Synthesis, Antimicrobial, and Brine Shrimps Lethality Assays of 3, 3-Diaryl-4-(1-methyl-1*H*-indol-3-yl)azetidin-2-ones

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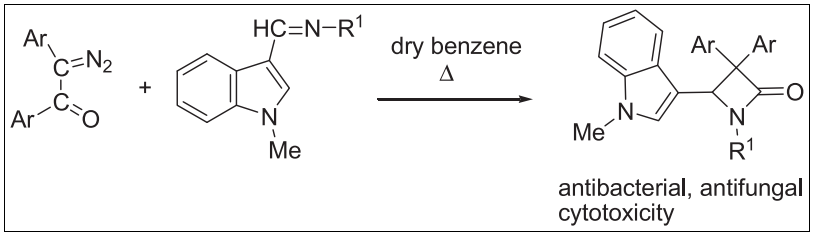

The paper describes the synthesis, characterization data, and biological activity (antibacterial, antifungal, and brine shrimps lethality) of new azetidin-2-ones. The compounds have been synthesized by the reaction of diarylketenes, generated in situ from thermal decomposition of the 2-diazo-1,2-diarylethanones, with *N*-(1-methyl-1*H*-indol-3-yl)methyleneamines. The compounds have been characterized by elemental analysis and spectral (IR, ^{1}H and ^{13}C NMR, and MS) data. The paper also reports the results of antibacterial, antifungal, and brine shrimps lethality assays of these compounds. Some of the compounds exhibited significant biological activity.

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INTRODUCTION

Azetidin-2-ones, commonly known as β-lactams, are well-known heterocyclic compounds of biological interest [1]. The potential antibacterial activity of the β-lactam group of antibiotics such as penicillins, cephalosporins, and carbapenems is attributed to the presence of azetidin-2-one ring in them. The growing concern about development of resistant pathogens due to β-lactamase enzyme led to extensive investigation on the isolation of β-lactams from natural resources, and design and synthesis of natural products-inspired β-lactams [1–3]. Once again, it was a β-lactam clavulanic acid that offered the major relief as a β-lactamase inhibitor. A monocyclic azetidin-2-one ezetimibe is now in clinical use as cholesterol absorption inhibitor [4]. Apart from these three main biological activities, studies are also in progress to synthesize new β-lactams and explore their anticancer activity, hypoglycemic activity, antileishmanial activity, antiplasmodial activity [5–7], and so forth.

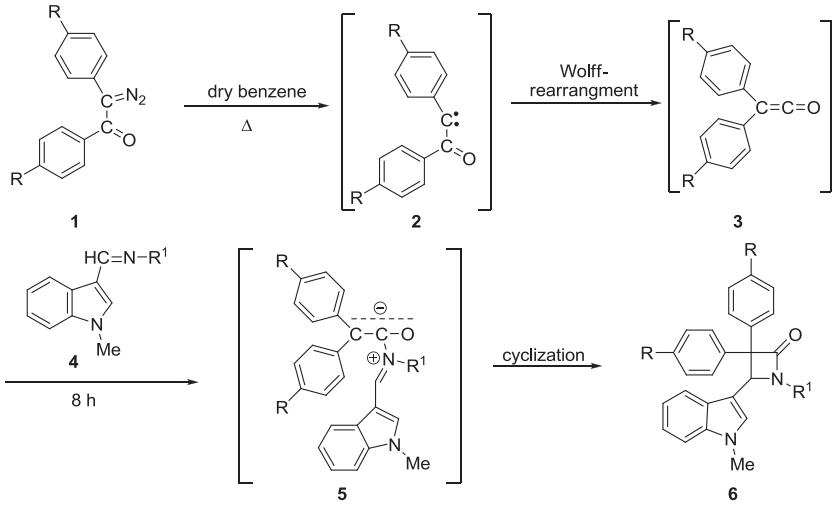
The synthesis of β-lactams involves different types of cycloaddition reactions and cyclization reactions involving β-amino acids or β-amino esters [1]. The Staudinger's ketene–imine cycloaddition is one of the most common cycloaddition reactions that is used for the synthesis of β-lactams [8]. This method, which involves generation of ketenes from acid chlorides in the presence of a tertiary base or directly from carboxylic acid employing an acid activator and a tertiary bases, has been used recently by several groups for the synthesis of diverse types of azetidin-2-ones [5–7,9–15]. Our group has been using thermal decomposition of 2-diazo-1,2-diarylethanones to generate diarylketenes *in situ* as a clean and convenient method requiring no base or any special reaction condition [16–19].

