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In-vitro cytotoxic activity of β-Sitosterol triacontenate isolated from *Capparis decidua* (Forsk.) Edgew

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ABSTRACT

Objective: To study the isolation and characterization of the constituent responsible for the cytotoxic activity of the ethanolic extract of stem of *Capparis decidua* (*C. decidua*). **Methods:** The preliminary cytotoxic effect of isolated compound (β-Sitosterol triacontenate) was investigated by MTT assay on A549 solid tumor cells. **Results:** IC_{50} value of the β-Sitosterol triacontenate was found to be 1 μM. The cytotoxic activity increased in a dose dependent manner in case of β-Sitosterol triacontenate. **Conclusions:** The data therefore provide direct evidence for the role of β-Sitosterol triacontenate as a potent antimetastatic agent, which can markedly inhibit the metastatic and invasive capacity of malignant cells.

1. Introduction

Chemotherapy is a major treatment modality for cancer and some of the plants like *Catharanthus roseus* (*C. roseus*), *Podophyllum peltatum* (*P. peltatum*), *Podophyllum emodii* (*P. emodii*), *Taxus brevifolia (T. brevifolia), Ochrosia elliptica (O. elliptica)* and *Campototheca acuminate (C. acuminata)*, have provided active principles which are in clinical use for controlling advanced stages of malignancies[1]. Moreover, many of the active molecules sold for the treatment of cancer, are highly expensive, mutagenic, carcinogenic and teratogenic. Hence, there is a need to find alternative drugs, which are highly effective at non-toxic doses, inexpensive and accessible to common man. A need is therefore felt to search newer remedies, which are cheaper economically and do not have severe side effects of the pure compounds[2].

The plant *C. decidua*[3] (green berries) is commonly known as kair in Hindi and the fruit of the plant is used in food preparations like pickles for over 2000 years[4]. In the traditional system of medicine, the bark has been shown to be useful in the treatment of coughs, asthma and inflammation[5]; roots used in fever and buds in the treatment of boils. According to the Unani system of medicine the plant has been used as a carminative, tonic, emmenagogue, aphrodisiac, alexipharmic; improves the appetite; good for rheumatism, lumbago, cough and asthma[6].

The aqueous extracts of the plant exhibits anthelmintic activity, seeds contain antibacterial principles-glucocapparin; isothiocyanate aglycone of glucocapparin[7].

In recent times the different parts of the plant have been evaluated for various activities. The aqueous and ethanolic extract of stem has antidiabetic[8-9] antimicrobial[10] and hepatoprotective[11] activities; the whole plant extracts have anthelmintic, antimicrobial & antifungal activities[12-13] while the fruit has anti atherosclerotic[14], hypolipidemic[15-16], hypocholesterolemic[17], sedative & anticonvulsant[18] activities. The flowers leave have analgesic, anti-Inflammatory and CNS depressant activity[19].

The main active constituents of the herb[20-23] have been reported to be diterpene alcohol (3-methyl-7-hydroxymethylene-10-(12, 16, 16-trimethylcyclohex-11-enyl) - dec-9-ene-5-one-8-ol), diterpenic ester (9-(11, 15, 15- trimethylcyclohex-11-ene-13-one-yl)-one-6- hydroxymethylene-7-one-yl, 4′-Me heptanoate), spermidine alkaloids like isocodonocarpine, capparisinine, capparadisine. Six oxygenated heterocyclic constituents capparis esterpenolide (3-carboxy-6, 17-dihydroxy-7, 11, 15, 19-tetramethyleicos-13-ene-d-lactone) and deciduaterpenolides (d-lactone derivatives of 1, 3, 3-trimethyl-1, 4-cyclohexadien-6-one) A, B, C,

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D and E had been obtained from alcoholic extract of root bark. The root bark also contains alkaloids, 14-N-acetyl isocodonocarpine, 15-N-acetyl capparisine, cadabicine, stachydrine, rutin, capparisine and codonocarpine, capparine, cappariline and capparinine. The stem contains two alkaloids n-triacontanol, 2-carboxy-1, 1-dimethylpyrrolodine, two acycylic terpenoids, four fatty acids, two sterols[24] and two lupine terpenoids. Flowers contain the hydrocarbons nonacosane and triacontane. Flowers and fruit husk contain phthalic acid.

