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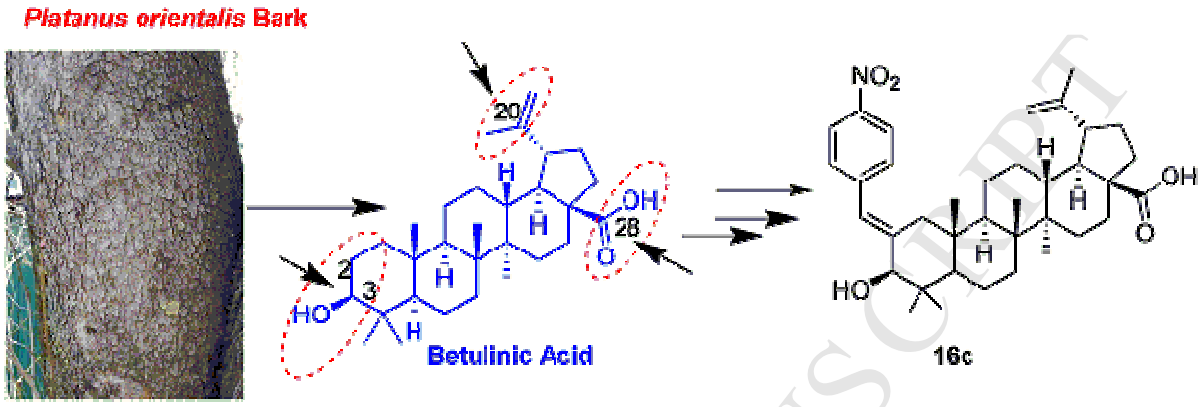

Design and synthesis of C-2 and C-3 derived benzylidene analogues of betulinic acid as potent cytotoxic agents

Synthesis of novel benzylidene analogues of betulinic acid as potent cytotoxic agents

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Abstract

Different benzylidene derivatives (**15a-o** and **16a-o**) of betulinic acid were designed and synthesized in an effort to develop potent anticancer agents. All the synthesized derivatives along with betulinic acid were evaluated for cytotoxicity against a panel of five different human cancer cell lines A-549 (Lung), PC-3 (Prostate), HCT 116 (Colon), MCF-7 (Breast) and MIA PaCa-2 (Pancreatic) using SRB assay. Pharmacological results showed that compounds **15b, 15c**, **15i, 15k**, **16a-c** and **16l** were found to have promising cytotoxic profile against various cancer cell lines tested (IC_{50} 1-2 µM). Best results were observed for compound **16c** with IC_{50} values 1.5, 1.6, 1.36, 3.5 and 3.2 µM against A-549, PC-3, HCT 116, MCF-7 and MIA PaCa-2 cell lines, respectively. Mechanistic study of compound **16c** revealed that it inhibits the colony formation and restrict the migration in HCT 116 cells *in vitro*. It also induces growth arrest with characterized morphological changes and loss of mitochondrial membrane potential (MMP) in a concentration dependent manner.

1. Introduction

Triterpenoids represents one of the major classes of plant derived natural products and some representative structural scaffolds shown in Fig. 1 such as lupane group betulinic acid (**1)** and lupeol (**2**), oleanane group oleanolic acid (**3**) and maslinic acid (**4**), ursolic acid (**5**) and ursane group β-boswellic acid (**6**) has gained considerable attention worldwide in recent past owing to their various pharmacological activities and substantial number of novel derivatives have been synthesized based on these scaffolds [1,2]. Betulinic acid (**1**), a pentacyclic lupane-type triterpenoid is known to possess a broad range of biological effects, particularly anticancer [3, 4]. Cytotoxic effects of betulinic acid has been studied on large variety of cancer cell lines [5-7] as well as applying xenograft mice models and primary tumor samples [8] and the compound

is currently under clinical evaluation in Phase I / II clinical trials (NCT00346502) for the treatment of dysplastic nevi (moderate to severe dysplasia) [9]. Further, mechanistic studies revealed that betulinic acid induces the cancer death via triggering the mitochondrial pathway of apoptosis. It increases mitochondrial membrane permeabilisation leading to caspase activation and nuclear fragmentation with the release of apoptogenic factors such as cytochrome c, Smac and AIF [10]. Compound **1** is also reported to modulate the expression level of Bcl-2 family proteins, induce apoptosis in p53 and CD-95 independent manner and inhibit the TNFα induced activation of NFκB in human prostate cancer cells (PC-3) [11,12].

Figure 1

Betulinic acid represents a biologically active scaffold with high safety profile in cancer therapy and is suitable to carry out chemical transformations because of the several key positions available on the molecule i.e. C-2, C-3, C-20 and C-28 (Fig. 1). Therefore, to investigate the structural features responsible for anticancer activity of betulinic acid and also to develop the structure activity relationship (SAR) studies, considerable structural modification has been done on betulinic acid for the improvement of its anticancer activity [13-15]. The oxidized product betulonic acid (**7**) having carbonyl moiety at C-3 position reportedly possess better anticancer profile than betulinic acid [16]. Also, several A-ring modified derivatives at C-2 and C-3 position of betulinic acid have been reported to possess potent anticancer activity. You et al. synthesized the various A-ring modified betulinic acid derivatives. Among them, compounds **8a** and **8b** showed potent cytotoxicity against M2 cell line with IC_{50} values 0.81 and 0.13µM respectively [17]. Grishko et al. reported the anticancer activity of ring-A fused azole derivatives of betulin and 1,2,4-triazine analog (**9**) was found as the most potent against HCT-116 cell line with an IC_{50} value of 1.4 µM [18]. Csuk et al. reported the anticancer activity of several alkylidene branched

lupane derivatives and some analogs including compounds **10** and **11** were found to be potent derivatives [19]. Borkova et al. reported the cytotoxic activity of 2,2-difluoroderivative of dihydrobetulinic acid (**12a**) [20]. Urban et al. reported the 2-bromo-3-oxo (**12b**) and 2-hydroxymethylene-3-oxo (**12c**) derivatives of betulinic acid and studied their cytotoxic effects on CEM cell line [21]. Ngoc reported the anticancer activity of **12d**, a diosphenol derivative of betulinic acid [22]. B10 (**13**) and NVX-207 (**14**) (Fig. 2.) are C-3 modified derivatives of betulinic acid having potent clinical applications [23, 24]. Kumar *et al*. [25] along with the work of Dar et al. [26] reported that introduction of benzylidene moiety into triterpene natural products (β-boswellic acid and ursolic acid) produced analogs with improved anticancer potential. Furthermore, benzylidene derivatives having α, β- unsaturated carbonyl moiety in biological systems interacts with targeted proteins or enzymes *via* Michael addition and exhibits various pharmacological activities [27, 28].

Based on these findings and as a part of our ongoing work on structural modification of natural products to get the anticancer lead molecules [5, 6, 14, 29], benzylidene derivatives of betulinic acid were designed to improve its antitumour potential. In the present paper, we report the synthesis of benzylidene derivatives **15a-o** of betulinic acid by aldol condensation reactions which were further reduced at C-3 position to produce derivatives **16a-o**. All these derivatives were evaluated for *in vitro* cytotoxic activity against five human cancer cell lines (A-549, PC-3, HCT 116, MCF-7 and MIA PaCa-2) and it was demonstrated that many analogs exhibited better anticancer potential than betulinic acid. All the active compounds including lead (**16c**) were tested on normal breast epithelial human cell line (fR2). Further, the mechanistic study of compound **16c** was carried out on colon cancer cell line (HCT 116).

Figure 2

2. Result and Discussion

2.1. Chemistry (Design and synthesis of betulinic acid benzylidene derivatives)

In the present study, betulinic acid was isolated from DCM:MeOH (1:1) extract of stem bark of *Plantanus orientalis* and was taken for structural modification studies. C-2 position was targeted and benzylidene derivatives **15a-o** were designed and synthesized. Reduction of carbonyl group at C-3 position of these derivatives was carried out to produce derivatives **16a-o.** The strategy behind this was to study the effect of benzylidene derivatives on the anticancer activity while retaining the C3 hydroxyl group. The procedure for the synthesis of benzylidene derivatives **15a-o** involved two steps (Scheme 1). In the first step, **1** was oxidized with Pyridinium chlorochromate (PCC) in dichloromethane (DCM) to provide betulonic acid **7**. In second step, compound **7** was reacted with different aldehydes to carry out aldol condensation reaction. Initially, **7** was reacted with 3-nitrobenzaldehyde and was taken as a model reaction to optimize reaction conditions. For this, we used different bases such as K_2CO_3, KOEt, NaOH, TEA, DMAP and NaH and best results were observed in case of NaH. Different solvents were also screened taking NaH as a base and THF was found to be best solvent (Supplementary information, Table S1). These optimized set of conditions then used for the condensation of various aromatic aldehydes with betulonic acid **7** to provide a series of benzylidene analogues **15a-o**. Further, reduction of **15a-o** with sodium borohydride ($NaBH_4$) in methanol produces derivatives **16a-o** with hydroxyl group at C-3 (Scheme 1).

Scheme 1

All the reactions were carried out at variable temperature conditions (0^oC to room temperature) and provided derivatives in good to excellent yields. Structures of all the derivatives were confirmed by spectroscopic techniques (^1H NMR, ^{13}C NMR and HRMS). In ^{13}C

NMR, signal at δ 204, 135 & 133 and in ^{1}H NMR, a singlet at δ 7.2-7.5 correspond to olefinic proton of α, β-unsaturated carbonyl system confirm the product formation of **15a-o**. For compounds, **16a-o**, ^{13}C NMR is distinguished from the spectrum of **15a-o** by the absence of signal from carbonyl group at δ 204 and appearance of signal from C-3 atom of lupane scaffold at δ 81 whereas in ^{1}H NMR, singlet from the proton at C-3 atom (δ 3.7-3.9) and upfield shift of olefinic proton signal (δ 6.4-6.7) is observed. Further confirmation for the formation of all the derivatives was done by DEPT and HRMS data.

2.2. Biology

2.2.1. Cell growth inhibition

All the synthesized derivatives were subjected to preliminary cytotoxicity screening at 10 µM concentration against a panel of five different human cancer cell lines namely, A-549 (Lung), PC-3 (Prostate), HCT 116 (Colon), MCF-7 (Breast) and MIA PaCa-2 (Pancreatic) along with compound **1** (parent molecule) taken as reference standard in present study to evaluate their cytotoxic potential using SRB assay. Results are summarized in table 1 and values given are the average of triplicate analysis. Betulinic acid **1** showed 26-49% growth inhibition against all the cancer cell lines. Compound **7** displayed better anticancer effects than **1** which was in agreement with the literature [16] and exhibited cytotoxicity against A-549, HCT 116, MCF-7 and MIA PaCa-2 cell lines with 62, 49, 43 and 36% inhibition respectively. Among benzylidene analogs, many of them exhibited ≥90% inhibition against various experimental cancer cell lines. Compounds **15f-j**, **15l-o** and **16a-c** displayed significant growth inhibition effects against all the experimental cancer cell lines. Compounds **15a**, **15b**, **15d**, **15e**, **15g**, **15h**, **15m-o**, **16a**, **16b** and **16d** affected A-549 the most whereas compounds **15c**, **15f**, **16e**, **16g-l**, **16n** and **16o** showed maximum inhibition effects against MCF-7 than other four experimental cancer cell lines.

Compounds **15k**, **15l** and **16c** exhibited maximum growth inhibition against colon cancer cell line with 92, 99 and 92% respectively. Compound **15i** affected MCF-7 and MIA PaCa-2 (97% inhibition) and **15j** affected A-549 and HCT 116 cell lines (89% inhibition) to same extent. Compound **16f** did not exhibit significant growth inhibition against any of the cancer cell line examined whereas compound **16m** showed fewer inhibition against MCF-7 and MIA PaCa-2 cell lines.

