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1,5-Bis (2-Hydroxyphenyl)Pent-1,4-Diene-3-One: A Lead Compound for the Development of Broad-spectrum Antibacterial Agents

Archna Rani[a,*], Sapna Jain[a], Rita Kumar[b] and Anil Kumar[b]

[a] *Department of Applied Chemistry, Delhi Technological University, Bawana Road, Delhi 110042, India.*
[b] *Environmental Biotechnology Division, Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India.*

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ABSTRACT

A systematic and comparative study has been made starting from a naturally-occurring chalcone nucleus to design effective antibacterial agents. The present investigation established 1,5-bis(2-hydroxyphenyl)pent-1,4-diene-3-one (1c) as a lead compound with potential against a panel of pathogenic bacterial strains, *Staphylococcus* (coagulase negative), *Escherichia coli, Pseudomonas aeruginosa, Acenetobacter* sp. and *Klebsiella pneumoniae.* Gentamycine and tetracycline were used as reference drugs. The mode of antibacterial action of 1c was also studied by scanning electron microscopy, which showed membrane disruption and cell lysis of the organisms during the exposure of the tested compound. *In vitro* toxicity tests demonstrated that all the bioactive compounds showed far less toxicity against human erythrocytes.

KEYWORDS

Chalcone, antibacterial agent, MIC, α,β-unsaturated carbonyl compounds.

1. Introduction

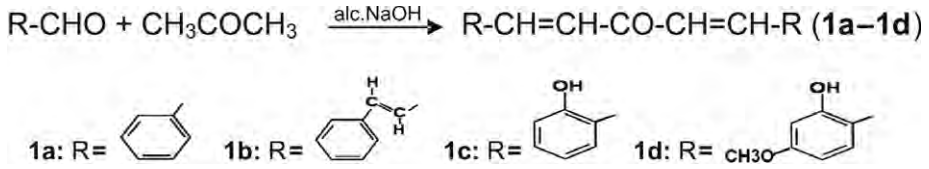
The development of untreatable resistant strains of pathogenic bacteria, because of extensive use of several types of antibiotics in clinical practice, is emerging as a serious problem before the medicinal chemists throughout the world.[1–3] Moreover, the use of a wide variety of synthetic and semi-synthetic antimicrobial chemotherapeutics is of prime concern for environmentalists, due to their hazardous synthetic pathways. To yield better pharmacologically acceptable antibacterial drugs with safer synthetic methodology, the current approach is based on findings such as: several naturally-occurring and synthetic compounds consisting of chalcone as the core nucleus possess broad-spectrum biological activity such as cytotoxicity,[4] antitrichomal,[5] antioxidant,[6–8] tyrosinase inhibitory,[9] anti-inflammatory,[9] cancer chemopreventive,[10] antimitotic,[11] analgesic,[12] antitumour,[13] antibacterial[14–17] and antifungal activity.[18] Many reports suggested that the number and position of the hydroxyl group in the chalcone nucleus showed tremendous influence on its biological activity.[19] It is also well documented that the carbonyl group in conjugation with the double bond shows a profound effect[20,21] on the activity of various pharmacologically important compounds.

Thus, a series of known and unknown analogues of chalcone, structurally modified with respect to the above mentioned parameters (Scheme **1a–1d** and Scheme **2a–2g**), has been evaluated against clinically isolated bacterial strains of Gram-positive [*Staphylococcus aureus* and *Staphylococcus* (cougulase negative)] and Gram-negative [*Escherichia coli, Pseudomonas aeruginosa, Acenetobacter* sp. and *Klebsiella pneumoniae*] by disc diffusion and microdilution methods. In addition, the mode of action of the most effective compound of the series, **1c**, was studied by scanning electron microscopy (SEM).

