Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Germinated Seeds of *Hordeum vulgare* Target Extrinsic Pathway of Apoptosis in Triple-Negative Breast Cancer Cells

G. H. Akhil, Bibu John Kariyil, G. D. Akshatha[1], S.V. Vasudhar Bhatt[2], G. Dhanusha, Reni John

Departments of Veterinary Pharmacology and Toxicology, [1]Animal Genetics and Breeding and [2]Veterinary Biochemistry, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Thrissur, Kerala, India

Submitted: 14-Apr-2020 Revised: 24-Apr-2020 Accepted: 06-Jul-2020 Published: 30-Nov-2020

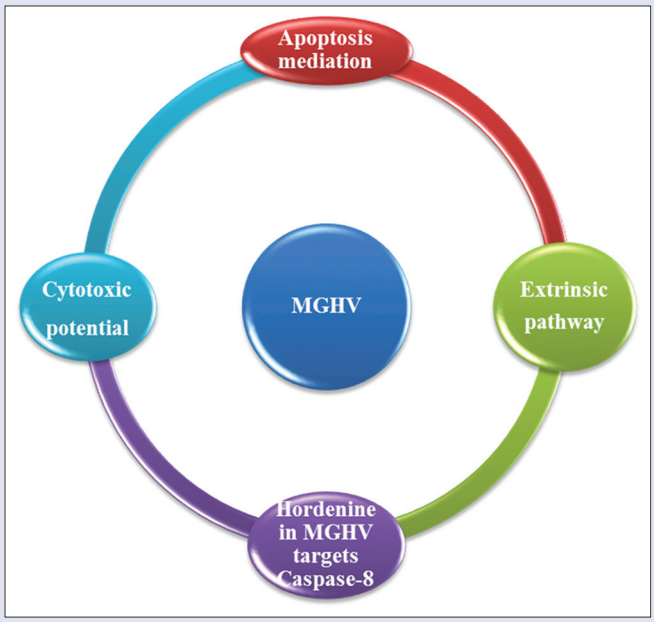
ABSTRACT

Background: The absence of receptors, lack of specific treatment regimen, and emergence of resistance against various currently available anticancer drugs have led to the development of lead molecules from botanicals for the mainstay treatment of triple-negative breast cancer (TNBC). *Hordeum vulgare,* commonly known as barley, has been reported to have many traditional uses, and the main alkaloid in its germinated seeds, hordenine, has been evaluated for many pharmacological properties. **Objectives:** The present study aimed to evaluate the anticancer activity of methanol extract of germinated seeds of *H. vulgare* (MGHV) against MDA-MB-231 TNBC cells, quantify hordenine in MGHV, and derive the probable target of action of the extract and hordenine. **Materials and Methods:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, morphological evaluation, acridine orange/ethidium bromide (AO/EB) staining, Hoechst 33258 staining, JC-1 staining, comet assay, and Western blot were conducted to evaluate the anticancer effect. High-performance liquid chromatography (HPLC) analysis was conducted to detect and quantify the presence of hordenine in the extract. Stimulation of extrinsic pathway of apoptosis was evaluated by studying the interaction of hordenine with Caspase-8 using *in silico* methods. **Results:** MTT assay revealed significant concentration-dependent cytotoxicity. AO/EB staining exhibited yellow-green fluorescence indicative of early apoptosis. Hoechst 33258 staining showed nuclear marginalization and fragmentation. The results of JC-1 staining showed a combination of red and green fluorescence indicative of partial reduction in mitochondrial membrane potential. Comet assay revealed that the extract did not produce deoxyribo nucleic acid damage. Western blotting analysis did not show any change in the expression of Bcl-2, whereas significant upregulation of Caspase-8 by 2.8 folds was noticed indicative of extrinsic pathway of apoptosis. HPLC analysis detected the presence of hordenine as the major constituent in the extract. Morphological assessment of MDA-MB-231 cells treated with hordenine showed cytotoxic changes similar to that of the extract. *In silico* studies with hordenine also substantiated the results of Western blot. **Conclusion:** Thus, it was concluded that MGHV targeted extrinsic pathway of apoptosis due to the presence of hordenine and hence hordenine could evolve as a potent anticancer molecule against TNBC.

Key words: Apoptosis, caspase-8, hordenine, *Hordeum vulgare*, *in silico*, MDA-MB-231

SUMMARY

- The present study revealed that the anticancer activity of methanol extract of germinated seeds of *Hordeum vulgare* in triple-negative breast cancer cells was due to the cytotoxic alteration produced by triggering the extrinsic pathway of apoptosis, without causing deoxyribo nucleic acid damage. High-performance liquid chromatography analysis has detected and quantified the presence of hordenine. *In silico* studies have also revealed the binding affinity of hordenine toward Caspase-8. Thus, it could be summarized that the anticancer mechanism exerted by methanol extract of germinated seeds of *Hordeum vulgare* was through stimulation of the extrinsic pathway of apoptosis, which might be due to the presence of hordenine in the germinated seeds.



Abbreviations used: TNBC: Triple-negative breast cancer; MGHV: Methanol extract of Germinated seeds of *Hordeum vulgare*; MDA: MB-231 – Monroe Dunaway Anderson-Metastatic Breast-231; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; AO/EB: Acridine orange/ethidium bromide; HPLC: High-performance liquid chromatography; Bcl-2:B cell lymphoma-2; FBS: Foetal bovine serum; RPMI: Rosewell Park Memorial Institute; EDTA: Ethylenediamine tetra acetic acid; CO_2: Carbondioxide; DMSO: Dimethylsulfoxide; IC_{50}: Inhibitory concentration 50; MMP: Mitochondrial membrane potential; DNA: Deoxyribo nucleic acid; NMPA: Normal melting point agarose; LMPA: Low melting point agarose; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: Polyvinylidene fluoride.

Correspondence:

Dr. Bibu John Kariyil,
Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode, Lakkidi P.O., Wayanad - 673 576, Kerala, India.
E-mail: bibujohn@kvasu.ac.in
DOI: 10.4103/pm.pm_123_20

Access this article online
Website: www.phcog.com
Quick Response Code:

Cite this article as: Akhil GH, Kariyil BJ, Akshatha GD, Bhatt SV, Dhanusha G, John R. Germinated seeds of *Hordeum vulgare* target extrinsic pathway of apoptosis in triple-negative breast cancer cells. Phcog Mag 2020;16:S531-9.

INTRODUCTION

One of the subtypes of breast cancer is the triple-negative breast cancer (TNBC) that lacks the expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2. TNBC constitutes 15%–20% of all the breast cancer cases diagnosed.[1] The absence of receptors on TNBC cells is one of the reasons being difficult to treat. The high expression of CD73,[2] overexpression of *IRAK*,[3] and mena invasive protein[4] have been reported to cause resistance of TNBC tumor cells to various chemotherapeutic agents. Moreover, there are no specific strategies or protocols implied for the treatment of TNBC although there are a few guidelines recommended by the National Comprehensive Cancer Network to use either single or combination of anticancer drugs, which have resulted in resistance. Due to the side effects and emergence of resistance against various anticancer drugs, botanicals and herbal extracts could be used as adjunctive or supportive therapy to favor additive or synergistic effect in the treatment of TNBC.

