Bioorganic & Medicinal Chemistry Letters 18 (2008) 5058–5062

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and cytotoxic activity of heterocyclic ring-substituted betulinic acid derivatives

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ARTICLE INFO

Article history:
Received 2 June 2008
Revised 8 July 2008
Accepted 1 August 2008
Available online 6 August 2008

Keywords:
Betulinic acid
Anticancer
Cytotoxicity

ABSTRACT

A new series of betulinic acid derivatives have been synthesized by introducing heterocyclic ring between C-2 and C-3 positions of betulinic acid. Further modifications were also carried out by reduction of C-20(29) unsaturated bond and substitution of C-28 carboxyl group by ester and amide linkage to enhance the selectivity. Compound **11** resulted in IC_{50} of 2.44, 2.5, and 2.7 μg/ml on MIAPaCa, PA-1, and SW620 cancer cell lines, respectively. Compound **38** resulted in IC_{50} of 0.67 μg/ml on MIAPaCa cell line.

Natural products played a major role in the anticancer drug discovery. Over 60% of the anticancer drugs are of natural origin. Betulinic acid (**1**), 3β-hydroxy-lup-20(29)-en-28-oic acid, a naturally occurring pentacyclic lupane-type triterpene, is widely distributed throughout the tropics. A variety of biological properties are ascribed to betulinic acid, but betulinic acid is recognized for its anticancer and anti-HIV activities.[1–4] Previous reports revealed that betulinic acid is a melanoma-specific cytotoxic agent,[5] but recent evidence has indicated that betulinic acid also possesses a broader spectrum of cytotoxic activity against other cancer cell lines.

Various modifications of substituents at positions 2, 3, 20, and 28 of betulinic acid have been the subject matter of all research efforts to obtain potent lead compounds.[6–11] All the above mentioned reports collectively disclose a large number of betulinic acid derivatives, with a vast majority of them found to possess antitumor activity. However, due to various reasons they are not particularly good candidates, clinically as well as do not have the best of pharmacokinetic properties. A need therefore exists for novel betulinic acid derivatives, which are not only potent, but also clinically safe and moreover, have better pharmacokinetic properties. In our efforts, we have found that in betulinic acid (**1**) heterocyclic ring-like indole, benzofuran and pyrrole at C-2 and C-3 positions, imparts the desired characteristics. Further changes were also carried out by reduction of C-20(29) unsaturated bond and substitution of C-28 carboxyl group by ester and amide linkage to enhance the selectivity (Table 1).[12]

Synthesis of betulinic acid derivatives has been described in Schemes 1–3.[13] Betulinic acid (**1**) was acetylated with acetic anhydride in presence of pyridine to afford 3-acetyloxy betulinic acid (**2**). Compound **2** upon hydrogenation with Pd/C in presence of hydrogen gas afforded 3-acetyloxy-20,29-dihydrobetulinic acid (**3**).[14] Dihydrobetulinic acid (**4**) was obtained by deacetylation of 3-*O*-acetyl 20,29-dihydrobetulinic acid (**3**) under basic conditions as shown in Scheme 1.

Both betulinic acid (**1**) and dihydrobetulinic acid (**4**) were oxidized in presence of Jones reagent to their respective betulonic acid (**5**) and 3-*O*-dihydrobetulinic acid (**6**), respectively.[15] Compounds **5** and **6** undergo Fischer indole synthesis with various arylhydrazines by loss of ammonia to afford compounds **7-19**.[16] *N*-Benzylpyrrolo substituted betulinic acid derivative (**20**) was afforded by the reaction of intermediate formed from the reaction of benzylamine with dihydrobetulinic acid (**6**), then with Michael acceptor nitroalkene.[17] Benzofuran derivatives (**21** and **22**) were synthesized from betulonic acid (**5**) and 3-*O*-dihydrobetulinic acid (**6**) with *O*-phenylhydroxylamine in presence of methanesulfonic acid as shown in Scheme 2.[18]

Ester derivatives **23–27** were synthesized by the reaction of compounds **7** and **11** with corresponding halides under basic condition. To synthesize amide derivatives (**28–37**), C-28 carboxylic group was converted to acyl chloride intermediate, which was further reacted with the corresponding amines. Compounds **28** and **29** upon hydrolysis under basic condition afforded compounds

