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Unveiling Antioxidant and Antiproliferative Effects of *Prosopis juliflora* Leaves against Human Prostate Cancer LNCaP Cells

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ABSTRACT

Herbal medications or formulations are regularly recommended by clinicians as a potential therapeutic method for a variety of human ailments, including cancer. Although *Prosopis juliflora* extracts have shown promise in anticancer activity, the effects on prostate cancer and the accompanying molecular mechanisms of action are still unexplored. This research aims at the antioxidant, antiproliferative, and apoptosis-inducing properties of *Prosopis juliflora* methanolic leaves extract in human prostate cancer LNCaP cells. The antioxidant ability of the extract was assessed using the DPPH (2, 2-diphenyl-2-picrylhydrazyl) and two additional reducing power tests. Antitumor activity was determined using MTT cell viability tests and LDH cytotoxicity assays. The probable mechanism of apoptotic cell death was further investigated utilizing a caspase-3 activation assay and qRT-PCR mRNA expression investigations of apoptotic-related genes. The results revealed that the methanol extract of *Prosopis juliflora* leaves contains alkaloids, flavonoids, tannins, glycosides, and phenols, all of which have substantial antioxidant activity. *In vitro* anticancer tests demonstrated that extract therapy resulted in a dose-dependent reduction in cell viability of LNCaP prostate cancer cells, but normal HaCaT cells showed no cytotoxic effects. Furthermore, plant extract therapy increased caspase-3 activation and mRNA expression of apoptotic-related genes, suggesting that this could be a mechanism for cancer cell growth suppression. The significance of *Prosopis juliflora* as a source of new antioxidant compounds against prostate cancer was emphasized in the current study. However, more study is needed to demonstrate the efficacy of *Prosopis juliflora* leaves extract in the treatment of prostate cancer.

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Introduction

Drug development using plant-derived phytocompounds has been immensely employed by researchers and clinicians (1). Medicinal plants are considered to be rich sources of antioxidants with enormous free radical scavenging potential and contributing significantly to both conventional and traditional drug formulations from ancient times (2). Identification and characterization of plant-derived antioxidant compounds are gaining considerable attention in protecting cells/tissues against free radicals-induced damage (3). These compounds play an important role as free radical scavengers and balance the effect of oxidative stress in several types of chronic diseases such as cancer and cardiovascular disease (4). This defensive role can be mainly accredited to the phytocompounds present in plants, which may be directly associated with their pharmacological as well as biological properties including antioxidant and anticancer activity (5). Antioxidant compounds may alter the progression of tumorigenesis by defending against DNA damage (6). Recent findings suggested the importance of plants and their products for their multifactorial biological properties including anticancer effects against various tumors. Various medicinal plants have proven significant anticancer potential for the inhibition of different kinds of carcinomas including prostate cancer (7-9). However, further research studies are still warranted to explore the anticancer potential of plant-derived products and their associated mechanisms for apoptosis induction. Prostate cancer is the most common type of tumor in men and is frequently diagnosed in elderly men (above 50 years) (10-11). Current treatment strategies available for prostate cancer include radiation therapy, chemotherapy, and hormone ablation therapy are some of the current treatment options available for prostate cancer. However, these traditional therapies have been demonstrated to help patients only to a limited extent and are associated with harmful side effects (12,13). In the prevention and management of prostate cancer, dietary supplements containing soy products, Vitamin E, selenium, lycopene, and catechin have been frequently employed (14). Bioactive chemicals originating from natural resources, such as

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plants, have contributed to the development of anticancer drugs to a great extent (about 60%), but there are still large plant species that need to be investigated for their therapeutic potential (15-17).