Hordeum vulgare, also known as barley, is the fourth highest cereal produced in the world next to maize, wheat, and rice. It is commonly used as animal fodder by hydroponics and an important ingredient in the production of alcoholic breweries. Juice of germinated seeds and young barley leaves are being used as diet and health supplements. Barley has been reported to have multiple pharmacological properties such as antioxidant,[5] anticancer,[6] antiproliferative,[7] antidiabetic,[8] anti-inflammatory,[9] immunomodulatory, anticholesterol,[10] antimicrobial, antihypertensive,[11] cognitive enhancer, and detoxifying agents. Conventionally, it has been used in treating diabetes, leprosy, hepatitis, respiratory and urinary tract infections, infected ulcers, arthritis, cystitis, syphilis, diarrhea, scrobutism, and obesity.[12] In addition, the flowers and infusion of dried seeds are reported to have contraceptive and galactogog properties, respectively.[12]

Hordenine is an important alkaloid present in high concentrations, particularly in the roots of germinated seeds of *H. vulgare,*[13] bitter orange, and peyote cactus.[14] Hordenine is absent in the seeds of barley, and its concentration increases as the seedlings grow. The concentration of hordenine is maximum during germination from days 3 to 12, and it declines as the plant grows.[13] The pharmacological effects reported for hordenine include anti-melanogenic, anticancer,[15] antibacterial, tachycardia, increased intestinal motility, increased respiratory rate, and increased hyperhidrosis.[16]

With these backgrounds, the current study was designed to investigate the anticancer effect of methanol extract of germinated seeds of *H. vulgare* (MGHV) against TNBC cells, quantify hordenine in MGHV, and derive the probable target of the extract and hordenine to induce apoptosis as no studies are reported till date showing the pharmacological effect of *H. vulgare* or hordenine from germinated seeds of barley in TNBC.

MATERIALS AND METHODS

Collection of plant sample

The seeds of *H. vulgare* were collected from Hebsur Herbal Store, Hubballi, Karnataka, India, in February–March 2018. The seeds were authenticated by Raw Material Herbarium and Museum, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, with authentication number NISCAIR/RHMD/CONSULT/2018/3232-33, and a voucher specimen with accession number HERB/VPT/CVASMTY/5/2019 was deposited in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India.

Preparation of methanol extract of germinated seeds of *Hordeum vulgare*

The seeds of *H. vulgare* were soaked in water for 24 h followed by spreading them on a plate at a thickness of 1 cm and sprayed with water every 4 h in order to keep them moist. The seeds were harvested on the 6th day of germination and were thoroughly dried in hot air oven at 50°C for 36 h. The germinated seeds were coarsely powdered using an electric pulverizer, and the powder obtained was extracted using a Soxhlet apparatus with methanol at 67°C. The methanol extract was then concentrated using a rotary vacuum evaporator under reduced pressure and temperature (40°C). MGHV was kept under refrigeration in an airtight container after complete evaporation of the solvent until further use.

Chemicals

Acridine orange, doxorubicin, Dulbecco's phosphate-buffered saline, ethidium bromide (EB), Hoechst 33258 stain, JC-1 stain, monoclonal anti-Bcl-2 antibody, monoclonal anti-β-actin antibody, standard hordenine, thiazolyl blue tetrazolium, and CelLytic^{M} were purchased from Sigma-Aldrich Limited, India. Antibiotic-antimycotic solution (×100), fetal bovine serum (FBS), and Rosewell Park Memorial Institute (RPMI) 1640 media were procured from Gibco ThermoFishcer India. Monoclonal anti-Caspase-8 antibody (MA141280) and 0.25% Trypsin-ethylenediamine tetraacetic acid (EDTA) were purchased from ThermoFischer, India. Agarose-low melting and bovine serum albumin (BSA) were procured from HiMedia Laboratories, India. Horseradish peroxidase-conjugated secondary anti-mouse antibody was procured from Abcam, Cambridge, MA, USA.

Cell lines

Authenticated adherent human breast adenocarcinoma cell line, MDA-MB-231, procured from NCCS (National Centre for Cell Science), Pune, Maharashtra, was used for *in vitro* anticancer studies. Cells were cultured in RPMI-1640 media supplemented with 10% FBS, 1% antibiotic-antimycotic solution containing penicillin-streptomycin, and amphotericin B. The cells were maintained in a humidified incubator at 37°C with 5% Carbondioxide (CO_2). Cell lines were subcultured by enzymatic digestion with 1% trypsin/1 mM EDTA solution when they reached approximately 70%–80% confluency, and these trypsinized cells were used for the studies.

High-performance liquid chromatography for detection of hordenine and its quantification in the methanol extract of germinated seeds of *Hordeum vulgare*

The UltiMate™ 3000 Rapid Separation Ultra high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, USA) equipped with LPG-3400RS pump with inbuilt vacuum degasser, a Rheodyne manual loop injector 20 µl, a thermostatted column compartment TCC-3000 RS (5°C to 110°C), a diode array detector (DAD)-3000RS (deuterium and tungsten light source), and a Chromeleon™ CDS version 6 software (Thermo Electron North America LLC, FL, USA) for data analysis were used.

HPLC technique for the quantitative detection of hordenine in MGHV was carried out using the method proposed by Viana *et al.* with slight modifications.[17] The mobile phase for detection of hordenine consisted of acetonitrile and 0.1% orthophosphoric acid in the ratio of 30:70. Orthophosphoric acid of concentration 0.1% was prepared by dissolving 350 µL in 349.65 mL of Milli-Q distilled water. The pH was adjusted

to 2.5 with triethylamine. The mobile phases were combined in the above-mentioned ratio and degassed using ultrasonication followed by filtration through 0.22 µm Whatman™ qualitative filter paper using filtration assembly.

The standard stock solution was prepared in HPLC grade methanol to attain a final concentration of one µg/mL and stored in amber-colored glassware wrapped in aluminum foil for protection from light. The stock solutions were stored in deep freezer at −20°C.

The linearity of the detector response was verified with hordenine standard solutions at different concentration levels. Working standard solutions were prepared by diluting the stock solution with methanol to obtain concentrations of 10, 12.5, 25, 50, and 100 µg/mL. Twenty microliters of these solutions was injected into HPLC system for analysis. A standard calibration curve was prepared by plotting peak area against standard concentrations. Least square linear regression analysis was performed to check the linearity of the data. The linear regression equation, coefficient of determination (R^2), and quantification of hordenine were determined from the calibration curve using GraphPad Prism Version 5 (GraphStats, Bengaluru, Karnataka, India). The mobile phase containing acetonitrile and 0.1% orthophosphoric acid (30:70) was used for the analysis of plant extract. The isocratic elution flow rate was fixed at 1 ml/min with Acclaim™ C_{18} column with 4.6 mm × 250 mm size, 120Å pore size, and 5 µm internal diameter column with column temperature set to 23°C. Hordenine was detected using a DAD at 220 nm. The total run time was 5 min [Table 1].

Sample preparation

The plant extract was solubilized in dimethylsulfoxide (DMSO) at a concentration of 20 mg/mL; this stock solution was further diluted with RPMI media to required concentrations.

Cytotoxicity studies using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The cytotoxic changes produced by MGHV in MDA-MB-231 cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as per Riss *et al.*[18] The MDA-MB-231 cells were seeded at a density of 5000 cells per well in 200 µL medium and were left overnight for attachment in a CO_2 incubator. Cells were treated with MGHV separately at concentrations of 2.5, 5, 10, 20, 40, 80, 160, and 320 µg/mL for a period of 24 h. After treatment with plant extract, the media was removed and 20 µL of MTT (5 mg/mL) in 150 µL medium was added and incubated at 37°C for 4 h. The media containing MTT was removed, and the formed purple formazan crystals were dissolved in 200 µL of DMSO and read at 570 nm in an ELISA plate reader (Varioskan Flash, Thermo Fischer Scientific, Finland). The percent cell inhibition was calculated, and inhibitory concentration 50 (IC_{50}) value was calculated by plotting the concentration against per cent cell inhibition using GraphPad prism version 5.

