

## 3-Butenyl isothiocyanate: a hydrolytic product of glucosinolate as a potential cytotoxic agent against human cancer cell lines

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**Abstract** The present study envisages the cytotoxic potential of 3-butenyl isothiocyanate isolated from *Brassica juncea* L. Czern var. Pusa Jaikisan against the human cancer cell lines viz. prostate, bone osteosarcoma, cervical, liver, neuroblastoma and breast cancer. As the compound was observed to be more effective against prostate cancer cell line, therefore, this cell line was further used to study the mechanism of cell death using neutral red assay, reactive oxygen species assay, mitochondrial membrane potential assay, microscopic and cell cycle analysis. The mechanistic analysis indicated that it induced the cell death of prostate cancer cells via apoptosis and hence made it an excellent choice as an effective anticancer compound.

**Keywords** Glucosinolates · 3-Butenyl isothiocyanate · Caspase-3 activity · Anticancer · Cell line

### Abbreviations

ITCs Isothiocyanates  
BITC 3-Butenyl isothiocyanate  
GC Gas chromatography

FID Flame ionization detector  
MS Mass spectrometer  
UHPLC Ultra high pressure liquid chromatography  
PDA Photo diode array  
NMR Nuclear magnetic resonance  
PC-3 Prostate cancer cell line  
MG-63 Human bone osteosarcoma cell line  
HeLa Human cervical cancer cell line  
HepG-2 Human liver cancer cell line  
IMR-32 Human neuroblastoma cell line  
MCF-7 Human breast cancer cell line  
FBS Fetal bovine serum  
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
MMP Mitochondrial membrane potential  
ROS Reactive oxygen species  
SEM Scanning electron microscopy  
DAPI 4',6-Diamidino-2-phenylindole  
LDH Lactate dehydrogenase

### Introduction

Glucosinolates are sulphur and nitrogen containing natural compounds, derived from glucose and amino acid. This organic compound in its intact form has low biological activity whereas its hydrolytic products formed by the action of myrosinase enzyme exert high biological activities (Arora et al. 2014a, b, 2016a; Bongoni et al. 2014). Among the variety of hydrolytic products formed, isothiocyanates (ITCs) emerges with unsurpassed activities (De Nicola et al. 2013; Arora et al. 2014c). They act as an external and important factor in maintaining cellular homeostasis in oxidative stress conditions (Ghasemzadeh and Ghasemzadeh 2011; Cabello-Hurtado et al. 2012).

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Since oxidative stress and cancer are correlated, so for the normal functioning of the body, both must be kept in control (Kryston et al. 2011). The cancer cells generated as a result of oxidative damage are mostly removed via xenobiotic metabolizing enzymes, which lead to the onset of apoptosis in these cells. The imbalanced enzyme system of body prevents the reduction of oxidative stress and hence alternate compounds are required to supplement the antioxidative and anticancer abilities of the body. Among the diverse range of ITCs, 3-butenyl ITC is an important hydrolytic product of gluconapin (parent glucosinolate). A thorough literature search has revealed that 3-butenyl ITC has not been explored for antiproliferative and anticancer activities. In line with these facts, the current study involves the evaluation of in vitro antiproliferative and anticancer activity of 3-butenyl ITC in human cancer cell lines.

## Materials and methods

### Plant material

Seeds of *Brassica juncea* (L.) Czern var. Pusa Jaikisan were procured from Indian Agricultural Research Institute (IARI), Karnal, India. The seeds were thoroughly cleaned for the removal of any pesticide or unwanted residue and were stored at  $-20\text{ }^{\circ}\text{C}$  until further use.

### Isolation and chemical characterization

The seeds after grinding were subjected to hydrodistillation with slight modifications (Arora et al. 2014b, 2016b). Extract was obtained in liquid form and was stored at  $-80\text{ }^{\circ}\text{C}$  until further use. The liquid extract was processed for the isolation of 3-butenyl isothiocyanate (BITC) using silica gel 60–120 mesh size and with hexane: ethyl acetate as mobile phase.

The isolated compound BITC was characterized using gas chromatography–flame ionization detector (GC–FID), gas chromatography–mass spectrometer (GC–MS) and ultra high pressure liquid chromatography–photo diode array (UHPLC–PDA). For GC–FID, the compound was dissolved in 1 ml GC-grade methylene chloride and was analyzed using Shimadzu (QP2010 series) gas chromatography–flame ionization detector (Tokyo, Japan). The instrument was equipped with an AOC-20i autosampler, which was coupled to a DB-5 MS capillary column ( $30\text{ m} \times 0.25\text{ mm i.d.}, 0.25\text{ }\mu\text{m}$ ). The analysis was started using the initial temperature gradient of  $40\text{ }^{\circ}\text{C}$ , held for 4 min and programmed to  $230\text{ }^{\circ}\text{C}$  at rate of  $4\text{ }^{\circ}\text{C}/\text{min}$  and was held for 15 min at  $230\text{ }^{\circ}\text{C}$ . The injection volume was  $2\text{ }\mu\text{l}$  with the temperature of  $40\text{ }^{\circ}\text{C}$ . An inlet pressure of

$97.1\text{ kPa}$  with helium as carrier gas (flow rate  $1.1\text{ ml}/\text{min}$  in split mode 1:50). The total run time of GC–FID was 46 min.