Traditionally the plant is used as anti-inflammatory, so that the plant was selected for cytotoxic activity. The present study represents the isolation, characterization and preliminary cytotoxic activity of the isolated compound from *Capparis decidua* (*C. decidua*) (Forsk.) Edgew.

2. Material and methods

2.1. Chemicals

Cell lines were procured from National Center for Cell Science, Pune, India. DMEM (Dulbecco's Modified Eagle Medium), MEM (Minimum Essential Medium), RPMI, L-glutamine, Penicillin and Streptomycin were procured from HIMEDIA, Mumbai. FCS (Fetal Calf Serum) was purchased from LONZA Belgium. The following chemicals were obtained from Sigma Aldrich (USA) - DMSO, Streptomycin, Penicillin, HEPES, L-glutamine, Propidium iodide. MTT reagent (Merck), Paclitaxel (Dabur India Ltd.), Chloroform, Ethanol, $CDCl_3$, β - Sitosterol triacontenate was isolated in house. All chemicals used are of analytical reagent grade or higher grade.

2.2. Instrumentation

ELISA plate reader (Bio Rad), Polyvinylidene difluoride (PVDF) membrane filters (Durapore), Rotary shaker (R 100/TW LUCKHAM, England), Microscope Olympus 1X51 with Prog Res Software (Jenoptix). Master cycler gradient (Eppendorf, Germany), LCMS: PE SCIEX, API 165 (Perkin-Elmer, USA); DSC: DSC-7 (Perkin-Elmer), GN-300 Omega NMR spectrometer, 6000 FT/IR Jasco Spectrometer and UV (Jasco- 640V).

2.3. Plant material

The stem of *C. decidua* was collected from Loharu surroundings; district Bhiwani (Haryana) in the month of August-September, 2008 depending upon its easy availability. The plant was authenticated by Dr. Minoo Parabia, Professor and Head, Department of Biosciences, Veer Narmad South Gujarat University against voucher specimen SA-1.

2.4. Extraction & isolation

The air dried and coarsely powdered stem (1.75 kg) was extracted with 95% ethanol (8 L) in a soxhlet apparatus. The extraction was continued till a few drops of the last portion of the percolate did not leave any precipitable residue on drying. It took about 46 hours for complete exhaustion. The extracts were filtered and evaporated to yield a dark green residue (195.0 g). The ethanolic extract was dissolved in chloroform, filtered and evaporated to dryness. A free flowing powder was made by mixing the organic extract with silica Gel and the free flowing powder of the crude extract was loaded in to column. Then it was eluted with the different eluents [pure chloroform, chloroform-ethyl acetate (99:1, 98:2, 95:5, 90:10, 75:25, 50:50), pure ethyl acetate to collect fractions of 200 mL each. Each fraction was evaluated by TLC and chemical test. Compound was isolated from fractions 5-12 with a flow rate drop by drop to yield 500 mg. Isolated compounds displayed >90% purity as estimated by examination of the NMR and Mass spectra.

2.5. Characterization of β-Sitosterol triacontenate

The β-Sitosterol triacontenate was then evaluated and characterized by comparing its physicochemical properties by physical methods (m.p., Solubility) and spectroscopic methods *viz*. NMR, Mass, IR, UV spectroscopy. Mass spectrum was recorded on a LCMS: PE SCIEX, API 165 (Perkin-Elmer, USA) instrument. ^{1}H-NMR (COSY) and ^{13}C-NMR measurements were recorded in $CDCl_3$ on a GN-300 Omega NMR spectrometer & IR Spectrum was recorded on 6000 FT/IR Jasco Spectrometer and UV (Jasco- 640V) in $CHCl_3$.

2.6. In vitro anticancer assays

2.6.1. Cell culture

A549 human Lung cancer cells were grown in DMEM supplemented with 100 U/mL, Penicillin G, 100 μg/mL Streptomycin, 0.25 μg/mL Amphotericin and 10% heat inactivated fetal bovine serum. Cultures were maintained at 37 ℃ in a 5% CO_2, 95% air atmosphere.

