

Original Article

## Evaluation of merits of petroleum ether extract of *Bunium bulbocastanum* on prostate cancer cell lines: An *in vitro* study

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### ABSTRACT

**Objectives:** This study aimed to investigate the potential anticancer effects of ethanolic seed extract and fractions derived from *Bunium bulbocastanum* on prostate carcinoma cell line 3 (PC-3).

**Materials and Methods:** *B. bulbocastanum* seeds were extracted using a Soxhlet apparatus and fractionated based on the solvent polarity. Phytochemical screening and quantitative analyses (total phenolic and flavonoid content) were conducted to assess the presence of bioactive compounds in each fraction. Cytotoxicity screening using the Sulforhodamine B assay was performed on PC-3 and verda reno (VERO) cell lines, with petroleum ether as the most potent cytotoxic fraction (half-maximal inhibitory concentration value). In addition, the antioxidant properties of the extract fractions were evaluated using a nitric oxide radical-scavenging assay.

**Results:** Among the tested fractions, petroleum ether exhibited the highest inhibition percentage. Gas chromatography-mass spectroscopy analysis of the petroleum ether fraction identified bioactive compounds that potentially contributed to its anticancer properties. The anti-proliferative effects were further evaluated using a scratch wound assay, which revealed significant inhibition of PC-3 cell migration by the petroleum ether fraction.

**Conclusion:** The petroleum ether fraction of *B. bulbocastanum* demonstrated notable cytotoxic, antioxidant, and antiproliferative activities against PC-3. These findings suggest its potential as a therapeutic candidate for the further exploration of cancer treatment strategies.

**Keywords:** Adverse effects, Anticancer, Antioxidants, Antiproliferation, *Bunium bulbocastanum*, Plant extract

### INTRODUCTION

India's flora is rich in variety and has great therapeutic significance for both treating and preventing disease. Plant-based chemicals are still employed in many low-income nations despite the availability of Western medicine because they are readily available, inexpensive, and have fewer side effects.<sup>[1]</sup> These therapeutic plants are abundant in phytoconstituents with established anti-cancer qualities, including flavonoids, phenolics, alkaloids, terpenoids, and glycosides. By reducing the growth of tumour cells and the production of molecules that cause tumour growth, they lower the risk of developing cancer.<sup>[2]</sup>

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Prostate cancer ranks third globally and is the eighth leading cause of death; in India, it ranks 12<sup>th</sup> most common and is the 14<sup>th</sup> leading cause of cancer-related death, according to Globocon 2020.<sup>[3]</sup> The most successful therapies are radiation therapy, cryotherapy, hormone therapy, and chemotherapy, depending on the Gleason score. Both systemic and local side effects are linked to these treatments.<sup>[4,5]</sup> In northern India, *Bunium bulbocastanum*, popularly referred to as kala or black jeera (cumin), is a member of the Apiaceae family and is frequently used as a spice in food preparation. Herbal extracts are made from the leaves, and the edible roots have a flavour akin to cocoa or chestnuts. According to studies, this plant has cytotoxic, anti-inflammatory, and anti-diabetic properties.<sup>[6]</sup> On cancer cell lines, ethyl acetate and fruit aqueous extracts have shown strong anti-oxidative and anti-cancer properties.<sup>[7]</sup> Considering the aforementioned results, this work aimed to assess the antiproliferative and antioxidant qualities of *B. bulbocastanum* seed petroleum ether extract in PC-3.

## MATERIALS AND METHODS

### Cell lines and growth media

PC-3 obtained from the National Centre for Cell Science, Pune, India, were cultured in Dulbecco's modified Eagle's medium supplemented with antibiotics (100 units/mL), antimycotics (10 µg/mL) and 10% foetal bovine serum from Thermo Fisher Scientific Ltd. Cells were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide.

### Plant collection and authentication

Seeds of *B. bulbocastanum* were obtained from Kashmir in December 2021. The seed sample was authenticated by the Associate Professor and Head, Department of Botany, from Mahatma Gandhi Memorial College, Udupi, Karnataka, India.

### Preparation of extracts and fractionation

To obtain the crude alcoholic extract, *B. bulbocastanum* seeds were initially dried under shade and finely ground. The powdered material was dissolved in ethanol and fractionated using a Soxhlet apparatus. Sequential fractionation was performed using water (350 mL), petroleum ether (3 × 500 mL), diethyl ether (3 × 500 mL) and ethyl acetate (3 × 500 mL). The fractions were then concentrated using a rotary evaporator at 40°C.