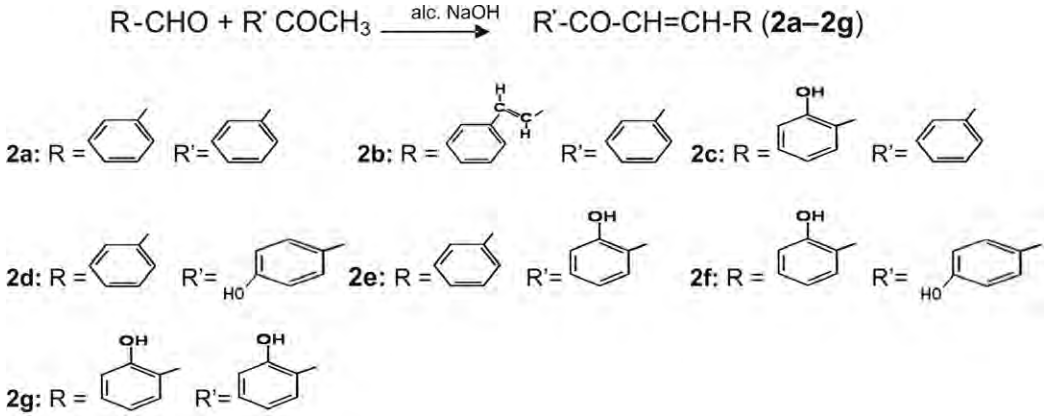
* To whom correspondence should be addressed. E-mail: archna.rani@gmail.com

2. Results and Discussion

The parent compound, chalcone (**2a**), showed moderate activity against two bacterial strains, *K. pneumoniae* (1) and *S. aureus* (MIC = 500 μg mL^{-1}) (Tables 1 and 2). Compound **1a** was slightly more active against these bacteria. Preliminary investigation for antibacterial activity of compound **2c** by the disc diffusion method showed significant reduction in bacterial growth in terms of inhibition around the disc against six test bacteria, *K. pneumoniae, Acenetobacter, S. aureus, Staphylococcus* (coagulase negative) and one strain of *E. coli* (1). The MIC was found in the range of 500 μg mL^{-1}. The biological activity data clearly revealed that compound **1c** had promising activity against all the test microbes with an MIC of 500 μg mL^{-1} against reference drug-resistant strains of *E. coli* (2), *Acenetobacter* sp., *Staphylococcus* (coagulase negative) and *P. aeruginosa* (also resistant to all the tested compounds); 250 μg mL^{-1} against *K. pneumonia* (1) (slightly sensitive to tetracycline with MIC 125 μg mL^{-1} for 50 % inhibition), *S. aureus* (sensitive to both the reference drugs with MIC 125 μg mL^{-1}) and another strain of *E. coli* (1) and 125 μg mL^{-1} against *K. pneumonia* (2) (resistant to both the reference drugs).



Scheme 1



Scheme 2

Table 1 Disc diffusion method: zone of inhibition (mm) for all strains.

Bacteria	1a	1b	1c	1d	2a	2b	2c	2d	2e	2f	2g	Gentamycine	Tetracycline
E. coli (1) [a]	–	–	8	–	–	–	7	–	–	–	–	–	–
E. coli (2) [a]	–	–	7	–	–	–	–	–	–	–	–	–	–
K. pneumoniae (1) [a]	8	–	11	–	7	–	7	–	–	–	–	–	8
K. pneumoniae (2) [a]	–	–	20	–	–	–	10	–	–	–	–	–	–
Acenetobacter	–	–	10	–	–	–	7	–	–	–	–	–	–
S. aureus	11	–	18	–	11	–	13	–	–	–	–	21	20
Staphylolcocus (coagulase negative)	–	–	8	–	–	–	6.5	–	–	–	–	–	–
P. aeruginosa	–	–	9	–	–	–	–	–	–	–	–	–	–

[a] Two different strains of same bacteria isolated from two different sources.

A series of scanning electron microscope pictures was taken of untreated cultures and also of cultures treated with compound **1c** during 30 min of incubation. Untreated organisms appeared as smooth and short rods (Figs. 1A and 1B). Bacterial cells treated with the compound were found ruptured and lysed, as is clear from Figs. 1C and 1D.

