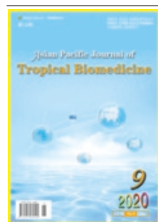

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In vitro antioxidant and anti-cancer activities and phytochemical analysis of *Commelina benghalensis* L. root extracts

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ABSTRACT

Objective: To explore antioxidant potential, anti-cancer activity, and phytochemicals of *Commelina benghalensis* L.

Methods: The roots of *Commelina benghalensis* were extracted in different solvents (methanol, ethanol, benzene, chloroform, *n*-hexane) with a range of polarity. Antioxidant activity was evaluated by reducing power assay, DPPH radical scavenging activity and phosphomolybdenum method, cytotoxicity by MTT assay, apoptotic and cell cycle analysis by flow cytometry, migratory and invasive potential by wound scratch assay and invasion assay, respectively, functional groups analysis by FT-IR spectroscopy and phytochemicals by aluminum chloride colorimetric and Folin-Ciocalteu methods.

Results: The extracts showed worthy antioxidant potential. The chloroform extract demonstrated the most significant cytotoxic effect on MDA-MB-231 (breast cancer) cell line, induced apoptosis and reduced migratory and invasive potential of MDA-MB-231 cells. Methanol and ethanol extracts presented good yield of total phenolic and total flavonoid contents. The FTIR spectroscopic studies revealed different characteristic peak values with various functional compounds such as alkenes, alkanes, aliphatic amines, aromatics, alkyl halides, carboxylic acid, alcohols, ester, aldehydes and ketones.

Conclusions: The results demonstrate the potential use of *Commelina benghalensis* as a good antioxidant with significant anti-cancer effect.

KEYWORDS: *Commelina benghalensis*; Anti-cancer; Antioxidant; FT-IR

1. Introduction

Medicinal plants are the chief source of natural products as alternatives to chemical products and offer a wealth of bioactive phytochemicals[1]. These phytochemicals have a wide array of biological activities such as antifungal, antioxidant, anticancer and antibacterial activities[2]. Free radicals generally cause DNA oxidation, degradation of protein and lipid peroxidation which are associated with several chronic diseases. Detailed researches have highlighted that medicinal plants are a rich source of antioxidants[3,4]. Flavonoids, tannins, phenolic acids, lignins and alkaloids as phenolic compounds found in plants are distinguished free radical scavengers possessing antioxidant activity[5]. Synthetic antioxidants were the main cause of liver damage and cancer in model animals[6]. When free radicals react with DNA, it results in different forms of cancer, consequently, a mutation arises that affects the regulation of the cell cycle and leads to tumor[7]. As cancer is a well-known disease in humans, scientific researchers and commercialists have shown considerable interest in exploring

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new anticancer compounds of natural origin[8]. Nowadays, the development of potential anticancer agents from plants has become an important part of cancer research to inhibit arrest or reverse the cellular and molecular processes of carcinogenesis[9].

Commelina benghalensis (*C. benghalensis*) Linn. (Commelinaceae), commonly known as Benghal dayfower or Dew fower, is a perennial herb native to tropical Africa and Asia. Traditionally, *C. benghalensis* is used to treat headache, fever, leprosy, constipation, jaundice and snake bite[10]. It is eaten by humans as a vegetable in Pakistan and used medicinally to cure inflammations of the skin, leprosy[11], cataracts, night blindness, conjunctivitis, skin diseases, mental disorders and insomnia[12]. Previously, it has been confirmed that *C. benghalensis* possessed significant antioxidant, anticancer and antitumor activities[10,13]. The plant root was also investigated in Wistar rats for protective effect against paracetamol-induced hepatic damage[14].

The objective of the current study was to explore the antioxidant potential and anti-cancer potency in tumor cells, and phytochemicals of *C. benghalensis* L.

2. Materials and methods

2.1. Collection of plants

C. benghalensis L. was harvested during summer from Balakot, Pakistan and identified and authenticated by Dr. Muhammad Zafar, taxonomist, Department of Botany, Quiad-i-Azam University, Islamabad, Pakistan. The plant with voucher number HPMPMBL-16-019 was kept in Herbarium of Plant Biochemistry and Molecular Biology Laboratory, Quaid-i-Azam University, Islamabad. Furthermore, the plant sample was carefully washed with running tap water, dried and grounded, and the powdered sample was placed in air-tight containers.

2.2. Preparation of extract

The maceration method was applied to obtain crude extracts of the plant using analytical grade solvents. The dried plant materials (25 grams) were soaked independently for 7 d in several polar and non-polar extraction solvents including ethanol (CBE), benzene (CBB), *n*-hexane (CBH), methanol (CBM) and chloroform (CBC). The soaked plant samples were filtered thrice *via* Whatman filter paper No. 1 and filtrate was subjected to evaporation in a rotary evaporator at 45 ℃ and under reduced pressure.

2.3. Phytochemical analysis

2.3.1. Determination of total phenolic contents

The Folin-Ciocalteu method was used to estimate the total phenolic content of the plant extracts[15]. Briefly, 20 μL of the extracted sample (dissolved in DMSO) was reacted with 90 μL Folin-Ciocalteu reagent followed by incubation for 5 min at room temperature. Subsequently, 7.5% of sodium carbonate solution (90 μL) was mixed with reaction mixture and absorbance was measured at 630 nm by a microplate reader (Biotek). The resultant data was expressed as microgram gallic acid equivalent per mg of the extracted sample.

