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ORIGINAL ARTICLE

New nano-chemotherapeutic chitosans-garlic oil-antibiotics against diabetic foot virulent *Proteus* spp.

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ABSTRACT

Background and Objectives: Diabetes foot ulcer is recognized to have a major side effect that raises the risk of amputation. Diabetic ulcer bacterial infections caused by virulent and resistant bacteria like *Proteus mirabilis* lead to serious wounds that are incurable with conventional medications.

Materials and Methods: In this study, we evaluated the antibacterial activity of a natural product nanochitosan - garlic oil against ten diabetic foot isolates of *Proteus mirabilis.* Various chitosans (Crab (CScr) - shrimp (CSsh) - squilla (CSsq)) in nano form were prepared and coated with garlic oil (GO). GC-MS analysis was carried out to determine the main components of the essential garlic oil. The physicochemical properties of GO-NCSsq were analyzed using dynamic light scattering (DLS), zeta potentials (ZP) and subsequently scan electron microscope (SEM). Additionally, the minimum inhibitory concentration (MIC) and fraction inhibitory concentration index (FICI) were determined.

Results: GC-MS analysis revealed the presence of major palmitic fatty acid. (GO) loaded on nanochitosan squilla (NCSsq) showed high activity. Although SEM showed lower nano-size, average size of the GO-NCSsq was 330.8 nm by DLS and its zeta potential formulation was +39.6 mV. The final formulation represented by GO-NCSsq + Pipercillin (Pi) inhibited the virulence factor of *P. mirabilis* and reduced the MIC value (p-value > 0.001). Moreover, the killing time at 9 h was found to be significantly higher for GO-NCSsq + pipercillin (Pi) against *P. mirabilis.*

Conclusion: In order to manage diabetic foot infections, GO-NCSsq is a legitimate antibacterial agent that can be coupled with other antibiotics.

Keywords: Antidiabeitic agents; Garlic oil; Nanochitosan; *Proteus* spp; Synergy

INTRODUCTION

Diabetic foot is one of the most dreaded consequences of diabetes and the primary cause of hospital admissions for diabetic people. Diabetic foot is linked to a number of pathological outcomes, such as neuropathy, peripheral vascular disease, ulceration of the foot, and infection with or without osteomyelitis. These illnesses have the potential to develop into gangrene and necessitate limb amputation. Diabetes increases a person's lifetime risk of foot ulcers by up to 25%; however, diabetic ulcers increase the risk of limb amputation by 15-46 times (1).

Proteus mirabilis is a member of the Gram-negative Enterobacteriaceae family that is motile and does not ferment lactose. Dimorphic short rods and elongated

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swarmer cells expressing a high number of flagella are characteristics of *P. mirabilis* (2). *P. mirabilis*, which accounts for roughly 18% of solitary cases, is one of the most dangerous pathogenic organisms that can cause diabetic foot ulcers. The virulence factors of *P. mirabilis* are diverse and range from the synthesis of extracellular enzymes and toxins such as hemolysins, urease, and protease to constitutional organelles like sticky fimbriae and swarming motility with the help of flagella (3). Moreover, *P. mirabilis* continuously becomes resistant to different drugs. *P. mirabilisis* is mostly contagious due to its propensity to build biofilms, which adds to the challenge of treating the infection with traditional antibiotics (4).

Antibiotic resistance is on the rise; thus natural plant-based products may be an intriguing substitute. Certain plant extracts and phytochemicals have been shown to possess antibacterial qualities, making them valuable tools for medical interventions. As an anti-infective, garlic (*Allium sativum*) has long been used in food and medicine (5). These applications are supported by *in vitro* evidence of fresh and freeze-dried garlic extracts' antibacterial efficacy against a variety of microorganisms. According to Tesfaye and Mengesha (6), garlic has high antibacterial potential against both Gram-positive and Gram-negative bacteria.

Because of its unique qualities, such as its biodegradability, biocompatibility, and non-toxicity, chitosan is one of the most intriguing polymeric compounds that scientists have been interested in studying in recent years (7). Chitosan has antibacterial properties that work against a variety of microbes. It attaches to the cell membrane and interferes with its ability to perform normal tasks, such as facilitating the leaking of intracellular components and obstructing the movement of nutrients into the cells (8). Furthermore, because of its easy and controlled extraction, biocompatibility, biodegradability, non-toxicity, antimicrobial properties, and ease of chemical modification—not to mention its capacity to form gels, films, and solid nanoparticles—this cationic polysaccharide holds great promise for use in nanotechnology (9). Moreover, studies have demonstrated the healing properties of both drug-loaded and drug-free chitosan in the treatment of wound injuries. Different polymers are frequently mixed to create new biomaterials with greater therapeutic potential in order to capitalize on their unique properties (10).

This study aims to assess the biological characteristics of garlic oil-loaded Chitosan nanoparticles and test their anti- *P. mirabilis* potential to treat diabetic foot infection. A new nano-chemotherapeutic chitosans - Garlic oil - antibiotics final formula was obtained after testing of minimum inhibitory concentrations (MIC) and fraction inhibitory concentration index resulting in procuring a natural diabetic foot drug treatment.

