Supporting materials

Development of Carboximidamide Small Molecule Nanogels As Potent Antimicrobial Functional Drug Delivery Systems

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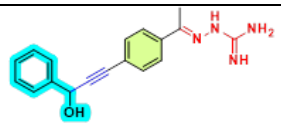
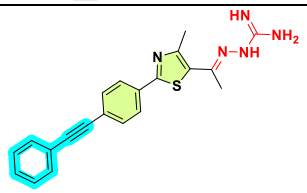
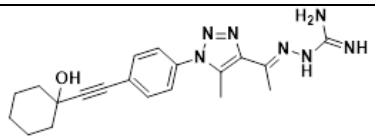
Experimental part and NMR and HPLC spectra

Table S1: Molecular compounds with corresponding molecular weights.

	Code	Structure	MWt
1	**ACE**		306
2	**TRZS**		373
3	**TRZO**		379

1.1. Chemistry of compounds

ACE: **2-(1-(4-(3-Hydroxy-3-phenylprop-1-yn-1-yl)phenyl)ethylidene)hydrazine-1-carboximidamide** (1). Light brown solid (85 mg, 69%); mp 183-185 ºC. ^{1}H NMR (DMSO-d_6); δ 7.97 (d, J = 7.6 Hz, 2H), 7.85 (d, J = 7.4 Hz, 2H), 7.81-7.33 (m, 5H), 6.24 (brs, 1H), 6.02 (brs, 2H), 5.86 (brs, 2H), 5.51 (s, 1H), 2.22 (s, 3H); ^{13}C NMR (DMSO-d_6); δ 159.9, 144.7, 135.7, 131.2, 129.1, 127,6, 127.2, 126.0, 121.9, 120.6, 92.5, 87.6, 67.5, 21.4; MS *m/z* (%): 306 (M+, 65.37); Anal. Calc. for: $C_{18}H_{18}N_4O$ (306): C, 70.57; H, 5.92; N, 18.29%; Found: C, 70.67; H, 6.07; N, 18.01%.

TRZS: 2-(1-(4-methyl-2-(4-(phenylethynyl)phenyl)thiazol-5-yl)ethylidene)hydrazine-1-carboximidamide (2)**:** Yellow solid (205 mg, 87%): mp = 190–192 °C. 1H NMR (DMSOd6) δ: 11.19 (brs, 1H), 7.98 (d, J = 8.9 Hz, 2H), 7.70 (d, J = 8.7 Hz, 2H), 7.58 (d, J = 8 Hz, 2H), 7.46 (m, 3H), 7.44 (brs, 3H), 2.63 (s, 3H), 2.43 (s, 3H). 13C NMR (DMSO-d6) δ: 163.68, 155.96, 152.42, 146.94, 132.37, 132.08, 131.32, 128.96, 128.68, 126.08, 124.06, 121.86, 121.86, 91.48, 88.73,

17.95, 17.90; HRMS (EI) m/z 373.1358 M+, calc. for C21H19N5S 373.1361; Anal. Calc. for: C21H19N5S (373): C, 67.54; H, 5.13; N, 18.75%; Found: C, 67.55; H, 5.12; N, 18.74%.

TRZO: 2-(1-(1-(4-((1-Hydroxycyclohexyl)ethynyl)phenyl)-5-methyl-1*H*-1,2,3-triazol-4-yl)ethylidene) hydrazine-1-carboximidamide . Brown solid (110 mg, 77%); mp 223-224 °C. ^{1}H NMR (DMSO-*d*$_6$); δ 8.12 (d, *J* = 8.2 Hz, 2H), 7.81 (d, *J* = 8.2 Hz, 2H), 6.22 (brs, 1H), 6.02 (brs, 4H), 2.65 (s, 3H), 2.26 (s, 3H), 2.20-1.15 (m, 10H); ^{13}C NMR (DMSO-*d*$_6$); δ 159.81, 155.76, 152.52, 149.00, 137.20, 136.12, 132.23, 126.93, 117.66, 93.05, 87.29, 58.38, 37.26, 29.23, 25.19, 21.47, 17.68, 16.25; MS *m/z* (%): 379 (M+, 79.67); purity *via* HPLC = 99.01%; Anal. Calc. for: $C_{20}H_{25}N_7O$ (379): C, 63.30; H, 6.64; N, 25.84%; Found: C, 63.41; H, 6.72; N, 25.77%.