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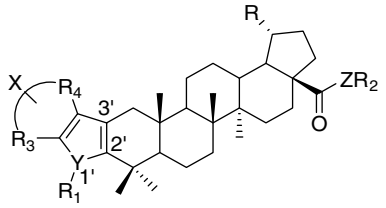
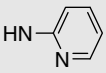
doi:10.1016/j.bmcl.2008.08.003

Table 1
Betulinic acid derivatives



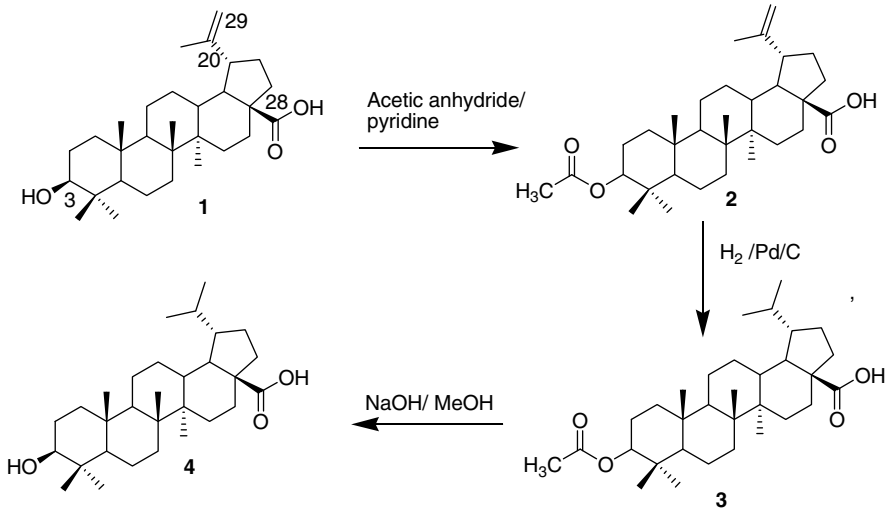
Compound	X	Y	R	R^1	Z	R^2	R^3	R^4
7	H	N	$C(=CH_2)CH_3$	H	O	H	Ph	
8	H	N	$CH(CH_3)_2$	H	O	H	Ph	
9	H	N	$C(=CH_2)CH_3$	CH_3	O	H	Ph	
10	H	N	$CH(CH_3)_2$	CH_3	O	H	Ph	
11	5′-Cl	N	$C(=CH_2)CH_3$	H	O	H	Ph	
12	5′-Cl	N	$CH(CH_3)_2$	H	O	H	Ph	
13	5′-F	N	$C(=CH_2)CH_3$	H	O	H	Ph	
14	5′-F	N	$CH(CH_3)_2$	H	O	H	Ph	
15	7′-Cl	N	$C(=CH_2)CH_3$	H	O	H	Ph	
16	7′-F	N	$C(=CH_2)CH_3$	H	O	H	Ph	
17	4′-Cl, 6′-Cl	N	$C(=CH_2)CH_3$	H	O	H	Ph	
18	5′-Cl, 7′-Cl	N	$C(=CH_2)CH_3$	H	O	H	Ph	
19	5′-OCH_3	N	$C(=CH_2)CH_3$	H	O	H	Ph	
20	—	N	$CH(CH_3)_2$	$C_6H_5CH_2$	O	H	H	Ph
21	H	O	$C(=CH_2)CH_3$	—	O	H	Ph	
22	H	O	$CH(CH_3)_2$	—	O	H	Ph	
23	H	N	$C(=CH_2)CH_3$	H	O	$CH_2C(O)OC(CH_3)_3$	Ph	
24	H	N	$C(=CH_2)CH_3$	H	O	$CH_2CH{=}CH_2$	Ph	
25	H	N	$C(=CH_2)CH_3$	H	O	CH_2Ph	Ph	
26	5′-Cl	N	$C(=CH_2)CH_3$	H	O	CH_2Ph	Ph	
27	H	N	$C(=CH_2)CH_3$	H	O	$CH_2PhNO_2(4)$	Ph	
28	H	N	$C(=CH_2)CH_3$	H	NH	$CH_2CO_2CH_3$	Ph	
29	5′-Cl	N	$C(=CH_2)CH_3$	H	NH	$CH_2CO_2CH_3$	Ph	
30	5′-Cl	N	$C(=CH_2)CH_3$	H	NH	$CH_2C{\equiv}CH$	Ph	
31	5′-Cl	N	$C(=CH_2)CH_3$	H	NH		Ph	
32	5′-Cl	N	$C(=CH_2)CH_3$	H	NH		Ph	
33	5′-Cl	N	$C(=CH_2)CH_3$	H	NH	$C_6H_4CF_3$ (4″)	Ph	
34	5′-Cl	N	$C(=CH_2)CH_3$	H	NH	$C_6H_4OCF_3$ (4″)	Ph	
35	H	N	$C(=CH_2)CH_3$	H	NH	CH_2Ph	Ph	
36	5′-Cl	N	$C(=CH_2)CH_3$	H	NH		Ph	
37	5′-Cl	N	$C(=CH_2)CH_3$	H	NH		Ph	
38	H	N	$C(=CH_2)CH_3$	H	NH	CH_2CO_2H	Ph	
39	5′-Cl	N	$C(=CH_2)CH_3$	H	NH	CH_2CO_2H	Ph	
40	H	N	$C(=CH_2)CH_3$	$C_6H_5CH_2$	NH	CH_2CO_2H	Ph	
41	5′-Cl	N	$C(=CH_2)CH_3$	$C_6H_5CH_2$	NH	CH_2CO_2H	Ph	

