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Article

Novel Chitosan Nanoparticles based 1,2,3-triazole as potent drugs against multi-drug resistant bacteria

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ABSTRACT: Drug resistance is a major global problem leading to higher morbidity and mortality rates especially among hospitalized patients. Hence, in the present study, 1,2,3-Triazole-acetophnone molecular conjugates **3a-c** were resynthesized and then used in nanoformulated composites (**3a-cCNPs)** using chitosan as a matrix to give nano compounds of particle sizes ranging from 20 to 40 nm. Antimicrobial activity of the synthesized compounds and the nanoformulated analogues against some multi-drug resistant microbes revealed that **3bCNPs** was the most potent candidate due to its high efficiency against multi-drug resistant (MDR) microbes that reached complete microbial cells eradication after 4 hours. The synthesized nanoparticles were fully characterized through TEM, FTIR and XRD which proved the homogeneity of the synthesized nanoformula with particle size ranged between 20 to 40 nm. The present findings discovered novel antimicrobial activities that may have therapeutic potentials. This discovery may lead to an assumption that the tested compounds can be used as candidates in biomedical applications against drug resistant microbes.

1. INTRODCTION

The most common illness in institutions and the general public is bacterial infection, which was also ranked as the global highest leading cause of death and morbidity [1]. Antibacterial drugs have revolutionized the treatment of bacterial infections, significantly lowering morbidity and death associated with infection [2]. Multi-drug-resistant gram-negative bacteria are among the list of drug-resistant bacteria for whom there are few effective drugs, therefore the design of novel antibacterial drugs is crucial [2]. Synthesized heterocyclic compounds exhibit distinctive properties and a wide range of applications in medicine, notably antimicrobials [2]. There are much more heterocyclic systems than ever before due to the steadily increasing rate of research and development in medicinal chemistry [3]. Among various nitrogen heterocyclic compounds, triazole derivatives have garnered much interest in medicinal chemistry, owing to their potential chemotherapeutical properties[4]. 1,2,3-Triazole derivatives have fascinating and unique structure allowing them to connect to a wide range of enzymes and receptors in biological systems *via* various interactions such as ion-dipole, coordination bonds, hydrogen bonds, hydrophobic effect, van der Waals force which inhibit the growth of some proteins and enhance a broad spectrum of biological activities [5], especially the antimicrobial activity [6]. As a consequence, nitrogen heterocyclic moieties have such diverse range of biological functions. Among the disclosed pharmacological activities of triazole compound [6] are antimicrobial [7], anti-inflammatory, local anesthesia [8], anticonvulsant [9], antineoplastic [10], antimalarial [11], antiviral [12], antiproliferative [13], and anticancer [14] activities.

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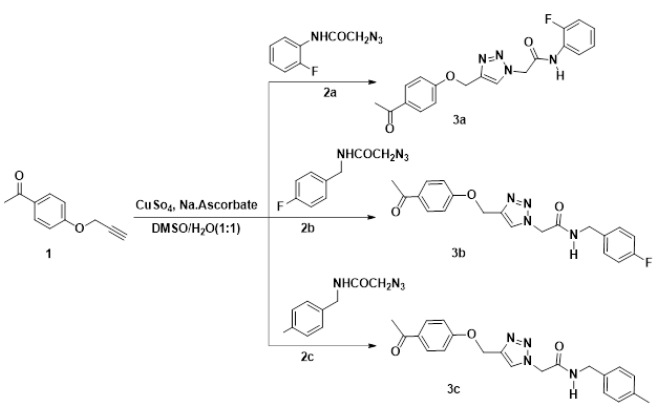
Recently, extensive studies in medicinal chemistry and drug delivery were conducted regarding the use of nanotechnology in the development of new candidates devoted with focused applications [15]. Distinctive physiochemical properties of nanomaterials including such exceptionally small size, high surface-to-mass ratio, high reactivity, and specific interactions with biological systems [16-20]. As a result, numerous drug delivery systems comprised of nanoparticles have approved for use in clinical trials to treat a variety of illnesses. Combination drug therapy requires delivering various therapeutic medicines to the same cells at the same time. Drug-loaded nanoparticles can also enter host cells via endocytosis and then release drug payloads to treat intracellular infections caused by bacteria [21]. Polymers derived from natural sources are widely used, not only in the food industry, but also in pharmaceutical area [20]. Chitosan, alginate, and carrageenan are the three most commonly used polysaccharide polymers in pharmaceutical formulations [20, 22]. Due to their distinct biochemical properties, chitosan nanoparticles (CNPs) have gained a lot of attention in the biomedical field [19]. In the context of medicine, non-toxicity, biodegradability, and biocompatibility of chitosan are some of its most remarkable characteristics [19]. The aim of the present study was to reconstruct three focused 1,2,3-triazole tethering acetophenone linkages using a click chemistry approach to involve them in nanoformulation with chitosan, producing new CNPs-based 1,2,3-triazole cores to investigate their activity against some multi-drug resistant microbes.

