter 103-143 nm (positive result when color change to colorless). Garlic nanoemulsion revealed significant effects and proved a lethal growth of *C. pseudotuberculosis* isolates.by cell wall lysis, of the bacterial cell





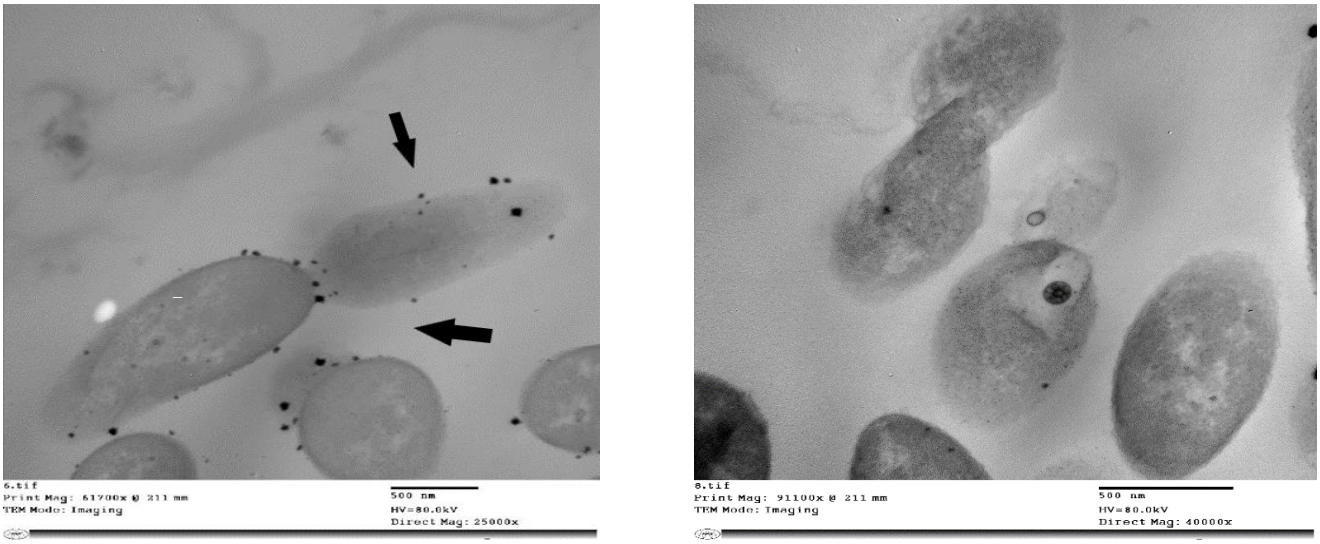


Fig. 8: HRTEM images for the antibacterial activity of garlic oil nanoemulsion (2%) for control and treated *C. pseudotuberculosis*. Control growth without treatment —TEM 15,000×. *C. pseudotuberculosis* treated with GO nanoemulsion (2%), structural changes in the cell wall (black arrows) with clusters on the surface —TEM 25,000×. The blue arrow indicates the cytoplasmic leakage, cell wall lysis, nanoparticle internalization to the bacterial cell —TEM 40,000×.

4.Discussion:

C. pseudotuberculosis is responsible for causing caseous lymphadenitis; the infectious, contagious, chronic disease, mainly characterized by the presence of caseous necrosis on the lymphatic glands or abscess formation in superficial lymph nodes and subcutaneous tissues.

The description of new bacterial species is based on the results of DNA-DNA hybridization and the description of phenotypic characteristics. However, it is expensive and technically complex to perform [Khamis et al. 2004]. The identification of clinical Corynebacterium isolates was compared with the classification using *rpo*B or *16S r*RNA gene sequencing. The majority of isolates

were correctly identified at the species level, except *C. aurimucosum* was misidentified as closely related to *C. minutissimum* (Khamis et al. 2004). *C. pseudotuberculosis* can be rapidly detected among diseased animals by using a multiplex PCR assay directed to the *16S r*DNA and the *rpo*B and *PLD* genes of this bacterium (Oreiby 2015).

The *rpo*B gene encodes the β subunit of bacterial RNA polymerase which plays the main role in the majority of the catalytic function of the RNA polymerase (Adékambi et al. 2009).

In our study, there were positive amplification of all isolates to *rpo*B gene that disagreed with the isolates in (Nabih et al. 2018). Our data, based on the *rpo*B sequences of these bacteria, confirm that this gene is as significant polymorphic as the *16S r*RNA gene, and We propose that it be used to replace or complement the *16S r*RNA gene for phylogenetic studies of C. pseudotuberculosis. Rather, in some non-tuberculosis mycobacteria, the *rpo*B gene provided a better phylogenetic resolution better than the *16S r*RNA gene (Abdallah 2016) and differentiates *C. pseudotuberculosis* from other pyogenic pathogens that present in pus discharge (Algammal 2016).

Phospholipase D (*PLD*) gene is the primary virulence factor of *C. pseudotuberculosis* [Dorella et al. 2006]. Studies with C. *pseudotuberculosis* strains with inactivated *PLD* demonstrated the necessity of *PLD* for CLA establishment (Nabih et al. 2018). In the current study, only 2 isolates were positively amplified for *PLD* gene that was not compatible with (Nabih et al. 2018).

Any bacteria need to respond rapidly to environmental stress like nitrosative stress due to the action of inducible nitric oxide synthase (Ehrt and Schnappinger 2009). Alternative sigma factors of the bacterial RNA polymerase especially the primary sigma (σ) factor subunit can face such stressful conditions (Pacheco et al. 2012). Genetic identification of *sig* E gene relieved negative amplification for all isolates.