2.3.2. Determination of total flavonoid contents

Total flavonoid content was analyzed *via* the aluminum chloride colorimetric method[16]. In short, 20 μL of extract solution (dissolved in DMSO) was mixed with potassium acetate (1 M), 10% aluminum chloride and 160 μL of deionized water followed by incubation at room temperature for 30 min. Moreover, absorbance was checked by a microplate reader (Biotek) at 415 nm. The data were expressed as microgram quercetin equivalent per mg of extract.

2.4. Antioxidant assays

2.4.1. Radical-scavenging activity (DPPH assay)

DPPH antioxidant evaluation is based on the ability of antioxidants to decolorize 1, 1-diphenyl-2-picryl-hydrazyl. The concentration used in reaction mixture for given samples was taken as 2 μg for 10 ppm-200 μg for 1 000 ppm. The antioxidant capacity of *C. benghalensis* extracts was ascertained through DPPH radical scavenging assay[15]. Firstly, 200 μg/mL of extract solution was quantified spectrophotometrically at 513 nm. Then, extracts showing more than 50% quenching activity were further analyzed to determine IC_{50} values using lower concentrations.

IC_{50} value of each extract was calculated as:

Scavenging percentage of each extract = $(1-Y_{ab}/Z_{ab})\times100$

Y_{ab} is the DPPH absorbance with test extract whereas Z_{ab} is the absorbance of reagent containing negative control without test sample.

2.4.2. Total antioxidant capacity assessment

The potential of the total antioxidant of the studied crude extracts was carried out by using the previously described phosphomolybdenum method[17]. This reaction was conducted by mixing reagent (900 μL) containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulfuric acid with hundred microliters of each extract solution followed by incubation at 95 ℃ for 90 min. Afterward, the resultant reaction mixture was cooled down at room temperature and a spectrophotometer (TECAN, Mannedorf, Switzerland) was used to measure absorbance at 695 nm. Ascorbic acid was employed as a reference standard. All the results were expressed as microgram ascorbic acid equivalent per mg of crude extract.

2.4.3. Reducing power assay

Reducing power of *C. benghalensis* extracts was determined according to previously described method[18]. Initially, phosphate buffer (40 μL) and 400 μL of potassium ferricyanide (1%) were gently mixed with each sample (200 μL) followed by incubation for 20 min at 50 ℃. Then, the obtained reaction mixture was blended

with 10% of trichloroacetic acid followed by centrifugation for 10 min at 3 000 rpm. Afterward, 500 μL of supernatant was mixed with distilled water (500 μL) and ferric chloride solution (100 μL) and reading was recorded at 700 nm. The results were expressed as microgram ascorbic acid equivalent per milligram of extract.

2.5. Cytotoxicity assay

The *in vitro* antiproliferative activity of plant extracts was gauged against cancer cell lines including Hep-2 (hepatic cancer), prostate cancer (DU-145), breast cancer (MDA-MB-231) and a normal cell line MCF 10A by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as done previously[19]. Cells suspensions were plated in 96 well microtiter plates at a density of 1×10^5 per mL and plates were incubated in a 5% CO_2 incubator for 24 and 48 h at 37 ℃. Then, medium in each well was replaced by different concentrations (50, 100, 200, and 400 μg/mL) of plant extract as well as the negative control (DMSO). The previously described tetrazolium-based colorimetric assay by Chua *et al.*[19], was used to detect cell growth inhibition in studied plant extracts after 24 and 48 h of incubation. After adding 10 μL of MTT to cells in each well for 4 h, 100 μL of solubilizing solution (DMSO) was added and absorbance was taken at 570 nm using a plate reader (TECAN, Mannedorf, Switzerland). The cytotoxic potential of each extract prepared in different solvents was calculated as:

% inhibitory percentage = (1-absorbance value of treated cell/ absorbance value of control)×100

2.6. Cell cycle analysis

MDA-MB-231 cells were seeded (2×10^5 cells) in duplicate and incubated for 24 h before extract treatment. The concentrations of plant extract (100 μg/mL and 200 μg/mL) were applied for 24 and 48 h. Afterward, cells were harvested, washed with PBS buffer and centrifuged for 5 min at 4 ℃ and 200×*g*, fixed with chilled 70% ethanol and incubated overnight at 4 ℃. Fixed cells were then centrifuged at 300×*g* for 5 min and re-suspended in 1 mL of 1×PBS. RNase A (0.5 mL) was added for 20 min and stained with propidium iodide (1 mM) for 15 min. DNA content in stained cells was measured by flow cytometry (BD LSRFortessa™ cell analyzer, USA) and population was quantified with Summit 4.3 software (Beckman Coulter, Inc).

2.7. Annexin V-FITC/PI double staining analysis

The necrotic and apoptotic cells were characterized by using annexin V-FITC kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The reactant cells were seeded (2×10^5 cells/well) in duplicate for 24 h. Subsequently, these cells were subjected with 100 and 200 μg/ mL of extract for 24 and 48 h followed by washing, trypsinization and staining with annexin V-FITC and PI binding buffer. Cells were examined by flow cytometry using 10 000 events per sample.