Prosopis juliflora selected for the present study is generally used for providing livestock feed, house wood, and pods are used in the human diet as a coffee substitute, pod syrup, fermented beverage, etc due to large sugar content (18,19). It is also used as a folk medicine for treating illnesses such as colds, inflammation, diarrhea, dysentery, and wound healing (20). *Prosopis sps.* leaves were reported to possess antioxidant, antiparasitic, antimicrobial, and antitumor potential (21-23). Generally, it has now been established fact that plants growing in extreme stress conditions are more effective in producing a large amount of stress-defensive plant secondary metabolites which could be used as therapeutics against various types of human diseases (24-26). With this considerable background of *P. juliflora* and the lack of substantial research reports of various leaf extracts on its anticancer potential, we designed a study for investigating the antioxidant and anticancer potential of *Prosopis juliflora* methanolic leaves extract (PJME) against prostate cancer cell lines.

Materials and Methods

Plant sample and extract preparation

Prosopis juliflora leaves were collected from the Noida Institute of Engineering and Technology Campus, Greater Noida, India. The leaves were shed dried and converted into fine powder. Dried powder (25 g) of leaves was extracted with methanol (250 mL) in the soxhlet apparatus (27). Subsequently, the solvent was evaporated by a rotary vacuum evaporator and dried at room temperature. Finally, leaf extract was stored and preserved under low temperatures for future use.

Qualitative analysis of Phytoconstituents

The methanolic extract of *Prosopis juliflora* leaves was subjected to various chemical tests for the detection of different phytoconstituents such as alkaloids, phenols, tannins, saponins, flavonoids, glycoside, and terpenoids as per previously described methods with some modifications (28, 29).

Analysis of *in vitro* antioxidant activity

DPPH Free Radical Scavenging Assay

The radical scavenging activity of PJME was determined by the DPPH method with minor modifications (30). 1 ml of different doses of PJME (15, 30, 60, 120 and 240 µg/mL) were added with 3 ml of DPPH (100 µM) solution and further, incubated for 30 min in dark at 37°C. The absorbance of the mixture was recorded at 515 nm and inhibition was calculated by:

Percent inhibition = [(control absorbance − sample absorbance)/control absorbance] × 100.

Superoxide Radical Scavenging Activity

The superoxide radical scavenging activity of PJME was assessed as per the previous protocol with some modifications (30). Different concentrations of PJME (15, 30, 60, 120 and 240 µg/mL) were added with EDTA (40.2 mg/ml), ethanol, riboflavin (0.2 mg/ml) and NBT (1 mg/ml) and were diluted to 3 ml with phosphate buffer. Furthermore, sample mixtures were incubated at room temperature for 15 min and absorbance was recorded at 560 nm. The superoxide percentage inhibition was calculated according to the following equation:

Percent inhibition= [(Absorbance of sample− Absorbance of control)/Absorbance of sample]× 100.

FRAP (Ferric Reducing Antioxidant Power) Assay

The ferric ions reducing the potential of PJME was carried out by using the previously described method (30). Briefly, 100 µL of different concentrations of PJME (15, 30, 60, 120 and 240 µg/mL) were added with 3 mL of FRAP reagent (300mM sodium acetate buffer with pH 3.6, 10 mM 2,4,6- Tri(2-pyridyl)-s-triazine solution and 20mM $FeCl_3$ in the ratio of 10:1:1). After that sample mixture was incubated at 37°C for 30 min and absorbance read at 593 nm. The results were represented as µM Fe (II)/mg dry weight of leaves extract.

Experimental analysis of anticancerous potential

Cell line and Culture

In this study, two cell lines were used LNCaP and HaCaT and obtained from National Centre for Cell Sciences in Pune, India. LNCaP, a human prostate cancer cell line was cultured at 37°C in a CO_2 incubator in RPMI media with 10% FBS (fetal bovine serum) and 1% antimycotic solution. As a normal control cell line, HaCaT (a human keratinocyte cell line) was used, and it was cultured in DMEM-F12 conditions with 10% FBS (fetal bovine serum) and 1% antimycotic solution.

