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Bioactivity-guided isolation of cytotoxic constituents from stem–bark of *Premna tomentosa*

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ABSTRACT

A bioassay-guided fractionation and chemical investigation of the stem bark of *Premna tomentosa* resulted in the isolation and characterization of four new icetexane diterpenes (**1–4**), along with the known compounds coniferaldehyde (**5**), syringaldehyde (**6**), lupeol (**7**), betulin (**8**), and 2-(4-methoxyphenyl)-2-butanone (**9**). 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. The new compounds exhibited diverse functionalities on a common icetexane diterpene skeleton. In addition, cytotoxic activities of the icetexanes (**1**–**3**) were evaluated by determining their inhibitory effects on the human cancer cell lines (MCF-7, HT-29, Hep-G2, A-431, and A-549). Compounds **1** and **3** showed selective inhibitory activity against MCF-7 (15.96 μg/mL and 15.84 μg/mL) and HT-29 cell lines (16.21 μg/mL and 14.57 μg/mL), respectively.

The genus Premna (Verbenaceae) comprises a group of more than 200 different trees, distributed in tropical and subtropical areas of the world.[1] *Premna tomentosa* (Verbanaceae) is a well-known medicinal plant used extensively for the treatment of various ailments. In Indian system of medicine, all parts of *P. tomentosa* have been employed for the treatment of various disorders. Its bark extract is claimed to have a lasting cure for hepatic disorders.[2] Extracts from *P. tomentosa* leaves are known to have diuretic,[3] hepatoprotective,[4] antioxidant,[5] lipid-lowering,[6] immunomodulatory activities,[7] and protective against acetaminophen-induced mitochondrial dysfunction properties.[8] In spite of the various pharmacological uses of *P. tomentosa* extracts, little is known about the chemical constituents. Previous studies on this species have resulted in the isolation of various compounds, including flavonoids, triterpenoids, and steroids.[9] As part of our continuing efforts directed towards the discovery of the structurally interesting and biologically active compounds from the Indian medicinal plants,[10] an initial screening procedure was conducted using the cancer cell lines. It was found that hexane extract of *P. tomentosa* showed cytotoxic activity against colon cancer (Colo-205), skin cancer (A-431), breast cancer (MCF-7), liver cancer (Hep-G2), and lung cancer (A-549) cell lines. Bioactivity-guided phytochemical analysis of hexane extract led to the isolation of four new icetexane type diterpenes (**1–4**) (Fig. 1) and the purification of the five known compounds (**5–9**). In this Letter, we describe the isolation, structural elucidation, and biological activity of the new icetexanes.

The dried stem bark (1 kg) were ground and extracted three times with hexane in a soxhlet apparatus. The combined extracts were concentrated under vacuum. Active hexane extract (2.5 g) was subjected to column chromatography (silica gel, 60–120 mesh) using step gradient of hexane/EtOAc to yield six major fractions (F1–F6). Fraction F1 was subjected to repeated silica gel (100–200 mesh) column chromatography (CC) by eluting with hexane–chloroform–acetone (5:3.2:1.8) to yield compound **3** (0.005 g). Fraction F2 was subjected to silica gel column chromatography eluting with hexane–chloroform–acetone (5:3.4:1.6) to get compound **4** (0.002 g). Fraction F3 was subjected to silica gel column chromatography with the elution of hexane–chloroform–acetone (5:3:2) to yield 0.080 g of compound **1**, with hexane–chloroform–acetone (5:3.8:1.2) to afford 0.005 g of compound **2.** Similarly, Fractions F4 and F5 were subjected to repeated column chromatography eluting with EtoAc:hexane (19:81) to yield 0.7 g of compound **5**, and 0.091 g, 0.098 g of compounds **6** and **7**, respectively. Finally, fraction F6 was purified by the silica gel column chromatography (100–200) with the elution of hexane:EtOAc (98:2) to afford the compound **8** (0.550 g) and **9** (0.025 g) in pure form.