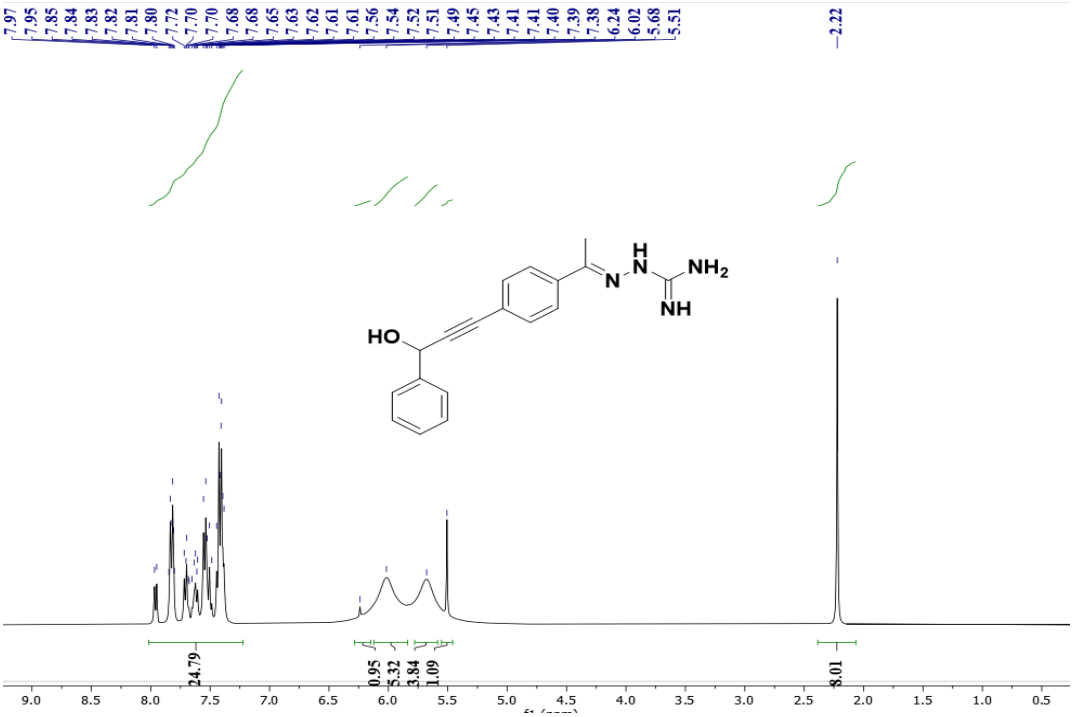


Figure S1: HNMR data of compound ACE

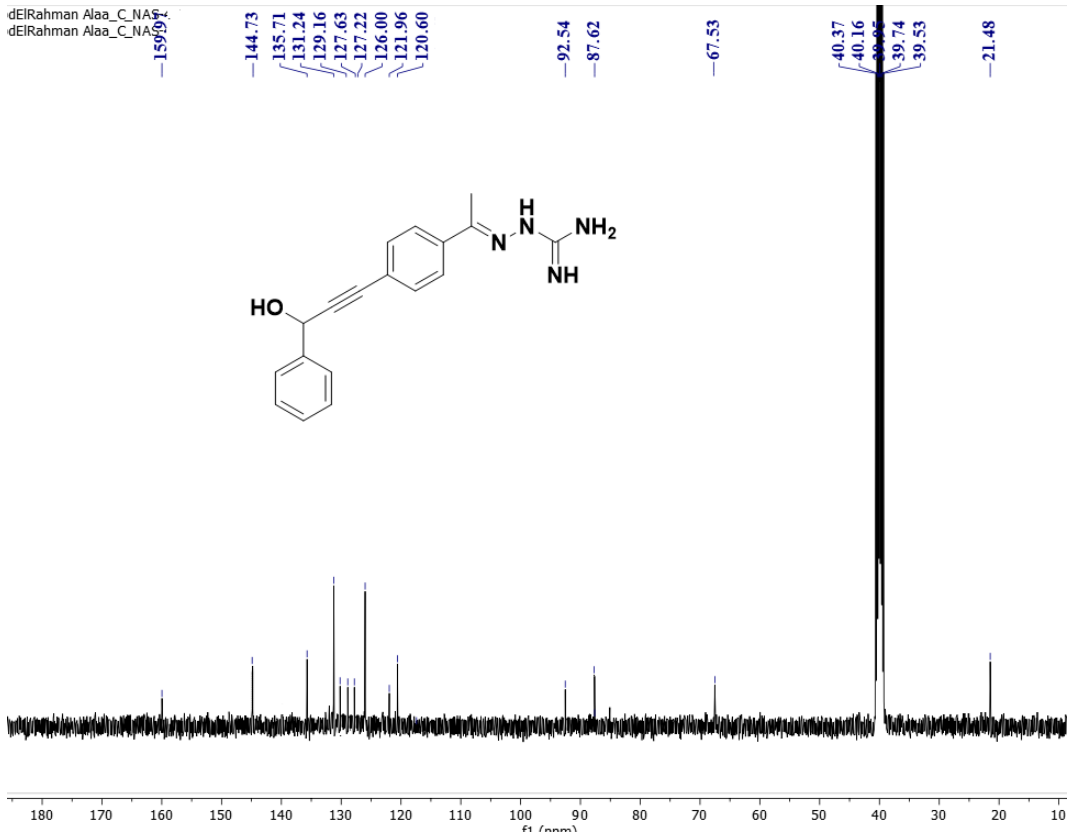