2. Results and Discussion

2.1. Chemical synthesis of the tested compounds

The targeted 1,2,3-triazole **3a-c** with acetophenone tethers were re-synthesized using our reported 1,3-dipolar cylcloaddition reaction [23, 24] of the *O*-propargylated acetophenone **1** and appropriate azides **3a-c** in the presence of copper sulfate and sodium ascorbate in a mixture of water and dimethyl sulfoxide as solvent (Scheme 1). The used alkynes **1** was in turn obtained *via* the propargylation of the *p*-hydroxyacetophenone in basic media aaccording to the reported procedure [23]. While the azides used in this study were prepared according to reported procedures [25-28].



Scheme 1: Click synthesis of 1,2,3-triazole-acetophenon molecular hybrids **3a-c**.

The success of the click reaction was supported by the investigation of the IR, ^{1}H-NMR and ^{13}C-NMR spectra of the resulted 1,2,3-triazoles. Thus, their IR spectra clearly the disappearance of the absorption bands belonging the alkyne group of their starting material **1** confirming their involvement on the formation of triazole rings. On the other hand, the absence of signals belong the acetylenic protons and acetylenic carbons in their ^{1}H-NMR and ^{13}C-NMR spectra, respectively, indicate in part that the cycloaddition reaction has tajen place. The diagnostic singlets resonating arround δ_H 8.20-8.25 ppm were assigned to the CH-1,2,3-triazole protons. While the two pairs of singlets observed between δ_H 5.14-5.41 ppm were attributed to the methylene protons of OCH_2 and NCH_2 linkages. Additional signals were also recorded in aromatic area in their ^{1}H-NMR and ^{13}C-NMR spectra of the phenyl rings. All remaining protons and carbons were listed in the experimental section confirming their structures.

2.2. Antimicrobial activity of the chemically synthesized compounds and nanoformulae

The chemically synthesized 1,2,3-triazole-acetophenone molecular hybrids **3a-c** were tested for their antimicrobial activity against multi-drug resistant strains of *Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Staphylococcus aureus and Candida albicans*. The largest inhibition zone was noticed with compound **3a** against *E.coli* (16 mm), while the most resistant organism was *K. pneumoniae* which was resistant to all the tested triazoles **3a-c** (Table 1**)**. On the other hand, the antimicrobial effects of the synthesized nanoformulae (CNPs-**3a-c**) were also assessed. The effect of the blank chitosan nanoparticles on bacteria was previously assessed showing no inhibition zones but improved the efficacy of the loaded drug. The results showed that the designed triazoles **3a-c** increased antimicrobial activity (Table 2). It was noticed that triazole **3b** and **3c** compounds showed no activity, while after loading on chitosan nanoparticles, the **3bCNPs** exhibited a higher activity against *E. coli and S. aureus* with inhibition zone diameter (IZ, mm) 22.5 and 29.5 mm, respectively. These promising results encouraged us to investigate further analysis for the most potent nanoformula **3bCNPs**. Microbial lethality curve was conducted to assess the optimum time needed to completely inhibit the *S. aureus* growth (**Figure 1**). Data revealed that **3b** caused complete lethality within 12 hours of incubation, while in the case of **3bCNPs**, only 4 hours were enough.

Higher antibacterial activity was recorded by the triazole loaded CNPs compared to their bulk triazoles **3a–c**. The superiority of the triazole congugated CNPs as an antimicrobial agent may be attributed to the synergistic effects of chitosan and triazole. The enhanced synergistic effect can be explained by the interaction between polycationic CNPs and the negative charge on the bacterial cell surface resulting in the disruption of the cell membrane and consequently lead to the leakage of the intracellular compounds and cell death. These results could confirm the aforementioned superiority of the formulated nanocarriers as promising antibacterial drug-delivery systems. Furthermore, the nanocarrier assembly is proposed to play a significant role to present the ingredient mixture in a nanometric size with high surface area, allowing better diffusion and intimate contact with bacteria.