The percentage yield of the extract was determined using the formula: Yield (%) = (weight of the dry extract/weight of the dry plant material) × 100.<sup>[7,8]</sup>

### Preliminary phytochemical analyses

Different qualitative tests were performed on the ethanolic seed extract fractions of *B. bulbocastanum* to determine the presence of various phytochemicals. The test procedure utilised to determine the presence of alkaloids, carbohydrates, polyphenols, flavonoids, tannins, terpenoids, saponins, proteins, polysterols and steroids in a sample which were as follows:<sup>[9,10]</sup>

1. Terpenoids test: A mixture of 2 mL chloroform and 5 mL alcohol seed extract was evaporated in a water bath, followed by the addition of 2 mL concentrated sulphuric acid. The appearance of a reddish-brown colour indicates the presence of terpenoids.
2. Flavonoid test (alkaline reagent test): A mixture of alcohol seed extract and 2 mL 20% NaOH produced a concentrated yellow colour, which turned colourless on the addition of two drops of diluted acid, confirming the presence of flavonoids.
3. Phenols/polyphenols test: Mixing 2 mL of the test solution in alcohol with one drop of 5% ferric chloride solution (pH 7) resulted in a strong blue colouration in the presence of phenols.
4. Saponin/fatty acid test: Five mL of distilled water was added to the alcohol seed extract in a test tube, vigorously mixed, and frozen with a few drops of olive oil. The presence of saponins was indicated by the formation of a foam.
5. Tannin test: Adding 2 mL of the test solution to bromine water and acetate resulted in discoloration of the bromine water, indicating the presence of tannins.
6. Steroids/polysterols test: Chloroform (2 mL) and concentrated sulphuric acid were added to 5 mL seed extract. The appearance of a red colour in the chloroform layer indicated the presence of steroids.
7. Alkaloids (Dragendorff's test): Ethanolic extract (2 mg), distilled water (5 mL), and hydrochloric acid (2 M). The addition of 1 mL of Dragendorff's reagent resulted in an orange or orange-red precipitate, indicating the presence of alkaloids.
8. Carbohydrates (anthrone test): 2 mg of ethanolic extract was shaken with 10 mL of water, filtered, and concentrated. The addition of 2 mL of anthrone reagent solution produced a green or blue colouration in the presence of carbohydrates.
9. Proteins (biuret test): The addition of a small amount of copper sulphate and sodium hydroxide (Biuret reagent) to the sample generated a blue complex in the presence of proteins.

### Total phenolic and flavonoid content

#### Total phenolic content

The Folin-Ciocalteu method was employed to determine the total phenolic content, which is a widely accepted

technique for quantifying phenolic compounds in materials. In this assay, the Folin-Ciocalteu reagent, containing sodium molybdate and sodium tungstate, oxidises phenolic substances, resulting in a blue-coloured complex detectable through spectrophotometry. The intensity of the colour was directly correlated with the concentration of the phenolic compounds. Total phenolic content was quantified using a gallic acid standard curve and expressed as milligrams of gallic acid equivalent per gram of dried plant material.<sup>[11,12]</sup>

#### **Total flavonoid content**

Total flavonoid content in the crude extracts was determined using the aluminium chloride (AlCl<sub>3</sub>) method. To prepare the sample, 4 mL of distilled water was added to 1 mL of the crude extract (1 mg/mL ethanol). After a 5-minute incubation, 0.4 mL of 10% AlCl<sub>3</sub> solution and 0.4 mL and 5% sodium nitrate solution were added, followed by an additional 6-minute incubation. The volume was adjusted to 10 mL by adding double-distilled water and 2 mL of a 1 mol/L NaOH solution. The absorbance was measured at 510 nm after a 15-min incubation. The total flavonoid content was determined using a calibration curve and expressed as milligram of quercetin equivalent per gram of dried plant material.<sup>[13]</sup>

#### **Sulforhodamine B (SRB) assay**

Plant extract cytotoxicity assays often employ the SRB assay, which evaluates the ability of plant extracts to inhibit cell growth *in vitro*. SRB, a protein-binding dye, binds to cellular proteins and can be quantified by spectrophotometry. SRB is a vivid pink amino xanthene dye with two sulfonic groups that dissociate under basic conditions and bind to basic amino acid residues under mildly acidic conditions. The amount of dye recovered from the stained cells is directly proportional to the number of cells in the sample, as SRB binding is stoichiometric. Cytotoxicity was determined by measuring the absorbance of SRB-stained cells treated with varying concentrations of plant extracts. The ethanolic seed extract and its fractions (petroleum ether, diethyl ether, ethyl acetate and aqueous) were evaluated for cytotoxicity using this method, and the results were compared with those of doxorubicin. In 96-well plates, the toxicity of the test extract against adherent cells was assessed. The cell monolayer was incubated and fixed with 10% trichloroacetic acid, and excess dye was removed with 1% acetic acid. Optical density (OD) at 540 nm was measured using a 2D microplate reader (Biotek EL-800 USA) after dissolving the protein-bound dye in 10 mM Tris base.<sup>[14]</sup> The cytotoxic potential of the ethanolic seed extract and its fractions on PC-3 and VERO normal cells was evaluated using the SRB assay.<sup>[15]</sup> Half-maximal inhibitory concentration (IC<sub>50</sub>) values were calculated for each fraction. The ethanolic seed extract fractions demonstrated significant dose-dependent cytotoxicity compared to both VERO cells and human PC-3.