The GC–MS analysis of the isolated compound was also performed using Shimadzu (QP2010 series) gas chromatography with mass spectrometer detector (Tokyo, Japan). The autosampler, column, temperature gradient and program were same as GC–FID. An inlet pressure of  $97.1\text{ kPa}$  with helium as carrier gas (flow rate  $1.1\text{ ml}/\text{min}$  in split mode 1:50). MS interface temperature was kept  $250\text{ }^{\circ}\text{C}$  at MS mode, detector voltage was set to  $0.9\text{ kV}$ , mass range was 40–800 u and scan speed was  $1666\text{ u/s}$  with an interval of  $0.50\text{ s}$  ( $2\text{ Hz}$ ).

The compound was also analyzed for its purity using UHPLC, Naxera model, Shimadzu Asia Pacific Ltd. The instrument was coupled with a PDA detector and a C18 column ( $150\text{ mm} \times 5\text{ }\mu\text{m i.d.}, 0.18\text{ }\mu\text{m}$ ). The compound ( $2\text{ }\mu\text{l}$ ) was dissolved in 1 ml HPLC grade acetonitrile. An injection volume of  $2\text{ }\mu\text{l}$  and a gradient mobile phase (acetonitrile: water) was programmed. The total run time for the UHPLC analysis was 20 min.

### Cell lines and culture

The anticancer activity of BITC was measured using in vitro assays involving human cancer cell lines. The human prostate cancer cell line (PC-3) and human bone osteosarcoma cell line (MG-63) were grown and maintained in RPMI-1640 medium supplemented with FBS (10 %), at  $37\text{ }^{\circ}\text{C}$  in a humidified incubator containing 5 %  $\text{CO}_2$ . While, the human cervical cancer cell line (HeLa), human liver cancer cell line (HepG-2), human neuroblastoma cell line (IMR-32) and human breast cancer cell line (MCF-7) were grown and maintained in DMEM medium supplemented with FBS (10 %), at  $37\text{ }^{\circ}\text{C}$  in a humidified incubator containing 5 %  $\text{CO}_2$ . All the cell lines were obtained from National Centre for Cell Science (NCCS), Pune and were passaged for less than 6 months in our laboratory. 3-Butenyl isothiocyanate was dissolved in DMSO for cellular treatment. All the cell lines were used for MTT assay, and prostate cancer cells (PC-3) were used for further mechanistic assays. The initial cytotoxicity assay involved seeding of  $3 \times 10^3$  cells per well, while the mechanistic assay involved the use of  $5 \times 10^5$  cells per well. The number of cells required for the experimentation was standardized in our laboratory.

### Cytotoxic activity

The cytotoxic activity of the compound was assessed using MTT and neutral red assay.

### *MTT assay*

It is a colorimetric assay employed for the determination of antiproliferative activity of BITC against a number of cell lines. The assay was performed using the protocol designed by Keepers et al. (1991). The cells were seeded at a density of  $3 \times 10^3$  cells per well in a 96 well plate. The results obtained from MTT assay were used for the calculation of IC<sub>50</sub> and IC<sub>70</sub> concentration of BITC in PC-3 cells, which were used for further mechanistic studies.

### *Neutral red assay*

This method involves the detection of intact lysosomes via neutral red uptake assay following the method given by Ohno et al. (1998). Briefly, PC-3 cells at a concentration of  $5 \times 10^5$  were seeded in a 24 well plate and incubated for 24 h. Following incubation, the cells were washed with 0.1 M PBS and later treated with camptothecin (positive control) and BITC (IC<sub>50</sub> and IC<sub>70</sub>) for 12–14 h. After incubation, the cells were again washed using PBS and were treated with neutral red dye (10 µg/ml) for 2–3 h. Later, the cells were washed using PBS and fixed using 4 % PFA for 30 min and then the extraction solution was added to the cells (1 % glacial acetic acid and 50 % ethanol). The supernatant was then analyzed using ELISA plate reader (Synergy HT Biotek instruments Inc, USA) at 540 nm.

### **Mechanistic studies**

The following mechanistic assays were performed to ascertain the mode of cell death.

#### *Mitochondrial membrane potential assay*

The mitochondrial membrane potential (MMP) assay is an important method that indicates the changes in membrane integrity of cancer cells following the treatment of BITC. It was assayed following the method proposed by Deng et al. (2013). Briefly, the cells ( $5 \times 10^5$ ) were seeded in a 24 well plate. The cells were treated with BITC (IC<sub>50</sub> and IC<sub>70</sub>) for 12–14 h. Following treatment, the dye rhodamine 123 (1 µM) was added in the wells and the fluorescence was measured using an excitation wavelength of 488 nm and emission wavelength 530 nm.

#### *Reactive oxygen species assay*

The reactive oxygen species generated by BITC (IC<sub>50</sub> and IC<sub>70</sub>) and camptothecin were measured in a 24 well plate seeded with  $5 \times 10^5$  cells per well. Following treatment, the dye 2,7-dichlorofluorescein diacetate (50 µM) was added in the wells and the fluorescence was measured

using an excitation wavelength of 488 nm and emission wavelength 530 nm (Wang and Joseph 1999).

