Pharmacogn. Res., 2025; 17(4):1328-1334.
https://www.phcogres.com

Original Article

Flavonoid Rich Fraction of Methanol Extract of *Capparis decidua* Attenuates MDA-MB-231 Breast Cancer Cells

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ABSTRACT

Background: The research investigated the mechanism behind the antiproliferative activity of *Capparis decidua* (Family: *Capparaceae*). Despite being tested for other cell lines, its mechanism of action is still not well established. **Objectives:** To evaluate the anticancer potential of the ethylacetate fraction of methanolic extract of *C. decidua* and explore the mechanism involved to validate its role in adjuvant therapy. **Materials and Methods:** This research, evaluated the ethylacetate fraction, through MTT, the cell viability assay. Reactive oxygen species, also known as ROS, generation was used to gauge the extent of oxidative stress in the cytosol. DAPI staining and mitochondrial membrane potential was also performed. **Results:** The methanolic extract and its ethylacetate fraction induced considerable ROS generation intracellularly, which might be ascribed to the several flavonoids as ascertained by total flavonoid content findings. Apoptosis mediated cell death was observed against MDA-MB231. The methanolic extract as well as the fraction exhibited cytotoxicity for both cell lines based on the dosage. It was discerned that ROS levels amplified, along with depletion of the mitochondrial membrane potential, led to consequent apoptosis of cancer cells. This study is the first report of its kind, that ethylacetate fraction induces breast cancer cell death via apoptosis *vis a vis* depletion in mitochondrial potential. **Conclusion:** We conclude better anticancer potential in ethylacetate fraction of methanolic extract, which may prove to be a cost-effective alternative for adjuvant therapy against breast cancer.

Keywords: Anticancer, MTT assay, flavonoid, apoptosis, Mitochondrial Membrane Potential, ROS.

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Received: 14-04-2025;
Revised: 09-06-2025;
Accepted: 25-08-2025.

INTRODUCTION

Cancer is a devastating metabolic disorder that is still one of the leading causes of death, even though diagnostic techniques, treatments, and preventive measures are now more effective.[1] In a study, done in 2018, it was claimed that There are around 100 distinct forms of cancer known to exist.[2] In accordance with data from the International Agency for Research on Cancer, mostly prevalent malignancies identified globally were breast, colorectum and lung.[3] Plants have traditionally been utilized for the treatment of dietary and pathogen-related ailments in indigenous populations. They are additionally believed to be an amazing source of potential pharmaceuticals. In the past few years, traditional and complementary medicine have been gaining acceptance all over the world. Herbal medicines have acquired recognition as a supplementary and alternative form of medicine because to their reduced price, effectiveness and minimal side effects.[4] The World Health Organization (WHO) reports that many countries, particularly developing ones, still employ natural products and plants for medical purposes.[5] Natural sources account about 60% of anticancer medications nationwide.[6] Natural ingredients can be found easily, generally more palatable, and believed to be safe for healthy human cells.[7]

In the sequence of the series of herbal drugs, this research work has added an anticancer herbal drug mostly known as karil, kair, karira etc., in different folk languages but it is globally accepted as *Capparis decidua*. In this investigation, an effort has been made to assess the drug's potential to combat breast cancer based on different standards or parameters.

It is believed that the Persian word kabar, meaning "caper," is the source of the Greek word kapparis. For more than two millennia, pickled capers have been traditionally consumed as a flavor.[8] A long-lived timber plant of the Capparaceae family, *Capparis decidua* is indigenous to tropical and subtropical areas.[9] Many

DOI: 10.5530/pres.20250003

disorders, including arthritic conditions, allergies and asthma, cough, lumbago, painful teeth, pyorrhea, diarrhea, hepatic diseases, diarrhea, fever, cardiovascular disease, constipation, intestinal ulcers, kidney problems, and skin diseases have been believed to be treated with this plant.[10-12] It has been demonstrated that the polyamines spermidine and spermine are crucial for the formation, proliferation, and expantion of breast carcinoma.[13] Furthermore, spermidine and spermine possessed anti-oxidant, anti-arteriosclerotic as well as antiallergenic effects.[14]