Table 1: High-performance liquid chromatography conditions for detection of hordenine in methanol extract of germinated seeds of *Hordeum vulgare*

Parameters	Specifications
Column	Acclaim™ C_{18} column with 4.6 × 250 mm size, 120 Å pore size and 5 µm internal diameter
Mobile phase	Acetonitrile:0.1% orthophosphoric acid (30:70)
Flow rate	1 ml/min
Detection wavelength	220 nm
Run time	5 min
Column temperature	23°C

Morphological assessment of cells treated with hordenine

Morphological alterations produced by hordenine and MGHV were assessed in MDA-MB-231 cells after 24 h of growth by treating the cells seeded at a density of 1×10^5 cells in a 6-well plate. The cells were incubated with hordenine and MGHV at a concentration of IC_{50} value of MGHV for 24 h followed by microscopic observation at ×40 magnification using phase-contrast inverted microscope. The morphological changes in the standard treated cells were compared with that of the extract treated cells.

Acridine Orange/Ethidium Bromide dual staining

The MDA-MB-231 cells were seeded in a 6-well plate at a density of 1×10^5 cells and allowed to grow for 24 h. The IC_{50} concentration of MGHV was treated for 24 h to differentiate the live, apoptotic, and necrotic cells. The spent media was discarded from the treated wells, and 200 µL of acridine orange (AO) (10 µg/mL)/EB (10 µg/mL) was added and analyzed under Trinocular Research Fluorescence Microscope (Axio Vert. A1 FL-LED, Carl Zeiss, Jena, Germany) with blue excitation (488 nm) and emission (550 nm) filters at ×20 and ×40 magnification.[19] Doxorubicin was used as positive control.

Analysis of morphological changes in the nucleus

The nuclear morphological changes produced by MGHV were assessed using Hoechst 33258 staining using the modified method of Chaudhary *et al.*[20] The MDA-MB-231 cells were seeded in 6-well plates at a density of 1×10^5 cells per well and allowed to grow for 24 h followed by which the cells were treated with IC_{50} concentration of MGHV and doxorubicin as positive control, for 24 h. Hoechst 33258 staining was performed by adding 200 µL of Hoechst 33258 stain (0.5 µg/mL) for 30 min in the dark and images were captured by Trinocular Research Fluorescence Microscope (DM 2000 LED, Leica, Germany) with filters having blue excitation (352 nm) and emission (461 nm) at ×20 and ×40 magnification.[20]

Analysis of mitochondrial membrane potential

MDA-MB-231 cells were plated at a concentration of 1×10^5 cells per well in 6-well plates and were allowed to grow for 24 h. After 24 h of treatment with MGHV at IC_{50} concentration and doxorubicin at 0.58 µg/mL concentration, the cells were incubated with 5 µM fluoroprobe, 5, 5', 6, 6'-tetrachloro-1,1', and 3,3'-tetra ethyl benzimidazole-carbocyanine iodide (JC-1) stain for 30 min at room temperature in the dark. The cells were analyzed using a trinocular fluorescent microscope with filters having blue excitation/emission of 540/570 nm and red excitation/emission of 590/610 nm (Axio Vert. A1 FL-LED, Carl Zeiss) at ×40 magnification.[21]

Measurement of deoxyribo nucleic acid damage by using comet assay

MDA-MB-231 cells were seeded at a density of 1×10^5 cells in a 6-well plate and were treated with IC_{50} concentration of MGHV for 24 h. The cells were then trypsinized and used for the basic alkaline comet assay as proposed by Nandhakumar *et al.*[22] Coating of slides with normal melting point agarose followed by double coating of low melting point agarose containing approximately 2000 cells were lysed in ice-cold lysis buffer. This was followed by subjecting the slides to electrophoresis. After electrophoresis, the slides were then treated with neutralization buffer and were allowed to dry and stained with EB (20 µg/mL) and analyzed within 1 to 2 h at ×20 magnifications on a Trinocular Research fluorescence Microscope (DM 2000 LED, Leica, Germany). Images of 100 randomly selected untreated MDA-MB-231

cells, MGHV-treated cells, and doxorubicin-treated cells were analyzed from each sample and the distribution of deoxyribo nucleic acid (DNA) between tail and head of the comet was used to evaluate the degree of DNA damage. The quantification of the DNA strand breaks of the stored images was done using comet assay software project lab (CASP). Using this software, comet length, per cent DNA in tail, tail length (TL), tail moment, and olive tail moment (OTM) were observed.

Western blot

Western blot analysis was performed according to the method of Shrivasthava *et al.*[23] MDA-MB-231 cells were treated with IC_{50} concentrations of MGHV for 24 h and were trypsinized followed by lysing the cells using CelLytic^{M}. Cell lysates were then centrifuged and supernatant containing proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis for protein separation. The separated proteins on the gel were transferred to polyvinylidene fluoride membrane (0.45 µm) using Hoefer semi-dry transfer apparatus. The membrane was initially blocked with 5% BSA followed by treating the membrane with respective primary antibodies overnight at 4°C. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody for one hour. Color reaction was carried out with 3, 3'-diaminobenzidine substrate buffer. To ensure equal protein loading, β-actin was used as an internal control. The strength of western blotting bands was determined by Image J density Measurement program (http://imagej.en.softonic.com).

In silico analysis

The ligand hordenine and the receptor Caspase-8 were prepared using PyMol software (pymol.org). The binding affinity studies of hordenine against Caspase-8 were done using Autodock vina.[24] The docking was done using Discovery Studio 2016 Client.

Statistical analysis

All results were expressed as mean ± standard error with "n" equal to the number of replicates. All the statistical analyses were conducted using SPSS software version 24 (IBM Corp., Armonk, N.Y., USA). Analysis of variance in a completely randomized design followed by Duncan's multiple range tests was used to compare any significant differences among various concentrations of the extract. The same technique was used for comparing any significant difference among various parameters of the comet assay and also to compare significant difference among normalized data of band intensity in Western blotting.

RESULTS

High-performance liquid chromatography for detection of hordenine and its quantification in methanol extract of germinated seeds of *Hordeum vulgare*

The results of chromatogram containing peak area, retention time, and spectra of hordenine and sample are depicted in Figure 1a and b, respectively. The study yielded hordenine with the retention time of 3.4 min detected by DAD at 220 nm. The standard calibration curve obtained by plotting different concentrations of standard hordenine of 10, 12.5, 25, 50, and 100 µg/mL against average areas is shown in Figure 1c. Simple linear regression analysis performed on the data revealed a coefficient of determination of 0.9982 [Table 2.]. A concentration of 48.29 µg/mL (4.82%) of hordenine was present in 1 mg/mL of MGHV.

Cytotoxicity studies using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The percent inhibition of cell proliferation as studied by MTT assay post 24 h of treatment with MGHV in MDA-MB-231 cell line is presented in Table 3 and Figure 2. The extract showed concentration-dependent cytotoxicity with IC_{50} value of 41.28 ± 0.30 µg/mL. The highest concentration of 320 µg/mL inhibited 55.07% ± 1.52% of cells.

Morphological assessment of cells treated hordenine

The results of morphological assessment of MDA-MB-231 cells treated with hordenine and MGHV are depicted in Figure 3. The results revealed that hordenine and MGHV showed cytotoxic alterations such as reduction in cell population, cell shrinkage, cell vacuolization, altered cell shape, and apoptotic body formation. The cytotoxic alterations in morphology produced by hordenine and MGHV treated cells at 41.28 ± 0.30 µg/mL for 24 h were similar indicative of apoptotic potential of hordenine in MGHV.