We have recently reported the synthesis and antileishmanial activity of new natural product-inspired azetidin-2-ones synthesized by the reaction of *N*-(1-methyl-1*H*-indol-3-yl) methyleneamines with 2-diazo-1,2-diarylethanones under thermal conditions as a short communication [20]. The present paper reports the full characterization data and results of antibacterial, antifungal, and brine shrimps lethality assays of these compounds. The brine shrimps lethality assay nowadays is commonly used as a quick and simple method to know the general toxicity and as a guide to determine the antitumor activity of the compounds [21,22].

RESULTS AND DISCUSSION

Chemistry. The reaction of 2-diazo-1,2-diarylethanones **1** with *N*-(1-methyl-1*H*-indol-3-yl)methyleneamines **4** by refluxing in dry benzene for 8 h afforded white crystalline compounds characterized as 3,3-diaryl-4-(1-methyl-1*H*-indol-3-yl)azetidin-2-ones **6** (Scheme 1) on the basis of satisfactory elemental analysis and spectral data. The IR spectra of the products showed the strong absorption band at $1733 \pm 6\,cm^{-1}$ corresponding to the azetidin-2-one carbonyl group. The ^{1}H NMR spectral spectra showed two characteristic singlet signals at around δ 3.4 ppm (three protons) and 6.0 ppm (one proton) corresponding to the *N*-methyl protons and β-lactam ring proton besides other

Scheme 1



6a. R = R^1 = Ph; **6b**. R = Ph, R^1 = 4-MePh; **6c**. R = Ph, R^1 = 4-EtOPh; **6d**. R = Ph, R^1 = 4-ClPh; **6e**. R = 4-MePh, R^1 = Ph; **6f**. R = R^1 = 4-MePh; **6g**. R = 4-MePh, R^1 = 4-EtOPh; **6h**. R = 4-MePh, R^1 = 4-ClPh

protons. The ^{13}C NMR spectra showed the carbonyl carbon around δ 167 ppm.

The formation of products is explained by the reaction of diarylketenes **3**, generated *in situ* by thermal decomposition of the 2-diazo-1,2-diarylethanones **1** followed by the Wolff rearrangement of the resulting α-ketocarbenes **2** [23], with the nitrogen atom of azomethine linkage in **4** leading to the formation of a *zwitterionic* intermediates **5** that cyclize to form the azetidin-2-ones **6**. The formation of the *zwitterionic* intermediate in the reaction of ketenes with imines has been evidenced earlier [24].

Pharmacology. ***Antibacterial activity.*** The compounds were screened for their *in vitro* antibacterial activity against three Gram-(+) strains: *Staphylococcus aureus*, *Bacillus subtilis*, and *Micrococcus luteus*, and two Gram-(−) strains: *Enterobacter aerogenes* and *Escherichia coli* by disk diffusion method (Kirby-Bauer method) using cefixime as the standard drug. In general, the compounds showed much better activity against the Gram-(+) strains in comparison to Gram-(–) strains (Table 1). The compound **6a** having unsubstituted phenyl rings showed significant activity against all three Gram-(+) strains. Its minimum inhibitory concentration (MIC) was determined as 125 μg/mL against *B. subtilis* and *S. aureus*, and 500 μg/mL against *M. luteus*. Azetidin-2-ones **6d** and **6g** also showed significant activity on *B. subtilis* whereas **6c** and **6g** showed significant activity on *M. luteus*.