2.6.2. MTT assay[25]

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] is taken up by the viable cells and reduced to formazan by the "Succinate-tetrazolium reductase" system that belongs to the mitochondrial respiratory chain functioning in metabolically active cells. Formazan formation takes place, is a purple colored water-insoluble product that is largely impermeable to cell membranes, thus resulting in its accumulation within the healthy cells which is solubilized by adding Dimethyl sulphoxide (DMSO). The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity, which, in turn, may be interpreted as a measure of viability and/or cell number. 5×10^4 cells/mL were seeded in a 96-well tissue culture plate with 200 μL of DMEM and incubated for 24 h. The compound was dissolved in minimum amount of Dimethyl sulphoxide (DMSO) and was prepared in DMEM without phenol red. All samples were first sterilized using PVDF membrane filters of 0.22 μm (Millipore, Ireland). Paclitaxel (Gift sample from Dabur) was used as positive control. Cells were treated with two different concentrations (5 μM, 10 μM) of isolated compound in 100 μL volume prepared in media without phenol red and with positive control (Paclitaxel-1 μM and 5 μM) and were incubated for 48 h. The cells in the control group received no drug treatment. Each treatment was performed in triplicates. After the treatment, drug containing media was removed and washed twice with 100 μL of PBS. To each well of the 96 well plate, 20 μL of MTT reagent (Merck, India) (Stock: 5 mg/mL) was added and incubated for 4 h at 37 ℃. Plates were shaken for 10 min. To solubilize formazan crystals in the wells, 100 μL of 100% DMSO was added to each well. Plates were placed on a Rotary shaker (R 100/TW LUCKHAM, England) and agitated for 15-20 min. The optical density (OD) was measured by an Enzyme Linked Immunosorbent Assay (ELISA) plate reader (Bio Rad plate reader) at 570 nm

with a reference wavelength of 630 nm. OD of each well was read and expressed as percentage cell survival (absorbance of treated wells/absorbance of control wells×100). Results were expressed as Mean ± S.E. OD values (proportional to cell survival) was plotted against the compound concentrations. From the results it can be interpreted that the activity of active constituent was significant which gave less than 50% survival at exposure time of 48 h. The active compound was further diluted in medium to produce 5 concentrations of 1, 5, 10, 15, and 20 μM. 100 μL/well of each concentration was added to the plates in triplicates and incubated for further 48 h. Its cytotoxic effect was determined by MTT assay. The inhibitory concentration IC_{50} of β-Sitosterol triacontenate was determined as the drug concentration that decreased 50% of the OD of the control (untreated) cells.

3. Result

The isolated compound was found to be β-Sitosterol triacontenate (stigmast-5-en-3 β-ol-3 β-n-triacont-15-enoate (Figure 1). It was a colorless crystalline mass, soluble in chloroform, petroleum ether, and ethyl acetate having melting point in the range of 69-70 ℃ and molecular formula $C_{59}H_{106}O_2$ & molecular weight 846. It responds positively to steroidal tests. The results of MTT assay indicate that the cytotoxic activity of β-Sitosterol triacontenate was found to be almost comparable to that of Paclitaxel at concentrations 5 μM and 10 μM.

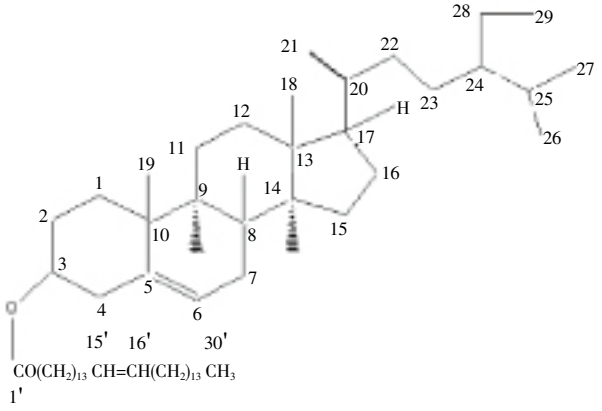

Figure 1. β-Sitosterol triacontenate.