Cell proliferation assay

The antiproliferative effects of different dosages of PJME on LNCaP and HaCaT cell lines were investigated using the MTT test. In a 96-well culture plate, $5x10^3$ cells/well were seeded for 24 hours (31). Cells were exposed to varying concentrations of PJME (0-240 µg/mL) for 24 hours period. After that, each well was filled with 20 µl of MTT dye (5 mg/mL) and incubated for 4 hours in the dark. Following incubation, the formazan crystal was dissolved in 100 µL DMSO, and the absorbance was measured at 490 nm with a microplate reader (BIORAD, USA). Finally, the findings were expressed as a percentage of cell viability compared to a control sample.

Cytotoxicity Assay

The manufacturer's protocol was followed while measuring extracellular LDH activity using the Cytotoxicity Cell Death Kit (Sigma, USA). Prostate cancer LNCaP cells were seeded at a density of $1x10^4$ cells per well on 96-well culture plates. Cells were exposed to PJME (0-240 µg/mL) for 24 h durations, and the supernatants were collected to determine LDH activity. On a microplate reader, the absorbances of all the samples were measured at 490 nm (Bio-Rad, USA).

Detection of apoptosis by Caspase-3 assay

The caspases-3 activity was measured using Colorimetric Assay Kits to assess the impact of PJME on caspase-mediated apoptosis (BioVision, USA). Treated and untreated cells ($3x10^6$) were lysed in an ice-cold cell lysis solution for 10 minutes before being centrifuged to collect the supernatant. In addition, 50 µl of lysate was transferred into a 96-well plate with 50 µl of reaction buffer contai-

ning 10 mM DTT. After that, 5 µl of each caspase's 4 mM substrate was added to each well, incubated for 1 hour at 37°C, and absorbance was measured at 405 nm using a microtiter plate reader. Then, the percent increase in caspase-3 activity was calculated by comparing the result to the uninduced control level. Furthermore cancer cells were pre-treated with 50 µM of caspase-3 inhibitor (Z-DEVD-FMK) for 2 hours, and afterwards exposed with PJME at selective dosages for 24 hours to determine the involvement of caspase activation in PJME-induced apoptosis. Finally, the MTT test was used to measure cell viability.

Real-time qPCR Analysis

The effect of PJME treatment on the mRNA expression level of apoptotic-related genes in the LNCaP cancer cell line was measured by real-time qPCR analysis. Cells were seeded in 6-well plates and treatment of PJME was done at 30, 60, and 120 µg/mL concentrations for 24 h. Thereafter, RNA was isolated by using the HiPurATM Total RNA Miniprep Purification Kit (Himedia, India) as per the manufacturer's protocol. The cDNA synthesis was done by Verso cDNA synthesis kit (Thermoscientific, USA) and the mRNA expression level of target genes was assessed by using DyNAmo ColorFlash SYBR Green qPCR Kit (Thermoscientific, USA). The primer sequences targets for this study were as follows: Bax (Forward Primer: 5'-AAGAAGCTGAGCGAGTGT-3' Reverse Primer: 5'-GGAGGAAGTCCAATGTC-3'), Bcl-2 (Forward Primer: 5'-TCCATGTCTTTGGACAACCA-3', Reverse Primer: 5'-CTCCACCAGTGTTCCCATCT-3'), caspase-3 (Forward Primer: 5'-ACCAAAGATCATACATGGAAGCGA-3', Reverse Primer: 5'-CGAGATGTCATTCCAGTGCT-3'), GAPDH (Forward Primer: 5'-GAAGGTCGGAGTCAACGGATTTGGT-3', Reverse Primer: 5'-CATGTGGGCCATGAGGTCCACCAC-3'). GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene was used as an internal control and the data was evaluated by the comparative $2^{-\Delta\Delta Ct}$ threshold cycle.

Experimental Data Processing

In this study, all the experiments were performed in triplicates and the respective data were depicted as the mean±SE of three individual experiments. One-way ANOVA was employed to perform the statistical analysis using Dunnett's multiple comparison test (*p < 0.01, **p < 0.001 represent significant difference compared with control).

