Synthesis and structure-activity relationship of 1-[(E)-3-phenyl-2-propenyl] piperazine derivatives as suitable antibacterial agents with mild hemolysis

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\begin{keywords}
1-[(E)-3-phenyl-2-propenyl]piperazine; Bromoacetyl bromide; Amides; Biofilm inhibition; Hemolysis.
\end{keywords}

\begin{abstract}
A new series of 1-[(E)-3-phenyl-2-propenyl]piperazine derivatives (5a-m) as antibacterial agents was designed and synthesized. The synthetic strategy was initiated by coupling different anilines (1a-m) with bromoacetyl bromide (2) in an aqueous basic medium to acquire different electrophiles, 3a-m, with good yields. These electrophiles further reacted with 1-[(E)-3-phenyl-2-propenyl]piperazine (4) to yield the desired compounds, N-(substituted)-2-{1-[(E)-3-phenyl-2-propenyl]-1-azirinyl} acetamides (5a-m). The structures of these compounds were established from their IR, \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR, EIMS, and CHN analysis data. The bacterial biofilm inhibitory potential of these piperazine derivatives was tested against two pathogenic strains, \textit{Bacillus subtilus}, and \textit{Escherichia coli}. Two compounds, 5d and 5h, were identified as suitable antibacterial agents. The cytotoxicity of these molecules was profiled through hemolytic assay, and it was inferred that all the compounds were nearly harmless for membrane of red blood cells.
\end{abstract}

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1. Introduction

Pipazene is a six-membered heterocyclic ring containing two nitrogen atoms in it and is a constituent part of several bioactive molecules. The polar nitrogen atoms in a piperazine ring assign bioactivity to molecules and ensure a favorable interaction with macromolecules. Slight changes in the substitution pattern in piperazine nucleus cause a distinguishable difference in their pharmacological activities [1-3]. Piperazine derivatives are classified to have privileged structure and are frequently found in biologically active compounds across a number of different therapeutic uses such as antimicrobial, anti-

tubercular, anticonvulsant, antidepressant, anti-inflammatory, antimalarial, antirhythmic, antioxidant, and antiviral [4-6]. Piperazine derivatives have been reported as promising enzyme inhibitors, and some molecules containing this moiety have also found applications in the field of engineering and polymers [7].

The polar and stable amide functionality is the key unit amongst organic molecules and, also, in naturally occurring materials, e.g., peptides and proteins. It has a wide range of applications where it is used as intermediates or as an active pharmaceutical product or predrug [8]. The stable and polar amide functionality is an important unit among the organic
molecules of natural occurrence (e.g., peptides and proteins). It is also found in many synthetic substances of therapeutic interests [9].

Based on these considerations and further to our previous effort for the antibacterial evaluation of piperazine-acetamides [10], hereby, this study reports the bacterial biofilm inhibition of a new series of piperazine-acetamides with a rationale that these molecules might overcome the overwhelming resistance of some microbes and can find application in antibiotic therapy.

2. Results and discussion

2.1. Chemistry

The synthetic route to new 1-[(E)-3-phenyl-2-propenyl] piperazine derivatives (5a-m) is outlined in Scheme 1, and varying groups are listed in Table 1. The procedures and conditions of the reactions are discussed in the experimental section. The synthesized compounds are subjected to structural analysis using IR, EI-MS, 1H-NMR, 13C-NMR, and CHN techniques. Initially, various electrophiles, 2-bromo-N-(substituted-phenyl)acetamides (3a-m), were synthesized by the reaction of bromoacetyl bromide (2) with various anilines (1a-m) in 10% aqueous Na2CO3 solution at room temperature. The reactions were accomplished only by vigorous stirring, which resulted in the formation of desired products in excellent yields.

These electrophiles were then coupled with 1-[(E)-3-phenyl-2-propenyl]piperazine (4) to achieve a series of new N-(substituted)-2-4-[(E)-3-phenyl-2-propenyl]-1-perazinyl] acetamides (5a-m), and their structures were corroborated with spectral analysis.

One of the compounds is discussed hereby in detail for the expediency of the readers. For example, compound 5b was obtained as light grey solid with a yield of 91% and melting points of 146-148°C. Its molecular formula, C20H27N3O, was ascribed by its CHN and EI-MS analysis data. The number of protons in its 1H-NMR spectrum and carbon resonances in its 13C-NMR spectrum was in agreement with its molecular formula. The presence of different functional groups was ascertained by its IR spectral data. The absorption band at ν 3419 cm⁻¹ was characteristic of N-H stretching. The other bands were observed at ν 3057 (C-H, str. of aromatic ring), 2893 (C-H, aliphatic str.), 1604 (aromatic C=C stretching), 1654 (C=O str.), 1640 (C=C, alkene str.), and 1301 (C-N aromatic str.) cm⁻¹. In 1H-NMR spectrum (Figure S1a in Supplementary Information), its aromatic region (Figure S1b in Supplementary Information) showed the highly deshielded singlet at δ 9.37, which was assigned to –NH proton of acetamide group (–NHCO). The N-(2-methylphenyl) moiety attached to acetamido group was demonstrated by three discrete and one merged resonances in the aromatic region. These three discrete resonances were observed at δ 7.83 (br.d, J = 7.9 Hz, 1H, H-9).
1H, H-6\textsuperscript{mii}), \(\delta 7.17\) (br. t, \(J = 7.6\) Hz, 1H, H-5\textsuperscript{mii}), and \(\delta 7.04\) (br. t, \(J = 7.4\) Hz, 1H, H-4\textsuperscript{mii}), while a singlet at \(\delta 2.24\) was characteristic of a methyl substituent (CH\textsubscript{3}-2). The 4-(E)-3-phenyl-2-propenyl moiety was ascertained by two resonances in the aromatic region at \(\delta 7.44\) (br. d, \(J = 7.3\) Hz, 2H, H-2\textsuperscript{mii} & H-6\textsuperscript{mii}) and \(\delta 7.32\) (br. t, \(J = 7.5\) Hz, 2H, H-3\textsuperscript{mii} & H-5\textsuperscript{mii}) for two ortho and two meta protons of phenyl group. While the signal of \(\alpha\)m proton (H-4\textsuperscript{mii}) of this phenyl ring was merged as multiplet with the signal of meta proton (H-3\textsuperscript{mii}) of N-(2-methylphenyl) group at \(\delta 7.23-7.21\) (m, 2H). Similarly, the signal of methylene (CH\textsubscript{2a}-2) in the 2-propenyl unit was merged as multiplet with the signal of acetalactic methylene (CH\textsubscript{2}-2) at \(\delta 3.15-3.13\) (m, 4H). However, the trans disposition of two methine protons in the 2-propenyl unit was clearly indicated by larger coupling constants in their respective signals at \(\delta 6.50\) (d, \(J = 15.9\) Hz, 1H, H-3\textsuperscript{m}) and \(\delta 6.30\) (td, \(J = 6.6, 15.8\) Hz, 1H, H-2\textsuperscript{m})

