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Original article

A new cytotoxic quinolone alkaloid and a pentacyclic steroidal glycoside from the stem bark of *Crataeva nurvala*: Study of anti-proliferative and apoptosis inducing property

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ABSTRACT

Chemical investigation of stem bark of *Crataeva nurvala* afforded 5,7-dimethoxy-3-phenyl-1-ethyl-1,4-dihydro-4-quinolone and a steroidal glycoside with unprecedented pentacyclic ring system named crataemine (**1a**) and crataenoside (**2**) respectively. The structures of the compounds were determined by spectroscopic analysis. A series of compounds with modification at position 1 of **1a** (**1a**–**1c**) were prepared. All compounds were screened for cytotoxic activity against HeLa, PC-3 and MCF-7 cells. Only **1a** and **2** showed potency against all three cells. Mechanism based study for activity of the compounds demonstrated that it could block the migration of more aggressive HeLa and PC-3 cells and prevent their colony formation ability as well. The compounds potentiated apoptosis in HeLa and PC-3 cells in a significant manner.

1. Introduction

Plant secondary metabolites and their semi-synthetic derivatives continue to play an important role in cancer chemopreventive studies. Few anticancer agents amongst a broad arsenal of naturally occurring compounds currently undergoing clinical trials include podophyllotoxin, camptothecin, vinblastine and paclitaxel. Considerable efforts have also been paid to generate promising lead compounds through high throughput screening (HTS) protocols, combinatorial chemistry and bioinformatics tools. However, natural products have an edge as they are evolved for some special biological purpose and exhibit remarkable HTS hit rates, much higher than to be expected. Usually these compounds possess unique built-in chirality which promotes most effective binding to complex proteins and other three-dimensional biological receptors. Hence, the best way to find new leads amid unexplored chemical space is to turn to those natural product scaffolds that are unrepresented.

Crataeva nurvala Buch. Ham. (Capparaceae), an important medicinal plant of Ayurvedic and Unani system of medicine has been studied mainly with the stem bark part for obstructive and nonobstructive uropathies. It is also used for the treatment of prostate enlargement and bladder sensitivity [1]. Phytochemical studies showed that it contained lupeol, betulinic acid, β-sitosterol, stigmasterol [2] and cabadicine [3] as major chemical constituents of the plant. Lupeol had been reported to reduce the deposition of calcium oxalate, the most common stone forming constituent in animals. It also possessed antifartility, anticancer, antitumor, anti-arthritic, anti-inflammatory, hepatoprotective, and cardioprotective activities [4]. In our continuous search for new pharmacologically active agents from Indian medicinal plants, we investigated *C. nurvala* for cytotoxic activity. Preliminary screening identified diethyl ether (Et_2O) and *n*-BuOH fractions of the methanolic extract (MeOH–H_2O; 9:1) of the plant as most active. Hence, subsequent analysis was carried out with these fractions separately. Herein, we report i) isolation and characterization of two new compounds viz., **1a** and **2**; ii) synthesis of compounds **1a**–**1c** and iii) screening of all these compounds for cytotoxic activity on human cervical cancer

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(HeLa), human prostate cancer (PC-3) and human breast cancer (MCF-7) cells. The active compounds were also studied for anti-proliferative and apoptosis inducing property.

2. Results and discussion

2.1. Chemistry

Repeated column chromatography of Et_2O fraction of stem bark of *C. nurvala* afforded crataemine (**1a**) while, *n*-BuOH fraction afforded crataenoside (**2**) in minor quantity. The structures of the compounds were elucidated by 1D and 2D NMR spectroscopy and mass spectrometry (Fig. 1).

The HRESIMS of **1a** gave $[M + H]^+$ ion peak at *m/z* 310.1434 indicating the molecular formula of $C_{19}H_{19}O_3N$ (calc. 310.1437). The presence of a conjugated carbonyl and an NR function attached to an olefinic moiety was revealed by IR absorption bands at 1641 and, 1635 and 1617 cm^{-1} respectively. The ^1H and ^{13}C NMR spectrum displayed signals for an N–Et function [δ 4.04 (q, 2H, $J = 7.2$ Hz) and δ 1.43 (t, 3H, $J = 6.6$ Hz); δ_C 48.6 and 13.9], a trisubstituted olefinic moiety [δ_H 7.44 (s, 1H); δ_C 139.4], a monosubstituted phenyl ring [δ 7.57 (d, 2H, $J = 8.4$ Hz), δ 7.30 (app t, 2H, $J_1 = 8.4$ Hz, $J_2 = 1.5$ Hz), δ 7.21 (dd, 1H, $J_1 = 8.4$ Hz and $J_2 = 1.5$ Hz); δ_C 128.8, 127.8 (each 2 × CH) and 126.6 (CH)] and a conjugated carbonyl at δ_C 175.5. Additionally, two aromatic methoxyl protons at δ 3.88 and 3.85 (each s, 3H) and two meta coupled aromatic protons at δ 6.30 and 6.27 (each d, 1H, $J = 2.1$ Hz) indicated the presence of a 1,2,3,5 tetra substituted phenyl moiety. It was further supported by the corresponding methoxyl carbons at δ_C 56.1, 55.3 and two meta coupled aromatic carbons at 94.1 and 89.8 respectively. These partial structures were in consistence with an N-Et-4-quinolone moiety un-substituted at C-2 position. The COSY and HSQC correlations showed connectivities between five aromatic carbons of a mono-substituted phenyl ring and between aromatic protons of the quinolone moiety (Fig. 2a). Moreover, the intense $^1H–^1H$ NOESY cross peaks between H-2/H_2-1″; H_2-1″/H-8 and H_2-1″/H_2-2″ confirmed that C-2 position of the compound was unoccupied while C-3 was linked to the phenyl ring. Consequently, the structure of **1a** was established as 5,7-dimethoxy-3-phenyl-1-ethyl-1,4-dihydro-4-quinolone. Comparison of the ^1H and ^{13}C NMR data of **1a** to those reported in the literature showed that it possesses striking similarity with the synthetically derived 5,7-dimethoxy-3-phenyl-1-methyl-1,4-dihydro-4-quinolone (**1c**). The two compounds differ essentially only by one methylene group, **1a** possessed N-ethyl functionality whereas **1c** contained N-Me group [5].