Figure S2: CNMR data of compound ACE

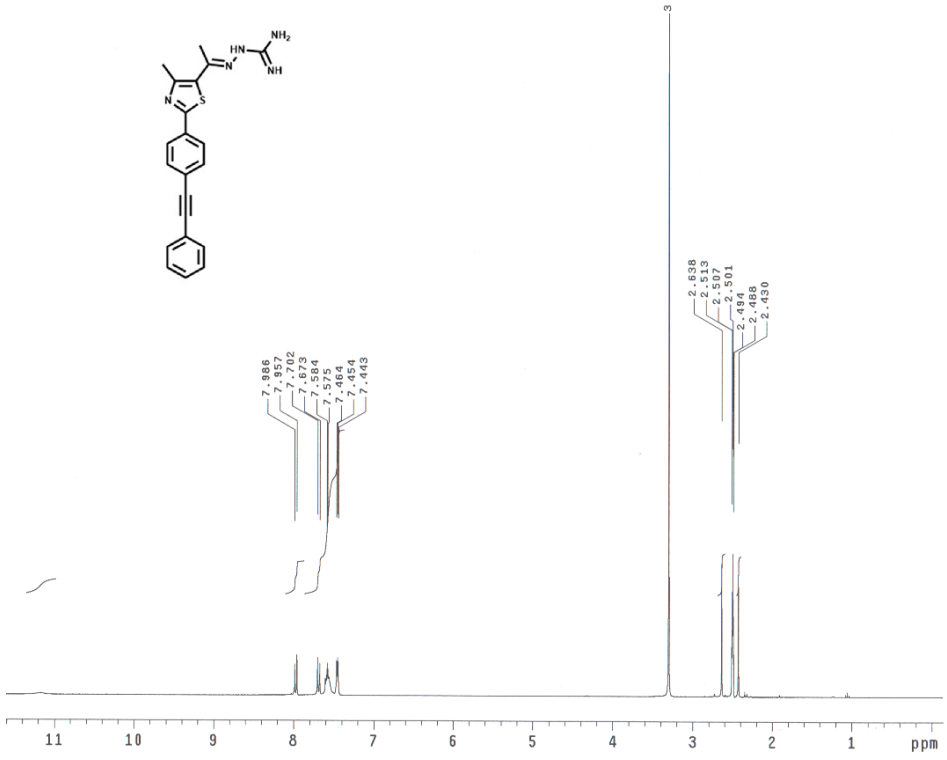


Figure S3: HNMR data of compound TRZS