Table1

2.2.2. IC_{50} and SAR (structure activity relationship)

Compounds showed significant inhibition effects at 10 µM concentration were further screened at three more concentrations 1, 2.5 and 5 µM and IC_{50} was calculated along with the parent molecule (Table 2). Conversion of **1** into **7** resulted in the improvement of activity with IC_{50} value 5.7 µM against A-549 cell line. In benzylidene analogs, compounds **15f-j**, **15l**, **15n, 15o**, **16a**, **16c and 16n** exhibit potent cytotoxic effects against all the cell lines with single digit IC_{50} value (<10 µM). Compounds (**15a-d**, **15f, 15h-m**, **15o**, **16a-d**, **16i-l** and **16n**) were found to be most promising against A-549 cell line with IC_{50} <5 µM among all the cell lines tested. In case of PC-3 and HCT 116 cell lines, compound **16c** displayed most potent activity with IC_{50} value 1.6 and 1.36 µM respectively. Compound **16l** displayed most potent activity against MCF-7 and MIA PaCa-2 cell lines with IC_{50} value 1.18 and 1.21 µM respectively. Overall, best results were observed for compound **16c** with IC_{50} values 1.5, 1.6, 1.36, 3.5 and 3.2 µM against A-549, PC-3, HCT 116, MCF-7 and MIA PaCa-2 cell lines respectively. All the active compounds were tested on normal breast epithelial human cell line (fR2) Table 2. Based on the results the selectivity index for cancer cells were determined Table 3. Moreover, IC_{50} value of **16c** was found to be high in normal human breast epithelial cells (fR2) which shows its selectivity for

cancer cells (Table 2 and 3) and justify its potency to develop as an anticancer agent. On the basis of growth inhibition and IC_{50} values, structure activity relationship of structural modifications of **1** can be summarized as follows:

a) Betulonic acid (**7**) showed better anticancer potential than the parent (**1**).

b) In general, benzylidene derivatives having α, β-unsaturated carbonyl moiety showed better anticancer effects than their corresponding derivatives with reduced keto group at C-3 position. However, some analogs with C3-*OH* (**16a-c**) exhibit cytotoxic effects better or comparable to that of their corresponding keto derivatives (**15a-c**) on certain cell lines examined.

c) Among the benzylidene derivatives, analogs containing *ortho* or *para* substitued electron withdrawing groups (-NO_2, -*Br* and -*F*) (**15b**, **15c**, **15g, 15l, 16b**, **16c** and **16l**) showed better anticancer effects than *meta*- substituted analogs (**15a, 15d, 16a** and **16d**). However, analogs with *meta*- and *para*- disubstituted halo groups (**15j** and **16j**) were found to be favourable for the improvement of cytotoxic activity for all cell lines examined compared to the *ortho* disubstituted analogs (**15e** and **16e**). Analog with electron releasing group (-OCH_3) (**15n** and **16n**) showed potent anticancer effects against all cell lines. Pyridine containing analog (**15i** and **16i**) exhibited better cytotoxic effects against colon, breast, lung and pancreatic cancer cells than analogs containing other heterocyclic moieties such as furan (**15f** and **16f**) and thiophene (**15h** and **16h**). Derivatives with extended conjugation (**15m**) and with polycyclic aromatic hydrocarbon moiety (**15o** and **16o**) also inhibited various cancer cell lines. It appears that many factors such as size, position, electronic effects (resonance and inductive) of the substituent and presence of heterocyclic groups are collectively responsible for anticancer effects of betulinic acid benzylidene derivatives. Compound **16c** was found to be most potent among synthesized series hence taken for further cell death mechanistic study.

Table 2

*2.2.3. Compound **16c** inhibited cell proliferation during colony formation assay in HCT 116 cells.*

Cell proliferation inhibiting ability of compound **16c** was measured using colony formation assay or clonogenic assay (Fig. 3.). Clonogenic assay is an *in vitro* cell survival assay depends on cell's ability to grow into a colony. Initially, this assay was used for studying the effect of radiations on cells, now it is also used for studying the effects of chemotherapeutic agents having potential clinical applications. This assay tests each cell in the population to undergo extensive divisions and also monitors the cells that have retained the capacity for producing colonies after treatment with cell death causing agents (radiations or chemotherapeutic agents) [30]. The effect of cytotoxic compounds on colony forming ability of cancer cells is measured. HCT 116 cells were treated with different concentrations of **16c** at 0.7, 1.4 and 2.8 µM in which colonies were formed after 14 days treatment. It was found that **16c** significantly decreased colony formation in HCT 116 cells in a concentration dependent manner as compared to untreated control (A).

Figure 3

*2.2.4. Compound **16c** inhibited cell migration during in vitro wound healing assay in HCT 116 cells*

In vitro wound healing assay or *in vitro* scratch assay is a method to study cell migration in which the cells on the edge of the newly created scratch or gap will move towards the opening to close this gap until the new cell-cell contacts are established again. Images were captured at the beginning and after termination in order to determine the rate of cell migration [31]. Herein, the monolayer of HCT 116 cells was scratched and treated with **16c** at various concentrations (1.4, 2.0 and 2.5 µM) for 24 h. The area of the wound was measured at two different time points 0 and

24 h and % reduction in cell migration was assessed by recovered area of the scratch as compared with 0 h post scratch. Rate of migration was calculated as compared to the corresponding control (A). It was observed that cell migration was inhibited in a dose dependent manner (Fig. 4). As higher concentration of **16c** correlates significantly in restricting metastasis by decreasing cell migration as well as inhibiting cell motility as data showed that the wound-healing rate in HCT 116 cells is slower at lower concentration in comparison to the control cells. So, migration analyzed by wound-healing assay revealed that **16c** clearly inhibited wound closure in HCT116 cells and displayed an anti-migratory effect in HCT 116 cells.

Figure 4

*2.2.5. Compound **16c** induced morphological changes in HCT 116 cells*

The HCT 116 cells were treated with **16c** at 0.7, 1.4, 2.0 and 2.5 µM for 24 h and observed for morphological changes by using phase contrast microscope. Cell growth arrest and characteristic changes were observed in the morphology of treated cells in a concentration-dependent manner (Fig. 5.).

Figure 5

*2.2.6. Compound **16c** triggered mitochondrial membrane potential (MMP) loss*

Loss of MMP causes depolarization of mitochondrial membrane with the release of apoptogenic factors and ultimately a cell death [32]. The changes of mitochondrial membrane were assessed by staining with rhodamine-123 (RH-123), a fluorescent dye. The rate of fluorescence decay is proportional to the loss in membrane potential. The loss of mitochondrial membrane integrity causes leakage of RH-123 which cause decrease in fluorescence intensity. HCT-116 cells were treated with different concentrations of **16c** at 0.7, 1.4, 2.0 and 2.5 µM and observed loss in

MMP in a concentration dependent manner whereas cytoplasm of untreated cells (control) were having intact mitochondria (Fig. 6).

Figure 6

3. Conclusion

To conclude, benzylidene derivatives of betulinic acid were synthesized at C-2 position by employing aldol condensation approach and screened against five human cancer cell lines (A-549, PC-3, HCT 116, MCF-7 and MIA PaCa-2) along with parent (**1**) as reference. Results demonstrated that C-2 position is favorable site to carry out modification as many analogs displayed better anticancer effects than betulinic acid. Compound **16c** was found as most potent analog among the synthesized series and further its mechanistic study through colony formation, wound healing, phase contrast microscopy and MMP loss experiments in HCT 116 cell line showed its potential to develop as potent anticancer agent.

4. Experimental

4.1. Chemistry

All the reagents and solvents for synthesis were purchased from Sigma-Aldrich. All the chemical reactions were monitored by TLC on silica gel 60 F_{254} plates (E. Merck) using 2% ceric ammonium sulphate solution as spraying reagent for detection of spots. Purification of all derivatives was carried out by column chromatography using silica gel 60-120 mesh as stationary phase. All NMR spectra were recorded on Bruker DPX 400 and DPX 500 instruments using $CDCl_3$ as the solvent taking TMS as the internal standard. The chemical shifts are expressed in δ and coupling constant in Hertz. High Resolution Mass Spectra (HRMS) were recorded on Agilent Technologies 6540 instrument.

*4.1.1. Isolation of betulinic acid (**1**)*

Betulinic acid was isolated in bulk quantity from DCM: MeOH (1: 1) extract of stem bark of *Plantanus orientalis* and characterized by spectroscopic techniques as reported previously [5].

*4.1.2. Preparation of betulonic acid (**7**)*

To a solution of compound **1** (5 g, 11 mmol) in DCM was added PCC (3.54 g, 16 mmol) dissolved in DCM dropwise till dark colour appears and kept it at r. t. for 2 h. After completion, reaction mixture was passed through celite and filtrate was concentrated at rotavapour. Purification was done through column chromatography with EtoAc: Hexane (1: 13) as the eluent to afford product **7** colourless solid (3.5 g, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.74 and 4.62 (1H each, s, H-29), 3.01 (1H, m, *H*-19), 2.5 and 2.39 (1H each, m, H-2), 2.29 and 1.99 (1H each, m, H-22), 2.21 and 1.97 (1H each, m, H-16), 1.9 (1H, m, H-13), 1.7 (3H, s, H-30), 1.74 and 1.62 (1H each, m, H-1), 1.63 (1H, m, H-5), 1.56 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.2 (1H each, m, H-11), 1.07 (3H, s, H-23), 1.02 (3H, s, H-24), 0.99 and 0.97 (3H each, s, H-26 and H-27), 0.93 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 219.75, 183.61, 151.75, 111.22, 57.83, 56.35, 51.26, 50.60, 48.77, 48.32, 43.91, 42.05, 41.03, 39.93, 38.47, 38.34, 35.56, 35.02, 33.52, 31.97, 31.10, 28.06, 26.90, 22.79, 22.43, 21.05, 20.79, 17.39, 17.25, 16.05. HRMS *m/z* calcd for $C_{30}H_{47}O_3$ $[M + H]^+$ 455.352, found 455.3507.

*4.1.3. General experimental procedure for preparation of benzylidene derivatives **15a-o***

To synthesize compounds **15a-o**, compound **7** (1 equiv) was dissolved in dry THF and NaH (1.2 equiv) was added to it at 0^{o}C. After 10 min, respective aldehyde (1.5 equiv) was added to reaction mixture. The reaction mixture was stirred at room temperature for 1.5-2 h till the

completion (monitored by TLC analysis) [25]. Workup of the reaction was done by diluting the reaction mixture with ice-cold water and extracting it with ethyl acetate (3 times). The combined organic layers were dried over sodium sulphate and concentrated on rotavapour. The crude product obtained was purified by column chromatography on silica gel 60-120 mesh with EtoAc: hexane (1: 10) as the eluent to afford the desired pure products **15a-o** in 90-93% yield. The spectral data of all the derivatives **15a-o** are given below.

*4.1.3.1. Synthesis of 2-(3-nitrobenzylidene)betulonic acid (**15a**)*. The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 3-nitrobenzaldehyde (50 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15a** colourless solid (120 mg, 93% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (2H, m, 2×Ar-C*H*), 7.54 (2H, m, 2 × Ar-C*H*), 7.41 (1H, s, -C*H*=C-CO-), 4.67 and 4.57 (1H each, s, H-29), 2.92 (1H, m, *H*-19), 2.89 and 2.16 (1H each, m, H-1), 2.21 and 1.93 (1H each, m, H-22), 2.18 and 1.89 (1H each, m, H-16), 2.13 (1H, m, H-13), 1.64 (3H, s, H-30), 1.58 (1H, m, H-18), 1.56 and 1.33 (1H each, m, H-6), 1.52 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.2 (1H each, m, H-11), 1.08 and 0.96 (3H each, s, H-23 and H-24), 0.91 (3H, s, H-27), 0.81 (3H, s, H-26), 0.77 (1H, m, H-5), 0.74 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 207.83, 181.59, 150.29, 148.30, 137.64, 137.03, 135.47, 134.42, 129.48, 124.60, 122.88, 109.82, 56.44, 52.95, 49.11, 48.33, 46.85, 45.41, 44.03, 42.52, 40.54, 38.39, 37.09, 36.70, 33.01, 30.04, 27.09, 25.44, 22.70, 22.37, 21.62, 20.31, 19.45, 15.84, 15.50, 14.62, 14.13. HRMS *m/z* calcd for $C_{37}H_{50}NO_5$ $[M + H]^+$ 588.3684, found 588.3678.

*4.1.3.2. Synthesis of 2-(4-bromobenzylidene)betulonic acid (**15b**)*. The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 4-bromobenzaldehyde (61 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15b** colourless solid (125 mg, 91%

yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 7.4 (1H, s, -C*H*=C-CO), 7.27 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 4.76 and 4.66 (1H each, s, H-29), 3.02 (1H, m, *H*-19), 2.96 and 2.16 (1H each, m, H-1), 2.29 and 1.99 (1H each, m, H-22), 2.22 and 1.98 (1H each, m, H-16), 1.96 (1H, m, H-13), 1.72 (3H, s, H-30), 1.68 (1H, m, H-18), 1.65 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.2 (1H each, m, H-11), 1.14 and 1.12 (3H each, s, H-23 and H-24), 1.02 (3H, s, H-27), 0.97 (3H, s, H-26), 0.78 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 207.92, 181.92, 150.44, 136.03, 134.88, 134.85, 131.70 × 2, 131.68 × 2, 122.66, 109.74, 56.43, 52.85, 49.18, 48.45, 46.84, 45.23, 44.39, 42.52, 40.54, 38.46, 37.03, 36.53, 33.06, 32.06, 30.60, 29.68, 29.43, 25.59, 22.34, 21.67, 20.33, 19.50, 15.80, 15.48, 14.62. HRMS *m/z* calcd for $C_{37}H_{50}BrO_3$ $[M + H]^+$ 621.2938, found 621.2921.