### *Microscopic studies*

The microscopic studies involved the use of phase contrast microscopy to assess the change in surface morphology, scanning electron microscopy (SEM) for observing the surface morphology and confocal microscopy for determining nuclear changes. The imaging of cell by phase contrast microscopy was done using Nikon Eclipse TS100 inverted microscope. In this, the untreated cells and cells given treatment with camptothecin (positive control) and BITC (IC<sub>50</sub> and IC<sub>70</sub>) were analyzed for structural changes following the treatment.

For SEM analysis, PC-3 cells were seeded in a 6-well plate at a concentration of  $5 \times 10^5$  cells per well in RPMI medium. After 24 h incubation, the cells were washed with 0.1 M PBS followed by the treatment with camptothecin (positive control) and BITC (IC<sub>50</sub> and IC<sub>70</sub>) for 12–14 h. Following incubation, the cells were fixed with 4 % osmium tetroxide for 4 h and then the cells were dehydrated using ethanol and acetone ranging from 10 to 100 % for 10 min each. The cells were later freeze dried using lypholyser and subjected to a single gold coating using Quorum Q150R ES Rotary-Pumped Sputter Coater/Carbon Coater. Finally, the cells were examined in EVO LS 10 scanning electron microscope (Carl Zeiss, Germany) (Ye et al. 2012).

In confocal microscopic, PC-3 cells were seeded at a concentration of  $5 \times 10^5$  cells per well in a 24 well plate for 24 h. Following incubation, the cells were washed with 0.1 M PBS and later treated with camptothecin (positive control) and BITC (IC<sub>50</sub> and IC<sub>70</sub>) for 12–14 h. After the incubation, the cells were again washed with 0.1 M PBS and fixed using 4 % PFA for 30 min. Finally, the cells were stained with DAPI and the slides were prepared. The confocal fluorescence images were taken using Nikon AiR laser scanning confocal microscope system (Nikon Corp., Japan) fitted with Nikon 40 × 0.95 NAD-ICM/N2 Plan objectives. The confocal image acquisition and analysis was performed using the built-in Nikon NIS Element AR software and the fluorescence was observed with a long-pass 488 emission filter (Bhushan et al. 2007).

#### *Cell cycle analysis*

The cell cycle analysis was done using the method proposed by Hu et al. (2010) to ascertain the arrest of cancer cells at different phases after the treatment with BITC (IC<sub>50</sub> and IC<sub>70</sub>). The cells in a density of  $5 \times 10^5$  cells per well were incubated in a 24 well plate. Following 24 h incubation, the cells were treated with camptothecin (positive

control) and BITC ( $IC_{50}$  and  $IC_{70}$ ) for 12–14 h. After the treatment, cells were trypsinized and harvested from plate by centrifugation. The pellet formed was resuspended in ice cold PBS and fixed with 70 % ethanol. The cells were fixed with propidium iodide and analyzed in FL-2 channel using BD Accuri C6 flow cytometer (BD Biosciences immunocytometry systems, San Jose, CA).

#### Caspase activity

Caspase 3 activity was analyzed using a kit based colorimetric assay with acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) as the substrate. This assay measures the amount of caspase released by cancer cells after BITC treatment, which is an indicator of early apoptosis.

#### Lactate dehydrogenase assay

The lactate dehydrogenase (LDH) assay measures the amount of lactate dehydrogenase released by the necrotic cells using the method proposed by Abe and Matsuki (2000). The cells in a density of  $5 \times 10^3$  cells per well were seeded in a 96 well plate. After a 24 h incubation in a  $CO_2$  incubator, the supernatant (50  $\mu$ l) was collected and then added 50  $\mu$ l solution A [2.5 mg lithium lactate, 2.5 mg NAD, 100  $\mu$ l of 2.5 mg/ml MTT, 1  $\mu$ l MPMS in 900  $\mu$ l tris-HCl (0.2 M, pH 8.2 with 0.1 % triton)]. The mixture was incubated for 2 h and the OD was taken in an ELISA reader (Synergy HT multi-mode microplate reader by BioTek Instruments Inc.) at 570 nm. Since, lactate dehydrogenase can only be released by necrotic cells and thus it differentiates between apoptosis and necrosis.

#### Annexin V-FITC/PI assay

Apoptosis was further confirmed using Annexin V-FITC apoptosis detection kit and using confocal microscopy. Briefly, cells in a density of  $5 \times 10^5$  cells per well were incubated in a 24 well plate. Following 24 h incubation, the cells were treated with camptothecin (positive control) and BITC ( $IC_{50}$  and  $IC_{70}$ ) for 12–14 h. After the treatment, the cells were co-stained with Annexin V-FITC and PI for 15 min. Later, the cells were trypsinized and the slides of different treatments were analyzed using Nikon AiR laser scanning confocal microscope system (Nikon Corp., Japan) fitted with Nikon 40  $\times$  0.95 NAD-ICM/N2 Plan objectives. The confocal image acquisition and analysis was performed using the built-in Nikon NIS Element AR software. This method further confirms the apoptotic mechanism of cell death and clearly differentiates it from necrosis. In this method, the viable cells are both annexin

V-FITC and PI negative, early apoptotic cells are annexin V-FITC positive and PI negative and dead cells are both annexin V-FITC and PI positive.