The *in vitro* cell cytotoxicity of **1c**, **2a** and **2c** was investigated using the haemolytic assay. The compounds were found to be less toxic up to the tested concentration, that is, 1000 μg mL^{-1} and lysed only 10.2 % **1c**, 6.9 % **2a** and 14 % **2c** of human erythrocytes, respectively, whereas at the same concentration, standard drugs tetracycline and gentamycine lysed 33.1 % and 28.5 % of erythrocytes, respectively (Fig. 2).

Chalcones are ubiquitous in plant cells as secondary metabolites. In order to identify the part of the molecule responsible for bioactivity, the basic nucleus of chalcone **2a** (Fig. 3), consisting of two benzene rings, A and B, was initially evaluated for antibacterial activity. Its activity was comparable with that of standard drugs (Tables 1 and 2).

To address the effect of structural differences on their respective antimicrobial activities we examined it in relation to the number and position of the double bond and/or the hydroxyl group. First we introduced an additional double bond in conjugation with benzene rings A (**1a**) and B (**2b**). Biological data clearly indicate that the introduction of a double bond in conjugation with ring B (**2b**) completely diminished the activity, however its symmetrical distribution around the carbonyl group (**1a**) showed a slightly positive influence on the activity of the chalcone nucleus against *K. pneumonia* (1) and *S. aureus*. It prompted us further to extend the double bond symmetrically around the carbonyl group (**1b**). Contrary to our expectation the compound was found to be inactive. We can say that the importance of this study lies in the optimization of the extension of the conjugation in the molecule. The structure of compound **1a**

Table 2 Microdilution method: MIC values (μg mL^{-1}) for all strains.

Bacteria	1a	1b	1c	1d	2a	2b	2c	2d	2e	2f	2g	Gentamycine	Tetracycline
E. coli (1) [a]	–	–	250	–	–	–	500	–	–	–	–	–	–
E. coli (2) [a]	–	–	500	–	–	–	–	–	–	–	–	–	–
K. pneumonieae (1) [a]	500	–	250	–	500 [b]	–	500 [b]	–	–	–	–	–	125 [b]
K. pneumonieae (2) [a]	–	–	125	–	–	–	500	–	–	–	–	–	–
Acenetobacter	–	–	500	–	–	–	500 [b]	–	–	–	–	–	–
S. aureus	500	–	250	–	500	–	500	–	–	–	–	125	125
Staphylolcocus (coagulase negative)	–	–	500	–	–	–	500 [b]	–	–	–	–	–	–
P. aeruginosa	–	–	500	–	–	–	–	–	–	–	–	–	–

[a] Two different strains of same bacteria isolated from two different sources.
[b] MIC for only 50 % inhibition.

