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Phytochemical investigation of labdane diterpenes from the rhizomes of *Hedychium spicatum* and their cytotoxic activity

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ABSTRACT

A comprehensive reinvestigation of chemical constituents from the rhizomes of *Hedychium spicatum* led to the isolation of two new labdane-type diterpene (**1**, **2**), together with six known compounds (**3**–**8**). Their structures were established on the basis of extensive spectroscopic (IR, MS, 2D NMR) data analysis and by comparison with the spectroscopic data reported in the literature. In addition, all the isolates were tested for their cytotoxicity against the THP-1 (human acute monocytic leukemia), HL-60 (human promyelocytic leukemia), A-375 (human malignant melanoma) and A-549 (human lung carcinoma) cancerous cell lines.

Labdane-type diterpenes constitute a significant class of natural products. They occur in several plants family of zingiberaceae and have been reported to possess a variety of biological activities such as antialgal, antibacterial, antifungal, antiprotozoal, enzyme inducing, anti-inflammatory, modulation of immune cell functions, as well as cytotoxic against human tumor cell lines.[1–3] *Hedychium spicatum* is a member of the zingiberaceae family, widely distributed in central Himalaya at 1100–2500 m altitude, East India, and hills of South India. It is locally known as 'kapura kachri' (Indian trade name) because of its camphor-like smell and a strong aromatic odor when fresh rhizomes are cut. Due to this reason its rhizomes are popular as an insect repellants and as a tobacco perfume.[4] Medicinally, essential oils of the rhizomes is used for the treatment of skin diseases, stomach ailments,[5] analgesic,[6] anti-emetic, blood purifier, anti-inflammatory,[7] antimicrobial,[8] antidote for snake bite, in vitro pediculicidal,[9] and mild tranquillizing[10] action of short duration, anthelmintic activity[11] and cytotoxic activity.[12]

As part of pharmacological–phytochemical integrated studies of medicinal plants from Indian flora,[13] we are investigating the chemical composition of plants from the genus *Hedichium* (zingiberaceae) as well as their cytotoxic activity. In the course, we have recently reported some labdane diterpenes from the *H. spicatum* which displayed moderate cytotoxicity against human cancer cells.[14] In the present work, to obtain the minor components, we decided to increase the amount of plant material and reinvestigate the chemical constituents of the rhizomes. This has led to the isolation of two new labdane diterpenes (**1**, **2**) and six known compounds (**3**–**8**). Herein, we describe the isolation, structure elucidation and anticancer activity of the labdane diterpene constituents of the *H. spicatum* (see Fig. 1).

Air dried rhizomes (1 kg) were ground and extracted at room temperature three times with DCM/MeOH (1:1). The combined extracts were concentrated under vacuum. The portion of active DCM/MeOH extract (6 g) was subjected to column chromatography (silica gel, 60–120 mesh) using step gradient of hexane/EtOAc to yield four major fractions (F_1–F_4). Fraction F_1 was subjected to repeated silica gel (100–200 mesh) column chromatography (CC) by eluting with EtOAc/ether/hexane (6:2:92) to yield compound **4** (1.89 g), with EtOAc/ether/hexane (8:5:87) to yield compound **2** (0.9 g). A portion of fraction F_2 was subjected to silica gel column chromatography with EtOAc/ether/hexane (15:5:80) to yield subfractions F_{2a} and F_{2b}. Fraction F_{2a} was purified by HPLC on a Phenomenex LUNA C18 column (250 mm × 10 mm,10 μ, 2 mL/min) with 50% acetonitrile in water as a eluent to give 0.069 g of compound **1**. Further, subfraction F_{2b} was eluted with EtOAc/ether/hexane (18:5:77) to yield compound **5**. Similarly, Fraction F_3 was subjected to repeated column chromatography eluting with EtOAc/ether/hexane (22:8:70) to yield 0.042 g of compound **3**, with EtOAc/ether/hexane (25:14:61) to yield 0.085 g of compound **8**. Fraction F_4 was subjected to repeated column chromatography eluting with EtOAc/ether/hexane (29:10:59) to yield 0.135 g of compound **6**, with EtOAc/ether/hexane (30:10:60) to yield 0.095 g of compound **7.**

The structures of the known compounds (**3**–**8**) were identified as yunnacoranarin D (**3**),[15] coronarin-E (**4**),[16] 8(12) drimene (**5**),[17]