*4.1.3.3. Synthesis of 2-(4-nitrobenzylidene)betulonic acid (**15c**).* The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 4-nitrobenzaldehyde (50 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15c** colourless solid (120 mg, 93% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.26 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 7.54 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 7.48 (1H, s, -C*H*=C-CO), 4.75 and 4.65 (1H each, s, H-29), 3.01 (1H, m, *H*-19), 2.97 and 2.3 (1H each, m, H-1), 2.24 and 2.0 (1H each, m, H-22), 2.21 and 1.98 (1H each, m, H-16), 1.96 (1H, m, H-13), 1.72 (3H, s, H-30), 1.69 (1H, m, H-18), 1.64 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.16 and 1.14 (3H each, s, H-23 and H-24), 1.03 (3H, s, H-27), 0.97 (3H, s, H-26), 0.79 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 209.29, 184.12, 151.79, 148.49,

143.89, 139.17, 135.95, 132.10 × 2, 125.13 × 2, 111.25, 57.86, 54.24, 50.51, 49.76, 48.26, 46.81, 45.82, 43.93, 41.93, 39.84, 38.46, 38.02, 34.40, 33.45, 31.97, 31.07, 30.77, 26.92, 23.80, 23.08, 21.71, 20.89, 17.29, 16.89, 16.03. HRMS *m/z* calcd for $C_{37}H_{48}NO_5$ $[M - H]^-$ 586.3538, found 586.3562.

*4.1.3.4. Synthesis of 2-(3-bromobenzylidene)betulonic acid (**15d**)*. The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 3-bromobenzaldehyde (61 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15d** colourless solid (125 mg, 91% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53 (1H, d, *J* = 12.0 Hz, Ar-C*H*), 7.45 (1H, m, Ar-C*H*), 7.38 (1H, s, -C*H*=C-CO), 7.31 (2H, m, 2 × Ar-C*H*), 4.76 and 4.64 (1H each, s, H-29), 3.0 (1H, m, *H*-19), 2.96 and 2.16 (1H each, m, H-1), 2.29 and 2.0 (1H each, m, H-22), 2.24 and 1.97 (1H each, m, H-16), 1.92 (1H, m, H-13), 1.72 (3H, s, H-30), 1.66 (1H, m, H-18), 1.63 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.14 and 1.12 (3H each, s, H-23 and H-24), 1.03 (3H, s, H-27), 0.97 (3H, s, H-26), 0.78 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 208.02, 181.89, 150.42, 138.10, 135.66, 133.22, 131.25, 130.66, 129.94, 128.08, 122.55, 109.74, 56.44, 52.91, 49.15, 48.36, 46.84, 45.31, 44.13, 42.52, 40.53, 38.42, 37.02, 36.62, 33.04, 32.05, 30.61, 29.68, 29.35, 25.52, 22.36, 21.63, 20.32, 19.49, 15.82, 15.50, 14.63. HRMS *m/z* calcd for $C_{37}H_{50}BrO_3$ $[M + H]^+$ 621.2938, found 621.2946.

*4.1.3.5. Synthesis of 2-(2, 6-dichlorobenzylidene)betulonic acid (**15e**)*. The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 2, 6-dichlorobenzaldehyde (57.75 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15e** colourless solid (124 mg, 92 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*),

7.25 (1H, s, -C*H*=C-CO), 7.21 (1H, t, *J* = 8.0 Hz, Ar-C*H*), 4.71 and 4.58 (1H each, s, H-29), 2.97 (1H, m, *H*-19), 2.41 and 1.84 (1H each, m, H-1), 2.26 and 1.99 (1H each, m, H-22), 2.19 and 1.95 (1H each, m, H-16), 1.93 (1H, m, H-13), 1.67 (3H, s, H-30), 1.61 (1H, m, H-18), 1.58 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.17 and 1.14 (3H each, s, H-23 and H-24), 0.97 (3H, s, H-27), 0.94 (3H, s, H-26), 0.81 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 208.46, 183.77, 152.0, 140.26, 135.75, 135.59 × 2, 133.96, 130.74, 129.38 × 2, 111.07, 57.83, 54.85, 50.50, 49.49, 48.27, 47.16, 44.51, 43.89, 41.88, 39.85, 38.44, 37.97, 34.58, 33.45, 31.95, 31.05, 30.14, 26.84, 23.85, 22.78, 21.62, 20.81, 17.06, 16.95, 16.01. HRMS *m/z* calcd for $C_{37}H_{47}Cl_2O_3$ $[M - H]^-$ 609.2908, found 609.293.

*4.1.3.6. Synthesis of 2-{(5-bromofuran-2-yl)methylene}betulonic acid (**15f**)*. The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 5-bromo-2-furaldehyde (57.75 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15f** colourless solid (124 mg, 92 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (1H, s, -C*H*=C-CO), 6.52 (1H, d, *J* = 4.0 Hz, Ar-C*H*), 6.44 (1H, d, *J* = 4.0 Hz, Ar-C*H*), 4.78 and 4.66 (1H each, s, H-29), 3.0 (1H, m, *H*-19), 2.96 and 2.1 (1H each, m, H-1), 2.32 and 2.01 (1H each, m, H-22), 2.28 and 1.99 (1H each, m, H-16), 1.8 (1H, m, H-13), 1.74 (3H, s, H-30), 1.68 (1H, m, H-18), 1.65 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.14 and 1.07 (3H each, s, H-23 and H-24), 1.04 (3H, s, H-27), 0.99 (3H, s, H-26), 0.82 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 208.71, 183.57, 155.88, 151.87, 133.31, 126.17, 124.67, 118.42, 115.56, 111.15, 57.87, 53.89, 50.58, 49.91,

48.28, 46.32, 43.92, 41.90, 39.89, 38.45, 37.43, 34.41, 33.48, 32.05, 31.13, 31.04, 28.34, 26.99, 23.63, 23.15, 21.79, 20.93, 17.75, 16.86, 16.07. HRMS *m/z* calcd for $C_{35}H_{48}BrO_4$ $[M + H]^+$ 611.273, found 611.2748.

*4.1.3.7. Synthesis of 2-(2-bromobenzylidene)betulonic acid (**15g**)*. The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 2-bromobenzaldehyde (61 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15g** colourless solid (125 mg, 91% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (1H, d, *J* = 8.0 Hz, Ar-C*H*), 7.49 (1H, s, -C*H*=C-CO), 7.33 (1H, t, *J* = 8.0 Hz, Ar-C*H*), 7.19 (2H, m, 2 × Ar-C*H*), 4.72 and 4.6 (1H each, s, H-29), 3.0 (1H, m, *H*-19), 2.82 and 1.97 (1H each, m, H-1), 2.28 and 1.98 (1H each, m, H-22), 2.2 and 1.96 (1H each, m, H-16), 1.93 (1H, m, H-13), 1.69 (3H, s, H-30), 1.63 (1H, m, H-18), 1.61 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.17 and 1.13 (3H each, s, H-23 and H-24), 0.99 (3H, s, H-27), 0.95 (3H, s, H-26), 0.8 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 209.67, 184.18, 151.93, 138.07, 137.77, 137.39, 134.30, 131.57, 130.89, 128.49, 126.28, 111.11, 57.87, 54.66, 50.52, 49.75, 48.26, 47.14, 44.78, 43.91, 41.95, 39.83, 38.47, 38.26, 34.56, 33.47, 31.99, 31.08, 30.36, 26.90, 23.87, 22.88, 21.62, 20.86, 17.17, 16.96, 16.03. HRMS *m/z* calcd for $C_{37}H_{50}BrO_3$ $[M + H]^+$ 621.2938, found 621.295.

*4.1.3.8. Synthesis of 2-(thiophen-3-ylmethylene)betulonic acid (**15h**)*. The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 3-thiophenecarboxaldehyde (37 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15h** colourless solid (108 mg, 90 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (2H, m, 2 × Ar-C*H*), 7.36 (1H, m, Ar-C*H*), 7.25 (1H, s, -C*H*=C-CO), 4.78 and 4.67 (1H each, s, H-29), 3.04 (1H, m, *H*-19), 3.0 and 2.2

(1H each, m, H-1), 2.32 and 2.01 (1H each, m, H-22), 2.29 and 1.99 (1H each, m, H-16), 1.97 (1H, m, H-13), 1.74 (3H, s, H-30), 1.68 (1H, m, H-18), 1.67 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.15 and 1.09 (3H each, s, H-23 and H-24), 1.03 (3H, s, H-27), 0.99 (3H, s, H-26), 0.81 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 208.14, 181.97, 150.58, 137.70, 132.71, 130.88, 129.66, 127.80, 125.63, 109.71, 56.45, 52.50, 49.16, 48.61, 46.85, 45.24, 44.96, 42.51, 40.50, 38.48, 37.04, 36.21, 33.02, 32.06, 30.62, 29.68 × 2, 25.69, 22.18, 21.81, 20.41, 19.52, 16.13, 15.43, 14.64. HRMS *m/z* calcd for $C_{35}H_{47}O_3S$ $[M - H]^-$ 547.3251, found 547.3276.

*4.1.3.9. Synthesis of 2-(pyridin-3-ylmethylene)betulonic acid (**15i**).* The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 3-pyridinecarboxaldehyde (35.31 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15i** colourless solid (108 mg, 90 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.67 (1H, s, Ar-C*H*), 8.54 (1H, d, *J* = 4.0 Hz, Ar-C*H*), 7.73 (1H, d, *J* = 8.0 Hz), 7.39 (1H, s, -C*H*=C-CO), 7.37 (1H, m, Ar-C*H*), 4.73 and 4.61 (1H each, s, H-29), 3.02 (1H, m, *H*-19), 2.97 and 2.21 (1H each, m, H-1), 2.3 and 2.01 (1H each, m, H-22), 2.27 and 1.98 (1H each, m, H-16), 1.96 (1H, m, H-13), 1.7 (3H, s, H-30), 1.61 (1H, m, H-18), 1.64 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.13 and 1.11 (3H each, s, H-23 and H-24), 1.0 (3H, s, H-27), 0.96 (3H, s, H-26), 0.76 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 207.76, 180.89, 150.60, 150.47, 148.30, 137.42, 136.78, 133.09, 132.13, 123.65, 109.70, 56.31, 52.78, 49.57, 48.41, 46.87, 45.26, 44.49, 42.52, 40.51, 38.35, 37.10, 36.52, 33.03, 32.17, 30.58,

29.70, 29.40, 25.57, 22.34, 21.68, 20.33, 19.42, 15.85, 15.49, 14.58. HRMS *m/z* calcd for $C_{36}H_{48}NO_3$ [M - H]$^-$ 542.364, found 542.3681.

*4.1.3.10. Synthesis of 2-(3-bromo-4-fluorobenzylidene)betulonic acid (**15j**).* The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 3-bromo-4-fluorobenzaldehyde (67 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15j** colourless solid (126 mg, 90 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58 (1H, m, Ar-C*H*), 7.36 (1H, s, -C*H*=C-CO), 7.32 (1H, m, Ar-C*H*), 7.16 (1H, m, Ar-C*H*), 4.76 and 4.65 (1H each, s, H-29), 3.01 (1H, m, *H*-19), 2.95 and 2.15 (1H each, m, H-1), 2.28 and 2.01 (1H each, m, H-22), 2.22 and 1.98 (1H each, m, H-16), 1.96 (1H, m, H-13), 1.72 (3H, s, H-30), 1.67 (1H, m, H-18), 01.64 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.14 and 1.12 (3H each, s, H-23 and H-24), 1.02 (3H, s, H-27), 0.98 (3H, s, H-26), 0.78 (3H, s, H-25).^{13}C NMR (125 MHz, $CDCl_3$): δ 207.78, 181.91, 159.96, 150.36, 135.45, 135.32, 134.71, 133.69, 130.14, 116.59, 109.75, 109.34, 56.44, 52.92, 49.19, 48.42, 46.84, 45.29, 44.10, 42.54, 40.56, 38.44, 37.02, 36.63, 33.05, 32.06, 30.62, 29.69, 29.36, 25.54, 22.33, 21.67, 20.32, 19.50,15.81, 15.50, 14.63. HRMS *m/z* calcd for $C_{37}H_{48}BrFO_3$ [M - H]$^-$ 639.2844, found 639.2838.