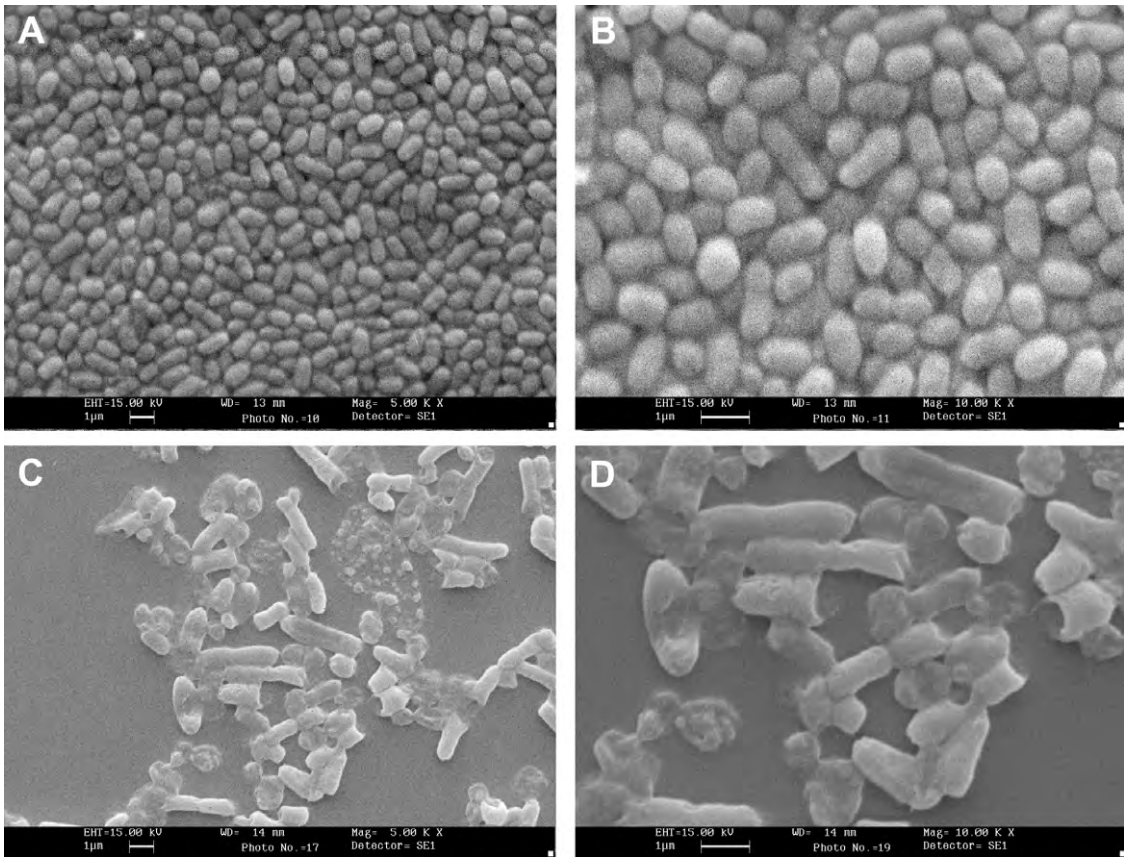


Figure 1 Scanning electron microscopy of cells from broth cultures; (**A**) untreated control culture of *E. coli* (1), × 5000; (**B**) untreated control culture of *E. coli* (1), ×10 000; (C) *E. coli* (1) treated with **1c**, 125 μg mL^{-1} 30 min, ×5000; (D) *E. coli* (1) treated with **1c**, 125 μg mL^{-1}, ×10 000, 30 min.

must be kept intact to preserve its efficacy during further structural modifications.

To ascertain the effect of the position and number of the hydroxyl group on the activity of the parent compound, **2c**, **2d** and **2e**, with hydroxylation at the 2, 2′ and 4′ positions, respectively, were screened against the bacteria. Compound **2c** was found to be more potent compared with the reference drugs, exhibiting varying broad-spectrum antibacterial activity against *K. pneumonia* (1 and 2), *Acenetobacter, S. aureus, Staphylococcus* (coagulase negative) and one strain of *E. coli* (1). This strongly suggests that hydroxylation at the 2 position somehow plays an important role in the induction of bioactivity in the parent compound. A comparative investigation of the bioactivity of compounds **2c**, **2f** and **2g** established that the presence of a hydroxyl group at the 2′ or 4′ positions completely inhibited the bioactivity of compound **2c**.

Based on the above positive findings, compound **1c,** bearing optimum extended conjugation around the carbonyl group and 2-hydroxyl groups, was studied for its antibacterial activity.

Surprisingly the compound showed tremendous activity against all the test bacteria. Encouraged by the results, its mode of action was further studied by SEM. Scanning electron microscopy of changes in the bacteria induced by **1c** revealed morphological alterations which correlated well with their mechanism of action. Knowledge of the morphological responses of bacterial species to a variety of tested compounds may allow some prediction of therapeutic potential and may also indicate organism and compound interaction.