MATERIALS AND METHODS

Bacterial samples. Fifty-two patients with diabetic ulcers who had foot wounds at the Diabetic Foot Unit and Vascular Surgery Unit of the Faculty of Medicine, Alexandria Main University Hospital, Egypt, were swabbed after taken their consent. Isolates of *Proteus* spp. were purified, stained with Gram, and biochemically identified (11). After being inoculated on macConkey, blood, and nutrient agar plates, the isolates were cultured for 24 hours at 37°C.

Antibiotics. Twelve antibiotics representing eight groups of antibiotics (aminoglycosides, carbapenems, cephalosporins, glycopeptides, macrolides, penicillin, quinolones, and tetracyclines) were used at different concentrations recommended as anti-*Proteus.* Antibiotics were tested against the ten *Proteus* isolates (MDR) to determine their resistance by MIC method. The antibiotic's concentrations used throughout the present work ranged from (8-256 µg/mL).

Chitosan (Cs). The shells of *Palaemon serratus* (shrimp) (CSsh), *Corystes cassivelaunus* (crab) and *Squilla mantis* (squilla) (CSsq) were used to produce chitosans. Preparation of chitosans was taken place in Microbiology and Botany Department, Alexandria University. The three forms of chitosan all had the same degree of deacetylation (DDA), which was 86%. There was an 18-22 kDa MWT range. The range of their viscosity was 12-16 × 10^2 cps. After giving the shrimp, crabs, and squilla a thorough wash, drying, and cutting into small pieces, 1% acetic acid was applied and allowed to sit at room temperature for two hours. Filtration, neutralization, and washing then were done respectively. At 65°C, an alkaline treatment employing a 2N sodium hydroxide solution was used to deproteinate the protein. Washing process was then done to neutralize the protein. 10% hydrochloric acid was added after demineralization and it

was left to sit at room temperature for five hours. Acetone was added to the chitin in order to eliminate the natural colors present. Deacetylation: after five hours, a suspension of one gram of chitin in fifty milliliters of aqueous sodium hydroxide was combined with 5% by weight of deacetylating reagent and mixed at the appropriate temperature of 95°C under nitrogen purging. The substance was filtered out and then cleaned with methanol and water to a pH of neutral, and it was finally dried at 85°C.

Gas chromatography–mass spectrometry analysis of garlic essential oil. Garlic oil (GO) was prepared by steam distillation of mashed garlic and tested against the ten selected isolates by MIC Method. Using a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness), a GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) was used to analyze the sample's chemical composition. The temperature of the column oven was first maintained at 60°C, then raised to 250°C with a 2-minute hold, and then raised to 300°C with a 30-minute hold. At 270°C, the injector temperature was maintained. As a carrier gas, helium was used at a steady flow rate of 1 milliliter per minute. Diluted samples containing 1 µl were automatically injected using an Autosampler AS3000 connected to a GC in split mode, with a 4-minute solvent delay. In full scan mode, EI mass spectra were obtained at 70 eV ionization voltages covering m/z 50-650. 200°C was the temperature at which the ion source and transfer line were set, respectively. By comparing the mass spectra of the constituent parts with those of the NIST14 and WILEY 09 mass spectral databases, the components were identified.

Preparation of garlic oil (GO) loaded chitosan-nanoparticles (NCSsq). For cross linking, squilla, shrimp and crab chitosan solution were prepared by dissolving 1.0 g of chitosan powder in 100 ml of 75% (v/v) of ethanol. A solution of 1ml (0.04 M) epichlorohydrin solution was added to chitosan solutions and stirred for 1.5 h to allow proper mixing at room temperature (12). This was followed by stirring vigorously under the treatment of ultrasonic for 20 min. One ml drop wise of prepared garlic oil was added to the treated chitosan nanoforms then subjected to stirring for 2 h, then these proper mixtures were centrifuged at room temperature. Finally, the obtained solid products were dried in an oven at 50°C for further analysis.

Dynamic light scattering and zeta potential analysis. A nanoPartica SZ-100V2 Series Nanoparticle Analyzer (HORIBA Scientific) was used to evaluate the size distribution of the chitosan nanoform droplets. Moreover, these droplets' zeta potential was determined with the same apparatus. After being diluted, the samples were examined three times. For characterization, the most transparent formulation was selected, and it was retained for further experiment.

SEM: scanning electron microscopy. SEM allowed the size and shape of the GO-loaded NCS to be identified. The JSM-IT200 InTouchScopeTM - JEOL (Germany) GmbH and Nordic (AB) SEM was used to evaluate the loaded NCS particles after they had been lyophilized and deposited onto SEM stubs.

Minimum inhibitory concentration (MIC). The broth dilution method was used to determine MICs. Broth served as the negative control and the bacterial suspension as the positive control. One mL nutrient broth was added in 10 test tubes. One mL of antibiotic or natural drugs solutions were added in the first one and successively transferred to the next tubes. After that, 0.1 mL test bacterium was added on each test tube. For 24 hours, each test tube was incubated at 34°C. In accordance with CLSI, minimum inhibitory concentrations (MIC) were noted. The higher the activity of the newly developed medications and tested antibiotics, the lower the MIC.

