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Synthesis, bioassay, crystal structure and ab initio studies of Erlenmeyer azlactones

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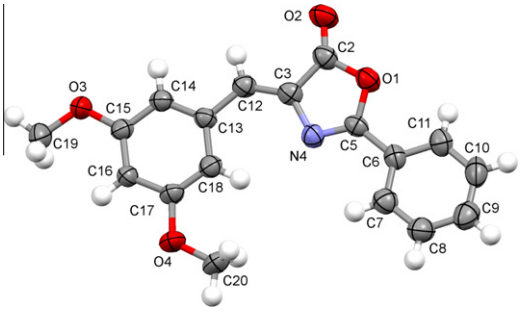
HIGHLIGHTS

- 4-Arylidene-2-phenyl-5(4H)-azlactones were synthesized and fully characterized.
- Antimicrobial and antioxidant activity.
- The structure of **5** & **6** were studied by X-ray study and compared to DFT calculations.
- DFT calculations of two compounds were suggested the stability of the Z-conformer.
- Crystal packing was stabilized by H-bond, weak C—H···π and π···π interactions were observed.

GRAPHICAL ABSTRACT

A series of 4-arylidene-2-phenyl-5(4H)-azlactones have been synthesized, characterized on the basis of systematic spectral studies and screened for their biological activity. Moreover, the *Z*-configuration and stability of compounds was ascertained on the basis of spectroscopy techniques, X-ray studies as well as DFT calculations.



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ABSTRACT

Several 4-arylidene-2-phenyl-5(4H)-azlactones have been synthesized *via* Erlenmeyer method. The synthesized compounds have been characterized on the basis of systematic spectral studies (IR, ^{1}H NMR, ^{13}C NMR, and MS). The compound (4*Z*)-4-(3,5-dimethoxybenzylidene)-2-phenyl-1,3-oxazol-5(4H)-one, $C_{18}H_{15}NO_4$, (**5**), crystallizes in the orthorhombic system, space group $P2_12_12_1$, with $a = 5.6793(3)$ Å, $b = 15.2038(7)$ Å, $c = 17.6919(10)$ Å, $Mr = 309.31$, $V = 1527.64(14)$ Å^3, $Z = 4$ and $R = 0.0547$. The compound (4*Z*)-2-phenyl-4-(3,4,5-trimethoxybenzylidene)-1,3-oxazol-5(4H)-one, $C_{19}H_{17}NO_5$, (**6**) crystallizes in triclinic geometry with space group *P-1*, having unit cell parameters $a = 7.3814(3)$ Å, $b = 8.1446(3)$ Å, $c = 13.9845(5)$ Å, $\alpha = 86.918(3)$, $\beta = 83.314(2)$, $\gamma = 82.462(3)$, $Mr = 339.34$, $V = 827.16(5)$ Å^3, $Z = 2$ and $R = 0.0433$. The DFT calculations of compounds (**5**) and (**6**) have been carried out to ascertain the stability of *Z*-conformer. The *in vitro* antimicrobial activity of all the compounds (**1–6**) was evaluated by the disk diffusion method against gram +ve and gram −ve microorganism and fungal strains. The MIC of the synthesized compounds was determined by agar well diffusion method in 96-well microtiter plate. All the synthesized compounds were also screened for their free radical scavenging activity by DPPH method.

Introduction

During the past few decades many research papers have been published in the area of Erlenmeyer synthesis by using different methods [1–5]. The synthesis of azlactones involves the condensation of aromatic or aliphatic aldehydes and hippuric acid with a

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stoichiometric amount of fused sodium acetate in presence of acetic anhydride as the dehydrating agent, the reaction is called Erlenmeyer Plöchl reaction [6]. The Erlenmeyer reaction was first described in 1893 by Friedrich Gustav Carl Emil Erlenmeyer [7] who reported the condensation of benzaldehyde with N-acetylglycine in the presence of acetic anhydride and sodium acetate. The Erlenmeyer azlactones are five membered heterocyclic compounds containing nitrogen and oxygen as hetero atoms. The C–2 and C–4 positions of the azlactones are significant for their various biological activities [8].

Azlactones, or 2,4-substituted oxazolin-5-ones, are important intermediates in the preparation of several fine chemicals, including amino acids, [9] peptides, [10] some heterocyclic precursors [11] as well as biosensors or coupling and photosensitive devices for proteins [12]. Erlenmeyer azlactone derivatives possess important biological activities such as an antimicrobial [13], antitumor [14], anti-inflammatory [15], anti-HIV [16,17], anticonvulsant [18] and antihypertensive [19]. They have been used in active site titrations of enzymes [20]. Recently, some new reagents have been explored for the synthesis of azlactones, such as Al_2O_3—H_3BPO_3 [21], $Bi(OAc)_3$ [22], $Bi(OTF)_3$ [23], and $Yb(OTF)_3$ [24]. Although these method are suitable, but some of them need elevated temperatures and hence possess difficult in handling.

In this work, we report the synthesis and the crystal structures of compounds (4Z)-4-(3,5-dimethoxybenzylidene)-2-phenyl-1,3-oxazol-5(4H)-one, $C_{18}H_{15}NO_4$, (**5**), and (4Z)-2-phenyl-4-(3,4,5-trimethoxybenzylidene)-1,3-oxazol-5(4H)-one, $C_{19}H_{17}NO_5$ (**6**), as determined by single-crystal X-ray analysis. To investigate the effect of the intermolecular interactions in the conformation of the molecules we have also performed the optimization of the geometries of the compounds using density functional theory (DFT) calculations. Moreover, the compounds (**1–6**) have also been screened for the antimicrobial and antioxidant properties.