*4.1.3.11. Synthesis of 2-(5-bromo-2-methoxybenzylidene)betulonic acid (**15k**).* The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 5-bromo-2-methoxybenzaldehyde (71 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15k** colourless solid (130 mg, 92 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57 (1H, s, -C*H*=C-CO), 7.37 (2H, m, 2 × Ar-C*H*), 6.76 (1H, m, Ar-C*H*), 4.74 and 4.62 (1H each, s, H-29), 3.83 (3H, s, -OCH_3), 3.0 (1H, m, *H*-19), 2.89 and 2.03 (1H each, m, H-1), 2.28 and 1.99 (1H

each, m, H-22), 2.22 and 1.95 (1H each, m, H-16), 1.93 (1H, m, H-13), 1.7 (3H, s, H-30), 1.66 (1H, m, H-18), 1.62 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.14 and 1.12 (3H each, s, H-23 and H-24), 1.01 (3H, s, H-27), 0.97 (3H, s, H-26), 0.8 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 207.68, 182.27, 157.19, 150.24, 135.20, 132.18, 131.33, 131.23, 127.14, 112.41, 111.88, 109.84, 56.45, 55.76, 53.24, 49.14, 48.23, 46.87, 45.47, 43.43, 42.51, 40.55, 38.40, 37.03, 36.80, 33.13, 32.08, 30.55, 29.71, 29.18, 25.42, 22.59, 21.44, 20.24, 19.48, 15.69, 15.57, 14.64. HRMS *m/z* calcd for $C_{38}H_{52}BrO_4$ $[M + H]^+$ 651.3043, found 651.3051.

*4.1.3.12. Synthesis of 2-(4-fluorobenzylidene)betulonic acid (**15l**).* The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 4-fluorobenzaldehyde (41 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15l** colourless solid (111 mg, 90 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): 7.56 (1H, s, -C*H*=C-CO), 7.32 (2H, m, 2 × Ar-C*H*), 7.17 (1H, t, *J* = 8.0 Hz, Ar-C*H*), 7.09 (1H, t, *J* = 8.0 Hz, Ar-C*H*), 4.74 and 4.63 (1H each, s, H-29), 3.0 (1H, m, *H*-19), 2.91 and 2.1 (1H each, m, H-1), 2.29 and 1.99 (1H each, m, H-16), 2.22 and 1.97 (1H each, m, H-1), 1.90 (1H, m, H-13), 1.71 (3H, s, H-30), 1.66 (1H, m, H-18), 1.62 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.15 and 1.14 (3H each, s, H-23 and H-24), 1.01 (3H, s, H-27), 0.97 (3H, s, H-26), 0.79 (3H, s, H-25). δ ^{13}C NMR (125 MHz, $CDCl_3$): δ 207.82, 182.16, 161.89, 150.58, 136.25, 130.10, 123.87 × 2, 122.79, 115.87, 115.69, 109.68, 56.43, 53.48, 53.01, 49.11, 48.34, 46.83, 45.41, 43.97, 42.50, 40.52, 38.41, 37.03, 33.09, 32.04, 30.57,

29.66, 29.22, 25.53, 22.46, 21.53, 20.27, 19.47, 15.79, 15.52, 14.61. HRMS *m/z* calcd for $C_{37}H_{48}FO_3$ $[M - H]^-$ 559.3593, found 559.3637.

*4.1.3.13. Synthesis of 2-((E)-3-phenylallylidene)betulonic acid (**15m**).* The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and cinnamaldehyde (43.5 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15m** colourless solid (113 mg, 90 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.49 (2H, m, 2 × Ar-C*H*), 7.32 (3H, m, 3 × Ar-C*H*), 7.23 (1H, d, *J* = 12.0 Hz, -CO-C=C*H*-), 6.93 (2H, m, 2 × Ar-C*H*=C*H*-), 4.79 and 4.67 (1H each, s, H-29), 3.06 (1H, m, *H*-19), 2.98 and 2.07 (1H each, m, H-1), 2.33 and 2.01 (1H each, m, H-22), 2.28 and 1.98 (1H each, m, H-16), 1.96 (1H, m, H-13), 1.74 (3H, s, H-30), 1.67 (1H, m, H-18), 1.65 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 0.97 (1H each, m, H-11), 1.11 and 1.09 (3H each, s, H-23 and H-24), 1.03 (3H, s, H-26), 1.0 (3H, s, H-24), 0.82 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 207.50, 182.52, 150.58, 140.66, 137.26, 136.70, 133.07, 128.77 × 3, 127.18 × 2, 123.35, 109.74, 56.48, 52.89, 49.17, 48.39, 46.90, 45.07, 42.72, 42.54, 40.53, 38.56, 37.08, 36.20, 33.17, 32.09, 30.58, 29.69, 29.41, 25.67, 22.35, 21.71, 20.33, 19.48, 15.91, 15.57, 14.62. HRMS *m/z* calcd for $C_{39}H_{53}O_3$ $[M + H]^+$ 569.3989, found 569.3985.

*4.1.3.14. Synthesis of 2-(4-methoxybenzylidene)betulonic acid (**15n**).* The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 4-methoxybenzaldehyde (45 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15n** colourless solid (113 mg, 90 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (1H, -C*H*=C-CO), 7.41 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 6.95 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 4.77 and 4.66 (1H each, s, H-29), 3.85 (3H, s, -OC*H*$_3$), 3.06 (1H, m, *H*-19), 3.02 and 2.2 (1H each, m, H-1), 2.31 and 2.01 (1H each, m, H-22),

2.28 and 1.98 (1H each, m, H-16), 1.96 (1H, m, H-13), 1.74 (3H, s, H-30), 1.68 (1H, m, H-18), 1.65 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 0.97 (1H each, m, H-11), 1.14 and 1.11 (3H each, s, H-23 and H-24), 1.03 (3H, s, H-27), 0.98 (3H, s, H-26), 0.79 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 208.13, 181.36, 159.82, 150.67, 137.31, 132.26 × 2, 131.96, 128.66, 114.01 × 2, 109.64, 56.42, 55.34, 52.63, 49.17, 48.51, 46.83, 45.01, 44.68, 42.51, 40.52, 38.45, 37.03, 36.40, 33.06, 32.06, 30.62, 29.68, 29.63, 25.66, 22.33, 21.69, 20.39, 19.54, 15.88, 15.48, 14.63. HRMS *m/z* calcd for $C_{38}H_{53}O_4$ $[M + H]^+$ 573.3938, found 573.3953.

*4.1.3.15. Synthesis of 2-(naphthalene-1-ylmethylene)betulonic acid (**15o**).* The title compound prepared by the reaction of betulonic acid (100 mg, 0.22 mmol) and 1-naphthaldehyde (51.5 mg, 0.33 mmol) as per the method described in Section 4.1.3 to furnish **15o** colourless solid (118 mg, 91 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.08 (1H, -C*H*=C-CO), 7.94 (1H, m, Ar-C*H*), 7.86 (2H, m, 2 × Ar-C*H*), 7.51 (3H, m, 3 × Ar-C*H*), 7.34 (1H, d, *J* = 8.0Hz, Ar-C*H*), 4.69 and 4.58 (1H each, s, H-29), 2.97 (1H, m, *H*-19), 2.91 and 2.04 (1H each, m, H-1), 2.26 and 1.97 (1H each, m, H-22), 2.18 and 1.94 (1H each, m, H-16), 1.93 (1H, m, H-13), 1.66 (3H, s, H-30), 1.59 (1H, m, H-18), 1.58 (1H, m, H-5), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 0.97 (1H each, m, H-11), 1.22 and 1.18 (3H each, s, H-23 and H-24), 0.97 (3H, s, H-27), 0.94 (3H, s, H-26), 0.79 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 207.95, 181.41, 150.41, 136.02, 135.95, 133.51, 133.13, 132.0, 128.62, 128.54, 126.48, 126.37, 126.12, 125.17, 124.68, 109.66, 56.36, 53.42, 49.13, 48.27, 46.79, 45.63, 43.52,

42.49, 40.53, 38.40, 37.0, 36.76, 33.21, 32.05, 30.56, 29.65, 29.14, 25.46, 22.64, 21.39, 20.27, 19.42, 15.64, 15.56, 14.58. HRMS *m/z* calcd for $C_{41}H_{51}O_3$ $[M - H]^-$ 591.3844, found 591.3849.

*4.1.4. General experimental procedure for synthesis of benzylidene derivatives **16a-o***

Compounds **16a-o** were prepared by addition of $NaBH_4$ (1.5 equiv) to stirring methanolic solution of corresponding benzylidene derivative **15a-o** (1 equiv) at 0^oC. After 10 min, reaction mixture was stirred at room temperature for 1-1.5 h till the completion (monitored by TLC analysis) [32]. Workup of the reaction was done by diluting the reaction mixture with ice-cold water and extracting it with ethyl acetate (3 times). The combined organic layers were dried over sodium sulphate and concentrated on rotavapour. The crude product obtained was purified by column chromatography on silica gel 60-120 mesh with EtoAc: Hexane (1: 10) as the eluent and recrystallized with hot methanol to afford the desired pure products **16a-o** in 70-75% yield. The spectral data of all the derivatives are given below.

*4.1.4.1. Synthesis of 2-(3-nitrobenzylidene)betulinic acid (**16a**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (2H, m, 2 × Ar-C*H*), 7.39 (2H, m, 2 × Ar-C*H*), 6.67 (1H, s, -C*H*=C-CHOH), 4.63 and 4.51 (1H each, s, H-29), 3.81 (1H, s, -C*H*OH), 2.89 (1H, m, *H*-19), 2.8 and 1.51 (1H each, m, H-1), 2.19 and 1.89 (1H each, m, H-22), 2.07 and 1.87 (1H each, m, H-16), 1.85 (1H, m, H-13), 1.6 (3H, s, H-30), 1.58 (1H, m, H-18), 1.56 and 1.33 (1H each, m, H-6), 1.52 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.2 (1H each, m, H-11), 1.06 (3H, s, H-26), 0.91 and 0.8 (3H each, s, H-27 and H-23), 0.67 (3H, s, H-24), 0.77 (1H, m, H-5), 0.62 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.3, 150.24, 148.2, 143.42, 139.89, 134.96, 129.01, 123.52, 120.99, 120.73, 109.77, 81.0, 56.36, 55.89, 49.78, 49.21, 46.89, 42.49, 41.88, 41.84, 40.91,

40.64, 38.35, 37.04, 34.1, 32.14, 30.56, 29.69, 28.55, 25.37, 20.95, 19.37, 18.38, 16.29, 15.9, 15.53, 14.66. HRMS *m/z* calcd for $C_{37}H_{50}NO_5$ $[M - H]^-$ 588.3694, found 588.3699.

*4.1.4.2. Synthesis of 2-(4-bromobenzylidene)betulinic acid (**16b**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 6.98 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 6.55 (1H, s, -C*H*=C-CHOH), 4.66 and 4.53 (1H each, s, H-29), 3.77 (1H, s, -C*H*OH), 2.91 (1H, m, *H*-19), 2.83 and 1.47 (1H each, m, H-1), 2.19 and 1.92 (1H each, m, H-22), 2.1 and 1.88 (1H each, m, H-16), 1.87 (1H, m, H-13), 1.61 (3H, s, H-30), 1.56 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.2 (1H each, m, H-11), 1.05 (3H, s, H-26), 0.92 and 0.81 (3H each, s, H-27 and H-23), 0.66 (3H, s, H-24), 0.69 (1H, m, H-5), 0.59 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 180.42, 149.86, 140.89, 136.51, 130.73 × 2, 130.01 × 2, 121.09, 119.36, 109.29, 80.58, 55.78, 55.37, 49.30, 48.71, 46.39, 41.98, 41.46, 41.13, 40.40, 39.89, 37.86, 36.54, 33.63, 31.62, 30.01, 29.18, 28.03, 24.89, 20.54, 18.84, 17.90, 15.71, 15.37, 15.03, 14.19. HRMS *m/z* calcd for $C_{37}H_{50}BrO_3$ $[M - H]^-$ 621.2949, found 621.2979.

*4.1.4.3. Synthesis of 2-(4-nitrobenzylidene)betulinic acid (**16c**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 7.27 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 6.71 (1H, s, -C*H*=C-CHOH), 4.75 and 4.65 (1H each, s, H-29), 3.83 (1H, s, -C*H*OH), 2.09 (1H, m, *H*-19), 2.84 and 1.55 (1H each, m, H-1), 2.22 and 1.91 (1H each, m, H-22), 2.09 and 1.89 (1H each, m, H-16), 1.88 (1H, m, H-13), 1.61 (3H, s, H-30), 1.54 (1H, m, H-18), 1.52 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 0.77 (1H each, m, H-11), 1.08 (3H, s, H-26), 0.93 and 0.81 (3H each, s, H-27 and H-23), 0.71 (1H, m, H-5), 0.68 (3H, s, H-24), 0.59 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 181.85, 150.29, 145.96, 145.38,

144.42, 129.51 × 2, 123.58 × 2, 121.35, 109.85, 81.01, 56.30, 55.77, 53.48, 49.79, 49.17, 46.89, 42.48, 41.93, 40.9, 40.7, 38.34, 37.04, 34.06, 32.10, 30.49, 29.71, 28.58, 25.31, 21.05, 19.35, 18.40, 16.23, 15.86, 15.58, 14.68. HRMS *m/z* calcd for $C_{37}H_{50}NO_5$ $[M - H]^-$ 588.3694, found 588.3717.