Antifungal activity. The antifungal bioassay was performed by the agar tube dilution method using miconazole as the reference drug, in which the test compounds were screened for activity against *Candida albicans*, *Microsporum canis*, *Fusarium solani*, and *Candida glabrata*. Some compounds of the series exhibited significant activity on all the strains except *F. solani* in which the compounds show good to moderate activity. The results of antifungal bioassay (Table 2) indicate significant activity of the compounds **6a**, **6e**, **6f**, and **6h** against *C. albicans*. The compounds **6b** and **6c** exhibit good activity against this fungus, whereas **6d**, **6g**, and **6i** show moderate activity toward same fungus.

The compounds **6e**, **6f**, and **6h** also show significant activity against *M. canis*. The compounds **6a**, **6c**, and **6d** show good activity against this fungus, and compound **6b** shows moderate activity.

Against *F. solani*, two azetidin-2-ones **6e** and **6h** exhibit good activity and three azetidin-2-ones **6a**, **6c**, and **6f** exhibit moderate activity. Azetidin-2-ones **6f** and **6h** exhibit significant activity against *C. glabrata*. The compounds **6c** and **6g** exhibit good activity against this strain, whereas the compounds **6a**, **6d**, **6e**, and **6f** exhibit only moderate activity.

Brine shrimps lethality assay. According to the data shown for brine shrimps lethality assays (Table 3), five compounds exhibit more cytotoxicity than the standard drug MS-222. In general, azetidin-2-ones **6e–h** bearing two *p*-tolyl groups on C-4 position were observed more toxic in comparison with those with unsubstituted phenyl rings on this position. Among the azetidine-2-ones **6a–d** obtained from the reaction of diphenylketene with imines, compound **6c** with a 4-ethoxyphenyl group on *N*-1 position of the azetidine-2-one ring was the most cytotoxic followed by **6b** containing a 4-methylphenyl group on *N*-1 position of azetidine-2-one ring. Among the azetidin-2-ones **6e–h** from di*p*-tolyl series, the compound **6h** bearing a 4-chlorophenyl group on the *N*-1 position of the azetidine-2-one ring was the most toxic.

Table 1

Antibacterial activity of azetidin-2-ones **6a–6h**.

No.	Average zone of inhibition(mm) ± SD				
	Staphylococcus aureus (ATCC6633)	*Bacillus subtilis* (ATCC6538)	*Micrococcus luteus* (ATCC10240)	*Enterobacter aerogenes* (ATCC13048)	*Escherichia coli* (ATCC5224)
6a	22 ± 0.01	23 ± 0.09	13 ± 0.04	12 ± 0.06	11 ± 0.01
6b	–	–	–	–	–
6c	21 ± 0.02	19 ± 0.12	12 ± 0.03	12 ± 0.02	–
6d	13 ± 0.02	24 ± 0.01	09 ± 0.05	–	–
6e	06 ± 0.01	07 ± 0.04	–	–	–
6f	12 ± 0.05	14 ± 0.01	10 ± 0.02	14 ± 0.01	08 ± 0.02
6g	20 ± 0.01	25 ± 0.02	12 ± 0.05	12 ± 0.02	11 ± 0.01
6h	13 ± 0.02	14 ± 0.01	09 ± 0.05	10 ± 0.07	–
Cefixime	25.1 ± 0.10	29.56 ± 0.02	15.4 ± 0.01	22.1 ± 0.03	26.6 ± 0.02
Vehicle control	–	–	–	–	–

The "–" sign indicates no activity. Vehicle control concentration: 1 mg/mL of DMSO. Reference drug cefixime concentration: 1 mg/mL.

Table 2

Percentage inhibition* of compounds (**6a–6h**) against different fungal strains.

No.	*Candida albicans*	*Microsporum canis*	*Fusarium solani*	*Candida glabrata*
6a	79	61	46	53
6b	66	54	–	51
6c	60	61	42	66
6d	51	64	–	58
6e	87	79	60	52
6f	76	80	55	78
6g	58		–	63
6h	89	77	62	87
DMSO	0	0	0	0
Standard drug MIC (mg/mL)				
Miconazole (2 mg/1 mL)	110.08	98.04	73.22	110.28

*Percentage inhibition activity: 0–39 = low (non-significant); 40–59 ([A-Za-z0-9,40–59 = moderate; 60–69 ([A-Za-z0-9,60–69 ; = Good; above 70 = significant activity.
MIC, minimum inhibitory concentration.

