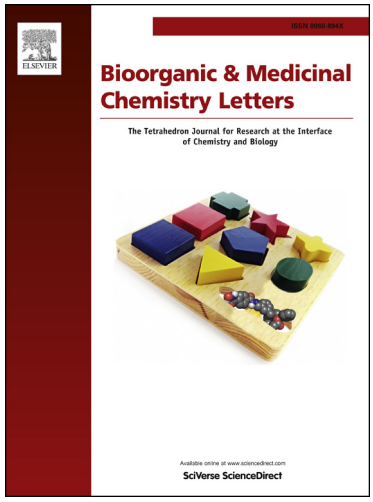

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Synthesis of β-Boswellic acid Derivatives as Cytotoxic and Apoptotic Agents

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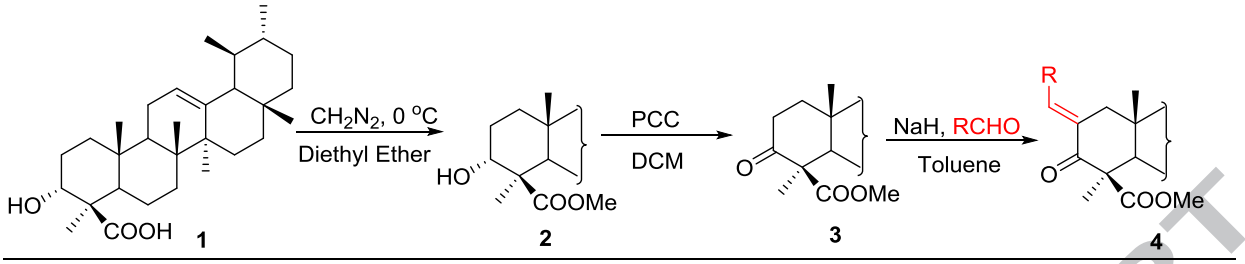
Keywords. *β*-boswellic acid, triterpenoid, anticancer, apoptosis.

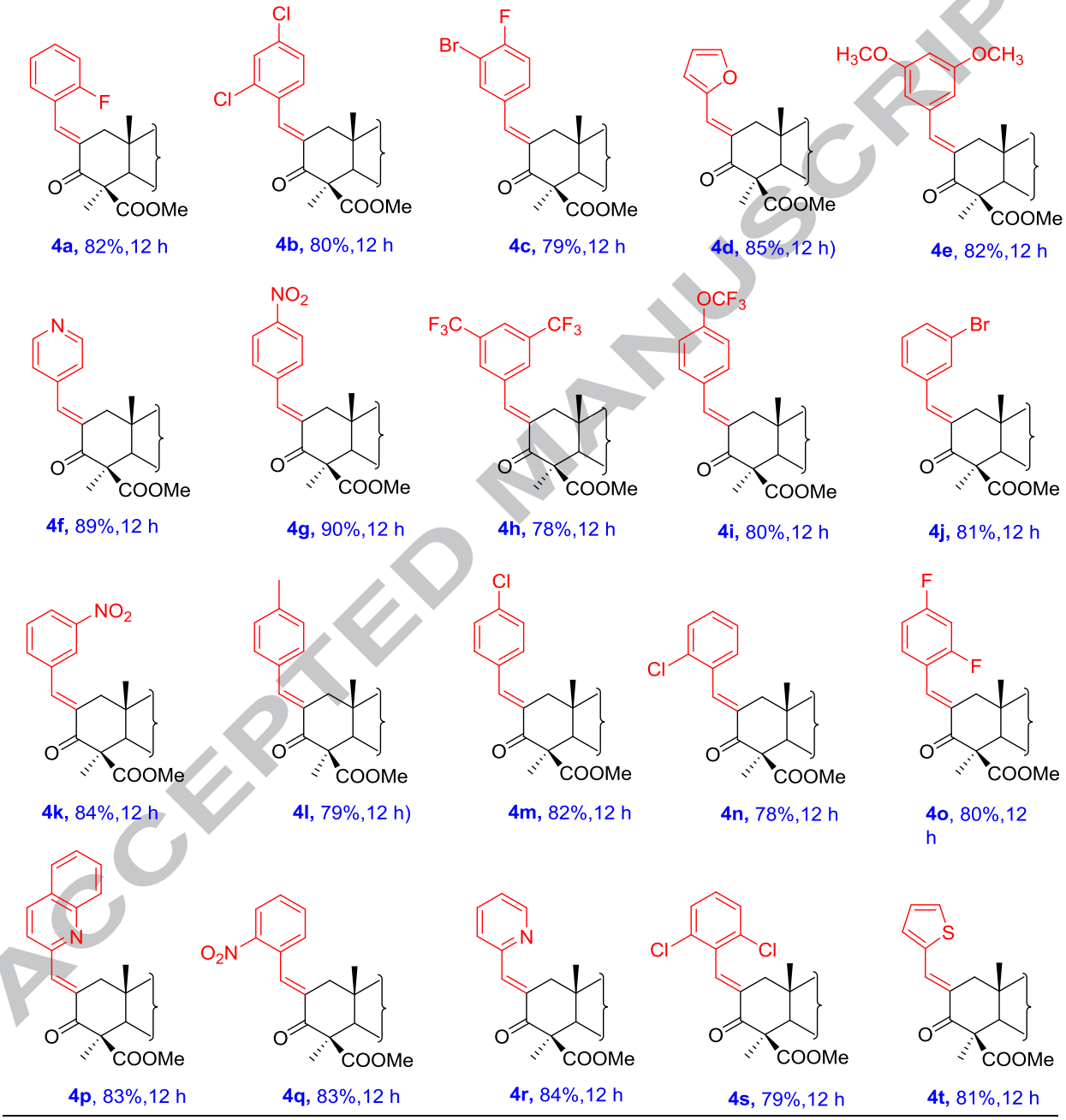
Abstract: A series of β-boswellic acid derivatives were synthesized and evaluated for anticancer activity. One of the lead analogue **4f** displayed significant anticancer activity against a panel of cancer cells as well as substantially inhibited colony formation in HCT-116 cells. Furthermore, **4f** was found to be a potent inducer of apoptosis confirmed by loss of mitochondrial membrane potential, DAPI staining, western blotting and ROS generation.