#### **Gas chromatography-mass spectroscopy (GC-MS) analysis of petroleum ether fraction of *B. bulbocastanum* ethanolic seed extract**

Following cytotoxic screening, GC-MS analysis (Shimadzu GC-MS-QP2010S) was conducted on the petroleum ether fraction of the ethanolic seed extract of *B. bulbocastanum* at Analytical Research and Metallurgical Laboratories Pvt. Ltd. (ARML), Bangalore, India. The analysis employed a mass spectrophotometer coupled with gas chromatography equipment featuring a triple-axis detector and an Hewlett-Packard-5 Microsoft (HP-5 MS) fused silica column interfaced with a 5675C Inert mass selective detector. Helium gas served as the carrier gas at a flow rate of 1 mm/min.

The GC-MS analysis procedure involved the following steps: Initially, the column temperature was held at 36°C for 5 min, then ramped up to 150°C at a rate of 4°C/min. Subsequently, the temperature was increased to 260°C at a rate of 20°C/min and maintained for 5 min. The total elution time was 47.5 min. The relative percentage of each component was determined by comparing its average peak area to the total area, and data acquisition and system management were facilitated using the National Institute of Standards and Technology (NIST) library. Data interpretation, peak identification, and quantitation were done using Shimadzu's GC-MS solution software.<sup>[16,17]</sup>

#### **Nitric oxide (NO) scavenging assay**

The Griess assay, also known as the N-(1-Naphthyl) ethylene diamine dihydrochloride NO scavenging assay, is a commonly used technique for determining a compound's capacity to scavenge NO radicals. Sodium nitroprusside (SNP), a stable source of NO radicals that releases NO in aqueous solution, was added to test compounds or extracts before testing.<sup>[18]</sup> To enable the production of NO radicals, the test materials were incubated with the NO donor (SNP) under particular circumstances.

The reaction mixture comprising sulphanilamide and N-1-naphthylethylenediamine dihydrochloride was mixed with Griess reagent after it had been incubated. This reaction then combines with nitrite ions (NO<sub>2</sub><sup>-</sup>), a stable oxidation product of NO, to create a reddish-purple azo compound that is easily detected spectrophotometrically. These outcomes were obtained by measuring the absorbance at 545 nm. Methanol served as the blank, and ascorbic acid, or Vitamin C, as the standard. The % antioxidation potential was calculated using the following formula: % inhibition = (A<sub>0</sub> - A<sub>t</sub>/A<sub>0</sub>) × 100

Where A<sub>t</sub> absorbance of the test samples and A<sub>0</sub> is the absorbance of the control reaction mixture without the extract.<sup>[19,20]</sup>

#### **Scratch wound assay**

One popular *in vitro* method for examining cell migration and wound-healing mechanisms is the scratch wound assay. In

this test, a confluent cell monolayer is deliberately scratched or punctured, and the migration of cells into the cleared region is tracked over time. Up until confluence, PC-3 and VERO cultured cells were cultivated in a monolayer. A sterile pipette tip that had the same length and width was used to make a scratch in the cell monolayer. Following the formation of the scratch, the cells were cleaned to get rid of any leftover material or separated cells, and new culture media was added. As a baseline for comparison, time-zero photos ( $\times 4$  and  $\times 10$  magnification) were obtained as soon as the scratch was produced. Following incubation, the cells were permitted to go to the area that had been scratched. Images were captured to monitor the migration of cells into the wound area at certain time intervals (6, 12 and 24 h). These photos were used to quantify the rates of cell migration and scratch closure.

### Cell cycle analysis

The cells underwent trypsinisation, harvesting, a full phosphate buffered saline (PBS) wash and a 30-min fixation in cold ethanol at 4°C. Drop by drop, ethanol was added to create a pellet, and the cells were vortexed to reduce clumping. Next, the cells underwent an  $850 \times g$  centrifugation. Subsequently, 50  $\mu\text{L}$  of a 100  $\mu\text{g}/\text{mL}$  stock of RNase was introduced into the mixture to guarantee deoxyribonucleic acid, staining through Ribonucleic acid elimination. Finally, 200  $\mu\text{L}$  of propidium iodide was added to the stock solution containing 50  $\mu\text{g}/\text{mL}$ . Petroleum ether extract fractions at doses of 50 and 100  $\mu\text{g}$  were applied to the cells. For every group, the experiment was run in triplicate.

### Search Tool for Interactions of Chemicals (STITCH) and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis-interaction networks of chemicals and function protein association networks

To learn more about the interactions of the phytochemicals present in the petroleum ether fraction and their associations with different protein molecules, and to gain insight into protein-protein interactions, all compounds detected by GC-MS analysis were screened in the STITCH (<http://stitch.embl.de/>) and STRING (<https://string-db.org/>) databases. This further aided the identification of different bioactive phytochemicals present in the petroleum ether fraction and various proteins that interact with them. For pathway analysis in GO Biological Processes to understand the function of the genes and their protein products in various biological pathways, data from ShineyGO 0.77 database (<http://bioinformatics.sdstate.edu>) was utilised.

