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# Synthesis and characterization of garlic oil nanoemulsion to detect its antibacterial effect against *Trueperella pyogenes*

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**ABSTRACT**

On account of the pivotal role of herbal compounds in all scientific research, this study aimed to detect the effect of garlic essential oil nanoemulsion (GON) on *Trueperella pyogenes* (*T. pyogenes)*. Four isolates of *T. pyogenes* were grown on brain heart infusion agar and used to extract the bacterial DNA to detect 16 *S*rRNA and *rpo*B genes. The physicochemical characteristics of GON were analyzed through GC-mass analysis and Nanotrac- Zeta sizer. Using the transmission electron microscope, the antibacterial activity was studied by minimum inhibitory concentration. All isolates used were positive for 16S rRNA *and rpo*B genes. The average Garlic oil nanoemulsion size was 118 nm with significant homogeneity. Garlic essential oil nanoemulsion has a negative charge with automatic polarity with 89 us/cm conductivity. Garlic essential oil nanoemulsion was safe to use, and the fatty acids, mainly oleic and stearic acids, were its major particulars. Garlic oil nanoemulsion has deleterious effects on *T. pyogenes* as it causes cell lysis, cytoplasmic leakage, and nanoparticle clusters on the bacterial cell. Furthermore, it resulted in alterations to the exterior surfaces, interior characteristics, and biological activity of the cells, so it should be studied on a large scale, and a trial to explore in vivo efficacy of this product should be done.

## 1. INTRODUCTION

*Trueperella pyogenes* (*T. pyogenes*) was initially classified as Corynebacterium; for many years it previously was classified as *Arcanobacterium pyogenes*, and belongs to Actinomycetaceae (Yassin et al., 2011; Yapicier et al., 2022; Fujimoto et al., 2023). It is a facultative anaerobic gram-positive rod, non-spore-forming, motile, and capsulated, distinguished by strong proteolytic activity and fermentative metabolism (Markey et al., 2013; Fujimoto et al., 2023). It is a worldwide pathogen that inhabits the skin and mucous membranes of the urogenital, respiratory, and gastrointestinal tracts of domestic animal species. This commensal opportunist bacterium is a common causative agent for a diversity of pyogenic infections encompassing abscesses, osteoarthritis, pneumonia, mastitis, metritis, subcutaneous abscesses lymphadenitis, and abortion in farm animals and is familiar with hybrid infections with *Fusobacterium necrophorum* 'Summer mastitis' (cattle), mixed infection with Peptoniphilusindolicus and Streptococcus dysgalactiae (Markey et al. 2013, Abdulmawjood et al. 2016; Rogovskyy et al. 2018; Beikzadeh et al. 2023). Among humans, sporadic cases of *T. pyogenes* infections have been reported in patients in contact with animals (Yapicier et al., 2022).

Despite the earlier studies on this bacterial pathogen segregated from cattle, goats, and sheep, there is still scarce data on its phenotypic and genotypic characteristics (Rogovskyy et al. 2018; Fujimoto et al. 2023). The preliminary recognition was based on the cell morphology and the criteria of colonies, surrounded by an area of beta-hemolysis on sheep blood agar. Then, the biochemical identification can be tested to the negative catalase one for species determination. (Hijazin et al. 2011; Markey et al. 2013; Rzewuska et al. 2019). Its virulence may be attributed to several elements encompassing pyolysin (PLO), fimbria A as an adhesive element, serine proteases with gelatinase and caseinase exertion, and DNases for the invasion of host cells. Also, it may be owing to the peptidoglycan cell wall results in a characteristic firm, impenetrable barrier that illustrates the induction of chronic granulomatous disease. The chemotaxonomic features described previously included a cell-wall peptidoglycan instituted on lysine as the

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dibasic amino acid and rhamnose as the distinctive cell-wall sugar (Yassin et al. 2011; Markey et al. 2013).