Checkerboard test. Using a checkerboard titration approach using 96-well polypropylene micro-titer plates, antibiotic-nanochitosan-garlic oil combinations were tested against the *P. mirabilisis* virulent strains in interaction tests. The ranges of natural medication dilutions were as follows: Antibiotics: 0.4-50 µg/mL; chitosans: 1-64 µg/mL; garlic oil: 0.1-16 µg/mL; chitosan–garlic oil nanoparticles: 0.1-8 µg/mL. Inoculums of every chosen virulent isolate were diluted in double-strength broth after being cultured in broth for an overnight period, achieving 10^7 CFU/mL. 25 µL of the antibiotic, 25 µL of either garlic oil or nanochitosan, 25 µL of garlic oil, and 75 µL of the inoculated double strength broth were all added to the micro-titer plate well. For twenty-four hours, the board was incubated at 37°C (13). The following formula was used to determine the fractional inhibitory concentration index (FICI) for combinations of three antimicrobials: antibiotics Ab,

chitosan CS (nanoforms), and garlic oil GO: FICI = (MIC_{Acombi}/MIC_{Aalone}) + (MIC_{Bcombi}/MIC_{Balone})

FIC index < 0.5 was used to indicate synergism, FIC index > 0.5 and ≤ 1 for additive effects, FIC index > 1 and ≤ 2 for indifferent effects, and FIC index > 4 for antagonistic effects.

Time-kill test. The microbroth dilution experiment was used to assess the time-kill curves of specific garlic oil - nanochitosan in combination with the antibiotic of choice (14). The tested combination agents were added to 0.04 mL of Müller-Hinton broth in microtiter wells, and the wells were then infected with 0.05 mL of a bacterial inoculum containing 10^5-10^6 cfu/mL. The growth control wells contained 0.05 mL of broth and just bacteria. Following that, the wells were incubated at 37°C, and viable counts were carried out after 0,2,4,6,8, and 24 hours. After being extracted from the wells, 0.01 mL of the sample was spread out on Müller-Hinton agar plates, diluted twice with normal saline (0.9% NaCl), and incubated for 24 hours at 37°C. The $\log_{10}$ CFU/mL and time (hr) were plotted to determine the colony count of bacteria and to create time-mortality curves. A ≥2 $\log_{10}$ CFU/mL decrease and a <2 $\log_{10}$ CFU/mL change in the average viable count at 24 hours for organisms treated with the combination, respectively, compared with the single medication, were considered as synergy and additively/indifference, respectively. When there was antagonistic interaction, the drugs under study had a substantially less combined effect than when they were taken separately (15).

Statistical analysis. The standard deviation, or mean ± SD, is used to present data. The means of three replicates comprise all the data. The Kolmogorov-Smirnov test was used to determine whether the data was normal. ANOVA, or one-way analysis of variance, was used to identify the significant variations between various antibiotic or bioactive substance groups. Tukey post hoc comparisons between the various groups were carried out to see if there was a significant difference between them. P value > 0.05 was regarded as not statistically significant for all statistical tests. The statistically significant result was defined as P value < 0.05, and the statistically moderately significant result as P value < 0.01, and the statistically highly significant result as P value < 0.001. Minitab version 19, GraphPad Prism version 8.3, and Microsoft Excel 365 (Microsoft Corporation, USA) were used to analyze all of the data.

RESULTS

Identification of diabetic foot *P. mirabilis* isolates. In this study, ten *P. mirabilis* isolates were identified as a Gram-negative, facultative anaerobic and rod-shaped bacterium. It can be diagnosed in the lab due to its characteristic swarming motility on nutrient agar, and its ability to generate yellowish lactose non-fermenting colonies on MacConkey's agar. It smells strongly like fish and has urease activity. Additionally, the pure isolates did not digest indole and turned black on triple sugar iron (TSI) agar as a result of hydrogen sulfide generation.

Determination of antibiotics resistance. Minimum inhibition concentration against 12 antibiotics representing 8 antibiotic groups (aminoglycosides, penicillin, carbapenems, cephalosporins, glycopeptides, macrolides, quinolones, and tetracyclines) showed highly significant MICs (p-value < 0.001) against the ten *P. mirabilis* isolates (Table 1). Higher MIC values (>200 µg/mL) had lower activities against Pm2,4,6,8,9 while, lower MICs (3 ± 2 – 48.3 ± 6.5 µg/mL) had higher activities against Pm1,3,5,7,10. Results illustrated in Fig. 1 show that the average minimum inhibitory concentration (MIC) for all antibiotics against the isolates ranged from 82.7 µg/mL for vancomycin to 259.7 µg/mL for ampicillin.