2.8. DNA fragmentation analysis

The DNA fragmentation was determined to evaluate the cellular apoptosis in MDA-MB-231 cells *via* the Cell Death Detection ELISA kit (Roche Diagnostic). Cells treated with different concentrations of extract for 24 and 48 h were suspended in 200 mL of lysis buffer for 30 min and centrifuged for 10 min at 5 000 rpm. The supernatants were transferred to 96-well streptavidin-coated microtiter plates with 80 mL of the immunoreagent mixture and incubated at 25 ℃ with continuous shaking at 200 rpm. Unbound antibodies were removed by washing and ABTS substrate developed color. Absorbance was read at 405 nm and percentage apoptosis in treated cells was obtained as given below:

Enrichment factor = % apoptosis= DNA fragments in treated sample/ DNA fragments in control cells

2.9. Migration assay

The migration capability of MDA-MB-231 cells was assessed by wound scratch assay[20]. A linear wound was generated with a sterile tip in confluent cells grown in a dish. After treatment with the extracts for 12 h, the wound area was photographed by field microscopy to estimate the migrated area. The gap distance of the wound scratch was measured at different time and calculated by ImageJ software. The data were normalized according to the average of the control.

2.10. Invasion assay

The invasive potential of *C. benghalensis* was gauged by BioCoat Matrigel invasion assay system (BD Biosciences, USA), following the manufacturer's guidelines. The suspension of MDA-MB-231 cells (2×10^5 cells/mL) in serum-free medium was plated in polycarbonate membrane of Matrigel transwell chambers with 8 μm pores. Subsequently, preincubated MDA-MB-231 cancer cells (for 12 h) in transwell chambers with or without the extract were placed precisely into a 24-well plate having basal medium. Followed by 12 h incubation, cells at the upper surface were swabbed with cotton while those that invaded through chamber pores were stained with crystal violet. The invaded cell number was counted in three randomly selected images.

2.11. Fourier transform infrared spectroscopy (FT-IR)

Dried plant material prepared in chloroform solvent was investigated for its FT-IR study. The luminous sample plate was prepared by using 100 mg KBr pellet including 10 mg of plant extract followed by FT-IR spectroscopic analysis of extract (Shimadzu, Japan) at a 400 to 4 000 cm^{-1} scan range and resolution of 4 cm^{-1}.

2.12. Statistical analysis

The data was presented as mean ± SEM. All the experiments were carried out in triplicate. SPSS Ver. 21 software was used for *post hoc* multiple comparison test in One Way ANOVA and IC_{50} was determined by using Table curve software 2D Ver. 4.

3. Results

3.1. Phytochemical analysis

CBM of *C. benghalensis* yielded the highest phenolic contents (74.10 ± 1.45) μg GAE/mg extract among all extracts (Figure 1) while CBH showed the least total phenolic content (24.75 ± 1.75) μg

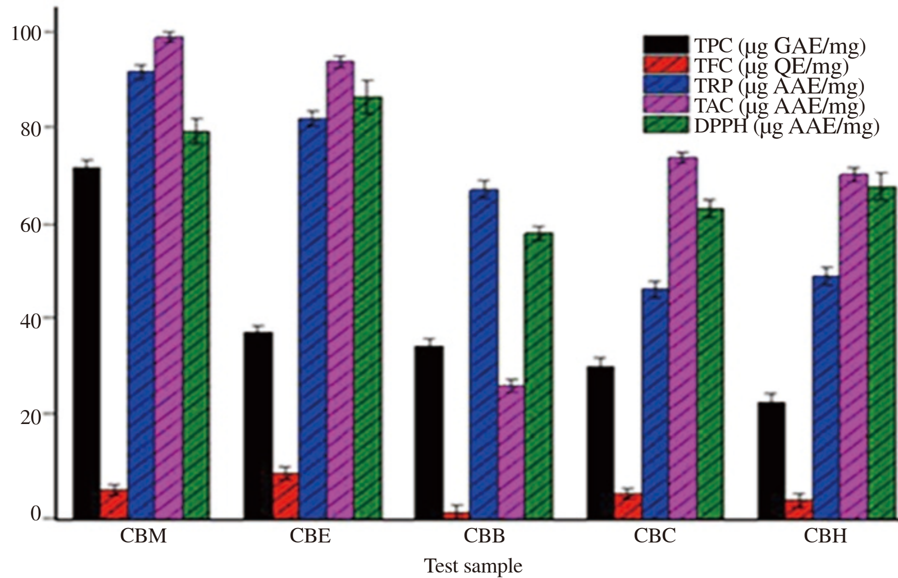

Figure 1. Total phenolic, flavonoid contents (TPC and TFC) and antioxidant activity of different solvent extracts of *Commelina benghalensis*. TRP: total reducing power; TAC: total antioxidant capacity; DPPH: 1,1-diphenyl-2-picrylhydrazyl. CBE, CBB, CBH, CBM, CBC: ethanol, benzene, *n*-hexane, methanol and chloroform extracts of *Commelina benghalensis*.