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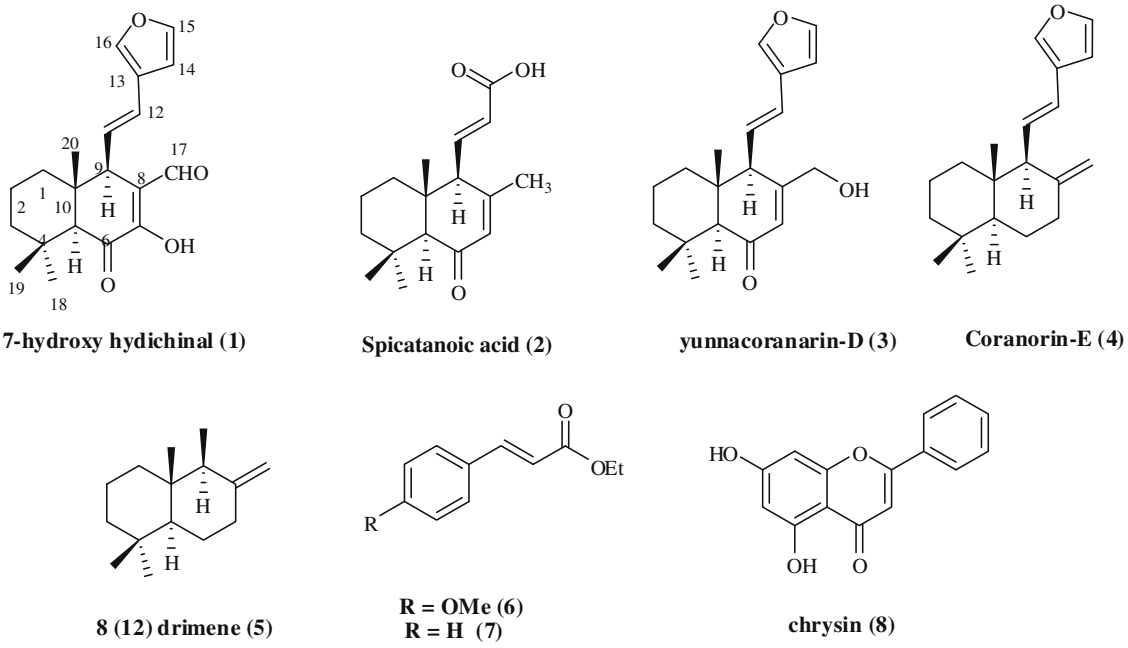


Figure 1. Compounds isolated from DCM/MeOH (1:1) extract of *H. spicatum*.

4-methoxy ethyl cinnamate (**6**),[18] ethyl cinnamate (**7**),[18] and chrysin (**8**)[19] from spectral data[20] comparison with those reported in the literature.

Compound **1** was isolated as a oil, with the positive optical rotation $[\alpha]_D^{25}$ +39.56 (c 1.89, $CHCl_3$). Electrospray-ionization MS showed a strong [M+H]+ ion peak at *m/z* 329 (100), and HRESIMS gave a [M]+ ion at *m/z* 329.1742 (calcd 329.1675), compatible with the molecular formula $C_{20}H_{24}O_3$, which is consistent with a diterpene structure. The IR spectrum displayed characteristic bands for α,β-unsaturated C=O (1647 cm^{-1}), α,β-unsaturated aldehyde (1682 cm^{-1}), and olefin (1622 cm^{-1}) functional groups. The ^1H NMR (Table 1) spectrum displayed three quaternary methyl singlets (δ 1.14, 1.21, 0.99), methine signals [δ 2.21 (H-5), 3.31 (d, *J* = 7.0 Hz, H-9)] and olefinic methine signals [δ 5.77 (dd, *J* = 15.8 Hz, 9.8 Hz, H-11), 6.37 (1H, d, *J* = 15.8 Hz, H-12)] suggesting the features of labdane diterpene skeleton. In addition, the analyses of the ^1H NMR spectrum ($CDCl_3$) (Table 1) indicated the existence of one aldehyde group [δ_H 9.81 (s, H-17)] and a furan moiety [δ_H 7.37 (1H, s), 7.45 (1H, s), 6.52 (1H, s)]. ^{13}C NMR spectrum of **1** (Table 1), together with the information from a DEPT spectrum, showed the presence of 20 carbon signals assigned to three methyls (tertiary), three methylenes (decalone moiety), seven methines (two olefinic, three furanoid methines, and two methines), six non protonated carbons (one carbonyl and three olefinic) and an aldehyde. Careful analysis and comparison of the ^1H and ^{13}C NMR spectra of **1** and **3** limited the differences to the substitution pattern around decalone nucleus. Complete assignment of protons and carbons was assisted by HMBC, COSY and