Compound **1** was obtained as a colorless gum ($[\alpha]_D^{25}$ +8.0 (*c* 0.05, chloroform)), with the molecular formula $C_{20}H_{30}O_4$, determined from the HRESIMS that revealed a molecular ion peak at *m/z*

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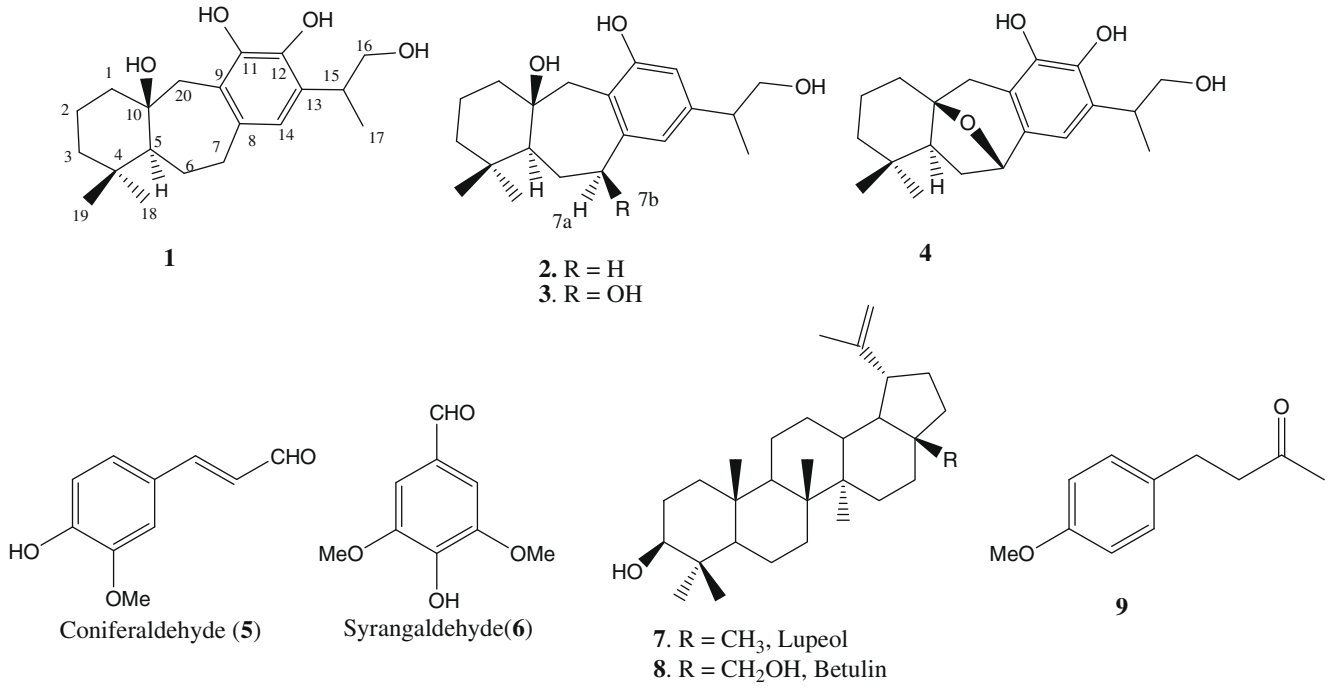

Figure 1. New icetexanes (**1**–**4**) and known compounds (**5**–**9**) isolated from hexane extract of *Premna tomentosa*.

334.2077 (calcd 334.2144), implying six degrees of unsaturation. The IR spectrum of **1** indicated the presence of hydroxyl groups (3280 cm^{-1}) and aromatic moiety (1634, 1560 cm^{-1}). The UV spectrum displayed absorption maxima at 280 nm indicative of simple phenyl ring. Initial interpretation of DEPT and ^{13}C NMR spectral data showed that majority of the proton signals were from methylene protons. The ^{1}H NMR spectrum of **1**, measured in $CDCl_3$ contained two methyl singlets at δ 0.95 (H_3-18) and 0.98 (H_3-19) and one methyl doublet at δ 1.76 (d, J = 7.2 Hz, H_3-17). It also displayed signals attributable to one oxygen-bearing methylene at δ 3.79–3.84 (m, H-16) and a multiplet at δ 3.41–3.46 from a deshielded methine (H-15). Furthermore, two proton resonances δ 6.08 and 8.12 (each, 1H, s), which did not show any correlation with all the carbons in the HSQC spectrum, were assigned to hydroxyl groups. The ^{1}H NMR also showed characteristic AB system of C-20 methylene protons at δ 2.47 (d, J = 14.1 Hz, H-20a), δ 3.17 (d, J = 14.1 Hz, H-20b), together with overlapped multiplets due to five methylenes between δ 1.28 and 2.60 were suggested that compound **1** is an icetexane type diterpene, with the structure similar to that of demethylsalvicanol.[11]