The symmetric 1,4-piperazinyl ring in the molecule was represented by two signals at \(\delta 2.88\) (br. s, 4H, H-2\textsuperscript{a} & H-6\textsuperscript{a}) and \(\delta 2.73\) (br. s, 4H, H-3\textsuperscript{a} & H-5\textsuperscript{a}). The \(^{13}C\)-NMR spectrum (Figure S2 in Supplementary Information) of this molecule also fully corroborated the subsistence of these moieties. Then, the N-(2-methylphenyl) group attached to the acetalidino group was verified by typical six resonances at \(\delta 136.03\) (C-1\textsuperscript{mii}), 126.03 (C-2\textsuperscript{mii}), 127.41 (C-3\textsuperscript{mii}), 124.19 (C-4\textsuperscript{mii}), 130.21 (C-5\textsuperscript{mii}), and \(\delta 121.61\) (C-6\textsuperscript{mii}) for the phenyl ring along with a signal at \(\delta 14.58\) for the methyl substituent attached at the 2-position (CH\textsubscript{2}-2). The acetalidino group was inferred clearly by two peaks at \(\delta 167.81\) (C-1) and \(\delta 61.46\) (C-2). The phenyl ring in the 4-(E)-3-phenyl-2-propenyl unit was ascertained by four signals at \(\delta 136.56\) (C-1\textsuperscript{m}), 126.18 (C-2\textsuperscript{m} & C-6\textsuperscript{m}), 128.53 (C-3\textsuperscript{m} & C-5\textsuperscript{m}), and \(\delta 127.41\) (C-4\textsuperscript{m}), while the propenyl part was evident with three signals at \(\delta 59.96\) (C-1\textsuperscript{p}), 128.46 (C-2\textsuperscript{p}), and 126.23 (C-3\textsuperscript{p}). The symmetrical 1,4-piperazinyl heterocycle was characterized by an overlapped signal at \(\delta 52.76\) (C-2\textsuperscript{a}, C-3\textsuperscript{a}, C-5\textsuperscript{a} & C-6\textsuperscript{a}). Moreover, on account of the aforementioned evidence, the structure 5b is named as N-(2-methylphenyl)-2-{4-[{(E)-3-phenyl-2-propenyl]-1-piperazinyl}acetamide. A similar pattern was adopted for the structural characterization of other derivatives in the series.

2.2. Bacterial biofilm inhibition and structure-activity relationship

The antibacterial activity of synthetic derivatives, 5a-m, was checked by the biofilm inhibition method using two bacterial pathogenic strains, i.e., *Bacillus subtilis* and *Escherichia coli*. Some of the compounds exhibited considerable antibacterial potential (Table 2) against these strains, relative to ampicillin (*B. subtilis*: 77.49% & *E. coli*: 78.88%), a standard drug to measure the extent of antibacterial activity.

![Figure 1. General structural features of compounds 5a-m.](image)

Although the observed antibacterial potential results from the whole molecule, a limited Structure-Activity Relationship (SAR) was rationalized by analyzing the effect of different aryl parts on the bacterial biofilm inhibition. Figure 1 displays the general structural features of the synthetic compounds.

Compound 5a featuring an unsubstituted phenyl ring showed the least activity against *B. subtilis* (5.52) and poor activity against *E. coli* (31.47). The presence of a methyl group on the phenyl ring (aryl part) in 5b and 5c enhanced their antibacterial activity, relative to 5a, against both strains. However, better antibacterial potential was observed when the methyl group was present at the 3-position in 5c (*B. subtilis*: 54.35% & *E. coli*: 62.63%), as compared to that of 5b (*B. subtilis*: 14.65% & *E. coli*: 58.80%), in which it was present at the 2-position (Figure 2). It means that in compound 5c when a small-sized group was present at the m-position, it behaved as a better antibacterial agent.