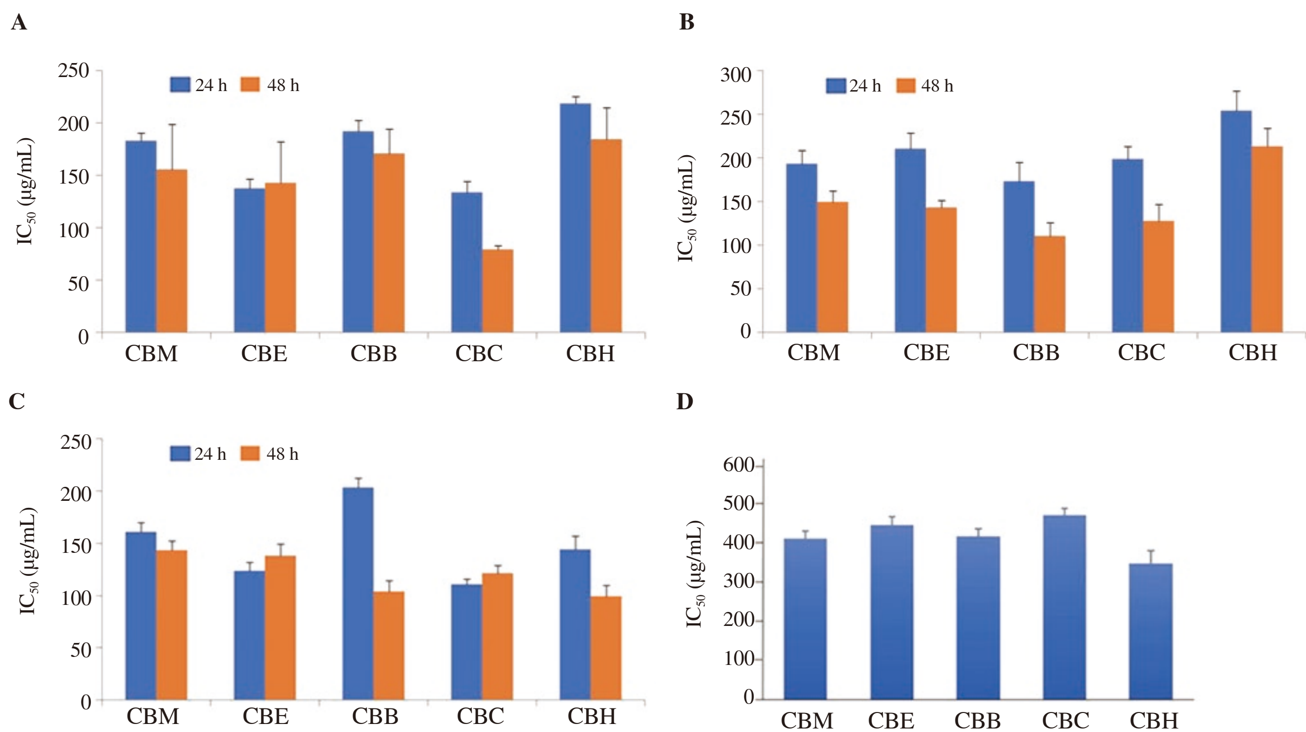

Figure 2. Cytotoxicity of *Commelina benghalensis* extracts in MDA-MB-231 (A), DU-145 (B), Hep-2 (C) and MCF 10A (D) cells treated with 50, 100, 200 and 400 μg/mL of *Commelina benghalensis* root extracts (CBM, CBE, CBB, CBC, CBH) for 24 and 48 h by MTT assay. Data presented as mean ± SEM (n=3).

GAE/mg extract. CBE presented good yield of total phenolic content (39.43 ± 1.42) μg GAE/mg and the highest quantity of flavonoid (9.86 ± 1.25) μg QE/mg extract followed by CBM, CBC, CBH, and CBB.

3.2. Antioxidant potential

3.2.1. DPPH radical scavenging activity

In this assay, the IC_{50} ranged from 11.76-48.52 μg/mL (Figure 1). CBE showed the highest antioxidant activity in DPPH assay (IC_{50}= 11.76 μg/mL, followed by CBM (IC_{50}= 14.42 μg/mL), CBH (IC_{50}= 48.52 μg/mL), CBC (IC_{50}= 42.65 μg/mL), and CBB (IC_{50}= 38.42 μg/mL) (Figure 1).

3.2.2. Evaluation of total antioxidant capacity

CBM showed the highest total antioxidant capacity (101.42 ± 1.00) μg AAE/mg among all extracts, followed by CBE, CBC, CBH, and CBB (Figure 1).

3.2.3. Reducing power assay

CBC showed much lower reducing potential of (48.44 ± 1.75) μg AAE/mg as compared to other analyzed samples of this plant. In