Table 1. Antimicrobial activity of the synthesized 1,2,3-triazoles **3a-c**.

Tested Compounds	*Escherichia coli*		*Klebsiella pneumonia*		*Proteus mirabilis*		*Staphylococcus aureus*		*Candida albicans*	
	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)
3a	16.0	128.0	0.0	256.0	11.0	128.0	10.0	128.0	9.5	128.0
3b	0.0	256.0	0.0	256.0	0.0	256.0	0.0	256.0	0.0	256.0
3c	0.0	256.0	0.0	256.0	10	256.0	0.0	256.0	0.0	256.0

Table 2. Antimicrobial activity of the synthesized nanoparticles

Tested nanoformula	*Escherichia coli*		*Klebsiella pneumonia*		*Proteus mirabilis*		*Staphylococcus aureus*		*Candida albicans*	
	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)
CNPs	6.0	256.0	6.0	256.0	6.0	256.0	6.0	256.0	6.0	256.0
3aCNPs	17.5	128.0	16.5	128.0	8.5	256.0	19.0	128.0	25.5	64.0
3bCNPs	22.5	64.0	17.5	128.0	10.5	256.0	29.5	32.0	20.0	128.0
3cCNPs	12.5	256.0	19.0	128.0	12.5	256.0	25.5	32.0	17.5	128.0

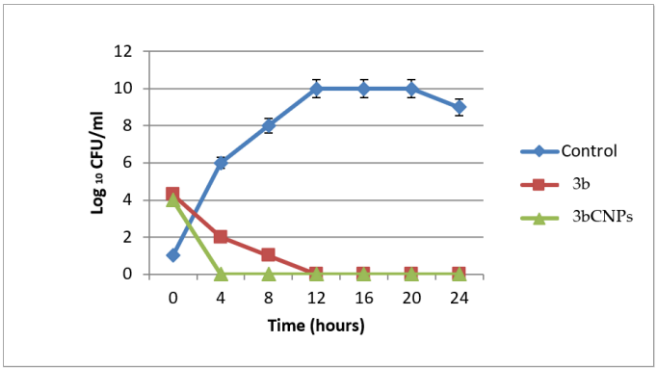


Figure 1. Microbial lethality curve of the tested nanoformula against *S.aureus*

2.3. Potent nanoparticles physico-chemical characteristics

The synthesized nanoparticles were subjected to X-ray analysis in a trial to investigate their diffraction patterns. Figure 2 showed the X-ray diffraction patterns of **3(a-c)CNPs**, which produced peaks at 2θ = 20-40° for each compound. The **3aCNPs** showed higher intensity than **3bCNPs** and **3cCNPs**, this was probably due to the presence of fluoride group in ortho position contrary to **3bCNPs** and **3cCNPs** with para substitution of fluoride and methyl substituents [20]. The FTIR spectra of the Loaded chitosan incorporating the 1,2,3-triazole **3b (CNPs-3b)** are presented in Figure 3. The absorption bands recorded at 3276.26 and 3408.19 cm^{-1} correspond to N-H stretch and O-H of chitosan matrix, respectively. While, the absorption bands observed at 1567.20 and 1679.33cm^{-1} correspond to two carbonyl groups. The decrement of their wave number is an evidance of their presence in highly polar chitosan matrix. In addition, bands from 1131.67 to 1210.72 cm^{-1} correspond to C-F stretch. The peaks observed at 1412.75 cm^{-1} were attributed to the C=C aromatic groups (Figure 3).The most potent nanoformula **(3bCNPs)** was further characterized through dynamic light scattering technique, which revealed that it has average size, PDI, zeta potential and encapsulation efficiency 23.4 nm, 0.37, +34.2 mV and 79.2%, respectively. These results proved the stability and homogeneity of the synthesized nanoparticles. In addition, transmission electron microscope study of the most potent nanoparticles of chitosan nano formula of **3b** showed the spherical shape of particles with homogenous particle size range (20-40nm) (Figure 4).