Table 1
^1H NMR and ^{13}C NMR data of compound **1** and **2**

Position	Compound **1**		Compound **2**	
	δc	δ_H multiplicity	δc	δ_H multiplicity
1	39.57	1.54 (2H, m)	40.20	1.52 (2H, m)
2	17.88	1.42 (2H, m)	18.18	1.44 (2H, m)
3	42.98	1.26 (2H, m)	43.04	1.22 (2H, m)
4	32.82	—	32.75	—
5	64.37	2.21 (1H, s)	63.33	2.09 (1H, s)
6	200.74	—	199.50	—
7	137.78	—	128.91	5.85 (1H, s)
8	150.24	—	154.37	—
9	54.92	3.01 (1H, d, *J* = 7.0 Hz)	61.73	3.01 (1H, d, *J* = 7.0 Hz)
10	43.37	—	43.27	—
11	125.31	5.77 (1H, dd, *J* = 15.8, 9.8 Hz)	148.24	6.94 (1H, dd, *J* = 15.8, 9.8 Hz)
12	123.96	6.37 (1H, d, *J* = 15.8 Hz)	125.77	6.01 (1H, d, *J* = 15.8 Hz)
13	124.19	—	170.33	—
14	143.60	7.37 (1H, s)	—	—
15	140.49	7.45 (1H, s)	—	—
16	107.44	6.52 (1H, s)	—	—
17	194.42	9.81 (1H, s)	21.84	1.79 (3H, s)
18	33.17	1.14 (3H, s)	33.75	1.13 (3H, s)
19	21.25	1.21 (3H, s)	22.95	1.16 (3H, s)
20	15.59	0.99 (3H, s)	16.04	1.01 (3H, s)

Assignments were based on 2D NMR including DQF-COSY, HSQC, HMBC and NOESY. Well-resolved couplings are expressed with coupling patterns and coupling constants in hertz in parentheses. For overlapped signals, only chemical shift values are given.

HSQC. The position of aldehyde group at C-17 was supported by HMBC correlations from H-17/C-8,C-9. Further, the presence of the furan ring in **1** was confirmed by its HMBC correlations from H-14/C-13, C-15; H-15/C-14; H-16/C-13 (Fig. 2).

The *trans* double bond at C-11/C-12 was confirmed by the coupling constant (15.8 Hz) from the ^{1}H NMR spectrum. In the ^{13}C NMR spectrum, signal at δ 199.50 was assigned to α,β-unsaturated carbonyl group at C-6, corresponding with the HMBC cross-peaks between H-5 (δ 2.21) /C-6 (δ 200.74). Relative configuration of **1** was determined from the analysis of the 2D NOESY data (Fig. 4). The configuration of the decalone portion of compound **1** was assumed to be the same as that of known diterpenes bearing the same skeleton such as yunnacoronarin D.[15] Further analysis of its NOESY spectrum showed the correlations between H_3-19 / H_3-20; H-5 /H-9, H_3-18 and H_3-20. These data were in accordance with the β-orientation of H_3-19, H_3-20, H-16 and α-orientation of H-5 and H-9. Thus, based on these discussions, the structure of **1** was confirmed as 7-hydroxy,15,16-epoxy-17-al-7,11,13(16),14-labdatetraene-6-one, a new labdane-type diterpene trivially named as 7-hydroxy hedichinal (**1**).

Compound **2** was obtained as a yellow semisolid, with the positive optical rotation $[\alpha]_D^{25}$ +83.16 (*c* 1.75, $CHCl_3$). The molecular formula was determined as $C_{17}H_{24}O_3$ based on the molecular ion peak at *m/z* 277.1712 $[M+H]^+$ (calcd 277.1725) in the HRESIMS. The ^{1}H and ^{13}C NMR spectra (Table 1) provided signals that were characteristic of a labdane-type diterpene. The IR spectrum showed the absorptions for hydroxyl (3425 cm^{-1}), α,β-unsaturated carbonyl (1691 cm^{-1}) and carboxylic acid (1664 cm^{-1}) groups, respectively. The ^{1}H NMR spectrum (recorded in $CDCl_3$) displayed four quaternary methyl signals as singlets at δ 1.01 (H_3-20), 1.13 (H_3-18), 1.16 (H_3-19), 1.79 (H_3-17). It also displayed signal attributed to one methine (H-5) adjacent to the carbonyl (C-6) carbon atom at δ 2.09 (1H, s) and a characteristic doublet for one proton at δ 3.01 (d, *J* = 7.0 Hz) indicating the presence of methine (H-9) group adjacent to olefinic double bond. The presence of *trans* double bond at δ 6.94 (1H, dd, *J* = 15.8, 9.8 Hz) and δ 6.01 (1H, d, *J* = 15.8 Hz) was suggested by the ^{1}H NMR, and NOESY spectrum. Comparison of NMR data with that of yunnacoronarin D[15] suggested the presence of trans double bond at C (11)/C (12) position. Further, also had a sharp singlet at δ 5.85 (1H, H-7) and a partially overlapped multiplets due to the three methylenes between δ 1.52 and 1.22 in ^{1}H NMR spectrum confirmed the labdane diterpene frame work.