In order to understand the significance of naturally occurring N-ethyl derivative we first prepared N-unsubstituted (**1b**) derivative by coupling of 3,5-dimethoxyaniline with ethyl α-formylphenyl acetate in inert atmosphere producing the intermediate, ethyl (*Z*)-3-(3,5-dimethoxyanilino)-2-phenyl-2-propenoate by following the reported procedure. Subsequent cyclization of the intermediate in a solution of biphenyl and diphenyl ether followed by heating at 250 °C produced 5,7-dimethoxy-3-phenyl-1,4-dihydro-4-quinolone (**1b**). Alkylation of **1b** with methyl iodide and ethyl iodide in the presence of potassium carbonate in anhydrous DMF under nitrogen atmosphere produced **1c** and **1a** respectively (Scheme 1). The crude residue was purified further by silica gel column chromatography. The structure of **1b** and **1c** was confirmed by comparing their physical and spectroscopic data with the reported literature values.

Crataenoside (**2**) was obtained as an amorphous solid. The molecular formula of **2** was established as $C_{33}H_{54}O_6$ by using positive ion HRESIMS exhibiting an $[M + H]^+$ peak at *m/z* 547.3979 and a quasi-molecular ion peak of $[M + Na]^+$ at 569.3901. A broad IR absorption band at 3385 cm^{-1} suggested the presence of hydroxyl functionality. The ^1H NMR spectrum showed signals for five methyl groups including two tertiary methyl groups at δ 1.02 and 0.7, one secondary methyl group at δ 0.93 and two geminal methyl groups at δ 0.86 and 0.83. Further, it showed one olefinic proton at δ 5.37, a broad methine multiplet of a carbinol proton at δ 3.59 and one anomeric proton at δ 4.40 (d, $J = 7.8$ Hz). The large coupling constant (7.8 Hz) of the anomeric proton along with five methine and one methylene carbons at δ_C 100.6, 76.1, 75.6, 73.1, 69.7 and 61.1 indicated the presence of a β-D-glucose moiety attached to an aglycon part. Analysis of ^{13}C and HMQC spectral data of **2** revealed that the aglycon, bearing 27 carbons contained three quaternary carbons including one olefinic; nine methine comprising an olefinic; ten methylenes, and five methyl groups. The long range HMBC correlations between H-1′ (δ 4.40) and C-3 (δ_C 78.3), and C-1′ (δ_C 100.6) to H-3 (δ 3.59) confirmed the position of attachment of the sugar moiety in the aglycon. Additionally, the HMBC connectivities of methyl protons at CH_3-19 to C-10, C-1, C-5 and C-9; CH_3-18 to C-13, C-17, C-14 and C-12; CH_3-21 to C-20, C-17 and C-22 showed exactly the same pattern to that of the partial structure contributed by ABCD rings of cholesterol [6]. The HMBC correlations also supported the relative configurations at A to B, B to C and C to D ring junctions, identical to that of cholesterol types. The $^1H–^1H$ COSY correlations between C-6 to C-7, C-3 to C-4 and C-2, and C-2′ to C-1′ and C-3′ further supported the above assumption. However, significant differences were observed mainly with the carbon signals and their splitting pattern, arising from the side chain of compound **2** to that of cholesterol. The comparative study showed that **2** contained ten methine and eight methylene carbons while cholesterol possessed nine methine and nine methylene carbons. It revealed the presence of one more degree of unsaturation. The HMBC spectrum showed prominent correlations between geminal methyl groups at δ 0.86 and 0.83 (each d, 3H, $J = 6.6$ Hz; H-26 and H-27) and corresponding carbons at δ_C 18.8 and 18.4. It also showed correlations between C-25 (δ_C 27.6) and C-24 (δ_C 45.3) (Fig. 2b). The COSY cross peaks

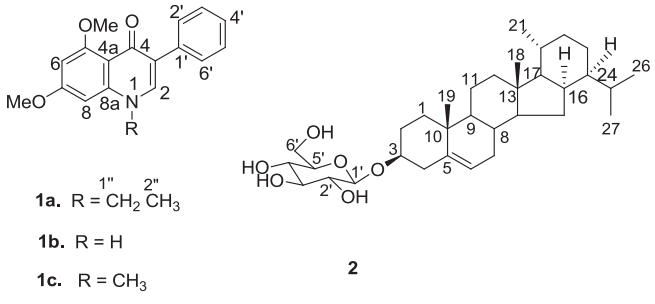


Fig. 1. Chemical structures of crataemine (**1a**), 1-unsubstituted (**1b**) and 1-Me (**1c**) substituted derivatives of **1a** and crataenoside (**2**).