Gas chromatographic analysis of garlic oil. GC-MS of garlic oil results were tabulated (Table 2 and Fig. 2) and showed a combination of fatty acids such as palmitic (major), 9-octadecenoic, 17-octadecynoic, Cis-vaccenic, docosatetraenoic, oleic & linolenic acid, 1-Heptatriacotanol. Organosulphur compounds such as 2-aminoethanethiol hydrogen sulfate (ester). Flavonoids derivatives such as 3H-cyclodeca[B]furan-2-one,4,9-dihydroxy-6-methyl-3,10-dimethylene-3A. Essential oil: baimuxinal and dotriacontane. Drugs and bioactive substances: carbamic Acid, diepoxyhexadecane, 2,2-dideutero octadecanal & 1-Docosanol. Clyc- and emulsifiers natural plant products: 1-methylpentyl cyclopropane & 1-hexadecanol.

MIC of chitosans and garlic oil. Minimum inhibitory concentration for 10 *Proteus* isolates were tested using different forms of chitosan concentration show-

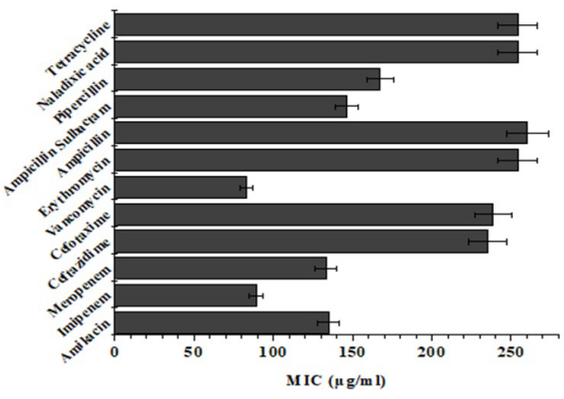

Fig. 1. Averages of MIC in µg/mL of different Antibiotics against ten *Proteus mirabilis* isolated strains

ing highly significant MIC values ranging from 12.3 ± 2.5 to 48 ± 3 µg/mL. Crab chitosan exhibited higher minimum inhibitory concentration (MIC) (lower activities) followed by shrimp chitosan while squilla chitosan showed lower MICs representing higher activities. Pm1 was the most resistant strain against the 3 types of chitosans while Pm4,6,7 were the most sensitive ones, especially when using CSsq (12.3 ± 2.5 - 13 ± 2.7 µg/mL). Garlic oil showed lower activity against Pm7 (127.7 ± 2.5 µg/mL) and higher activity against Pm4 (11.7 ± 2.5 µg/mL) (Table 3). Calculated MICs averages for all CS (crab, shrimp and squilla) were 36.1, 26.3 and 22.6 µg/mL, respectively.

MIC and FICI of garlic oil loaded nanochitosans. Garlic oil was loaded on different nanochitosan forms and an increase in MICs activities against *P. mirabilis* isolated strains was observed. Calculated MICs averages for all GO loaded NCS (crab, shrimp and squilla) were 24, 24 and 12 µg/mL, respectively (Table 4). FICIs for these natural drug combinations were determined showing indifference effect using GO-NCS Squilla, however, Crab and Shrimp nanoforms combination exhibited antagonistic effect.

DLS, ZP and SEM analysis of GO-NCSsq. Dynamic light scattering (DLS) and Zeta Potential were used to measure the GO-NCSsq's size, dispersion, and homogeneity. The particles' average mean diameter and charge, as indicated by Figs. 3a and b were 330.8 nm, and +39.6 mV, respectively. This was further supported by SEM, which showed that GO-NCSsq contained distinct, spherical particles with a dense structure. In contrast to the average particle size found by DLS analysis, these particles appeared to be smaller