The ^{13}C NMR (Table 1) spectrum of compound **2** showed the presence of 17 carbon atoms and were further classified by DEPT experiments into the categories of four methyls (δ 16.04, 21.84, 22.95 and 33.75), three methylenes (δ 18.18, 40.20 and 43.04), five methine groups (δ 61.73, 63.33, 125.77, 128.91 and 148.24) and five quaternary carbon atoms including two carbonyls (δ 199.50 and 170.33). The signal at δ 199.50 is due to α,β-unsaturated C=O at C-6 and another signal at δ 170.33 is due to C=O of carboxylic acid (C-13). The major portion of the labdane skeleton frame work could be assembled through the interpretation of COSY, HSQC, HMBC and NOESY correlations. Interpretation of ^{1}H–^{1}H COSY experiment revealed the sequential correlations of H-1 through H-2 to H-3 and H-11 to H-12. In the HMBC spectrum, the olefinic proton at δ 6.01(d, *J* = 15.8 Hz, H-12) showed strong correlation with C-11 (δ 148.24), C-9 (δ 61.73) and acid carbonyl group at δ 170.33. The characteristic methine H-9 (δ 3.01) showed correlations with C-11, C-12 and C-8. In addition to the above correlations, carbon skeleton suggested several diagnostic correlations H-5/C-4, C-6, C-10; H-11/C-9, C-12, C-13; H-17/C-8, C-9 and H-18, H-3, H-19/C-4 (Fig. 3).

The relative stereochemistry at chiral centers was consistent with that of **1** based on the analysis of NOESY spectrum as well as biogenetic considerations (Fig. 4). NOE effects between H_3-19/H_3-20; H-5/H-9, H_3-18 and H-5/H-9 suggested the β-orientation of H_3-19, H_3-20 and α-orientation of H-5 and H-9. Based on these data, compound **2** was identified as 14,15,16-trinor-7,11-labdadien-13-oicacid, a new labdane-type diterpene trivially named as spicatanoic acid (**2**).

Biological activities of the isolates were evaluated by in vitro cytotoxicity test, which was carried out with the four panel of cancerous cell lines that comprises human acute monocytic leukemia (THP-1), human promyelocytic leukemia (HL-60), human malig-

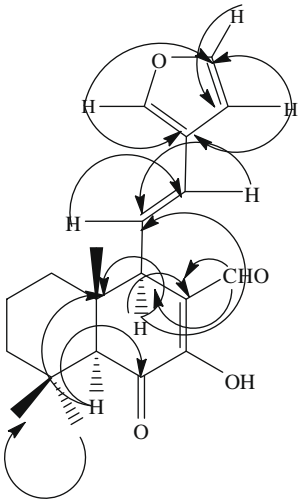
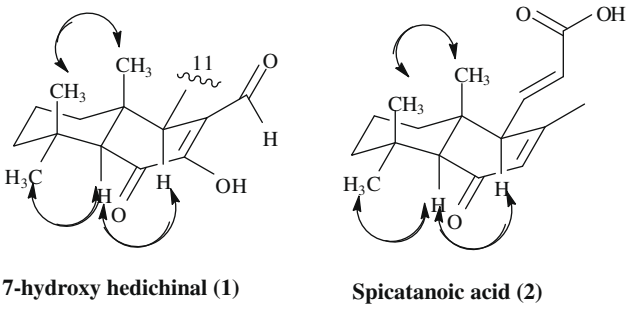


Figure 2. Key HMBC correlations of compound **1**.



7-hydroxy hedichinal (1) **Spicatanoic acid (2)**

Figure 4. NOESY relations of compounds **1** and **2**.