### Statistical analysis

All experiments were conducted in triplicate, and data were expressed as mean  $\pm$  standard deviation. Statistical significance was assessed using one-way analysis of variance followed by Tukey's *post hoc* test for multiple comparisons. A statistical

significance was set at  $P < 0.05$ . GraphPad Prism software (version 9.0) was used for data analysis and visualisation.

## RESULTS

The ethanolic extract (140 g) was subjected to further fractionation with petroleum ether, diethyl ether, ethyl acetate and water to yield 56 g, 44 g, 31 g and 18 g fractions, respectively.

### Preliminary phytochemical analysis

Table 1 summarises the phytochemical analysis of fractions of *B. bulbocastanum* ethanolic seed extract. Proteins were absent in all the fractions and alkaloids, terpenoids, flavonoids, carbohydrates, polysterols and tannin were present in petroleum ether and diethyl ether extracts.

### Total phenolic and flavonoid content

Table 2 provides a summary of the total phenolic and flavonoid contents across the different fractions of the plant extract. The analysis revealed significant variability in the phenolic content among the fractions ( $P < 0.01$ ). The aqueous extract exhibited the highest total phenolic content, measured at  $46.0 \pm 0.26$  mg gallic acid equivalent (GAE)/g of plant extract, followed by the petroleum ether fraction with  $13.5 \pm 0.19$  mg GAE/g of plant extract, the diethyl ether fraction with  $18.5 \pm 0.28$  mg GAE/g of plant extract and the ethyl acetate fraction with  $26.0 \pm 0.07$  mg GAE/g of plant extract.

Significant differences were also observed in the flavonoid content among the various fractions ( $P < 0.05$ ). The ethyl acetate fraction, with a flavonoid content of  $8.97 \pm 0.12$  mg quercetin equivalents per gram (QE/g) of plant extract, was followed by the diethyl ether fraction at  $4.2 \pm 0.17$  mg QE/g of plant extract, and the petroleum ether fraction at  $2.02 \pm 0.26$  mg QE/g of plant extract, in comparison to the highest flavonoid content observed in the aqueous extract, which was  $19.99 \pm 0.03$  mg QE/g of plant extract.

### Effect of fractions of ethanolic extract of *B. bulbocastanum* using SRB assay

Doxorubicin was utilised as a positive control in this study, yielding an  $\text{IC}_{50}$  value of 1.052  $\mu\text{g}/\text{mL}$  for PC-3 cells and 80.37  $\mu\text{g}/\text{mL}$  for normal cells. The petroleum ether fraction exhibited the most pronounced cytotoxic effects, with an  $\text{IC}_{50}$  value of 63.58  $\mu\text{g}/\text{mL}$  against PC-3 cells and 542.5  $\mu\text{g}/\text{mL}$  against normal cells [Table 3].

### GC-MS analysis of petroleum ether fraction of *B. bulbocastanum*

The top 5 constituents present in the highest concentration are listed in Figure 1. Triterpenoids are present in highest concentration in the petroleum ether fraction.

**Table 1:** Phytochemical analysis of fractions of *Bunium bulbocastanum* ethanolic seed extract.

Phytochemical constituents	Aqueous	Petroleum Ether (PEF)	Diethyl Ether (DEF)	Ethyl acetate (EAF)
Alkaloids	–	+	+	–
Terpenoids	+	+	+	+
Flavonoids	+	+	+	+
Carbohydrates	+	+	+	+
Polyphenols	+	+	+	+
Tannins	–	+	+	–
Proteins	–	–	–	–
Saponins	–	–	+	+
Polysterols	–	–	+	+
Steroids	–	–	+	+

**Table 2:** Percentage of inhibition of fractions of *Bunium bulbocastanum* seeds at different concentrations with IC<sub>50</sub> value on PC-3 cell lines.

Concentration (microgram/mL)	% Inhibition of different fractions			
	Aqueous	Diethyl ether	Petroleum ether	Ethyl acetate
500	41.65	96.4	96.67	96.23
250	18.79	95.27	96.70	89.74
125	19.03	94.19	82.61	23.68
62.5	0.186	87.94	43.75	10.95
31.25	8.40	55.81	22.90	2.86
15.625	–11.4	4.16	8.47	9.28
IC <sub>50</sub>	645.5	30.27	63.58	159.5

IC<sub>50</sub>: Half-maximal inhibitory concentration

### NO radical-scavenging assay

The results of the NO scavenging assay are presented in Figure 2. The petroleum ether fraction demonstrated significant antioxidant activity, achieving a scavenging rate of 30.34% at a concentration of 0.5 mg/mL, which was notably higher than the antioxidant potential of the standard, which exhibited a 25.60% scavenging rate.