*4.1.4.4. Synthesis of 2-(3-bromobenzylidene)betulinic acid (**16d**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32 (2H, m, 2 × Ar-C*H*), 7.14 (2H, m, 2 × Ar-C*H*), 6.63 (1H, s, -C*H*=C-CHOH), 4.73 and 4.6 (1H each, s, H-29), 3.84 (1H, s, -C*H*OH), 2.92 (1H, m, *H*-19), 2.8 and 1.56 (1H each, m, H-1), 2.28 and 1.97 (1H each, m, H-22), 2.25 and 1.95 (1H each, m, H-16), 1.93 (1H, m, H-13), 1.68 (3H, s, H-30), 1.6 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.12 (3H, s, H-26), 0.99 and 0.9 (3H each, s, H-27 and H-23), 0.73 (3H, s, H-24), 0.81 (1H, m, H-5), 0.69 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 179.06, 150.58, 142.13, 140.67, 131.55, 129.54, 128.71, 127.32, 121.96, 121.31, 109.34, 80.56, 56.14, 55.98, 49.80, 49.06, 46.96, 42.42, 41.89, 41.5, 40.81, 40.32, 38.21, 37.02, 34.09, 32.15, 30.43, 29.58, 28.15, 25.42, 20.93, 18.94, 18.33, 16.07, 15.55, 15.43, 14.41. HRMS *m/z* calcd for $C_{37}H_{50}BrO_3$ $[M - H]^-$ 621.2949, found 621.2974.

*4.1.4.5. Synthesis of 2-(2, 6-dichlorobenzylidene)betulinic acid (**16e**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.28 (2H, m, 2 × Ar-C*H*), 7.1 (1H, t, *J* = 8.0 Hz, Ar-C*H*), 6.42 (1H, s, -C*H*=C-CHOH), 4.69 and 4.59 (1H each, s, H-29), 3.92 (1H, s, -C*H*OH), 2.96 (1H, m, *H*-19), 2.25 and 1.48 (1H each, m, H-1), 2.12 and 1.95 (1H each, m, H-22), 2.06 and 1.92 (1H each, m, H-16), 1.91 (1H, m, H-13), 1.67 (3H, s, H-30), 1.56 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.03 (1H each, m, H-11),

1.14 (3H, s, H-26), 0.97 and 0.81 (3H each, s, H-27 and H-23), 0.88 (1H, m, H-5), 0.81 (3H, s, H-24), 0.56 (3H, s, H-25).^{13}C NMR (125 MHz, $CDCl_3$): δ 182.33, 150.58, 144.25, 135.7 × 2, 135.51, 128.15 × 2, 127.95, 117.67, 109.64, 80.77, 56.40, 55.72, 49.80, 49.17, 46.93, 43.85, 42.41, 41.78, 40.79, 39.94, 38.32, 37.03, 34.06, 32.12, 30.54, 29.68, 28.36, 25.32, 20.84, 19.35, 18.47, 15.78, 15.71, 15.52, 14.67. HRMS *m/z* calcd for $C_{37}H_{49}Cl_2O_3$ $[M - H]^-$ 611.3064, found 611.3089.

*4.1.4.6. Synthesis of 2-{(5-bromofuran-2-yl)methylene}betulinic acid (**16f**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.37 (1H, s, -C*H*=C-CHOH), 6.26 (1H, d, *J* = 4.0 Hz, Ar-C*H*), 6.17 (1H, d, *J* = 4.0 Hz, Ar-C*H*), 4.76 and 4.63 (1H each, s, H-29), 3.79 (1H, s, -C*H*OH), 3.03 (1H, m, *H*-19), 3.37 and 1.73 (1H each, m, H-1), 2.3 and 1.98 (1H each, m, H-22), 2.26 and 1.96 (1H each, m, H-16), 1.94 (1H, m, H-13), 1.71 (3H, s, H-30), 1.68 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.1 (3H, s, H-26), 1.02 and 0.94 (3H each, s, H-27 and H-23), 0.76 (3H, s, H-24), 0.81 (1H, m, H-5), 0.66 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.79, 155.32, 150.40, 140.89, 119.94, 112.54, 110.57, 110.54, 109.77, 81.17, 56.45, 56.26, 49.75, 49.21, 46.92, 42.84, 42.51, 42.40, 41.03, 40.98, 38.46, 37.08, 34.21, 32.16, 30.59, 29.75, 28.43, 25.55, 21.0, 19.44, 18.45, 15.98, 15.89, 15.37, 14.74. HRMS *m/z* calcd for $C_{35}H_{46}BrO_4$ $[M - H]^-$ 611.2571, found 611.2621.

*4.1.4.7. Synthesis of 2-(2-bromobenzylidene)betulinic acid (**16g**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55 (1H, m, Ar-C*H*), 7.24 (1H, d, *J* = 8.0 Hz, Ar-C*H*), 7.1 (2H, m, 2 × Ar-C*H*), 6.62 (1H, s, -C*H*=C-CHOH), 4.7 and 4.59 (1H each, s, H-29), 3.89 (1H, s, -C*H*OH), 2.96 (1H, m, *H*-19), 2.66 and 1.54 (1H each, m, H-1), 2.23 and 1.95 (1H each, m, H-22), 2.14 and 1.93 (1H each, m, H-16), 1.92 (1H, m, H-13), 1.66 (3H, s, H-30), 1.61 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-

6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.06 (1H each, m, H-11), 1.13 (3H, s, H-26), 0.97 and 0.84 (3H each, s, H-27 and H-23), 0.8 (3H, s, H-24), 0.88 (1H, m, H-5), 0.6 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 181.03, 149.45, 140.36, 137.35, 131.38, 129.86, 126.8, 125.81, 123.49, 121.9, 108.63, 79.99, 55.33, 54.83, 48.84, 48.19, 45.88, 41.42, 41.31, 40.71, 39.84, 39.29, 37.36, 36.0, 33.11, 31.11, 29.51, 28.65, 27.43, 24.37, 19.92, 18.32, 17.40, 14.81 × 2, 14.44, 13.65. HRMS *m/z* calcd for $C_{37}H_{50}BrO_3$ $[M - H]^-$ 621.2949, found 621.2954.

*4.1.4.8. Synthesis of 2-(thiophen-3-ylmethylene)betulinic acid (**16h**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25 (1H, m, Ar-C*H*), 7.05 (2H, m, 2 × Ar-C*H*), 6.59 (1H, s, -C*H*=C-CHOH), 4.74 and 4.61 (1H each, s, H-29), 3.81 (1H, s, -C*H*OH), 3.13 and 1.64 (1H each, m, H-1), 3.0 (1H, m, *H*-19), 2.29 and 1.98 (1H each, m, H-22), 2.26 and 1.96 (1H each, m, H-16), 1.94 (1H, m, H-13), 1.68 (3H, s, H-30), 1.61 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.11 (3H, s, H-26), 1.0 and 0.91 (3H each, s, H-27 and H-23), 0.76 (3H, s, H-24), 0.83 (1H, m, H-5), 0.7 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.44, 150.37, 140.1, 138.6, 128.76, 124.83, 121.77, 116.79, 109.76, 81.3, 56.4, 56.21, 49.76, 49.29, 46.98, 42.51, 41.81, 40.96, 40.53, 39.68, 38.48, 37.07, 34.22, 32.19, 30.59, 29.74, 28.47, 25.5, 21.14, 19.36, 18.42, 16.24, 15.92, 15.46, 14.74. HRMS *m/z* calcd for $C_{35}H_{49}O_3S$ $[M - H]^-$ 549.3408, found 549.3409.

*4.1.4.9. Synthesis of 2-(pyridin-3-ylmethylene)betulinic acid (**16i**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.44 (2H, m, 2 × Ar-C*H*), 7.52 (1H, d, *J* = 8.0 Hz, Ar-C*H*), 7.27 (1H, m, Ar-C*H*), 6.68 (1H, s, -C*H*=C-CHOH), 4.72 and 4.58 (1H each, s, H-29), 3.88 (1H, s, -C*H*OH), 3.04 (1H, m, *H*-19), 2.8

and 1.54 (1H each, m, H-1), 2.3 and 2.01 (1H each, m, H-22), 2.18 and 1.98 (1H each, m, H-16), 1.96 (1H, m, H-13), 1.68 (3H, s, H-30), 1.61 (1H, m, H-18), 1.56 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.13 (3H, s, H-26), 1.13 and 0.98 (3H each, s, H-27 and H-23), 0.85 (3H, s, H-24), 0.89 (1H, m, H-5), 0.74 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 180.53, 150.71, 148.76, 146.04, 143.73, 136.94, 134.44, 123.43, 118.89, 109.57, 80.94, 56.28, 55.83, 49.81, 49.32, 47.04, 42.49, 41.93, 41.79, 40.89, 40.56, 38.41, 37.12, 34.12, 32.42, 30.71, 29.75, 28.55, 25.4, 21.02, 19.41, 18.41, 16.27, 15.92, 15.56, 14.70. HRMS *m/z* calcd for $C_{36}H_{52}NO_3$ $[M + H]^+$ 546.3942, found 546.3957.

*4.1.4.10. Synthesis of 2-(3-bromo-4-fluorobenzylidene)betulinic acid (**16j**)*. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (1H, m, Ar-C*H*), 7.06 (2H, m, 2 × Ar-C*H*), 6.6 (1H, s, -C*H*=C-CHOH), 4.73 and 4.6 (1H each, s, H-29), 3.83 (1H, s, -C*H*OH), 2.99 (1H, m, *H*-19), 2.83 and 1.5 (1H each, m, H-1), 2.26 and 1.97 (1H each, m, H-22), 2.15 and 1.95 (1H each, m, H-16), 1.94 (1H, m, H-13), 1.68 (3H, s, H-30), 1.65 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.12 (3H, s, H-26), 0.98 and 0.89 (3H each, s, H-27 and H-23), 0.72 (3H, s, H-24), 0.85 (1H, m, H-5), 0.67 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 179.23, 158.26, 150.62, 141.98, 135.85, 133.51, 129.17, 120.42, 116.02, 109.5, 108.49, 80.67, 56.14, 55.8, 49.74, 49.08, 46.89, 42.41, 41.81, 41.55, 40.81, 40.36, 38.16, 37.06, 34.06, 32.16, 30.47, 29.6, 28.34, 25.37, 20.95, 19.22, 18.34, 16.19, 15.69, 15.5, 14.56. HRMS *m/z* calcd for $C_{37}H_{49}BrFO_3$ $[M - H]^-$ 639.2855, found 639.2883.

*4.1.4.11. Synthesis of 2-(5-bromo-2-methoxybenzylidene)betulinic acid (**16k**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.28 (1H, m, Ar-C*H*), 7.23 (1H, m, Ar-C*H*), 6.72 (1H, d, *J* = 8.0 Hz, Ar-C*H*), 6.56 (1H, s, -C*H*=C-CHOH), 4.74 and 4.62 (1H each, s, H-29), 3.85 (1H, s, -CHOH), 3.76 (3H, s, -OCH_3), 2.98 (1H, m, *H*-19), 2.71 and 1.49 (1H each, m, H-1), 2.25 and 1.97 (1H each, m, H-22), 2.13 and 1.94 (1H each, m, H-16), 1.92 (1H, m, H-13), 1.67 (3H, s, H-30), 1.62 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.12 (3H, s, H-26), 0.98 and 0.88 (3H each, s, H-27 and H-23), 0.74 (3H, s, H-24), 0.84 (1H, m, H-5), 0.67 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.39, 156.38, 150.36, 141.51, 132.49, 130.08, 129.18, 117.52, 112.24, 112.07, 109.79, 81.2, 56.39, 55.93, 55.68, 49.76, 49.2, 46.9, 42.46, 42.26, 41.58, 40.86, 40.32, 38.34, 37.07, 34.12, 32.16, 30.56, 29.69, 28.49, 25.39, 20.89, 19.4, 18.4, 16.08, 15.92, 15.54, 14.69. HRMS *m/z* calcd for $C_{38}H_{52}BrO_4$ $[M - H]^-$ 651.3054, found 651.3084.