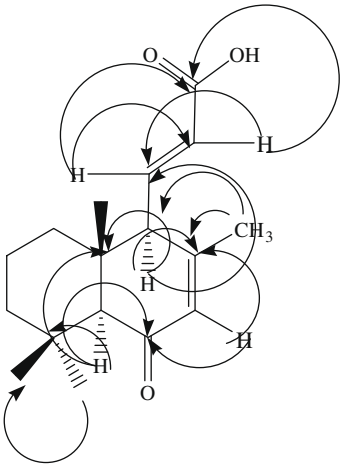


Figure 3. Key HMBC correlations of compound **2**.

Table 2
Cytotoxicity effects of DCM/MeOH extract and constituents from *H. spicatum*

Compound	HL-60	Cell lines (IC_{50} μM)		
		THP-1	A-375	A-549
DCM/MeOH extract	58.14 ± 1.12[*]	37.45 ± 0.9[a]	58.08 ± 0.15[a]	63.21 ± 1.19[a]
1	15.95 ± 0.09	23.36 ± 1.05	21.58 ± 0.06	37.85 ± 1.01
2	69.39 ± 1.09	38.96 ± 1.01	56.08 ± 0.05	51.97 ± 1.09
3	36.43 ± 0.01	59.49 ± 0.09	41.91 ± 0.18	51.24 ± 1.21
4	31.21 ± 0.06	33.99 ± 0.07	36.58 ± 0.02	53.26 ± 1.09
5	NA	53.89 ± 0.06	50.19 ± 0.80	61.29 ± 1.11
6	29.64 ± 0.02	33.52 ± 0.04	31.29 ± 0.81	NT
7	35.69 ± 1.10	41.23 ± 0.80	31.59 ± 0.15	50.35 ± 1.29
8	33.98 ± 0.01	23.38 ± 0.09	29.65 ± 0.06	19.88 ± 1.01
Etoposide[*]	2.16 ± 0.18	1.83 ± 0.26	3.92 ± 0.15	9.51 ± 1.29

NA = not active, NT = not tested, each value represents the mean ± standard deviation.
[*] Etoposide was considered as positive control.
[a] Values expressed in μg/mL.

nant melanoma (A-375) and human lung carcinoma (A-549), using Etoposide as a standard. The screening procedure was based on the standard MTT assay.[21] The experiments were repeated in triplicate, and the IC_{50} values were expressed as mean ± standard deviation (Table 2).[22] As observed from Table 2, structural differences of the labdane diterpenes significantly affected the anticancer activity. Among the isolates, compound **1** exhibited potent activity and **2** showed moderate activities on the tested cell lines with the IC_{50} value ranging from 14.14 μg/mL to 36.56 μg/mL. The furan moiety and hydroxyl group at C-7 position, are markedly affected the activity profile of the labdane diterpenes class compounds. It is interesting that yunnacoronarin D (**3**), the structural analog of **1**, showed weak activity, this may be due to lack of hydroxyl group at C-7.

In this study, we have focused to investigate anti cancer principles from the *H. spicatum* and found two new labdane diterpenes with good cytotoxic activity against tested cancer cell lines. Based on these preliminary reports obtained by us, further study directed towards the synthesis of derivatives, SAR/lead identification towards the cancer is currently being investigated.