The major portion of the icetexane skeleton frame work, which is composed of mostly methylene carbons, assembled through the interpretation of 2D NMR (COSY, HMBC, and HSQC) correlations. Interpretation of ^{1}H–^{1}H COSY spectra revealed the connectivities between H-1/H-2/H-3, H-5/H-6, and H-6/H-7 protons. The low field quaternary carbon at δ 70.24 (C-10) was attributed to a hydroxyl group bearing carbon due to its HMBC correlations from H-1, H-20, and H-5 (Fig. 2). In the HMBC spectrum (Fig. 2), two hydroxyls resonating at δ 6.08 and 8.12 were assigned to C-11 and C-12 by the HMBC correlations from 11-OH to C-9 (δ 118.67), C-12 (δ 139.03), C-20 (δ 41.21) and from 12-OH to C-11 (δ 143.32), C-13 (δ 135.33), respectively.

Moreover, aromatic proton at δ 6.34 (s, 1H) could be assigned to H-14 by its HMBC correlations with C-13 (δ 135.33), C-8 (δ 127.21) and C-9 (δ 118.67). The ^{1}H–^{1}H COSY correlations from H_3-17 through H-15 to H-16, in combination with HMBC correlations from H-15 to C-12, C-13 and C-14 were suggestive of isopropanol moiety in **1**. Finally, methine proton at δ 1.54–1.61 (1H, m) was assigned to H-5 as a result of its HMBC correlations with C-10 (δ 70.24), C-6 (δ 28.37) and correlation of CH_3-19, CH_3-18 to C-5. In addition, relative configuration of **1** was determined from biogenetic basis[11] and by analysis of the 2D NOESY data. The configuration of the basic icetexane portion of compound **1** was assumed to be the same as that of known diterpenes bearing the same skeleton such as demethylsalvicanol. Its NOESY spectrum showed the correlations between H_3-18/H-5; H-10/H-19. These data were in accordance with the β-orientation of H_3-19, OH-10 and α-orientation of H-5 and H-18. Based on these data, the structure of **1** was identified as 8,11,13 icetexatriene-10,11,12,16-tetrol, a new diterpene and trivially named as icetexane-1.

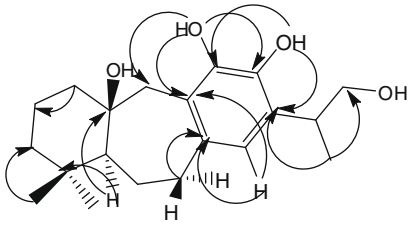

Figure 2. Key HMBC correlations of compound **1**.

Compound **2** was isolated as a colorless gum with $[\alpha]_D^{25}$ +5 (*c* 0.05, chloroform). The molecular formula of **2** was defined as $C_{20}H_{30}O_3$ from the sodiated molecular ion peak at *m/z* 341.2096 $[M+Na]^+$ in the HRESIMS (calcd 341.2084), indicating one oxygen atom less than in the case of **1**. The IR spectrum proved the existence of hydroxyl (3280 cm^{-1}) and aromatic functionalities (1634 and 1560 cm^{-1}). Both ^{1}H and ^{13}C NMR spectral data (Table 1) disclosed the same key structural features of **1**, including the same icetexane diterpene skeleton with isopropanol moiety. The difference in the ^{1}H NMR spectrum of **2** with that of **1** was the substitution pattern of phenyl ring in which two aromatic methines at δ 6.89 (1H, s) and at δ 6.42 (1H, s) were shown. The DEPT experiment also proved the presence of two aromatic methine signals instead of one in case of **1**, which was associated with the absence of ^{1}H