*4.1.4.12. Synthesis of 2-(4-fluorobenzylidene)betulinic acid (**16l**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.17 (2H, m, 2 × Ar-C*H*), 7.03 (2H, m, 2 × Ar-C*H*), 6.6 (1H, s, -C*H*=C-CHOH), 4.71 and 4.59 (1H each, s, H-29), 3.89 (1H, s, -C*H*OH), 2.97 (1H, m, *H*-19), 2.71 and 1.6 (1H each, m, H-1), 2.27 and 1.96 (1H each, m, H-22), 2.24 and 1.94 (1H each, m, H-16), 1.90 (1H, m, H-13), 1.71 (3H, s, H-30), 1.62 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 1.08 (1H each, m, H-11), 1.15 (3H, s, H-26), 1.14 and 1.03 (3H each, s, H-27 and H-23), 1.01 (3H, s, H-24), 0.84 (1H, m, H-5), 0.79 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.31, 161.14, 150.48, 142.81, 130.79, 128.01, 125.77, 123.57, 115.79, 115.41, 109.7, 81.12, 56.41, 55.84, 49.81, 49.18, 46.92, 42.55, 42.45, 41.59, 40.86,

40.21, 38.38, 37.05, 34.11, 32.13, 30.52, 29.73, 28.49, 25.38, 20.94, 19.33, 18.42, 15.94, 15.84, 15.48, 14.69. HRMS *m/z* calcd for $C_{37}H_{66}FO_2$ [M - H]$^-$ 561.5052, found 561.5091.

*4.1.4.13. Synthesis of 2-((E)-3-phenylallylidene)betulinic acid (**16m**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39 (2H, m, 2 × Ar-C*H*), 7.3 (2H, m, 2 × Ar-C*H*), 7.2 (1H, t, *J* = 8.0 Hz, Ar-C*H*), 6.96 (1H, m, Ar-C*H*=C*H*-), 6.58 (1H, d, *J* = 16.0 Hz, Ar-CH=C*H*-), 6.41 (1H, d, *J* = 12.0 Hz, C*H*=C-CHOH), 4.76 and 4.62 (1H each, s, H-29), 3.8 (1H, s, -C*H*OH), 3.02 (1H, m, *H*-19), 2.94 and 1.79 (1H each, m, H-1), 2.28 and 2.0 (1H each, m, H-22), 2.21 and 1.97 (1H each, m, H-16), 2.04 (1H, m, H-13), 1.7 (3H, s, H-30), 1.64 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 0.97 (1H each, m, H-11), 1.09 (3H, s, H-26), 1.01 and 0.93 (3H each, s, H-27 and H-23), 0.72 (3H, s, H-24), 0.69 (1H, m, H-5), 0.65 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.62, 150.41, 141.32, 137.92, 131.9, 128.6 × 2, 127.19, 126.25 × 2, 124.25, 122.19, 109.87, 81.25, 56.46, 56.06, 49.91, 49.25, 47.0, 42.51, 42.18, 40.98, 40.6, 38.42, 37.1, 34.2, 32.17, 31.47, 30.52, 30.41, 28.34, 25.44, 21.18, 19.32, 18.53, 15.98, 15.89, 15.45, 14.74. HRMS *m/z* calcd for $C_{39}H_{53}O_3$ [M - H]$^-$ 569.4, found 569.4025.

*4.1.4.14. Synthesis of 2-(4-methoxybenzylidene)betulinic acid (**16n**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.12 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 6.84 (2H, d, *J* = 8.0 Hz, 2 × Ar-C*H*), 6.61 (1H, -C*H*=C-CHOH), 4.73 and 4.6 (1H each, s, H-29), 3.83 (1H, s, -C*H*OH), 3.8 (3H, s, -OCH_3), 3.01 (1H, m, *H*-19), 2.98 and 1.6 (1H each, m, H-1), 2.26 and 1.97 (1H each, m, H-22), 2.17 and 1.94 (1H each, m, H-16), 1.92 (1H, m, H-13), 1.68 (3H, s, H-30), 1.66 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 0.97

(1H each, m, H-11), 1.11 (3H, s, H-26), 0.99 and 0.89 (3H each, s, H-27 and H-23), 0.72 (3H, s, H-24), 0.87 (1H, m, H-5), 0.68 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 182.11, 157.81, 150.47, 139.27, 130.49, 2 × 129.92, 121.99, 2 × 113.57, 109.71, 81.22, 56.38, 55.97, 55.32, 55.21, 49.77, 49.23, 46.44, 42.47, 41.94, 41.52, 40.9, 40.3, 38.43, 37.07, 34.17, 32.16, 30.55, 28.51, 25.45, 21.04, 19.35, 18.39, 16.21, 15.9, 15.51, 14.71. HRMS *m/z* calcd for $C_{38}H_{53}O_4$ [M - H]$^-$ 573.3949, found 573.396.

*4.1.4.15. Synthesis of 2-(naphthalene-1-ylmethylene)betulinic acid (**16o**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.97 (1H, m, Ar-C*H*), 7.83 (1H, m, Ar-C*H*), 7.73 (1H, d, *J* = 12.0 Hz, Ar-C*H*), 7.43 (3H, m, 3 × Ar-C*H*), 7.21 (1H, d, *J* = 8.0 Hz, Ar-C*H*), 7.06 (1H, -C*H*=C-CHOH), 4.66 and 4.54 (1H each, s, H-29), 4.0 (1H, s, -CHOH), 2.91 (1H, m, *H*-19), 2.69 and 1.54 (1H each, m, H-1), 2.23 and 1.93 (1H each, m, H-22), 2.04 and 1.94 (1H each, m, H-16), 2.08 (1H, m, H-13), 1.63 (3H, s, H-30), 1.59 (1H, m, H-18), 1.54 and 1.33 (1H each, m, H-6), 1.51 and 1.29 (1H each, m, H-15), 1.48 and 1.41 (1H each, m, H-7), 1.46 and 1.24 (1H each, m, H-21), 1.43 and 1.25 (1H each, m, H-12), 1.38 (1H, m, H-9), 1.35 and 0.97 (1H each, m, H-11), 1.17 (3H, s, H-26), 0.96 and 0.87 (3H each, s, H-27 and H-23), 0.78 (3H, s, H-24), 0.75 (1H, m, H-5), 0.53 (3H, s, H-25). ^{13}C NMR (125 MHz, $CDCl_3$): δ 181.11, 150.35, 141.79, 135.39, 133.60, 132.41, 128.29, 126.77, 126.42, 125.61 × 2, 125.27, 125.18, 120.89, 109.62, 81.28, 56.28, 55.9, 49.9, 49.24, 46.85, 42.77, 42.43, 41.55, 40.86, 40.01, 38.34, 37.0, 34.17, 32.12, 30.53, 29.67, 28.47, 25.36, 20.87, 19.32, 18.46, 16.13, 15.79, 15.69, 14.65. HRMS *m/z* calcd for $C_{41}H_{53}O_3$ [M - H]$^-$ 593.4, found 593.4.

4.2. Biology

4.2.1. Cell culture and growth conditions

Human cancer cell lines PC-3 (Prostate), MCF-7 (Breast), A-549 (Lung), HCT 116 (Colon) and MIA PaCa-2 (Pancreatic) were obtained from National Cancer Institute (NCI), USA. Non tumor human breast epithelial cell line fR2 (ECACC 98031102) were procured from European Collection of Authenticated Cell Cultures (ECACC), UK. The cells lines are maintained and serially passaged as per the NCI protocols. The authenticity and integrity of cancer cell lines are checked on regular basis by comparing genetic profiling with established database (NCI-FREDERICK Cancer DCTD cell line repository, NCBI'S sky). The cell lines which found with genetic variability are replaced with the frozen master/primary stock. The human cancer cell lines were grown in tissue culture flasks in complete growth medium (RPMI-1640) supplemented with 10% fetal bovine serum, 100µg/mL streptomycin and 100 units/mL penicillin (New Brunswick, Galaxy 170R, Eppendorf) at 37°C, 5% CO_2 and 98% RH. Paclitaxel used as positive control have been purchased from Sigma-Aldrich (Bangalore, India).

4.2.2. Cytotoxicity Assay

The SRB assay was executed to assess the cytotoxic potential of the potent inhibitors in which optimum cell density per well was seeded in 96 well flat bottom plates. 100 µL of cell suspension of various panel of human cancer cell lines PC-3 (7000), MCF-7 (8000), A-549 (7500), HCT 116 (7000), and MIA PaCa-2 (12000) was plated. Following 24 h of incubation under culture conditions, the cells were exposed to different concentration (1, 2.5, 5 and 10 µM) of test materials containing complete growth medium along with Paclitaxel as positive control. The plates were kept under incubation under the same conditions for 48 h at 37°C. Further, cells were fixed with ice cold TCA for 1 h at 4°C. After 1 h, the plates were washed three times with water and allowed to air dry. Afterwards, 100 µL of 0.4% SRB dye was added for half an hour at room temperature. Plates were then washed three times with water followed by 1% v/v acetic

acid to remove the unbound SRB. After drying at room temperature, the bound dye was solubilised by adding 100 µL of 10 mM Tris buffer (pH-10.4) to each well. The plates were retained on the shaker for 5 min in order to dissolve the protein bound dye. OD was taken at 540 nm in a microplate reader (Thermo Scientific) and IC_{50} was determined by using GraphPAD Prism Software Version 5.0 (1).

$$\text{The \% of cell viability} = \frac{\text{Absorbance of treated cells - Absorbance of Blank}}{\text{Absorbance of control cells - Absorbance of Blank}} \text{ X } 100$$

$$\text{\% Growth inhibition} = 100 - \text{\% of cell viability}$$

4.2.3. Colony formation assay

HCT 116 cells ($8x10^4$/mL/well) were seeded and treated with compound **16c** at different concentrations of 0.7, 1.4 and 2.8 µM and incubated for 24 h. Then, the treated cells were trypsinized, counted and re-seeded at 1000 cells/well in a six well plate. The cells were left in order to give colonies of >50 cells to assess the clonogenic ability. After that, cells were fixed with 1 mL of 4% formaldehyde and stained with 0.5% crystal violet. Finally, crystal violet was aspirated carefully and rinsed with water. Clonogenic survival was expressed as the number of colony-forming units in treated cultures in comparison to untreated controls.

4.2.4. In vitro cell migration assay

HCT 116 cells were plated at a concentration of $8x10^5$cells/well and allowed to reach confluency up to 70-80%. Then, the cells were serum starved for 24 h and monolayer was scraped in a straight horizontal line with a sterile 200 µL tip. Eventually, cells were treated with different concentrations of **16c** for 24 h. Wounded areas were gradually photographed (20x magnification) at 0 and 24 h and the percentage of wound closure was estimated by the following equation: % Wound closure = [1-(wound area at 0 h /wound area at 24 h) × 100%]

4.2.5. Phase contrast microscopy

HCT 116 cells were treated with 0.7, 1.4, 2.0 and 2.5 µM concentration of compound **16c** for 24 h. Cells were simply photographed in order to observe the changes in the morphology of the cells after treatment using microscope.

4.2.6. Mitochondrial membrane potential (MMP)

Cells ($1x10^6$/1.5mL/well) in 12 well plates were treated with **16c** at different concentration of 0.7, 1.4, 2.0 and 2.5 µM and Paclitaxel at 100 nM for 24 h. Rhodamine-123 (2.5 µg/mL) was added 1 h before experiment termination for 30 min at 37^{o}C in dark. Afterwards, cells were washed with PBS and immediately analyzed under the fluorescence microscope. The fluorescence intensity was determined at an excitation wavelength of 488 nm under confocal microscope (Olympus, 40X) representing the mitochondrial membrane potential.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at

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Legends to Figures

Fig.1. Some representative triterpene scaffolds having anticancer potential

Fig. 2. Structures of potent anticancer ring A (C-2 and C-3) modified betulinic acid derivatives

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Tables

Table 1

In vitro cytotoxic activity of betulinic acid and its derivatives against various cancer cell lines

Cell Line			A-549 (Lung)	PC-3 (Prostate)	HCT 116 (Colon)	MCF-7 (Breast)	MIA PaCa-2 (Pancreatic)
S. No.	Code	Conc. (μM)	% growth inhibition				
1	**1**	10	40	49	42	28	26
2	**7**	10	**62**	43	49	43	36
3	**15a**	10	**75**	41	**69**	**70**	31
4	**15b**	10	**72**	**66**	**61**	**68**	27
5	**15c**	10	**82**	37	**75**	**83**	**61**
6	**15d**	10	**71**	**56**	**57**	**56**	35
7	**15e**	10	**62**	25	33	**54**	15
8	**15f**	10	**91**	**79**	**87**	**92**	**75**
9	**15g**	10	**90**	**77**	**86**	**80**	**81**
10	**15h**	10	**95**	**82**	**91**	**92**	**76**
11	**15i**	10	**94**	**67**	**96**	**97**	**97**
12	**15j**	10	**89**	**71**	**89**	**85**	**82**
13	**15k**	10	**88**	**81**	**92**	**85**	28
14	**15l**	10	**92**	**95**	**99**	**95**	**96**
15	**15m**	10	**93**	**71**	66	**84**	48
16	**15n**	10	**94**	**81**	**69**	**88**	**71**
17	**15o**	10	**90**	**71**	**62**	**67**	**64**
18	**16a**	10	**92**	**80**	**88**	**77**	**80**
19	**16b**	10	**80**	48	**66**	**64**	48
20	**16c**	10	**73**	**56**	**92**	**85**	**81**
21	**16d**	10	**90**	**59**	46	**65**	22
22	**16e**	10	40	38	40	**56**	38
23	**16f**	10	0	8	25	22	34
24	**16g**	10	47	19	43	**57**	49
25	**16h**	10	**72**	35	**62**	**79**	**70**
26	**16i**	10	**71**	26	**65**	**72**	**63**
27	**16j**	10	**65**	31	**57**	**72**	40
28	**16k**	10	**65**	7	**54**	**67**	16
29	**16l**	10	**59**	48	**67**	**77**	**78**
30	**16m**	10	26	30	40	49	49
31	**16n**	10	**54**	**52**	**63**	**69**	**67**
32	**16o**	10	36	36	**55**	**64**	**62**

Bold values indicate ≥ 50% growth inhibition against cancer cell lines.