### Scratch wound assay

After 24 h, the cells treated with the standard (doxorubicin) and petroleum ether fraction at a concentration of 100 µg/mL exhibited significant anti-proliferative effects, whereas the control group showed continued cell growth and complete healing [Figure 3].

### Cell cycle arrest by flow cytometry

Significant variations in the flavonoid content were observed among the different fractions ( $P < 0.05$ ). The aqueous extract

**Table 3:** Percentage of inhibition of fractions of *Bunium bulbocastanum* seeds at different concentrations with IC<sub>50</sub> value on VERO cell lines.

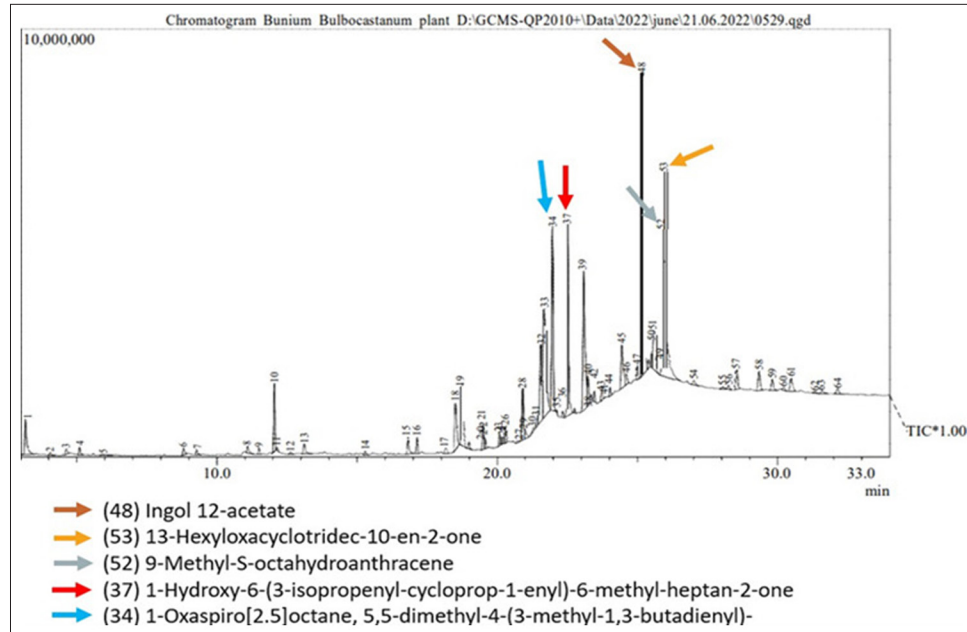
Concentration (microgram/mL)	% Inhibition of different fractions			
	Aqueous	Diethyl ether	Petroleum ether	Ethyl acetate
500	86.03	86.76	40.91	92.95
250	22.71	89.63	–1.05	91.66
125	–29.89	90.64	–31.63	57.69
62.5	–35.80	93.0	–21.49	14.83
31.25	–24.25	82.36	1.145	11.28
15.625	–3.30	57.69	19.45	16.24
IC <sub>50</sub>	333.1	1.473	542.5	110.9

IC<sub>50</sub>: Half-maximal inhibitory concentration, VERO: Verda reno

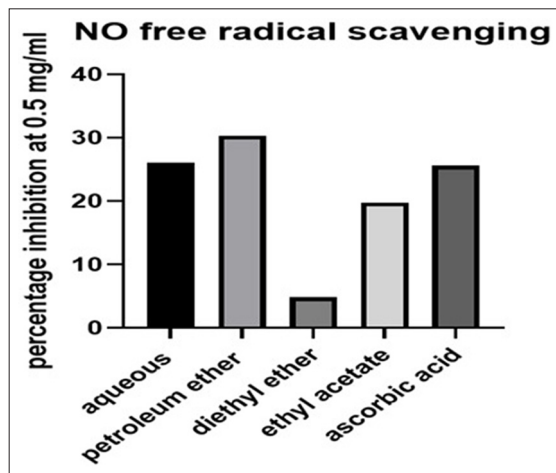
exhibited the highest flavonoid concentration at  $19.99 \pm 0.03$  mg QE/g of plant extract. The fractions with the next highest flavonoid concentrations were the ethyl acetate fraction ( $8.97 \pm 0.12$  mg QE/g of plant extract), the diethyl ether fraction ( $4.20 \pm 0.17$  mg QE/g of plant extract) and the petroleum ether fraction ( $2.02 \pm 0.26$  mg QE/g of plant extract) [Figure 4].