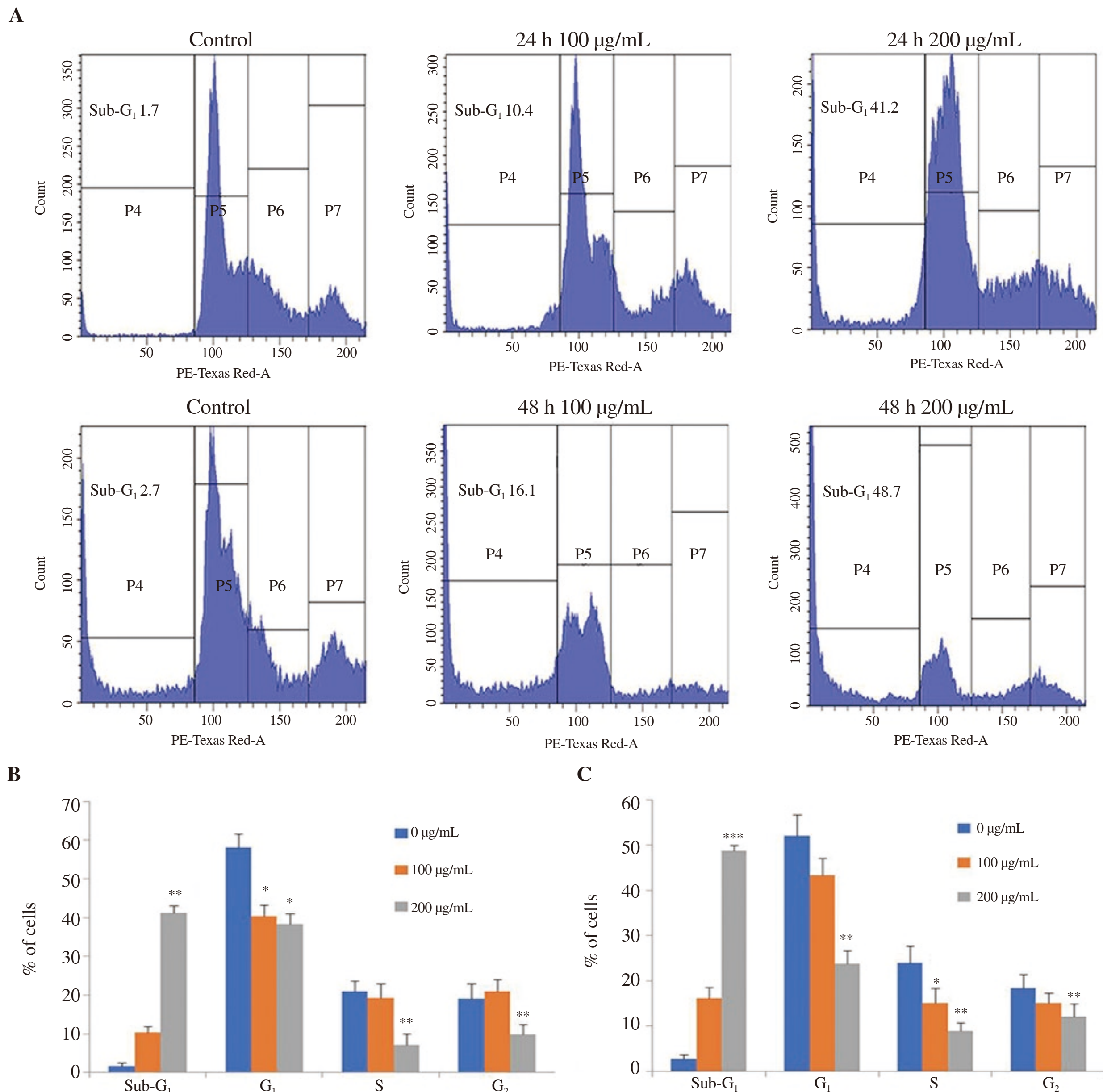

Figure 3. Effects of the *Commelina benghalensis* extracts on MDA-MB-231 breast cancer cell line after treatment for 24 and 48 h. MDA-MB-231 cells were treated with 100 μg/mL and 200 μg/mL of chloroform extract of *Commelina benghalensis*, washed, fixed, stained with propidium iodide, analyzed by flow cytometry (A) and represented in bar graphs for 24 h (B) and 48 h (C). Values are expressed as mean ± SEM ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

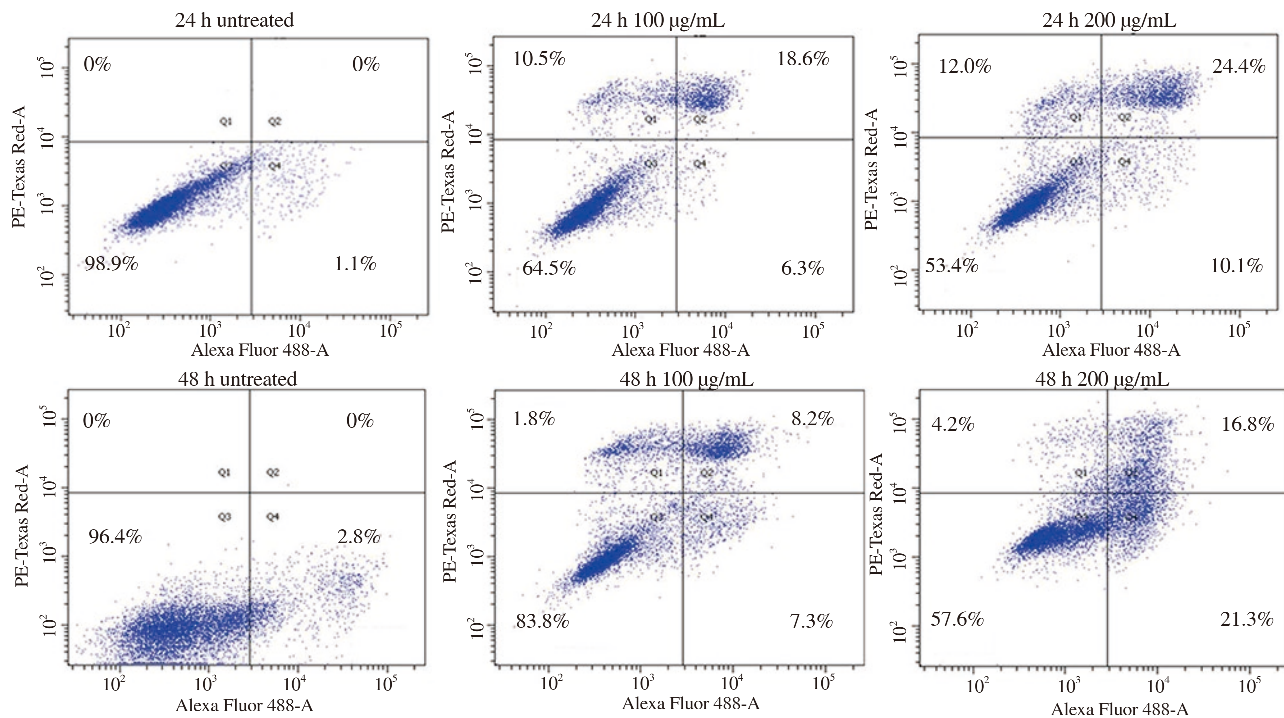

Figure 4. Induction of apoptosis in MDA-MB-231 cells after treatment of chloroform extract of *Commelina benghalensis* for 24 and 48 h followed by propidium iodide and annexin V-FITC staining. Data is presented as mean ± SEM (*n*=3).

contrast, CBM showed the best antioxidant potential as revealed by the highest value of total reducing power (Figure 1).