Considering antibiotic therapy is the leading choice for the treatment of bacterial infection, its usage in farm animals has been restricted due to the genesis of drug-resistant bacterial species resulting from its long-term misuse. Nanotechnology has made a new age in biological and pharmaceutical uses possible in terms of better bioavailability, increased therapeutic effectiveness, and increased penetrative power. Nanoemulsion has widths ranging between 20 and 200 nm and can be translucent or milky white, depending on the size of its droplets. Nanoemulsions are more than traditional emulsions and robust against gravity separation, coalescence, and flocculation. Owing to their tiny droplet size and large surface area, they can increase the bioavailability of the bioactive substances. The use of dynamic light scattering and zeta potential techniques to determine particle size and surface charge has grown in favor (Al Siraj et al., 2023). Curcumin, garlic, thymol, ginger, and eugenol are phyto-molecules from medicinal and aromatic plants with distinct bioavailability, effectiveness, and solubility restrictions. It restricts its biological activity and, thus, its use in biomedicine. After these strong phyto-molecules were enclosed in the nanocarriers, an increase in physical activity and sustained delivery was seen. In that case, essential oils, which are natural essences yielded by plants, have been widely applied in cosmetics and pharmaceutical industries due to their antioxidant, anti-parasitic, antiviral, and antimicrobial features. These merits have prompted new studies that have reported variations in their efficacy against bovine mastitis and respiratory and reproductive disorders of dairy animals. (Sampaio et al. 2022; Al Siraj et al. 2023; Paiano et al. 2023). To our knowledge, there are no previous studies on treating *T. pyogenes*. Therefore, this inquiry pointed to clarify the antibacterial effect of garlic oil nanoemulsion against *T. pyogenes* recorded by the transmission electron microscope.

## 2. MATERIAL AND METHODS

### *2.1. Ethical Approval:*

All live samples were taken from animals in the rest stage. The entire samples were assembled in consonance with the National Institutes of Health's Guidelines for the animal care and authorized by the Ethical Committee of Research at Benha University, Faculty of Veterinary Medicine Egypt. (Approval no.: BUFVTM121022)

### *2.2. Isolation and Identification of Bacteria:*

Four positive isolates of *T. pyogenes* (two from does and two from cows) were identified from 215 total samples (144 sheep, 61 goats, and 10 cattle) from different abscess locations. They were cultured on Brain Heart Infusion agar (BHI) with fresh sheep blood 5% and on paired barker media, then incubated at 37 °C for 24 h (HIMEDIA). The isolated colonies were characterized macroscopically, stained by the Gram stain, and subjected to biochemical tests. All tested isolates were immotile, negative for oxidase and Urease, while acid phosphatase and nitrate reduction were positive.

### *2.3. DNA extraction and PCR amplification according to Sambrook et al. (1989); WHO (2002):*

The selected isolates were grown on BHI agar for 24 h at 37 °C then extracted the bacterial DNA using QIA amp DNA Mini Kit Catalogue according to the manufacturer's instructions. Using the extracted bacterial DNA, amplification of 16S rRNA, rpoB, and phospholipase D (pld) genes were done using the primers included in Table (1) below.

Table 1 Amplification cycling conditions to 16S rRNA, rpoB, and pld genes with their primers.

| Gene | Sequence | Size (bp) | No. of cycles (35) Primary denaturation | Secondary denaturation | Annealing | Extension | Final extension |
|---|---|---|---|---|---|---|---|
| RpoB | CGWATGAACATYGGBCAGGT | 406 bp | 94˚C | 94˚C | 52˚C | 72˚C | 72˚C |
| | TCCATYTCRCCRAARCGCTG | | 5 min. | 30 sec. | 40 sec | 45 sec | 10 min. |
| 16Sr | ACCGCACTTTAGTGTGTGTG | 816 bp | 94˚C | 94˚C | 58˚C | 72˚C | 72˚C |
| RNA | TCTCTACGCCGATCTTGTAT | | 5 min. | 30 sec. | 40 sec | 50 sec | 10 min. |
| pld | ATAAGCGTAAGCAGGGAGCA | 203 bp | 94˚C | 94˚C | 56˚C | 72˚C | 72˚C |
| | ATCAGCGGTGATTATCTTCCAGG | | 5 min. | 30 sec. | 30 sec | 30 sec | 10 min. |

The isolates were further investigated for the presence of the pld gene specific to C. pseudotuberculosis. It was not detected in any isolates to ensure they were not misdiagnosed with other bacteria (Rogovskyy et al. 2018). The amplification reactions were equipped in 25 µl containing PCR master-mix (2X premix) (Emerald Amp GT PCR master, Takara kit, code RR310A) 12.5 µl, 1 µl for each primer (forward and reverse), 5 µl template DNA completed to 25 µl by 5.5 µl PCR grade water. The amplified PCR products were electrophoresed in Gel Casting Apparatus (Biometra) through 1.5 Agarose gel in TBE buffer for 1 h. The bands were stained with Ethidium bromide and photographed by a gel documentation system, and the data analysis was done through computer software.