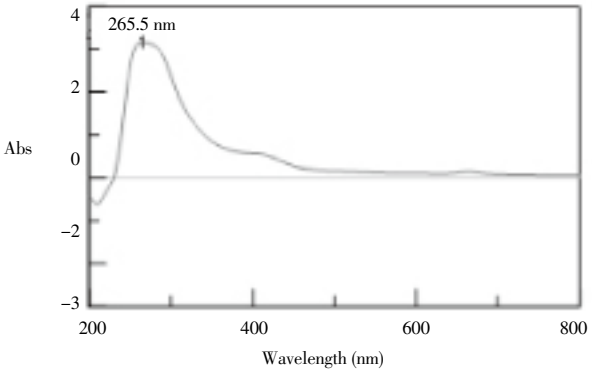

Figure 2. UV absorption spectrum of β-Sitosterol triacontenate in $CHCl_3$.

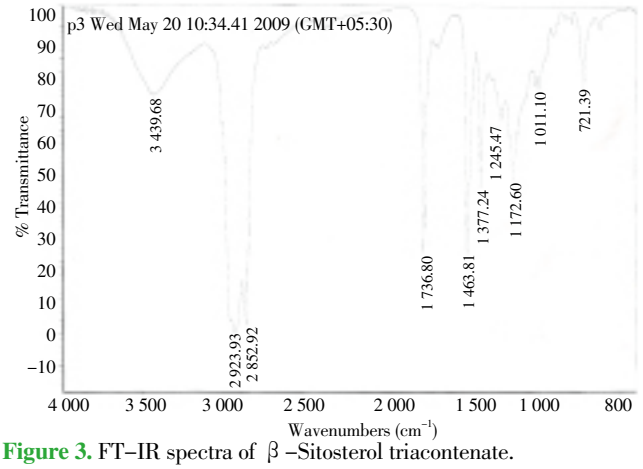

Figure 3. FT-IR spectra of β-Sitosterol triacontenate.

Table 1

^1H and ^{13}C- NMR data for isolated compounds (300 MHz, $CDCl_3$, J in Hz, δ in ppm, TMS as internal standard).

S. No	δ C	δ H
1	173.61 (C-1′)	5.35 (1H, m, H-6)
2	141.36 (C-5)	5.33 (1H, m, H-15′)
3	130.01 (C-15′)	5.31 (1H, m, H-16′)
4	127.98 (C-16′)	4.10 (1H, brm, H-3 σ)
5	122.56 (C-6)	2.31 (1H, d, j=8.3Hz, H_2-2′a)
6	73.64 (C-3)	2.28 (1H, d, j=8.1 Hz, H_2-2′b)
7	56.65 (C-14)	2.25 (2H, m, H_2-4)
8	55.99 (C-17)	2.05 (2H, m, H_2-7)
9	49.99 (C-9)	2.03 (2H, m, H_2-14′)
10	45.79 (C-24)	2.01 (2H, m, H_2-16′)
11	42.27 (C-13)	1.86 (2H, m, CH_2)
12	39.69 (C-4)	1.82 (2H, m, CH_2)
13	38.12 (C-12)	1.67 (1H, m, H-9)
14	37.04 (C-1)	1.62 (4H, m, 2×CH_2)
15	36.56 (C-10)	1.60 (1H, m, H-17)
16	36.13 (C-20)	1.58 (1H, m, H-20)
17	34.66 (C-21)	1.55 (1H, m, H-8)
18	34.36 (C-22)	1.49 (1H, m, H-24)
19	33.88 (C-8)	1.47 (1H, m, H-25)
20	32.73 (C-7)	1.28 (4H, m, 2×CH_2)
21	31.88 (C-2)	1.26 (44H, brs, 22×CH_2)
22	29.67 (21×CH_2)	1.22 (4H, brs, 2×CH_2)
23	29.12 (C-25)	1.09 (3H, brs, Me-19)
24	28.21 (C-16)	0.93 (3H, d, j=6.1Hz, Me-29)
25	27.78 (C-14′)	0.87 (3H, d, j=6.3Hz, Me-26)
26	27.14 (C-17′)	0.85 (3H, t, j=6.5Hz, Me-30′)
27	25.99 (C-23)	0.82 (3H, t, j=6.2Hz, Me-29′)
28	25.59 (CH_2)	0.71 (3H, d, j=6.5Hz, Me-30′)
29	24.99 (C-28)	0.68 (3H, brs, Me-18)
30	24.26 (C-15)	
31	22.65 (CH_2)	
32	20.98 (C-11)	
33	19.72 (C-19)	
34	19.26 (C-26)	
35	18.99 (C-27)	
36	18.74 (C-21)	
37	14.08 (C-30′)	
38	11.84 (C-18)	
39	11.82 (C-29)	