A reverse trend was observed when a medium-sized ethyl group was present at ortho position of the phenyl ring in 5d. In fact, this compound is the best antibacterial agent (66.77%) among the synthetic series against *B. subtilis* and also exhibits a promising antibacterial potential against *E. coli* (63.15%). The presence of an additional methyl group at the 6-position in 5m enhanced its activity against *E. coli* (66.77%), and it behaved as the second most active compound among the synthetic derivatives. However, relative to 5d, a slight decrease in antibacterial activity was observed in 5m against *B. subtilis* (56.79%). Both para-group bearing molecules 5e (*B. subtilis*: 25.05% & *E. coli*: 7.14%) and 5f (*B. subtilis*: 13.69% & *E. coli*: 26.29%) displayed considerably weak antibacterial potential. According to the results, a medium-sized group at ortho-position of the phenyl ring was going to render a promising antibacterial potential to the molecule relative to other synthetic analogues (Figure 3).

Among the di-methylated region-isomers, two compounds, 5g and 5i, showed very moderate and much resembling antibacterials (*B. subtilis*: 43.42% & *E. coli*: 43.79%) and (*B. subtilis*: 45.75% & *E. coli*: 41.61%), respectively. The methyl groups were present at the 2- and 3-position in 5g, while they were present at the 2- and 5-position in 5i. Compound 5j with sym-
Table 2. Percentage (%) of biofilm inhibition against *Bacillus subtilis/Escherichia coli* and hemolytic activity of 1-[(E)-3-phenyl-2-propenyl]piperazine derivatives (5a-m).

| Compound | Aryl part | B. subtilis | E. coli | Hemolysis |
|----------|-----------|-------------|---------|-----------|
| 5a       | ![5a](image) | 5.52        | 31.47   | 9.26      |
| 5b       | ![5b](image) | 14.65       | 58.80   | 9.47      |
| 5c       | ![5c](image) | 54.35       | 62.63   | 15.26     |
| 5d       | ![5d](image) | 66.77       | 63.15   | 7.79      |
| 5e       | ![5e](image) | 25.05       | 7.14    | 19.68     |
| 5f       | ![5f](image) | 13.69       | 26.29   | 9.79      |
| 5g       | ![5g](image) | 43.42       | 43.79   | 6.00      |
| 5h       | ![5h](image) | 63.06       | 71.95   | 13.79     |
| 5i       | ![5i](image) | 45.75       | 41.61   | 9.79      |
| 5j       | ![5j](image) | 9.24        | 21.33   | 5.05      |
| 5k       | ![5k](image) | 39.49       | 50.93   | 7.89      |
| 5l       | ![5l](image) | 27.60       | 61.59   | 12.32     |
| 5m       | ![5m](image) | 56.79       | 66.77   | 15.58     |

Ampicillin | 77.49 | 78.88 | -
Triton-X-100 | Positive control | 89.00
PBS | Negative control | 0.54

Note: Ampicillin was used as a positive control. Negative control (% inhibition) = 1.021.
metrical di-ortho methyl groups at the 2- and 6-position possessed very weak antibacterial potential (B. subtilis: 9.24% & E. coli: 21.33%); however, the isomer 5h with methyl groups at the 2- and 4-position displayed excellent antibacterial potential. Indeed, this compound is the most active (71.95%) among the whole series against E. coli and is the second most active (63.06%) against B. subtilis. It means that when methyl groups are at ortho and para positions, these impart better antibacterial potential to the molecule (Figure 4).

The regioisomers, 5k and 5l, exhibited moderately weak antibacterial potential against B. subtilis (39.49% and 27.60%, respectively) and considerably good potential against E. coli (50.93% and 61.59%, respectively). A closer look at the comparative percentage biofilm inhibition data exposed that the former with 3,4-dimethyl groups was a better antibacterial agent against B. subtilis, while the latter with symmetrical 2,6-dimethyl groups displayed better antibacterial potential against E. coli (Figure 5).

Therefore, it was inferred from the structure-activity relationship that two molecules, one with an ortho-ethyl group (5d) and the other with ortho and para-methyl groups (5h), generally behaved as suitable antibacterial agents against both strains. The phase-contrast microscopic view of inhibition of Bacillus subtilis biofilm is given in Figure S3 (in Supplementary Information), while that of Escherichia coli biofilm is given in Figure S4 (in Supplementary Information).

### 2.3. Hemolytic activity

All the synthesized compounds were subjected to hemolytic assay to determine their cytotoxicity profile. Results of percentage hemolysis are shown in Table 2, indicating that all the compounds were nearly nontoxic for membrane of red blood cells, and their hemolysis values ranged from 5.05% to 19.68%, which were much lower than Triton-X (positive control) having %hemolysis of 89%.

### 2.4. Conclusion

In conclusion, a series of 1-[(E)-3-phenyl-2-propenyl] piperazine derivatives was synthesized successfully and evaluated for their biofilm inhibition against two
Figure 4. Structure-activity relationship of 5g, 5h, 5i, and 5j.

Figure 5. Structure-activity relationship of 5k and 5l.

pathogenic bacterial strains. SAR studies were carried out to investigate the role of various groups attached to the phenyl ring, exerting imperative influence on the antibacterial potential of these molecules. From the hemolytic activity, it was ascertained that these molecules were nearly nontoxic for membrane of red blood cells. Particularly, two molecules, one with an ortho-ethyl group (5d) and the other with ortho and para-methyl groups (5h), were explored as suitable antibacterial agents against both of the studied strains.