The alcoholic extract of the gum resin of *Boswellia serrata* has been traditionally used for the treatment of adjuvant arthritis.[1] The gum resin comprises of four major triterpenic acids known as boswellic acids i.e., β-boswellic acid (BA), 11-keto-β-boswellic acid (KBA), acetyl-β-boswellic acid (ABA) and acetyl-11-keto-β-boswellic acid (AKBA).[2] The boswellic acids are known for their biological properties like inflammation,[3,4] arthritis,[5] ulcerative colitis,[6] chronic colitis,[7] asthma[8] and hepatitis.[9] However it's their anticancer activity which has garnered considerable interest in recent past as they have been shown to inhibit the growth and induce apoptosis in brain tumors,[10] malignant glioma cells,[11] colon cancer cells[12] and leukemic cells.[13] Thus, in continuation of our work regarding development of anticancer leads based on natural products,[14] and boswellic acids[15] in particular, herein we report synthesis of a novel series of semi-synthetic analogues of β-boswellic acid modified at C-2 position and their bio-evaluation against a panel of human cancer cell lines. The synthesized analogues thereby had a α,β-unsaturated ketone moiety, which is not only a significant precursor for synthetic manipulations but also forms a major component of the natural products having anti-cancer activity.[16] Notably,

the incorporation of a Michael-acceptor into a molecular structure has previously been revealed to enhance the biological activity of natural products.[17,18] The study lead to the identification of a lead compound capable of inhibiting colony formation in HCT-116 cells as well as induces apoptosis evidenced by loss of mitochondrial membrane potential, DAPI staining, western blotting and ROS generation.

The synthesis of new semi-synthetic analogues of β-boswellic acid derivatives is outlined in Scheme 1. The compound **2** was prepared by esterification of **1** with diazomethane, which on oxidation at C-3 using pyridinium chlorochromate in dichloromethane lead to the synthesis of **3**, the target compound for carrying out aldol reaction with different aldehydes. To begin with we chose reaction of 4-nitrobenzaldehyde with **3** as model reaction to establish the optimum conditions. For optimization and standardization of reaction conditions, we tried different bases such as NaOH, KO*t*Bu, KOH, K_2CO_3, NaH, TEA, DIPEA and DMAP (Supporting information, Table S1), and observed that only sodium hydride resulted in formation of aldol product, which subsequently gets dehydrated to afford the desired product having an α,β-unsaturated ketone system. The failure of other bases might be because of the steric hindrance caused by the cyclic system, whereas dehydration is possibly facilitated by the extended conjugation with aromatic system. Thus, use of NaH (1.5 equiv), **3 (**1.0 equiv) and aldehyde (1.5 equiv), in toluene was found to be condition of choice. It would be pertinent to mention here that we screened several solvents to find toluene as most suitable. After optimizing the reaction conditions, we extended the substrate scope to different aromatic aldehydes. The reaction proceeded well with a range of electron deficient, electron rich as well as heterocyclic benzaldehydes to give a library of 20 analogues **4a-t** in good yields (Scheme 1).