Results

Screening of phytoconstituents

Prosopis juliflora leaves extract was first analyzed for the qualitative presence of plant active compounds by the previously described method (28). The preliminary analysis showed the presence of alkaloids, flavonoids, tannins, glycoside, and phenols whereas saponins and terpenoids were not detected in plant extract (Table 1).

Effect of *Prosopis juliflora* extract on DPPH free radical

The DPPH test was used to determine the free radical scavenging activity of PJME. The conversion of the colourless stable DPPH radical to yellow-colored diphenylpicrylhydrazine is the basis for this test. The results demonstrated that leaf extract administration increased free radical scavenging activity in a dose-dependent manner (Figure 1A).

Effect of *Prosopis juliflora* extract on Superoxide radical

Superoxide radicals are a major source of reactive oxygen species (ROS), which may be linked to a variety of illnesses (32). As a result, we calculated PJME's superoxide radical scavenging activity in this study. Our findings showed that PJME dosing inhibited superoxide free radical formation in a dose-dependent manner and had

Table 1. Qualitative determination of phytoconstituents present in PJME.

S. No.	Screened Phytochemicals	PJME
1	Alkaloids	+
2	Flavonoids	+
3	Saponins	-
4	Tannins	+
5	Glycoside	+
6	Phenols	+
7	Terpenoids	-

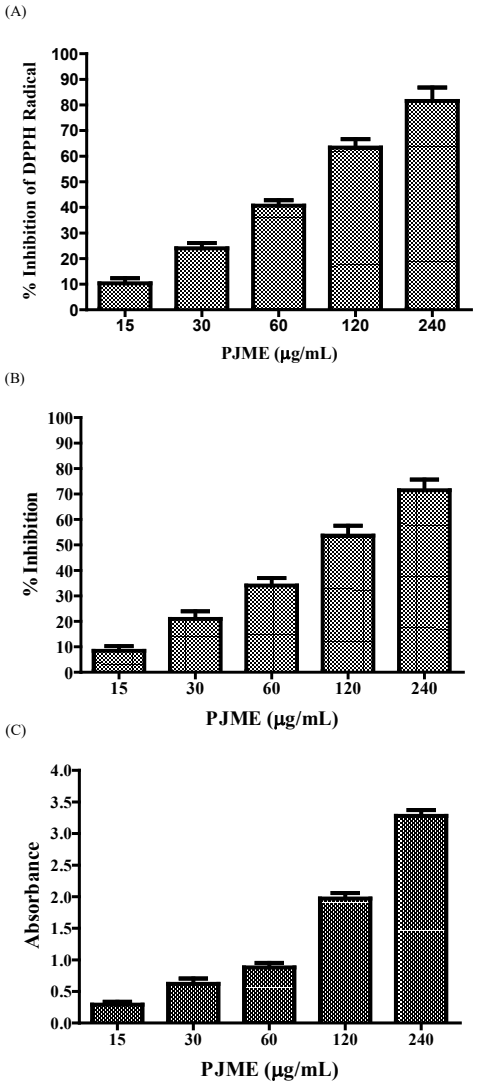

Figure 1. *In vitro* antioxidant activities of PJME were assessed by different assays. (A) Dose-dependent effect of PJME on free radical scavenging (DPPH) activity, (B) superoxide free radical and (C) ferric reducing antioxidant power. Data represented here are the means ± SEM of three independent experiments performed in triplicates.

considerable superoxide radical scavenging ability (Figure 1B).

Effect of *Prosopis juliflora* leaves extract on Ferric ion reducing power

Further to confirm the antioxidant potential of PJME, a FRAP assay was performed and represented in terms of equivalence of ferrous sulphate (mM/g of sample). Results illustrate that the ferric-reducing antioxidant potential of PJME was in increasing order with the dose (Figure 1C). In this assay, the absorbance recorded was found to be 0.29, 0.62, 0.88, 1.97, 3.28 at 15, 30, 60, 120 and 240 µg/mL respectively. These observations of FRAP analysis also indicated a positive correlation with the findings of the reducing power and DPPH radical scavenging assay.