#### Statistical analysis

The experiments was carried out in triplicate and expressed as mean  $\pm$  SE of  $IC_{50}$  and  $IC_{70}$ . One way analysis of variance (ANOVA) and Tukey's HSD post hoc test were carried to determine significant differences between the mean at  $p \leq 0.05$  using SigmaStat v3.5.

#### Results

A compound 3-Butenyl ITC (BITC) was isolated from the extract of *Brassica juncea* (L.) Czern var. Pusa Jaikisan using hexane: ethyl acetate as solvent system. The purity of compound was confirmed by GC-MS, GC-FID and UHPLC-PDA and was found to be  $\geq 90$  % pure (Online resource 1a, b and c). Mass spectra of the compound was  $m/z$  113, 72, 55, 39, which was further confirmed by NMR ( $^1H$ -NMR (600 MHz,  $CDCl_3$ ):  $\delta$  5.75–5.82 (m, 1H), 5.16–5.20 (m, 2H), 3.51–3.56 (m, 2H), 2.41–2.45 (m, 2H);  $^{13}C$  NMR (400 MHz,  $CDCl_3$ ): 135.4, 131.9, 117.4, 43.2, 33.0).

After the chemical characterization and identification, the compound were analyzed its cytotoxic activity using MTT and neutral red assays. In MTT assay, seven different human cancer cell lines viz. prostate, bone, osteosarcoma, cervical, liver, neuroblastoma and breast cancer cell lines were used (Table 1). The  $IC_{50}$  and  $IC_{70}$  values of the compound were ascertained in each of the cell lines using MTT assay. It was observed that the compound was most effective against prostate cancer cell line ( $IC_{50}$ : 0.041  $\mu$ l/ml;  $IC_{70}$ : 0.060  $\mu$ l/ml). These values were found to be even lower than the positive control camptothecin ( $IC_{50}$ : 121.597  $\mu$ M). The antiproliferative activity was further confirmed using neutral red assay in prostate cancer cell line. The initial 100 % cell viability of PC-3 cells in control was reduced to 12 % at  $IC_{50}$  and 5 % at  $IC_{70}$  (Fig. 1a). Overall, the antiproliferative activity of BITC in both MTT and neutral red assay was found to be good in prostate cancer cell line.

To understand the mechanism of cell death as indicated by MTT and neutral red assay, a number of other assays viz. MMP, ROS, phase contrast microscopy, confocal microscopy, SEM, cell cycle analysis, caspase activity, LDH assay and annexin V-FITC/PI assay were also performed. In MMP assay, a better bioactivity in case of  $IC_{50}$  and  $IC_{70}$  concentration of BITC in PC-3 cells compared to positive control was observed. The BITC treatment in ROS

**Table 1** The antiproliferative activity of 3-butenyl isothiocyanate (IC<sub>50</sub> and IC<sub>70</sub> value (μl/ml)) against a number of cancer cell lines

Treatment	Cell lines						
	PC-3	A-549	HeLa	HepG-2	IMR-32	MCF-7	MG-63
BITC (IC <sub>50</sub> )	0.041	167.49	0.072	0.049	0.133	0.666	0.065
BITC (IC <sub>70</sub> )	0.060	185.550	1.217	0.967	1.314	20.649	1.098
Camptothecin (μM)	121.597	65.556	12.749	10.219	78.337	57.559	60.614
Regression Eqn	y = 1049x + 6.791	y = 2.853ln(x) + 35.39	y = 7.072ln(x) + 68.61	y = 6.707ln(x) + 70.23	y = 8.725ln(x) + 67.62	y = 5.823ln(x) + 52.37	y = 716.3x + 3.244
Correlation coefficient	0.969	0.963	0.841	0.841	0.881	0.839	0.919

The cells are treated with different concentrations of 3-butenyl isothiocyanate ( $5 \times 10^{-4}$ – $35 \times 10^{-3}$  μl/ml)

PC-3 human prostate cancer cell line, A-549 human lung cancer cell line, HeLa human cervical cancer cell line, HepG-2 human liver cancer cell line, IMR-32 human neuroblastoma cell line, MCF-7 human breast cancer cell line, MG-63 human osteosarcoma cell line