Scheme-1. Reagents and conditions: (i) CH_2N_2, diethyl ether (94%); (ii) PCC, DCM, RT (82%); (iii) NaH, Toluene, RCHO, 0°C to RT (78%-90%).

All the synthesized analogues were evaluated for their anticancer activity against a panel of human cancer cell lines viz., A549 (Lung cancer), PC-3 (Prostate cancer), HCT-116 (Colon cancer) and HT-1080 (Fibro sarcoma) cells (Table 1). The results of preliminary screening shown that aromatic aldehydes with halo-substitutions (mono- and disubstituted) were not favourable groups to improve antitumor activity, as these compounds lead to lower cell growth inhibition compared to parent compound **3** (Table 1). However, the derivative having 2, 4-chloro-substitution (**4b)** displayed better growth inhibition values in various cancer cell lines. Furthermore, results also disclosed that aromatic aldehydes having electron withdrawing groups (-NO_2, -CF_3 and –OCF_3) as well as electron donating groups (-CH_3 and -OCH_3) were not suitable substitutions, which was clearly indicated by lower growth inhibition values of respective compounds in various cancer cell lines (Table 1). Based on the growth inhibition data of these compounds in various cell lines, it had been find out that products having pyridine ring (**4b** and **4f**) were found to be more active as compare to the products having other heterocyclic rings (quinoline, furan and thiophene) (Table 1). Therefore, preliminary screening led to the identification of three lead compounds **4b**, **4f** and **4r**, displaying promising growth inhibition. These compounds were then further taken up for IC_{50} value determination.

Table 1. In *vitro* cytotoxicity of compounds (**4a-t)** against different cancer cells by SRB assay[19]

Cell line			A549 (Lung cancer)	PC-3 (Prostate cancer)	HCT-116 (Colon cancer)	HT-1080 (Fibrosarcoma cancer)
S.no.	Code	Conc.(μM)	% growth inhibition			
1.	3	100	56	44	58	21
2.	4a	100	47	44	0	23
3.	**4b**	**100**	**97**	**79**	**49**	**100**
4.	4c	100	47	40	27	43
5.	4d	100	48	44	18	16
6.	4e	100	55	41	21	17
7.	**4f**	**100**	**99**	**100**	**100**	**95**
8.	4g	100	47	52	30	36
9.	4h	100	5	7	10	0
10.	4i	100	14	35	0	78
11.	4j	100	14	6	8	15
12.	4k	100	0	10	6	13
13.	4l	100	28	11	20	26
14.	4m	100	7	10	0	6
15.	4n	100	0	25	0	21
16.	4o	100	33	20	32	0
17.	4p	100	37	44	37	43
18.	4q	100	42	11	21	100
19.	**4r**	**100**	**100**	**100**	**100**	**95**
20.	4s	100	74	24	57	99
21.	4t	100	42	19	40	37

Among the three lead molecules, **4f** was found to be most cytotoxic against various cancer cell lines viz., A549, PC-3 HCT-116 and T47D having IC_{50} values of 2.13, 1.96, 1.84 and 1.26 µM respectively (Table 2 and Figure 1). The other selected molecule **4b** was found to be comparatively less toxic in all the four cancer cell lines, whereas **4r** displayed moderate activities against various cancer cell lines having IC_{50} values of 26.1, 24.4, 7.2 and 15.6 µM respectively against A549, PC-3, HCT-116 and T47D cell lines (Table 2). It would be pertinent to mention here that IC_{50} values of compounds **4b**, **4f** and **4r** were considerably high in normal human breast epithelial cells (FR-2) (Table 2). Thus, based on IC_{50} values we chose **4f** for further studies to determine if the cell death is caused by apoptosis or necrosis.