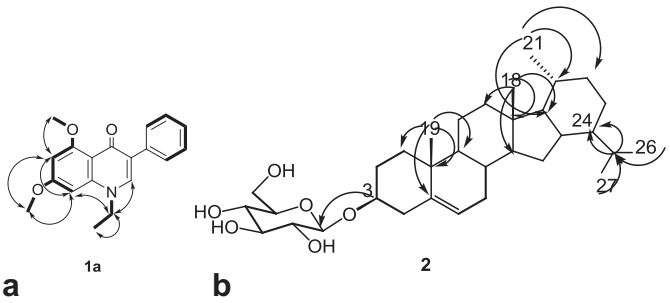
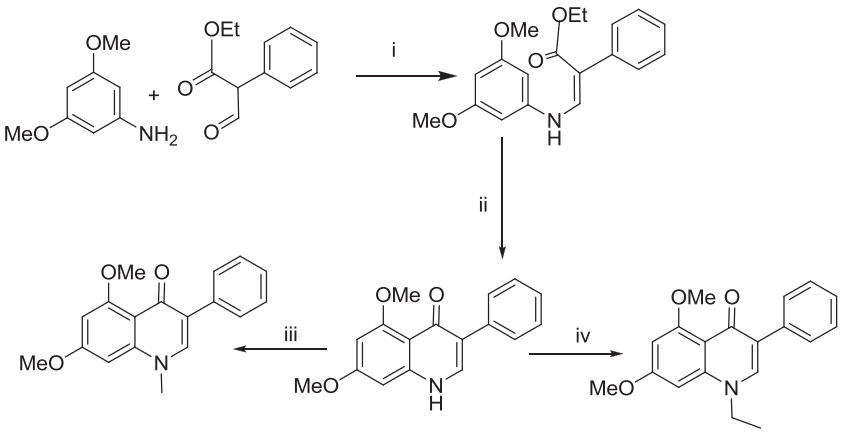


Fig. 2. 2D NMR correlations of **1a** and **2**. (a) Showing HSQC and COSY correlations of **1a** in bold lines and important NOESY correlations by arrows. (b) Showing HSQC and COSY correlations in bold lines and important HMBC correlations by arrows.

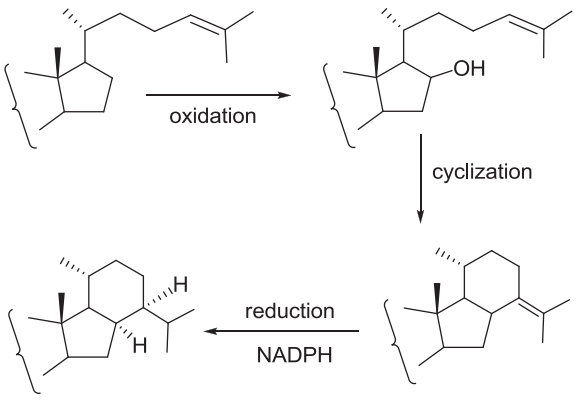


Scheme 1. Synthesis of compounds **1a**–**1c**. Reagents and conditions: (i) reflux, 18 h, N_2 atm, toluene, 73%; (ii) biphenyl + diphenyl ether, 250 °C, 10 min, 60%; (iii) CH_3I + anhydrous K_2CO_3, DMF, N_2 atm, rt, 18 h, 75%; (iv) C_2H_5I + anhydrous K_2CO_3, DMF, N_2 atm, rt, 18 h, 70%.

indicated the presence of sequence C-26, C-27 to C-25 and C-25 to C-24. Finally, as there was no additional olefinic carbon, the extra one degree of unsaturation could be devoted only by the formation of an additional ring. Biogenetically, cholesterol and other phytosterols can undergo a variety of structural transformation in plants, especially involving side chains but formation of pentacyclic structures [7,8] is not common in plant kingdom. A study on the mode of hydroxylation of steroids at non-activated secondary carbons in higher plants demonstrates that the introduction of the 16β hydroxyl group in the biosynthesis of gitoxiginin from pregnenolone in *Digitalis pupurea* proceeds by the direct displacement of the 16β proton [9]. A chemical investigation conducted on aerial part of *Polianthes tuberose* reported a bisdesmosidic cholestane glycoside with an apiofuranoside group at C-16 [10]. On the basis of all these observations a plausible biogenetic pathway for crataenoside was proposed as shown in Scheme 2. Compound **2** might be derived from a steroidal derivative by sequential enzymatic hydroxylation at C-16 followed by cyclization via C-16–C-24 bond formation and subsequent reduction of C-24–C-25 bond [11]. Two methine signals at δ_C 48.6 and δ_C 45.3 supported the above contention in the favor of C-16 to C-24 linkage. Acid hydrolysis of **2** with 0.2 M HCl/MeOH furnished the aglycon and one monosaccharide unit which was identified as D-glucose by co-HPTLC with the authentic sample [12,13].

2.2. Biological evaluation

Compounds **1a**–**1c** and **2** were screened against HeLa, PC-3 and MCF-7 cell lines purchased from European Collection of Cell Culture (ECACC). Staurosporine was used as positive control. Compounds **1a** and **2** were observed to be active on all three cells whereas **1b** and **1c** were completely inactive. Various derivatives of **1a** have been studied widely for remarkable antibacterial and antitumor activity [14]. Structural modifications in reference to position and change in functionality and SAR study with respect to these activities have been examined extensively. Previous studies have shown that **1b** as well as **1c** in combination with standard anticancer drugs is capable of stimulating the recruitment of clonogenic cells in tumor, making it more sensitive to the conventional treatment with cytotoxic agents.



Scheme 2. Probable biosynthesis for compound **2**.