Acridine orange/ethidium bromide dual staining

The results of apoptotic stages analyzed by AO/EB staining upon treatment of cells with MGHV and doxorubicin are depicted in the Figure 4. Control cells emitted uniform green fluorescence with circular nucleus at the center, whereas MGHV (41.28 ± 0.30 µg/mL) treated cells showed early apoptosis characterized by yellow-green fluorescence and crescent shaped nucleus. The doxorubicin-treated cells exhibited late apoptosis emitting orange to red fluorescence. Cellular changes such as fragmentation, condensation, and marginalization of nucleus were also noticed in cells treated with MGHV.

Analysis of morphological changes in nucleus

The nuclear morphological changes analyzed by Hoechst 33258 staining upon treatment of cells with MGHV and doxorubicin are shown in the Figure 5. Control cells emitted uniform blue fluorescence, whereas cells

Table 2: Parameters of linearity for hordenine detected using high-performance liquid chromatography with acetonitrile and 0.1% orthophosphoric acid as mobile phase

Parameter	Values for hordenine
Retention time (min)	3.4
Linear regression equation	Y=0.2582× − 0.2321
Regression coefficient (R^2)	0.9979
Linear range (µg/mL)	10-100

Table 3: The per cent cell viability of Monroe Dunaway Anderson-metastatic breast-231 cells after 24 h treatment with methanol extract of germinated seeds of *Hordeum vulgare*

Concentrations (µg/mL)	Percentage inhibition
2.5	33.56±0.96^{a}
5	37.21±0.23^{b}
10	37.46±0.73^{b}
20	44.10±1.40^{c}
40	42.94±0.40^{c,d}
80	46.17±0.36^{c,d}
160	47.81±2.54^{d}
320	55.07±1.52^{e}
IC_{50} (µg/mL)	41.28±0.30

Values are expressed as mean±SE (n=6). Means bearing the different superscript (a-e) vary significantly at P<0.05. SE: Standard error

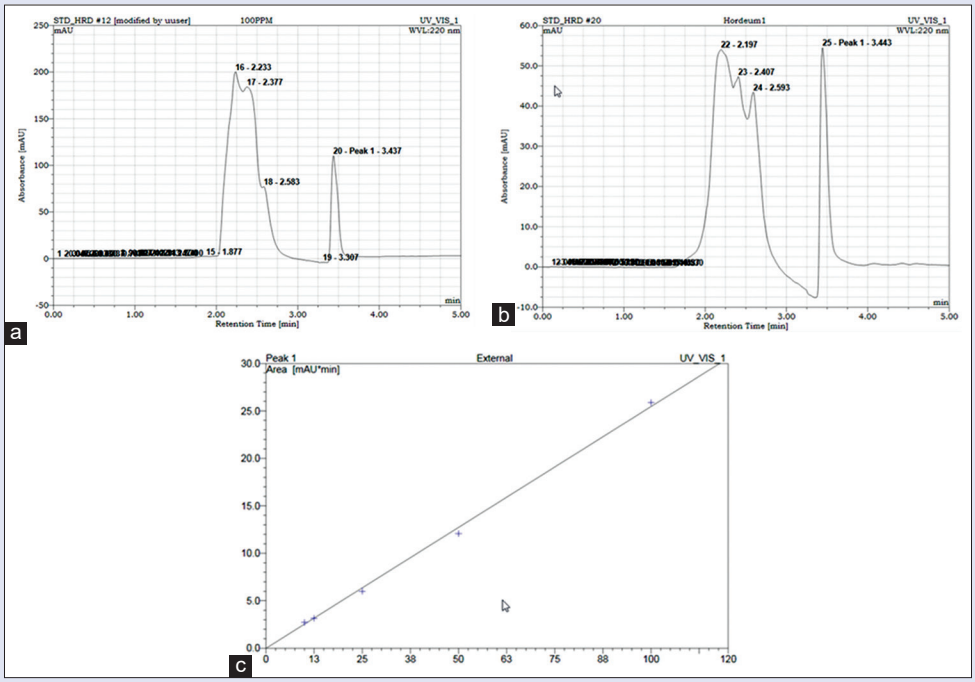


Figure 1: (a) High-performance liquid chromatography chromatogram of standard hordenine at 100 µg/mL concentration. (b) High-performance liquid chromatography chromatogram of MGHV at 1 mg/mL concentration. (c) Calibration curve of different standard hordenine concentrations

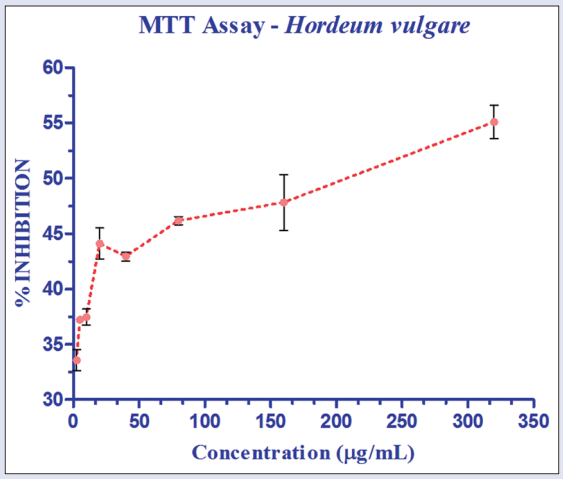


Figure 2: Graph showing the results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (% inhibition versus concentration [µg/mL])

treated with doxorubicin and MGHV showed bright blue fluorescence with apoptotic features such as fragmentation and marginalization of nuclei.

Analysis of mitochondrial membrane potential

The results of MMP evaluated by JC-1 staining upon treatment of cells with MGHV and doxorubicin are shown in Figure 6. Cells treated with MGHV showed a combination of cells with red and green fluorescence indicating a partial low-grade effect on mitochondrial membrane potential, whereas those treated with doxorubicin at a concentration of 0.58 µg/mL showed complete green fluorescence indicating a potential drop in mitochondrial membrane potential.

Measurement of deoxyribo nucleic acid damage using comet assay

The comet assay results are shown in Figure 7. The cells treated with MGHV did not show comets when compared with control cells. Doxorubicin-treated cells showed comets with very long tail. The mean values of tail DNA per cent, TL, tail moment, and OTM were estimated and given in Table 4. All the parameters of comet assay in doxorubicin treated cells were significantly higher when compared with MGHV-treated and control cells. No significant difference was noticed between MGHV-treated and control cells.

Western blot

The relative expressions of Bcl-2 and Caspase-8 in MDA-MB-231 cells on treatment with MGHV and doxorubicin were compared with control cells and the results are presented in Table 5 and Figure 8. Bcl-2 expression was significantly ($P < 0.01$) downregulated by 0.67 ± 0.05 folds in doxorubicin-treated cells, whereas no significant difference was observed in MGHV-treated and control cells. Caspase-8 expression was significantly ($P < 0.01$) upregulated by 2.52 ± 0.30 and 3.77 ± 0.38 folds in MGHV and doxorubicin treated cells, respectively.

In silico analysis

The binding affinity studies conducted revealed that hordenine showed binding energy of 4.9 kcal/mol against Caspase-8. The results are presented in Figure 9.