Table 2. In *vitro* cytotoxicity of lead compounds against different human cancer cell lines[19]

Cell line		A549 (Lung cancer)	PC-3 (Prostate cancer)	HCT-116 (Colon cancer)	T47D (breast cancer)	FR-2 (Normal epithelial)
S. No.	Code			IC_{50}(µM)		
1	4b	56.1	69.5	>100	>100	>100
2	**4f**	**2.13**	**1.96**	**1.84**	**1.26**	**21.4**
3	4r	26.1	24.4	7.2	15.6	59
4	AKBA	25.8	32.7	13.8	43.4	67.2

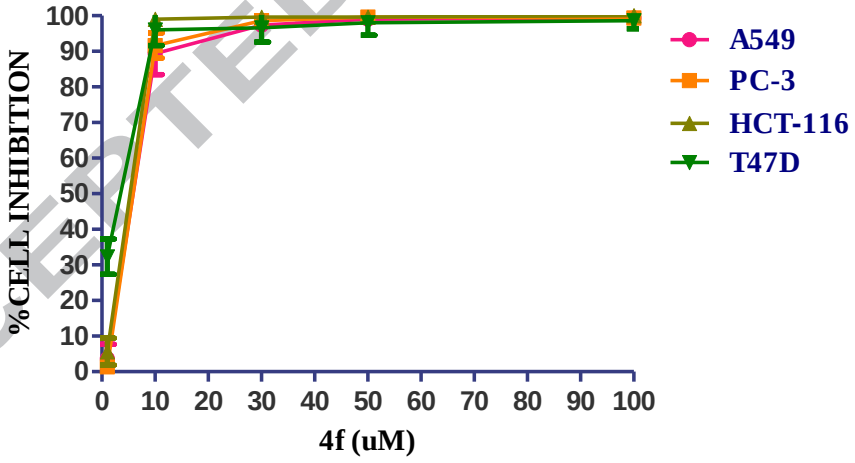


Figure 1. Graphical representation of cell inhibition on different concentrations of **4f** against various cancer cell lines (A549, PC-3, HCT-116, T47D) determined by SRB assay. The inhibitory values were calculated by using Graph PAD Prism software version 5.0.The results represent mean ± SD of three experiments.

To gain further insight into the anti-proliferative activity, we investigated the effect of compound **4f** on HCT-116 cells by clonogenic assay. Colony formation assay is a cell survival assay based

on the ability of a single cell to grow into a colony. It primarily tests each cell in the population for its ability to undergo extensive division and monitors all cells that have retained the capacity for producing a large number of progeny after various treatments that can cause cell death as well as evaluate the reproductive integrity of different cells. This assay is used to determine the effect of cytotoxic compounds on colony forming ability of cancer cell lines. The HCT- 116 cells were treated with compound **4f** at different concentrations of 1, 2.5 and 5 µM. After treatment, cells were seeded in appropriate dilution to form colonies in 1-3 weeks.[20,21] The results of the colony formation assay revealed that **4f** significantly inhibited colony formation in HCT-116 cells (Figure 2) and the inhibitory effect was found to be increased with increasing doses of **4f**.