Experimental

Physical measurements

All the solvents and chemical were purchased from commercial sources (Sigma–Aldrich, Merck) and others and used as received or dried using standard procedures. Melting points were determined on a Kofler apparatus and uncorrected. Elemental analysis (C, H, N) were conducted using Carlo Erba analyzer model 1108. The IR spectra were recorded on KBr pellets with Interspec 2020 (FT-IR) spectrometer, Spactro Lab UK and its values are given in cm^{-1}. The UV spectra were recorded with UV VIS-1800 spectrophotometer (Shimadzu). ^1H and ^{13}C NMR spectra were run in $CDCl_3$ on a Bruker Avance-II 400 MHz and 100 MHz instrument respectively. TMS was used as an internal standard; *J* values are given in Hertz. Mass spectra were recorded on a JEOL D-300 mass spectrometer. Thin layer chromatography (TLC) glass plates (20 × 5) were coated with silica gel (E-Merck G_{254}, 0.5 mm thickness) and exposed to iodine vapors to check the purity as well as the progress of the reaction.

*General method for the preparation of (4Z)-2-phenyloxazol-5(4H)-ones (**1–6**)*

An equimolar mixture of hippuric acid and suitable aldehyde (15 mmol) in freshly distilled acetic anhydride (10 mL) containing fused anhydrous sodium acetate (1.2 g) was heated on an oil bath at 140–150 °C for 2 h and then cooled. Progress of the reaction was monitored by TLC. After completion, the compounds were filtered, washed with light petroleum ether (60–80 °C) and air-dried. They were triturated with cold saturated solution of sodium carbonate and filtered, washed with water, air dried and recrystallized from suitable solvent to yield the representative compounds.

*(4Z)-4-(2-methoxybenzylidene)-2-phenyloxazol-5(4H)-one (**1**)*

It was recrystallized from $CHCl_3$—EtOH as bright yellow solid; Yield: 80%, m.p. 154–55 °C (lit. m.p. 154 °C) [2]; Anal. Calc. for $C_{17}H_{13}NO_3$: C, 73.11; H, 4.69; N, 5.02. Found: C, 72.98; H, 4.64; N, 4.98. IR ν_{max}^{KBr} cm^{-1}: 1788 (C=O), 1669 (C=N), 1653 (C=C), 1248 (C—O Lactone); ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 3.78 (s, 3H, CH_3), 6.98 (d, 1H, *J* = 8.2, H-6″), 7.18–7.28 (m, 2H, H-4″,5″), 7.35 (s, 1H, =CH=), 7.40–7.46 (d, 1H, *J* = 8.4, H-3″), 7.48–7.52 (m, 2H, H-3′,5′), 8.12–8.14 (m, 2H, H-2′,6′), 8.70 (dd, 1H, *J* = 7.4 H-4′); 13C NMR (100 MHz, $CDCl_3$, δ, ppm): 55.8 (CH_3), 113.6 (C3″), 121.3 (C5″), 127.9 (C3′&5′), 128.7 (C2′&6′), 129.0 (C6″), 131.1 (C1″), 132.2 (C4′), 133.8 (C1′), 135.8 (CH=C), 144.0 (C4), 161.1 (C2″), 164.4 (C2), 181.6 (C5); MS (ES+) m/z: 280 $(M+H)^+$.

*(4Z)-4-(3-methoxybenzylidene)-2-phenyloxazol-5(4H)-one (**2**)*

Compound (**2**) was recrystallized from $CHCl_3$—MeOH as yellow solid; Yield: 80%, m.p. 102–03 °C (lit. m.p. 102–04 °C) [25]; Anal. Calc. for $C_{17}H_{13}NO_3$: C, 73.11; H, 4.69; N, 5.02. Found: C, 73.10; H, 4.67; N, 5.04. IR ν_{max}^{KBr} cm^{-1}: 1795 (C=O), 1665 (C=N), 1652 (C=C), 1249 (C—O Lactone); ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 3.80 (s, 3H, CH_3), 7.02 (d, 1H, *J* = 8.4, H-4″), 7.16 (s, 1H, —CH=), 7.30 (m, 2H, H-3′,5′), 7.42 (m, 1H, H-5″), 7.52 (d, 1H, *J* = 8.4, H-6″), 7.80 (s, 1H, H-2″), 8.10–8.06 (m, 2H, H-2′,6′), 8.40 (dd, 1H, *J* = 7.4 H-4′); 13C NMR (100 MHz, $CDCl_3$, δ, ppm): 55.2 (CH_3), 115.5 (C2″), 117.1 (C4″), 122.5 (C6″), 127.6 (C3′&5′), 128.1 (C2′&6′), 132.2 (C4′), 133.1 (C5″), 133.4 (C1″), 134.2 (C1′), 135.8 (—CH=C), 145.3 (C4), 155.1 (C2), 159.3 (C3″), 184.5 (C=O); MS (ES+) m/z: 280 $(M+H)^+$.

*(4Z)-4-(4-methoxybenzylidene)-2-phenyloxazol-5(4H)-one (**3**)*

It was recrystallized from $CHCl_3$—MeOH as orange colored solid; Yield: 90%, m.p. 155 °C (lit. m.p. 157 °C) [26]; Anal. Calc. for $C_{17}H_{13}NO_3$: C, 73.11; H, 4.69; N, 5.02. Found: C, 73.10; H, 4.68; N, 5.06. IR ν_{max}^{KBr} cm^{-1}: 1791 (C=O), 1668 (C=N), 1650 (C=C), 1245 (C—O Lactone); ^1H NMR (400 MHz, DMSO, δ, ppm): 3.87 (s, 3H, CH_3), 7.10–7.18 (d, 2H, *J* = 8.2, H-3″,5″), 7.32 (s, 1H, —CH=), 7.50, (d, 2H, *J* = 8.2, H-2″,6″), 7.70–7.75 (m, 2H, H-3′,5′), 8.10 (dd, 1H, *J* = 7.4, H-4′), 8.30 (d, 2H, *J* = 7.2, H-2′,6′); 13C NMR (100 MHz, DMSO, δ, ppm): 55.4 (CH_3), 114.5 (C3″&5″), 126.6 (C2″&6″), 127.6 (C3′&5′), 128.0 (C1″), 128.5 (C2′&6′), 132.3 (C4′), 133.4 (C1′), 135.5 (—CH = C), 142.3 (C4″), 144.4 (C4), 162.0 (C2), 182 (C=O); MS (ES+) m/z: 280 $(M+H)^+$.