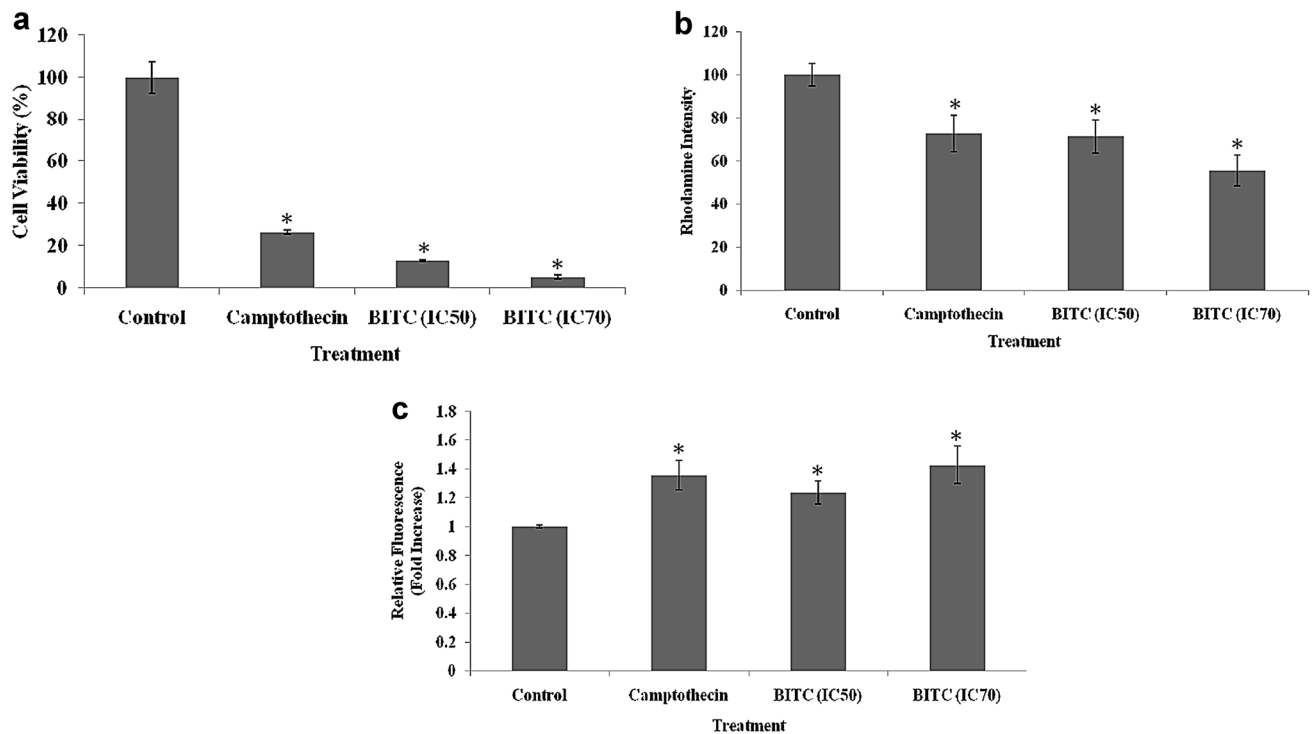
assay caused an increased generation of reactive oxygen species in prostate cancer cells as seen by the IC<sub>50</sub> and IC<sub>70</sub> values thus showing a positive correlation with MMP assay (Fig. 1b, c).

The above studies were further strengthened by microscopic studies which indicated the changes in cell and nuclear morphology following the treatment with BITC. Online resource 2a clearly shows the rounding of PC-3 cells following the treatment with BITC (IC<sub>50</sub> and IC<sub>70</sub>) and camptothecin (positive control) in phase contrast microscopy. The cancer cells began to die via apoptosis mechanism and the apoptotic bodies were formed. The surface morphology of PC-3 cells showed an enhanced apoptosis in BITC (IC<sub>50</sub>) treated cells as compared to positive control in SEM studies as seen in Online resource 2b. The formation of apoptotic bodies started, leading to cellular death (Online resource 2bIII). The confocal microscopic imaging of PC-3 cells treated with BITC (Fig. 2aIII, IV) showed changes in nuclear morphology compared to control (Fig. 2aI).

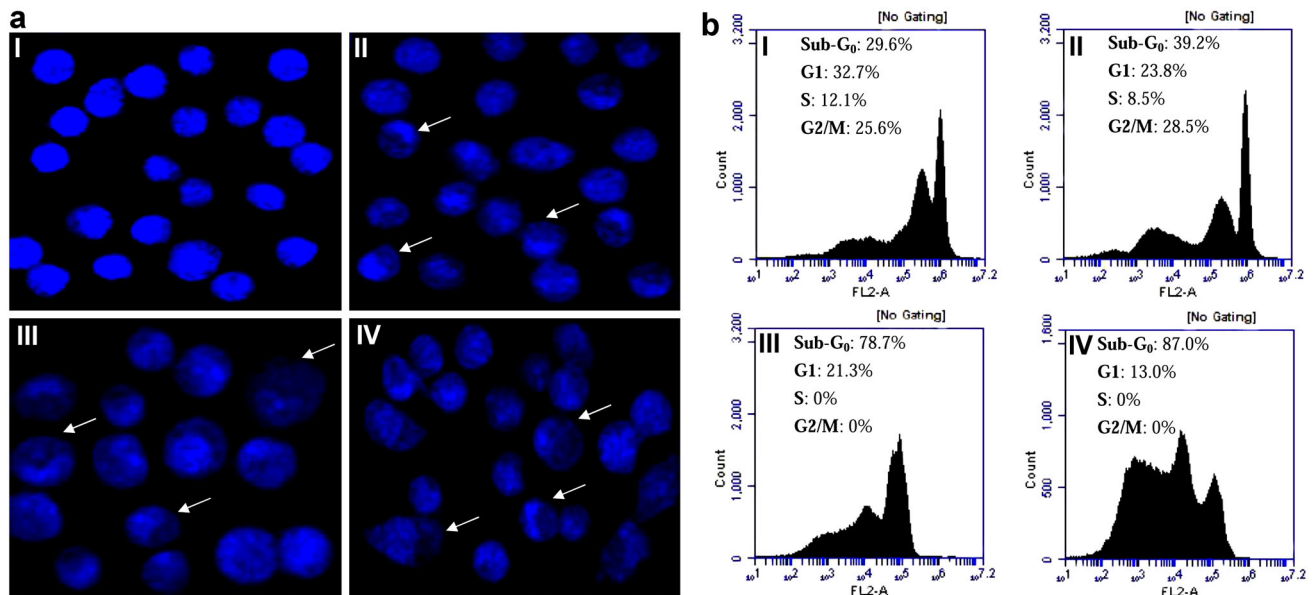
The cell cycle analysis using flow cytometry was also conducted. It was seen that the BITC treatment caused the arrest of PC-3 cells at hypo-diploid (sub G<sub>0</sub>) phase of cell cycle (78.7 and 87 % in IC<sub>50</sub> and IC<sub>70</sub> respectively) (Fig. 2bIII, IV). In contrast, the cells in G1 phase considerably decreased following the treatment with BITC (21.3 % at IC<sub>50</sub> and 13.0 % at IC<sub>70</sub>). In addition, no cells were seen at S and G<sub>2</sub>/M phase, after the treatment with BITC. The positive control camptothecin on the other hand had 39.2 % cells at sub G<sub>0</sub> phase, 23.8 % cells at G<sub>1</sub> phase, 8.5 % cells at S and 28.5 % cells at G<sub>2</sub>/M phase of cell cycle.