Table 1
^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectral data of icetexanes **1–4**

Position	1		2		3		4	
	δ (^{1}H)	δ (^{13}C)	δ (^{1}H)	δ (^{13}C)	δ (^{1}H)	δ (^{13}C)	δ (^{1}H)	δ (^{13}C)
1	1.22–1.28 (m)	33.04	1.22–1.28 (m)	33.02	1.21–1.28 (m)	33.09	1.22–1.28 (m)	33.08
2	1.24–1.29 (m)	17.34	1.23–1.29 (m)	17.45	1.23–1.31 (m)	19.96	1.24–1.32 (m)	19.92
3	1.46–1.52 (m)	20.32	1.42–1.49 (m)	20.32	1.44–1.50 (m)	28.56	1.40–1.48 (m)	26.58
4	—	29.66	—	29.49	—	36.55	—	36.59
5	1.54–1.61 (m)	56.71	1.54–1.60 (m)	56.73	1.52–1.58 (m)	51.33	1.51–1.59 (m)	51.32
6	1.81–1.87 (m)	28.37	1.82–1.88 (m)	28.37	1.84–1.91 (m)	32.01	1.83–1.88 (m)	32.01
7	2.56–2.64 (m)	39.65	2.52–2.59 (m)	39.66	4.84 (d, 12)	72.64	4.84 (d, 12)	80.72
8	—	127.21	—	127.32	—	128.21	—	128.32
9	—	118.67	—	118.90	—	134.88	—	134.80
10	—	70.24	—	70.57	—	69.84	—	76.26
11	6.08 (s, OH)	143.32	6.04 (s, OH)	143.33	6.07 (s, OH)	142.88	6.08 (s, OH)	142.81
12	8.12 (s, OH)	139.03	6.42 (s)	112.32	6.45 (s)	112.12	8.13 (s, OH)	144.19
13	—	135.33	—	135.26	—	140.52	—	141.52
14	6.34 (s, OH)	115.43	6.32 (s)	116.46	6.34 (s)	118.01	6.32 (s)	116.02
15	3.41–3.46 (m)	34.60	3.40–3.46 (m)	35.76	3.42–3.48 (m)	30.92	3.43–3.49 (m)	30.54
16	3.79–3.84 (m)	68.46	3.78–3.85 (m)	68.43	3.77–3.84 (m)	70.14	3.79–3.83 (m)	70.44
17	1.76 (d, 7.2)	14.24	1.74 (d, 7.2)	14.26	1.76 (d, 7.2)	16.24	1.75 (d, 7.2)	16.29
18	0.95 (s, Me)	22.86	0.96 (s, Me)	22.89	0.95 (s, Me)	15.48	0.94 (s, Me)	15.23
19	0.98 (s, Me)	31.09	0.98 (s, Me)	32.70	0.98 (s, Me)	26.98	0.98 (s, Me)	26.31
20	2.47 (d, 14.1)	41.21	2.46 (d, 14.1)	41.24	2.47 (d, 14.1)	41.21	2.47 (d, 14)	41.22
	3.17 (d, 14.1)		3.18 (d, 14.1)		3.17 (d, 14.1)		3.17 (d, 14.1)	

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 Hz in parentheses. For overlapped signals, only chemical shift values are given.

NMR signal of 12-OH group. This finding was further confirmed by its HMBC correlations from H-12 (δ 6.42, s) to C-13 (δ 135.26) and C-11 (δ 143.33). Isopropanol group was found to be attached at C-13 as evidenced by the HMBC correlations from H-15 (1H, m, 3.40–3.46) to C-12 (δ 112.23) and C-14 (δ 116.46). The proposed structure was further confirmed by the key HMBC correlations between H-2/C-1; H-2/C-3; H-20/C-10; H_3-18/C-5; H_3-19/C-3; H_3-18/C-4; H-5/C-6; H-5/C-10; H-14/C-9, C-12; OH-11/C-11, C-12, C-9; H_3-17/C-15, C-16, C-13 and H-15/C-13, C-12, C-14. The relative stereochemistry at chiral centers was consistent with that of icetexane-1(**1**) based on the analysis of NOESY spectrum as well as biogenetic considerations. NOE effects between H-5 with H_3-18 and absence of the correlations between H-19 and H-5 indicated that β-orientation of H_3-19 and α-orientation of H_3-18 and H-5. Based on these discussions, compound **2** was identified as 8,11,13 icetexatriene-10,11,16-triol, which is a new diterpene and trivially named as icetexane-2.