*(4Z)-4-(2, 5-dimethoxybenzylidene)-2-phenyloxazol-5(4H)-one (**4**)*

Its recrystallized from $CHCl_3$—MeOH as bright yellow colored solid; Yield: 80%, m.p. 140–41 °C; Anal. Calc. for $C_{18}H_{15}NO_4$: C, 69.89; H, 4.89; N, 4.53, Found: C, 69.85; H, 4.86; N, 4.54. IR ν_{max}^{KBr} cm^{-1}: 1795 (C=O), 1651 (C=N), 1570 (C=C), 1274 (C—O Lactone); ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 3.90–3.93 (s, 6H, 2 × CH_3), 7.02 (m, 2H, H-4″,6″), 7.51 (t, 2H, *J* = 7.4, H-3′,5′), 7.60 (t, 1H, *J* = 7.2, H-3″),7.71 (s, 1H, —CH=), 8.17 (d, 2H, *J* = 8.2, H-2′,6′), 8.46 (m, 1H,H-4′); ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 56.4 (CH_3), 118.3 (C6″), 119.1 (C5″) 124.1 (C4″), 127.2 (C3′&5′), 128.5 (C2′&6′), 131.6 (C1″), 132.4 (C4′), 133.5 (C1′), 135.1 (—CH=C), 145.0 (C4), 151.8 (C3″), 154.7 (C2″), 160.8 (C2), 184.2 (C=O); MS (ES+) m/z: 310 $(M+H)^+$.

*(4E)-4-(3, 5-dimethoxybenzylidene)-2-phenyloxazol-5(4H)-one (**5**)*

It recrystallized from $CHCl_3$—EtOH as yellow color crystal; yield: 75%, m.p. 128–30 °C; Anal. Calc. for $C_{18}H_{15}NO_4$: C, 73.11; H, 4.69; N, 5.05. Found: C, 73.15; H, 4.88; N, 5.08. IR ν_{max}^{KBr} cm^{-1}: 1799 (C=O), 1655 (C=C), 1591 (C=N), 1270 (C—O Lactone); ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 3.88 (s, 6H, 2 × CH_3), 7.17 (s, 1H, —CH=), 7.40 (m, 2H, H-2″,6″), 7.49 (s, 1H, H-4″), 7.53 (m, 2H, H-3′,5′), 7.62 (t, 1H, *J* = 7.4,H-4′) 8.14 (m, 2H, H-2′,6′); ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 56.1 (CH_3), 107.2 (C2″&6″), 108.0 (C4″), 127.0 (C3′&5′), 128.3 (C2′&6′), 132.1 (C4′), 133.1 (C1′), 135.0 (—CH=C), 138.5 (C1″), 144.3 (C4), 161.3 (C3″&5″), 162.7 (C2), 184.0 (C=O); MS (ES+) m/z: 310 $(M+H)^+$.

*(4Z)-4-(3, 4, 5-trimethoxybenzylidene)-2-phenyloxazol-5(4H)-one (**6**)*

It was crystallized from $CHCl_3$—MeOH as yellow color crystal; Yield: 70%, m.p. 204–05 °C (lit. mp 205 °C) [27]; Anal. Calc. for $C_{19}H_{17}NO_5$: C, 67.25; H, 5.05; N, 4.13. Found: C, 67.15; H, 5.18; N, 4.08. IR ν_{max}^{KBr} cm^{-1}: 1783 (C=O), 1655 (C=C), 1578 (C=N), 1244 (C—O Lactone); ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 3.95–3.97 (s, 9H, 3 × CH_3), 7.17 (s, 1H, —CH=), 7.60 (t, 1H, *J* = 7.4, H-4′), 7.48–7.53 (m, 4H, H-2′,3′,5′,6′), 8.11 (m, 2H, H-2″,6″); ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 56.6 (CH_3), 106.8 (C2″&6″), 127.4 (C3′&5′), 128.5 (C2′&6′), 131.8 (C1″), 132.4 (C4′), 133.3 (C1′) 135.2 (—CH=C), 143.2 (C4″), 144.5 (C4), 153.5 (C3″&5″), 162.9 (C2), 185.0 (C=O); MS (ES+) m/z: 340 $(M+H)^+$.

Crystal structure determination

A crystal of (**5**) with a needle shape and having approximate dimensions of 0.47 × 0.07 × 0.05 mm was glued on a glass fiber and mounted on a Bruker Apex II diffractometer. The same procedure was done for a crystal of (**6**) with plate habit and approximate dimensions 0.44 × 0.29 × 0.09 mm. Diffraction data were collected at room temperature 293(2) K using graphite monochromated Mo Kα (λ = 0.71073 Å). Data reduction was performed with APEX II [28]. Lorentz and polarization corrections were applied. A multi-scan absorption correction was applied using SADABS [29]. The crystallographic structure was solved by direct methods (SHELXS-97) [30]. Refinements were carried out with SHELXL-97 package [30]. All refinements were made by full-matrix least-squares on F^2, with anisotropic displacement parameters for all non-hydrogen atoms. All the hydrogen atoms could be located in a difference Fourier synthesis but were placed at calculated positions and then, included in the structure factor calculation in a riding model using SHELXL-97 defaults. For compound (**5**), the final least-squares cycle was based on 2074 observed reflections [$I > 2\sigma(I)$], 211 variable parameters, 0 restraints, converged with $R = 0.0547$ and $wR = 0.0884$.