C. decidua grows to a height of 4 to 5 meters as a bushy shrub. It occasionally appears as a small tree with thick bundles of dark glossy green branches that appear to be leafless, tiny caducuous leaves that are only present on new shoots, and two spines at each twig node. In excessively dry conditions, the glabrous, thin, or spike-shaped leaves minimize water loss through transpiration.[15] Caper plants can absorb water from the ground up to 4 M deep because to the tap root structure. Even with little rainfall, the plant was able to take water because of the secondary root system that was present nearby the ground surface.[16]

The CDM extract, and its ethyl acetate (CDE) fraction has not yet been assessed against breast tumours. Thus, the goal of the current study was to investigate the anticancer potential of CDM extract on MDA-MB 231 and further effect of CDE fraction on the triple (-ve) MDAMB231 cells.

MATERIALS AND METHODS

Collection and Authentication

Capparis decidua (capperaceae) plant aerial parts were collected in September, 2023 from Agra, Uttar Pradesh, India. A herbarium sheet was created and authenticated at taxonomic division of Banaras Hindu University, Varanasi (Specimen no. Cappara.2023/01 preserved the specimens of the plants and properly documented them for use in the future).

Preparation of Methanol extract (CDM) Ethyl acetate fraction (CDE)

The Soxhlet method was employed to extract 200 g of dried powdered *Capparis decidua* using methanol. The solvent was subsequently concentrated using a vacuum solvent evaporator and it was stored at 4ºC to allow for further assessments.[17]

A few grams of methanol extract were suspended in distilled water and then fractionated in chloroform and ethyl acetate. For each passage, the aqueous suspension was vigorously shaken, then let rest for 20 min while removing the organic phase. Organic phases were concentrated to produce chloroform, ethyl acetate, and aqueous fractions. These crude extracts and fractions were kept in a tightly capped container at 4ºC until required. They had tests conducted for total phenols, flavonoids, and their ability to scavenge DPPH. The most effective crude extract and active fraction i.e. ethyl acetate were chosen for *in vitro* anticarcinogenic efficacy.[18]

The Total Phenolic Content (TPC) of *Capparis decidua* Methanolic extract (CDM) and different fractions was measured using the Folin-Ciocalteu. To begin, equal amount (0.5 mL) of all plant extractives and fractions and Folin Ciocalteu reagent was combined. After 5 to 8 min sodium carbonate solution was mixed at 25ºC, and adjusted the volume with water. After 2 hr, absorbance was measured at 725 nm. Gallic acid was employed as the reference material in the calibration curve. The total Phenolic Content was reported as GAE/g (GAE: Gallic acid equivalents).[19]

The Total Flavonoid Content (TFC) The flavonoids assay was carried out in accordance with the method previously reported.[20] A spectrophotometer was utilized to read the absorbance at 510 nm. The procedure was conducted more than three times, and the finding had been represented in mg equivalents of quercetin per gram of extract.

The extracts' and fractions' free radical scavenging capability was assessed using the DPPH Radical Scavenging Assay, as described by Nguelefack-Mbuyo *et al.*,[21] Ascorbic acid was employed as a standard.

By performing above mentioned assays for methanolic extract of *Capparis decidua* (CDM) and its fraction i.e. Chloroform Fraction (CDC), Ethyl acetate fraction (CDE) and water fraction (CDW), CDE was found most flavonoid rich fraction after methanolic extract of *Capparis decidua* (CDM). Therefore, further assays are done to estimate the anticancer potential for most flavonoid rich extract CDM as well as its fraction CDE.

Culture of Cell Lines and Cytotoxicity Assay

The MDA-MB 231 breast cancer cell lines were acquired from CDRI in Lucknow. The cells were maintained in 96-well tissue culture plates employing Dulbecco's Minimum Essential Medium (DMEM) fortified with foetal bovine serum, Penicillin, Streptomycin and TPVG solution. CO2 incubator (produced by Haier Electric Co. Ltd.) was used to incubate the required cells at 37ºC in a moistened atmosphere with 95% CO_2 and sub cultured.[22]