3.3. Cytotoxicity

All the studied plant extracts demonstrated anticancer activity on selected cell lines in a time dependent manner. Based on IC_{50} values, the highest anticancer effect was observed in MDA-MB-231 cells by CBC that inhibited cell proliferation at the lowest IC_{50} of 134 μg/mL at 24 h treatment and 79 μg/mL at 48 h treatment. In contrast, CBH presented a weaker inhibitory effect for cell mortality as compared to other tested extracts of *C. benghalensis* with IC_{50} of 219 μg/mL at 24 h and 185 μg/mL at 48 h treatment of cancer cells. CBE and CBM also showed considerable inhibitory action in breast cancer cells. CBC was most active in the inhibition of Hep-2 cell proliferation at 24 h with IC_{50} of 111 μg/mL, but its efficacy decreased at 48 h with IC_{50} of 121 μg/mL. For DU-145 cell inhibition, CBB was most active at 24 h with IC_{50} of 173 μg/mL and at 48 h with 128 μg/mL. All extracts were found non-cytotoxic to normal cell line at quite higher concentrations which were found cytotoxic to cancer cell lines. However, based on strong cytotoxic potential, CBC which showed the most potent anti-proliferative effect on breast cancer cell line (MDA-MB-231) was selected for further downward assays including cell cycle analysis, apoptotic effect, wound healing, invasion, and FT-IR analysis.

3.4. CBC induced G_0/G_1 phase arrest in MDA–MB–231 cells

CBC was investigated for its effect on the distribution of cell cycle in MDA-MB-231 cells by flow cytometric analysis using propodium iodide staining for 24 and 48 h of extract-treated cells. After cancer cells were treated with CBC for 24 h, a gradual increase in sub-G_1 population was observed at both concentrations applied (100 μg/mL and 200 μg/mL). It was found that by increasing the concentration of extract, the population in sub-G_1 was significantly increased from 10.4% to 41.2% at 24 h treatment and decreased in G_0/G_1 and S phase populations. Cell cycle analysis showed an increasing trend of DNA accumulation in sub-G_1 from 16.1% to 48.7% at 48 h with a decrease in G_0/G_1 and S phase population. This data suggested that by increasing the concentrations and time for treatment of cancerous cells, there was an increase in sub-G_1 phase population and cell cycle arrest at G_0/G_1 phase (Figure 3).

3.5. Induction of apoptosis in MDA–MB–231 cells

The role of CBC extract was also studied on cellular apoptosis of treated MDA-MB-231 cells double-labeled with propodium iodide and annexin V-FITC using flow cytometry. The results showed that CBC induced apoptosis in a dose and time-dependent manner in breast cancer cells (Figure 4). At 24 h exposure to CBC, increasing dose of the plant extract resulted in increased apoptotic cells from 6.3% to 10.1%. Following 48 h of CBC treatment, the proportion of annexin V-FITC positive cells raised from 7.3% to 21.3%. Thus, these results demonstrated the ability of CBC to induce apoptosis in breast cancer cells (MDA-MB-231).

3.6. DNA fragmentation in MDA–MB–231 cells

Both 100 and 200 μg/mL of CBC were effective on MDA-MB-231 cells. The fold change in fragmentation increased in a time and dose-

dependent manner (Figure 5).

3.7. Inhibition of migration ability in MDA-MB-231 cells

The treatment of cancer cells by CBC showed considerable reduction of migration in MDA-MB-231 cells that was proportional to the concentrations of extract (Figure 6).

3.8. Inhibition of invasive potential in MDA-MB-231 cells

After 24 h treatment of CBC (100 μg/mL and 200 μg/mL), the invasion of treated MDA-MB-231 cells was suppressed through the chamber (Figure 7).

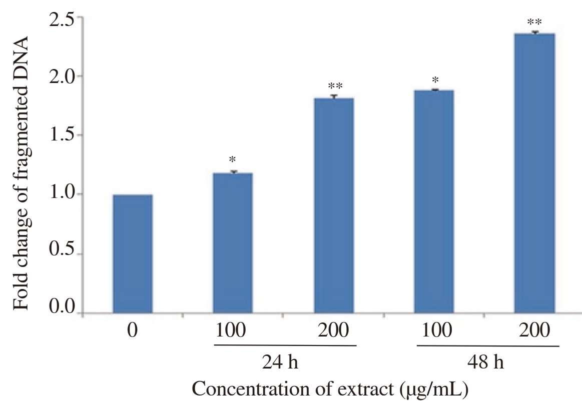

Figure 5. DNA fragmentation after treatment of 100 μg/mL and 200 μg/mL of chloroform extract from *Commelina benghalensis* for 24 h and 48 h in MDA-MB-231 cells. Results are presented as mean ± SEM ($^*P$<0.05; $^{**}P$<0.01).

3.9. FT-IR spectrophotometry analysis

The presence of some compounds in CBC was confirmed by FT-IR analysis. The existence of alkenes, alkanes, aliphatic amines, aromatics, alkyl halides and carboxylic acid (O-H stretch) was confirmed by detecting their peak at 3008 cm^{-1}, 2917 cm^{-1}, 1051 cm^{-1}, 1461 cm^{-1}, 1163 cm^{-1}, and 2850 cm^{-1}, respectively. Furthermore, the peak for alcohols and ester was also found at 1220 cm^{-1} and for α, β-unsaturated aldehydes and ketones at 1711 cm^{-1} (Figure 8).