Initially, MTT assay was performed to define the optimum concentration at which the compounds were nontoxic to cells. The cancer cells treated with 50 μM concentration of **1a** and 80 μM of **2** (IC_{50} values) affect the viability of HeLa and PC-3 (data not shown) cells significantly, whereas less aggressive MCF-7 cells were affected even at much lower concentration of the compounds. To test the effect of **1a** and **2** on growth kinetics of cancer cells, colony formation assay were performed in a dose dependent manner as shown in Figs. 3a and 4a respectively. Sub-lethal doses of the compounds decreased colony formation ability of HeLa and PC-3 (data not shown) cells in statistically significant manner ($P < 0.05$) while MCF-7 cells were unaffected. To examine the effect of the compounds on cell migration, wound healing assay was performed on confluent monolayers of HeLa, PC-3 and MCF-7 cells. After making the wound with a pipette tip, the cells were cultured in the presence or absence of different concentrations of the compounds along with vehicle DMSO. The wounded areas were progressively photographed with Olympus c-7070 with 700M camera. The vehicle DMSO treated cells (control) were able to completely fill in the cleared area, whereas treatment with 50 and 80 μM of **1a** and **2** significantly ($P < 0.05$) inhibited the migration of HeLa (Figs. 3b and 4b) and PC-3 (data not shown) cells, thereby reducing their migration capability. The experimental data strongly demonstrated that both **1a** and **2** could block the migration of more aggressive HeLa and PC-3 cells in a dose dependent manner but not MCF-7 cells, which exhibited less IC_{50} values compared to HeLa and PC-3 cells.

To further elucidate the underlying mechanism for cytotoxicity of **1a** and **2** we performed apoptosis studies on HeLa and PC-3 (data not shown) cells. After treating the cells with different concentrations of the compounds, the percentage of apoptotic cells was assessed by propidium iodide staining, followed by flow cytometric analysis (Figs. 3c and 4c). Interestingly, compounds **1a** and **2** observed 76.8% and 63.5% apoptotic cells at 50 μM and 80 μM concentrations compared to 68.6% with 1 μM concentration of

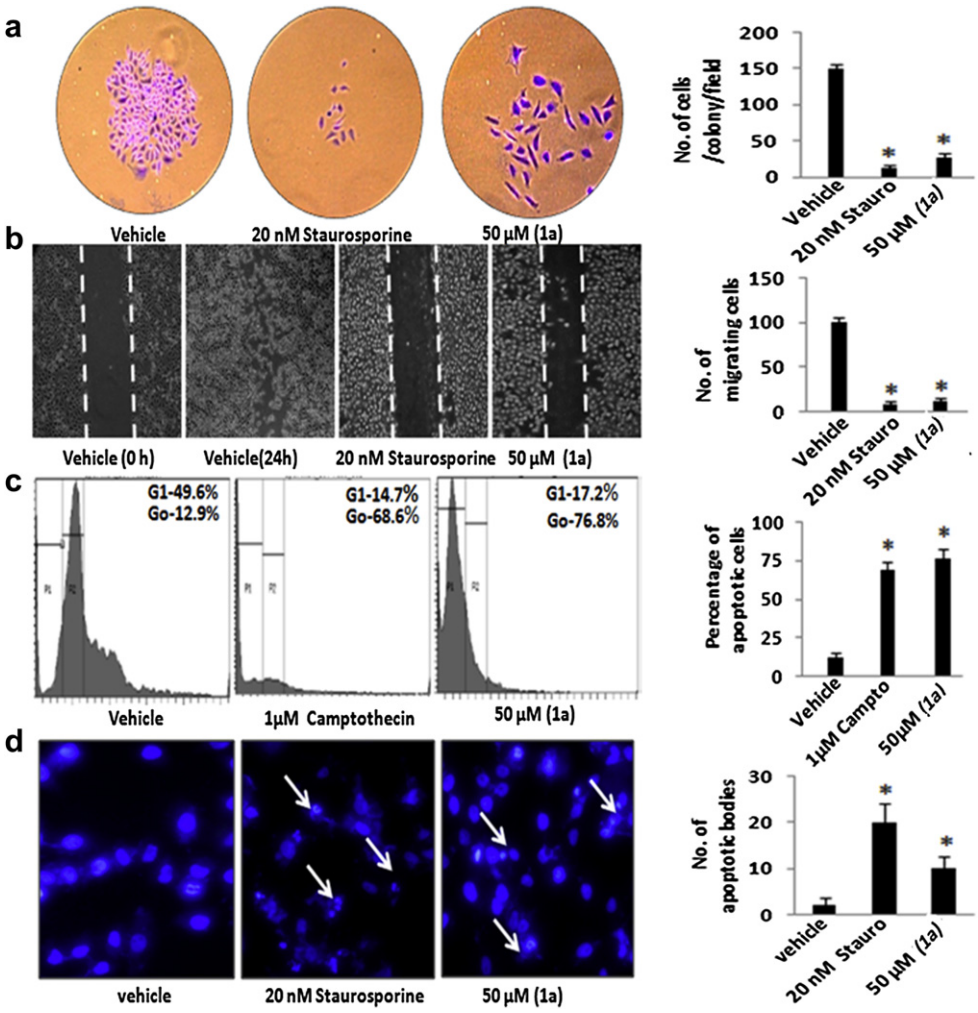


Fig. 3. Assessment of cytotoxicity and cell motility of **1a** at a concentration of 50 μM along with vehicle DMSO and positive control staurosporine (25 nM) or camptothecin (1 μM) against HeLa cells. (a) Colony formation assay was performed with 1×10^3 cells/well and the number of stained cells per colony was counted randomly and images were captured in 100× magnifications under inverted microscope. (b) Wound healing assay (0.5×10^5 cells/well) to assess the degree of wound healing. The scratched areas were quantified in three random fields in each treatment, and data were calculated from three independent experiments. $^*P < 0.05$ compared with untreated control. (c) Cell cycle analysis to determine cell cycle distribution. Data were calculated from three independent experiments. (d) HeLa cells (5×10^4) after fixation were stained with nuclear stain DAPI reagent and were photographed under fluorescence microscope (100× magnification) and data were calculated from three independent experiments. Columns mean; bars SD of three independent experiments. $^*P < 0.05$ compared with untreated control.

positive control camptothecin. By using 4,6-diamidino-2-phenylindole (DAPI) staining we observed that **1a** and **2** induced apoptosis in HeLa cells within 24 h of incubation as compared to positive control staurosporine as shown in Figs. 3d and 4d respectively. The data indicated the occurrence of early apoptosis with chromatin condensation around the nuclear periphery, accompanied by nuclear size reduction (white arrow heads).