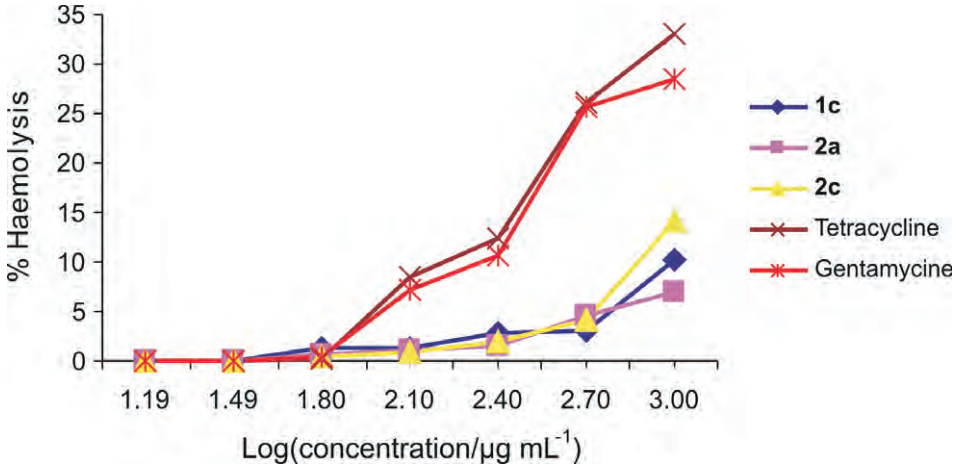


Figure 2 Haemolytic activities of tested bioactive compounds.

Table 3 Physical characterization of the compounds.

Compound	Molecular formula	IUPAC name	Melting point/°C	Yield/%	Reference
1a	$C_{17}H_{14}O$	1,5-diphenyl pent-1,4-diene-3-one	110	88	24
1b	$C_{21}H_{18}O$	1,9-diphenylnon-1,3,6,8-tetraene-5-one	96	78	24
1c	$C_{17}H_{14}O_3$	1,5-bis(-2-hydroxyphenyl)pent-1,4-diene-3-one	112	77	18
1d	$C_{19}H_{18}O_5$	1,5-bis(2-hydroxy-4-methoxyphenyl)penta-1,4-dien-3-one	116	76	
2a	$C_{15}H_{12}O$	1,3-di(phenyl)prop-2-ene-1-one	53	80	18
2b	$C_{17}H_{14}O$	1,5-di(phenyl)pent-2,4-diene -1-one	70	86	
2c	$C_{15}H_{12}O_2$	1-phenyl, 3-(2-hydroxyphenyl) prop-2-ene-1-one	115	83	18
2d	$C_{15}H_{12}O_2$	1-(4-hydroxyphenyl) 3-phenyl prop-2-ene-1-one	80	78	25
2e	$C_{15}H_{12}O_2$	1-(2-hydroxyphenyl) 3-phenylprop-2-ene-1-one	89	85	26
2f	$C_{15}H_{12}O_3$	1-(4-hydroxyphenyl), 3-(2-hydroxyphenyl) prop-2-ene-1-one	200	74	27
2g	$C_{15}H_{12}O_3$	1-(2-hydroxyphenyl), 3-(2-hydroxyphenyl) prop-2-ene-1-one	166	79	26

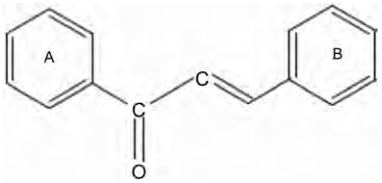


Figure 3 The basic structure of chalcone.

Furthermore a methoxy group on both the rings (**1d**) in the active compound **1c** retarded the activity, as mentioned previously by other authors[22] in their studies.

The significance of the present investigation lies in the fact that the syntheses of the basic nucleus of chalcone and all reported modifications were conducted at ambient temperature and pressure with no use of hazardous chemicals as catalysts or solvents. To the best of our knowledge such systematic and comparative studies, starting from a bioactive nucleus of natural origin to obtain potent antibacterial agents through safer synthetic routes, has never been made before. The information gained in the present approach will help us to discover new potential anti-Gram-positive and anti-Gram-negative bacterial agents with **1c** as the lead compound.