The test results for CDM and CDE indicate that dead cells or their products do not reduce tetrazolium. The assay is modified by cell count and mitochondrial activity per cell. The assay relies on the efficient cleavage of MTT by cells that are alive to produce a blue formazan derivative. Cell count correlates with formazan production. The crystals were allowed to dissolve in DMSO, and absorbance was measured with an ELISA plate reader at 540 nm. To calculate IC_{50} values, GraphPad Prism 5.1 was used. Morphological alterations were observed using inverted phase contrast imaging.[23,24]

Mitochondrial Membrane Potential (MMP) (Δψm)

A cationic probe called rhodamin 123 is easily absorbed and builds up in a living cell's mitochondria.[25] One clear sign of early apoptotic processes is a decrease in mitochondrial membrane potential (DY).[26] After exposing the quantity of rhodamine in each cell was assessed in both treated and untreated cells (MDA MB 231).

Determination of Intra-cellular ROS generation

Since ROS is necessary for decrease in cell proliferation and death, the intracellular ROS formation in MDAMB 231 cell lines was measured using DCFH-DA.[27]

DAPI analysis for nuclear morphology

Following the recommended procedure, the fluorescent nuclear dye DAPI was used to investigate the effects of CDM and CDE treatment on nuclear alterations into MDA-MB-231 cells. Zeiss Axio Vert 135, a type of inverted fluorescence microscope, was employed for identification of the programmed cell death.[28]

Statistical analysis

The Mean±SD of three separate studies was used to express the results. The Tukey Multiple Comparison Test was conducted using GraphPad Prism software after a one-way ANOVA. Statistical significance was represented by *p*-values which should be less than 0.05.

RESULTS

Total Flavonoid Content (TFC) and Total Phenolic Content (TPC) in Methanolic extract and its fractions of *Capparis decidua*

The methanol extract and fractions of *Capparis decidua* were assessed for Phenolics, flavonoids and radical scavenging activity (Table 1).

Cell viability assay for MDAMB 231 by *Capparis decidua* methanolic extract and its fraction

The IC_{50} dose of Methanolic extract of *Capparis decidua* (CDM), was observed to be at 15 µg/mL for cancer cell lines i.e. MDAMB 231 respectively, with respect to control as well as standard 5-Fluorouracil. MTT assay was also performed for Ethyl acetate fraction (CDE) and the IC_{50} was observed as 10 µg/mL. There was no discernible effect of DMSO treatment on the cells' ability to maintain vitality. A dose-dependent tendency toward decreased cell viability was observed (Figure 1).

When exposed to modest doses, a sizable number of MDAMB 231 cell lines displayed non-adherent, detached, and spherical shapes. CDM had a notable impact on MDAMB 231 cells and a dose-specific decline in cell viability was shown. MDAMB 231 cells were chosen for additional research because of the notable impact that CDM had on the former. Additionally, an active fraction of methanolic extract, Ethyl acetate also has shown considerable impact on inhibition of cell proliferation of MDA-MB-231 Cell lines.

Mitochondrial Membrane Potential (MMP) of *Capparis decidua* methanolic extract and its Ethyl acetate fraction

To probe probable participation of mitochondrial pathway in *C. decidua* -induced apoptosis, fluorescent probe Rh-123 was used to assess MMP changes via flow cytometry. The results indicated towards a considerable MMP loss through CDM and CDE with respect to control and 5-FU as shown in Figures 2 and 3.

Capparis decidua methanolic extract and its Ethyl acetate fraction promotes ROS generation intracellularly

Usually, mitochondrial dysfunction is linked with amplified ROS generation, henceforth, DCFDA (2,7-Dichlorodihydrofluorescein Diacetate) fluorescence was utilized to detect changes in ROS levels. It was suggestive of the fact that cytotoxicity is linked to the mitochondrial dysfunction through increased levels of reactive oxygen species (Figure 4).

DAPI staining to analyse nuclear changes revealed apoptotic patterns

The study analyzed chromatin condensation, a key feature of apoptosis, using DAPI staining and fluorescence microscopy to observe nuclear changes and apoptotic body formation. Cells treated with CDM and CDE displayed apoptotic morphology in MDA-MB-231 carcinoma cells. Cells which are under treatment, showed characteristics like chromatin condensation, apoptosis, nucleus and cytol shrinkage. In contrast, untreated exhibited rounded, homogeneously stained nuclei with DAPI.