3. Conclusion

A critical analysis of compounds **1a**–**1c** in reference to cytotoxic activity revealed that **1a** possessing an N-ethyl functionality showed much higher activity against HeLa and PC-3 cells affording important information about structure based drug designing. The study also afforded a unique pentacyclic steroidal glycoside with antiproliferative and apoptosis inducing property. Though few pentacyclic polyoxygenated steroids have been reported from marine microorganisms and plants, however this is the first report of a cholestane type pentacyclic steroidal glycoside from higher plants.

4. Experimental

4.1. Chemicals

All solvents used were of analytical grade (CDH Laboratory Reagents, India). Silica gel 60, 100–200 (Merck, India) and Sephadex LH-20 (Pharmazia) was used for gravity column and silica gel 300–400 (Merck, India) was used for flash column chromatography. Pre-coated silica gel 60 GF_{254} plates were used for analytical TLC. HPTLC (Merck) were used for co-TLC assay. D-Glucose (Merck, India) was used as a standard. The spots were visualized under UV light (254 and 366 nm) and also by spraying with Dragendorff's reagent or with anisaldehyde sulfuric acid followed by heating. IR (KBr) spectra were recorded using a Nicolet model Protégé 460 spectrophotometer. $^1H/^{13}C$-NMR spectra were recorded on 300/75 and 600/125 MHz Bruker spectrometer using TMS as internal standard. The chemical shift values are reported in ppm (δ) units and the scalar coupling constants (J) are in Hz. HRESIMS was performed on Agilent-6220 accurate mass LC-TOF system attached

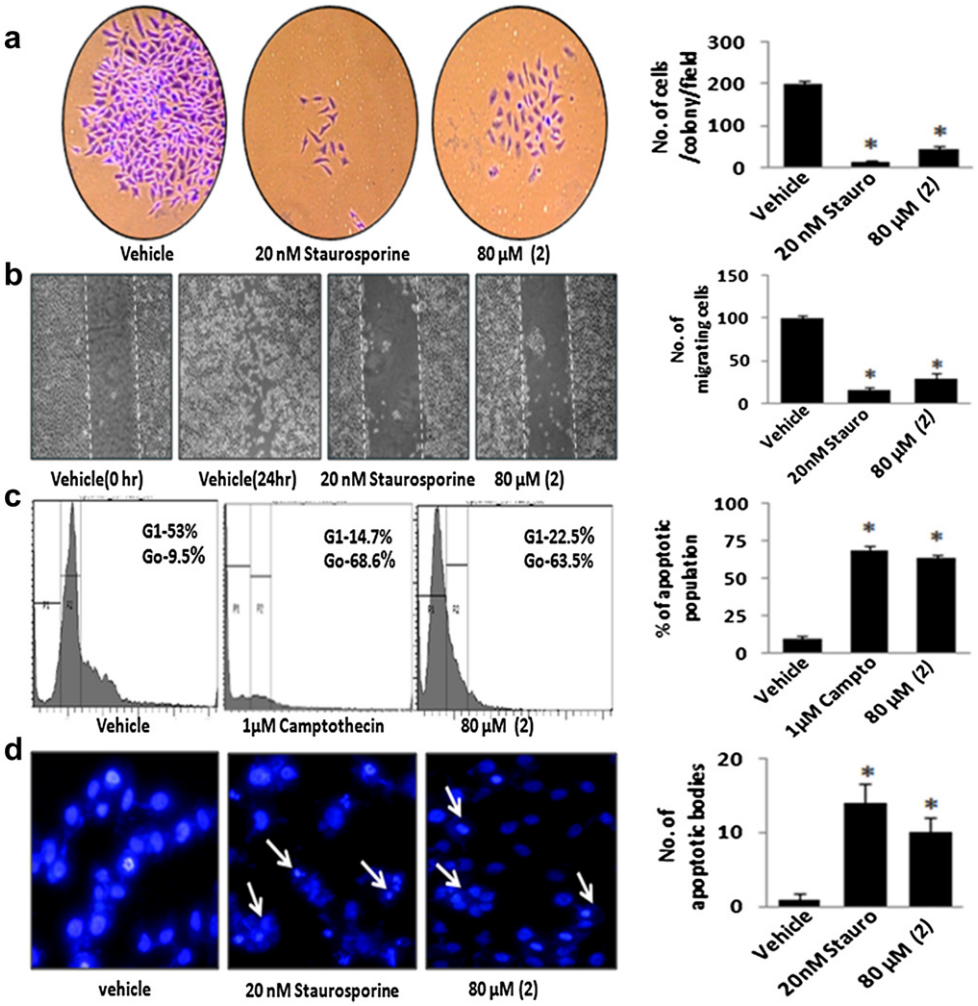


Fig. 4. Cytotoxicity and cell motility assessment of **2** at a concentration of 80 μM along with vehicle DMSO and positive control staurosporine (25 nM) or camptothecin (1 μM) against HeLa cells. (a) Colony formation assay was performed with 1×10^3 cells/well and the number of stained cells (crystal violet) per colony was counted randomly and images were captured in 100× magnifications under inverted microscope. (b) Wound healing assay (0.5×10^5 cells/well) to assess the degree of wound healing. The scratched areas were quantified in three random fields in each treatment, and data were calculated from three independent experiments. $^*P < 0.05$ compared with untreated control. (c) Cell cycle analysis to determine cell cycle distribution. Data were calculated from three independent experiments. (d) HeLa cells (5×10^4) after fixation were stained with nuclear stain DAPI reagent and were photographed under fluorescence microscope (100× magnification) and data were calculated from three independent experiments. Columns mean; bars SD of three independent experiments. $^*P < 0.05$ compared with untreated control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with Agilent 1200 Series HPLC with an eluent of 0.3 mL/min acetonitrile.