Compound **3** was isolated as a colorless gum with $[\alpha]_D^{25}$ +9 (*c* 0.05, chloroform). The molecular formula of **3** was determined to be $C_{20}H_{30}O_4$ by HRESIMS (*m/z* 333.2077) [M^+−H], implying six degrees of unsaturation. IR absorption bands at 3286, 1632, and 1560 cm^{-1} suggested the presence of hydroxyl group and aromatic moiety. The IR, ^{1}H and ^{13}C NMR spectra of **3** was almost identical to those of **2** except the difference due to hydroxyl group at C-7. This was indicated by the presence of an additional hydroxymethine group (δ 4.8 dd; δ 72.64, CH-OH), which was confirmed by its HMBC correlations to C-6 (δ 32.01), and in turn the correlations of H-6 (δ 1.84–1.91, m) to C-5 (δ 51.33) and C-7 (δ 72.64) (Fig. 3). Further, a set of oxymethylene protons resonated at δ 3.77–3.84 (H-16, m) and exhibited HMBC correlations of H-15 (1H, m, 3.42–3.48) to C-12 (δ 112.12) and C-14 (δ 118.01) indicating presence of propanol group in **4**. Relative stereochemistry of **4** was determined from the NOESY correlations between H-5/H_3-18, H-7/H-5 which were in agreement with the β-orientation of H_3-19 and α-orientation of H_3-18, H-7a, and H-5. Thus, structure of **3** was confirmed as 8,11,13 icetexatriene-7,10,11,16-tetrol, which is a new icetexane diterpene and trivially named as icetexatriene-3.

Compound **4** was isolated as a gum with $[\alpha]_D^{25}$ −26.0 (*c* 0.05, chloroform). The molecular formula of **4** was determined as $C_{20}H_{28}O_4$ on the basis of HRESIMS ion at *m/z* 333.1992 [M+H]$^+$ (calcd 333.2066), corresponding to seven degrees of unsaturation. The IR absorptions implied the presence of hydroxyl (3250 cm^{-1}), ether (1230 cm^{-1}), and aromatic (1615 and 1510 cm^{-1}) moieties. Extensive analysis of the NMR spectroscopic data revealed that the structure of **4** was closely related to **1**, possessing isopropanol group and aromatic ring. The presence of the aromatic ring was concluded from its ^{13}C NMR spectrum (δ 128.32, 134.80, 142.81, 144.19, 141.52, and 116.02), and isopropanol group located on the aromatic ring confirmed on basis of the HMBC correlations (Fig. 4). On the basis of the molecular formula $C_{20}H_{28}O_4$ revealed by HRESIMS, there were three remaining oxygen atoms in the molecule other than those associated with isopropanol. Two of these were due to hydroxyl groups resonating at δ 6.08 and δ 8.12 attributable to C-11 and C-12 by the HMBC correlations 11-OH to C-12 (δ 144.19), C-20 (δ 41.22) and from 12-OH to C-11 (δ 142.81), C-13 (δ 141.52), respectively. From the ^{13}C NMR spectrum, the carbon at δ 80.72 (C-7) should be attached to the oxygen atom as in the case of **3** and the carbon at δ 76.26 located at C-10 was also attached to an

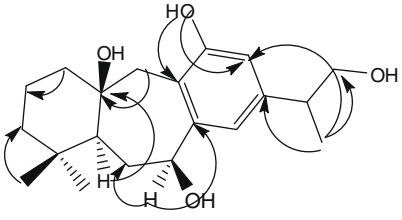

Figure 3. Key HMBC correlations of compound **3**.