The apoptotic mechanism of cell death induced by BITC in PC-3 cells was further investigated using a battery of other assays in order to differentiate the necrotic and apoptotic mechanism of cell death. The caspase 3 activity was increased after the BITC (IC<sub>50</sub>: 185.19 % and IC<sub>70</sub>: 214.81 %) treatment as compared to the control (Fig. 3a). The LDH activity in PC-3 cells following BITC (IC<sub>50</sub> and IC<sub>70</sub>) treatment was also studied. An increased LDH activity following the treatment with the compound was considered as the marker of tissue damage (Fig. 3b). This is an important marker for identifying the actual mechanism of cellular death. Finally, annexin V-FITC and PI assay was performed to confirm the apoptotic cellular death. The co-incubation of PC-3 cells and BITC with and without these dyes (IC<sub>50</sub> and IC<sub>70</sub>) (Fig. 3cI) showed no red coloration, revealing an absence or decreased cellular death. On the other, hand BITC (IC<sub>50</sub> and IC<sub>70</sub>) treatment showed cellular death via apoptosis at IC<sub>50</sub> concentration, while slight necrosis could be seen at IC<sub>70</sub> concentration (Fig. 3cIII, IV).





**Fig. 1** Anticancer activity of 3-butenyl isothiocyanate: **a** cell viability using neutral red assay; **b** MMP assay; **c** ROS assay. Experiment was performed in triplicates and *asterisk* represents  $p \leq 0.05$  compared to control