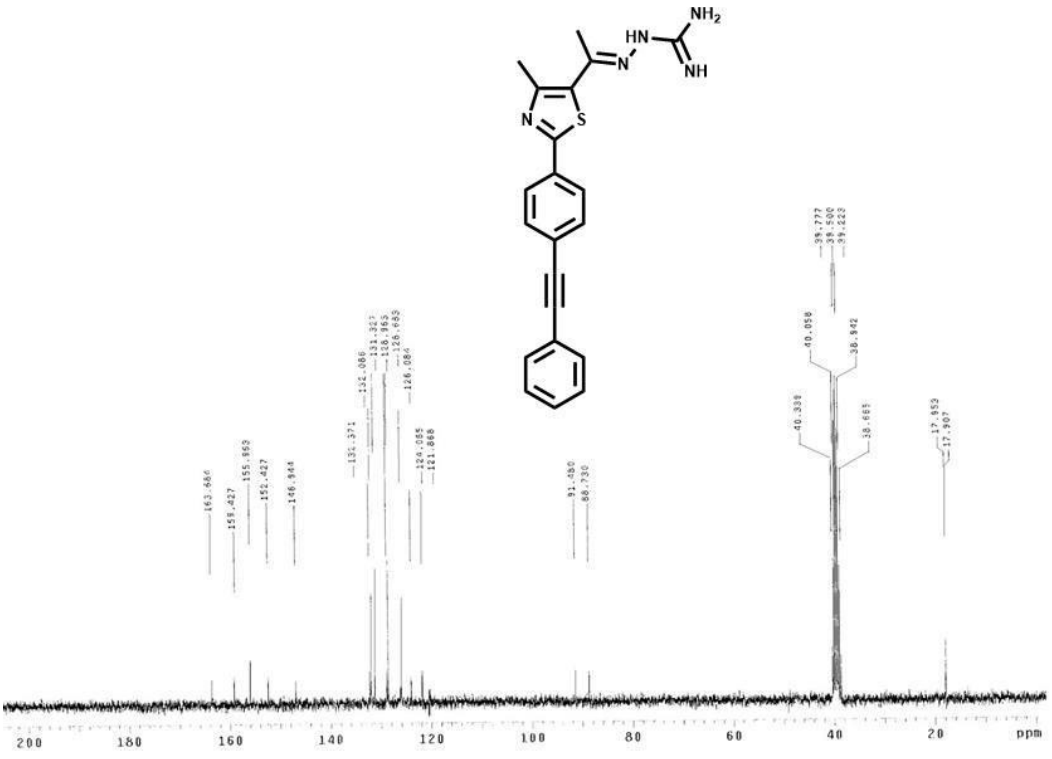


Figure S4: CNMR data of compound TRZS

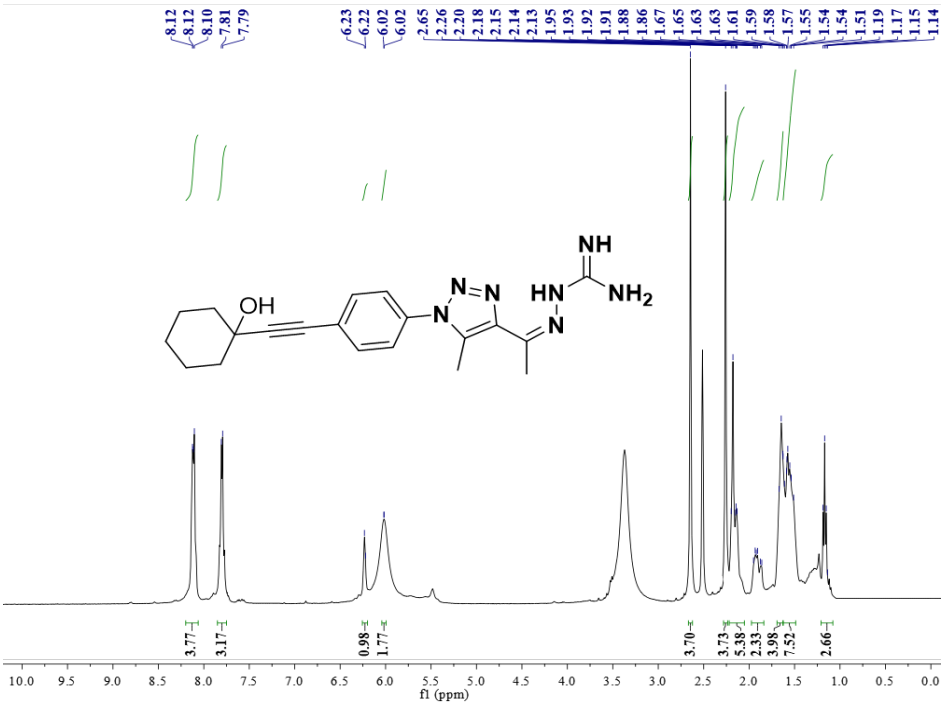