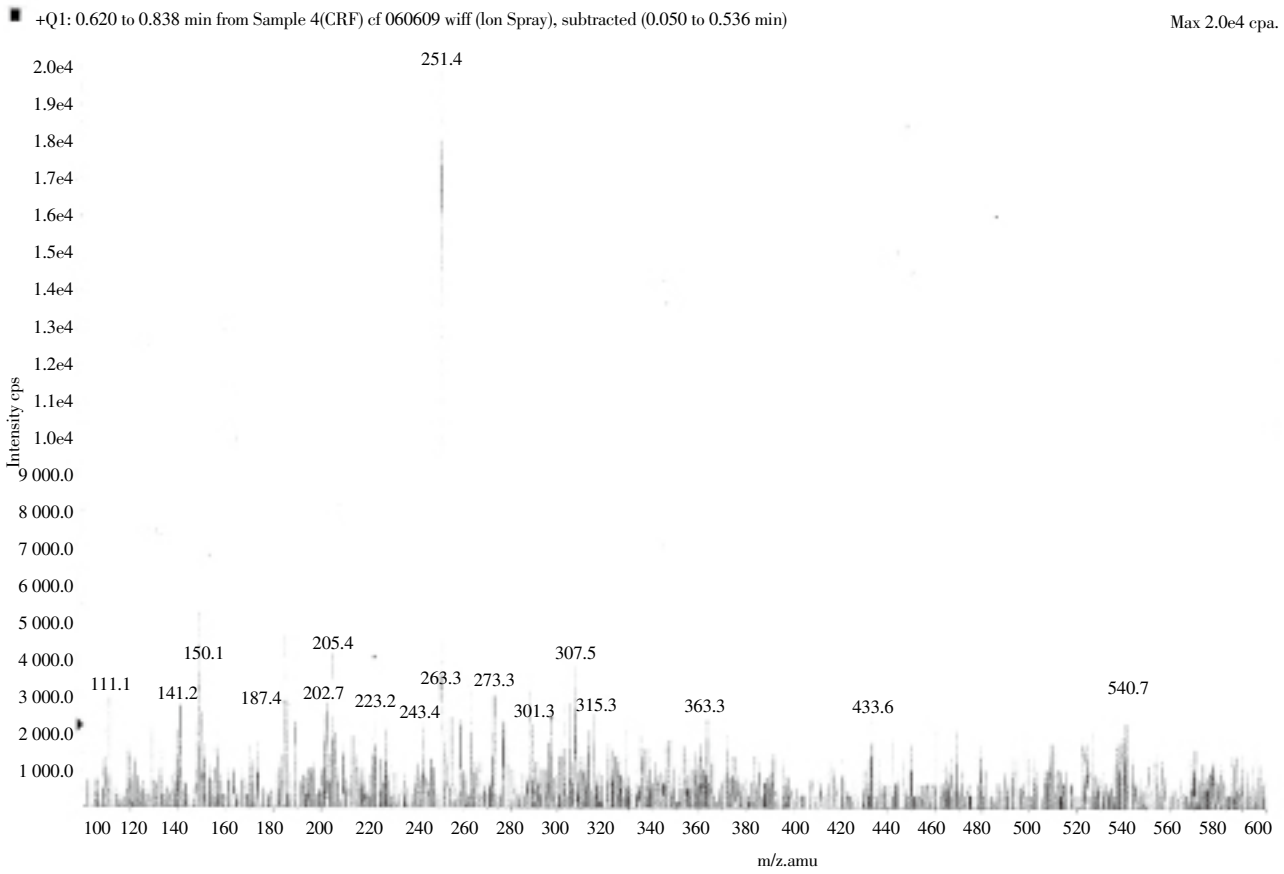

Figure 4. Mass spectra of β -Sitosterol triacontenate.