Effect of *Prosopis juliflora* leaves extract on cell viability

The antiproliferative efficacy of PJME on prostate cancer LNCaP cells was assessed by MTT assay after 24 h of treatment. Figure 2A clearly revealed a significant decrease in cell viability of PJME-treated LNCaP cells in a concentration-dependent. Our results showed that PJME (15, 30, 60, 120 and 240 µg/mL) treatment significantly reduced LNCaP cell viability by 82.41%, 70.66%, 64.48%, 46.53%, 27.09% and 11.31%, respectively manner in comparison to untreated control cells. However, the cytotoxic effects of PJME on normal HaCaT cells were found to be insignificant as compared to LNCaP prostate cancer cells (Figure 2B).

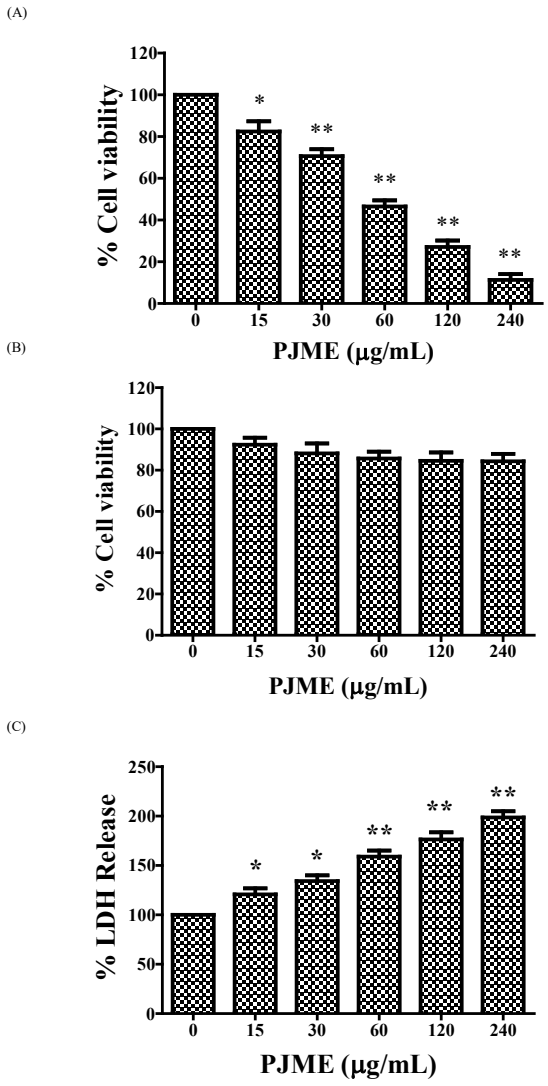

Figure 2. Cytotoxic effects of PJME against prostate cancer LNCaP and normal HaCaT cells. (A) effect of PJME on cell viability of prostate cancer LNCaP cells assessed by MTT assay (B) effect of PJME on cell viability of normal HaCaT cells assessed by MTT assay (C) effect of PJME on PJME-treated LNCaP cells analyzed by LDH release assay. Results represented as Mean ± SEM of three independent experiments (*p<0.01, **p<0.001).

Effect of *Prosopis juliflora* leaves extract on LDH release

LDH release test was used to investigate the effect of PJME on lactate dehydrogenase release from LNCaP prostate cancer cells. Figure 2C shows the results of LDH leakage after treatment with PJME at various doses (0-240 µg/mL). In LNCaP prostate cancer cells, LDH release increased considerably after treatment with PJME compared to control. At 24 hours, LDH release rose by 20.76%, 34.11%, 58.97%, 76.23%, and 98.53% in the LNCaP cell line. The antiproliferative activity of PJME against prostate cancer cell lines, as demonstrated by the MTT assay, is further supported by these findings.