### *2.4. Garlic nano-emulsion:*

#### *2.4.1. Garlic nano-emulsion Preparation:*

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

Nanoemulsion (2%) was prepared in the nanomaterials Research and synthesis unit (El Oksh et al., 2022).

*2.4.2. Characterization of oil Nanoemulsion:*

The prepared oil nanoemulsion was measured using the NANOTRAC-WAVE II Zeta-sizer (MICROTRAC, USA) for Particle size analysis (Dynamic light scattering), similarity, polydispersity indexes (PDI), surface charge, and electrical conductivity. GC-MS analysis was measured at Nawah Scientific Inc.

*2.4.3. Cytotoxicity test:*

Cell viability was evaluated using the Sulforhodamine B assay (SRB) test at various doses (0.01,0.1,1,10,100 ug/ml), coinciding with Allam et al. (2018). Cell culture: the oral epithelial cells were purchased from Mokatam, Egypt-basedNawah Scientific Inc. They were cultured in Dulbecco's Modified Eagle Medium with additives such as 100 mg/mL streptomycin, 100 unit/mL penicillin, and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified 5% (v/v) CO2 air.

*2.4.4. Determination of the antibacterial activity by Minimum Inhibitory Concentration (MIC) (Panphut, 2017):*

100 µl Mueller-Hinton broth was dispensed in each well of the 96-well plate. 100 µl of stock solution of GON and serial two-fold dilutions were done in rows on the plate, with resulting concentrations ranging from 100 to 0.195 mg/ml. A 100 µl of *T. pyogenes* of concentration 3×10 5 CFU/ml was added in each well. 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 2 hours.

*2.4.5. Transmission electron microscopy for detection of garlic nanoemulsion antibacterial effect coinciding with (Mohamed et al., (2016); El Sayed et al., (2022).*

The morphology of the GON was explored by transmission electron microscopy (TEM). A drop of garlic nanoemulsion was thinned with deionized water and relocated into a carbon-coated 400-mesh copper grid. The replica was lifted to dry, and then the picture was envisioned with J EM-1400 TEM at a beam energy of 80 K.V. TEM was performed to detect the detrimental changes of Garlic nanoemulsion on *T. pyogenes*.

## 3. RESULTS

The amplification of 16 SrRNA and rpoB genes is positive for each of the four *T. pyogenes* isolates that were tested, as illustrated in Figures 1 and 2. While, all isolates exhibit negative pld gene amplification, as shown in Figure 3.

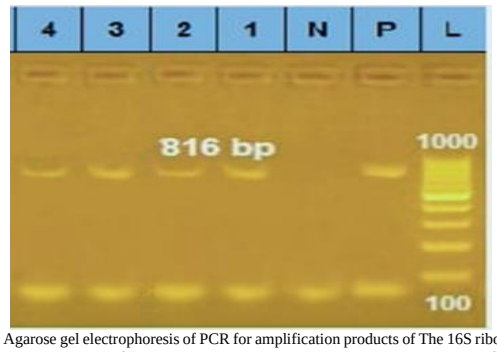


Fig. 1 Agarose gel electrophoresis of PCR for amplification products of The 16S ribosomal RNA gene (16S rRNA) for 4 *T. pyogenes* isolates. All Lanes show positive amplification of16S rRNA gene at 816 bp. Lane L: DNA ladder at 100-1000bp. N.: Negative control. P.: Positive control.

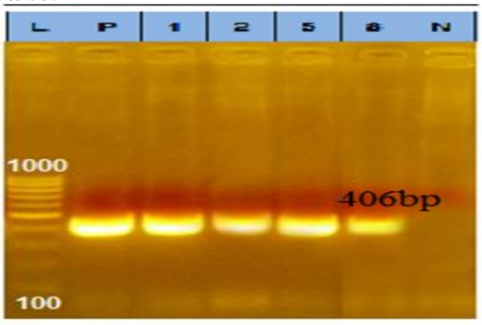


Fig. 2 Agarose gel electrophoresis of PCR for amplification products of the RNA polymerase beta subunit-encoding gene (rpoB) for the isolates. All Lanes show positive amplification of rpoB gene at 406 bp. Lane L: DNA ladder at 100-1000bp. N.: Negative control P.: Positive control.