38 and **39**. In a similar way, compounds **40** and **41** were synthesized by benzylation at *N*-1 position using sodium hydride, followed by hydrolysis as shown in Scheme 3.

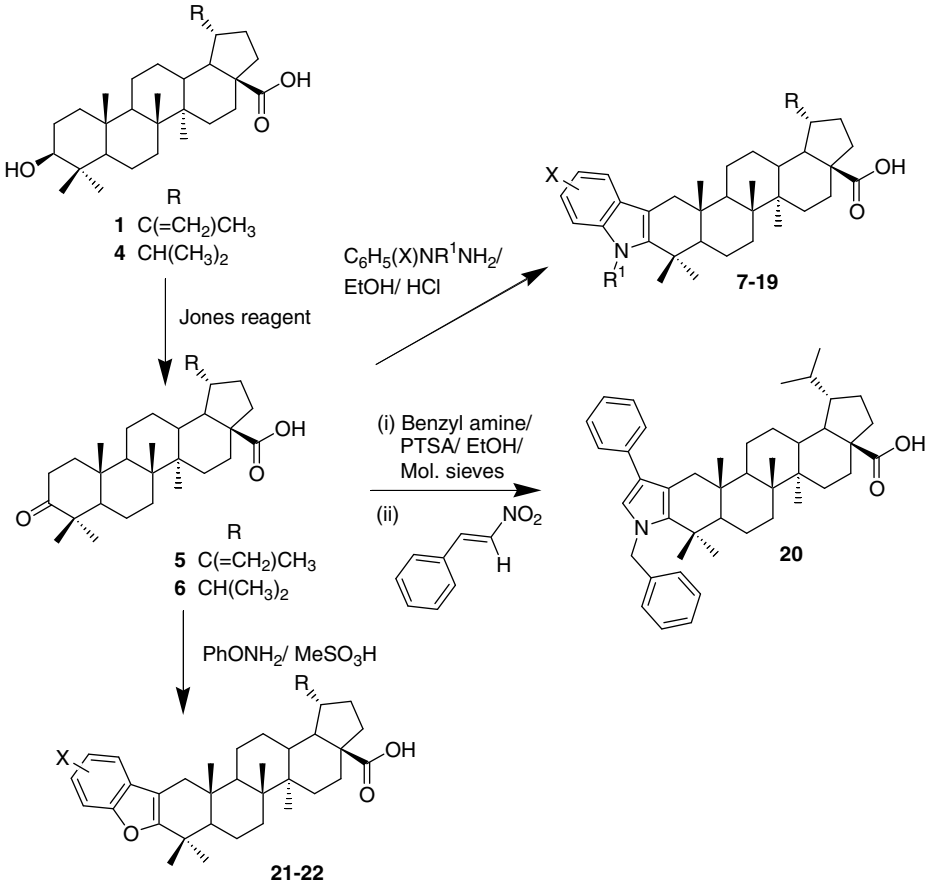
Results and discussion. All the synthesized betulinic acid derivatives (**7–41**) were tested for in vitro cytotoxicity on seven tumor cell lines as well as on one non-tumorous cell line, and IC_{50} values were calculated in micromole (μg/ml).[19,20] The human tumor cell lines used in the study are ovary (PA-1), prostate (DU145), colon (SW620), breast (HBL100), pancreas (MIAPaCa2), lung (A-549), and leukemia (K562) cancers. Compounds (**7–41**) were also screened against normal mouse fibroblast (NIH3T3) to evaluate their cancer cell specificity (safety index).[19] The cytotoxicity data are summarized in Table 2.