Figure S5: HNMR data of compound TRZO

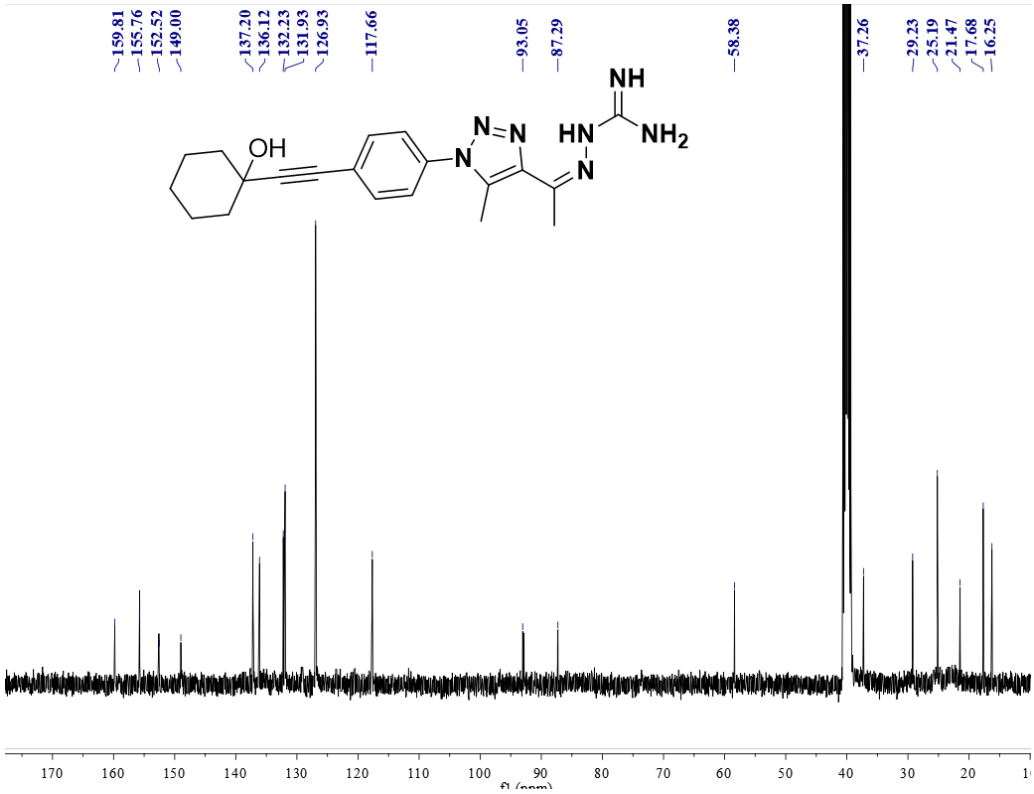
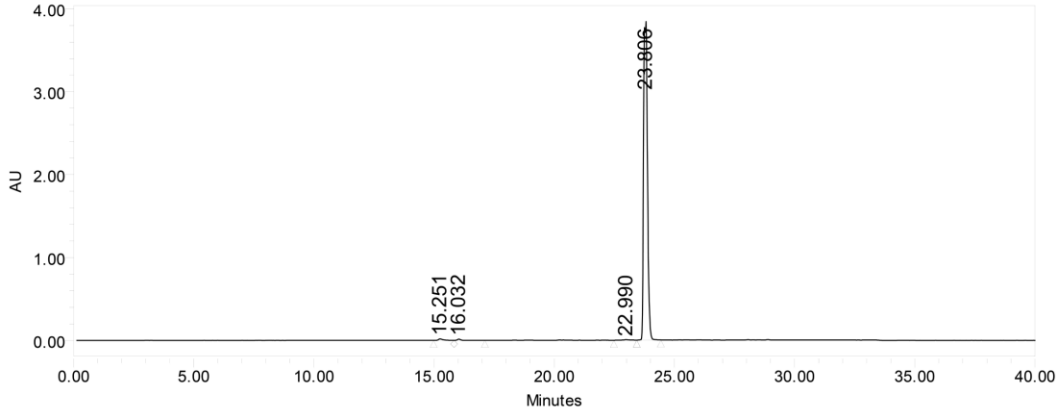