Table 2 IC_{50} value in µM of betulinic acid and its analogs on the panel of human cancer cell lines and normal breast epithelial

Compound	Lung	Prostate	Colon	Breast	Pancreatic	Normal breast epithelial
	A549	PC-3	HCT-116	MCF-7	MIA PaCa-2	fR2
1	>10	>10	>10	>10	>10	30.09
7	5.7	>10	>10	>10	>10	16.18
15a	4.7	>10	7.6	7.1	>10	12.18
15b	**1.71**	8.3	4.4	7.1	>10	10.52
15c	**1.5**	>10	**1.4**	3.1	6.5	14.03
15d	**3.04**	8.7	4.9	5.2	>10	16.90
15e	**7.7**	>10	>10	**9.07**	>10	18.17
15f	2.9	5.7	2.9	5.6	5.2	13.62
15g	6.1	7.6	4.3	7.7	4.04	18.98
15h	3.9	6.7	5.1	2.9	5.3	20.74
15i	3.1	6.6	**1.97**	2.6	4.9	100.0
15j	3.9	8.02	5.8	7.07	6.9	47.17
15k	**1.7**	7.1	4.7	6.3	>10	13.10
15l	2.6	6.4	3.6	4.4	5.4	71.06
15m	3.4	7.8	5.8	7.2	>10	13.14
15n	5.1	6.8	5.4	6.8	7.07	23.95
15o	3.09	5.3	8.4	8.6	7.9	12.29
16a	**1.22**	4.1	3.9	4.5	6.7	12.06
16b	**1.8**	8.2	5.7	8.1	>10	35.34
16c	**1.5**	**1.6**	**1.36**	3.5	3.2	16.80
16d	3.6	5.5	>10	8.06	14.4	15.01
16e	>10	>10	>10	>10	>10	13.34
16g	>10	>10	>10	8.2	9.6	21.67
16h	>10	>10	>10	**1.71**	>10	11.05
16i	3.4	>10	5.04	3.9	5.8	12.42
16j	4.9	>10	8.4	6.4	>10	25.74
16k	2.8	>10	5.44	5.2	>10	10.53
16l	4	>10	**1.5**	**1.18**	**1.21**	14.43
16n	4.3	7.4	4.9	6.9	6.4	21.57
16o	>10	>10	3.6	7.5	7.09	10.04

Bold values indicate compounds with IC_{50} value <2 µM.

Table 3 Selectivity Index of the compounds towards various human cancer cell lines

Compound	A-549	PC-3	HCT 116	MCF-7	MIA PaCa-2
1	ND	ND	ND	ND	ND
7	2.838	ND	ND	ND	ND
15a	2.591	3.009	1.602	1.715	ND
15b	6.15	1.267	2.390	1.481	ND
15c	9.353	ND	10.02	4.525	2.158
15d	5.55	1.94	3.44	3.25	ND
15e	2.359	ND	2.003	ND	ND
15f	4.69	2.389	4.696	2.432	2.619
15g	3.111	2.497	4.413	2.464	4.698
15h	5.317	3.095	4.066	7.151	3.913
15i	32.25	15.15	50.76	38.46	20.40
15j	12.09	5.881	8.132	6.671	6.836
15k	7.705	1.854	2.802	2.090	ND
15l	27.33	11.10	19.73	16.15	13.15
15m	3.864	1.684	2.265	1.825	ND
15n	4.696	3.522	4.435	3.522	3.387
15o	3.977	2.318	1.463	1.429	1.555
16a	9.88	2.941	3.092	2.68	1.8
16b	19.63	4.30	6.2	4.36	ND
16c	**11.2**	**10.5**	**12.35**	**4.8**	**5.25**
16d	4.16	2.72	ND	1.86	1.041
16e	ND	ND	ND	ND	ND
16g	ND	ND	ND	2.642	2.257
16h	ND	ND	ND	6.461	ND
16i	3.65	ND	2.46	3.18	ND
16j	5.253	ND	3.064	4.021	ND
16k	3.76	ND	1.93	2.025	ND
16l	3.60	ND	9.62	12.22	8.702
16n	5.016	2.914	4.402	3.126	3.370
16o	ND	ND	2.788	1.338	1.416

ND stands for not determined because compound was having IC_{50} >10 µM.

Figures

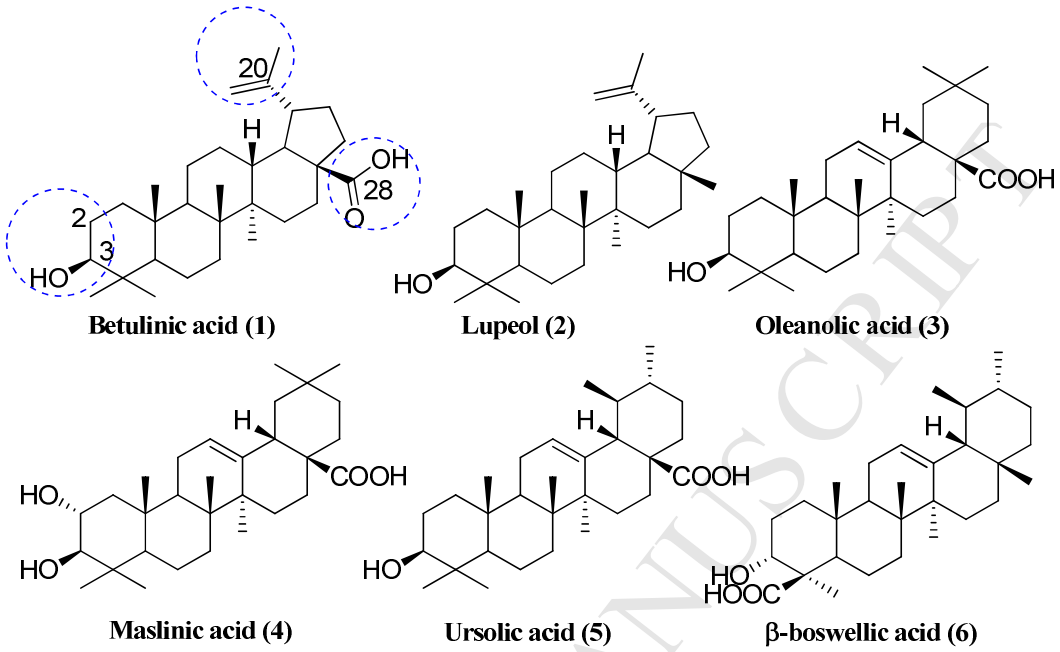
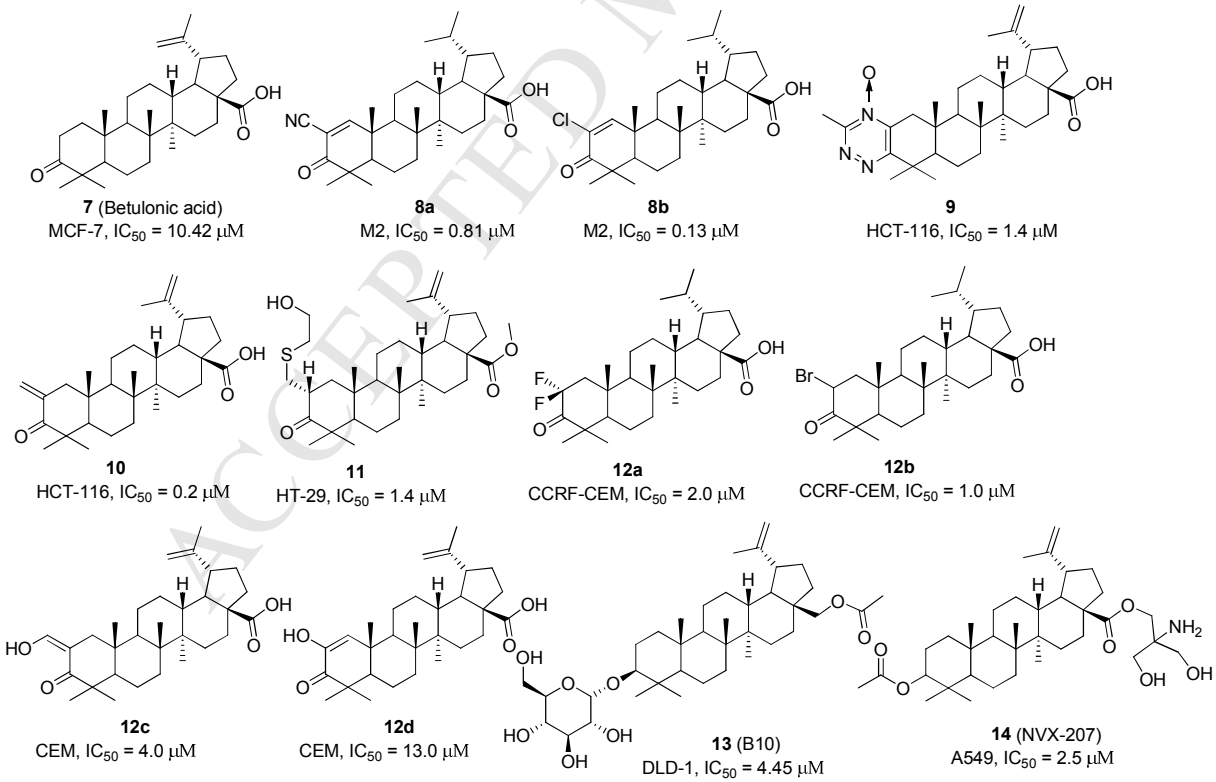


Fig.1. Some representative triterpene scaffolds having anticancer potential



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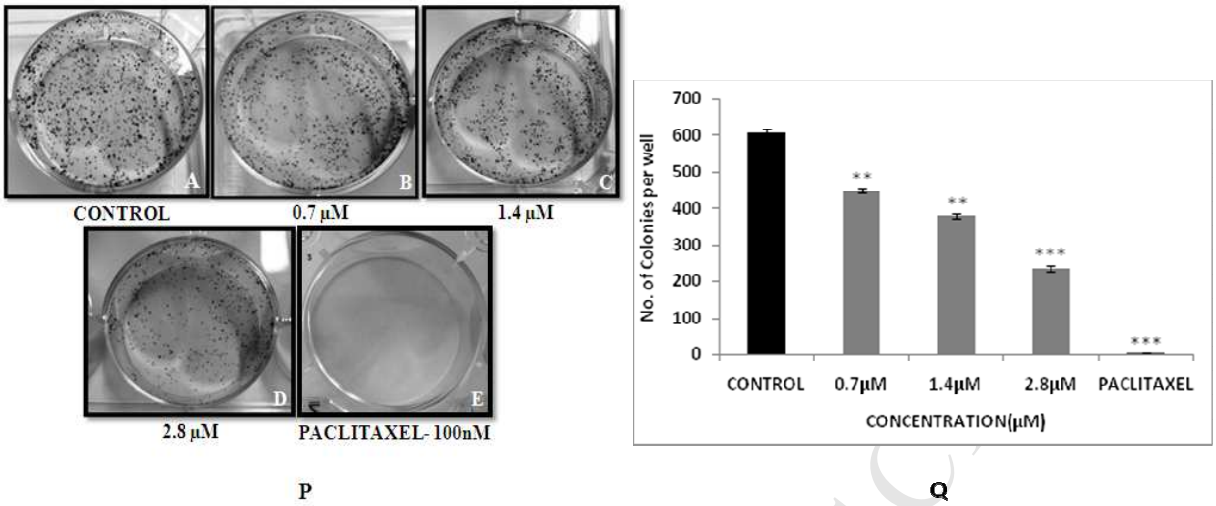