Unsubstituted indolo betulinic acid derivative (**7**) resulted in broad spectrum of cytotoxicity with IC_{50} of 5.15, 6.01, and 6.7 μg/ml on SW620, PA-1, and MIAPaCa cancer cell lines, respectively. Reduction of C-20(29) double bond led to inactive compound **8**. Introduction of electron-donating methyl group at N-1 position in indolo betulinic acid derivative (**9**) showed selectivity toward MIAPaCa cancer cell line with IC_{50} of 5.34 μg/ml. No major affect on activity was observed (**10**) upon reduction of C-20(29) double bond.

Introduction of the electron-withdrawing halogen group in indolo ring imparted potent cytotoxicity. Compound **11** having chloro group at C-5′ position is the most potent compound of this series. Compound **11** showed IC_{50} of 2.44, 2.5, and 2.7 μg/ml on MIAPaCa, PA-1, and SW620 cancer cell lines, respectively, with safety index of ~2 on MIAPaCa. Reduction of C-20(29) double bond (**12**) caused 2- to 3-fold decrease in the activity. However, when we replaced the chloro group with fluoro at C-5′ position (**13**), major fall in activity was observed. Reduction of double bond (**14**) caused some improvement in activity on MIAPaCa and A549 cancer cell lines. On changing the position of halo group from C-5′ to C-7′ in indolo ring, chloro-substituted derivative **15** resulted in maximum



Scheme 1.