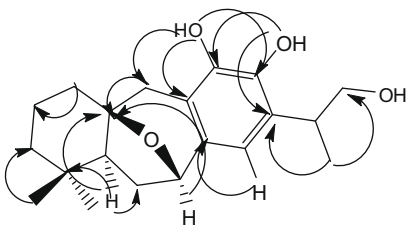

Figure 4. Key HMBC correlations of compound **4**.

oxygen atom on the basis of the HMBC spectrum (Fig. 4). Since there was only oxygen left, these two carbons were concluded to be attached to the same oxygen atom to form the cyclic ether functionality,[12] which is commonly found in taxane diterpenes.[13] The HMBC spectrum demonstrated the correlations between H-5/C-10, H-7/C-8 and H-7/C-10 verifying the presence of cyclic ether ring. Other key HMBC correlations were observed between H-2/C-1; H-2/C-3; H-20/C-10; H_3-18/C-5; H_3-19/C-3; H_3-18/C-4; H-5/C-6; H-5/C-10; H-14/C-9, C-12; OH-11/C-11, C-12, C-9 and H_3-17/C-15, C-16, C-13.

Investigation of 2D NMR data including HMBC and NOESY spectra enabled the assignment of all substituent groups and the relative configuration of **4**, which was comparable to compounds **1–3**. As observed previously for all the above compounds, NOESY spectra revealed the interactions between H-5/H_3-18, and H-5/H-7, which were in agreement with the β-orientation of H_3-19 and α-orientation of H_3-18, H-7, and H-5. Based on the data, structure of **4** was identified as a 7,10-epoxy-8,11,13 icetexatriene-11,12,16-triol, which is new icetexane diterpene and trivially named as icetexane-4.

In addition to the above compounds, five known compounds were also isolated from the hexane extract. By comparison of their physical and spectroscopic data with the literature they were characterized as coniferaldehyde (**5**),[14] syringaldehyde (**6**),[15] lupeol (**7**),[16] betulin (**8**),[17] 2-(4-methoxyphenyl)-2-butanone[18] (**9**). To the best of our knowledge, this is the first report on chemical analysis of compounds **5–9** from *P. tomentosa*.

Compounds **1–3** were tested for in vitro cytotoxicity on cancer cell lines and IC_{50} values were calculated in microgram (μg/mL).[19] Due to the insufficient quantity of the compound **4**, we were unable to test for its activity. The cell lines used for this study are the colon cancer (Colo-205), skin cancer (A-431), breast cancer (MCF-7), liver cancer (Hep-G2), and lung cancer (A-549). The results (Table 2) showed that all the tested icetexanes (**1–3**) exhibited consistent activity with an IC_{50} value ranging from 14.57 μg/mL to 123.1 μg/mL. It was found that compounds **1** and **3** are

Table 2
Cytotoxic activities of compounds **1–3**

Compound	Cell lines (IC_{50}, μg/mL)				
	HT-29	MCF-7	Hep-G2	A-549	A-431
Hexane extract	41.04 ± 6.08	75.77 ± 1.61	45.01 ± 0.60	61.65 ± 0.04	123.1 ± 14.7
1	16.21 ± 0.00	15.96 ± 0.21	18.63 ± 0.73	18.62 ± 0.02	NA
2	NA	80.75 ± 4.65	NA	43.65 ± 0.32	NA
3	14.57 ± 0.69	15.84 ± 0.37	34.41 ± 0.46	21.37 ± 0.10	NA

NT, not tested; NA, not active.