Table 1. MIC of tested antibiotics against *Proteus mirabilis* isolates

Antibiotic group	Antibiotics	Pm1	Pm2	Pm3	Pm4	Pm5	Pm6	Pm7	Pm8	Pm9	Pm10
Aminoglycosides	Amikacin	32.3 ± 2.5 ^{e,f}	252 ± 10.6 ^{a}	3 ± 2 ^{e}	253.3 ± 8.3 ^{a}	5.3 ± 2.1 ^{d,e}	254 ± 13.1 ^{a}	32.3 ± 2.5 ^{c,d}	252 ± 10.6 ^{a}	253.3 ± 8.3 ^{a}	6 ± 2 ^{f}
Carbapenems	Imipenem	23.3 ± 3.1 ^{f}	252 ± 10.6 ^{a}	12 ± 2 ^{e}	23.7 ± 3.5 ^{b}	12.3 ± 2.5 ^{d,e}	23.7 ± 2.5 ^{b}	23 ± 2.6 ^{d}	253.3 ± 8.3 ^{a}	252 ± 10.6 ^{a}	12 ± 2 ^{e,f}
	Meropenem	23.7 ± 3.5 ^{e,f}	254.3 ± 9.6 ^{a}	3 ± 2 ^{e}	254 ± 13.1 ^{a}	3.3 ± 1.5 ^{e}	254 ± 13.1 ^{a}	23.3 ± 2.1 ^{d}	252 ± 10.6 ^{a}	254 ± 13.1 ^{a}	3 ± 2 ^{f}
Cephalosporins	Ceftazidime	64.3 ± 4.5 ^{d}	254 ± 13.1 ^{a}	252 ± 10.6 ^{a}	253.7 ± 7.8 ^{a}	254 ± 10.1 ^{a}	252 ± 10.6 ^{a}	254 ± 9.2 ^{a}	252 ± 10.6 ^{a}	255 ± 11.5 ^{a}	255.3 ± 5 ^{a}
	Cefotaxime	91.7 ± 3.5 ^{c}	253.3 ± 8.3 ^{a}	253 ± 11.8 ^{a}	253.7 ± 7.8 ^{a}	255.3 ± 5 ^{a}	257.3 ± 8.1 ^{a}	254 ± 13.1 ^{a}	253 ± 7.9 ^{a}	253.3 ± 8.3 ^{a}	256 ± 0 ^{a}
Glycopeptides	Vancomycin	127.7 ± 7.5 ^{b}	93.3 ± 6.1 ^{b}	92.7 ± 6 ^{c}	47.7 ± 2.5 ^{b}	93.3 ± 6.1 ^{c}	48.7 ± 6 ^{b}	47.7 ± 3.5 ^{c}	91.7 ± 3.5 ^{b}	92 ± 4 ^{b}	92.3 ± 2.5 ^{d}
Macrolides	Erythromycin	254 ± 13.1 ^{a}	252 ± 10.6 ^{a}	253.7 ± 7.8 ^{a}	254 ± 10.1 ^{a}	252 ± 10.6 ^{a}	254 ± 9.2 ^{a}	252 ± 10.6 ^{a}	255 ± 11.5 ^{a}	255.3 ± 5 ^{a}	252 ± 10.6 ^{a}
Penicillin	Ampicillin	253.7 ± 7.8 ^{a}	254 ± 10.1 ^{a}	196.3 ± 3.5 ^{b}	252 ± 10.6 ^{a}	195 ± 4.6 ^{b}	253.7 ± 7.8 ^{a}	93.7 ± 6.7 ^{b}	254 ± 9.2 ^{a}	252 ± 10.6 ^{a}	168 ± 2.6 ^{c}
	Ampicillin Sulbactam	47.7 ± 2.5 ^{d,e}	252 ± 10.6 ^{a}	48.3 ± 6.5 ^{d}	253.7 ± 7.8 ^{a}	24 ± 4 ^{d}	254 ± 9.2 ^{a}	47.7 ± 2.5 ^{c}	252 ± 10.6 ^{a}	254 ± 10.1 ^{a}	24.3 ± 2.5 ^{e}
	Pipercillin Naladixic acid Tetracycline	252 ± 10.6 ^{a}	254 ± 10.1 ^{a}	195 ± 4.6 ^{b}	23.3 ± 3.1 ^{b}	195 ± 4.6 ^{b}	23.7 ± 3.5 ^{b}	23.7 ± 2.5 ^{d}	254 ± 9.2 ^{a}	252 ± 10.6 ^{a}	195 ± 4.6 ^{b}
Quinolones		254 ± 13.1 ^{a}	252 ± 10.6 ^{a}	253.7 ± 7.8 ^{a}	254 ± 10.1 ^{a}	252 ± 10.6 ^{a}	254 ± 9.2 ^{a}	252 ± 10.6 ^{a}	255 ± 11.5 ^{a}	255.3 ± 5 ^{a}	252 ± 10.6 ^{a}
Tetracyclines	F-value	254 ± 13.1 ^{a}	252 ± 10.6 ^{a}	253.7 ± 7.8 ^{a}	254 ± 10.1 ^{a}	252 ± 10.6 ^{a}	254 ± 9.2 ^{a}	252 ± 10.6 ^{a}	255 ± 11.5 ^{a}	255.3 ± 5 ^{a}	252 ± 10.6 ^{a}
One way ANOVA test	p-value	483.6	61.2	784.6	418.1	786.6	374.9	645.6	67.7	80.6	1077.6
		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

F-value = value of one-way ANOVA test, p-value < 0.001 is considered highly significant. Means that do not share the same letter are significantly different (Tukey's test, p < 0.05).

Table 2. Major components of garlic oil using GC-MS analysis

RT	Area %	Compounds
2.14	1.33	1-Methylpentyl cyclopropane
3.00	0.28	Carbamic Acid
18.07	0.06	3H-Cyclodeca[B]Furan-2-ONE,4,9-Dihydroxy-6-Methyl-3,10-Dimethylene-3A
19.69	0.26	Diepoxyhexadecane
23.04	0.96	Baimuxinal
24.22	0.47	1-Heptatriacotanol
24.41	0.41	Dotriacontane
24.60	0.24	Docosatetraenoic Acid
24.70	0.23	Linolenic Acid
26.59	0.68	2,2-Dideutero Octadecanal
27.25	0.69	9-Octadecenoic Acid
27.90	0.14	17-Octadecynoic Acid
28.90	0.12	Cis-Vaccenic Acid
31.06	7.14	Palmitic Acid
32.31	0.65	1-Hexadecanol
34.04	1. 50	Oleic Acid
35.11	0.28	1-Docosanol
41.85	0.12	2-Aminoethanethiol Hydrogen Sulfate (Ester)