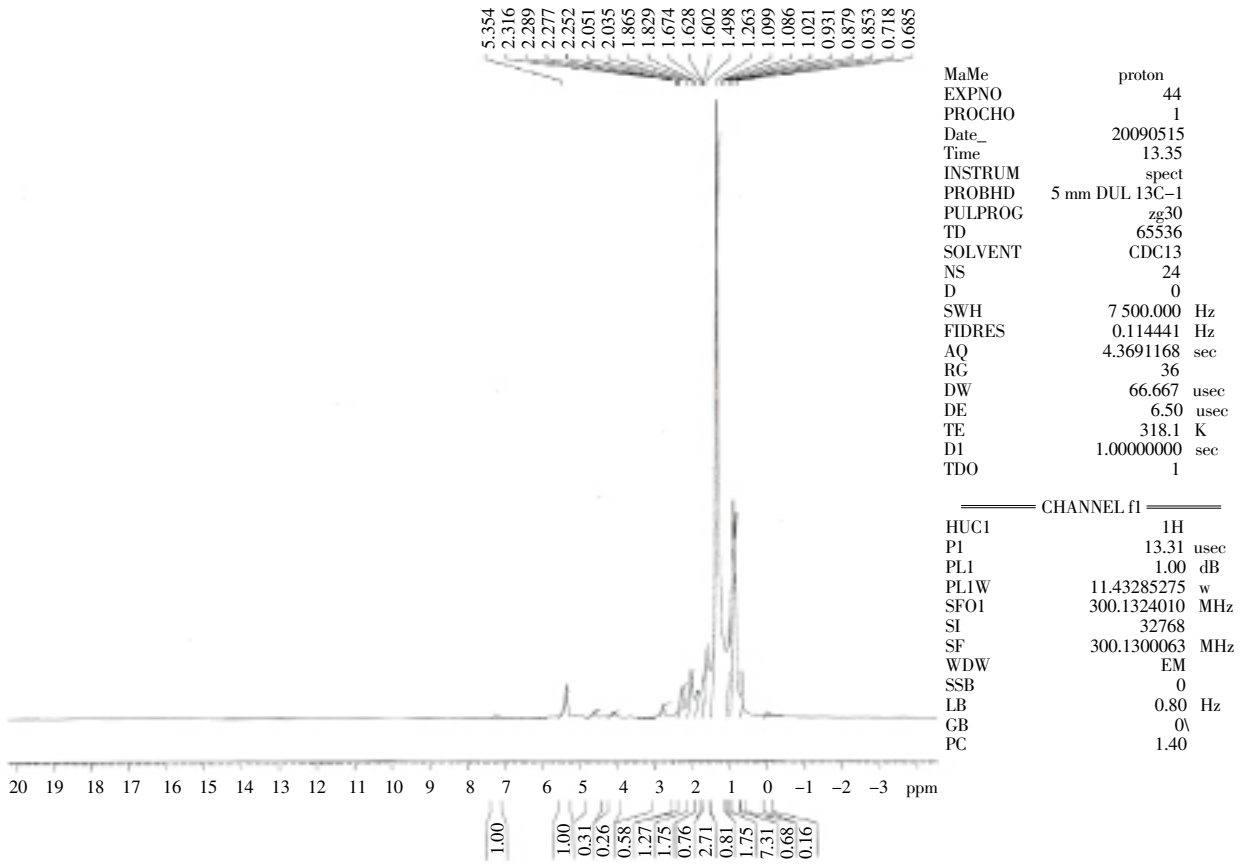

Figure 5. ^{1}H-NMR spectra of β -Sitosterol triacontenate.