### STITCH and STRING analysis

The STITCH and STRING analysis revealed that 5 active phytochemicals had potential biological activity. Squalene monooxygenase, Lanosterol 14-alpha demethylase, Lanosterol synthase, and Delta (24)-sterol reductase were just a few of the proteins of the lipid and cholesterol synthesis pathways that were discovered to interact with phytochemicals [Figure 5a and b]. A deeper analysis of the pathways revealed that they were based on cholesterol metabolism in accordance to GO Biological Processes database [Figure 5c].



**Figure 1:** Gas chromatography-mass spectroscopy analysis of petroleum ether fraction of *Bunium bulbocastanum*. Numbers in parenthesis are the number given to each peak starting from left hand side of the chromatogram.

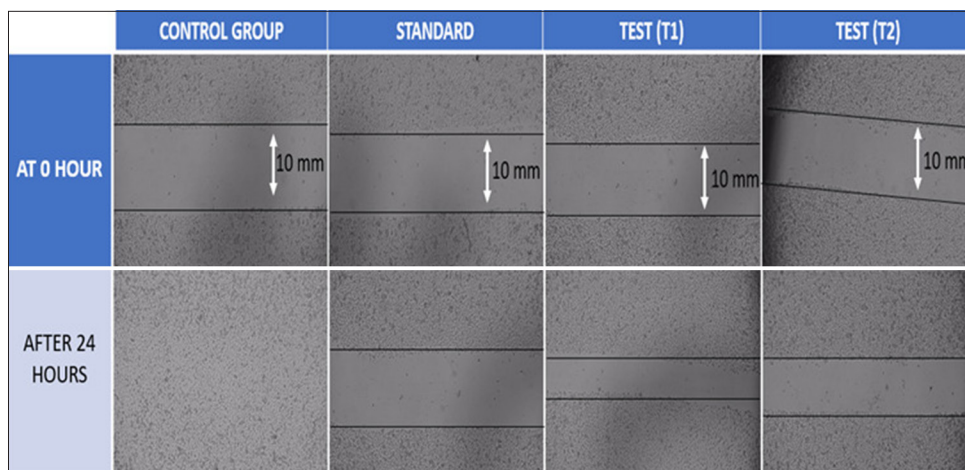


**Figure 2:** Antioxidant activity of fractions of *Bunium bulbocastanum*.

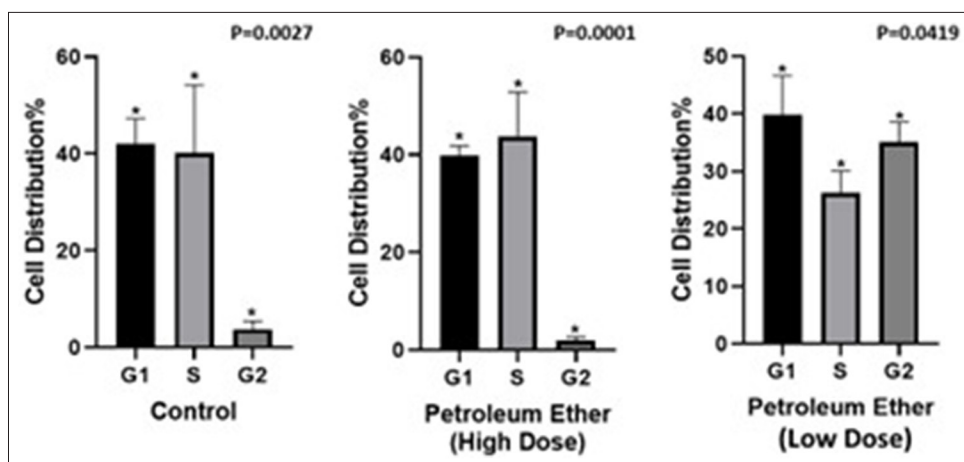
## DISCUSSION

*B. bulbocastanum* has demonstrated significant therapeutic potential in various medicinal applications. Despite its traditional use, the medicinal properties of this herb remain unclear. However, emerging research suggests that *B. bulbocastanum* may exert beneficial effects across multiple therapeutic domains, primarily due to its potent antioxidant properties.<sup>[19]</sup> In a previous study, the methanolic extracts of *B. bulbocastanum* and their fractions were evaluated against various human cancer cell lines, including Henrietta Lacks (HeLa), human epithelial cell line (Hep 2), michigan cancer