4.2. Plant material

The stem-bark of *C. nurvala* was procured from a registered vendor 'Accolent Dried Herbs' Parkvale Street, Victoria Point, Queensland 4165, Australia. A voucher specimen (SG/01/2010) was authenticated by Dr. M.P. Sharma, Department of Botany, Hamdard University, New Delhi and has been deposited in the herbarium of Amity Institute of Biotechnology, Amity University, Noida, India.

4.3. Chemistry

4.3.1. Extraction, isolation and characterization

The dried stem bark part of *C. nurvala* (0.25 kg) was extracted with MeOH–H_2O (9:1, 1 L × 3 × 24 h) followed by H_2O (0.5 L) at room temperature. The concentrated MeOH extract was suspended in water and successively partitioned into Et_2O (1 L, 8 g) and *n*-BuOH (0.5 L, 1.5 g) fractions. Successive chromatography of Et_2O fraction (5.7 × 85 cm, silica gel 60; 200 mL each) using *n*-hexane and stepwise 10–100% gradient of EtOAc followed by solvent distillation under vacuum, afforded five fractions (F-1 to F-5) by TLC monitoring under UV light and Dragendorff's reagent. Further, chromatography of F-2 (2 × 70 cm, silica gel 100–200) with *n*-hexane–EtOAc (90:10) eluent afforded previously reported white precipitate of lupeol in excess (1 g). Additionally, fraction F-3 on elution with *n*-hexane–EtOAc (80:20) produced formerly reported semisolid precipitate of stigmasterol. Repeated column chromatography of F-5 with *n*-hexane–EtOAc (1:1) afforded light yellow solid of **1a** (TLC solvent: *n*-hexane–EtOAc, 1:2; 6 mg). The *n*-BuOH soluble fraction was purified by flash chromatography with DCM and 10% stepwise gradient of MeOH affording five fractions (100 mL; S-1 to S-5) monitored by EtOH–H_2SO_4 spray reagent (7% H_2S0_4 in EtOH). Further chromatography of S-2 on Sephadex LH-20 with MeOH as eluent afforded partially purified **2** (9 mg). As the compound was invisible under PDA detector, it could not be separated by HPLC. The final separation was achieved by silica gel chromatography using gradient elution of DCM–MeOH (99:1 to 95:5). The 5% MeOH–DCM fraction afforded **2** (7 mg) as white solid.

Compound **2** was visualized as bright pink spot on TLC (DCM–MeOH; 7:3) with the spray reagent.

*4.3.1.1. 5,7-Dimethoxy-3-phenyl-1-ethyl-1,4-dihydro-4-quinolone (**1a**).* IR (KBr) ν_{max} in cm^{-1}, 1641,1635, 1617, 1560, 1508, 1445, 1340, 1320, 1242, 1215, 1027, 932, 858, 830, 783; ^1H NMR (600 MHz, $CDCl_3$): $\delta = 7.57$ (d, 2H, $J = 8.4$ Hz, H-2′, H-6′), 7.44 (s, 1H, H-2), 7.30 (app t, 2H, $J_1 = 8.4$ Hz, $J_2 = 1.5$ Hz, H-3′, H-5′), 7.21 (dd, 1H, $J_1 = 8.4$ Hz, $J_2 = 1.5$ Hz, H-4′), 6.30 (d, 1H, $J = 2.1$ Hz, H-6), 6.27 (d, 1H, $J = 2.1$ Hz, H-8), 4.04 (q, 2H, $J = 7.2$ Hz, H_2-1″), 3.88 (s, 3H, 5-OMe), 3.85 (s, 3H, 7-OMe), 1.43 (t, 3H, $J = 7.2$ Hz, H_3-2″); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 175.5$ (C-4), 163.1 (C-5), 162.5 (C-7), 142.9 (C-8a), 139.4 (C-2), 135.7 (C-1′), 128.8 (C-2′, C-6′), 127.8 (C-3′, C-5′), 126.6 (C-4′), 123.8 (C-4a), 112.9 (C-3), 94.1 (C-6), 89.8 (C-8), 56.1 (5-OMe), 55.3 (7-OMe), 48.6 (C-1″), 13.9 (C-2″); HRESI MS: *m/z* calc. for $C_{19}H_{19}NO_3$ $[M + H]^+$ 310.1437, $[M + Na]^+$ 332.1359; found 310.1434 and 332.1354 respectively.