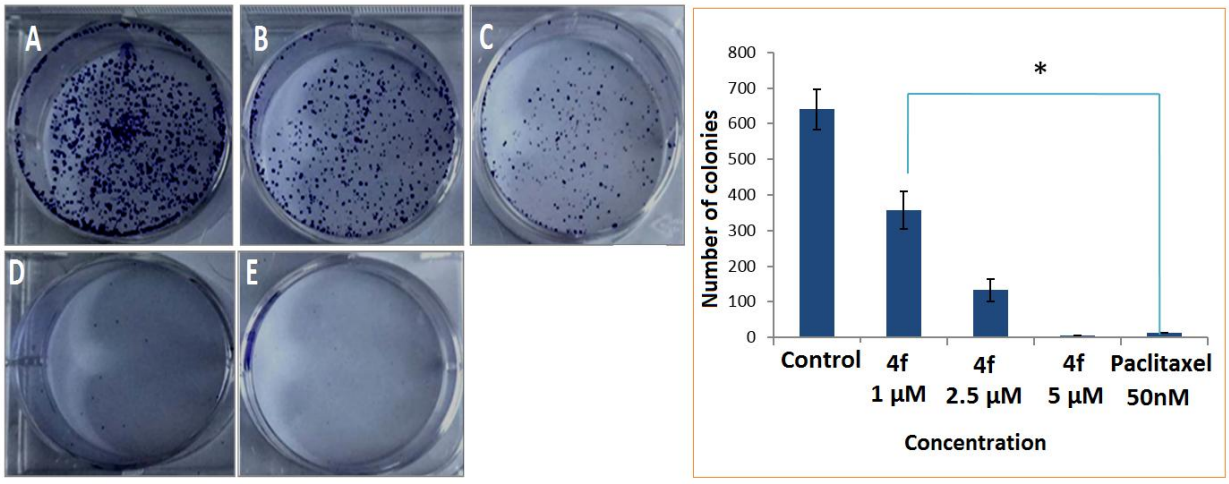


Figure 2. Clonogenic assay was performed in six well plate, with clones produced by HCT-116 colon cancer cells for 48h. (a) Untreated control with 600 clones, respectively, formed after seeding 500 cells. Treated HCT-116 cells with compound **4f** at different concentrations of 1µM, 2.5µM, 5µM (B, C, D). Paclitaxel was taken as a positive control (E); (b) bar diagram representation of survival fraction of HCT-116 cells treated with **4f** compound in a dose dependent manner for each analysis versus control. The inhibitory effect of **4f** compound on the relative clonogenicity of the control and **4f** treated HCT-116 colon cancer cell line.*P value<0.05 *Vs* control.

Apoptotic bodies' formation is a distinguishing feature of cells undergoing apoptosis.[22] Induction of apoptotic bodies by chemotherapeutic agents has always been a preferred choice in developing anti-cancer therapeutics. 4',6-Diamidino-2-phenylindole (DAPI) is a fluorescent nuclear stain which binds to minor groove of A-T regions of DNA. Characteristic apoptotic morphology like chromatin condensation, nuclear shrinkage and formation of apoptotic bodies can easily be observed under fluorescence microscopy, after staining of nuclei with DNA

specific DAPI.[23,24] The HCT-116 cells were treated with test compound **4f** at 1, 2.5 and 5 µM for 48 h, stained with DAPI and investigated for morphological changes. It was observed that nuclei of HCT-116 cells treated with **4f** compound increased formation of apoptotic bodies and chromatin condensation in concentration dependent manners compared to untreated control (Figure 3). This data suggests that **4f** inhibits growth of HCT-116 cells by arresting the proliferation of cells and subsequent induction of apoptosis.