DISCUSSION

Hordenine, an alkaloid, present particularly in the germinated seeds of *H. vulgare* is highest during its germination from days 3 to 9.[13] Hence, germinated seeds of day 6 was harvested and used for the study. The HPLC analysis performed to detect and quantify hordenine in the extract yielded 4.82% of hordenine in 1 mg/mL of MGHV. Hoult and Lovett obtained limit of detection of 2.5 µg/mL of hordenine

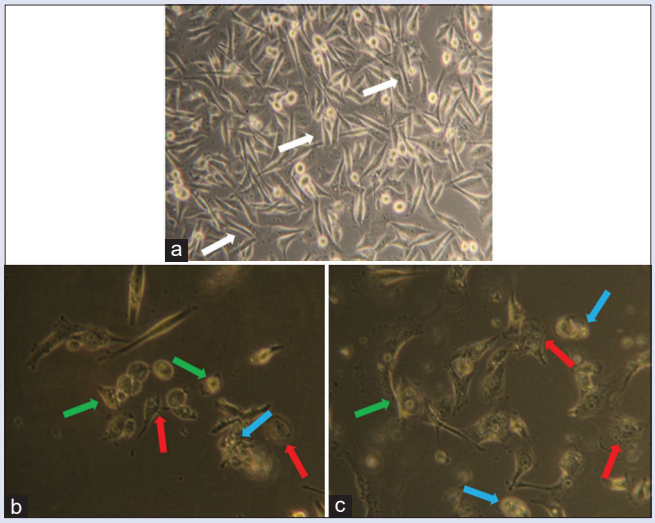


Figure 3: Morphological assessment of cells treated with hordenine and methanol extract of germinated seeds of *H. vulgare* (MGHV). (a) Control cells; (b) Cells treated with hordenine; (c) Cells treated with MGHV; White arrow – Normal cells; Blue arrow– Cell vacuolization; Green arrow – Altered cell morphology; Red arrow – Apoptotic body formation

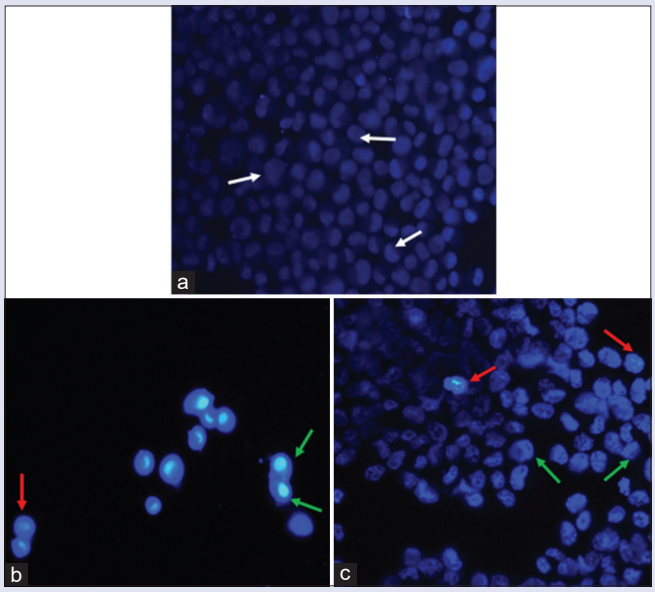


Figure 5: Morphological changes in MDA-MB-231 cells by Hoechst staining, ×40. (a) Control cells; (b) Cells treated with doxorubicin at 0.58 µg/mL; (c) Cells treated with MGHV inhibitory concentration 50 concentration; White arrow – Live cells, Red arrow – Fragmentation of nuclei; Green arrow– Marginalization of nucleus

in germinated seeds of barley with retention time of 2.93 min and regression coefficient of 0.9994.[25] Hence, Nelson *et al.* quantified the presence of hordenine in bitter orange fruit, its extract, and solid dosage form by LC-MS/MS technique and found that the hordenine was present in the concentration of 12.2, 19.1, and 5.9 mg/kg (w/w), respectively.[26] Hordenine showed anticancer effect against mammalian ovarian cancer cell line, A2780.[15] Thus, from the results of HPLC analysis, it could be inferred that the presence of hordenine in MGHV along with other phytochemicals could contribute to the anticancer property of MGHV.

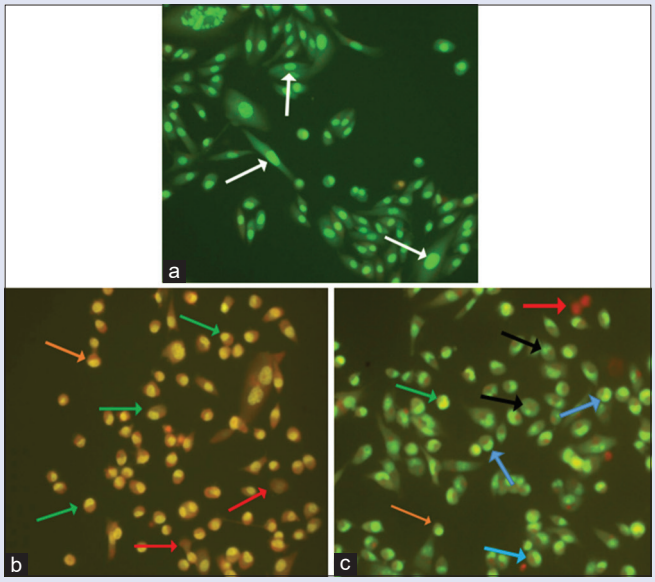


Figure 4: Morphological changes of MDA-MB-231 cells by acridine orange/ethidium bromide staining, ×20. (a) Control cells; (b) Cells treated with doxorubicin at 0.58 µg/mL; (c) Cells treated with MGHV inhibitory concentration 50 concentration; White arrow – Normal cells; Blue arrow – Early apoptotic cells; Green arrow – Late apoptotic cells; Red arrow – Necrotic cells; Black arrows – Apoptotic body formation; Orange arrow – Nuclear condensation and marginalization; Black – Nuclear fragmentation

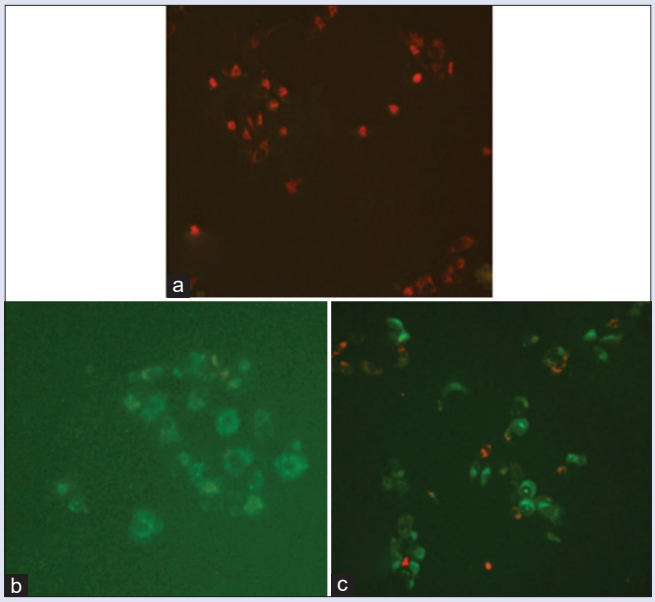


Figure 6: Morphological changes in MDA-MB-231 cells by JC-1 staining, ×40. (a) Control cells; (b) Cells treated with doxorubicin at 0.58 µg/mL; (c) Cells treated with MGHV inhibitory concentration 50 concentration