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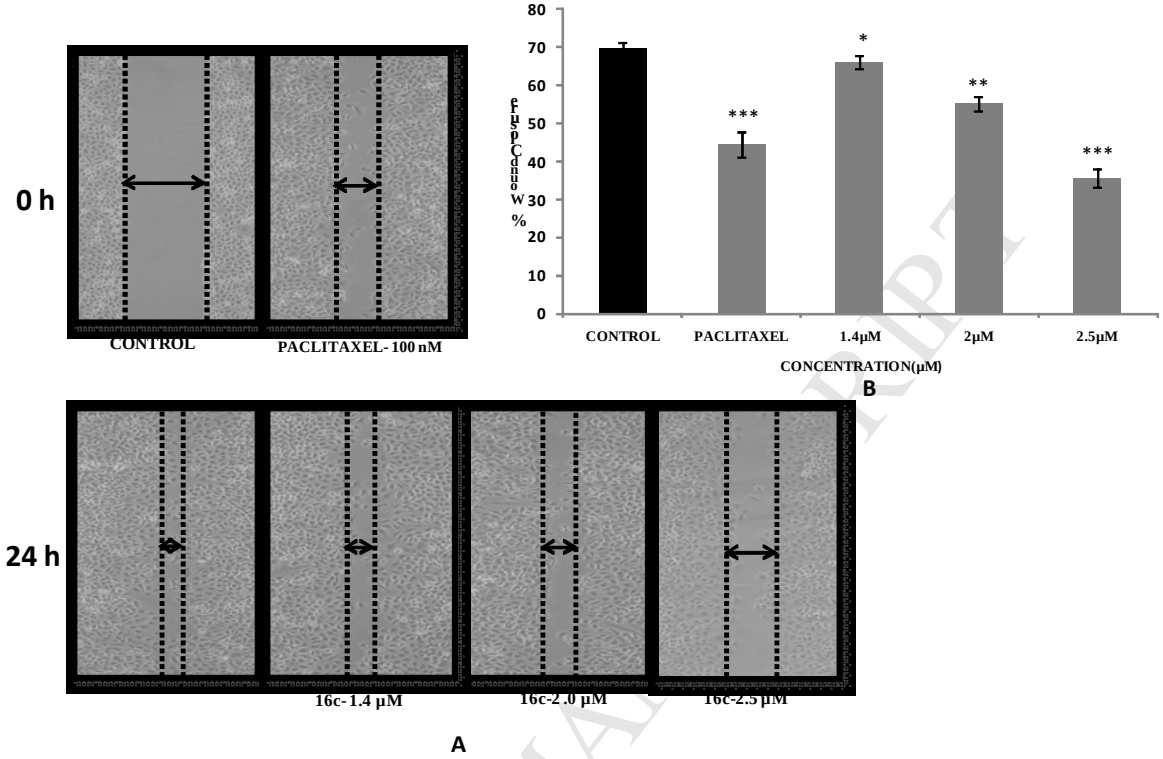

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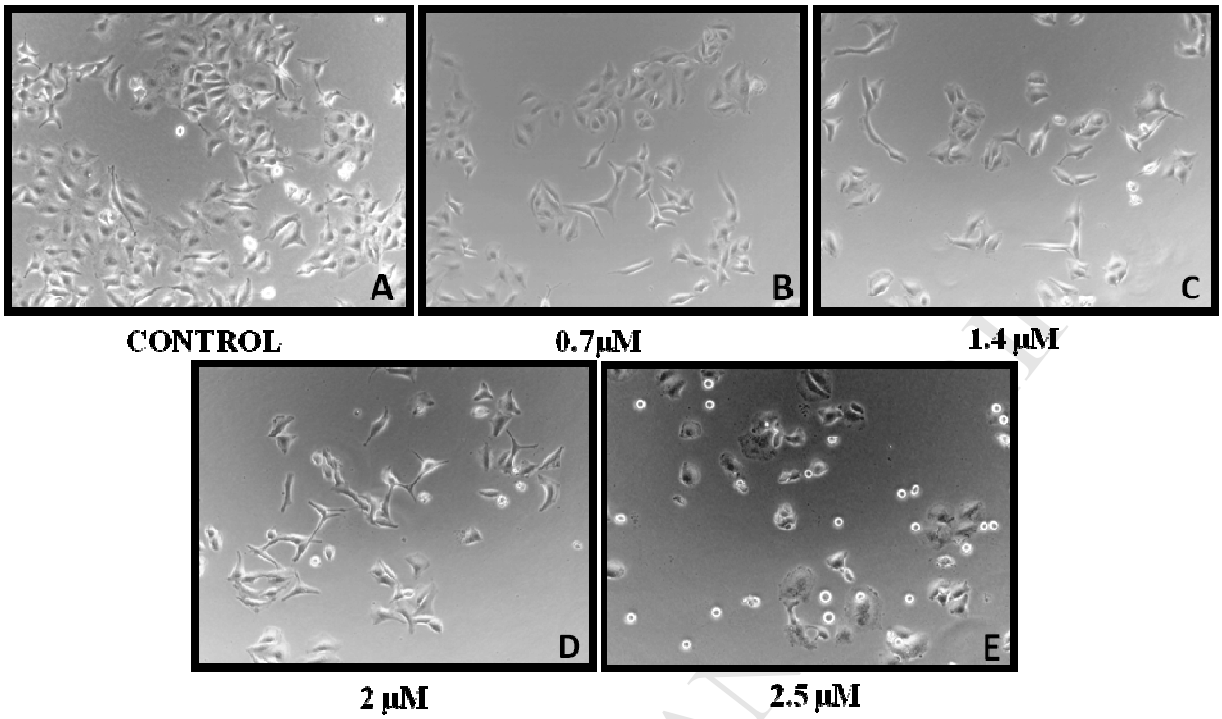

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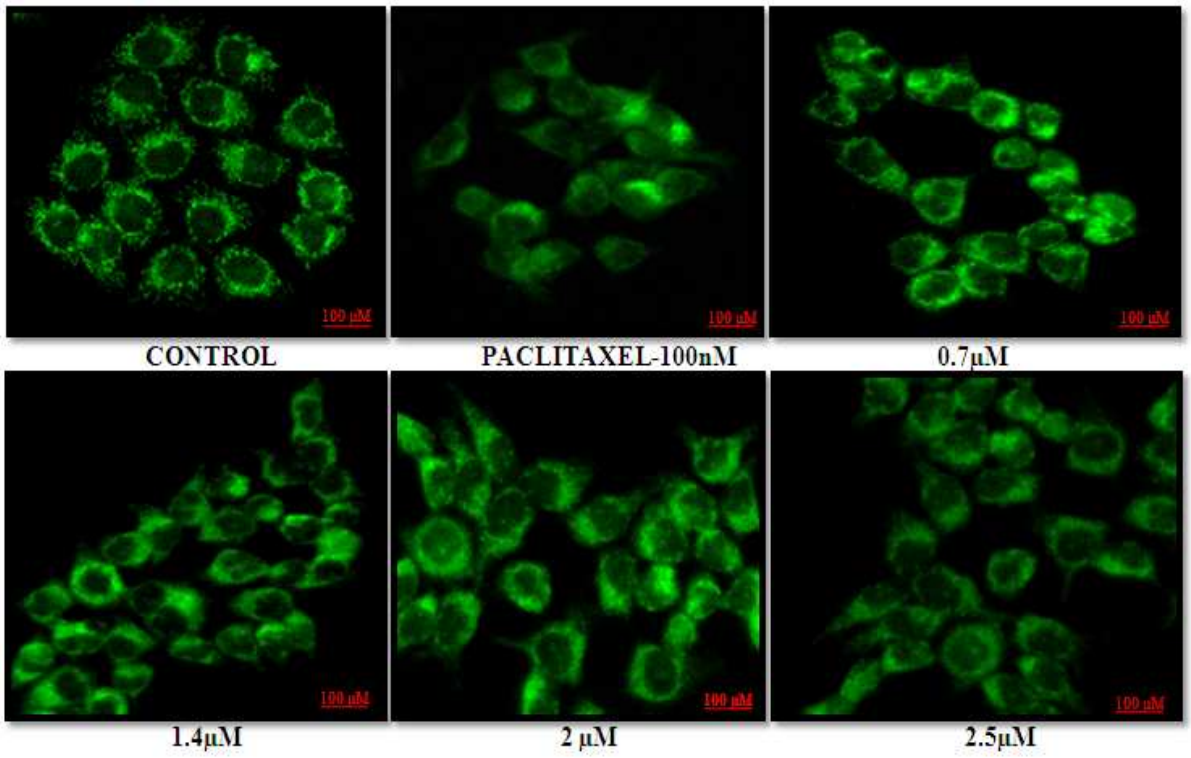

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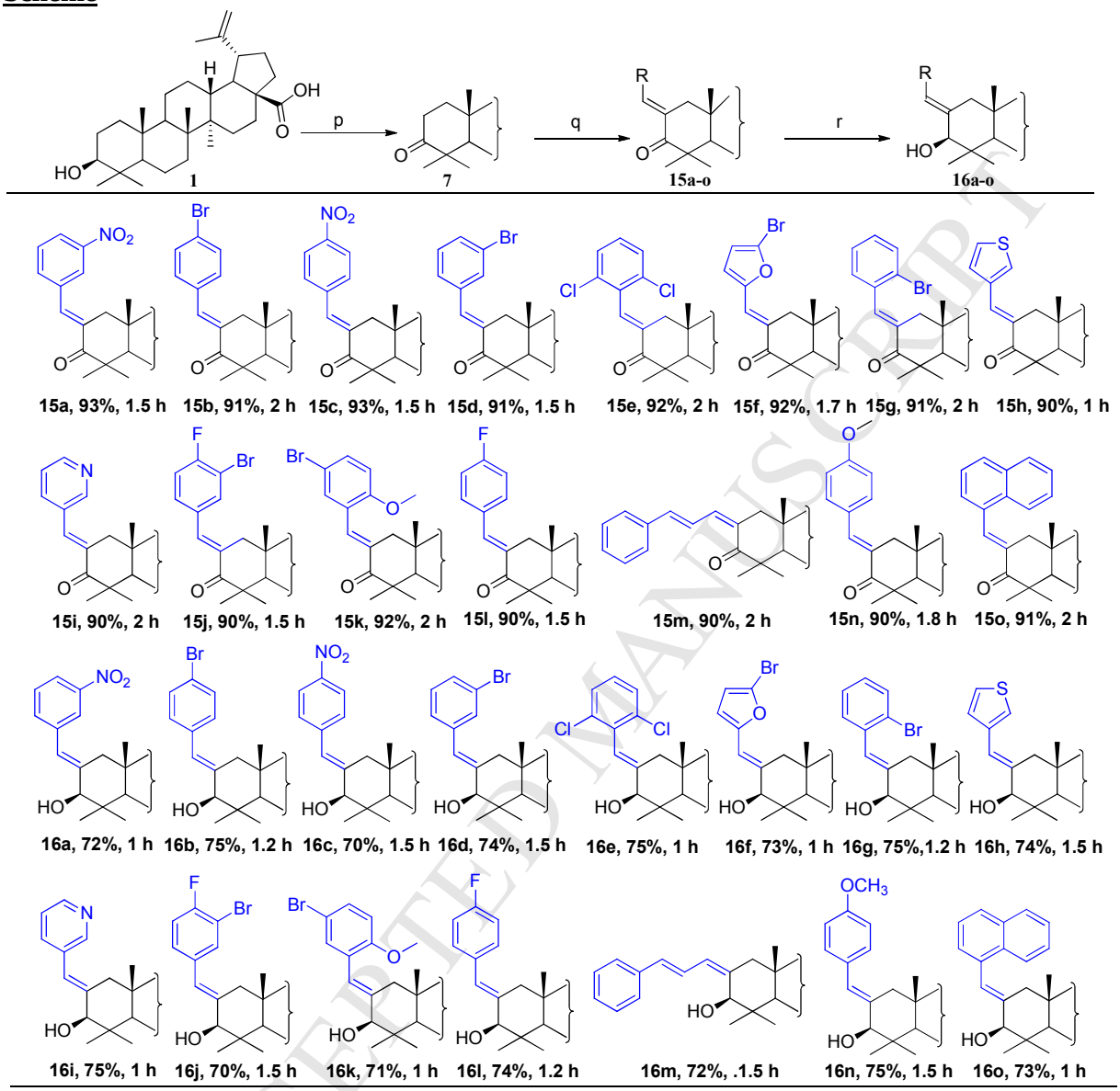
Scheme



Reagents and conditions: p) PCC, DCM, rt, 2h, 70% q) RCHO, NaH, THF, 0°-rt, 1.5-2h, 90-93% r) $NaBH_4$, MeOH, 0°-rt, 1-1.5h, 70-75%

Scheme 1. Synthesis of betulinic acid benzylidene derivatives (**15a-o** and **16a-o**)

Research highlights

- Synthesis of 31 betulinic acid derivatives.
- Several derivatives were active against human cancer cell lines.
- The molecules inhibits the colony formation and restrict the migration of HCT-116 cells