Table 1: TFC, TPC and DPPH values of methanolic extract and its fractions of *Capparis decidua*.

Sl. No.	Test extract and fractions	TFC value (mg/g)	TPC value (mg/g)	DPPH Assay (µg/mL)
1	Methanol Extract	2.30±0.11	1. 92±0.14	54.53±2.07
2	Chloroform fraction	0. 74±0.10	0.67±0.04	24.47±1.10
3	Ethyl acetate fraction	3.42±0.14	1.79±0.19	51. 68±1.41
4	Water	1.45±0.20	2.25±0.15	42.20±1.19

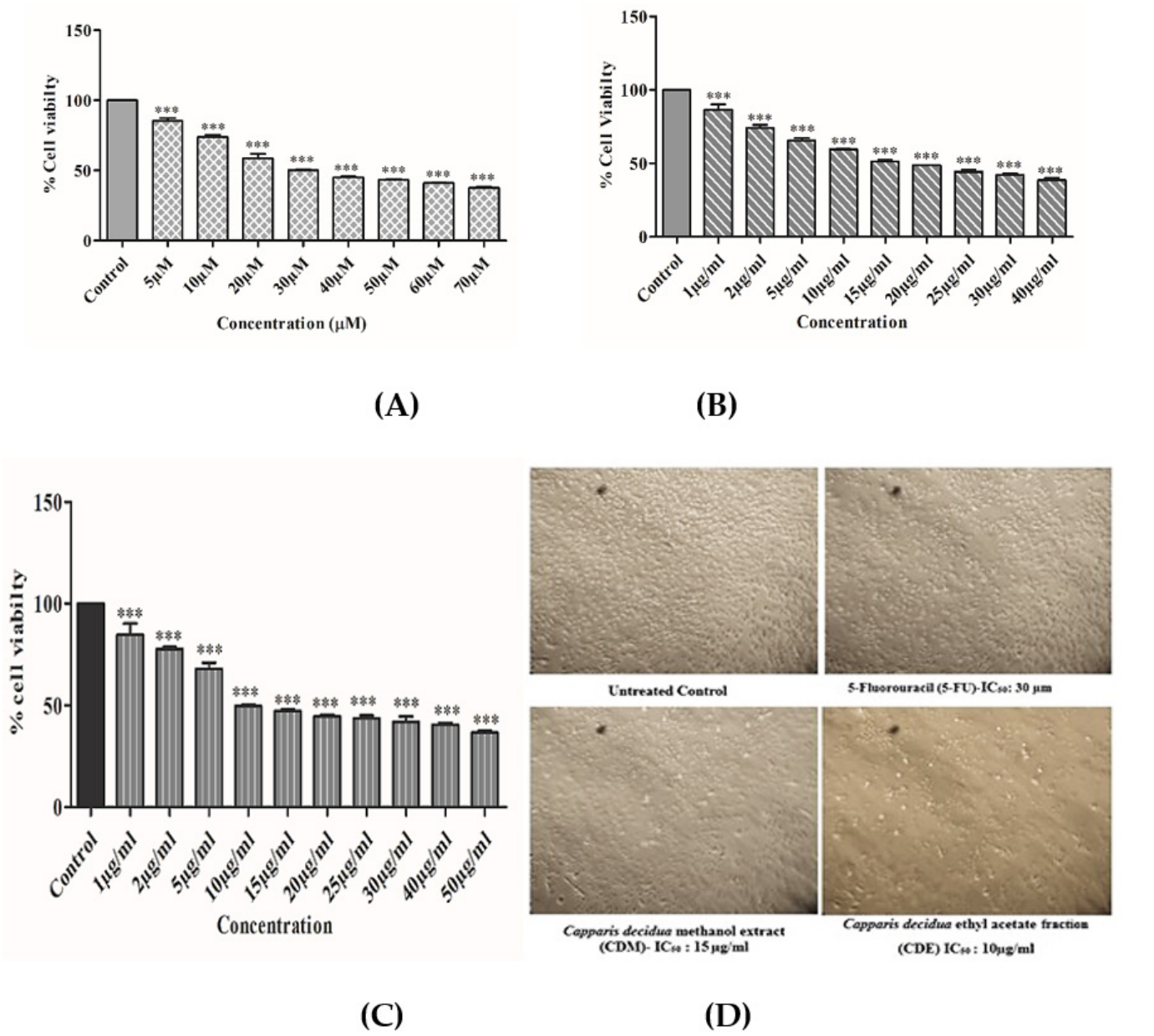

Figure 1: Cell Viability Assay by using the MTT for MDA-MB 231 (A) Graphs represents IC_{50} (30 μm) for 5-FU on MDA-MB- 231 (B) Graph represents IC_{50} (15 μg/mL) for CDM on MDA-MB-231 (C) Graphical presentation of estimation of IC_{50} (10 μg/mL) for CDE on MDA-MB-231 respectively at various concentrations. ***$p<0.05$ as compared to control and (D) Morphological changes by CDM and CDE on MDA-MB-231 cells at IC_{50} doses vs control.

DISCUSSION

Cancer is one of the leading causes of mortality in the world, affecting more than 30% of the population and accounting for more than 20% of all fatalities worldwide.[29] The World Health Organization (WHO) expects that varied types of cancer will cause more deaths worldwide by 2030-34.[30] Drugs used for chemotherapy should have a deadly impact on new tumor cells; but, in actuality, this therapy induces certain basic modifications in the patient.[31] The best type of programmed cell death is engaged in regulating the quantity of normal cells and their replication during stages of growth.[32] Thus, in order to avoid the obstacles of chemical or synthetic treatments, a novel approach of treating metastatic breast carcinoma has increased reliance on herbal drugs.

Capparis decidua has been claimed to have widespread ethnomedicinal benefits due to the presence of a diverse range of active plant constituents. It establishes the fact that the plant has numerous pharmacological actions, including anti-diabetic, anti-bacterial, anti-fungal, antiaging, antitumor, anti-atherosclerotic, hepato-protective, anti-oxidant, anti-hypertensive, hypo-lipidemic, and anti-inflammatory activities.[33-35]