Figure S6: CNMR data of compound TRZO

SAMPLE INFORMATION

Sample Name:	SN-3	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	dsrtrr
Vial:	41	Acq. Method Set:	Trial 1 gradient
Injection #:	1	Processing Method:	Default
Injection Volume:	10.00 ul	Channel Name:	380.0nm
Run Time:	40.0 Minutes	Proc. Chnl. Descr.:	W2996 PDA 380.0 nm (PDA 210.0 tc
Date Acquired:	9/23/2021 9:10:13 PM EEST		
Date Processed:	9/25/2021 10:21:44 AM EEST		



	RT	Area	% Area	Height
1	15.251	212107	0.49	17149
2	16.032	135351	0.31	14260
3	22.990	84749	0.19	5648
4	23.806	43255800	99.01	3841452

Figure S7: HPLC data of compound ACE.

1.2. Cell Culture Protocol and MTT assay

The human diploid fibroblast (WI-38) cell line was obtained from the American Type Culture Collection. The cultured cells were grown using DMEM (Invitrogen/Life Technologies) in 10% fetal bovine serum (FBS), 10 µg/ml of insulin (Sigma), and 1% penicillin-streptomycin antibiotics. Other chemicals and reagents were obtained from Sigma or Invitrogen. Cell density was 1.2 – 1.8 × 103 cells/well in a volume of 100 µl complete growth medium + 100 µl of the tested compound/well in a 96-well plate for 24 hours, performing assay. After treatment of cells with the tested compounds' serial concentrations, 48 h at 37ºC is incubated. Human diploid fibroblast cell

line (WI-38) cells were plated in 96-well microplates at a density of 1 × 104 (8000- 10000) cells per well (100 µL per well). Control wells contained no drugs, and blank wells contained only growth medium for background correction. After cell attachment, the medium was removed, and cells were incubated with a serum-free medium containing 10000 µg/mL of the synthetic compounds by 1/2 serial dilutions according to these concentrations: 16, 32, and 64 μg/mL. Nanogels and free compounds were first dissolved in DMSO and then diluted in medium, therefore, the maximum concentration of DMSO in the wells did not exceed 0.5%. Cells were further incubated for 24 h. At the end of the incubation time, the medium was removed, and MTT solution was added to each well at a final concentration of 5 mg/mL, and the plates were incubated for another 4 h at 37 °C. Then, formazan crystals were solubilized in 150 µL DMSO. The optical density was measured at 570 nm with background correction at 655 nm using a (STAT-fax 2100) microplate reader (Serial 2100-4019, UK).

1.3. Inhibition zone data

The antibacterial activity of three compounds—TRZS, TRZO, and ACE—was evaluated in both nanogel (Ng) and free sample (S) forms against ten standard bacterial strains using the agar well diffusion method. The inhibition zone diameters (mm) revealed that the nanogel formulations consistently outperformed their free counterparts across most strains. ACE-Ng exhibited the highest overall activity, notably against *Bacillus subtilis* (31 mm), *Staphylococcus aureus* MRSA (25 mm), and *Escherichia coli* (20 mm). TRZS-Ng also showed potent effects, particularly against *B. subtilis* (24 mm) and *A. baumannii* (21 mm). TRZO-Ng demonstrated moderate efficacy, with its strongest inhibition observed against *E. coli* (19 mm) and *S. aureus* MRSA (18 mm). In contrast, the free forms generally showed reduced activity, with inhibition zones ranging from 6 to 16 mm. The positive control, chloramphenicol (50 µg/mL), yielded the highest inhibition zones in most cases, confirming assay validity.

The enhanced antibacterial activity of nanogel formulations compared to free samples aligns with recent findings on nanocarrier-mediated drug delivery, which improves bioavailability and penetration through bacterial membranes. ACE-Ng's superior performance, especially against Gram-positive strains like *B. subtilis* and *S. aureus* MRSA, suggests a strong interaction with peptidoglycan-rich cell walls, possibly due to optimized release kinetics and surface charge interactions.