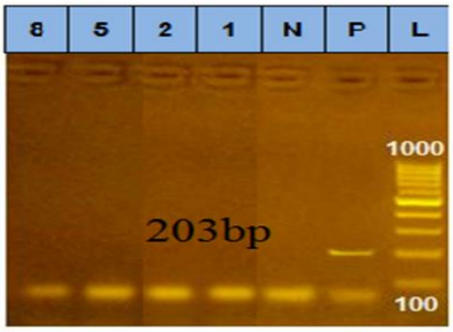


Fig. 3 Agarose gel electrophoresis of PCR for amplification products of Phospholipase D gene (plD) for 4 *T. pyogenes* isolates. All Lanes show negative amplification of plD gene at 203 bp. Lane L: DNA ladder at 100-1000bp. N.: Negative control. P.: Positive control.

*Characterization of garlic nanoemulsion:*

The nano-emulsion garlic oil was measured at 118 nm average in size according to the HRTEM (Figure, 4). Its conductivity and zeta potential were measured to be 89 us/cm and 13.2Mv, respectively. In addition, it was stable with a negative charge, and there was no clustering, and its nature is spherical with homogeneous dimensions. Gas mass spectrometry (GC-MS) showed that the emulsion consisted of 29 chemical compounds with different ratios, and the highest percentages were identified as 21.08% oleic acid, 12.7% stearic acid, and 6.62% Myristic acid.

Table 2 Chemical components analysis of garlic oil nanoemulsion using GC-Mass analysis (R T: retent ion time (as minutes)).

| No. | RT | Area% | Compounds |
|---|---|---|---|
| 1 | 9.93 | 0.59 | [1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester |
| 2 | 12.68 | 1.59 | Cycloheptasiloxane, tetradecamethyl- |
| 3 | 15.31 | 3.24 | Benzene, (1-butylheptyl)- |
| 4 | 15.5 | 1.53 | 10,13-Octadecadiynoic acid, methyl ester |
| 5 | 15.88 | 2.85 | Cyclooctasiloxane, hexadecamethyl- |
| 6 | 16.59 | 1.78 | 1,3,5-triazine-2,4-diamine, 6-chloro-n-ethyl- |
| 7 | 17.02 | 2.40 | Benzene, (1-pentylheptyl)- |
| 8 | 17.11 | 3.51 | Benzene, (1-butyloctyl)- |
| 9 | 17.32 | 1.48 | Benzene, (1-propylnonyl)- |
| 10 | 17.48 | 1.22 | 5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)- |
| 11 | 17.73 | 1.13 | 10,13-Octadecadiynoic acid, methyl ester |
| 12 | 18.4 | 2.04 | Dotriacontane |
| 13 | 18.68 | 4.04 | Cyclononasiloxane, octadecamethyl- |
| 14 | 18.86 | 0.94 | Pentacosane, 13-phenyl- |
| 15 | 19.08 | 0.94 | 2H-Pyran, 2-(7-heptadecynyloxy) tetrahydro- |
| 16 | 20.69 | 6.62 | Pentadecanoic acid, 14-methyl-, methyl ester |
| 17 | 21.17 | 3.09 | Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15 -hexadecamethyl- |
| 18 | 23.33 | 21.08 | 9-Octadecenoic acid (Z)-, methyl ester |
| 19 | 23.47 | 12.7 | 10-octadecenoic acid, methyl ester |
| 20 | 25.57 | 3.51 | Silicone oil |
| 21 | 27.51 | 3.51 | 1h-purin-6-amine, [(2-fluorophenyl) methyl]- |
| 22 | 29.33 | 4.40 | 1h-purin-6-amine, [(2-fluorophenyl) methyl]- |
| 23 | 31.05 | 3.63 | Silicone oil |
| 24 | 32.65 | 3.12 | Silicone oil |
| 25 | 33.7 | 3.58 | Silicone oil |
| 26 | 34.3 | 2.10 | 9,12-octadecadienoic acid (z,z)-, 2,3-bis[(trimethylsilyl)oxy]propyl ester |
| 27 | 34.8 | 1.22 | 4h-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy- |
| 28 | 35.35 | 0.70 | Ethyl iso-allocholate |
| 29 | 37.04 | 0.76 | Ethyl iso-allocholate |