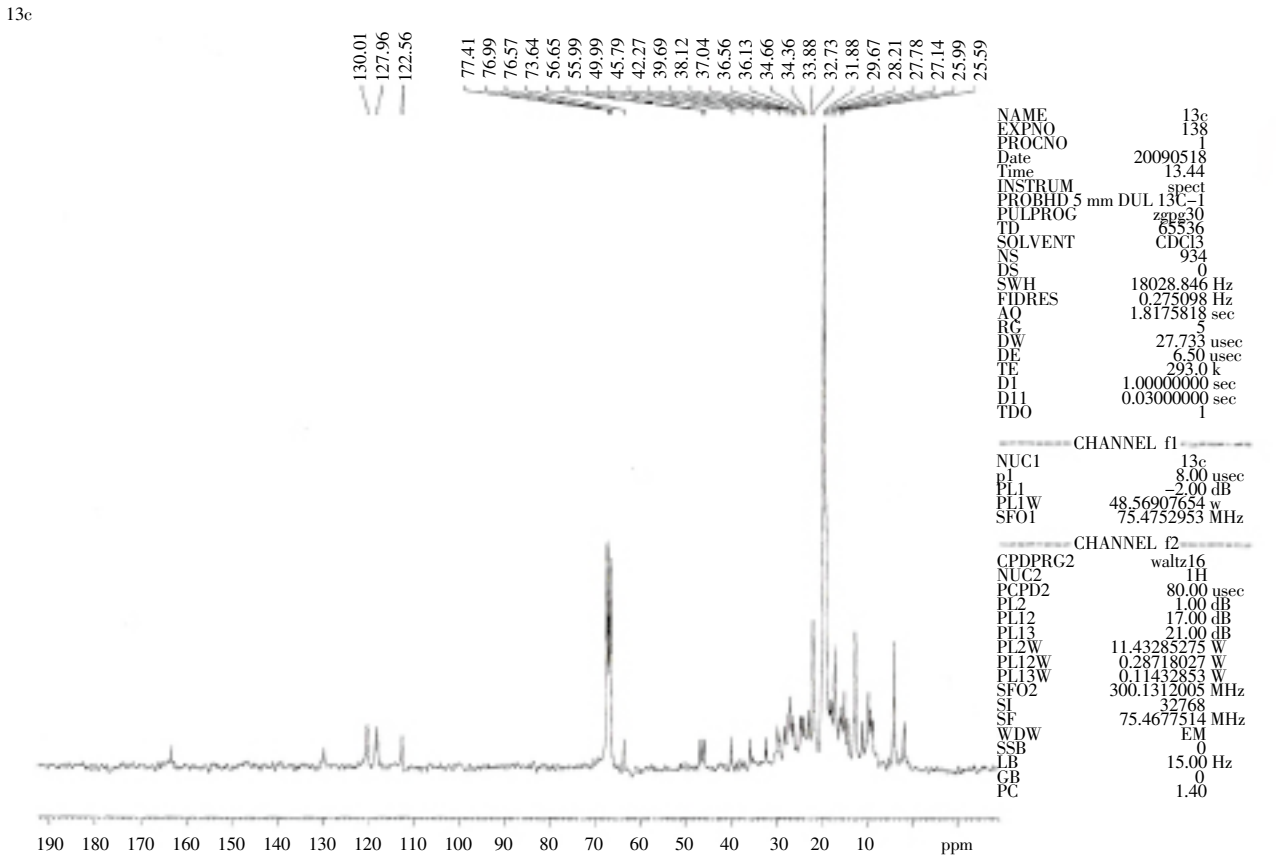

Figure 6. ^{13}C-NMR spectra of β-Sitosterol triacontenate.

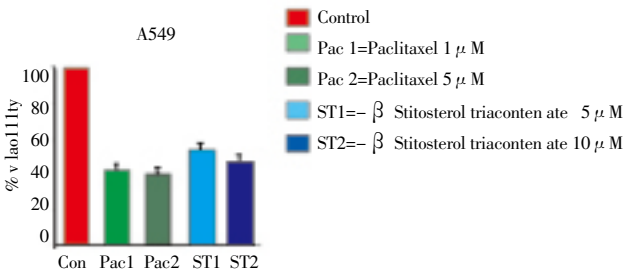

Figure 7. *In-vitro* cytotoxic activity was evaluated by MTT assay. Cells were treated with 5 μM and 10 μM of β-Sitosterol triacontenate and positive control (Paclitaxel-1 μM and 5 μM) for 48 h, thereafter cultures were evaluated as described in text. Percentage cell viability of isolated compound on lung cancer cell lines was calculated. Con: Control; Pac: Paclitaxel; ST: β - Sitosterol triacontenate.