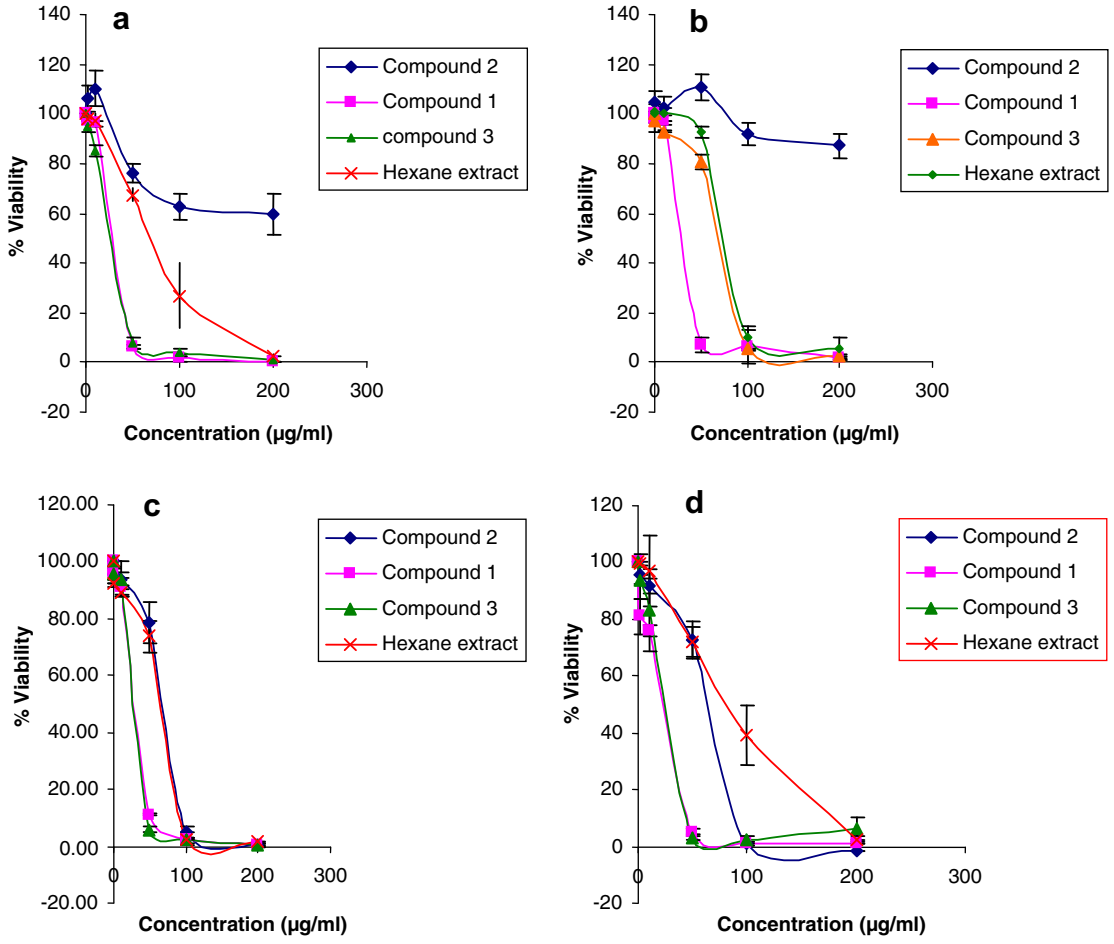

Figure 5. Cell viability studies after treatment of icetexanes at various concentrations. (a) Hep-G2 cells; (b) HT-29 cells; (c) MCF-7 cells; (d) A-549 cells.

showing potent activity against A-549 (lung cancer) HT-29 (colon cancer), and MCF-7 (breast cancer) cell lines. To compare cytotoxic activity between crude hexane extract and isolated compounds, cells were exposed to a series of concentrations of either extract or isolated compounds for 48 h and the percentage of viability was determined by MTT method which was presented in Figure 5. The concentration of isolated compounds causing 50% cytotoxicity (IC_{50}) was much lower for the isolated compounds compared to that of crude extract (Table 2).

The icetexane family of natural products encompasses a variety of structurally unique and bioactive natural products. Most of the known icetexane type diterpenes have been isolated from Salvia species, and only a few originated from another genus.[11,20] The present work implies the isolation and characterization of the four new icetexane diterpenes from the *P. tomentosa* and their cytotoxic activity against human cancer cell lines. It is noteworthy to mention that icetexane diterpenes represent the first examples from the Premna genus. Thus, isolation of the above compounds from Premna species is of interest from a chemotaxonomic perspective. As suggested by the Simmons et al.[21] biosynthetic connection of these structurally similar icetexanes involved a series of oxidative or reduction of the abietane [6-6-6] tricyclic skeleton, which serves as a biosynthetic precursor to the icetexanes via ring expanding rearrangement.

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