*4.3.2. General procedure for the synthesis of **1b** and **1c***

1.54 g (10.05 mmol) of 3,5-dimethoxyaniline and 2.12 g (1.1 equiv) of ethyl-α-formylphenyl acetate in 20 mL of toluene was stirred at reflux condition for 18 h under nitrogen atmosphere. After cooling, the reaction mixture was diluted with toluene (20 mL), acidified with 10% HCl and then extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$, filtered and then evaporated under reduced pressure. The residue was dissolved in methanol and was stored for crystallization in a freezer at −80 °C. Repeated crystallization of the residue afforded 2.2 g (73%) of ethyl (*Z*)-3-(3,5-dimethoxyanilino)-2-phenyl-2-propenoate as intermediate. The intermediate (1.20 g, 3.67 mmol) was added portion wise and rapidly with stirring, to a solution of biphenyl (1.52 g) and diphenyl ether (11.60 g) heated to 250 °C for 10 min. The precipitate of **1b** was obtained in excess from the reaction mixture on cooling. The product was collected by filtration and then rinsed with petroleum ether. White crystals of **1b** (500 mg) were collected after drying. 200 mg (0.7 mmol) of the intermediate was added to 10 mL of anhydrous N,N-dimethylformamide (DMF) under nitrogen atmosphere. 734 mg of anhydrous K_2CO_3 (7.5 equiv) and 0.13 mL of methyl iodide (3 equiv) were added consecutively to the suspension. The mixture was stirred at room temperature for 18 h. Then the solvent was evaporated and the residue was extracted with CH_2Cl_2 and washed with water. The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude residue was purified on a column of silica (CH_2Cl_2–EtOAc; 9:1) to produce 150 mg (71%) of **1c**.

The structure of **1b** and **1c** was established as 5,7-dimethoxy-3-phenyl-1,4-dihydro-4-quinolinone and 5,7-dimethoxy-3-phenyl-1-methyl-1,4-dihydro-4-quinolinone respectively by analysis of NMR and MS spectral data.

*4.3.2.1. 5,7-Dimethoxy-3-phenyl-1,4-dihydro-4-quinolinone (**1b**).* ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.79$ (s, 1H, H-2), 7.56 (d, 2H, $J = 7.5$ Hz, H-2′, H-6′), 7.3 (app t, 2H, $J = 7.5$ Hz, H-3′, H-5′), 7.19 (app t, 1H, $J = 7.8$ Hz, H-4′), 6.50 (d, 1H, $J = 2.3$ Hz), 6.30 (d, 1H, $J = 2.3$ Hz), 3.80 (s, 3H, 5-OMe), 3.76 (s, 3H, 7-OMe); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 175.3$ (C-4), 162.9 (C-5), 162.5 (C-7), 142.8 (C-8a), 139.4 (C-2), 135.6 (C-1′), 128.8 (C-2′, C-6′), 127.6 (C-3′, C-5′), 126.5 (C-4′), 123.8 (C-4a), 112.7 (C-3), 94.1 (C-6), 89.8 (C-8), 55.9 (5-OMe), 55.4 (7-OMe); HRESI MS: *m/z* calc. for $C_{17}H_{16}NO_3$ $[M + H]^+$ 282.1130; found 282.1125.

*4.3.2.2. 5,7-Dimethoxy-3-phenyl-1-methyl-1,4-dihydro-4-quinolinone (**1c**).* ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.77$ (s, 1H, H-2), 7.53 (d, 2H, $J_1 = 7.6$ Hz, H-2′, H-6′), 7.26 (app t, 2H, $J = 7.6$ Hz, H-3′, H-5′), 7.15 (app t, 1H, $J = 7.6$ Hz, H-4′), 6.46 (d, 1H, $J = 2.3$ Hz), 6.27 (d, 1H, $J = 2.3$ Hz), 3.78 (s, 3H, 5-OMe), 3.73 (s, 3H, 7-OMe), 3.29 (s, 3H, N-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 175.6$ (C-4), 162.8 (C-5), 162.3 (C-7), 142.5 (C-8a), 140.9 (C-2), 135.8 (C-1′), 128.6 (C-2′, C-6′), 127.5 (C-3′, C-5′), 126.5 (C-4′), 123.9 (C-4a), 112.6 (C-3), 94.4 (C-6), 90.2 (C-8), 55.8 (5-OMe), 55.3 (7-OMe), 42.2 (N-Me); HRESI MS: *m/z* calc. for $C_{18}H_{18}NO_3$ $[M + H]^+$ 296.1287; found 296.1282.

*4.3.2.3. Crataenoside (**2**).* ^1H NMR ($CDCl_3$–CD_3OD; v/v, 2:1, 600 MHz): δ 5.37 (d, 1H, $J = 4.9$ Hz, H-6), 4.40 (d, 1H, $J = 7.8$ Hz, H-1′), 3.85 (dd, 1H, $J_1 = 12$ Hz, $J_2 = 2.4$ Hz, H-6′α) 3.71 (dd, 1H, $J_1 = 12$ Hz, $J_2 = 5.2$ Hz, H-6′β), 3.59 (br m, 1H, H-3), 3.37 (m, 1H, H-3′), 3.32 (m, 1H, H-4′), 3.27 (m, 1H, H-2′), 3.20 (t, 1H, $J = 6$ Hz, H-5′), 2.42 (dd, 1H, $J_1 = 13.1$ Hz, $J_2 = 1.2$ Hz, H_2-4), 2.27 (t, 1H, H_2-4) 1.95 (m, 1H, H-7) 1.66 (m, 1H, H-7), 1.63 (t, 1H, H-25), 1.61 (m, 2H, H_2-2), 1.25 (m, 1H, H-24), 1.02 (s, 3H, H-19), 0.93 (d, 3H, $J = 6.4$ Hz, H-21), 0.86 (d, 3H, $J = 6.6$ Hz, H-25), 0.83 (d, 3H, $J = 6.6$ Hz, H-26), 0.7 (s, 3H, H-18).