For compound (**6**), the final least-squares cycle was based on 3654 observed reflections [$I > 2\sigma(I)$], 229 variable parameters, 0 restraints, converged with $R = 0.0433$ and $wR = 0.0961$. Additional information to the structure determination is given in Table 1 and 2 (Supplementary data), respectively. Selected structural parameters can be seen in Tables 3 and 4. Supplementary data have been deposited at the Cambridge Crystallographic Data Centre (CCDC No. 876047 & 876048).

Ab initio calculations

The geometry optimizations of (**5**) and (**6**) were performed using the Firefly QC package [31], which is partially based on the GAMESS (US) source code [32], starting from the experimental X-ray geometry (*Z*-conformations). We have also performed the geometry optimization of the corresponding *E*-conformations of (**5**) and (**6**). The *E*-conformations were generated from the X-ray geometries performing a rotation of 180° around the C3—C12 bound with the aid of the software Ascalaph Designer version 1.8.50 [33].

The calculation was performed within density functional theory (DFT) using B3LYP (Becke three–parameter Lee–Yang–Parr) for exchange and correlation, which combines the hybrid exchange functional of Becke [34,35] with the correlation functional of Lee, Yang and Parr [36]. The calculation was performed with the Pople's 'triple split' 6-31G(d,p) basis set, which includes a set of p-polarization functions for the H atoms and a set of d-polarization functions for the C, N and O atoms. Each self-consistent field calculation was iterated until a $\Delta\rho$ of less than 10^{-5} $bohr^{-3}$ was achieved. The final equilibrium geometries at the minimum energy had a maximum gradient in internal coordinates of 10^{-5} Hartree $bohr^{-1}$ or Hartree rad^{-1}. At the end of these geometry optimizations, Hessian calculation were performed to guarantee that the final structures correspond to true minima, using the same level of theory as in the geometry optimizations. A vibrational analysis was performed for compounds (**5**) and (**6**) during the Hessian calculation to calculate the IR spectra. We also calculated the single point energy for the optimized structures of the *Z*- and *E*-conformations of compounds (**5**) and (**6**) using the B3LYP/6-31G(d,p) level of theory.

Bioassay

Antibacterial studies

The *in vitro* antimicrobial activities of Erlenmeyer azlactones (**1–6**) were tested using the bacterial cultures of *Pseudomonas aeruginosa* (ATCC-9029), *Staphylococcus Pyogenes* (clinically isolated), *Klebsiella pneumonia* (clinically isolated), Methicillin resistant *Staphylococcus aureus* (MRSA + Ve), *Escherichia coli* (ATCC-25922) bacterial strains by disk diffusion method [37,38]. A standard inoculums (1–2 × 10^7 c.f.u./mL 0.5 McFarland standards) was introduced on to the surface of sterile agar plates, and a sterile glass spreader was used for even distribution of the inoculums. The disks measuring 6 mm in diameter were prepared from What-

Table 1
Crystallographic data and structure refinement of compound (**5**).

Empirical formula	$C_{18}H_{15}NO_4$
Formula weight	309.31
Temperature (K)	293(2)
Wavelength (Å)	0.71073
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
a (Å)	5.6793(3)
b (Å)	15.2038(7)
c (Å)	17.6919(10)
α (deg.)	90
β (deg.)	90
γ (deg.)	90
Volume ($Å^3$)	1527.64(14)
Z	4
Calculated density (g/cm^3)	1.345
Absorption coefficient (mm^{-1})	0.096
Extinction coefficient	0.0102(17)
F(000)	648
Crystal size (mm)	0.47 × 0.07 × 0.05
θ range for data collection (deg.)	1.77–27.65
Index ranges	$-7 < h < 7$, $-19 < k < 19$, $-23 < l < 22$
Reflections collected/unique	19930/3561 [$R(int) = 0.147$]
Completeness to $\theta = 25.00°$	100%
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	2074/0/211
Goodness-of-fit on F^2	0.965
Final *R* indices [$I > 2\sigma(I)$]	$R1 = 0.0473$ $wR2 = 0.0814$
R indices (all data)	$R1 = 0.1133$ $wR2 = 0.1642$
Largest diff. peak and hole (e $Å^{-3}$)	0.162 and −0.165

Table 3
Comparison of selected geometrical parameters for (**6**) as determined by X-ray diffraction and from DFT geometry optimization (Å,°).

	Experimental	DFT
O2—C2	1.195(4)	1.2007
O1—C2	1.399(5)	1.4094
O1—C5	1.384(4)	1.3754
N4—C5	1.279(4)	1.2964
N4—C3	1.403(4)	1.3971
C2—C3	1.452(6)	1.4812
C3—C12	1.341(5)	1.3583
C12—C13	1.447(6)	1.4520
O1—C2—C3	105.2(4)	103.98
C2—C3—N4	108.3(4)	108.52
C3—N4—C5	105.5(4)	105.63
N4—C5—O1	116.1(4)	115.98
C5—O1—C2	104.9(3)	105.88
C3—C12—C13	130.3(4)	130.06
O1—C5—C6—C11	−9.0(6)	−0.12

Table 4
Comparison of selected geometrical parameters for (**6**) as determined by X-ray diffraction and from DFT geometry optimization (Å,°).