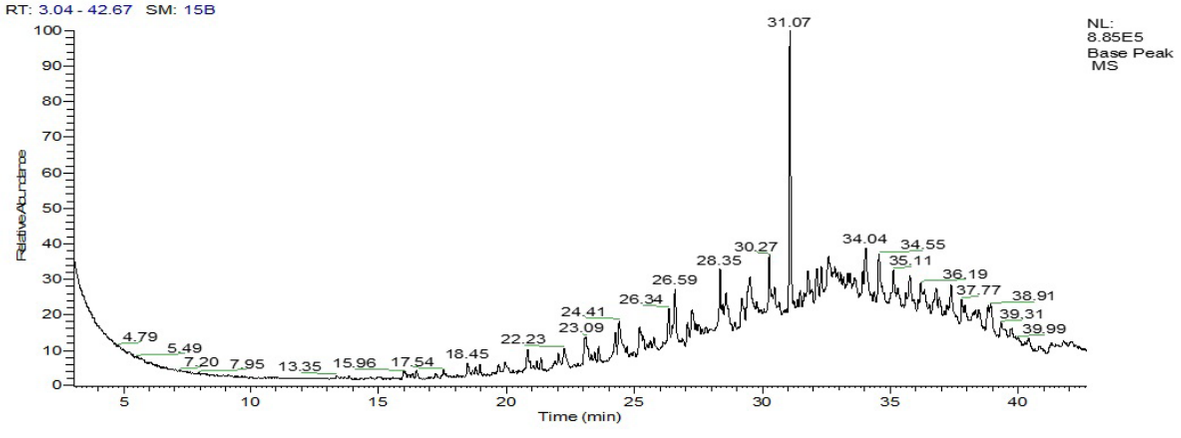

Fig. 2. Gas chromatography-mass spectrometry (GC-MS) of garlic oil

Table 3. MIC of the chitosans (crab, shrimp and squilla) and garlic oil against ten *P. mirabilis* isolates

Natural drugs	Pm1	Pm2	Pm3	Pm4	Pm5	Pm6	Pm7	Pm8	Pm9	Pm10
CS_{Cr}	47.3 ± 3.1^{b}	48 ± 3^{b}	23.7 ± 3.5^{b}	23.7 ± 2.5^{a}	23.7 ± 1.5^{b}	23.7 ± 2.5	47.7 ± 2.5^{b}	48 ± 1^{a}	47.3 ± 3.1^{b}	23.3 ± 3.1^{b}
CS_{Shr}	47.3 ± 3.1^{b}	23.7 ± 3.5^{c}	23.7 ± 2.5^{b}	23.7 ± 1.5^{a}	23.7 ± 2.5^{b}	23.3 ± 3.1	23.7 ± 2.5^{c}	23.7 ± 1.5^{b}	23.7 ± 2.5^{c}	23.7 ± 2.5^{b}
CS_{Sq}	48 ± 3^{b}	23.3 ± 3.1^{c}	23.7 ± 2.5^{b}	12.3 ± 2.5^{b}	23.7 ± 1.5^{b}	13 ± 2.7	12.3 ± 2.5^{d}	23.7 ± 3.5^{b}	23.7 ± 2.5^{c}	23.7 ± 1.5^{b}
Gar_{oil}	96.7 ± 3.1^{a}	72.3 ± 2.5^{a}	48 ± 3^{a}	11.7 ± 2.5^{b}	94.7 ± 5.1^{a}	23.7 ± 1.5	127.7 ± 2.5^{a}	47.3 ± 3.1^{a}	72.3 ± 2.5^{a}	96.3 ± 2.5^{a}
F-value	195.6	177.36	52.25	25.56	405.1	13.38	1281.7	92.2	228.78	653.0
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001

F-value = value of one-way ANOVA test, p-value < 0.001 is considered highly significant. Means that do not share the same letter are significantly different (Tukey's test, $p < 0.05$)

Table 4. Average MICs and FICI of Garlic oil-loaded nanochitosans

GO-NCS	MIC_{Avg} (µg/mL)	FICI	FIC effect
Crab	24 ± 1.2[b]	3 ± 0.15[b]	Antagonistic
Shrimp	24 ± 1.2[b]	3.06 ± 0.153[b]	Antagonistic
Squilla	12 ± 0.6[a]	2 ± 0.1[s]	Indifference

F-value = value of one-way ANOVA test, p-value < 0.001 is considered highly significant. Means that do not share the same letter are significantly different (Tukey's test, $p < 0.05$)

(166 nm), round, smooth, and uniformly distributed (Fig. 3c).

FICI of garlic oil- nanochitosan squilla - antibiotics formula. Synergistic action was observed for *Proteus mirabilis* when combined GO loaded-NCS_{Sq} with piperacillin (Pi) at maximum FICI 0.16 (Fig. 4). This was the first report that showed the combination of GO loaded-NCSSq with piperacillin (Pi) reduced the MIC value from 166.77 to 17.2 µg/mL. As a blind preliminary chemotherapeutic dose, combined GO loaded-NCSSq with piperacillin for diabetic foot *Proteus mirabilis* pathogen showed reduction of MIC value by 100%. Accordingly, suggested formulae and recommended dose were: Formula for *Proteus* sp.: GO loaded-NCSSq with piperacillin (12 + 17.2 µg/mL).