Table 3

Cytotoxicity data[a] of compounds **6a–h**.

No.	No. of shrimps killed out of 30 per dilution			
	1000 μg/mL	100 μg/mL	10 μg/mL	LD_{50}
6a	21	20	11	33.61
6b	25	23	17	3.01
6c	30	26	23	0.95
6d	24	19	12	27.18
6e	30	26	22	1.60
6f	24	20	16	6.37
6g	28	25	21	0.85
6h	30	27	24	0.56
Vehicle control	0	0	0	

[a]The data are based on mean value of three replicates each of 10, 100, and 1000 μg/mL compared with the standard drug MS-222 (LD_{50} = 4.30 μg/mL).

CONCLUSIONS

In conclusion, the paper reports synthesis and characterization data of some new natural product-inspired azetidin-2-ones. The evaluation of compounds for their antibacterial, antifungal, and cytotoxicity activity led to discovery of some significantly bioactive azetidin-2-ones. The compound **6h**, bearing two *p*-tolyl groups on C-3 and a 4-chlorophenyl group on the ring nitrogen, exhibited highly significant cytotoxicity. This study opens avenue for further investigation on azetidin-2-ones bearing diverse types of indole rings in order to develop potential antitumor compounds.

EXPERIMENTAL

Chemistry. Melting points have been recorded on a Stuart Scientific melting point apparatus and are uncorrected. The IR spectra were recorded on a Perkin-Elmer-781 IR spectrophotometer using KBr disc of the sample. The ^1H and ^{13}C NMR spectra were recorded in a $CDCl_3$ solution at 300 MHz and 75.4 MHz, respectively, on a Brucker™ 300 MHz spectrometer. The mass spectra were recorded on a Finnigan LCQ Deca mass spectrometer by electrospray ionization. *N*-methylindole-3-carboxaldehyde and amines used in the study were Aldrich products. The imines of *N*-methylindole-3-carboxaldehyde were prepared by refluxing it with appropriate amine in ethanol for 2–6 ([A-Za-z0-9,2–6 h [20]. 2-Diazo-1,2-diphenylethanones were prepared by oxidation of appropriate benzyl monohydrazone with bis(acetylacetonato)copper(II) according to the reported method [25].

General procedure for the synthesis of azetidin-2-ones (6a–j). An equimolar amount of an appropriate 2-diazo-1,2-diarylethanone **2a** or **2b** and appropriate imine 1 mmol of each in 8 mL of dry benzene was refluxed for 8 h under an atmosphere of N_2. The reaction mixture was allowed to stand overnight at room temperature. The solvent was evaporated under reduced pressure and residue was triturated with ethanol to afford the white crystalline product that was recrystallized with ethanol. The characterization data of the products are as follows.

4-(1-Methyl-1H-indol-3-yl)-1,3,3-triphenylazetidin-2-one (6a). Yield 82%; mp 108–110°C; IR (KBr, cm^{-1}): 1737, 1493, 1376, 739; ^1H NMR ($CDCl_3$, δ ppm): 7.84 (m, 3H), 7.59 (dd, *J* = 7.5, 1.2 Hz, 2H), 7.49 (m, 3H), 7.32 (m, 7H), 7.10 (m, 4H), 6.57 (s, 1H), 6.23 (s, 1H), 3.56 (s, 3H); ^{13}C NMR ($CDCl_3$, δ ppm): 167.6, 141.3, 138.5, 137.8, 136.9, 129.0, 128.9, 128.6, 128.2, 127.7, 127.4, 127.3, 127.2, 126.8, 124.0, 121.9, 119.8, 118.6, 117.7, 109.7, 108.5, 72.3, 61.1, 32.8; MS *m/z* (%): 428 (M^+, 20), 234 (72), 194 (100), 77 (30). *Anal*. calcd for $C_{30}H_{24}N_2O$: C, 84.08; H, 5.65; N 6.54. Found: C, 83.73; H, 5.97; N, 6.55.