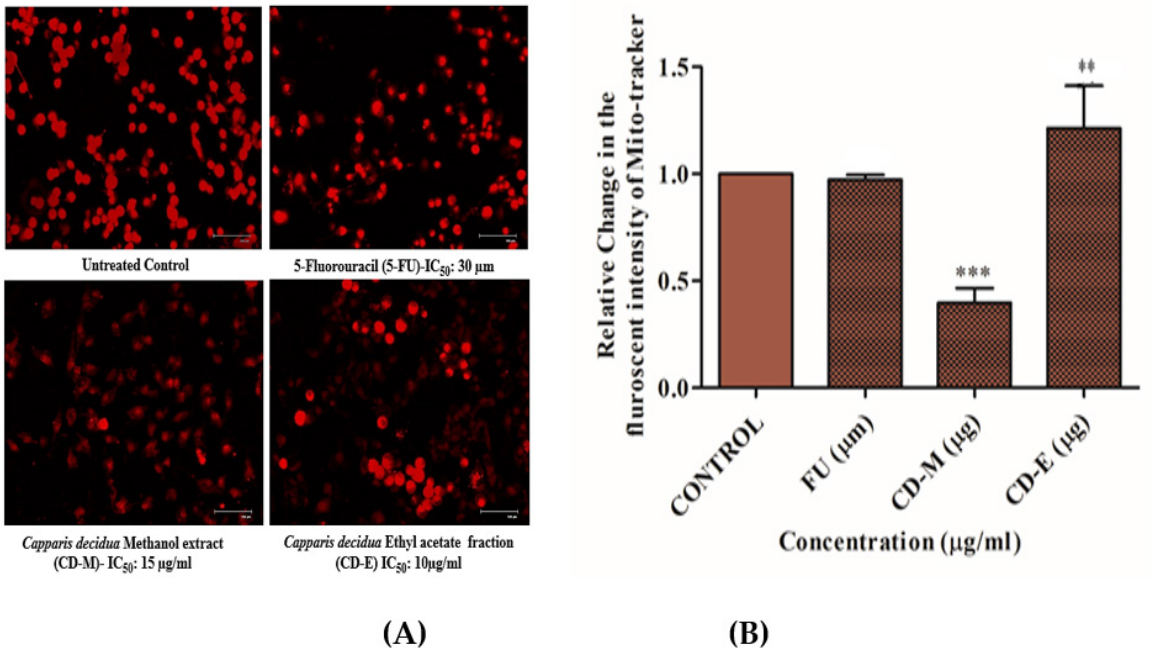

Figure 2: The mitochondrial potential depletion is depicted. The absorption of the Rhodamine, a hydrophobic cationic dye, while it enters mitochondria, was visualized in order to assess the MMP in MDAMB 231 cells. (A) The findings demonstrated that the treatment with CDM and CDE at their IC_{50} dose significantly reduced the rhodamine 123 fluorescence signal related to reduced MMP in comparison to the control. (B) fluorescence intensity variation is shown graphically.