^{13}C NMR ($CDCl_3$–CD_3OD; v/v, 2:1, 600 MHz): 139.8 (C-5), 121.3 (C-6), 100.6 (C-1′), 78.3 (C-3), 76.1 (C-5′), 75.6 (C-3′), 73.1 (C-2′), 69.7 (C-4′), 61.1 (C-6′). 56.2 (C-14), 55.5 (C-17), 49.7 (C-9), 48.6 (C-16), 45.3 (C-24), 41.7 (C-13), 39.2 (C-12), 38.0 (C-4), 36.7 (C-1), 36.1 (C-10), 35.5 (C-20), 33.3 (C-22), 31.3 (C-7), 31.3 (C-8), 28.5 (C-2), 27.6 (C-15), 27.6 (C-25), 25.4 (C-23), 20.4 (C-11), 18.8 (C-27), 18.4 (C-26), 18.0 (C-19), 17.9 (C-21), 11.0 (C-18); HRESI MS: *m/z* calc. for $C_{33}H_{54}O_6$ $[M + H]^+$ 547.3984, $[M + Na]^+$ 569.3906; found 547.3979 and 569.3901 respectively.

*4.3.3. Acid hydrolysis of **2***

Compound **2** (3 mg) was refluxed at 90°C with 2 N HCl in MeOH (1:4, 1 mL) for 2 h under argon atmosphere. The reaction mixture was diluted with water and extracted with $CHCl_3$ (10 mL). The aqueous layer was neutralized with Amberlite MB-3 (Organo Co.) and then evaporated under reduced pressure to produce the monosaccharide as residue. The residue was extracted with MeOH and was analyzed by silica gel HPTLC developed with Me_2CO and 2 mM NaOAc (17:3, v/v) and detected by spraying with 0.2% naphthoresorcinol in Me_2CO and 3 N H_3PO_4 (5:1, v/v) followed by heating at 105 °C for 5 min (R_f 0.6). D-Glucose was used as standard.

4.4. Biology

4.4.1. Cell culture

All cell lines were purchased from European Collection of Cell Culture (ECACC), Fetal Bovine Serum (FBS), RPMI-1640, Minimum Essential Medium (MEM), Penicillin G, Streptomycin, Trypsin-EDTA were obtained from Invitrogen Corp. 5-Diphenyltetrazolium bromide (MTT), paraformaldehyde, staurosporine, camptothecin, dimethylsulfoxide (DMSO), Bradford reagent were obtained from Sigma Chemicals Co. (St. Louis, MO). Propidium iodide and Ultracruz DAPI mounting medium were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

4.4.2. Cell proliferation assay

The cell viability was determined by standard MTT dye uptake method. Briefly, HeLa, PC-3 and MCF-7 cells (3×10^3 cells/well) were plated into 96 well plate and were treated with different concentrations of compounds **1a**–**1c** and **2** in triplicate so that the final concentration of DMSO was 0.2 %. After 48 h incubation, MTT (0.5 mg/mL) solution was added and was further incubated at 37 °C for 4 h. The amount of colored formazan derivatives was determined by measuring optical density (OD) using a microplate reader (Infinite M200 PRO) at 570 nm. The percentage viability was determined according to the protocol described in Ref. [15].

4.4.3. Clonogenic assay

The assay was performed according to the previously described method [16]. Briefly, HeLa, PC-3 (data not shown) and MCF-7 cells were plated at a seeding density of (1×10^3 cells/well) in 6 well tissue culture grade plates. After 24 h the culture medium was changed and new medium was added and cells were exposed to various concentration of compounds **1a** and **2** along with vehicle DMSO for 5 days in 5% CO_2 incubator at 37 °C. Later on, the obtained colonies were fixed with 4% paraformaldehyde and were stained with 0.5% crystal violet solution. The colonies from the plates were counted and averaged from the observed fields randomly ($n = 3$) and photographed with Olympus c-7070 wide 700M inverted microscope camera.

4.4.4. Wound healing assay

Briefly, HeLa, PC-3 (data not shown) and MCF-7 cells were plated in 6 well plates at a seeding density of 5.5×10^5 cells/well and grown overnight to form a confluent monolayer. An artificial wound was made by a sterile pipette tip (200 μL) after 24 h of serum starvation and washed with serum free medium to remove floated and detached cells. A photograph was recorded (time 0 h). Thereafter, the cells were successively treated in a medium containing low serum (1%), in the presence of different concentrations of compounds **1a** and **2** along with vehicle DMSO for 24 h. The wounded areas were progressively photographed with Olympus c-7070 with 700M camera (100× magnification). The percentage of wound closure was estimated by the following equation: wound closure % = [1 − (wound area at t_1/wound area at t_0) × 100%], where t_1 is the time after wounding and t_0 is the time immediately after wounding [17].

4.4.5. Cell cycle analysis

HeLa and PC-3 cells (5×10^5) were seeded in 6 well plate and were treated with various concentrations of **1a** and **2** along with vehicle DMSO for 24 h. Cells were collected by trypsinization and washed with PBS. Cells were incubated in hypotonic solution (0.1% sodium citrate, 25 μg/mL propedium iodide, 0.03% Triton-X100, 40 μg RNase-A) for 25 min at room temperature and then acquired for FACS analysis in BD Aria [18].

4.4.6. Apoptosis by DAPI staining

Following the treatments with **1a** and **2** in DMSO, adherent HeLa and PC-3 cells (1×10^5 cells/well) were harvested by trypsin digestion and washed twice with chilled PBS. Cells were fixed with 4% paraformaldehyde for 10 min followed by incubation with DAPI, containing mounting medium for 15 min at rt in the dark and apoptosis was detected by fluorescence microscopy (100× magnification) [19].

Conflict of interest

None of the authors of the above manuscript have declared any conflict of interest within the last three years which may arise from being named as an author on the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.12.017.

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