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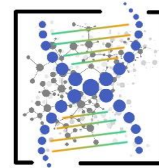

FLAVONOIDS OF CAPPARIS CARTILAGINEA FRUIT EXTRACT EFFECT ON WOUND HEALING IN HUMAN PROSTATE CANCER CELL LINE

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ABSTRACT

Prostate cancer (PCa) is the most prevalent cancer in men who live in developed nations. Even with the recent advancements in surgery, radiation, hormonal therapy, and medical management, medical treatment for advanced-stage PCa is being developed. Alternative strategies for reducing the prevalence of PCa include dietary cancer chemopreventive agents, it has been suggested that medicinal plants can treat cancer. Active composites of Capparis Cartilaginea (*C. cartilaginea*) are alkaloids, tannins, steroids, terpenoids, and flavonoids. This study aims to investigate the cytotoxic effect of *C. cartilaginea* fruit extract on the 22RV1 human prostate cancer cell line. Under an inverted light microscope, a dose- and time-dependent morphological alteration and cell migratory capacity were observed after exposure to the ethanol extract of *C. cartilaginea* fruit. The cytotoxicity of the *C. cartilaginea* fruit ethanol extract on the prostate cancer 22RV1 cells was performed by using the 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. The effect of *C. cartilaginea* fruit extract on cell migration was assessed using an in vitro wound-healing scratch assay. The MTT assay showed that C. cartilaginea fruit extract had a significant cytotoxic effect on 22RV1 cells after 72 h treatment with a value of (0.088 ± 0.031) µg/mL. Following exposure to *C. cartilaginea* fruit extract, morphological changes in the cells showed that the extract had a remarkable impact on cell death, and the mortality correspondingly increased with the increase in concentration. The findings suggested that *C. cartilaginea* fruit ethanol extract might be used as a medicinal plant for treating prostate cancer.

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Introduction

Prostate cancer (PCa) accounts for 6.6% of total deaths in males, making it the fifth leading cause of cancer mortality in men [1]. The Saudi cancer registry recorded 1739 cases in the Kingdom of Saudi Arabia during 7 years. The highest rate (10.1 per 100,000 men) was recorded in the eastern region of Saudi Arabia. The rate of PCa cases was 7.1 in Riyadh, 5.2 in Makkah, 1.4 in Jizan, and 2 in Najran [2]. In the Middle East, the incidence rate of PCa is significantly lower than in Europe and the United States [3]. In Saudi Arabia, PCa is the sixth most common cancer among men of all ages [4]. In 2018, the age-standardized incidence rate for PCa was 7.7 per 100,000 men, and the age-standardized mortality rate was 5.1 per 100,000 men, as estimated by the International Agency for Research on Cancer. When comparing Persian Gulf countries with European countries, the age-standardized incidence rate of PCa in Saudi Arabia is generally very low [2].

The early diagnosis of PCa by screening tumor markers is vital since the effectiveness of the ordinary treatment for PCa is useful for early-stage cancer when a tumor is localized within the prostate [5]. The common treatment of localized PCa with radical prostatectomy or radiotherapy may result in a serious negative impact on quality of life, including sexual dysfunction, urinary leakage, and compromised bowel function [6]. The choice of therapy is made based on clinical staging and the general health of the patient. Active investigation is typically used to manage localized PC a with low risk. More invasive therapy is necessary for intermediate and especially high-risk, diseases, for which radiotherapy or surgery is currently the standard treatment option, regardless of multimodal approaches. Androgen deprivation therapy (ADT), which focuses on androgen receptor signaling, is the go-to treatment for metastatic cancer [7]. PCa tumors grow very slowly before symptoms appear. As a result, there is a relatively long window for therapeutic interventions. Even a small delay in the disease's progression could