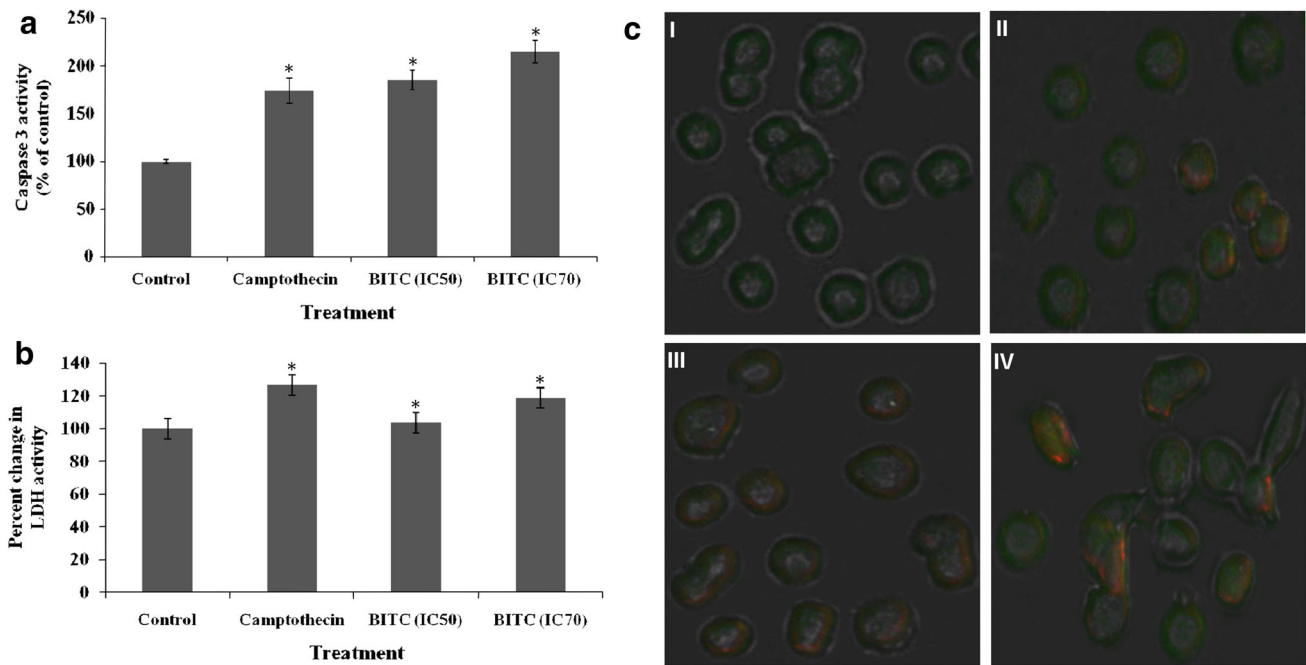


**Fig. 2** Nuclear changes following 3-BITC treatment: **a** confocal microscopy *I* control, *II* positive control, *III* BITC (IC<sub>50</sub>), *IV* BITC (IC<sub>70</sub>); **b** cell cycle analysis *I* control, *II* positive control, *III* BITC (IC<sub>50</sub>), *IV* BITC (IC<sub>70</sub>)

## Discussion

*Brassica juncea* is a member of family Brassicaceae and is a rich source of glucosinolates (GSLs) (Fahey et al. 2001). The evaluation of hydrolytic products of *Brassica juncea* var. Pusa Jaikisan showed 3-butenyl ITC as the major

product. It was then isolated using silica 60–120 mesh and ethyl acetate: hexane as solvent system. The isolated product BITC was obtained with a purity  $\geq 90\%$ . The results were confirmed via a number of analytical instruments viz. GC–MS, GC–FID, UHPLC–PDA and NMR. All these instruments validated the compound along with its purity.



**Fig. 3** Assays for differentiating between apoptosis and necrosis: **a** Caspase 3 activity; **b** LDH assay; **c** annexin V-FITC/PI assay *I* control, *II* positive control, *III* BITC (IC<sub>50</sub>), *IV* BITC (IC<sub>70</sub>). Experiment was performed in triplicates and *asterisk* represents  $p \leq 0.05$  compared to control

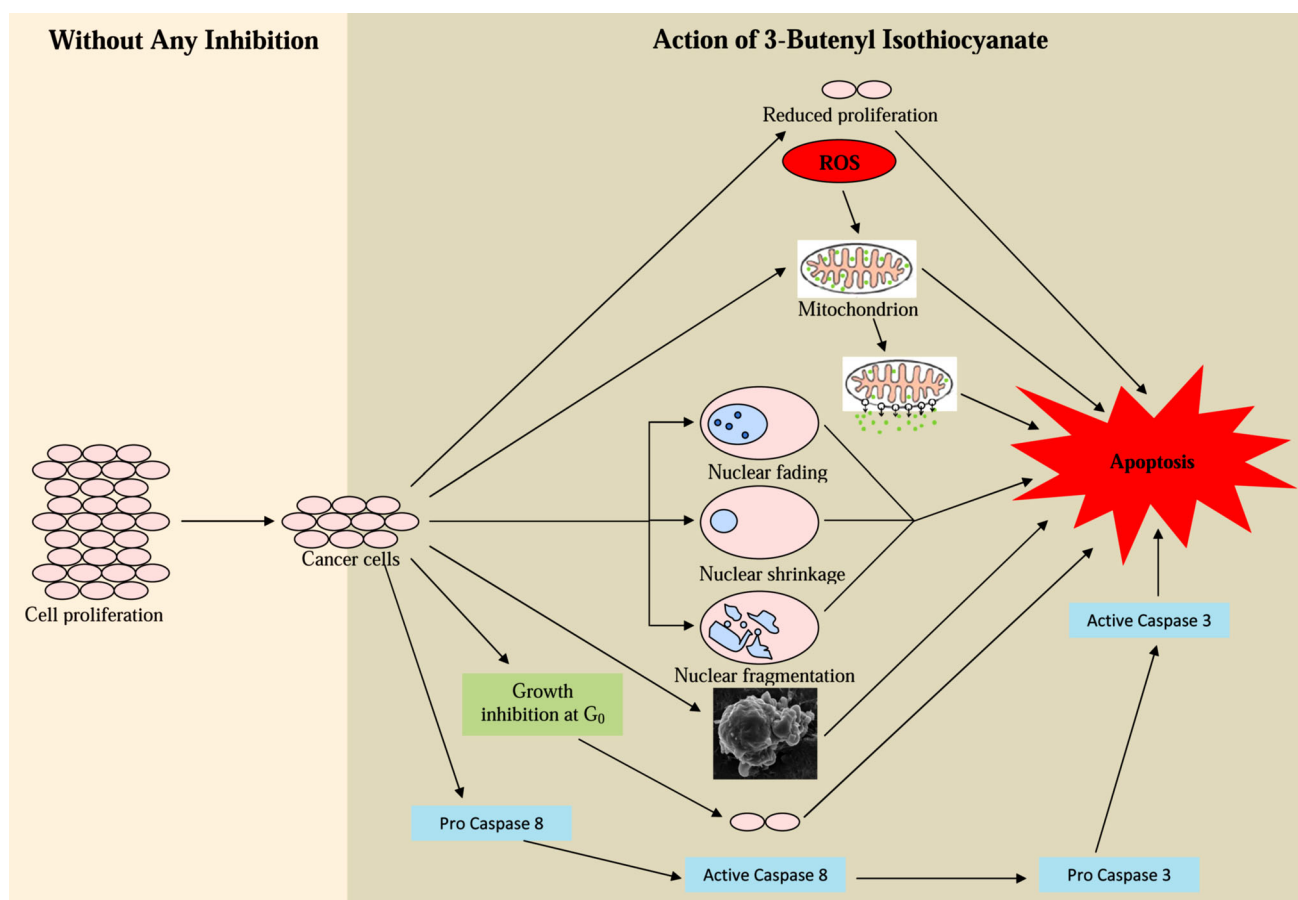
Although, BITC showed antiproliferative activity in all cell lines but it was found to be more effective in prostate cancer cells. It was seen that IC<sub>50</sub> value of BITC was as low as 0.041  $\mu\text{l/ml}$  in PC-3 cells compared to 167.49  $\mu\text{l/ml}$  in A-549 cells. The antiproliferative activity points towards the ability of BITC to prevent/inhibit the cancer cell growth. A number of manuscripts have reported ITCs as potential antiproliferative agents (Ferrarini et al. 2011; Melchini et al. 2013). An initial test of cytotoxicity using neutral red assay was performed for reconfirming the activity of BITC. The test showed a low cellular viability at IC<sub>50</sub> concentration. A potent antiproliferative activity plays a keystone for assessing the possible role of BITC as an anticancer agent. For this purpose, a number of assays were performed to confirm the apoptotic mechanism of cellular death and thus testing the anticancer activity of this compound. The mitochondrial membrane potential of PC-3 cells was reduced following the treatment with BITC. On the other hand, an increase in the reactive oxygen species was seen after the treatment. The decreased MMP and an increased mitochondrial ROS is often linked to initiation of apoptosis related cellular death (Gottlieb et al. 2003; Chen et al. 2010; Kwang-Youn et al. 2011).