4. Discussion

Polyphenols of plants are well known for their biological activities that showed a linear correlation with total antioxidant potential and free radical scavenging capability[21]. CBM and CME presented maximum total phenolic contents and total flavonoid contents while CBH extract recorded the lowest polyphenols. Studies have shown that certain diseases can be combated by increasing levels of flavonoids in the diet[22]. Hernandez-Hernandez *et al.*[23] reported antioxidant potency of flavonoids as they promote health and have stronger ability for electron donation. All the plant extracts of *C. benghalensis* were subjected to reducing power assay, DPPH radical scavenging activity and phosphomolybdenum method for antioxidant evaluation. The DPPH assay has been suggested as a precise method for measurement of antioxidant activity of plants or plant extracts and obtained results are comparable to other free radical scavenging methods[24]. CBE presented the highest DPPH scavenging ability and good reducing power while CBM showed the best total antioxidant activity. Plants with higher amount of polyphenols were found with significant antioxidant potential as well. In this manner,

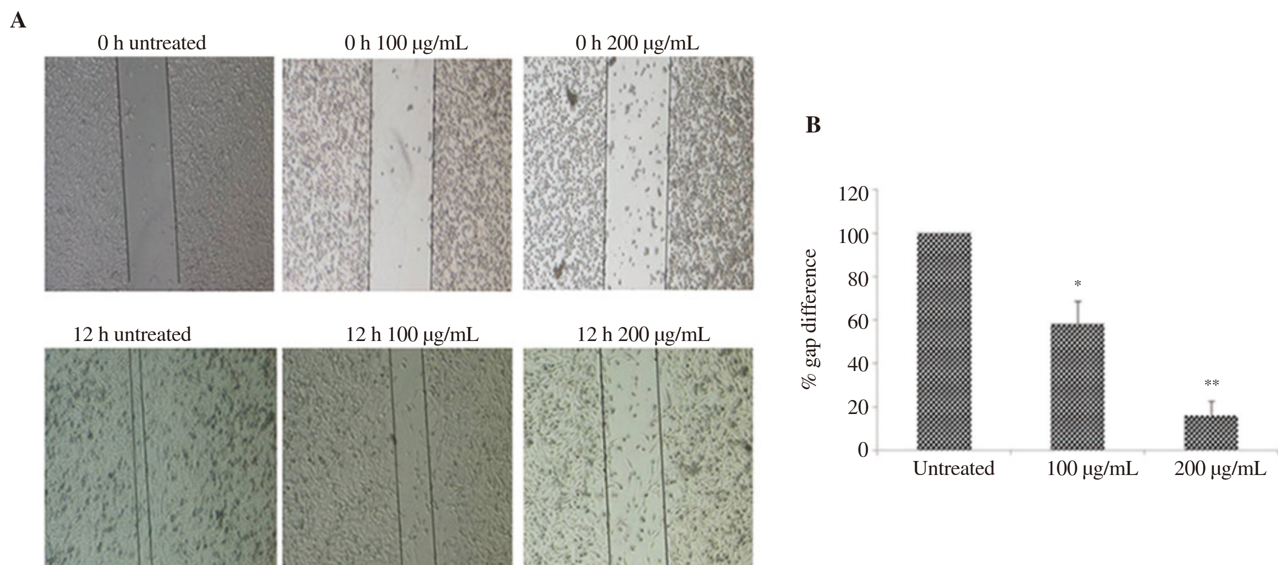

Figure 6. % gap difference covered by MDA-MB-231 cells treated with indicated concentrations of chloroform extract measured at 0 h and 12 h. (A) The images show (×4 magnification and scale bar ~500 μm) the same area at 0 h and after 12 h of treatment with chloroform extract of *Commelina benghalensis*. (B) Columns indicated mean ± SEM (*n*=3), $^*P$<0.05, $^{**}P$<0.01.