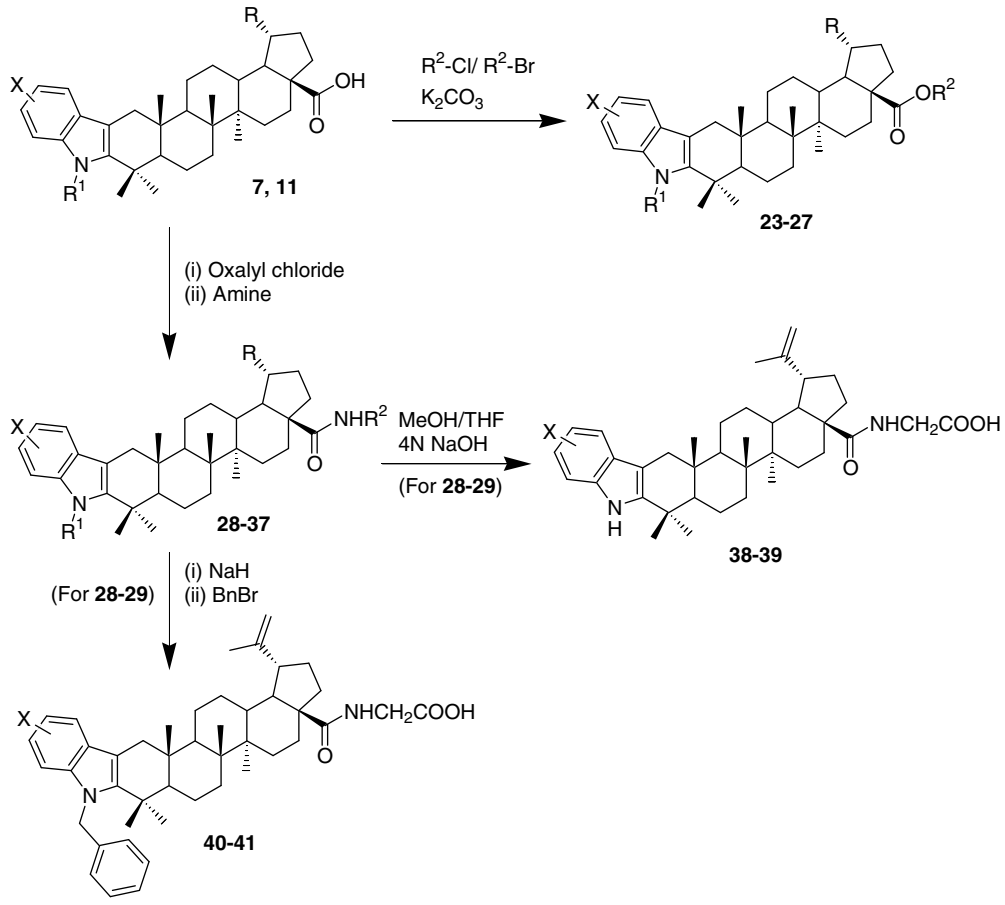


Scheme 2.

activity on SW620 cancer line with IC_{50} of 2.00 μg/ml, with safety index of ~6. Exchange of chloro with fluoro led to compound **16**, which showed potent activity with IC_{50} of <3.5 μg/ml on three cancer cell lines. Substitution of the indolo ring with dihalo electron-withdrawing group provided the selectivity toward A549 cancer cell line in compound **17**, but compound **18** was inactive. Replacing the electron-withdrawing halo group with electron donating methoxy group (**19**) did not result in substantial change in the activity.

N-Benzyl pyrrolo-substituted betulinic acid derivative (**20**) led to complete loss in the activity. Replacement of the indolo part with isosteric benzofuran led to inactive compound **21**. While reduction of C-20(29) double bond in benzofuran derivative (**22**) resulted in moderate activity on A549 cancer cell line.

As indolo-substituted betulinic acid derivatives **7** and **11** might have broad spectrum of cytotoxicity, their C-28 carboxyl group was further replaced with different ester and amide linkage. Replacement of the C-28 carboxyl group with ester groups like Boc (**23**),



Scheme 3.

Table 2
IC_{50} values of in vitro cytotoxicity of betulinic acid (BA) derivatives

Compound	IC_{50} (μg/ml) for cell lines							
	PA-1	DU145	SW620	HBL100	MIAPaCa	A549	K562	NIH3T3
1	11.53 ± 0.8	>20	13.26 ± 0.64	5.02 ± 0.7	>20	3.00 ± 0.7	3.25 ± 1.2	4.37 ± 0.7
7	6.01 ± 0.9	8.86 ± 0.2	5.15 ± 0.7	10.3 ± 0.9	6.7 ± 0.59	7.6 ± 0.57	10.05 ± 0.61	6.9 ± 0.76
9	>20	20 ± 0.6	>20	19.5 ± 2.1	5.34 ± 0.96	6.9 ± 0.21	7.73 ± 0.17	15.12 ± 3.04
10	>20	>20	>20	14.14 ± 0.8	5.4 ± 0.43	6.5 ± 3.4	10.85 ± 1.5	>20
11	2.5 ± 0.6	4.9 ± 0.9	2.7 ± 0.2	11.75 ± 1.65	2.44 ± 0.26	7.14 ± 0.5	9.61 ± 0.78	4.6 ± 0.14
12	6.6 ± 0.9	6.5 ± 1.2	5.9 ± 0.8	>20	15.19 ± 1.06	8.8 ± 0.74	>20	19.03 ± 0.21
13	>20	>20	9.16 ± 0.8	>20	17.4 ± 2.6	17.8 ± 1.79	10.95 ± 0.93	>20
14	>20	>20	7.28 ± 0.3	>20	8.4 ± 0.76	8.89 ± 0.84	14.01 ± 0.75	14.59 ± 0.33
15	6.39 ± 0.1	11.66 ± 0.71	2.00 ± 0.3	11.8 ± 0.35	11.6 ± 0.49	8.7 ± 0.19	9.26 ± 1.6	11.8 ± 0.19
16	ND	>20	>20	3.5 ± 0.9	3.3 ± 0.2	3.0 ± 0.6	8.7 ± 0.9	2.1 ± 0.55
17	>20	>20	>20	>20	>20	5.5 ± 1.1	>20	4.10 ± 0.9
19	5.8 ± 0.9	5.75 ± 0.75	8.4 ± 0.9	12.8 ± 0.39	6.4 ± 0.44	8.2 ± 0.14	>20	7.5 ± 0.48
22	>20	>20	>20	>20	8.8 ± 0.36	7.1 ± 1.6	15.28 ± 0.18	20 ± 5.6
38	3.0 ± 0.9	7.0 ± 0.7	8.7 ± 0.1	>20	0.67 ± 0.03	3.53 ± 0.82	11.92 ± 1.37	0.68 ± 0.003
39	6.66 ± 0.43	10.32 ± 0.9	10.42 ± 0.5	16.5 ± 0.6	10.7 ± 0.63	12.4 ± 1.78	>20	11.2 ± 0.63