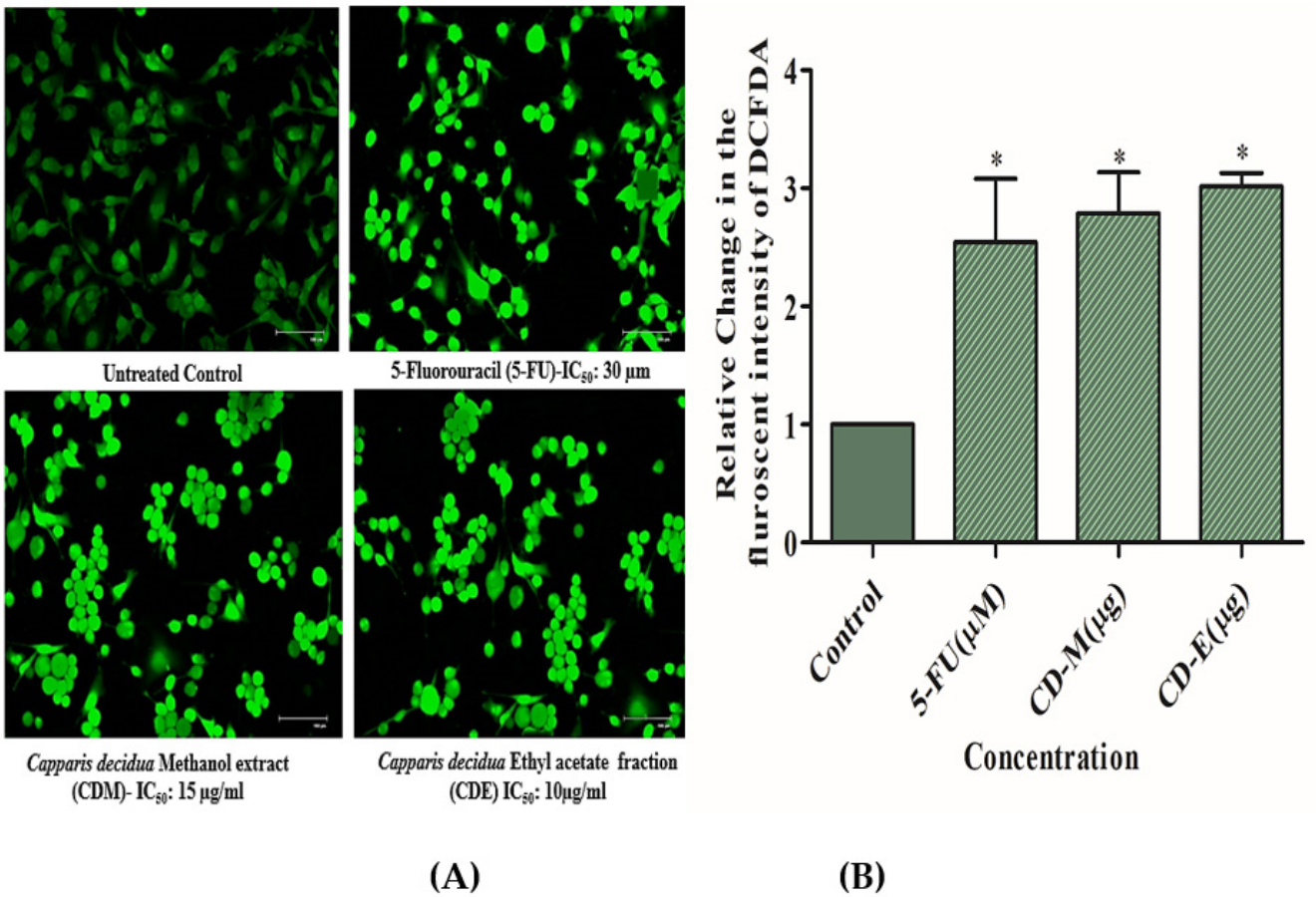

Figure 3: The DCFDA fluorescent probe was used to measure the ROS levels in MDAMB 231 cells, after treating 24 hr with the IC_{50} dose (CDM and CDE), and optical microscopy was used for analysis. (A) After treatment, MDA-MB-231 cells generate more ROS, as seen in the representative photographs. (B) A graphic representation of the relative change in DCFDA fluorescence intensity is provided. In this * signifies $p<0.05$.