Comparing these results with studies from the past four years, particularly those investigating 1,2,4-triazole derivatives, supports the observed efficacy. Strzelecka and Świątek (2021) emphasized the broad-spectrum antibacterial potential of triazole-based compounds, noting their effectiveness against resistant strains such as MRSA and A. baumannii (3) Similarly, Jacob et al. demonstrated that triazole derivatives exhibited significant inhibition zones against S. epidermidis, B. subtilis, and S. aureus, consistent with the current data (4) More recently, Zhang et al. (2023) synthesized triazolo[4,3-a]pyrazine derivatives with notable activity against E. coli and S. aureus, reinforcing the relevance of nitrogen-containing heterocycles in combating multidrug-resistant pathogens (5) The nanogel-enhanced delivery of TRZS, TRZO, and ACE appears to amplify these intrinsic antibacterial properties, offering a promising route for future antimicrobial development. These findings advocate for further exploration into nanocarrier systems to potentiate the efficacy of triazole-based therapeutics, especially against WHO-priority pathogens.

Table S2: Antibacterial activities of nanogels (Ng) and free sample (S) of target compounds against different bacterial strains, by agar well diffusion assay (IZD=mm)

Bacterial strains	**TRZS 1000µg/mL**		**TRZO 1000µg/mL**		**ACE 1000µg/mL**		**Positive control**
	(Ng)	(S)	(Ng)	(S)	(Ng)	(S)	chloramphenicol 50µg/Ml (Positive control)
	Inhibition zone diameter (mm)						
E. coli ATCC 25922	17	9	19	8	20	6	24
A. baumannii ATCC 17978	21	8	14	16	22	12	22
K. pneumoniae ATCC 700603	6	6	9	6	18	6	22
P. aeruginosa ATCC 25668	21	12	12	6	21	12	23
P. mirabilis ATCC 29906	11	10	15	8	17	6	20
MRSA ATCC 43300	17	14	18	19	25	16	27
S. epidermidisa ATCC 12228	20	11	20	20	23	26	22
B. subtilis ATCC 6633	24	15	18	12	31	19	32

Micrococcus luteus ATCC 9341	21	15	19	7	20	15	27
E. feacalis ATCC 29212	13	12	10	8	20	6	22

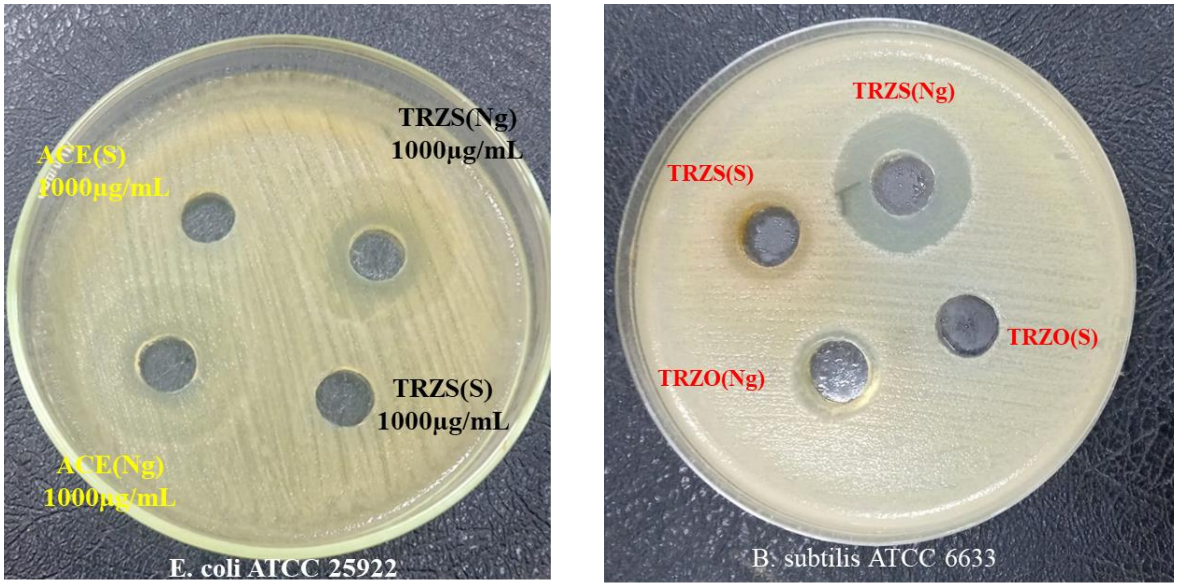


Figure S8: Antibacterial activities of nanogels (Ng) and free sample (S) of target compounds against *E. coli* ATCC 25922 (left) and *B. subtilis* ATCC 6633 (right) by agar well diffusion assay (IZD=mm).