4-(1-Methyl-1H-indol-3-yl)-3,3-diphenyl-1-(p-tolyl)azetidin-2-one (6b). Yield 88%; mp 152–154°C; IR (KBr, cm^{-1}): 1736, 1511, 1372, 739; ^1H NMR ($CDCl_3$, δ ppm): 7.6 (m, 3H), 7.25 (m, 4H), 7.10 (m, 6H), 6.88 (m, 4H), 6.35 (s, 1H), 5.98 (s, 1H), 3.35 (s, 3H), 2.11 (s, 3H); ^{13}C NMR ($CDCl_3$, δ ppm): 167.3, 141.4, 138.6, 136.9, 135.3, 133.5, 129.5, 128.9, 128.6, 128.3, 127.7, 127.4, 127.3, 126.3, 121.9, 119.8, 118.6, 117.6, 109.6, 108.7, 72.2, 61.1, 32.8, 21.0; MS *m/z* (%): 442 (M^+, 16), 248 (65), 194 (100), 91 (25). *Anal*. calcd for $C_{31}H_{26}N_2O$: C, 84.13; H, 5.92; N 6.33. Found: C, 83.86; H, 6.20; N, 6.28.

1-(4-Ethoxyphenyl)-4-(1-methyl-1H-indol-3-yl)-3,3-diphenylazetidin-2-one (6c). Yield 71%; mp 222–224°C; IR (KBr, cm^{-1}): 1735; 1510, 1241, 744; ^1H NMR ($CDCl_3$, δ ppm): 7.77 (t, *J* = 7.5 Hz, 3H), 7.45 (m, 4H), 7.29 (m, 6H), 7.03 (m, 3H), 6.79 (d, *J* = 9.0 Hz, 2H), 6.51 (s, 1H), 6.11 (s, 1H), 3.97 (q, *J* = 7.0 Hz, 2H), 3.58 (s, 3H), 1.39 (t, *J* = 7 Hz, 3H); ^{13}C NMR ($CDCl_3$, δ ppm): 166.8, 155.4, 141.3, 138.5, 136.8, 131.1, 128.8, 128.6, 128.2, 127.7, 127.3, 127.2, 126.7, 121.8, 119.7, 118.9, 118.5, 114.8, 109.6, 108.5, 72.1, 63.6, 61.0, 32.8, 14.0; MS (*m/z*, r. i.): 472 (M^+, 100). *Anal*. calcd for $C_{32}H_{28}N_2O_2$: C, 81.87; H, 5.97; N, 5.93. Found: C, 81.52; H, 6.25; N, 5.85.

1-(4-Chlorophenyl)-4-(1-methyl-1H-indol-3-yl)-3,3-diphenylazetidin-2-one (6d). Yield 62%; mp 109–112°C; IR (KBr, cm^{-1}): 1739, 1490, 1373, 739; ^1H NMR ($CDCl_3$, δ ppm): 7.73 (d, *J* = 7.5 Hz, 3H), 7.35 (m, 12H), 7.05 (m, 3H), 6.53 (s, 1H), 6.11 (s, 1H), 3.60 (s, 3H); ^{13}C NMR ($CDCl_3$, δ ppm): 167.5, 141.1, 138.2, 136.9, 136.2, 129.1, 128.9, 128.6, 128.1, 127.8, 127.5, 127.2, 127.1, 126.9, 122.0, 119.9, 118.9, 118.5, 109.7, 108.1, 72.5, 61.2, 32.9; MS (*m/z*, r. i.): 462 (M^+, 100). *Anal*. calcd for $C_{30}H_{23}ClN_2O$: C, 77.83; H, 5.01; N, 6.05. Found: C, 77.35; H, 5.40; N, 5.80.