3. Experimental

All melting points were determined in open capillary tubes and are uncorrected (Table 3). IR spectra were recorded on a Perkin Elmer BX-II spectrophotometer (Singapore) using KBr pellets. The spectra showed characteristic bands at 3100–2900 cm^{-1} and 1610–1570 cm^{-1}, due to aromatic ring skeleton vibrations. The shift in wavenumber from the normal range of the C=O band of 1700 cm^{-1} to the range of 1698–1650 cm^{-1} confirmed the presence of a conjugated carbonyl group. A band in the 3100–2900 cm^{-1} range confirmed the -CH=CH- skeleton. For **1c**, **2c**, **2d**, **2e, 2f** and **2g,** bands near 778 cm^{-1} and 696 cm^{-1} signified the presence of monosubstituted benzene. A band near 830 cm^{-1} due to a disubstituted benzene was observed for **1d**. The presence of an OH group in **1c**, **1d**, **2c, 2d**, **2e, 2f** and **2g** was confirmed by a band in the range of 3455–3350 cm^{-1}. The UV spectra were recorded on a UNICAM UV-4 spectrophotometer (Cambridge, UK) using ethanol as solvent. The UV spectra showed a $n \rightarrow \pi^*$ transition (λ_{max} in the range of 300–340 nm) and a $\pi \rightarrow \pi^*$ transition (λ_{max} in the range of 220–280 nm) with an increment of wavelength indicating the presence of a conjugated carbonyl group. ^{1}H NMR ($CDCl_3$) analysis was done with a Bruker model ACP 300 spectrometer (Zurich, Switzerland) and CHN analysis was done with Element Analysensysteme GmbH VarioEL (Hanau, Germany). The ^{1}H NMR spectra of lead compound **1c** showed a peak at δ 7.2–7.6 (m, 8H, Ar), δ 7.4–7.8 (s, 4H, HC=CH) and δ 6.9–7.0 ppm (s, 2H, OH). The ^{1}H NMR spectra of other compounds showed medium intensity peaks for aromatic protons in the range of δ 7.2–7.9 ppm, strong peaks for -HC=CH- protons and -OH protons in the ranges of δ 7.5–8 and δ 6.5–7.3 ppm, respectively. The spectral data confirmed the assigned structures. The CHN results were found to be in accordance with their expected values.

All the reported structural modifications were carried out by the classical aldol condensation involving base-catalyzed condensation of the desired carbonyl compounds followed by dehydration forming α,β-unsaturated carbonyl compounds. The detailed synthetic route is described below.

3.1. 1,5-Diarylpent-1,4-diene-3-one/1,9-diarylnon-1,3,6,8-tetraene-5-one (1a–1d)

In a two-necked round-bottom flask equipped with a mechanical stirrer, a solution of the desired aldehyde (0.01 mol) was taken in the required amount of alcoholic NaOH. Acetone (0.02 mol) was added slowly from a dropping funnel with vigorous shaking. The temperature of the reaction mixture was maintained at 25 °C. Reaction was completed in 40–45 min with the formation of a yellow precipitate. The precipitate was filtered out and recrystallized from the appropriate solvent[23] (Scheme 1).

3.2. 1,3-Diarylprop-2-ene-1-one/1,5-diarylpent-2,4-diene-1-one (2a–2g)

Acetophenone or substituted acetophenone (0.01 mol) was taken in alcoholic NaOH in a bolt head flask, provided with a mechanical stirrer. The desired aldehyde (0.01 mol) was then added from a dropping funnel with stirring at 25 °C. The reaction mixture was further stirred vigorously for 2–3 h and was kept in a refrigerator overnight. The compound was filtered, washed and recrystallized from the appropriate solvent[23] (Scheme 2).