4. Discussion

4.1. Characterization of isolated compound

The UV absorption spectrum shows a band at 265.5 nm in $CHCl_3$. Its IR spectrum showed characteristic absorption bands for the ester group (1 736.80 cm^{-1}), unsaturation (1 645 cm^{-1}), and long aliphatic chain (721.39 cm^{-1}). The prominent ion peaks arising at m/z 223.2 $[CH_3(CH_2)_{13}CH{=}CH]^+$, 433.6 $[CH_3(CH_2)_{13}CH{=}CH(CH_2)_{13}CO]^+$ and 450 $[CH_3(CH_2)_{13}CH{=}CH(CH_2)_{13}COOH]^+$ indicated that a C_{30} fatty acid containing a vinylic linkage at C_{15} was esterified to the steroid. The ion peaks arising at m/z 413 [M- 433]$^+$, 277 [413-$C_{10}H_{21}$ side chain]$^+$, 257 [272-Me]$^+$, 242 [257-Me]$^+$, 396 [M-450]$^+$, 255 [396-$C_{10}H_{21}$ side chain]$^+$, 240 [255-Me]$^+$, 225 [240-Me]$^+$, 213 [255-ring D fussion]$^+$ and 198 [213-Me]$^+$ suggested that the steroidal moiety was β-Sitosterol[26].

The ^{1}H NMR spectrum of compound displayed three one proton multiplets at δH 5.35, 5.33 and 5.31assigined to vinylic H-6, H-15′ and H-16′ respectively. A one proton multiplet at δH 4.10 with half width of 18.5 Hz was attributed to an α- oriented C-3 carbinol proton. Two one proton doublets at δH 2.31 (J=8.3 Hz) and 2.28 (J=8.1 Hz) were ascribed to methylene H_2-2′ protons adjacent to the ester group. Four two proton multiplets at δH 2.25, 2.05, 2.03 and 2.01 were due to the C-4, C-7, C-14′ and C-16′ methylene protons adjacent to the vinylic carbon. Two three proton broad signals at δH 1.09 and 0.68 were accounted C-19 and C-18 tertiary methyl proton adjacent respectively. The C-21, C-26 and C-27 secondary methyl proton appeared as doublets at δH 0.93 (J=6.1 Hz), 0. 87 (J= 6.3 Hz), and 0.85 (J=6.5 Hz) respectively. Two three proton triplets at δH 0.82 (J=6.2 Hz) and 0.71 (J=6.5 Hz) were associated with C-29 and C-30′ primary methyl protons. Its ^{13}C NMR spectra gave resonances of the ester carbon at δC 173.61 (C-1′), the vinylic carbon at δC 141.36 (C-5), 130.01 (C-15′), 127.98 (C-16′) and 122.56 (C-6), carbinol carbon at δC 73.64 (C-3), the primary methyl carbon at δC 14.08 (C-30′) and 11.82 (C-29), the secondary methyl carbon at δC 18.74 (C-21), 19.26 (C-26) and 18.99 (C-27), the tertiary methyl carbon at δC 19.72 (C-19) and 11.84 (C-18) and the remaining methylene and methane carbon between δC 56.65-80.98.

The ^{1}H & ^{13}C-NMR values of the steroidal nucleus were

confirmed with β-Sitosterol[27], β-Sitosterol esters[27] and related sterols[28]. Alkaline hydrolysis of the compound yielded β-Sitosterol and *n*-triacontenoic acid. On the bases of above information, the isolated compound possessed the steroidal skeleton esterified with a C_{30} fatty acid. Its molecular formula indicated six double bond equivalents. Four of them were adjusted in the tetracyclic carbon framework of the steroidal nucleus, one in the vinylic linkage and the remaining one in the ester group. Thus, the structure of compound was confirmed as β-Sitosterol triacontenate (stigmast-5-en-3 β-ol-3 β-n-triacont-15-enoate.

4.2. MTT assay

The assay data showed that the activity of isolated compound on lung cancer cell line A549 was found to be satisfactory, and activity was comparable to that of positive control. IC_{50} value of the β-Sitosterol triacontenate was found to be 1 μM and the cytotoxic activity increased in a dose dependent manner in case of β-Sitosterol triacontenate.

One new β-Sitosterol ester (β-Sitosterol triacontenate) was isolated from *C. decdua* plant stem. The isolated compound, β-Sitosterol triacontenate exhibits cytotoxic effect on lung cancer cells however, its mechanism of action still remains elusive.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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