	Experimental	DFT
O2—C2	1.195(2)	1.2028
O1—C2	1.397(2)	1.4097
O1—C5	1.384(2)	1.3751
N4—C5	1.280(2)	1.2961
N4—C3	1.394(2)	1.3966
C2—C3	1.465(3)	1.4786
C3—C12	1.446(3)	1.3605
C12—C13	1.446(2)	1.4485
O1—C2—C3	104.80(17)	103.98
C2—C3—N4	108.18(16)	108.59
C3—N4—C5	105.97(15)	105.62
N4—C5—O1	115.82(17)	115.94
C5—O1—C2	105.21(15)	105.87
C3—C12—C13	130.5(18)	130.14
O1—C5—C6—C11	11.2(3)	−0.18

man No. 1 filter paper and sterilized by dry heat at 140 °C for 1 h. Ciprofloxacin was used as positive control (standard drug) while the disk poured in DMSO was used as negative control. The susceptibility was assessed on the basis of diameter of zone of inhibition against gram +ve and gram –ve strains of bacteria. Inhibition zones were measured and compared with the controls. The bactericidal zones of inhibition values are given in Fig. 1.

Minimum inhibitory concentrations (MICs) were determined by broth dilution technique. The nutrient broth, which contained logarithmic serially two fold diluted amount of test compound and controls were inoculated with approximately 5×10^5 c.f.u./mL of actively dividing bacteria cells. The lowest concentration (highest dilution) required to arrest the growth of bacteria was regarded as minimum inhibitory concentration (MIC). To obtain the minimum bacterial concentration (MBC), 0.1 mL volume was taken from each tube and spread on agar plates. The number of c.f.u. was counted after 18–24 h of incubation at 35 °C. MBC was defined as the lowest drug concentration at which 99.9% of the inoculums were killed. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration are given in Table 5.

Antifungal studies

The antifungal activity was also done by disk diffusion method [39–40]. For assaying antifungal activity *Candida albicans, Aspergillus fumigatus, Penicillium marneffei* and *Trichophyton mentagrophytes* were used. Sabourands agar media was prepared by dissolving peptone (1 g), D-glucose (4 g) and agar (2 g) in distilled water (100 mL) and adjusting the pH to 5.7. Normal saline was

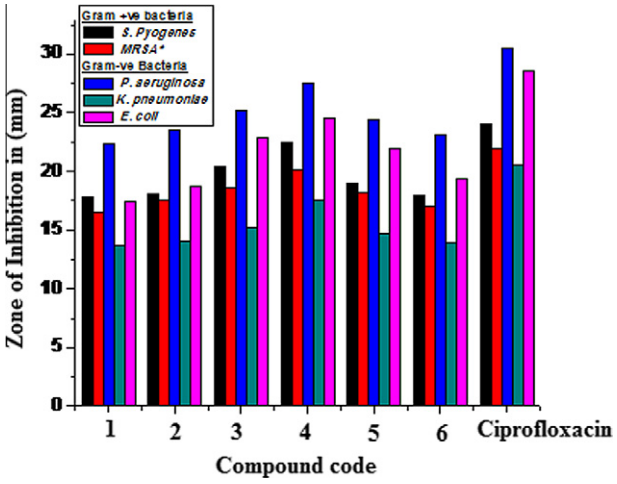


Fig. 1. Antibacterial activity of azlactones (**1–6**), Diameter of zone of inhibition (mm), Positive control (standard): Ciprofloxacin and negative control (DMSO) measured by the Halo Zone Test (Unit, mm). * Methicillin resistant *Staphylococcus aureus* (MRSA + Ve).

used to make a suspension of spore of fungal strain for lawning. A loopful of particular fungal strain was transferred to 3 mL saline to get a suspension of corresponding species. Twenty millilitres of agar media was poured into each petri dish. Excess of suspension was decanted and the plates were dried by placing in an incubator at 37 °C for 1 hour. Using an agar punch, wells were made and each well was labelled. A control was also prepared and maintained at 37 °C for 3–4 days. The fungal activity of each compound was compared with Amphotericin B as positive control (standard drug), while the disk containing DMSO was used as negative control. Inhibition zones were measured and compared with the controls. The fungal zones of inhibition values are given in Fig. 2.

The nutrient broth, which contained logarithmic serially two fold diluted amount of test compound and controls was inoculated with approximately 1.6×10^4–6×10^4 c.f.u./mL. The cultures were incubated for 48 h at 35 °C and the growth was monitored. To obtain the minimum fungicidal concentration (MFC), 0.1 mL volume was taken from each tube and spread on agar plates. The number of c.f.u. was counted after 48 h of incubation at 35 °C. MFC was defined as the lowest drug concentration at which 99.9% of the inoculums were killed. The minimum inhibitory concentration and minimum fungicidal concentration are given in Table 6.

Antioxidant studies

The Erlenmeyer azlactones (**1–6**) were tested for their antioxidant property by 1,1-diphenylpicrylhydrazyl (DPPH) method [41–43]. In this procedure drug stock solution (1 mg/mL) was diluted to final concentration of 2, 4, 6, 8, 10 and 12 in methanol. Methanolic DPPH solution (1 mL, 0.3 mmol) was added to 3.0 mL of drug solution of different concentrations. The tube was kept at an ambient temperature for 30 min and the absorbance was measured at 517 nm in UV VIS-1800 spectrophotometer. The scavenging activity was calculated by following formula:

$$[\%\text{inhibition} = [(A_{\text{Control}} - A_{\text{Sample}})/A_{\text{Control}}] \times 100]$$

where A_{Control} is the absorbance of the L-ascorbic acid (Standard) and A_{Sample} is the absorbance of different compounds.

The methanolic DPPH solution (1 mL, 0.3 mM) was used as control. The inhibitory concentration (IC_{50}) value represents the concentration required to exhibit 50% antioxidant activity (Fig. 3). The IC_{50} values were calculated by the linear regression of plots where the abscissa represented the concentration of the

Table 5
MIC and MBC results of azlactones (**1–6**) positive control Ciprofloxacin.