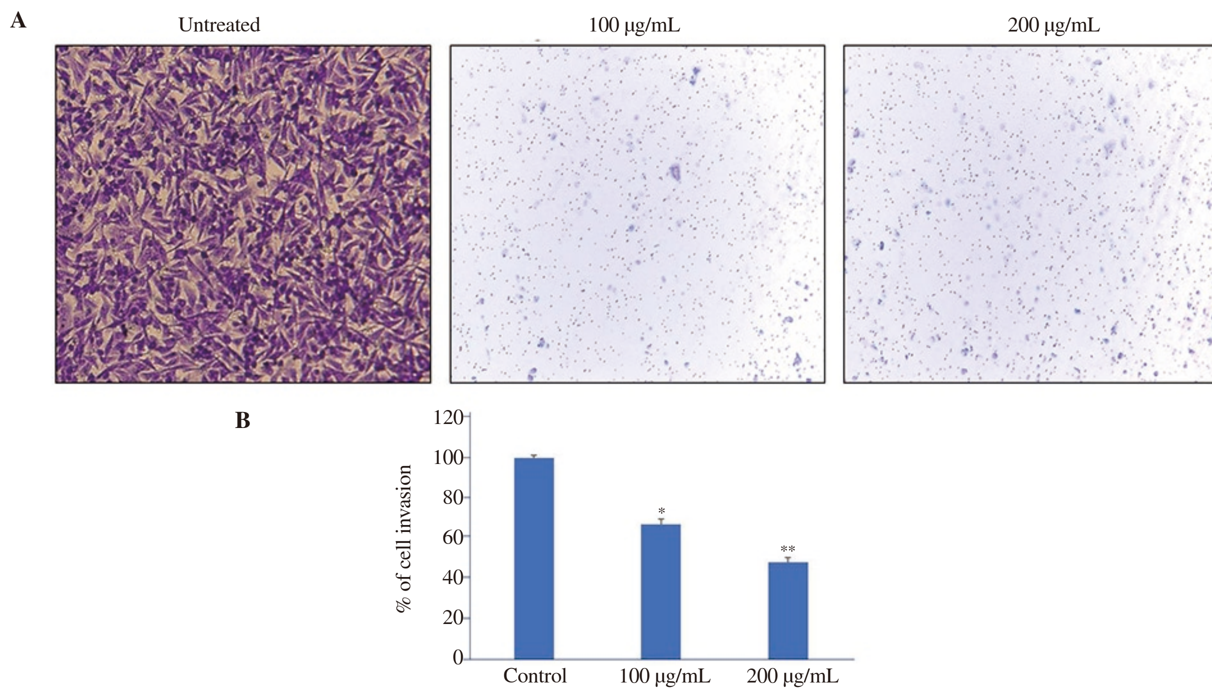

Figure 7. Inhibition of invasive potential of MDA-MB-231 cells treated with indicated concentrations of chloroform extract of *Commelina benghalensis*. (A) Representative images of invaded cells (magnification, ×100). (B) mean percentage of invaded cells ± SEM ($^{*}P$<0.05, $^{**}P$< 0.01).

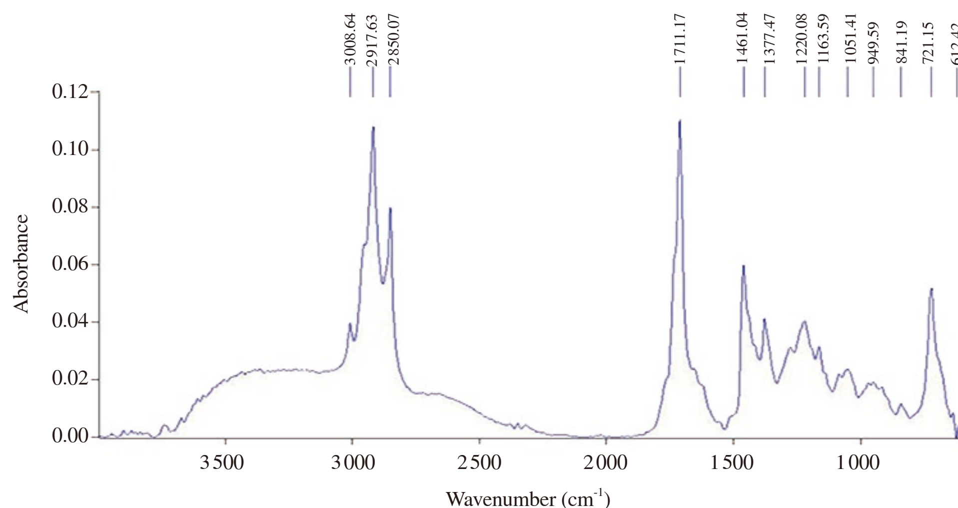

Figure 8. FT-IR spectral peaks obtained from chloroform extract of *Commelina benghalensis*.

the present study suggested that the different polyphenols in plant extract are responsible for the antioxidant potential.

Many anticancer medications have been developed from plant materials. Induction of apoptosis in malignant cells has been considered as an important tool to cure breast cancer[25,26]. Screening of apoptotic inducers from several plants has been estimated as a valuable source for cancer prevention, both in the form of isolated bioactive compounds as well as crude extracts extracted from the plant[27]. Therefore, the current study investigated the cytotoxic and apoptotic effects of *C. benghalensis* extracts against human cancer cell lines including MDA-MB-231, DU-145, and Hep-2. CBC possessed the highest cytotoxic activity for MDA-MB-231 cells as demonstrated in MTT assay and was selected for further analysis. CBC triggered cell cycle arrest at the sub-G_1 phase and reduced the DNA accumulation in the S phase. This effect might be responsible for the proliferation of inhibition of MDA-MB-231 cells. CBC was also found efficient in apoptotic induction in MDA-MB-231 cells and numbers of apoptotic cells were significantly increased after treatment with increased concentrations of extracts. Migration and invasion are considered the key metastatic events for the progression of early-stage breast cancer to its aggressive point. Our experiments show evidence for higher potential of CBC for a significant reduction in migratory and invasive potential of breast cancer cells. FT-IR spectrophotometry was also carried out to confirm the active functional groups of compounds in the studied plant. The study concluded that the CBC has potential bioactive components including alkanes, alcohols, aromatics, *etc.* Jemilat *et al.*[28] reported the existence of tannins, carbohydrates, volatile oils,

phlorotannins, glycosides, saponins, balsams, flavonoids, and resins in *C. benghalensis*. In the future, a detailed study should be carried out to isolate the active compounds that are responsible for the antioxidant and anticancer effect of this plant.

Conflict of interest statement

The authors declare no conflict of interests.

Authors' contributions

RB executed all expiremantal work and compiled the data. EA helped write manuscript and conduct experiment. JI helped in plant collection and interpretation of data. HS and BKHT made substantial contribution in biological evaluation of sample and revision of manuscript. ST contributed in study design. TM supervised the execution of experiments and revised the manuscript. All authors read and approved the final manuscript.

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