3. Experimental

3.1. General

All the chemicals, along with analytical grade solvents, were purchased from Sigma Aldrich, Alfa Aesar (Germany), or Merck through local suppliers. Pre-coated silica gel Al-plates were used for TLC with ethyl acetate and n-hexane as the solvent system (20:80). Spots were detected by UV254. Gallenkamp apparatus was used to detect melting points (uncorrected) in capillary tubes. IR spectra (ν, cm⁻¹) were recorded by the KBr pellet method in the Jasco-320-A spectrophotometer. Elemental analyses were performed by a Foss Hemaus CHN-O-Rapid instrument and were within ±0.4% of the theoretical values. EI-MS spectra were measured by a JEOL JMS-600H instrument with a data-processing system. ¹H-NMR spectra (δ, ppm) were recorded at 600 MHz (¹³C-NMR spectra, at 150 MHz) in DMSO-d₆ using the Bruker Advance III 600 Ascend spectrometer using BBO probe. The coupling constant (J) is given in Hz and chemical shift (δ) in ppm. The abbreviations used in the interpretation of ¹H NMR spectra are as follows: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; br, broad triplet; q, quartet; quint, quintet; sext, sextet; sep, septet; m, multiplet; dist., distorted.

3.2. Preparation of 2-bromo-N-(substituted-phenyl) acetamides (3a-m)

Equimolar amounts (0.001 moles) of various anilines (1a-m, one in each reaction) were added to the round bottom flask with distilled water at room temperature and stirred for 30 minutes. Herein, 10% aqueous Na₂CO₃ solution was added into the reaction mixture to adjust pH to 9-10. Gradually, 0.001 moles of bromoacetyl bromide (2) were added into the reaction mixture. The completion of reaction was monitored by TLC. HCl was added drop-wise to set pH to 5 until precipitates were formed. The product was filtered, washed with distilled water, and dried to obtain 2-bromo-N-(substituted-phenyl) acetamides (3a-m) as electrophiles.

3.3. General procedure for the synthesis of N-(substituted)-2-{4-[((E)-3-phenyl-2-propenyl]-1-piperazinyl}acetamides (5a-m)

The calculated amount of 1-[(E)-3-phenyl-2-propenyl] piperazine (4; 0.1 mmol) was taken in a round bottomed flask (50 mL); then, dimethyl formamide DMF
(10.0 mL) was added to dissolve it, followed by the addition of lithium hydride (0.1 mmol) to the mixture. The mixture was stirred for 30 minutes at room temperature and, then, slowly an electrophile from the aforementioned 2-bromo-N-(substituted-phenyl)
acetamides (3a-m, one in each reaction) was added to the mixture; next, the solution was further stirred for three hours. The progress of reaction was monitored via TLC till single spot. The product was precipitated by adding water. It was filtered, washed with distilled water, and crystallized from aqueous methanol.

3.4. Structural characterization

N-phenyl-2-{4-[[(E)-3-phenyl-2-propenyl]-1-perazinyl]acetamides (5a)

Light brown solid. Yield 72%, m.p. 138-139°C, C_{22}H_{25}N_{0.5}O, Mol. Mass: 335 g mol⁻¹; IR (KBr, cm⁻¹) ν: 3410 (N-H, str.), 3057 (C-H, str. of aromatic ring), 2803 (C-H, aliphatic str.), 1604 (C=C, aromatic str.), 1654 (C=O str.), 1640 (C=C, alkene str.). 1301 C-N aromatic str.). ¹H-NMR (600 MHz, DMSO-d₆, δ/ppm): 9.37 (s, 1H, -NHC)), 7.83 (br.d, J = 7.9 Hz, H-6″), 7.44 (br. d, J = 7.3 Hz, 2H, H-2‴ & H-6‴), 7.32 (br. t, J = 7.5 Hz, 2H, H-3‴ & H-5‴), 7.23-7.21 (m, 2H, H-4‴ & H-3‴), 7.17 (br. t, J = 7.6 Hz, 1H, H-5‴), 7.04 (br. t, J = 7.4 Hz, 1H, H-4‴), 6.5 (d, J = 15.9 Hz, 1H, H-3‴), 6.30 (td, J = 6.6 & 15.8 Hz, 1H, H-2‴), 3.15-3.13 (m, 4H, CH₂-2 & CH₂-1‴), 2.88 (br.s) & 2.73 (br.s, 8H, H-2′, H-3′, H-2‴ & H-3‴), 2.24 (s, 3H, 2-CH₃); ¹³C-NMR (DMSO-d₆, 150 MHz, δ/ppm): 167.81 (C-1), 136.56 (C-1‴), 136.03 (C-1‴), 130.21 (C-5‴), 128.53 (C-1‴″ & C-5‴″), 128.46 (C-2‴″), 127.41 (C-4‴″ & C-3‴″), 126.23 (C-3‴), 126.18 (C-2‴″ & C-6‴), 126.03 (C-2‴), 124.19 (C-4‴), 121.69 (C-6‴), 61.46 (C-2), 59.96 (C-1‴), 52.76 (C-2‴, C-3‴ & C-6‴), 14.58 (2-CH₃); Anal. Calc. for C_{22}H_{25}N_{0.5}O: C, 75.61; H, 7.79; N, 12.02. Found: C, 75.51; H, 7.63; N, 11.96; EI-MS: m/z [M⁺]^+, 215 [C_{14}H_{10}N_{0.5}]^+, 134 [C₆H₅NO]^+, 117 [C₆H₅]^+, 91 [C₆H₄]^⁺.