foundation (MCF) 7, and National Institutes of Health 3 day transfer (NIH 3T3).<sup>[20]</sup> This study aimed to assess the anticancer effects of *B. bulbocastanum* on PC-3 *in vitro*. The anticancer potential of *B. bulbocastanum* seed extract is closely associated with its flavonoid and polyphenol content. These bioactive compounds exert their effects through multiple mechanisms, including free radical scavenging, apoptosis induction, and inflammation reduction.<sup>[21,22]</sup> In addition to their ability to selectively target rapidly proliferating cells, flavonoids and polyphenols modulate aberrant molecular pathways, mitigate oxidative stress, regulate cellular growth factors, inhibit angiogenesis in tumour tissues, and induce apoptosis in cancerous cells.<sup>[23,24]</sup> Previous studies employing the MTT assay for cytotoxic screening demonstrated that ethyl acetate and aqueous fractions of *B. bulbocastanum* fruit exhibit potent cytotoxic activity with low IC<sub>50</sub> values against HeLa, Hep 2 and MCF 7 cancer cell lines. Notably, these fractions showed minimal toxicity toward the normal NIH 3T3 cell line (IC<sub>50</sub> > 1000 g/mL).<sup>[20]</sup> In the present study, preliminary cytotoxic screening of *B. bulbocastanum* ethanolic seed extract and its fractions was conducted using the SRB assay against PC-3 prostate cancer cells and normal human VERO cell lines. Among the tested fractions, the petroleum ether fraction exhibited notable cytotoxicity, while the diethyl ether fraction demonstrated severe toxicity toward both PC-3 cancer cells and VERO normal cells, with IC<sub>50</sub> values of 1.5 g/mL and 30 g/mL, respectively. Given its nonspecific cytotoxicity, the diethyl ether fraction was deemed unsuitable for further anticancer investigation.



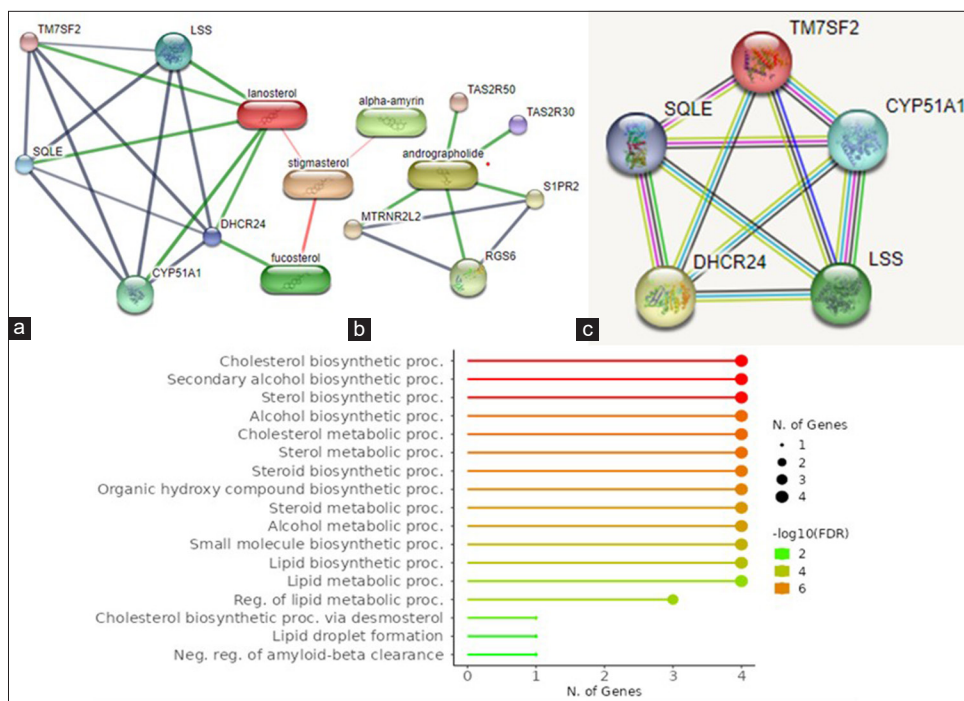
**Figure 3:** The prostate cancer cell lines cells treated with petroleum ether fraction was subjected to scratch wound assay and anti-proliferative activity was compared at 0 h and at 24 h. Control group: Untreated cells. Standard: Doxorubicin-treated cells. T1 (test at low concentration): Cells treated with 50 ug petroleum ether extract fraction. T2 (test at high concentration): Cells that have been exposed to a 100 ug extract of petroleum ether.



**Figure 4:** Analysis of cell cycle distribution of cell lines. Cell lines are treated with high (50 ug extract fraction) and low doses (100 ug extract fraction) of petroleum ether extract. Distribution of cells in the G0/G1, S and G2/M phases in prostate cancer cell lines cell lines. Data are expressed as mean values standard deviation ( $n = 3$ ) \* $P < 0.05$  indicating a significant difference compared with untreated cells. G1: Gap 1 phase, S: Synthesis phase, G2: Gap 2 phase.