A Few years ago, food scientists have discovered a new type of oil in water nanoemulsion: antimicrobial nanoemulsions. They are active against yeast, fungi, viruses, mold, spores, Gram-positive, and harmful bacteria. The antimicrobial nanoemulsion's particle size ranged from 200 to 600 nm and they are formed by oil, water, and surfactant without the addition of any natural and synthetic antimicrobial material (Zadeh et al. 2018).

So, On the way to solving the nightmare of *C. pseudotuberculosis*, we decided to try nanoemulsion of essential oils derived from plants rather than metallurgic nanomaterials as gold nanoparticles (Mohamed et al. 2017) or biogenic silver particles (Sekar et al. 2020).

In this investigation, GO nanoemulsion was used for further identification (DLS and TEM) and antimicrobial effects on *C. Pseudotuberculosis*.

The Morphology OF GE nanoemulsion (2%) particles were imagined by TEM (Fig.6) showing a spherical shape and a good dispersion which agreed with that reported in Mossa et al. 2018.

The resulting data illustrated that the average zeta potential was 13.2mv lower than reported in El oksh et al. 2022 with droplet size in-

between 103-143 nm which is lower than reported in Mossa et al. 2018. Smaller droplet sizes with semi-uniformity prevent adhesion and aggregation of particles that indicate the formation of well-dispersed emulsion and enhance stability (Zhang et al. 2017; Ibrar et al. 2022). In agreement with our study, negatively charged zeta potential automatic polarity was significantly associated with nano-delivery system uptake (Honary et al. 2013). A negatively charged GE nanoemulsion is the target for gram +ve bacteria (*C. Pseudotuberculosis*) with automatic polar reaction.

The herbal essential oil contains various chemical molecules with antimicrobial activities such as ethers, ketones, polyphenols, terpenes, aldehydes, and alcohols (Mishra et al. 2020).

Besides, the presence of hydrophilic functional groups and the lipophilicity of EOs favor their antibacterial activities. Gram-positive bacteria are publicized to be much more sensitive to essential oil than Gram-negative ones given due to the weak resistance to the penetration of hydrophobic compounds (Elian et al. 2022).

The antimicrobial activity of garlic essential oil is exhibited by the chemical composition of sulfur constituents and non-sulfur constituents including fatty acids.

In the current study, GC mass analysis revealed fatty acids and ethers as 9-Octadecenoic acid (Z)-, methyl ester (oleic acid), 10-octadecenoic acid, methyl ester (stearic acid), Pentadecanoic acid, 14-methyl-, methyl ester (Myristic acid) that represent 40.4%. That result coincided with the GC-MS of the different extracts of Chrozophora

tinctoria which possess a wide range of pharmacological properties (Mariyammal et al. 2023).

Likewise, oleic acid was the major fatty acid of Gypsophila tuberculosa and Gypsophila eriocalyx (42.0%, 36.0%), as Gypsophila species have industrial, medicinal applications (SERVİ et al. 2019). Pentadecanoic acid, 14-methyl-, methyl ester is used as an antioxidant, antifungal, and antimicrobial (Iqbal et al. 2022)

Furthermore, our study showed that the MIC of garlic oil nanoemulsion revealed a bactericidal effect against *C. pseudotuberculosis* isolates. MIC value as the lowest concentration that prevented the change in the color of blue tetrazolium giving a direct, computed measure of bacterial metabolic activity (Mohamed et al. 2016).

TEM was found to be an enormous method to illustrate the morphological changes between treated and untreated *C. pseudotuberculosis* that exemplified the effect of GO nanoemulsion on the bacterial structure. The morphology of the untreated cells as observed revealed that they are intact cocco-bacilli bacteria. The degradation of the cell wall might be proposed due to the weakening of the peptidoglycan layer due to exposure to GO which agrees with the effect of garlic extract (Booyens et al. 2014). Cell wall lysis was also reported for Listeria monocytogenes treated with garlic shoot juice (Booyens et al. 2014). It is well-established that phenotypic changes in prokaryotes can indicate important changes in biological function (Mihoub et al. 2010). GO nanoemulsion was able to lysis the

cell wall leading to cell leakage and autolysis, thereby preventing bacterial growth and causing cell death (Mihoub et al. 2010).

Conclusion

The current study is the first to reveal the effect of garlic essential oil nanoemulsion on *C. pseudotuberculosis*. GO has been detected to have an anti-corynebacterial effect, resulting in changes to the outer surfaces, internal properties as well as the biological activity of the cells. It is considered a novel approach to antimicrobial treatment.

5- Declarations:-

1. Ethics approval

All live samples were taken from animals in the resting stage without stress. Samples from dead animals were taken under complete aseptic conditions. All experiments were performed in accordance with relevant guidelines and regulations at Benha University. The entire samples were assembled in consonance with the Standard Issues of Animal Rights and Medical Research at the Faculty of Veterinary Medicine, Benha University, Egypt. (The approval no. we obtain: BUFVTM121022)

b) Consent for publication: not applicable.

c)Availability of data and materials: The information supporting datasets are included in this article.

d) Authors & contributions: all authors contributed to the design of the study, sample collection, and data analysis. They performed the initial examination of the isolates as well as read and approved the final manuscript.

e) Competing interests: the authors declare that they have no competing interests.

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Author details:

[1]Aya, M. Soliman, [1]Ashraf, A. Abd El-Tawab, and [2]Amany, O. Selim*

1 Department of Bacteriology, Immunology and Mycology, Faculty of Veterinary Medicine, Benha University, Egypt.

ashraf.awad@fvtm.bu.edu.eg

Aya.Suleiman20@fvtm.bu.edu.eg

2 Department of Bacteriology, Benha Provincial Laboratory, Animal Health Research Institute, Agriculture Research Center, Egypt.

Amany.omar@ahri.gov.eg

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