N-(3-methylphenyl)-2-{4-[(E)-3-phenyl-2-propenyl]-1-perazinyl}acetamides (5c)

Light yellow solid. Yield 88%, m.p. 143-144°C, C_{22}H_{25}N_{0.5}O, Mol. Mass: 349 g mol⁻¹; IR (KBr, cm⁻¹) ν: 3413 (N-H, str.), 3061 (C-H, str. of aromatic ring), 2899 (C-H, aliphatic str.), 1621 (C=C, aromatic str.), 1654 (C=O str.), 1647 (C=C, alkene str.), 1312 (C-N aromatic str.); ¹H-NMR (600 MHz, DMSO-d₆, δ/ppm): 8.32 (s, 1H, -NHC)); 7.46-7.40 (m, 5H, H-2‴, H-6‴, H-3‴, H-4‴, H-6‴″), 7.34 (br. t, J = 7.5 Hz, 2H, H-3‴″ & H-5‴″), 7.23 (br. t, J = 7.5 Hz, 1H, H-4‴″), 7.18 (br. t, J = 7.8 Hz, 1H, H-5‴″), 6.61 (d, J = 16.3 Hz, 1H, H-3‴), 6.32 (td, J = 6.7 & 15.9 Hz, 1H, H-2‴″), 6.36 (m, 4H, CH₂-2 & CH₂-1‴″), 2.88 (br.s) & 2.73 (br.s, 8H, H-2′, H-3′, H-2‴ & H-3‴), 2.23 (s, 3H, 3-CH₃); ¹³C-NMR (DMSO-d₆, 150MHz, δ/ppm): 167.78 (C-1), 141.62 (C-1‴), 138.09 (C-3‴), 137.39 (C-4‴), 136.24 (C-1‴″), 128.93 (C-2‴″), 128.50 (C-3‴″ & C-5‴″), 127.59 (C-1‴″), 127.19 (C-3‴″), 126.24 (C-2‴″ & C-6‴″), 124.01 (C-5‴″), 119.83 (C-6‴″), 116.30 (C-3‴″), 61.17 (C-2), 59.59 (C-1‴), 59.33 & 58.62 (2-CH₂, C-3′, C-5′ & C-6′), 20.03 (3-CH₂); Anal. Calc. for C_{22}H_{25}N_{0.5}O: C, 75.61; H, 7.79; N, 12.02. Found: C, 75.49; H, 7.61; N, 11.88; EI-MS: m/z [M⁺]^+, 215 [C_{14}H_{10}N_{0.5}]^+, 134 [C₆H₅NO]^+, 117 [C₆H₅]^+, 91 [C₆H₄]^⁺.

N-(2-ethenylphenyl)-2-{4-[(E)-3-phenyl-2-propenyl]-1-perazinyl}acetamides (5d)

Light pink solid. Yield 92%, m.p. 149-151°C, C_{22}H_{25}N_{0.5}O, Mol.Mass: 363 g mol⁻¹; IR (KBr, cm⁻¹) ν: 3436 (N-H, str.), 3032 (C-H, str. of aromatic ring), 2903 (C-H, aliphatic str.), 1627 (C=C, aromatic str.), 1654 (C=O str.), 1650 (C=C, alkene str.). 1319 (C-N aromatic str.); ¹H-NMR (600 MHz, DMSO-d₆, δ/ppm): 9.45 (s, 1H, -NHC)); 7.85 (br.d, J = 7.8 Hz, 1H, H-6‴″), 7.44 (br. d, J = 7.3 Hz, 2H, H-2‴ & H-6‴″), 7.32 (br. t, J = 7.5 Hz, 2H, H-3‴ & H-5‴″), 7.23-7.21 (m, 2H, H-4‴ & H-3‴), 7.17 (br. t, J = 7.6 Hz, 1H, H-5‴″), 7.04 (br. t, J = 7.4 Hz, 1H, H-4‴″), 6.5 (d, J = 15.9 Hz, 1H, H-3‴″), 6.30 (td, J = 6.6 & 15.8 Hz, 1H, H-2‴″), 3.15-3.13 (m, 4H, CH₂-2 & CH₂-1‴″), 2.88 (br.s) & 2.73 (br.s, 8H, H-2′, H-3′, H-2‴ & H-3‴), 2.24 (s, 3H, 2-CH₃); ¹³C-NMR (DMSO-d₆, 150 MHz, δ/ppm): 167.94 (C-1), 136.49 (C-1‴), 135.33 (C-
White solid. Yield 177%, m.p. 160-161 °C. C₇₂H₁₂N₂O₂. Mol. Mass: 363 g/mol; IR (KBr, cm⁻¹): ν: 3441 (N-H str.); 3067 (CH str. of aromatic ring); 2909 (C-H aliphatic str.); 1633 (C=C, aromatic str.); 1654 (C=O str.); 1652 (C=C, alkenic str.); 1323 (C-N aromatic str.); 4-H-DMO-d₄, 150 MHz, δ (ppm): 6.87 (s, 1H, -NICO), 7.55 (br.d, J = 8.4 Hz, 2H, H-3' & H-5') 7.44 (d, J = 7.3 Hz, 2H, H-2' & H-6''). 7.32 (br.t, J = 7.5 Hz, 2H, H-3'' & H-5''). 7.3 (br.t, J = 7.3 Hz, 1H, H-2''), 7.13 (d, J = 8.4 Hz, 2H, H-2'' & H-6''). 6.53 (d, J = 15.9 Hz, 1H, H-3''). 6.30 (td, J = 6.6 & 15.9 Hz, 1H, H-2''). 3.12 (d, J = 6.4 Hz, 2H, CH₃-1). 3.10 (s, 2H, CH₂-2). 2.88 (br.s) & 2.73 (br.s, 8H, H-2', H-3', H-5' & H-6'). 2.55 (q, J = 7.5 Hz, 2H, CH₂-CH₃-1, 1.11 (t, J = 7.5 Hz, 3H, CH₃-CH₂-2). 11.06 (C-1). 13.85 (C-4''), 136.59 (C-1''). 136.21 (C-1''). 132.11 (C-2''). 128.51 (C-3'' & C-5''). 127.79 (C-3'' & 5''). 127.36 (C-4''), 126.84 (C-3''), 126.16 (C-2'' & C-6''). 119.45 (C-2'' & C-6''). 116.72 (C-2). 60.04 (C-1'). 52.71-52.45 (C-2', C-3', C-5' & C-6'). 27.57 (CH₃-4'). 15.68 (CH₂-4'); Anal. Calc. for C₇₂H₁₂N₂O₂ (363.23): C: 79.00; H: 8.04; N: 11.56. Found: C: 78.84; H: 7.96; N: 11.33; El-MS: m/z 303 [M]+, 215 [C₁₄H₁₀N₂]+, 148 [C₆H₄NO]+, 117 [C₈H₆]+, 105 [C₆H₅]+.