The MTT assay is used to assess the metabolic activity to investigate the cytotoxic potential of any chemicals on the cells. In normal live cells, the nicotinamide adenine dinucleotide phosphate-dependent cellular oxidoreductase enzyme are effective in reducing the yellow-colored tetrazolium dye MTT to insoluble purple formazan crystals which can be solubilized by DMSO. When there is cytotoxicity, decrease in the number of cells causes decreased purple formazan development

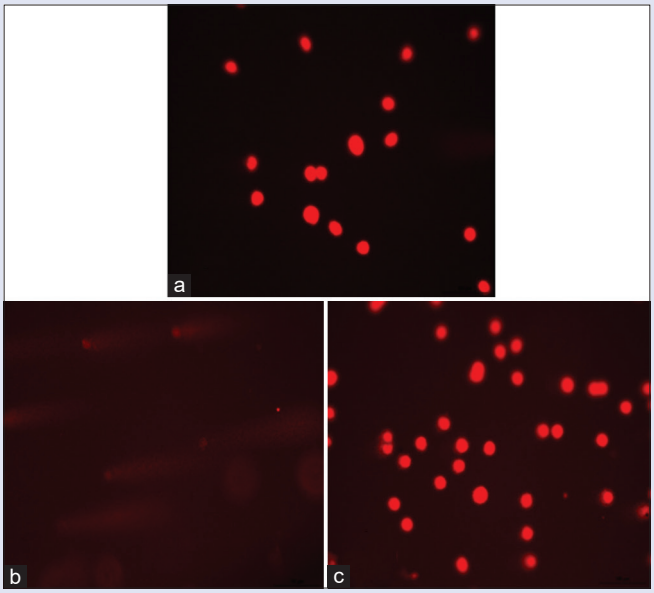


Figure 7: Assessment of deoxyribo nucleic acid damage in MDA-MB-231 by comet assay. (a) Control cells; (b) Cells treated with doxorubicin at 0.58 µg/mL; (c) Cells treated with MGHV inhibitory concentration 50 concentration

Table 4: Comet parameters of Monroe Dunaway Anderson-metastatic breast-231 cells treated with methanol extract of germinated seeds of *Hordeum vulgare* and doxorubicin

	Tail DNA (%)	Tail length (µm)	Tail moment	Olive tail moment
Control	3.6±0.27[a]	0.09±0.04[a]	0.003±0.001[a]	0.05±0.03[a]
MGHV	4.15±0.59[a]	0.11±0.06[a]	0.01±0.006[a]	0.08±0.04[a]
Doxorubicin	68.48±2.32[b]	444.30±27.06[b]	313.91±27.65[b]	145.75±14.91[b]

Values are expressed as mean±SE (n=20). Means bearing the different superscript (a-b) vary significantly at P<0.01. SE: Standard error; MGHV: Methanol extract of germinated seeds of *Hordeum vulgare*

Table 5: Relative expression of Bcl-2 and caspase-8 in Monroe Dunaway Anderson-metastatic breast-231 cells treated with methanol extract of germinated seeds of *Hordeum vulgare* and doxorubicin

Treatment group	Normalized protein levels	
	Bcl-2	Caspase-8
Control cells	1	1
MGHV	0.91±0.08	2.52±0.30**
Doxorubicin	0.67±0.05**	3.77±0.38**

Values are expressed as mean±SE (n=3); **Significant (P<0.01) difference compared with control, SE: Standard error; MGHV: Methanol extract of germinated seeds of *Hordeum vulgare*

whose absorbance is measured by an ELISA plate reader.[27] The extract produced a concentration dependent inhibition of MDA-MB-231 cells with IC_{50} value of 41.28 ± 0.30 µg/mL. The highest concentration of 320 µg/mL produced 55.07% ± 1.52% of cells. Woo *et al.* discovered anticancer potential of barley grass extract on MDA-MB-231 cells by MTT assay and found significant concentration-dependent decrease in cell viability with IC_{50} value of 100 µg/mL.[28] The findings of the present study revealed that MGHV could be grouped as cytotoxic anticancer agent for TNBC. As MTT is a preliminary assay that provide results only based on the viability of the cells, the mechanism

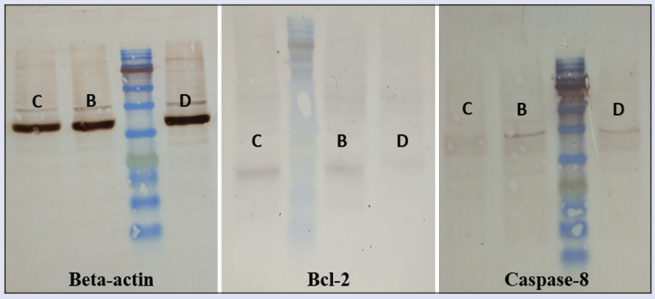


Figure 8: Western blot images of β-actin, Bcl-2, and caspase-8 proteins in MDA-MB-231 cells. (C) Control cells; (B) Cells treated with MGHV at its inhibitory concentration 50 concentration; (D) Doxorubicin-treated cells at their inhibitory concentration 50 concentration

of cell death and the stage of apoptosis has to be analyzed using various other assays.

Studies performed by Bahçeevli *et al.* revealed anticancer activity of hordenine against A2780 cell line.[15] Hence, in order to assess the anticancer potential of hordenine in MDA-MB-231 cells, morphological assessment was made by treating the cells with standard hordenine at 41.28 ± 0.30 µg/mL (IC_{50} concentration of MGHV), and the results of the morphological analysis showed cytotoxic changes similar to that of the extract. From this, it can be substantiated that hordenine in MGHV could be one of the contributing phytochemical toward the cytotoxic activity of plant extract.

AO/EB staining is a technique for identification of stage of apoptotic cells and necrotic cells. AO dye diffuses into all the cells and binds to DNA emitting green fluorescence whereas EB enters only those cells with compromised cell integrity as seen in late apoptotic and necrotic cells.[29] Early apoptotic cells are characterized by granular, yellow-green, crescent-shaped marginalized nucleus, while late apoptotic cells has concentrated or asymmetrically localized nucleus that stains orange in color. Necrotic cells are distinguished by increased cell volume in disintegrating manner and uneven orange red fluorescence at periphery.[19] The MDA-MB-231 cells treated with MGHV showed early apoptotic changes characterized by yellow-orange fluorescence and crescent-shaped nucleus. Doxorubicin was used as a positive control which showed late apoptotic changes (secondary necrotic changes) characterized by orange to red fluorescence. The AO binds to condensed or fragmented chromatin in early apoptotic cells, producing yellow green fluorescence. The integrity of cell membrane of late apoptotic cells is compromised because of which EB enters the cells and binds to concentrated DNA and apoptotic bodies producing orange to red fluorescence.

Hoechst 33258 staining involves binding of nonintercalating Hoechst dye to A-T rich regions of minor groove of double-stranded DNA, producing blue fluorescence when excited by ultraviolet light (~360 nm). In normal cells, the dye is distributed evenly producing uniform blue fluorescence whereas in apoptotic cells, due to chromatin condensation and nuclear marginalization, the dye is concentrated producing intense bright blue fluorescence. The extracted treated cells showed nuclear morphological changes such as nuclear fragmentation and marginalization that are indicative of apoptosis which can be correlated with the results of AO/EB staining.