Time-kill assay. The first inoculum had an average bacterial density of 8-8.5 $\log_{10}$ CFU/mL. Control and perpercilin (Pi) isolates grew up to 8 $\log_{10}$ CFU/ mL over 24 h. Fig. 5 summarizes the time-kill curves for *Proteus mirabilis* after exposures to pipercillin (Pi) combined with natural drugs showing synergistic action of GO-NCSsq alone and with perpercilin (Pi). Highly significant difference was observed between control, and Pipercillin (Pi) alone and both GO loaded NCS_{sq} and GO loaded NCS_{sq} + Pi (p-value > 0.001). The bactericidal effect of GO-NCSsq against *Proteus*

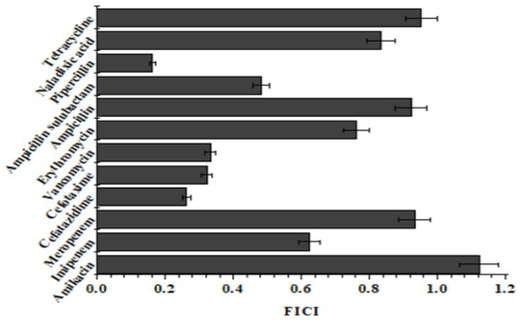

Fig. 4. Fractional inhibitory concentration index (FICI) of GO-NCSsq- antibiotics- combination against *Proteus mirabilis*

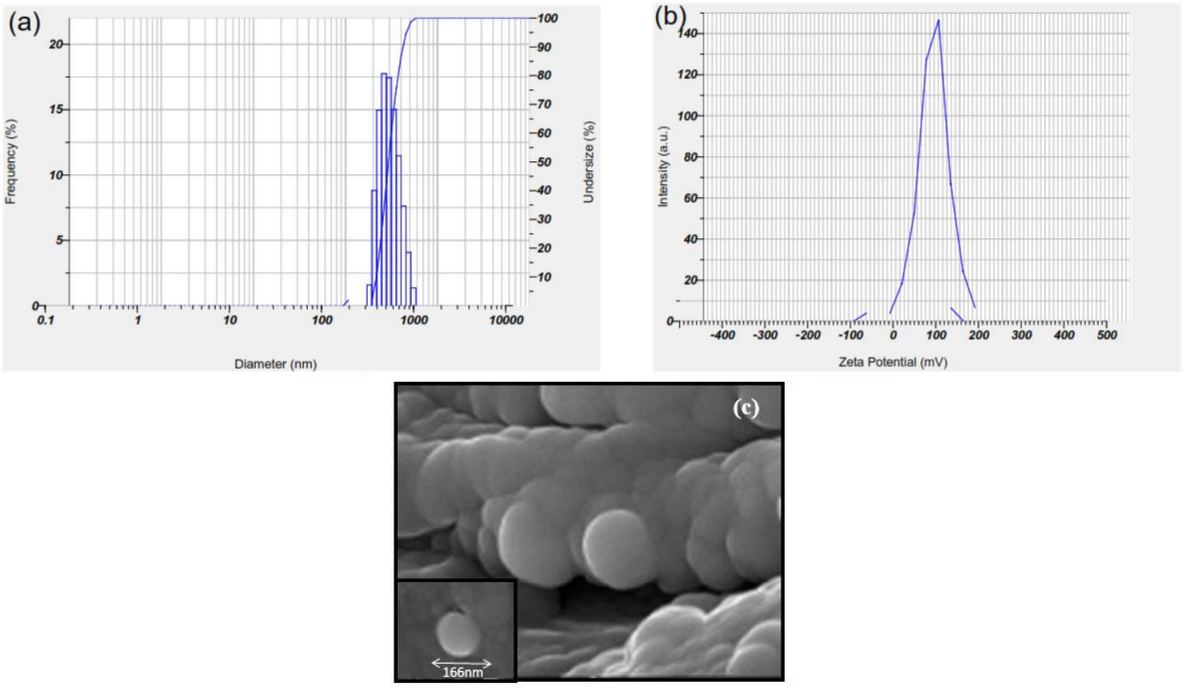

Fig. 3. Size characterization by DLS and ZP (a&b) of GO-NCSsq. Shape characterizations by SEM image of aggregation GO loaded NCSsq

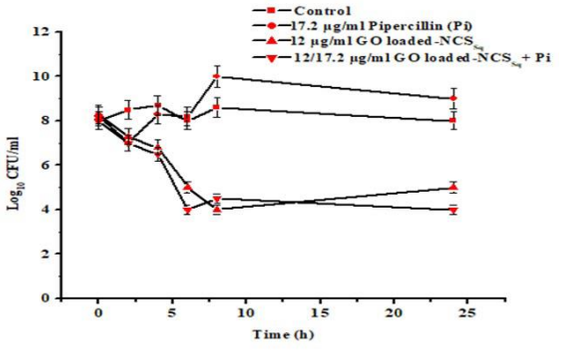

Fig. 5. Representative time-kill curve showing the averages of log_{10} CFU/ mL over 24 h for *Proteus mirabilis* clinical isolates, growth control; Piprecillin and NCSsq monotherapy; NCSsq + pipercillin (Pi) combination therapy.

mirabilis was confirmed at 12 µg/ mL concentration. When used together, the GO-NCSsq and pipercillin (Pi) killed *P. mirabilis* at a rate of 9 hours. This implies that there was a greater bactericidal impact than the combo therapies.