*Cell viability assay:*

Using the SRB assay, cell viability was estimated as 100.48, 100.43, 62.73, 14.60, and 0.17% for 5 concentrations as 0.01, 0.1, 1, 10 and 100 ug/ml individually which means that the IC50 was 1.69 ug/ml as in figure 5.

MIC results for GO nanoemulsion against *T. pyogenes*:

The antimicrobial activity and micro-dilution susceptibility test of GO nanoemulsion was estimated using the MIC test as the lowest concentration that prevented the change in color and killed *T. pyogenes*. The resultant of the two-fold serial dilation of GON on *T. pyogenes* betrayed that the concentration 1:3 coincides with the MIC values that betrayed significant effects and proved lethal to bacterial cell (Figure, 6 and 7).

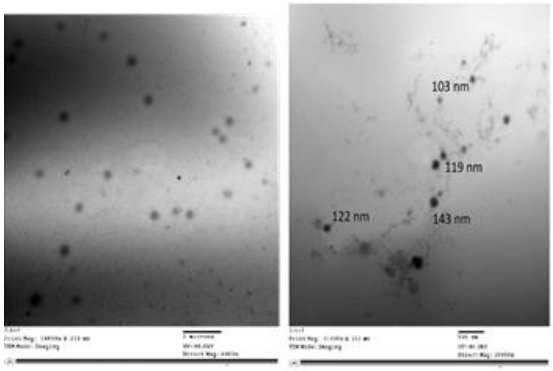


Fig. 4 Garlic nanoemulsion under HRTEM shown the average of nano-droplet size 118 nm with a complete disparity and greater homogeneity.

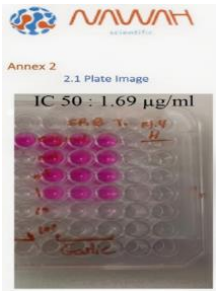


Fig 5 Plate image of SRB assay.

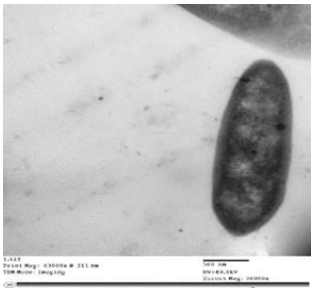

Fig 6 Control growth without treatment —TEM 30,000×.

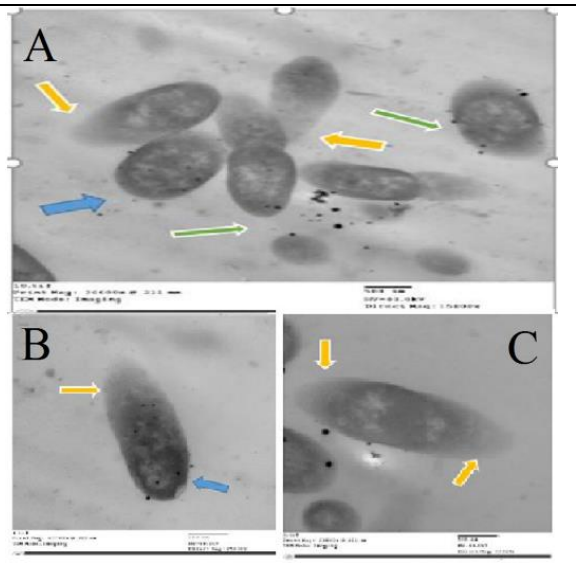


Fig. 7 *T. pyogenes* treated with GO nanoemulsion (2%), A: The green arrows elucidate clusters of GON on the surface of the bacterial cell -TEM 15,000×. The blue arrow indicates nanoparticle internalization to the bacterial cell —TEM 25,000×. The yellow arrows elucidate structural changes in the cell wall (cell wall lysis) with cytoplasmic leakage —TEM 30,000×. B: The blue arrow indicates nanoparticle internalization to the bacterial cell —TEM 25,000×. The yellow arrows elucidate structural changes in the cell wall (cell wall lysis) with cytoplasmic leakage —TEM 30,000×. C: The yellow arrows elucidate structural changes in the cell wall (cell wall lysis) with cytoplasmic leakage —TEM 30,000×.