Light yellow solid. Yield 60-70%, m.p. 157-159 °C. C₇₂H₁₂N₂O₂, Mol. Mass: 363 g/mol; IR (KBr, cm⁻¹): ν: 3441 (N-H str.); 3077 (C-H, str. of aromatic ring); 2945 (C-H aliphatic str.); 1643 (C-C, aromatic str.); 1654 (C=O str.); 1672 (C=C, alkenic str.); 1343 (C-N aromatic str.); 1H-NMR (600 MHz, DMSO-d₆, δ (ppm): 9.37 (s, 1H, -NICO), 7.53 (br.d, J = 7.9 Hz, 1H, H-6''), 7.44 (d, J = 7.3 Hz, 2H, H-2'' & H-6''). 7.32 (br.t, J = 7.32 Hz, 2H, H-3'' & H-5''). 7.23 (br.t, J = 7.3 Hz, 1H, H-2''), 7.05 (br.t, J = 7.8 Hz, 1H, H-5''), 6.97 (br.d, J = 7.4 Hz, 1H, H-4''). 6.54 (d, J = 15.9 Hz, 1H, H-3''). 6.30 (td, J = 6.6 & 15.8 Hz, 1H, H-2''), 3.33 (br.s, 2H, CH₂-2). 3.12 (disd, J = 6.6 Hz, 2H, CH₂-1). 2.95-2.92 (m, 8H, H-2', H-3', H-5' & H-6'). 2.25 (s, 3H, 2-CH₃). 2.10 (s, 3H, 3-CH₃); 13C-DMO-D₄, 150 MHz, δ (ppm): 167.80 (C-1). 136.60 (C-4''), 136.53 (C-1''). 135.75 (C-3''). 131.94 (C-2''). 128.45 (C-3'' & C-5''). 127.93 (C-3'' & 5''). 127.28 (C-4''), 126.95 (C-3''), 126.08 (C-2'' & C-6''). 126.00 (C-4''), 125.25 (C-6''). 120.51 (C-5''). 61.44 (C-2), 60.02 (C-1'), 52.82 & 52.71 (C-2', C-3', C-5' & C-6'). 20.10 (3-CH₃), 13.33 (2-CH₃); Anal. Calc. for C₇₂H₁₂N₂O₂ (363.23): C: 79.00; H: 8.04; N: 11.56. Found: C: 78.79; H: 7.94; N: 11.46; El-MS: m/z 303 [M]+, 215 [C₁₄H₁₀N₂]+, 148 [C₆H₄NO]+, 117 [C₈H₆]+, 105 [C₆H₅]+.
δ/ ppm): 167.60 (C-1), 136.58 (C-1′′′′), 134.49 (C-1′′′), 133.08 (C-2′′′′), 132.00 (C-2′′′), 130.68 (C-3′′′), 128.46 (C-3′′′′ & C-5′′′′), 128.31 (C-4′′′′), 127.93 (C-4′′′), 126.90 (C-3′′), 126.59 (C-5′′′′) 126.11 (C-2′′′′ & C-6′′′′), 121.69 (C-6′′′′), 61.44 (C-2), 60.05 (C-1′′′), 52.86 & 52.81 (C-2′, C-3′, C-5′ & C-6′), 20.35 (4-CH₃), 17.34 (2-CH₃);