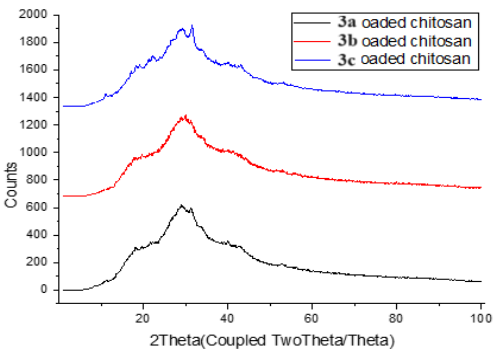


Figure 2. XRD of the investigated nanoformulae **3(a-c)CNPs.**

3. Materials and methods

3.1. Chemistry

All used chemicals and solvents were of the highest analytical reagent grade and were not further purified. The melting points of the synthesized compounds were uncorrected and were determined using Stuart Scientific SMP1. Thin layer chromatography (TLC) was carried out on UV fluorescent Silica gel Merck 60 F254 plates, and the spots were revealed with a UV lamp (254 nm) SHIMADZU.

The FTIR-Affinity-1S spectrometer was used to identify functional groups in the 400-4000 cm^{-1} area. The NMR spectra were obtained using Bruker spectrometers (500 and 400 MHz) with TMS as an internal reference.

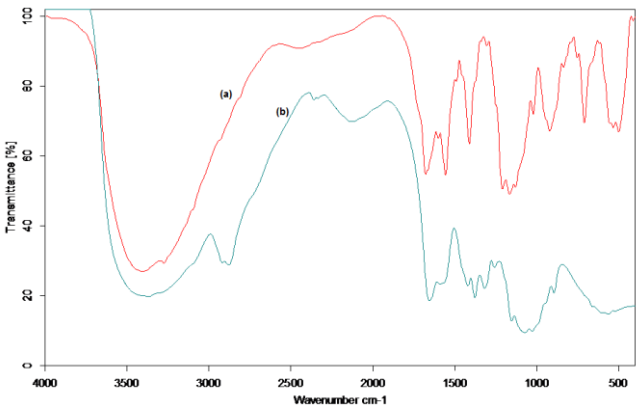


Figure 3. FTIR of compound of **3bCNPs (a)** and chitosan **(b).**

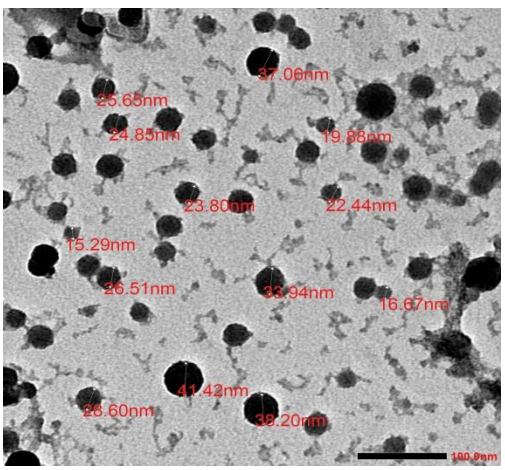


Figure 4. TEM of the most potant compound **(3bCNPs)**

Synthesis of 1-(4-(prop-2-yn-1-yloxy)phenyl)ethan-1-one 1

A mixture of 4-hydroxyacetophenone (1) (10 mmol) in dimethylformamide (10 mL) and potassium carbonate (12 mmol) was stirred for 1 hThen, propargyl bromide (1.2 mmol) was added, and the stirring was continued for 4 h at 80 °C until the consumption of the starting material as indicated by TLC (hexane-ethyl acetate). After cooling, the mixture was poured onto crushed ice water and the precipitate formed was filtered, washed with water, dried and crystallized from ethanol to give the targeted *O*-propargylatedacetophenone **2** as pale yellow powder in 91% yield, mp: 150–151 °C IR (υ, cm^{-1}): 1250 (C-O), 1600 (C=C), 1680 (C=O), 2100 (C≡C), 2900 (CH-Al), 3070 (CH-Ar), 3210 (≡CH)^{1}H NMR (400 MHz, $CDCl_3$) δ_H = 2.57 (bs, 4H, CH_3 and ≡CH), 4.77 (d, 2H, J = 4.0 Hz, OCH_2), 7.03 (d, 2H, J = 4.0 Hz, Ar-H), 7.97 (d, 2H, J = 12.0 Hz, Ar-H)^{13}C NMR (100 MHz, $CDCl_3$): δ_C= 21.70 (CH_3); 51.08 (OCH_2); 71.48 (C≡CH); 73.00 (C≡CH); 109.68, 109.80, 125.21, 125.50, 125.80, 125.95, 126.23, 156.51, 156.51 (Ar-C); 192.23 (C=O).