3.3. Antibacterial Activity

3.3.1. *Preliminary Screening: Disc Diffusion Method*

This is one of the most widely used methodologies for the rapid screening of antibacterial agents.[28] A loopful of culture was taken from the slants of individual bacterial strain and inoculated in 25 mL of sterile nutrient broth. Cultures were incubated at 37 °C and 120 rpm for 4–6 h until the culture exhibited exponential growth. 100 μL of actively growing culture (an OD_{650} of 1.0) was evenly spread with the help of a spreader onto the surface of a nutrient agar plate. A concentration of 20 mg mL^{-1} of each compound (**1a–1d** and **2a–2g**) and a blank (solvent in which each compound was dissolved) was taken. The discs (5 mm diameter), made by using a standard punching machine from Whatman filter paper (#3) were sterilized by autoclaving them

in a glass bottle. A solution of the compounds was added to each disc (6 μL disc^{-1}) placed on a sterile petriplate and dried for 3–5 min. The impregnated discs were placed on a pre-inoculated agar surface with sterile forceps and gently pressed down to ensure contact.

The experiment was done in triplicate. The plates were incubated at 37 °C for 16–18 h. The degree of inhibition of the test compounds was observed by the formation of zones around the spotted compounds and compared with the zone of the blank.

3.3.2. *Determination of Minimum Inhibitory Concentration: Microdilution Method*

Antimicrobial susceptibility testing was carried out using the National Committee for Clinical Laboratory Standards (NCCLS) microdilution broth assay. Briefly, the bacterial strains were grown in nutrient broth (HIMEDIA) until exponential growth was achieved. Test compounds were dissolved in DMSO/water to make a series of two-fold dilutions.

90 μL of 2–7 $\times$ 10^{-5} CFU mL^{-1} of bacterial sample per mL of nutrient broth (HIMEDIA) was dispensed into a 96-well polypropylene microtitre plate (SIGMA). Then 10 μL of serially diluted compound was added. The microtitre plates were incubated overnight at 37 °C and the absorbance was read at 630 nm. Uninoculated nutrient broth was used as a negative control. The tests were carried out in triplicate. The minimum inhibitory concentration (MIC) was taken as the lowest concentration of compound that inhibited more than 90 % growth of the microorganism.

3.4. Scanning Electron Microscopy

For the SEM study, nutrient broth (HIMEDIA) was inoculated with *E. coli* (1) and incubated at 37 °C for 24 h. A sample was then subcultured into fresh broth to obtain cultures in the logarithmic phase of growth. A specified concentration of 125 μg mL^{-1} of **1c** was added to the culture in the logarithmic phase of growth (4 h).The test organism was exposed to **1c** for 30 min at 37 °C. In this study, the control culture was manipulated in an identical manner except that it was not exposed to the compound. To prepare the specimens of *E. coli* (1) for SEM, a sample (1.0 mL) of the broth culture was washed three times with phosphate buffer (pH 6.8) by centrifuging. All specimens were treated simultaneously so that observed differences between specimens were not due to differences in preparation. These specimens were examined with a LEO 435VP, Version V3.0, Oxford Instruments INCA X-Sight scanning electron microscope (Oxford, UK).

3.5. Haemolytic Assay

The haemolytic activities of the compounds were determined using human red blood cells (hRBC).[29] The hRBC were centrifuged for 15 min to remove the buffy coat and washed three times with phosphate-buffered saline (PBS: 35 mmol L^{-1} phosphate buffer, pH 7.0/150 mmol L^{-1} NaCl). The suspended hRBC (100 μL, 4 % (v/v)) in PBS was plated into sterilized 96-well plates and then 100 μL of the compound solution was added to each well. The plates were incubated for 1 h at 37 °C and centrifuged at 1500 rpm for 5 min. Aliquots (100 μL) of the supernatant were transferred to 96-well plates, where haemoglobin release was monitored using ELISA plate reader (Benchmark Plus Microplate Spectrophotometer, Bio-Rad Laboratories, Hemel Hempstead, UK) by measuring the absorbance at 414 nm. Percentage haemolysis was calculated by the following formula:

$$\% \text{ Haemolysis} = [(Abs_{414\,nm} \text{ in the compound solution} - Abs_{414\,nm} \text{ in PBS})/(Abs_{414\,nm} \text{ in 0.1 \% Triton X-100} - Abs_{414\,nm} \text{ in PBS})] \times 100$$

Zero percentage and 100 % haemolysis were determined in PBS and 0.1% Triton X-100, respectively.

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