Effect of *Prosopis juliflora* leaves extract on caspase-3 activation

To further confirm the apoptotic events in LNCaP prostate cancer cells, PJME-treated cells were examined for caspase-mediated apoptosis by using a caspase-3 colorimetric assay. PJME-treated LNCaP cells exhibited significant caspase-3 activity in a dose-responsive manner. The results revealed that *Prosopis juliflora* administration resulted in a significant quantity of caspase-3 activities in LNCaP cells of about 66.47%, 79.36% and 101.37%, at 30, 60 and 120 µg/mL respectively as compared to control (Figure 3A).

Caspase-3 inhibitor attenuated PJME–mediated apoptosis in LNCaP Cells

Further, in order to delineate the role of caspase-mediated apoptosis induction, we performed the cell viability assay with a caspase-3 inhibitor (Z-DEVD-FMK). We detected a significant decrease in the cell viability inhibition percentage of PJME–exposed LNCaP cancer cells pretreated with caspase-3 inhibitors (Figure 3B).

Effect of *Prosopis juliflora* leaves extract on modulation of apoptotic-related genes

To further illuminate the anticancer potential of PJME, qRT-PCR was employed to observe the mRNA expression level of Bax, Bcl-2 and caspase-3 genes in LNCaP cells. PJME-treated cells exhibited downregulated expression of Bcl-2 level as compared to the control. In contrast to Bcl-2, PJME treatment resulted in the upregulated mRNA expression level of Bax and caspase-3 genes in LNCaP cells in a dose-responsive manner (Figure 4A, B and C).

Discussion

Despite major advancements in cancer research, interest in finding new anticancer drugs has not been reduced, since cancer remains a major health issue throughout the world (33). Amongst several cancers, prostate cancer is one of the most common cancer in men and the first-line

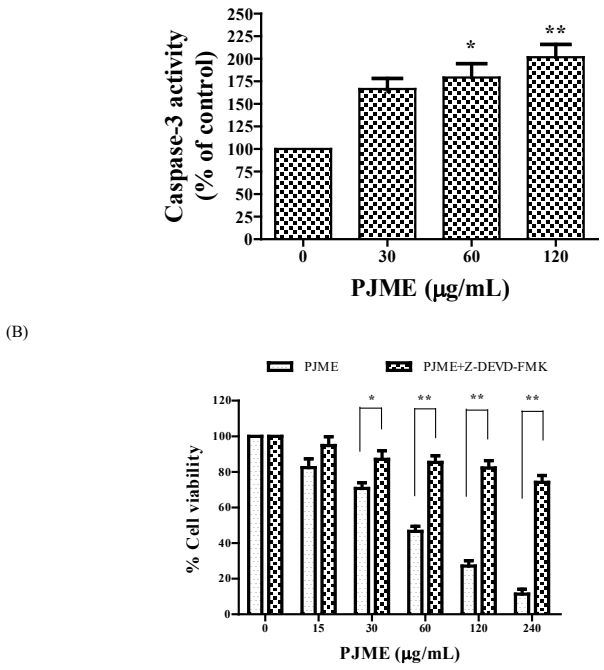

Figure 3. Effect of PJME on caspase-3 activation. (A) Increased activity of Caspase-3 in PJME-treated LNCaP cells inspected by caspase-3 colorimetric kit (B) Percent cell viability of LNCaP cells pretreated with caspase-3 inhibitor then treated with various concentrations of PJME (15–240 µg/mL) for 24 h assessed by MTT assay. Results represented as Mean ± SEM of three independent experiments (*p<0.01, **p<0.001).