Microscopic studies are another important visual study for proving the anticancer activity of BITC. The rounding and distortion in the normal shape of the cells along with the budding of apoptotic bodies in phase contrast microscope pointed towards the beginning of apoptosis (Chiang

et al. 2011). This mechanism of apoptosis seen in phase contrast microscopy was further confirmed using scanning electron microscope. A clear and detailed formation and budding of apoptotic bodies was seen in the PC-3 cells treated with IC<sub>50</sub> concentration of BITC.

The confocal microscopy of PC-3 cells was also conducted. This study revealed the change in nuclear morphology following the treatment with BITC. The cells showed swelling, clear morphological and nuclear deformation and shrinkage of nuclear material as compared to untreated control. A cell cycle analysis acts as another important factor adding to the apoptotic mechanism of cellular death. The PC-3 cells treated at IC<sub>50</sub> concentration of BITC showed a restriction at sub G<sub>0</sub> (78.7 %) phase of cell division as compared to the untreated control (29.6 %). After the treatment with BITC, the cells in the G<sub>1</sub> phase considerably decreased, while an increase in the cells at G<sub>0</sub> phase was observed. The cells were therefore entering the quiescent state and thus an inhibition of cell cycle was observed. This was further confirmed by the absence of any cells in the S and G<sub>2</sub>/M phase of cell cycle. This increase in the number of cells at sub G<sub>0</sub> phase and decrease in the cells at G<sub>1</sub>, S and G<sub>2</sub>/M phase is an indicator that the cancer cells are prevented from undergoing cellular division and thus pushing these towards apoptotic cell death.

The difference between the apoptotic and necrotic cell death following the treatment with BITC was also confirmed using a number of other assays. Among these,



**Fig. 4** Mechanism of action of 3-butenyl isothiocyanate

caspase 3 is an important enzyme responsible for chromatin condensation and DNA fragmentation. An increase in caspase 3 activity was recorded in PC-3 cells treated with BITC. This increase showed the apoptotic cellular death occurring due to the compound in hand. LDH is another important marker of tissue damage (Gonzalez-Flecha et al. 1993). A low level of LDH in BITC treated cells is a clear indicator of the ongoing apoptotic cellular death (Wrobel et al. 2003). The confirmation was further strengthened by annexin-V FITC and PI assay. The  $IC_{50}$  dose and the positive control showed apoptotic cellular death (Fig. 4). Thus, BITC is an important and potent anticancer agent leading to cellular death by apoptosis.

## Conclusion

Isothiocyanates have emerged as a ray of hope against a number of biological stresses including cancer. The current study involved the isolation of an important isothiocyanate

viz. 3-butenyl isothiocyanate with a purity of  $\geq 90\%$ . The compound exhibited exceptional anticancer activity among the different human cancer cell lines. Among these, the prostate cancer (PC-3) cells showed great sensitivity against this compound. The mechanistic pathway involved in the cellular death of the prostate cancer cells was evaluated using a number of assays and it was concluded that the cells were killed following apoptosis. This study exhibited the potent anticancer activity of 3-butenyl isothiocyanate and hence makes it a suitable candidate for animal model study.

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## Compliance with ethical standards

**Conflict of interest** Authors pose no conflict of interest.



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