DISCUSSION

P. mirabilis uses peritrichous flagella to facilitate its innate translocation capabilities, which leads to a broad range of infections. *P. mirabilis* possesses a robust capacity for biofilm formation in addition to a well-rounded arsenal of exoenzymes, including hemolysins, proteases, and ureases (2). MIC test using different antibiotics against the ten isolated *P. mirabilis* showed average values from 82.7 µg/mL (for Vancomycin) to 259.7 µg/mL (for Ampicillin). Similarly, MICs sensitivity test of different antibiotics against *Proteus mirabilis* from diabetic foot diseased patients was recently studied (4).

Using different forms of chitosan, MIC values represented higher significant activities for squilla chitosan compared with the other forms that showed lower MICs. The minimum inhibitory concentrations (MIC) of high molecular weight chitosan against *Proteus mirabilis* was recorded at 31 µg/mL (15) and MIC values for chitosan against *P. vulgaris* was recorded for being active from 100 to 50 µg/mL (16). Moreover, garlic oil showed high activity against all *Proteus mirabilis* tested strains. The MIC values of garlic extract (allicin) against *P. mirabilis* isolates were found to be 128 µg/mL using the microdilution technique (17). Out of all the five essential oils, slight reduction in *P. mirabilis* MTCC 425 biofilm formation was carried out using garlic extract (18). Many reports have explored the antibacterial and antibiofilm activities of garlic extract and its potential to be used in medicinal treatment (19, 5, 20). Calculated MICs averages for all CS (crab, shrimp and squilla) were 36.1, 26.3 and 22.6 µg/mL, respectively.

Using DLS analysis, the average mean diameter of the NCSsq-GO particles was 330.8 nm, and the zeta potential was observed at +39.6 mV. The destiny of delivered nanoparticles is significantly influenced by both surface charge and particle size. Zeta-potential controls the stability of nanoparticles in the system, whereas particle size controls their distribution inside the diseased tissues. The optimal zeta potential of the nanoparticles is more than ±30 mV in order to achieve a stable nano-suspension (21). SEM investigation showed that the size of garlic oil-NCSsq was 166 nm, similarly, garlic oil-loaded Nano-chitosans were observed by Elghobashy et al. (22) with a uniform distribution, spherical shape, and smooth surface measuring 145.5 nm. Furthermore, our results aligned with the size distribution data published by Natrajan et al. (23), who stated that the NPs loaded with essential oil (EO) had a spherical form and that the mean size of the NPs was less than 300 nm following aggregation. The smooth surface of NPs indicated that the EOs was appropriately enclosed in NPs, according to Alghuthaymi et al. (24). According to our results, the average size of coated NCSsq with GO was higher than anticipated based on dynamic light scattering (DLS) analysis; this suggests that the presence of an organic layer may have contributed to the observed rise in coated NCSsq's size (25).

FICIs for these natural drug combinations (Garlic oil loaded on different nanochitosan forms) showed indifference effect using GO-NCS Squilla, which encouraged the authors to use them for further tests. Chitosans or its nanoforms incorporated with essential oil for potential applications of wound healing or antimicrobial activities have been studied by many authors (26, 27, 9).

When combined GO was loaded-NCS_{Sq} with piperacillin (Pi) at maximum, synergy was observed at FICI 0.16 for *Proteus mirabilis.* Similarly, the combination between piperacillin and metal complex-CS_{Sq}-nanosilver showed maximum FICI average of 0.28 for Gram-negative infected diabetic foot (28). Akhlaq et al. (29) showed that the FICI of ciprofloxacin and doxorubicin with carvacrol loaded on chi-

tosan nanoparticles was ≤ 0.5 against *S. aureus, E. coli* and *S. typhi* revealed the synergistic effect. A recent study investigated synergistic bactericidal combinations between gentamicin and chitosan capped ZnO nanoparticles (30).

The GO-NCSsq + pipercillin (Pi) resulted in a rate of killing at 9 hr, in combination against *Proteus mirabilis*. This suggests that the combination treatments exerted a stronger bactericidal effect. Chitosan and silver nanoparticles have been suggested separately or together as components of antimicrobial burn dressings to prevent the growth of the Gram-negative bacteria *P. mirabilis in vitro* and to cause synergistic killing as determined by a CFU assay after 30 hrs of incubation (31). Many recent reports showed the potent effect of chitosan nanoparticles for their antimicrobial activity against many pathogenic bacteria, using a time-kill test (32, 33).

CONCLUSION

Using of chitosan nanofrom with Garlic oil revealed that they have a great potential effect instead of using the antibiotics as a sole regimen against diabetic foot pathogens *Proteus mirabilis* which acts as an excellent natural antibiotic drug compared with the antibiotic itself and makes triple GO-NCSsq-pipercillin combination therapy.

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