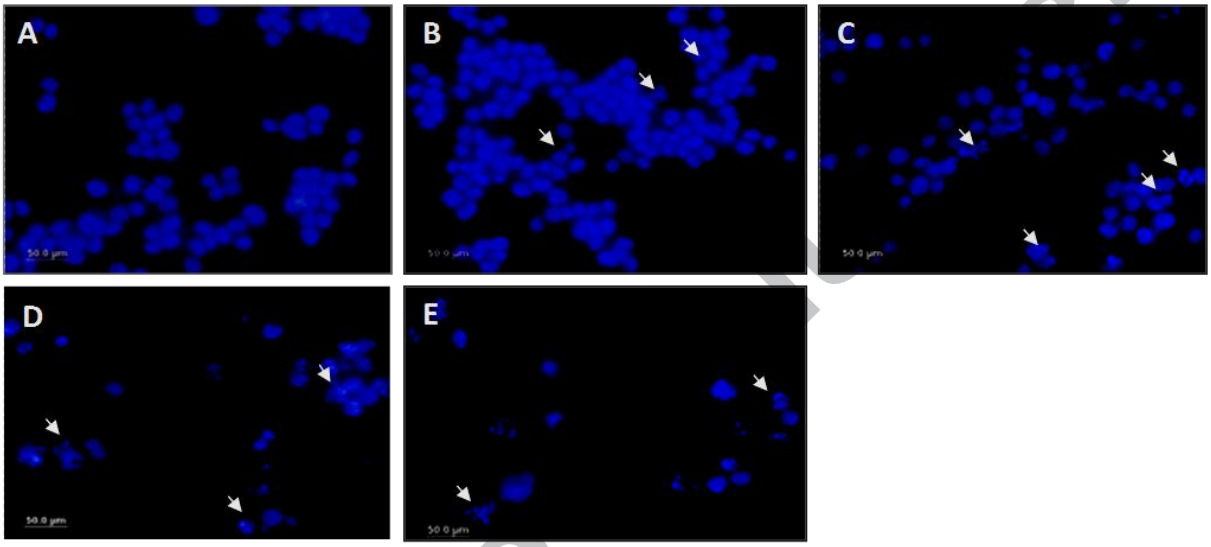


Figure 3. DAPI staining in HCT-116 cells. The cells were treated with test compound **4f** at 1, 2.5 and 5µM for 48 h, stained with DAPI and observed to determine morphological changes. Untreated HCT-116 cells showed normal nuclear morphology while Paclitaxel showed typical apoptotic bodies. Cells treated with test compound **4f** showed concentration dependent effect. The arrow in each case showed the appearance of apoptotic bodies.

The initiation of apoptosis is investigated by the mitochondria compartmentalization. The changes in mitochondrial membrane potential were determined by using RH-123 in a population of apoptotic cells. RH-123 is a fluorescent dye and the electrochemical potential of the proton gradient across the mitochondrial membrane was assessed by monitoring fluorescence quenching of RH-123. Its rate of fluorescence decay is proportional to the mitochondrial membrane potential. The loss of mitochondrial integrity results in leakage of the RH-123 from mitochondria results in consequent decrease in fluorescence.[25] HCT-116 cells were treated with compound **4f** at different concentrations of 1, 2.5 and 5 µM. RH-123 staining results revealed loss of mitochondrial membrane potential in **4f** treated HCT-116 cells. HCT-116 treated cells with **4f** displayed a remarkable increase in mitochondrial membrane potential loss with increase in

concentration as compared to the untreated cells which exhibited intact mitochondria (Figure 4). Compound **4f** induced concentration dependent depolarization of the mitochondrial membrane potential (low $\Delta\Psi mt$).

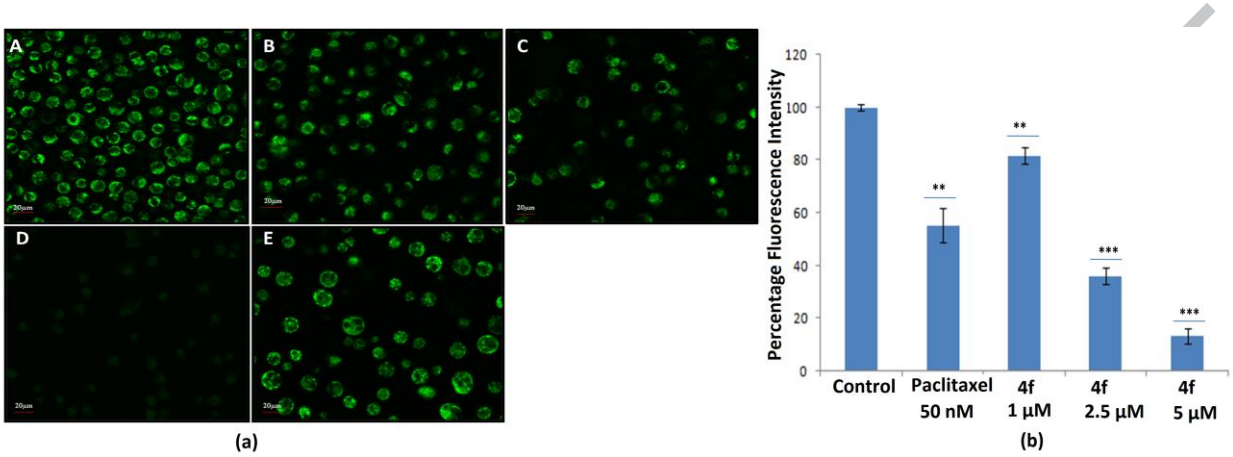


Figure 4. (a) Mitochondrial membrane potential of **4f** compound treated HCT-116 cells were measured by Rhodamine-123 staining and visualized by confocal microscopy. Untreated cells showed intact mitochondria as compared to treated cells which induced a remarkable increase in mitochondrial membrane potential loss with increase in concentration. Treated HCT-116 cells with compound **4f** at different concentrations of 1µM, 2.5µM, 5µM (B, C, D). Paclitaxel (50 nM) was taken as a positive control (E); (b) effect of **4f** on $\Delta\psi m$ measured with laser scanning confocal microscope. Fluorescence intensity (FI) indicates membrane potential of mitochondria in the cells. FI decreased in a concentration-dependent manner. Data are presented as mean ± SD, statistical analysis was done with **P<0.01 and ***P<0.001.