Synthesis of 2-(4-((4-acetylphenoxy)methyl)-1H-1,2,3-triazol-1-yl)-N-(4-substitutedbenzyl)acetamide 3(a-c)

To a solution of copper sulfate (0.10 g) and sodium ascorbate (0.15 g) in water (10 mL) was added with stirring to a solution of propargylated compound **1** (1 mmol) in DMSO (10 mL). Thereafter, the appropriate azide **2a-c** was added, and the reaction mixture was stirred at 80 °C for 6 h. The reaction was monitored *via* TLC (hexane-ethylacetate). After the completion of the reaction, the mixture was poured onto iced-water. The precipitate thus formed was collected by filtration, washed with saturated solution of ammonium chloride then water and recrystallized from ethanol/DMF to give the targeted 1,2,3-triazole hybrids **3a-c** [29].

***2-(4-((4-acetylphenoxy)methyl)-1H-1,2,3-triazol-1-yl)-N-(2-fluorophenyl)acetamide* (3a)**

Pale yellow solid was obtained in 87 % yield, mp: 179-180 ºC. IR (KBr; υ): 3300 (NH), 3060 (CH-Ar), 1705, 1670 (C=O), 1550 (C=C) cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d_6): δ_H = 10.29 1H, (s, N*H*), 8.25 (1H, s, C*H*-1,2,3-triazole), 7.91 (2H, d, J =10 Hz, Ar-*H*), 7.86-7.89 (1H, m, Ar-*H*), 7.13-7.25 5H, (m, Ar-*H*), 5.41 (2H, s, NCH_2), 5.24 (2H, s, OCH_2), 2.48 (3H, s, CH_3 overlapped with DMSO-d_6). ^{13}C NMR (125 MHz, DMSO-d_6): δ_C =196.89 (*C*=O). 115.34, 115.89, 116.40, 124.16, 124.97, 125.96, 126.08, 126.19, 126.32, 127.12, 130.84, 142.49, 162.40, 165.23 (Ar-*C*, *C*ONH); 61.74 (NCH_2); 52.96 (OCH_2); 27.16 (CH_3). ^{19}F NMR (377 MHz, DMSO-d_6): δ_F = -124.75 to -124.69 (m, 1F, Ar-*F*).

***2-(4-((4-acetylphenoxy)methyl)-1H-1,2,3-triazol-1-yl)-N-(4-fluorobenzyl)acetamide* (3b)**

Pale yellow solid was obtained in 88 % yield, mp: 165-166 ºC. IR (KBr; υ): 3290 (NH), 3070 (CH-Ar), 1695, 1670 (C=O), 1540 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ_H = 8.89 (1H, s, N*H*), 8.25 (1H, s, C*H*-1,2,3-triazole), 7.94 (2H, d, J = 8.0 Hz, Ar-*H*), 7.30-7.33 (2H, m, Ar-H), 7.15-7.16 (4H, m, Ar-*H*), 5.25 (2H, s, NCH_2), 5.19 (2H, s, OCH_2), 4.30 (2H, d, J = 8.0 Hz, NHCH_2), 2.51 (3H, s, CH_3 overlapped with DMSO-d_6). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C = 197.07 (*C*=O); 114.97, 115.46, 115.67, 127.02, 129.74, 129.80, 130.51, 130.97, 135.31, 160.48, 162.31, 162.93, 165.93 (Ar-*C*, *C*ONH); 61.71 (NCH_2); 52.05 (OCH_2); 42.35 (NHCH_2); 26.88 (CH_3). ^{19}F NMR (172 MHz, DMSO-d_6): δ_F = -118.56 to -118.48 (m, 1F, Ar-*F*).