N-(2,5-dimethylphenyl)-2-{4-[(E)-3-phenyl-2-propenyl]-1-perazinyl} acetamide (5i)
White solid, Yield 85-86%, m.p. 122-124°C, C₂₉H₂₀N₂O₂ Mol. Mass: 363 g/mol⁻¹, IR (KBr, cm⁻¹) ν: 3444 (N-H, str.), 3065 (C-H, str. of aromatic ring), 3006 (C-H, aliphatic str.), 1632 (C=C, aromatic str.), 1654 (C=O, str.), 1657 (C=C, aliphatic str.), 1321 (C-N aromatic str.); 1H-NMR (600 MHz, DMSO-d₆, δ/ppm): 9.31 (s, 1H, -NHCO), 7.65 (s, 1H, H-6′′′′), 7.44 (d, J = 7.49Hz, 2H, H-2′′′ & H-6′′′′), 7.32 (br.t, J = 7.5 Hz, 2H, H-3′′′ & H-5′′′′), 7.23 (br.t, J = 7.3 Hz, 1H, H-4′′′′), 7.08 (d, J = 7.6 Hz, 1H, H-3′′′′), 6.84 (br.d, J = 7.44 Hz, 1H, H-4′′′′), 6.54 (td, J = 15.9 Hz, 1H, H-1′′′′), 3.11 (2H, CH₂), 3.10 (s, 2H, CH₂), 2.33-2.50 (m, 8H, H-2′, H-3′, H-5′ & H-6′), 2.24 (s, 3H, 5-CH₃), 2.18 (s, 3H, 2-CH₃); 13C-NMR (DMSO-d₆, 150 MHz, δ/ppm): 167.66 (C-1), 136.54 (C-1′′′′), 135.77 (C-5′′′′′), 135.19 (C-1′′′′′), 131.94 (C-2′′′), 129.93 (C-4′′′′′), 128.45 (C-3′′ & C-5′′′), 127.28 (C-4′′′), 126.95 (C-3′′′), 126.08 (C-2′′ & C-6′′′), 125.12 (C-2′′′′), 124.70 (C-3′′′′′), 122.03 (C-6′′), 61.44 (C-2), 60.01 (C-1′′′), 52.81 & 52.79 (C-2′, C-3′, C-5′ & C-6′), 20.69 (5-CH₃), 16.94 (2-CH₃); Anal. Calc. for C₂₉H₂₀N₂O₂ (363.23): C, 79.00; H, 8.04; N, 11.56. Found: C, 78.76; H, 7.99; N, 11.51; El-MS: m/z 363 [M]+, 215 [C₁₅H₁₀N₂]+, 148 [C₁₄H₁₀NO]+, 117 [C₁₃H₉]+, 105 [C₉H₆]+.

N-(3,4-dimethylphenyl)-2-{4-[(E)-3-phenyl-2-propenyl]-1-perazinyl} acetamide (5k)
Light yellow solid, Yield 75-77%, m.p. 133-135°C, C₂₉H₂₀N₂O₂ Mol. Mass: 363 g/mol⁻¹, IR (KBr, cm⁻¹) ν: 3444 (N-H, str.), 3064 (C-H, str. of aromatic ring), 3004 (C-H, aliphatic str.), 1629 (C=C, aromatic str.), 1652 (C=O str.), 1656 (C=C, aliphatic str.), 1321 (C-N aromatic str.); 1H-NMR (600 MHz, DMSO-d₆, δ/ppm): 9.49 (s, 1H, -NHCO), 7.44 (br.d, J = 7.4 Hz, 2H, H-2′′ & H-6′′′), 7.36 (br.s, 1H, H-2′′′), 7.33 (br.s, 1H, H-3′′′), 7.03 (br.d, J = 8.0 Hz, 1H, H-5′′′′), 6.52 (d, J = 15.9 Hz, 1H, H-3′′′′), 6.30 (td, J = 6.5 & 15.7 Hz, 1H, H-2′′′′), 3.11 (br.d, J = 7.0 Hz, 2H, CH₂-1′′′′), 3.05 (s, 2H, CH₂-2′′′′), 2.58 & 2.73 (br.s, 2H, 8H, H-2′, H-3′, H-5′ & H-6′), 1.98 (s, 3H, 4-CH₃), 1.69 (s, 3H, 3-CH₃); 13C-NMR (DMSO-d₆, 150 MHz, δ/ppm): 167.84 (C-1), 136.63 (C-1′′′′), 136.21 (C-1′′′), 132.28 (C-3′′′), 131.84 (C-2′′′′), 131.12 (C-2′′′′′), 129.74 (C-3′′′′), 128.51 (C-3′′′′′ & C-5′′′′′), 127.33 (C-4′′′′′), 126.09 (C-3′′′′′′′), 126.15 (C-2′′′′′′′ & C-6′′′′′), 120.56 (C-5′′′′′), 116.85 (C-4′′′′′), 61.76 (C-2), 60.12 (C-1′′′′′), 52.81 & 52.62 (C-2′, C-3′, C-5′ & C-6′), 19.55 (CH₃-3′), 18.72 (CH₃-4′); Anal. Calc. for C₂₉H₂₀N₂O₂ (363.23): C, 79.00; H, 8.04; N, 11.56. Found: C, 78.97; H, 7.94; N, 11.42; El-MS: m/z 363 [M]+, 215 [C₁₅H₁₀N₂]+, 148 [C₁₄H₁₀NO]+, 117 [C₁₃H₉]+, 105 [C₉H₆]+.
(C-1), 138.31 (C-1"′′′), 137.62 (C-3′′′ & C-5′′′), 136.62 (C-1′′), 132.02 (C-2′′), 128.51 (C-3′′ & C-5′′), 127.34 (C-4′′), 126.97 (C-3′), 126.51 (C-2′′ & C-6′′), 124.87 (C-4′′′), 117.06 (C-2′′′ & C-6′′′) 61.73 (C-2), 60.06 (C-1′′′), 52.74 & 52.51 (C-2′, C-3′, C-5′ & C-6′), 21.00 (3-CH₃ & 5-CH₃); Anal. Calc. for C₇₀H₅₂NO₆ (363.23): C, 79.00% H, 8.04%; N, 11.56%. Found: C, 78.87%; H, 7.95%; N, 11.42. EI-MS: m/z 363 [M]+, 215 [C₁₉H₁₉N₂]+, 148 [C₉H₁₀NO⁺], 117 [C₉H₇]+, 105 [C₈H₆]+.