Our preliminary data indicated the role of mitochondrial membrane potential loss in the cell death induced by **4f** in HCT-116 cells. PARP cleavage is considered as a sign of apoptosis and is associated with mitochondrial cell death. PARP is a nuclear enzyme involved in DNA repair whose cleavage is considered to be a hallmark of the apoptotic mode of cell death. The PARP is cleaved into 89 kDa fragment upon the induction of apoptosis. The cleavage inactivates the enzyme by destroying its ability to respond to DNA strand breaks.[26] Therefore, we tried to investigate if PARP cleavage has any role to play in the apoptosis induced by **4f**, by western blot analysis. HCT-116 cells were incubated with **4f** at 1, 2.5 and 5µM concentrations for 48 h. Results demonstrated that **4f** treatment significantly induced PARP cleavage in HCT-116 cells; and was comparable to positive control paclitaxel (Figure 5). All these data indicated that **4f** induced apoptosis in HCT-116 cells.

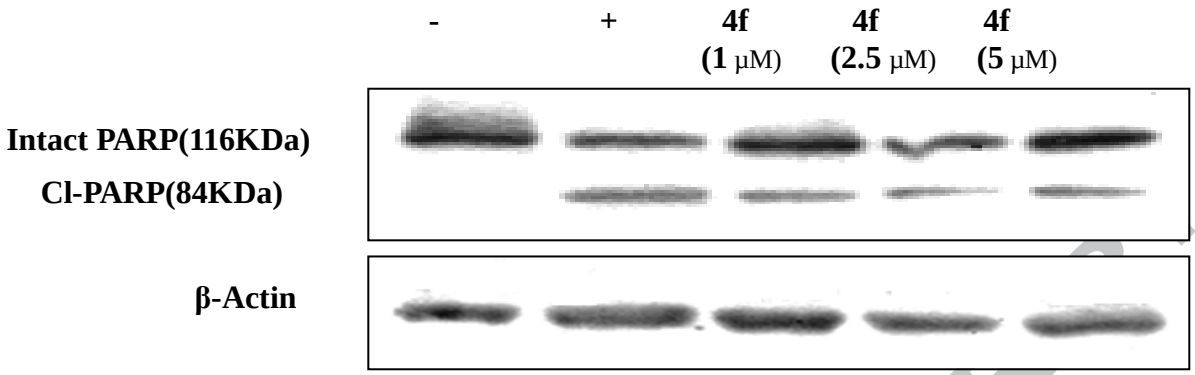


Figure 5. Western blot analysis revealed the expression levels of intact and cleaved PARP-1 in HCT-116 colon cancer cells on treatment with varying concentrations (1, 2.5 and 5μM) of **4f** compound for 48 h. Paclitaxel was used as positive control. The housekeeping protein β–actin served as a loading control.

Reactive Oxygen Species (ROS) are the common mediator of apoptotic signalling. It is generated as a by-product of cellular metabolism through leakage of electrons by mitochondrial electron transport. The total cellular ROS level was measured using 2', 7'-dichlorofluorescien diacetate (DCFDA) dye which is oxidized in the presence of free radicals to form a green fluorescent by product dichlorofluorescein (DCF).[27] Numerous anticancer agents have confirmed to put forth their cytotoxic effects by the generation of reactive oxygen species (ROS).[28] Therefore, we examined the role of compound **4f** in inducing the production of ROS that could potentially lead to the cytotoxic effect in the HCT-116 cells. After treatment with **4f** at 1 and 2.5 μM concentrations, fluorescence intensity of DCF was increased in a dose dependent manner after 48h exposure to the test compound. The increase of fluorescent product dichlorofluorescein (DCF) was observed in treated cells as compared to untreated cells (Figure 6). The dose dependent increase in fluorescent product dichlorofluorescein (DCF) confirmed the ROS generation.