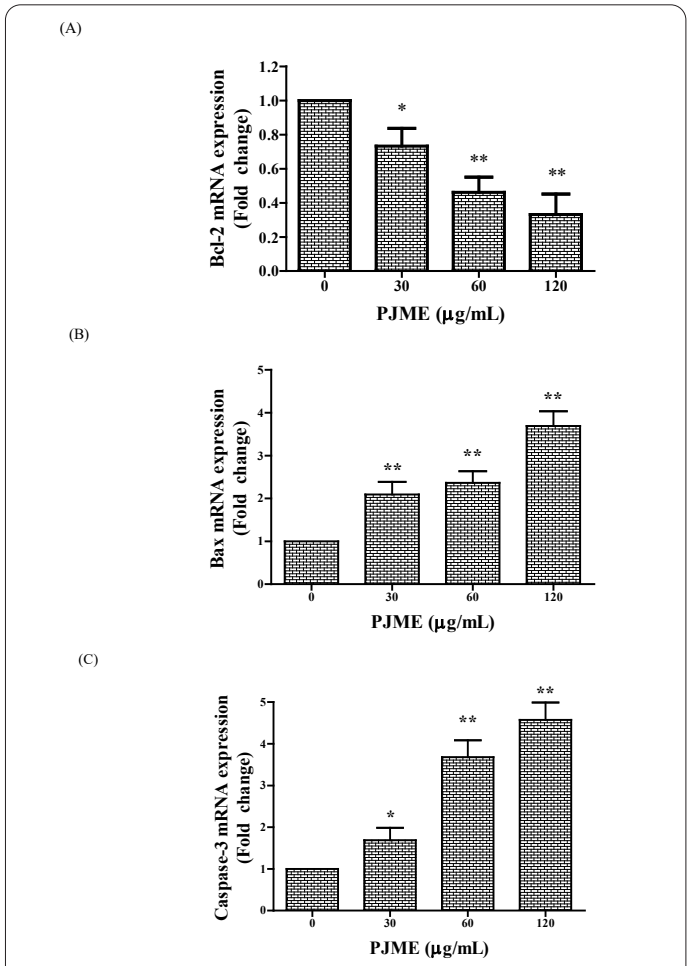

Figure 4. Effect of PJME on apoptosis induction determined by analysis of mRNA expression of apoptotic-related genes. The mRNA expression of (A) Bcl-2, (B) Bax and (C) caspase-3 in PJME-treated LNCaP cells (30, 60, and 120 µg/mL) was examined by qRT PCR. Results represented as Mean ± SEM of three independent experiments (*p<0.01, **p<0.001).

treatment for prostate cancer produces numerous adverse effects as well as ineffective after long-term use (34). Therefore, it is essential to explore novel chemotherapeutic drugs which are more effective, multi-targeted, and cause minimal side effects. Natural products have been considered a vast source of new drug development due to their potent biological properties such as antioxidants, antiviral, anti-inflammatory, antimicrobial, and antitumor potential (35-37). The usage of plant extract is coming up as a positive hope for the development of effective anticancer therapeutics due to their multi-targeted approach and combating drug resistance phenomena (38,39). Thus, these strategies could immensely help researchers in finding and developing leading antitumor therapeutic agents with improved efficacy and less toxicity (40). The present investigation was designed to evaluate the antioxidant and antitumor potential of the methanolic leaf extract of *Prosopis juliflora* against the androgen-independent LNCaP prostate cancer cells. We have performed several *In vitro* assays like antioxidant assays, cell viability assays, cytotoxicity assays, caspase-3 colorimetric assay, quantitative apoptotic analysis, etc. for assessing the effect of plant leaves extract against the selected LNCaP and normal HaCaT cells.

Different kinds of plant phytocompounds such as flavonoids, saponins, alkaloids, phenols and terpenes are considered strong antioxidants due to their beneficiary medicinal properties including antimicrobial and anticancerous activities (41, 42). Therefore, we have carried out qualitative analysis for phytochemicals in *Prosopis juliflora* methanolic leaves extract and the results revealed the presence of alkaloids, flavonoids, tannins, glycoside, and phenols in the leaves extract.