Because phenolic compounds and flavonoids are known to exhibit anticancer properties through diverse mechanisms, quantitative analysis was performed. The results indicated that phenolic and flavonoid contents varied across the fractions, with higher concentrations observed in more polar solvents. The total phenolic content decreased from the aqueous extract to the ethyl acetate, diethyl ether, and petroleum ether fractions. These findings align with prior research suggesting that polar solvents are more effective for extracting phenolic compounds than non-polar solvents.<sup>[25,26]</sup> Free radicals contribute to cellular damage and play significant roles in cancer progression. Antioxidants function as biological

scavengers by neutralising free radicals and preventing cellular degradation. A NO radical-scavenging assay was used to evaluate the antioxidant potential of different fractions of the ethanolic *B. bulbocastanum* seed extract. Previous studies have demonstrated that methanolic fractions of *B. bulbocastanum* exhibit potent antioxidant activity in HeLa, Hep 2, and MCF 7 cell lines.<sup>[21]</sup> Similarly, in the present study, the antioxidant activity was assessed across different fractions of *B. bulbocastanum* seeds at various concentrations. The petroleum ether fraction exhibited the highest percentage of inhibition, indicating superior antioxidant activity compared with the other fractions. GC-MS is a well-established



**Figure 5:** STITCH and STRING analysis. (a) STITCH chemical and Protein association network of the phytochemicals detected in gas chromatography-mass spectroscopy analysis, (b) STRING function protein association network and (c) the correlated transcripts and enrichment analysis using ShinyGO, the lollipop plot illustrating the enriched pathways [false discovery rate (FDR) 0.05].

analytical technique widely employed for phytochemical identification and chemotaxonomic research of medicinal plants containing biologically active compounds.<sup>[27]</sup> GC-MS analysis was conducted in the present study to identify the bioactive constituents of the petroleum ether fraction of *B. bulbocastanum*. The results revealed a diverse array of phytochemicals, with triterpenoids constituting the most abundant class, accounting for 21.18% of the total detected compounds. Triterpenoids are widely recognised for their anticancer efficacy, exerting their effects through multiple biological mechanisms.<sup>[28,29]</sup> To further investigate the anticancer potential of the petroleum ether fraction, a scratch wound assay was performed to assess its effect on the proliferation of PC-3 prostate cancer cells.<sup>[30]</sup> Treatment with the petroleum ether fraction resulted in significant suppression of cell migration and proliferation, indicating potent antiproliferative properties. Additional cell cycle analysis using flow cytometry revealed that the petroleum ether fraction primarily affected the S phase of the cell cycle at a lower dose, further supporting its role in inhibiting cancer cell proliferation. To elucidate the underlying molecular mechanisms, *in silico* analysis was conducted using the STITCH and STRING databases. This analysis identified key interactions between the bioactive compounds present in the petroleum ether fraction and various metabolic pathways, particularly those involved in cholesterol

metabolism. Cholesterol serves as a crucial precursor for steroid hormone biosynthesis, including androgens, such as testosterone.<sup>[31,32]</sup> Androgens play a fundamental role in prostate gland development and maintenance; however, in prostate cancer, they contribute to tumour progression by activating androgen receptor (AR) signalling.<sup>[33]</sup> Prostate cancer cells utilise cholesterol to synthesise androgens, thereby promoting tumour growth.<sup>[33]</sup> Moreover, cholesterol and its metabolites have been shown to influence prostate cancer cell survival by modulating AR signalling pathways.<sup>[34]</sup> Given that AR signalling is a critical driver of prostate cancer progression, targeting cholesterol metabolism may represent a promising therapeutic strategy.<sup>[35]</sup> The findings of this study suggest that the anticancer activity of *B. bulbocastanum* petroleum ether fraction may be attributed to its ability to interfere with cholesterol metabolism and AR signalling. This aligns with emerging evidence that drugs targeting cholesterol metabolism can exert significant anticancer effects in prostate cancer models.<sup>[36]</sup> Further preclinical investigations are warranted to explore the full therapeutic potential of *B. bulbocastanum* and its bioactive constituents for prostate cancer treatment. The present study highlights the potential of *B. bulbocastanum* seed extract, particularly the petroleum ether fraction, as a promising source of anticancer agents against prostate cancer. These findings provide compelling evidence that the extract exhibits

cytotoxicity against PC-3 prostate cancer cells, possesses strong antioxidant properties, and interferes with cholesterol metabolism. These results underscore the need for further studies to elucidate the precise molecular mechanisms and to evaluate the *in vivo* efficacy of *B. bulbocastanum*-derived compounds in prostate cancer treatment.

## CONCLUSION

The *B. bulbocastanum*'s ethanolic seed extract's petroleum ether fraction possesses strong *in vitro* cytotoxic, anti-proliferative, and antioxidant effects on PC-3 cells. The petroleum ether fraction showed significant antioxidant qualities by scavenging NO radicals. Triterpenoids, which are most concentrated in the petroleum ether fraction of the seed, were thought to be responsible for these activities. The antioxidant, antiproliferative, and cytotoxic qualities of the fraction are responsible for the inhibition of cancer growth. Plant derivatives can be used to produce commercial anticancer drugs more quickly and with greater potency.

**Ethical approval:** The research/study was approved by the Institutional Review Board at Kasturba Medical College and Kasturba Hospital, approval number 775/2021, dated 9th November 2021.

**Declaration of patient consent:** Patient's consent was not required as there are no patients in this study.

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