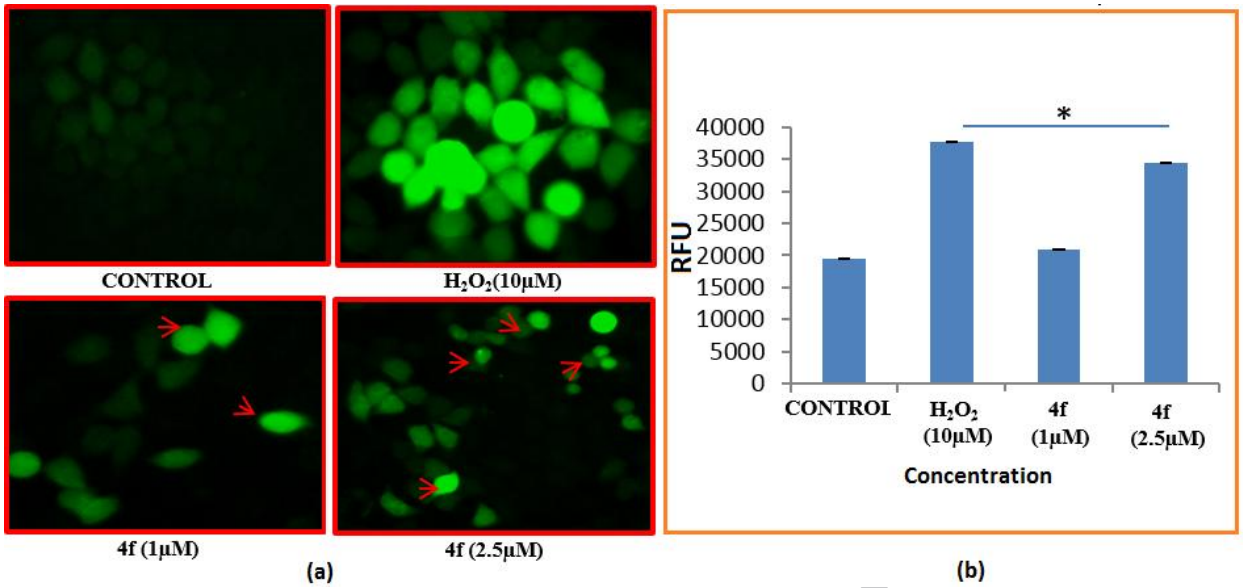


Figure 6. (a) Detection of ROS in HCT-116 cells. The cells were treated with the indicated concentrations of **4f** for 48h. H_2O_2 taken as a positive control. The fluorescence intensity of DCF was increased in a dose dependent manner after 48h exposure to the test compound (*P<*0.05 vs.* control); (b) bar diagram representation of ROS generation following 48h exposure of various concentrations (1 and 2.5µM) of **4f** in HCT-116 cells assessed by spectrofluorometirc analysis.

In conclusion, we synthesized a series of β-boswellic acid derivatives using aldol condensation approach. The synthesized analogues were evaluated for their anticancer activities using growth inhibition assay. The lead compound **4f** displayed significant anti-proliferative activity against various cancer cell lines viz., A549, PC-3 HCT-116 and T47D having IC_{50} values of 2.13, 1.96, 1.84 and 1.26 µM respectively. The lead analogue **4f** substantially inhibited colony formation in HCT-116 cells. The induction of apoptosis was further confirmed by loss of mitochondrial membrane potential, DAPI staining, western blotting and ROS generation in HCT-116 cells. These data suggest that compound **4f** can be transformed into promising lead molecule and warrant further detailed investigation and optimization.

Supporting Information:

Experimental procedures, biological materials and methods, compound characterization data, NMR spectra (^{1}H and ^{13}C) and HRMS spectra.

Author Contribution

‡These authors contributed equally

Acknowledgment

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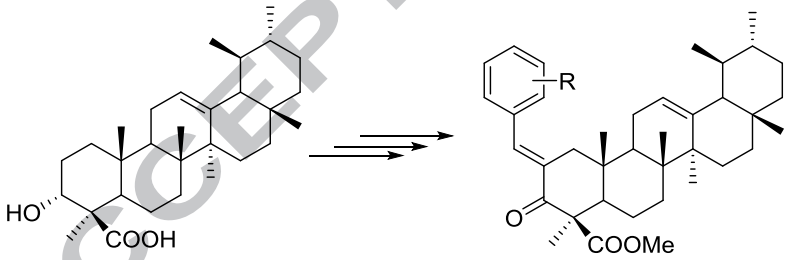
Graphical abstract



R= Substituted benzaldehydes
20 examples
Yields upto 90%