Furthermore, *Prosopis juliflora* methanolic leaf extract was assessed for *in vitro* antioxidant activities by using different assays. Results indicated that there was increased DPPH scavenging activity with increasing concentration of the leaves extracts which may represent an augmented ability to donate hydrogen ions in the solution (Figure 1A) (43).The capability of plant extract to reduce Fe^{3+} (ferric) ions to Fe^{2+} (ferrous) ions is commonly employed as an index to calculate the antioxidant potential of the extract (44). Our results suggested that the treatment of the extract resulted in a reduced amount of Fe^{3+} ion which also depicted the antioxidant activity of leaves extract (Figure 1B). Moreover, the superoxide radical scavenging potential of leaves extract was also elucidated which is considered an important precursor of reactive oxygen species generation and responsible for the development of various diseases. Our data demonstrated that the treatment of leaf extract suggested a dose-responsive increase in superoxide radical scavenging activity (Figure 1C). Thus, these results confirm the significant antioxidant potential of *Prosopis juliflora* methanolic leaves extract which may be due to the presence of various phytochemicals in the plant extract.

Plant extracts and their active components have been shown to have anticancer properties against a variety of cancer cell lines, including prostate cancer, in several in-

vestigations (45,46). In triple-negative breast cancer cells, a new study highlighted the *in vitro* and *in vivo* potential of *Prosopis juliflora* plant leaves extract (47). Thus, we investigated the antiproliferative and apoptosis-inducing properties of *Prosopis juliflora* methanolic leaves extract against prostate cancer LNCaP cells in this aspect. The results of our MTT and LDH assays clearly showed that leaf extract induces growth-inhibitory effects in a concentration-dependent manner. Surprisingly, normal HaCaT cells showed just a minor level of cytotoxicity in response to extract treatment (Figure 2A B and C).

Apoptosis has been represented as an important pathway of cytotoxic drug-mediated cell death in different types of cancers and is associated with several morphological alterations like cell shrinkage, nuclear condensations and fragmentation (48). Cysteine proteases (caspases) are recognized as an important player in apoptosis induction and execution. Caspases are activated as a series of events in response to a death signal during apoptosis induction (49). Our results suggested that PJME induced caspase-3 mediated apoptosis induction which is evident by caspase-3 activation and elevated level of caspase-3 mRNA expression in LNCaP cells. Further, the substantial reduction of the PJME-mediated cytotoxicity in the presence of the caspase-3 inhibitor established the caspase-3 association in apoptosis execution in LNCaP cells.

Several studies have reported an important role of the Bcl-2 family protein in the regulation of apoptosis and cell survival (50). In this regard, the effect of medicinal plants has also been considered (51-60). In further elucidating the molecular mechanism of PJME-induced apoptosis, the mRNA expression of Bax, Bcl-2 and caspase-3 genes were measured. At the mRNA level, PJME treatment significantly decreased the Bcl-2 level and increased Bax and caspase-3 expression (Figure 4). Thus, we hypothesized that modulation of mRNA expression of Bcl-2 family proteins and activation of caspase-3 may contribute to PJME-induced apoptosis in LNCaP prostate cancer cells.

Conclusions

In conclusion, the present investigation suggested the significant antioxidant potential of the methanolic leaf extract of *Prosopis juliflora*. PJME also demonstrated potent antiproliferative and apoptotic effects in prostate cancer LNCaP cells through modulation of Bcl-2 family proteins expression as well as caspase activation. Thus, current experimental findings showed that Prosopis leaf extract could be used as a potent therapeutic agent in human prostate cancer. However, further *in vivo* and clinical studies are still required to confirm its anticancer mechanism.

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Interest conflict

The authors declared that they have no conflict of interest.

Consent for publications

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material

All data generated during this study are included in this published article

Authors' Contribution

All authors had equal roles in study design, work, statistical analysis and manuscript writing.

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Ethics approval and consent to participate

No human or animals were used in the present research.

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