***2-(4-((4-acetylphenoxy)methyl)-1H-1,2,3-triazol-1-yl)-N-(4-methylbenzyl)acetamide* (3c)**

Pale yellow solid was obtained in 89 % yield, mp: 180-182 ºC. (KBr; υ): 3295 (NH), 3040 (CH-Ar), 1695, 1670 (C=O), 1575 (C=C). ^{1}H NMR (500 MHz, DMSO-d_6): δ_H = 8.77 (1H, t, J = 5 Hz, N*H*), 8.20 (1H, s, C*H*-1,2,3-triazole), 7.91 (2H, d, J = 5 Hz, Ar-H), 2.23 (3H, s, CH_3), 7.09-7.13 (6H, m, Ar-*H*), 5.23 (2H, s, NCH_2), 5.14 (2H, s, OCH_2), 4.24 (2H, d, J = 5 Hz, NHCH_2), 2.43 (3H, s, COCH_3 overlapped with DMSO-d_6). ^{13}C NMR (125 MHz, DMSO-d_6): δ_C = 196.85 (*C*=O), 115.05, 126.94, 127.93, 129.43, 129.45, 130.63, 131.00, 136.17, 136.64, 142.44, 162.41, 165.82 (Ar-*C*, *C*ONH); 61.78 (NCH_2); 52.15 (OCH_2); 40.38 (NHCH_2); 26.96 (COCH_3); 21.19 (CH_3).

3.2. Microorganisms

Four multi-drug resistant strains belonging to the species *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis and Candida albicans* were kindly identified and provided by the Microbiology Department's Strain Bank, at the main University Hospital, Alexandria, Egypt.

3.3. Antibacterial activity of the prepared compounds

The antibacterial activities of the prepared compounds were evaluated against different bacterial strains Disc-diffusion method (using 25 µg/mL of the tested compounds). Further investigations of the antibacterial activity were done by determining the minimum inhibitory concentration (MIC) values and the bacterial lethality curve [1].

3.4. Synthesis of nanoformulation of 4(a-c)

0.5 g of chitosan (100–150 kDa, DDa 85%, Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in 100 mL of acetic acid solution (2% v/v) then stirred for 30 min and filtered using Whatman filter paper no1. Sodium tri-poly phosphate (TPP) solution (0.2% w/v) was prepared using deionized water then the **3a**, **3b** and **3c** solutions (30 mg) were added to the prepared TPP solution individually. Each mixture was then poured drop-wisely to the prepared chitosan solution followed by continuous stirring for 20 min. The resultant precipitate was stored at 4°C in sterile falcon tubes for further investigations.

3.5. Physicochemical characterization of the nanoformulations

3.5.1. Fourier-transform infrared spectroscopy

Perkin-Elmer R79521 (USA) Fourier-transform infrared (FTIR) was used for the detection of FTIR spectra of chitosan and the most promising nanoformula at wave number ranged from 4000 cm^{-1} to 450 cm^{-1} during 64 scans, with 2 cm^{-1} resolution [30]. The experiment was carried out at the Central Lab, Faculty of Science, Alexandria University, Egypt.

3.5.2. X-ray diffraction studies

X-ray diffraction patterns of the **3a-cCNPs** was measured using X-ray powder diffractometer (Rigaku SCXmini, USA) at scan rate = 10°/min, coupled with Ni filter and Cu Kα radiation source (λ = 0.154 nm) [31]. The experiment was carried out at the Central Lab, Faculty of Science, Alexandria University, Egypt.

3.5.3. Transmission electron microscopic examination

The ultra-structure, size and shape of the promising nanoformula were examined microscopically using transmission electron microscopic (TEM) examination (JEM-100 CX, JOEL, Japan), with resolution 3 nm at 30 kV [32].

4. Conclusion

In the present study**,** the focused 1,2,3-triazole-acetophnone molecular conjugates **3a-c** and their corresponding nanoformulated **3a-cCNPs** have been prepared and fully characterized confirming their particle size ranging from 20 to 40 nm. Their antimicrobial activity against multi-drug resistant strains showed an improvements in the term of the nano formula rather than the free 1,2,3-triazoles. Moreover, **3a** showed the highest activity for all strains and the most resistant organism was *Klebsiella pneumonia*. The nanoformulae **3bCNPs** showed the highest activity for all micro-organisms with microbial lethaltion time against *Staphylococcus aureus* of four hours instead of the twelve hours required by its free 1,2,3-triazole analogue **3b**. The results of this work uncovered compounds with considerable antimicrobial activities that may possess therapeutic potential appropriate for medical applications.

Author Contributions: Conceptualization MH, HM; B.H.E., ZO, YE, methodology, HM., B.H.E., MZ., validation, B.H.E., MH, HM, and E.A.M.; writing—original draft preparation, B.H.E., MZ, HM, and M.H.; writing—review and editing, B.H.E., MH, HM, NR, MR; visualization, B.H.E.; project administration, MJ; funding acquisition, MJ. All authors have read and agreed to the published version of the manuscript.

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