Compounds	Gram positive bacteria				Gram negative bacteria					
	S. Pyogenes		*MRSA*∗		*P. aeruginosa*		*K. pneumoniae*		*E. coli*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	25	50	25	50	25	100	25	100	25	100
2	25	50	25	50	25	100	25	50	25	100
3	25	50	25	50	25	50	25	50	25	50
4	12.5	25	12.5	25	12.5	25	25	50	12.5	25
5	25	50	50	100	50	>100	50	100	50	100
6	50	100	50	100	50	>100	50	100	50	100
Standard	6.25	12.5	6.25	12.5	12.5	25	6.25	25	6.25	25

MIC (μg/ml) = minimum inhibitory concentration, i.e the lowest concentration of the compound to inhibit the growth of bacteria completely; MBC (μg/ml) = minimum bacterial concentration, i.e., the lowest concentration of the compound for killing the bacteria completely.

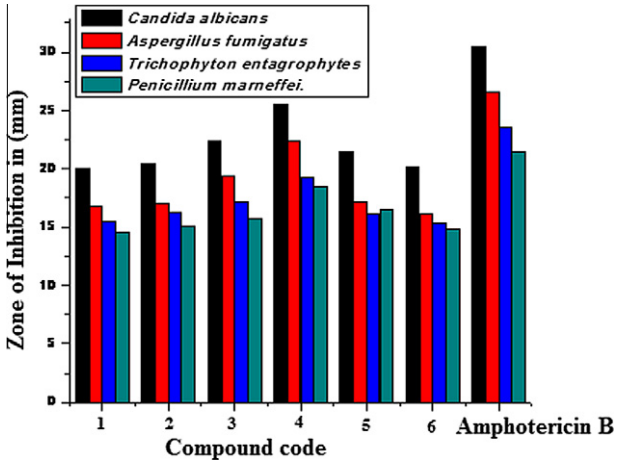


Fig. 2. Antifungal activity of azlactones (**1–6**) Positive control (Amphotericin B) and negative control (DMSO) measured by the Halo Zone Test (Unit, mm). Diameter of zone of inhibition (mm), **CA**: *Candida albicans*, **AF**: *Aspergillus fumigatus*, **TM**: *Trichophyton entagrophytes*, **PM**: *Penicillium marneffei*.

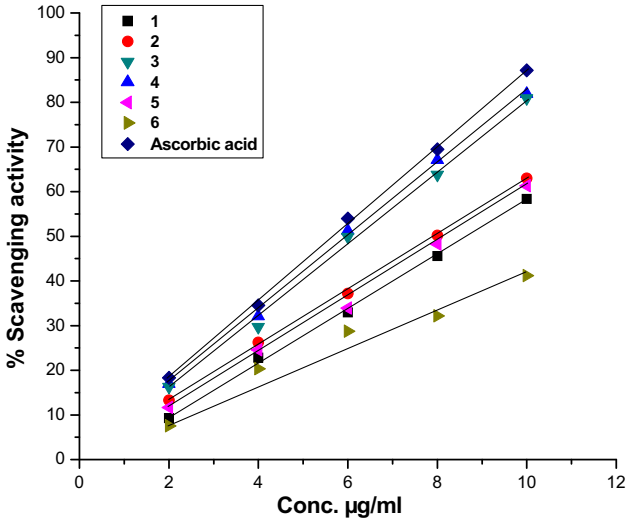


Fig. 3. Antioxidant activity of compounds (**1–6**).

Table 6
MIC and MFC results of azlactones (**1–6**) positive control Amphotericin B.

Compounds	**CA**		**AF**		**TM**		**PM**	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
1	25	50	25	50	25	100	25	100
2	50	100	50	100	50	100	50	100
3	25	50	25	50	25	50	50	100
4	12.5	25	12.5	25	12.5	25	12.5	50
5	25	50	25	50	25	50	50	100
6	50	100	50	100	50	100	50	100
Standard	6.25	25	12.5	25	6.25	25	12.5	25

CA; *Candida albicans*, **AF**; *Aspergillus fumigatus*, **TM**; *Trichophyton mentagrophytes*, **PM**; *Penicillium marneffei*. MIC (μg/ml) = minimum inhibitory concentration, i.e., the lowest concentration of the compound to inhibit the growth of fungus completely; MFC (μg/ml) = minimum fungicidal concentration, i.e., the lowest concentration of the compounds for killing the fungus completely.

compounds (μg/mL). Explicitly, IC_{50} is the average percentage of antioxidant activity. Results in the form of percent inhibition are tabulated in the Table 7. The experiments were done in triplicate.

Results and discussion

Chemistry

The ^{1}H NMR spectra of the compounds (**1–6**) exhibited a characteristic sharp down field singlet at δ 7.16–7.71 attributed to the olefinic proton which is in agreement with the *Z*-configuration as reported in the literature [2]. The *Z*-configuration has less steric repulsion as compare to *E*-configuration. The phenyl protons attached at the position 2 of azlactones were found to resonate in the range of 8.70–7.30 ppm. The other signals of substituted benzylidines attached at the position 4 of azlactones displayed at δ 7.48–6.98 for four protons (**1**), 7.70–7.02 for four protons (**2**), 7.50–7.10 for four protons (**3**), 7.60–7.02 for three protons (**4**), 7.40–7.49 for three protons (**5**) and a singlet at δ 8.11 ppm for two protons (**6**). The methoxy groups of benzylidines substituted at different positions displayed singlet singles in the range of δ 3.87–3.97 ppm. ^{13}C NMR spectra provided a firm support for the formation of compounds and their signals were in good agreement with proposed structures. All the compounds exhibited signals at δ 182–185.0 due to (C=O) while the signals at δ 135.0–135.8 ppm may be attributed to the olefinic carbon. Further support of structures (**1–6**) was given by (+)-ESI mass spectroscopy. The mass spectra of (**1**), (**2**), (**3**), (**4**), (**5**) and (**6**) showed the molecular ion peak at m/z 280 $(M+H)^+$, m/z 280 $(M+H)^+$, m/z 280 $(M+H)^+$, m/z 310 $(M+H)^+$, 310 $(M+H)^+$ and m/z 340 $(M+H)^+$ respectively. The complete chemical shifts for synthesized compounds are listed in experimental part.