JC-1 staining is performed to analyze the mitochondrial membrane potential (MMP) of the cell. JC-1 stain being a cationic dye particularly enters the mitochondria in normal cells due to high membrane potential and produces intense red fluorescence as they forms complexes known as J-aggregates. In apoptotic cells, there are alterations in electron transport and release of cytochrome c in mitochondria leading to

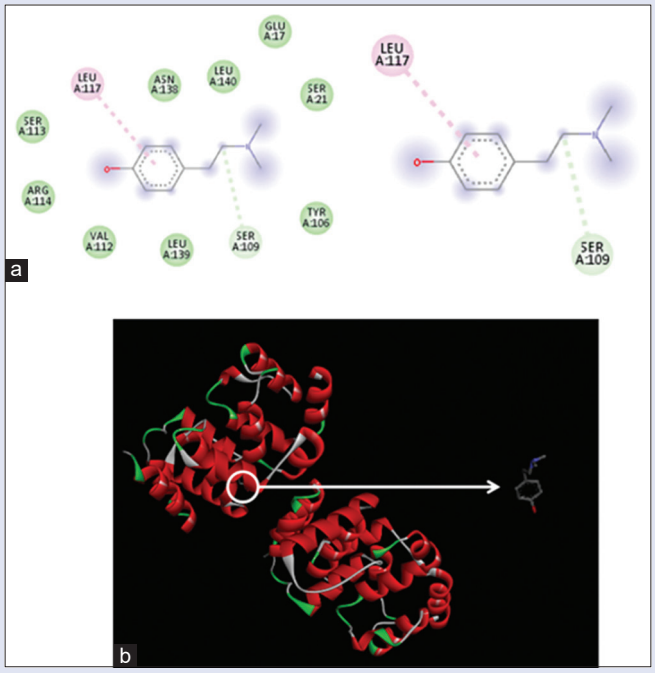


Figure 9: *In silico* analysis of binding of hordenine with Caspase-8. (a) Chemical structure of hordenine molecule and its possible binding sites with caspase-8 molecule; (b) three-dimensioanl structure of caspase-8 and position and binding of hordenine molecule to caspase-8

depolarization of mitochondrial membrane causing DNA fragmentation and nuclear chromatin condensation ultimately leading to decreased MMP. This decrease in MMP causes the JC-1 dye to leak out from the mitochondria into the cytosol where it remains in its monomeric form producing green fluorescence.[30] Studies performed by Robles-Escajeda *et al*. on Nalm-6 cells after incubation with green barley powder for 4 h, found that green barley powder induced cytotoxicity was independent of the formation of J-aggregates indicating intact mitochondria and an apoptotic mechanism not mediated by the intrinsic pathway.[7] However, in the present study, combination of green and red fluorescence could be indicative of low-grade effect of the extract on MMP, which could possibly be due to the presence of phytochemicals that were produced during germination. The other reason could be that disruption of MMP might have occurred during the final stages of apoptosis that might have occurred as a result of DNA fragmentation and ATP depletion.

Comet assay is a popular technique to identify single- and double-stranded breaks in DNA at individual cell level. The parameters such as TL of comet indicate the extent of DNA damage. Thus, increase in TL of the comets indicates that there is frequent strand breakage, leading to formation of several small molecules of DNA. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet TL) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail). OTM is the product of the distance between the center of gravity of the head and the center of gravity of the tail and percentage DNA in the tail.[31] The results of the comet assay showed absence of comets in cells treated with MGHV but slight insignificant increase in the values of comet assay parameters were noticed when compared to negative control. This could be confirmed by increasing the duration of incubation of cells treated with extract (more than 24 h) to observe if the duration of incubation is associated with the DNA damage. Similar results were obtained by Ghavami *et al.* who studied the effect of barley β-glucan on radiation damage in human hepatoma cell line, HepG2 where they found that the cells pretreated with 1 μg/mL of barley β-glucan upon radiation treatment at six Gy X-rays did not produce DNA damage in HepG2 cells.[32] The extract did not cause DNA damage in MDA-MB-231 cells. The extract-induced DNA damage is selective for each cell line which has been described in the studies performed by Robles-Escajeda *et al.* using green barley extract.[7] The authors found the selective DNA damaging action of green barley extract on BJAB cells but not on Nalm-6 cells. This selective action was mainly attributed to disrupted cell-cycle progression causing increment in sub-G0/G1 phase and G2/M phase arrest finally leading to DNA fragmentation.

An important anti-apoptotic protein known as Bcl-2 belonging to the family of proteins called Bcl-2 proteins is associated with intrinsic pathway of apoptosis. The Bcl-2 protein family is made of two groups, namely, pro-apoptotic proteins and anti-apoptotic proteins. The anti-apoptotic proteins inhibit apoptosis by blocking the mitochondrial release of cytochrome-c. Therefore, increased expression of anti-apoptotic protein such as Bcl-2 contributes to the development of cancer by inhibiting apoptosis. The Bcl-2 expression was significantly ($P < 0.01$) downregulated by 0.67 ± 0.05 folds in doxorubicin-treated cells, whereas no significant difference was observed in MGHV-treated and control cells. The partial disruption of mitochondrial membrane potential produced by MGHV could be suggestive of involvement of other Bcl-2 related anti-apoptotic proteins like Bcl-X, Bcl-B, Bcl-W, and myeloid cell leukemia 1. The results of the present study did not show evidence of involvement of intrinsic pathway in MGHV-induced apoptosis, which is in agreement with the results obtained by Robles-Escajeda *et al*.[7] The extrinsic pathway of apoptosis is mediated by Caspase-8 and is triggered by activation of death receptor complex, tumor necrosis factor (TNF) alpha-TNF receptor (TNF-α-TNF-R). Caspase-8 is a cysteine-aspartic acid protease protein encoded by *CASP8*. Initially, caspases are present as proenzymes and the activation of caspases occurs by first apoptotic signal and external apoptotic stimuli induced by dimerization and cleavage of internal aspartic residues thereby forming heterodimeric enzyme consisting of large and small protease subunits. The activated caspase-8 initiates apoptosis directly by cleaving and activating executioner caspases such as caspase 3, 6 and 7.[33] The results revealed significant ($P < 0.01$) upregulation of caspase-8 by 2.52 ± 0.30 and 3.77 ± 0.38 folds in MGHV and doxorubicin treated cells respectively. The results obtained by Woo *et al.* (2018) who experimented the effect of barley extract on MDA-MB-231 and DU145 cells reported that the extract at 100 μg/mL induced activation of Caspase-8 and cleavage of PARP as visualized by western blotting.[25] Robles-Escajeda *et al*. (2013) revealed aqueous extract of green barley powder activated caspase-8 in Nalm-6 cells as early as 4 h treatment.[7]

The results of *in silico* analysis showed that hordenine had binding energy of 4.9 kcal/mol against Caspase-8. So far, this is the first report of interaction of hordenine with caspase-8. *In silico* study performed by Bucur *et al*. had revealed that CaspPro, a caspase-8 activator bound to Caspase-8, potentiated TRAIL-induced activation of Caspase-8.[34] The activated Caspase-8 initiated apoptosis directly by cleaving and activating executioner caspases such as caspase-3, 6, and 7.[33] Thus, the findings of the Western blot analysis and *in silico* analysis confirmed that the extract activated caspase-8 and produced cell death by extrinsic pathway of apoptosis.

CONCLUSION

The present study revealed that the anticancer activity of MGHV in TNBC cells was due to the cytotoxic alteration produced by triggering the extrinsic pathway of apoptosis, without causing DNA damage. Because hordenine is abundantly found in the germinated seeds and 4.82% of hordenine had been detected in MGHV, it could be assumed

that the anticancer mechanism on MDA-MB-231 cells could be due to hordenine. Hordenine also showed binding affinity toward caspase-8. Further in-depth studies for the anticancer property of hordenine for the treatment of TNBC are warranted.