N-(2-ethyl-6-methylphenyl)-2-{[4-(E)-3-phenyl-2-propenyl]-1-perazinyl} acetamides (5m)

Light pink solid. Yield 92-93%, m.p. 136-138°C, C₉₂H₈₂N₂O, Mol. Mass: 377 g/mol; IR (KBr, cm⁻¹): ν 3445 (N-H, str.), 3008 (C-H, str. of aromatic ring), 2955 (C-H, aliphatic str.), 1633 (C=C, aromatic str.), 1684 (C=O str.), 1567 (C=C, alkeno str.), 1323 (C-N aromatic str.); ¹H-NMR (600 MHz, DMSO-d₆, δ/ppm): 9.28 (s, 1H, -NHCO), 7.44 (br.d, J = 7.3 Hz, 2H, H-3′ & H-6′), 7.32 (br.t, J = 7.5 Hz, 2H, H-3′′ & H-5′′), 7.23 (t, J = 7.3 Hz, 1H, H-4′′′), 7.12 (dd, J = 8.2 & 8.2 Hz, 1H, H-4′′′), 7.07 (dis.d, J = 7.5 Hz, 2H, H-3′ & H-5′′′), 6.6 (d, J = 15.9 Hz, 1H, H-3′), 6.31 (td, J = 6.6 & 15.9 Hz, 1H, H-2′), 3.15-3.13 (m, 2H, CH₂-2 & CH₂-1′′), 2.88 (br.s & 2.73 (br.s, 8H, H-2′, H-3′, H-5′ & H-6′), 2.50 (q, J = 7.5 Hz, 2H, CH₂CH₂), 2.13 (s, 3H, 6-CH₃). 1.08 (t, J = 7.5Hz, 5H, CH₃CH₂-2); ¹³C-NMR (DMSO-d₆, 150 MHz, δ/ppm): 168.23 (C-1), 140.82 (C-1″′), 136.52 (C-1″), 135.45 (C-2″′), 134.29 (C-4′′′), 132.17 (C-2″), 128.45 (C-3″′ & C-5″′), 127.84 (C-6′′′), 127.54 (C-2′), 126.59 (C-3′), 126.12 (C-2″ & C-6′′′), 125.84 (C-5′′′), 61.23 (C-2), 59.94 (C-1″′), 52.84 & 52.34 (C-2′, C-3′, C-5′ & C-6′), 24.39 (CH₂-2), 18.19 (CH₂-6), 14.52 (CH₂-2); Anal. Calc. for C₁₉H₁₉N₂O (377.25): C, 76.35; H, 8.28; N, 11.13%. Found: C, 76.27; H, 8.21; N, 11.09. EI-MS: m/z 377 [M]+, 215 [C₁₉H₁₉N₂]+, 162 [C₁₀H₁₂NO]+, 119 [C₉H₇]+, 117 [C₈H₆]+.

3.5. Assessment of bacterial biofilm inhibition

The microtiter-plate method was used for the assessment of the inhibition of bacterial (Bacillus subtilis/Esherichia coli) biofilm formation, as described in [11,12]. The 24-well flat-bottomed plastic tissue culture plates of sterile were filled with 100 µL of nutrient broth (Oxoid, UK). Concentration, which was 1.0 µg of the testing sample (dissolved in 1 mL of DMSO), was added in different wells. At last, 20 µL of the bacterial suspension containing 1 × 10⁶ CFU/mL was inoculated. The well of positive control contained ampicillin and nutrient broth (Oxoid, UK), whereas the well of negative control contained nutrient broth and microbial strain. Afterwards, plates were covered and aerobically incubated for 24 hours at 37°C. Subsequently, by applying sterile phosphate buffer (pH: 7.2) of 220 µL, the contents of each well were held thrice. Plates were vigorously shaken to remove all non-adherent bacteria. Then, the bacteria attached to plates were fixed with 220 mL of 99% methanol per well. After every 15 min, the plates were emptied and left to dry. Then, by using 220 mL of 50% crystal violet per well, the plates were stained for 5 min. Surplus stain was rinsed with distilled water. Then, plates were re-solubilized with 220 µL of 33% (v/v) glacial acetic acid per well after air-dried and the bound dye. By using a 630 nm microplate reader (Biotek, USA), the Optical Density (OD) of each well was measured. Against selected bacterial strains, all the tests were carried out thrice, and the results were averaged. The bacterial growth inhibition (inhibition%) was calculated through the following formula:

Inhibition% = 100 - (ODsample of sample × 100)

3.6. Hemolytic activity

Bovine blood samples were collected in EDTA, diluted with saline (0.9% NaCl), and centrifuged at 1000g for 10 min. The separated erythrocytes were diluted in phosphate buffer saline of pH 7.4, and a suspension was made. Then, 20 µL of the synthetic compound solution (10 mg/mL) in 180 µL of RBCs suspension was added and incubated for 30 min at room temperature. PBS was used as negative control and Triton 100-X was taken as positive control [13,14]. The %age of hemolysis was taken through the formula:

(% of hemolysis =

absorbance of sample - absorbance of negative control

absorbance of positive control

× 100).

Supplementary Information

Supplementary Information is available at: http://scientiarica.ofarshi.edu/julfe/ar_file/123581

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**Biographies**

Muhammad Athar Abbasi secured his PhD degree in 2005 from International Center for Chemical and Biological Sciences (ICCBS), HEJ, Research Institute of Chemistry, Karachi, Pakistan. He has published more than two hundred research papers in well-reputed journals. His research papers have been acknowledged and cited by various authors. He is working in collaboration with Dr. Aziz-ur-Rehman in Organic Pharmaceutical Research group.

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**Muhammad Shahid** is working as an Associate Professor at the Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. He has published several research papers in valued journals. The present study carried out bacterial biofilm inhibition and cytotoxicity in his laboratory.