Crystal structure

Compound (**5**) crystallizes in the orthorhombic system with space group $P2_12_12_1$ Fig. 4. The molecule as a whole is approxi-

Table 7
Quantitative screening of antioxidant activity of azlactones (**1–6**) by DPPH assay method ($n = 3$).

S. No	Compounds	Absorbance	Absorbance at 517 nm					IC_{50}
			2 μg/mL	4 μg/mL	6 μg/mL	8 μg/mL	10 μg/mL	
1.	**Control**	($Abs_{control}$)	0.9420 ± 0.04	0.9420 ± 0.04	0.9420 ± 0.03	0.9420 ± 0.04	0.9420 ± 0.04	
2.	**1**	Abs_{sample}	0.8542 ± 0.02	0.7265 ± 0.04	0.6314 ± 0.07	0.5125 ± 0.05	0.3919 ± 0.04	7.78
		(AA%)	9.28	22.84	32.94	45.57	58.37	
3.	**2**	Abs_{sample}	0.8164 ± 0.05	0.6945 ± 0.07	0.5867 ± 0.05	0.4689 ± 0.06	0.3485 ± 0.08	6.94
		(AA%)	13.29	26.24	37.21	50.20	62.96	
4.	**3**	Abs_{sample}	0.7820 ± 0.05	0.6394 ± 0.06	0.4589 ± 0.04	0.3098 ± 0.04	0.1705 ± 0.03	5.35
		(AA%)	16.94	32.09	51.26	67.09	81.89	
5.	**4**	Abs_{sample}	0.7883 ± 0.05	0.6619 ± 0.08	0.4720 ± 0.03	0.3412 ± 0.06	0.1800 ± 0.05	5.15
		(AA%)	16.28	29.70	49.87	63.79	80.91	
6.	**5**	Abs_{sample}	0.8318 ± 0.08	0.7105 ± 0.07	0.6224 ± 0.08	0.4873 ± 0.04	0.3646 ± 0.04	7.45
		(AA%)	11.66	24.54	33.89	48.24	61.27	
7.	**6**	Abs_{sample}	0.8703 ± 0.02	0.7498 ± 0.04	0.6709 ± 0.07	0.6138 ± 0.07	0.5540 ± 0.03	10.95
		(AA%)	7.57	20.36	41.16	32.15	28.74	
8.	**Standard**	Abs_{sample}	0.7694 ± 0.04	0.6168 ± 0.07	0.4336 ± 0.06	0.2878 ± 0.06	0.1208 ± 0.05	4.78
		(AA%)	18.32	34.52	53.97	69.44	87.17	

mately planar, with the oxazolone ring making a dihedral angle of 10.3(2)° with the 2-phenyl ring and 2.9(2) with the methoxyphenyl ring. This planarity presumably results from the effects of conjugation. The molecule adopts the *Z* configuration about the central olefinic bond C3═C12 bond [1.342(4) Å] and the coplanarity and conjugation of the π-electron systems in the aromatic rings are reflected in the C—C bond lengths between the oxazolone and methoxyphenyl rings. The C3—C12 bond is significantly shorter than the C12—C13 bond. The methoxy groups lie in the plane of the methoxy phenyl ring.

The crystal structure of (**5**) is determined by rather weak C—H···O interactions Table 3. The C19—H19B···O2 interaction forms helical chains along the *a* axis with descriptor C(10) according to Etter's graph-set theory [44,45]. The weaker C14—H14···O3 interaction also delineate helical chains along the *a* axis with graph-set C(4). Additionally, there is one intramolecular C—H···N hydrogen bond between an H atom of the methoxyphenyl ring and the N atom of oxazolone ring and one intramolecular C—H···O hydrogen bond between one H atom of the phenyl ring and the O atom of the oxazolone ring [Table 8; Fig. 5 (Supplementary data)]. The crystal packing is also stabilized by weak C—H···π interactions and π···π interactions.

Compound (**6**) crystallizes in the triclinic system with space group P-1 [Fig. 6 and 7 (Supplementary data)]. The structure of this compound was originally reported [27]. We obtained this compound from a different synthesis route of that used by Sun and Cui.

Results of the ab initio calculations

In order to gain some insight on the influence of the intermolecular interactions on the molecular geometry we have performed

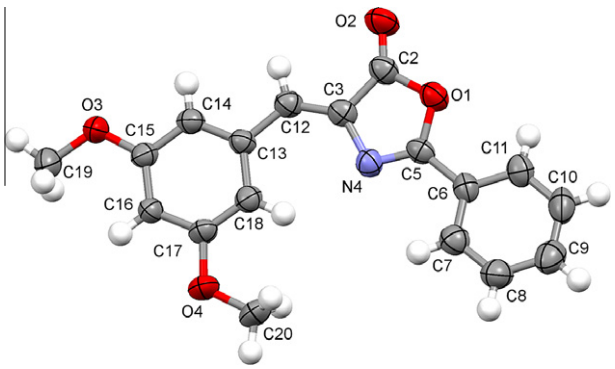


Fig. 4. Asymmetric unit of the compound (**5**) with the ellipsoids drawn at the 50% probability level, with the atomic labelling scheme (Mercury, version 3.0 [46]).

Table 8
H-bond geometry (Å,°) of azlactones (**5**).