## 4. DISCUSSION

This work aimed to approach *T. pyogenes* therapy using garlic oil nanoemulsion. Regarding *T. pyogenes* identification, the 16S rRNA gene amplification was done as a standard to simplify the taxonomy and identification of bacteria, especially those with scarce distinguishable phenotypic characteristics. More accurate and acceptable phylogenetic resolution can be accomplished by investigation of the rpoB gene, which is widely used for an accurate differentiating species of genera Arcanobacterium and Trueperella (Abdallah, 2016; Park et al., 2022).

The obtained data, based on the rpoB and the 16S rRNA genes detection as molecular targets, confirm that all isolates were positive for these genes and the bands of rpoB gene were more obvious than 16S rRNA. These results may

propose that the rpoB gene may be used to supersede or correlate the 16S rRNA gene for phylogenetic studies of Trueperella as supposed in (Khamis et al., 2004; Park et al., 2022).Nanoemulsions have attracted global attention to their submicron size with numerous advantages, such as improved treatment efficiency, prolonged release profile, and high kinetic stability when effectively targeted to the bacteria (Moghassemi et al., 2022). Herbal essential oils (EOs) have low toxicity, are affordable, and are broadly utilized in the pharmaceutical, cosmetic, food, and beverage industries, besides antibacterial applications. Notably, Gram-positive bacteria are noted to be more sensitive than Gram-negative ones to EOs given that they possess frail resistance to the penetration of hydrophobic compounds (Liang et al., 2022).Garlic essential oil was noted to have higher antibacterial activity against *T. pyogenes*, like cinnamon essential oil, unlike oregano or thyme essential oil. It is influential to point out that the interactions of the minor components with the major ones can pitch in the antibacterial exertion of each essential oil (Paiano et al., 2023). The Morphology of GE nanoemulsion (2%) particles showed a spherical shape with high homogeneity, which agreed with that reported by (Mossa et al. 2018). The obtained data elucidates that the average zeta potential was 13.2mv lower than what has been reported by El oksh et al. (2022) and higher than that has been reported by Ibrar et al. (2022). The droplet size was in-between 103-143 nm which is lower than that has been reported by Ibrar et al. (2022). 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). Moreover, negatively charged zeta potential automatic polarity was significantly associated with nano-delivery system uptake. A negatively charged GE nanoemulsion is the target for gram +ve bacteria (*T. pyogenes*) with automatic polar reaction (Honary and Zahir 2013).In the current study, GC mass analysis revealed fatty acids and ethers as oleic, stearic, and myristic acids, representing more than 40 % of the GEO. This 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 (Servi et al. 2019). Pentadecanoic acid, 14-methyl-, methyl ester is used as an antioxidant, antifungal, and antimicrobial (Iqbal et al. 2022).Transmission electron microscopy was found to be an enormous method to illustrate the morphological changes between treated and untreated *T. pyogenes*; therefore, it was used to exemplify the effect of GO nanoemulsion on the bacterial structure. The morphology of the untreated cells, as observed by TEM, revealed that they are intact cocco-bacilli bacteria. The GO-treated bacteria showed degradation of its wall, which might be proposed owing to the weakening of the peptidoglycan layer due to exposure to GO, which agrees with the effect of garlic extract (Tavares et al. 2021). Cell wall lysis was also documented for Listeria monocytogenes treated with garlic shoot juice (Booyens et al. 2014). It is well-established that phenotypic alterations in prokaryotes can signify vital alterations in biological function (Mihoub et al. 2010). GO nanoemulsion was reported to lyse the cell wall, spawning cell leakage and autolysis, thereby preventing bacterial growth and causing cell apoptosis (Mihoub et al. 2010).

## 5. CONCLUSIONS

GO has been detected to have an anti-bacterial effect, spawning in alterations to the outer surfaces, internal properties as well as the biological activity of the cells. It is considered a novel candidate for antimicrobial treatment and further studies should be applied.

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