Cytotoxicity was determined by MTT assay, as described.[19] Data shown are IC_{50} ± SD of three independent experiments. If IC_{50} was not achieved, it was represented as greater than highest concentration tested, that is, 20 μg/ml.
ND, not done.

allyl (**24**), and benzyl groups (**25–27**) led to the complete loss of the activity. Similarly, replacement of the C-28 carboxyl group with amide groups like amino acid ester (**28** and **29**), alkyl (**30**) cycloalkyl (**31** and **32**), aryl (**33–35**), and heteroaryl (**36** and **37**) amide led to the inactive compounds.

However, when we carried out the hydrolysis of ester compound **28**, it led to potent molecule **38**, which resulted in IC_{50} of 0.67, 3.0, and 3.53 μg/ml on MIAPaCa, PA-1 and A549 cancer cell lines, respectively. While compound **39** showed moderate activity with IC_{50} of 6.66 μg/ml on PA-1 cancer cell line. Substitution of N-1 position with benzyl group along with hydrolysis in compounds **28** and **29** resulted in inactive compounds **40** and **41**.

In the present study, several derivatives have shown better cytotoxicity than betulinic acid. The halo-substituted hetrocyclic ring (indolo) at C-2 and C-3 positions in betulinic acid afforded highly potent cytotoxic compounds **11**. Substitution of N-1 posi-

tion of indolo ring led to the loss of activity. So, N-1 position should be kept unoccupied. In most of the cases, hydrogenation of C-20(29) double bond decreased the cytotoxicity. At C-28 position, carboxylic group is essential for activity, replacement of carboxyl group with ester and amide ester leads to inactive compounds. However, hydrolysis of amide ester provided highly potent compound **38**. Compounds **11** and **38** have been selected for further studies.

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19. Derivatives of betulinic acid (**7–41**) were screened for cytotoxic activity at the highest soluble concentration of 20 μg/ml and at four lower concentrations on seven human tumor cell lines and one non-tumorous cell line. Briefly, cells were collected from 70–80% confluent adherent cultures by trypsinization (0.25% trypsin and 0.02% EDTA) and seeded in 96-well plates at a density of 5000 cells/well, except K562 which was seeded at15,000 cells/well in cell culture medium (DMEM) for 24 h in a CO_2 incubator. The test substance was dissolved in DMSO (Merck, India), and further dilutions were made in cell culture medium such that the final DMSO concentration in the well even at the highest concentration is less than 1%. After 24 h, the cells were incubated with the above-mentioned test substance to obtain drug concentrations in the range of 0.5–20 μg/mL. After 72 h of incubation in a CO_2 incubator, cytotoxicity was measured by the tetrazolium-based MTT assay adapted from previously published methods.[20] Briefly, 25 μL of MTT (5 mg/ml, Sigma, USA) was added to each well of the 96-well plate, and the plate was incubated at 37 °C for 3 h. MTT was converted to greenish-brown colored formazan by mitochondrial dehydrogenase enzyme present in viable cells. For adherent cells, the medium in the wells was gently pipetted out and replaced with 150 μL of DMSO and kept with gentle shaking for 15 min to dissolve formazan crystals. For suspension cultures, formazan was dissolved by direct addition of 50 μL of sodium dodecyl sulfate (SDS) acidified with 1N HCl, added to the wells followed by incubation for 1 h and the contents were mixed using a pipetman. The optical density (O.D.) in the wells was measured at 540 nm (for adherent cells) or 570 nm (for suspension cells) using a multi-well spectrophotometer. Percentage cytotoxicity was calculated using the formula given below $\%\text{Cytotoxicity} = 1 - (X/R_1) * 100$ where X = O.D. of wells containing the test substance and R_1 = O.D. of control wells. Each experiment was repeated thrice and IC_{50} values (half-maximal cytotoxicity) were calculated by employing non-linear regression analysis using Prism® software.
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