	D—H	H···A	D···A	D—H···A
C14—H14···O3^{i}	0.93	2.57	3.441(4)	155
C19—H19B···O2^{ii}	0.96	2.43	3.377(4)	168
C11—H11···O1 (intra)	0.93	2.48	2.794(4)	100
C20—H20···O2 (intra)	0.93	2.43	3.062(5)	126

(Symmetry codes i: $-1/2 + x$, $1/2 - y$, $-z$; ii: $3/2 + x$, $1/2 - y - 1/2$, $-z$).

quantum mechanical calculations of the equilibrium geometry of the free molecule. The DFT calculations closely reproduce the solid-state geometry of the molecule is (Tables 3 and 4). The agreement between the experimental and calculated bond lengths and valence angles is very good, but the calculated torsion angle O1—C5—C6—C11 in the free molecule differs by 8.88° from that of the molecules in the crystal for (**5**) and 11.38° for (**6**). The differences between the calculated and experimental bond lengths are smaller than 0.0292 and 0.0205 Å for (**5**) and (**6**), respectively (Figs. 8 and 9).

Overall, our data suggest that the supramolecular aggregation does not play a major role in stabilizing the observed geometries of (**5**) and (**6**), in agreement with the absence of strong intermolecular interactions.

The calculated energy difference between the optimized *E*- and *Z*-conformations of compound (**5**) is 2.21 kcal/mol, with the *Z*-conformation having the lower energy. In compound (**6**), the *Z*-conformation is again the more stable, by 2.08 kcal/mol.

The calculated IR spectra of (**5**) and (**6**) describe the main features of the experimental spectra Figs. 9 and 10 (See Supplementary data).

Antimicrobial study

The *in vitro* antimicrobial activities of Erlenmeyer azlactones **(1–6)** is presented in Figs. 1 and 2 and Tables 5 and 6. The *in vitro* study results demonstrated that the compound (**4**) was most active among all compounds in terms of antibacterial as well as antifungal activity. The MIC of compound (**4**) is 12.5 μg/mL with zone of inhibition 24.5 ± 0.5 against *Escherichia coli* (ATCC-8739) (bacterial strain) while the zone of inhibition was 25.5 ± 0.2 against *Candida albicans* (fungal strain). The MIC results for both bacterial and fungal strains are shown in (Tables 5 and 6). It is concluded that compound (**4**) bearing CH_3O-group at position 2,4 of the benzene ring is most potent followed by (**3**), (**2**), (**5**), (**1**) and (**6**) compounds.

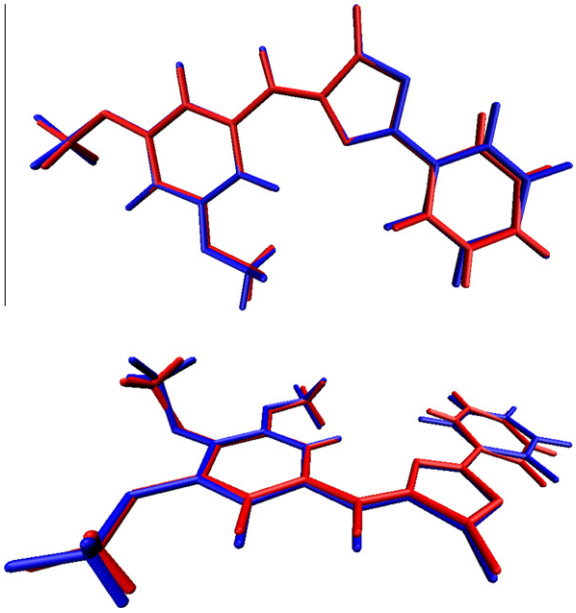
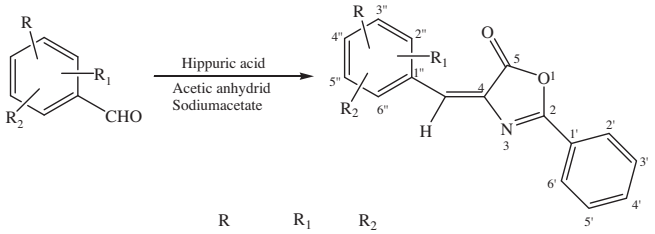


Fig. 8. Comparison of the molecular conformation of (**5 & 6**), as established from the X-ray study (red) with the optimized geometry (blue) (Software used for visualization: VMD, version 1.9.1, January 29, 2012 [47]).



	R	R_1	R_2
1	H	H	2-OMe
2	H	H	3-OMe
3	H	H	4-OMe
4	H	2-OMe	5-OMe
5	H	3-OMe	5-OMe
6	3-OMe	4-OMe	5-OMe

Scheme 1. *Z*-configuration of the products (**1–6**).

Antioxidant sturdy

The synthesized azlactones (**1–6**) were subjected to free radical scavenging activity by DPPH method. This model of scavenging activity by DPPH radical is extensively applied to evaluate antioxidant activity in shorter as compared with other methods. The odd electron in the DPPH free radical gives a strong absorption band at λ 517 nm, which is purple in color. This property makes it suitable for spectrometric studies. The DPPH assay has often been used to estimate the antiradical activity of a given antioxidant. The free radical scavenging capabilities of the compounds were measured in term of hydrogen donating of free radical scavenging ability after adding methanolic solution of DPPH to the sample solution of different concentrations. The synthesized azlactones reacted with DPPH and converted to it 1,1 diphenyl-2-picrylhydrazine. The extent of decolourizing is indicative of antioxidant behavior of a particular compound. Ascorbic acid was used as the reference compound. All the tests were performed in triplicate. The compound (**4**) showed the highest IC_{50} value followed by compounds (**3**), (**2**), (**5**), (**1**) and (**6**) respectively and results are reported in Table 7 (see Scheme 1).

Conclusions

The present work reports the synthesis, spectral characterization, bioassay of synthesized azlactones obtained from condensation of aldehydes and hippuric acid. All the compounds showed substantial antibacterial and antifungal activity against different strains of bacteria and fungi respectively. The compounds also exhibited good antioxidant activity by the DPPH method. The *Z*-configuration of synthesized Erlenmeyer azlactones was confirmed on the basis of spectroscopy techniques, X-ray crystallographic studies as well as DFT calculations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.saa.2012.11.054.

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