4-(1-Methyl-1H-indol-3-yl)-1-phenyl-3,3-dip-tolylazetidin-2-one (6e). Yield 67%; mp 182–184°C, IR (KBr (cm^{-1}): 1732, 1498, 1370, 746; ^1H NMR ($CDCl_3$, δ ppm): 7.66 (d, *J* = 7.8 Hz, 1H), 7.48 (d, *J* = 8.1 Hz, 2H), 7.37 (dd, *J* = 7.8, 0.9 Hz, 2H), 7.1

(m, 7H), 6.98 (d, $J = 8.1$ Hz, 2H), 6.90 (t, $J = 7.5$ Hz, 1H), 6.70 (d, $J = 8.1$ Hz, 2H), 6.37 (s, 1H, CH), 5.97 (s, 1H, CH), 3.42 (s, 3H), 2.23 (s, 3H), 2.03 (s, 3H); ^{13}C NMR ($CDCl_3$, δ ppm): 167.9, 138.6, 137.8, 136.9, 136.8, 136.2, 135.6, 129.5, 128.9, 128.6, 128.4, 128.0, 127.3, 127.1, 123.8, 121.8, 119.7, 118.5, 117.6, 109.6, 108.6, 71.7, 61.0, 32.0, 21.1, 20.9; MS (*m/z*, r. i.): 456 (M^+, 100). *Anal*. calcd for $C_{32}H_{28}N_2O$: C, 84.18; H, 6.18; N, 6.14.; Found: C, 83.80; H, 6.35; N, 5.96.

4-(1-Methyl-1H-indol-3-yl)-1,3,3-trip-tolylazetidin-2-one (6f). Yield 72%; mp 197–198°C; IR (KBr, cm^{-1}): 1731, 1511, 1371, 741; ^{1}H NMR ($CDCl_3$, δ ppm): 7.66 (d, $J = 7.8$ Hz, 1H), 7.48 (d, $J = 8.1$ Hz, 2H), 7.26 (d, $J = 8.4$ Hz, 2H), 7.10 (m, 5H), 6.98 (d, $J = 8.4$ Hz, 2H), 6.92 (d, $J = 8.4$ Hz, 2H), 6.70 (d, $J = 8.1$ Hz, 2H), 6.37 (s, 1H, CH), 5.94 (s, 1H, CH), 3.44 (s, 3H), 2.24 (s, 3H), 2.15 (s, 3H), 2.05 (s, 3H); ^{13}C NMR ($CDCl_3$, δ ppm): 167.6, 138.6, 136.9, 136.1, 135.6, 135.3, 133.3, 129.41, 129.39, 128.6, 128.4, 128.1, 127.3, 127.1, 121.7, 119.7, 118.5, 117.6, 109.5, 108.7, 71.6, 61.0, 32.8, 21.0, 20.9; MS (*m/z*, r. i.): 470 (M^+, 100). *Anal*. calcd for $C_{33}H_{30}N_2O$: C, 84.22; H, 6.43; N, 5.95. Found: C, 84.25; H, 6.40; N, 5.65.

1-(4-Ethoxyphenyl)-4-(1-methyl-1H-indol-3-yl)-3,3-dip-tolylazetidin-2-one (6g). Yield 76%; mp 128–130°C; IR (KBr, cm^{-1}): 1728, 1506, 1231, 749; ^{1}H NMR ($CDCl_3$, δ ppm): 7.66 (d, $J = 7$ Hz, 1H), 7.48 (d, $J = 8.1$ Hz, 2H), 7.30 (dd, $J = 6.9$, 2.1 Hz, 2H), 7.18–7.05 (m, 5H), 6.99 (d, $J = 8.1$ Hz, 2H), 6.71 (dd, $J = 8.1$, 2.2 Hz, 2H), 6.65 (dd, $J = 7.1$, 2.0 Hz, 2H), 6.37 (s, 1H), 5.93 (s, 1H), 3.84 (q, $J = 6.9$ Hz, 2H), 3.45 (s, 3H), 2.25 (s, 3H), 2.05 (s, 3H), 1.26 (t, $J = 7.1$ Hz, 3H); ^{13}C NMR ($CDCl_3$, δ ppm): 167.2, 155.3, 138.7, 136.8, 136.1, 135.7, 131.3, 129.4, 128.6, 128.4, 128.0, 127.3, 127.1, 121.7, 119.7, 118.9, 118.5, 114.8, 109.5, 108.7, 71.6, 63.7, 61.1, 32.8, 21.1, 20.9, 14.8; MS (*m/z*, r. i.): 500 (M^+, 100). *Anal*. calcd for $C_{34}H_{32}N_2O_2$: C, 81.57; H, 6.44; N, 5.60. Found: C, 81.22; H, 6.76; N, 5.80.