Acknowledgements

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20. *Spectral data for the known compounds* (**3**–**8**):*Yunnacoronarin D* (**3**): IR (KBr) ν_{max} 3418, 1666, 1507, 1377, 1231, 1160 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.42 (1H, s, H-16), 7.38 (1H, s, H-15), 6.49 (1H, s, H-14), 6.30 (1H, d, J = 9.8 Hz, H-12), 6.12 (1H, s, H-8), 5.75 (1H, dd, J = 9.8 Hz, 15.6 Hz, H-11), 4.15 (1H, dd, J = 1.8, 3.7 Hz, H-17), 4.09 (1H, dd, J = 1.8, 3.7 Hz, H-17), 3.01 (1H, d, J = 7.0 Hz, H-9), 2.16 (1H, s, H-5), 1.71 (2H, m, H_2-2), 1.45 (2H, m, H_2-1), 1.23 (2H, m, H_2-3), 1.21, 1.18, 0.99 (3H each, all s, 20, 18, 19-H_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 200.01 (C-6), 158.43 (C-8), 143.83 (C-16), 140.58 (C-15), 125.33 (C-7), 124.71 (C-12), 124.13 (C-11), 123.60 (C-13), 107.35 (C-14), 63.77 (C-17), 63.71 (C-5), 58.62 (C-9), 43.27 (C-3), 43.07 (C-10), 39.98 (C-1), 32.73 (C-4), 33.48 (C-19), 21.68 (C-20), 18.03 (C-2), 15.76 (C-18). ESI-MS: m/z 315 (M^++H).*Coronarin E* (**4**): IR (KBr) ν_{max}: 3080, 2940, 2860, 1645, 1460, 1440, 1390, 1370, 1160, 1070, 1025, 970, 895, 595 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.35 (1H, s, H-16), 7.34 (1H, s, H-15), 6.54 (1H, s, H-14), 6.19 (1H, d, J = 9.8 Hz, H-12), 5.76 (1H, dd, J = 9.8, 15.6 Hz, H-11), 4.76 (1H, dd, J = 1.8, 3.7 Hz, H-17), 4.53 (1H, dd, J = 1.8, 3.7 Hz, H-17), 2.40 (1H, d, J = 9.8 Hz, H-9), 1.78–1.05 (m, CH_2's), 0.90, 0.85 and 0.84 (3H each, all s, 20, 19, 18-H_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 150.13 (C-8), 143.21 (C-16), 139.57 (C-15), 128.21 (C-12), 124.47 (C-13), 121.74 (C-11), 107.97 (C-17), 107.61 (C-14), 61.44 (C-9), 54.78 (C-5), 42.29 (C-3), 40.74 (C-1), 39.12 (C-10), 36.75 (C-7), 33.55 (C-18), 33.53 (C-4), 23.36 (C-6), 21.94 (C-19), 19.11 (C-2), 14.99 (C-20). ESI-MS: m/z 285 (M^++H). *8(12)-Drimene* (**5**): IR (KBr) ν_{max}: 3080, 1645, 1381, 1372, 1225, 1164 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 4.68 (1H, dd, J = 1.8, 3.7 Hz, H-17), 4.51 (1H, dd, J = 1.8, 3.7 Hz, H-17), 2.21 (1H, q, J = 1.8, 3.7 Hz, H-9), 1.78–1.05 (m, CH_2's), 1.11, 0.90, 0.85 and 0.84 (3H each, all s, 11, 20, 19, 18-H_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 150.13 (C-8), 21.74 (C-11), 59.44 (C-9), 51.78 (C-5), 42.21 (C-3), 40.04 (C-1), 38.12 (C-10), 36.15 (C-7), 33.12 (C-18), 33.01 (C-4), 23.36 (C-6), 21.94 (C-19), 19.11 (C-2), 14.99 (C-20). ESI-MS: 207 (M^++H). *Ethyl cinnamate* (**6**): IR (KBr) ν_{max} 3415, 1618, 1440, 1162, 835, 625 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 7.54 –7.29 (aromatic protons, m), 7.59 (1H, d, J = 16.5 Hz), 6.38 (1H, d, J = 16.5 Hz), 4.23 (2H, q), 2.12 (3H, t). ESI-MS: m/z 177 (M^++H). *p-Methoxy ethyl cinnamate* (**7**): IR (KBr) ν_{max} 3450, 2925, 1654, 1621, 1016 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) (δ) 7.31–7.52 (aromatic protons, m), 7.65 (1H, d, J = 16.5 Hz), 6.41 (1H, d, J = 16.5 Hz), 4.23 (2H, q), 3.14 (3H, s, –OMe), 2.12 (3H, t). ESI-MS: m/z 207 (M^++H). *Chrysin* (**8**): IR (KBr) $\nu_{max:}$ 3450, 2925, 1654, 1621, 1016 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 7.92–7.82 (2H, m, H-2′, 6′), 7.58–7.44 (3H, m, H-3′, 4′, 5′), 6.64 (1H, d, J = 2 Hz, H-8), 6.44 (1H, s, H-3), 6.24 (1H, d, J = 2 Hz, H-6). ESI-MS: m/z 255 (M^++H).
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22. *In vitro cytotoxicity evaluation*: All the isolates were tested for in vitro cytotoxicity on different cancer cell lines. The cell lines used in this study were THP-1 (human acute monocytic leukemia), HL-60 (human promyelocytic leukemia), A-375 (human malignant melanoma) and A-549 (human lung carcinoma) cancerous cell lines. All the cells were obtained from National Center for cellular Sciences (NCCS), Pune, India. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, at 37 °C with 5% CO_2. The cells were seeded at 1×10^4 cells/well. After 24 h, cells were treated with the test compound and IC_{50} values were calculated in μM/mL.