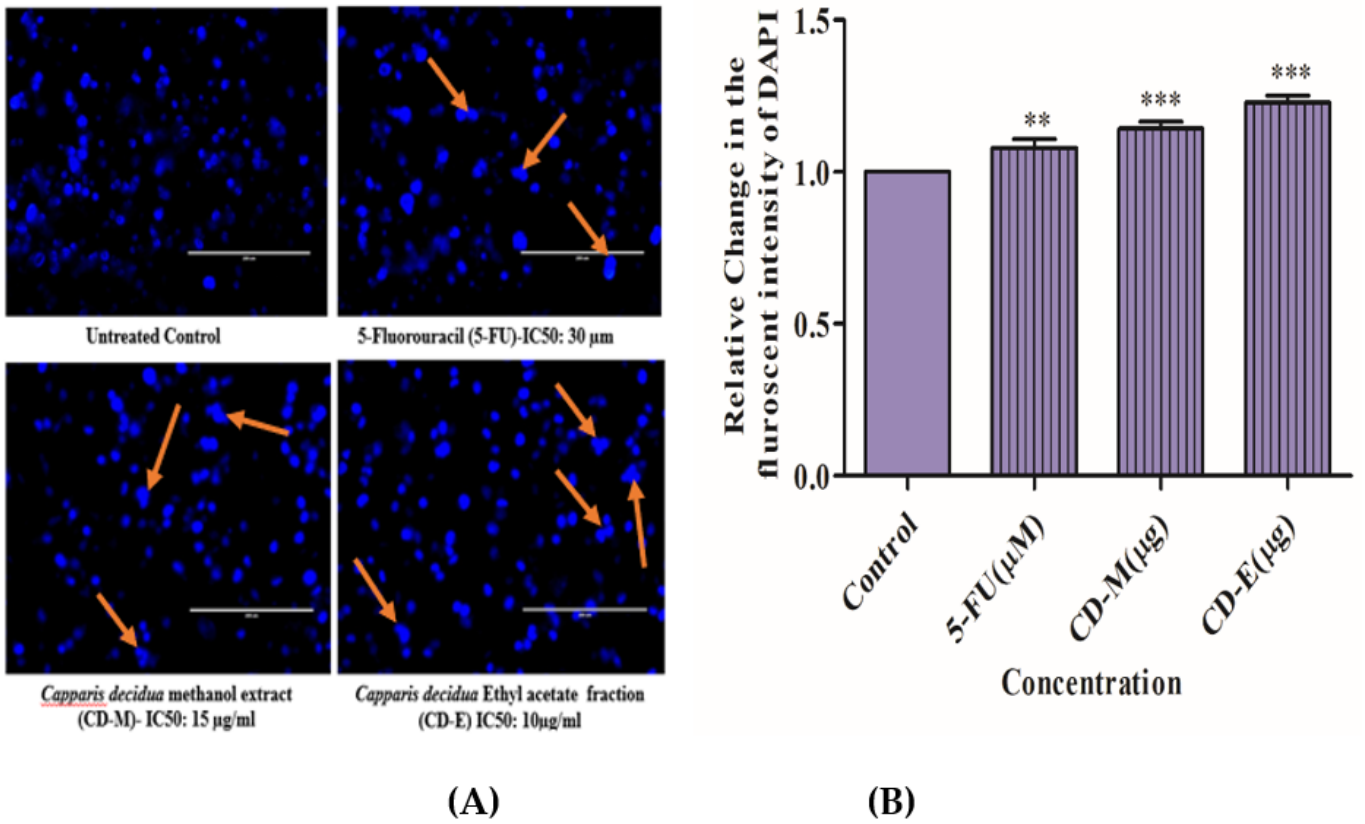

Figure 4: A) The MDAMB 231 cells with nuclear staining using DAPI dye are shown in the photomicrographs. Proapoptosis was seen in the cells administered CDM and CDE. The cells exposed to ethyl acetate fraction and methanol extract (IC_{50} dosages) The cells treated with CDM and CDE revealed clear signs of proapoptosis i.e. blebbing of cellular membrane and the changes in the chromatin structure. Condensed and de-structured nuclei. The arrows demonstrate the chromatin dissolution, breakdown and fragmentation. B) Graphical representation of percentage of apoptotic indices. ** represents p=0.01 and *** represents p=0.001.

CONCLUSION

Methanol extract of *Capparis decidua* (CDM) and ethyl acetate fraction of methanolic extract contains a noticeably greater concentration of flavonoids and polyphenolic contents, which may be associated with its anti-cancer activities.[36-38] Most oxidizing substances and several other free radicals linked to numerous disorders are typically significantly scavenged by flavonoids.[39,40] In the current investigation, tested extract was found to have significant quantities of polyphenolics and flavonoids, ascertained through TFC and TPC determination The MTT assay, A sensitive, accurate, and trustworthy colorimetric test for determining cell viability, is employed for breast cancerous cell lines; MDA-MB-231.

ACKNOWLEDGEMENT

The authors are thankful to the Chancellor Integral University, Lucknow, Uttar Pradesh, India for his sustained encouragement, meticulous supervision, and valuable suggestions at all the stages of completion of the current research article. All authors are also thankful to Prof. (Dr.) Juber Akhtar, Head, Faculty of Pharmacy, for providing technical support.