1-(4-Chlorophenyl)-4-(1-methyl-1H-indol-3-yl)- 3,3-dip-tolylazetidin-2-one (6h). Yield 70%; mp 216–218°C; IR (KBr, cm^{-1}): 1731, 1491, 1370, 747; ^{1}H NMR ($CDCl_3$, δ ppm): 7.63 (d, $J = 7$ Hz, 1H), 7.48 (d, $J = 8.1$ Hz), 7.31 (m, 2H), 7.1 (m, 7H), 6.96 (d, $J = 8.1$ Hz, 2H), 6.70 (d, $J = 7.8$ Hz, 2H), 6.37 (s, 1H), 5.95 (s, 1H), 3.47 (s, 3H), 2.25 (s, 3H), 2.01 (s, 3H); ^{13}C NMR ($CDCl_3$, δ ppm): 167.8, 138.4, 137.1, 136.9, 136.3, 135.4, 129.5, 129.0, 128.9, 128.5, 128.4, 127.9, 127.2, 127.0, 121.9, 119.8, 118.8, 118.5, 109.6, 108.2, 72.0, 61.2, 32.8, 21.1, 20.9; MS (*m/z*, r. i.): 504 (M^+, 100). *Anal*. calcd for $C_{32}H_{27}ClN_2O$: C, 78.27; H, 5.54; N, 5.71. Found: C, 78.30; H, 5.40; N, 5.40.

Pharmacology. The bacterial and fungal cultures, identified earlier by 16S and 18S rRNA, were obtained from the H. E. J. University, Karachi, Pakistan.

In vitro antibacterial assay. *In vitro* antibacterial activity was assayed by disc diffusion method [26] against three Gram (+) strains: *S. aureus*, *B. subtilis*, and *M. luteus*, and two Gram (−) strains: *E. aerogenes* and *E. coli*. Each test compound (1 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO). The bacterial cultures were prepared fresh in the nutrient broth medium over 24 h. In order to compare the turbidity of bacterial culture, the McFarland 0.5% barium sulfate solution was used (as turbidity standard). To perform the antibacterial assay, nutrient agar Petri plates were prepared with sterile cotton swabs; respective bacterial colony lawns were prepared with sterile cork-borer (4 mm). Using a micropipette, 30 µL of test solution was poured into the respective wells and the Petri plates were incubated at 37°C for 24 h. After 24 h of incubation, the radius of the clear zone showing no bacterial growth was measured around each well. The zone of inhibition (mm) was calculated and compared with the standard drug cefixime.

In vitro antifungal assay. The antifungal activity was evaluated by the Agar tube dilution method [27] against *C. albican*, *M. canis*, *F. solani*, and *C. glabrata*. The concentration of the test compounds was 200 µg/mL of DMSO. A reference antifungal standard drug miconazole and DMSO were used as positive and negative controls, respectively. Incubation temperature was maintained at 27°C for 7 days. The inculcation of fungus was carried out with a 4.0 mm diameter piece of fungus removed from a seven-days-old culture. The growth in the compound-amended media was determined by measuring linear growth (mm) and growth inhibition calculated with reference to the negative control and % inhibition is reported.

Brine shrimps lethality assay. The cytotoxicity was studied by the brine-shrimps bioassay lethality method [28]. Brine shrimp (*Artemia salina*) larvae, used as test organisms, were hatched at 37°C in artificial seawater prepared by dissolving commercial sea salt (28 g) in distilled water (1.0 L). For each sample, the test was performed in three replicates at 10, 100, and 1000 µg/mL concentrations in DMSO. The survival rate of the larvae was observed against all concentrations of test compounds. For this purpose, 0.5 mL sample of each compound was taken and the solvent from each vial was evaporated followed by an addition of 2 mL of artificial seawater. Thirty shrimps were transferred to each vial and the final volume was adjusted to 5 mL by artificial seawater. The uncovered vials were placed under florescence light at 25°C for 24 h after which the number of survivors were counted and recorded. The data were analyzed with the Finney computer program (Finney, 1971) for probit analysis to determine the LD_{50} values with 95% confidence intervals.

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