Financial support and sponsorship

The authors acknowledge the Kerala Veterinary and Animal Sciences University for the M. V. Sc research grant and facilities provided to the first author. The authors also acknowledge Government of Kerala for providing the financial assistance as Kerala Government State Plan Fund RSP/18-19/VII-6.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L. Triple-negative breast cancer: Challenges and opportunities of a heterogeneous disease. Nature Rev Clin Oncol 2016;13:674-90.
2. Beavis PA, Divisekera U, Paget C, Chow MT, John LB, Devaud C, *et al.* Blockade of A2A receptors potently suppress the metastasis of CD73+tumors. Proc Natl Acad Sci USA 2013;110:14711-6.
3. Wee ZN, Yatim SM, Kohlbauer VK, Feng M, Goh JY, Bao Y, *et al.* IRAK1 is a therapeutic target that drives breast cancer metastasis and resistance to paclitaxel. Nature Comm 2015;6:1-16.
4. Oudin MJ, Barbier L, Schäfer C, Kosciuk T, Miller MA, Han S, *et al.* MENA confers resistance to paclitaxel in triple-negative breast cancer. Mol Cancer Ther 2017;16:143-55.
5. Anwar F, Qayyum HM, Hussain AI, Iqbal S. Antioxidant activity of 100% and 80% methanol extracts from barley seeds (*Hordeum vulgare* L.): Stabilization of sunflower oil. Grasas y Aceites 2010;61:237-43.
6. Czerwonka A, Kawka K, Cykier K, Lemieszek MK, Rzeski W. Evaluation of anticancer activity of water and juice extracts of young *Hordeum vulgare* in human cancer cell lines HT-29 and A549. Ann Agric Environ Med 2017;24:345-9.
7. Robles-Escajeda E, Lerma D, Nyakeriga AM, Ross JA, Kirken RA, Aguilera RJ, *et al.* Searching in mother nature for anti-cancer activity: Anti-proliferative and pro-apoptotic effect elicited by green barley on leukemia/lymphoma cells. PLoS One 2013;8:e73508.
8. Minaiyan M, Ghannadi A, Movahedian A, Hakim-Elahi I. Effect of *Hordeum vulgare* L. (Barley) on blood glucose levels of normal and STZ-induced diabetic rats. Res Pharm Sci 2014;9:173-8.
9. Gul S, Ahmed S, Kifli N, Uddin QT, Tahir NB, Hussain A, *et al.* Multiple pathways are responsible for anti-inflammatory and cardiovascular activities of *Hordeum vulgare* L. J Tansl Med 2014;12:316.
10. Madhujith T, Shahidi F. Antioxidative and antiproliferative properties of selected barley (*Hordeum vulgare* L.) cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. J Agric Food Chem 2007;55:5018-24.
11. Lee NY, Kim Y, Choi I, Cho SK, Hyun JN, Choi JS, *et al.* Biological activity of barley (*Hordeum vulgare* L.) and barley by-product extracts. Food Sci Biotechnol 2010;19:785-91.
12. Singh R, Singh M, Kotecha M. Yava (*Hordeum vulgare* LINN.): A review. Int Res J Pharm 2016;7:5-9.
13. Mann JD, Steinhart CE, Mudd SH. Alkaloids and plant metabolism V. The distribution and formation of tyramine methylpherase during germination of barley. J Biol Chem 1963;238:676-81.
14. Duke AJ, Bogenschutz-Godwin MJ, Ottesen AR. Duke's Handbook of Medicinal Herbs of Latin America. Boca Raton (BR): CRC Press; 2009.
15. Bahçeevli KA, Kurucu S, Kolak U, Topcu G, Adou E, Kingston DG. Alkaloids and aromatics of *Cyathobasis F.* ruticulosa (Bunge) Aellen. J Nat Prod 2005;68:956-8.
16. Frank M, Weckman TJ, Wood T, Woods WE, Tai CL, Chang SL, *et al.* Hordenine: Pharmacology, pharmacokinetics and behavioural effects in the horse. Equine Vet J 1990;22:437-41.
17. Viana C, Zemolin GM, Lima FO, de Carvalho LM, Bottoli CB, Limberger RP. High-performance liquid chromatographic analysis of biogenic amines in pharmaceutical products containing Citrus aurantium. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2013;30:634-42.
18. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, *et al.* Cell viability assays: In: Sittampalam GS, Grossman A, Brimacombe K, Arkin M, Auld D, Austin CP, *et al.*, editors. Assay Guidance Manual. Bethesda (MD): Eli Lily and Company and the National Centre for Advancing Translational Sciences; 2004.
19. Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. BMC Biotechnol 2005;5:12.
20. Chaudhary S, Chandrashekar KS, Pai KS, Setty MM, Devkar RA, Reddy ND, *et al.* Evaluation of antioxidant and anticancer activity of extract and fractions of Nardostachys jatamansi DC in breast carcinoma. BMC Complement Altern Med 2015;15:50.
21. Ovadje P, Chatterjee S, Griffin C, Tran C, Hamm C, Pandey S. Selective induction of apoptosis through activation of caspase-8 in human leukemia cells (Jukart) by dandelion root extract. J Ethnopharmacol 2011;133:86-91.
22. Nandhakumar S, Parasuraman S, Shanmugam MM, Rao KR, Chand P, Bhat BV. Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). J Pharmacol Pharmacother 2011;2:107-12.
23. Shrivasthava S, Kulkarni P, Thummuri D, Jeengar MK, Naidu VG, Alvala M, *et al.* Piperlongumine, an alkaloid causes inhibition of PI3K/Akt/mTOR signalling axis to induce apoptosis in triple negative breast cancer cells. Apoptosis 2014;19:1148-64.
24. Trott O, Olson AJ. Auto dock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 2010;31:455-61.
25. Hoult AH, Lovett JV. Biologically active secondary metabolites ofbarley. III. A method for identification and quantification of hordenine and gramine in barley by high-performance liquid chromatography. J ChemEcol 1993;19:2245-54.
26. Nelson BC, Putzbach K, Sharpless KE, Sander LC. Mass spectrometric determination of the predominant adrenergic protoalkaloids in bitter orange (Citrus aurantium). J Agric Food Chem 2007;55:9769-75.
27. Stockert JC, Horobin RW, Colombo LL, Blazquez-Castro A. Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging and labelling perspectives. Acta Histochemical 2018;120:159-67.
28. Woo SM, Kwon SC, Ko SG, Cho SG. Barley grass extract causes apoptosis of cancer cells by increasing intracellular reactive oxygen species production. Biomed Rep 2017;6:681-5.
29. Dolai N, Islam A, Haldar PK. Antiproliferative activity and apoptosis inducing mechanism of *Anthocephalus cadamba* on Dalton's Lymphoma ascites cells. Iran J Pharm Res 2016;15:505-14.
30. Sun Y, Zong WX. Cellular apoptosis assay of breast cancer. In: Breast Cancer: Methods and Protocols. New York (USA): Humana Press; 2016. p. 139-49.
31. Kumaravel TS, Vilhar B, Faux SP, Jha AN. Comet assay measurements: A perspective. Cell Biol Toxicol 2009;25:53-64.
32. Ghavami L, Goliaei B, Taghizadeh B, Nikoofar A. Effects of barley β-glucan on radiation damage in the human hepatoma cell line HepG2. Mut Res 2014;775:1-6.
33. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 2013;7:a008656.
34. Bucur O, Gaidos G, Yatawara A, Pennarun B, Rupasinghe C, Roux J, *et al.* A novel caspase 8 selective small molecule potentiates TRAIL-induced cell death. Sci Rep 2015;5:9893.