We also extend gratitude to office of Research and development cell, Integral University for providing Manuscript Communication Number (MCN): IU/R&D/2024- MCN0002358.

ABBREVIATIONS

MDA-MB-231: M D Anderson - Metastatic Breast-231; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **ROS:** Reactive Oxygen Species; **DAPI:** 4',6-diamidino-2-phenylindole; **CDM:** Methanol extract of *Capparis decidua*; **CDE:** Ethyl acetate fraction of *Capparis decidua*; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **CDRI:** Central Drug Research Institute; **DMSO:** Dimethyl Sulfoxide; **DCFH-DA:** 2',7'-dichlorodihydrofluorescein diacetate; **FU:** Fluorouracil; **MMP:** Mitochondrial Membrane Potential; **TFC:** Total Flavonoid Content; **TPC:** Total Phenolic Content.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

This research has included investigation of anticancer property for breast cancer of *Capparis decidua*. By performing various tests for phenolic content, flavonoid content and other general phytochemical tests, the methanolic extract and its ethyl acetate fraction are found to be rich in flavonoids and showed more antioxidant quality than other fractions. Both the methanolic extract and ethyl acetate fraction again undergo MTT assay and their IC_{50} value were estimated against MDA-MB-231; the triple negative breast cancer cell lines. Cell viability was observed to be dependent on given dose concentration. Furthermore MMP, ROS and DAPI assays are performed to identify the pattern of cell toxicity especially apoptosis.

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Cite this article: Shukla N, Usmani S, Siddiqui S, Wahab S, Ahmad R, Khatoon S, *et al*. Flavonoid Rich Fraction of Methanol Extract of *Capparis decidua* Attenuates MDA-MB-231 Breast Cancer Cells. Pharmacog Res. 2025;17(4):1328-34.