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greatly enhance the quality of life for the patient. Dietary substance consumption can pose a significant clinical challenge in the quest to improve nontoxic chemopreventive strategies [8]. Plant-based drugs have lately received extensive research attention [9], largely due to significant advancements in organic chemistry, ground-breaking modifications to the extraction and purification processes, and a better understanding of the natural compounds found in plants. In addition, using plant sources as less expensive raw materials might help the pharmaceutical sector to create better drugs at significantly lower costs [10].
Capparis cartilaginea (*C. Cartilaginea*) (flower buds, fruits, and shoots) have been used in traditional medicine as effective treatments for numerous human diseases over the past thousand years. It can also be used as a drug, cosmetics, food, and animal nutrition [11, 12]. *C. cartilaginea* is widely distributed in the Arabian Peninsula (Saudi Arabia, and Yemen); North Africa (Egypt), Western Asia (Iraq and southern Iran); tropical Northeast Africa (Somalia and Sudan); tropical East Africa; and the Indian subcontinent [13]. Plants of this family are annual, perennial, and sometimes climbers or trees [14]. Six flavonoids isolated from *C. cartilaginea* are identified as kaempferol-3-O-rutinoside, quercetin-3-O-rutinoside, kaempferol 3-neohesperidoside, kaempferol 3-(2 G-rhamnosylrutinoside), quercetin 3-(2 G-rhamnosylrutinoside), and isorhamnetin-3-O-rutinoside [15]. Additionally, this genus includes non-nutritive plant chemicals known as phytochemicals, such as glycosides, tannins, flavonoids, alkaloids, saponins, unsaturated sterols, and coumarins [16]. Flavonoid and alkaloid compounds are used as drugs to prevent many diseases. Scientists have suggested that various types of cancer can be prevented and efficiently inhibited by these compounds [17]. According to a study carried out in an animal model, flavonoids and alkaloids can block the progression of the cell cycle, inhibit mutations, and prevent multistage carcinogenesis, thus breaking lipid peroxide chain reactions. They can also scavenge proxy radicals, superoxides, and hydroxides, as well as protect cells from X-ray damage [18]. Flavonoids have anticancer, antioxidant, anti-inflammatory, and antiviral properties, among other therapeutic advantages. Additionally, they have cardio-protective and neuroprotective effects [19]. Quercetin (3, 3′, 4′, 5, 7-Penta-hydroxy-flavone) Several cancers, including breast, lung, prostate, cervical, liver, and colon cancer, are prevented from spreading by quercetin, and it works by a variety of mechanisms, including cellular signaling, binding to cellular receptors and proteins, and inhibiting enzymes that activate carcinogens [20]. Glucosinolates (S-glucopyranosyl thiohydroximates) are a large group of secondary metabolites consisting of S-linked glucosides. The potential role of isothiocyanates as anti-carcinogenic agents has been thoroughly investigated as cytotoxic against a variety of cancer cell lines and is currently being utilized for the creation of new anticancer drugs [21].

Materials and Methods

Fruit Preparation

The fresh fruit of *C. cartilaginea* was collected from Wadi Molham, Wadi Abu Al-Haza, and Wadi Al-Quraine in Tuwaiq Mountains, Saudi Arabia in October 2018. The fresh fruit of *C. cartilaginea* was freeze-dried by freeze dryer (ilShin BioBase, Korea) at -60 °C under 5 mTorr pressure for 24 hr, then grind into powder by using Waring blender (USA). The powdered sample was kept at -80°C in an airtight container until required. *C. cartilaginea* fruit extract was ethanol extracted by using Al-Goufi and Sonbol methods [22]. Varying concentrations of the extract were prepared from stock solutions (0.5-20 μg/ml) and made up with complete RPMI-1640 medium to a final volume of 100 μl. The criteria of cytotoxicity for the crude extract, which was established by the US National Cancer Institute, is IC50 <20 μg/ ml in the preliminary assay [23].

Cell Culture and Subculture Procedures

The prostate cancer 22RV1 cell line was maintained in RPMI-1640 medium supplemented with 10% (w/v) FBS and 1% (w/v) penicillin. Cells were grown in 25 cm2 tissue culture flasks in a humidified atmosphere containing 5% (v/v) CO2 at 37 °C. Once the cells reached 80% confluency, the medium was removed, and the cell monolayer was washed with PBS. Then, trypsin–EDTA (1 mL) was added to the flasks for 5–7 min to detach the cells' monolayer. The cells were occasionally observed under the inverted light microscope (Nikon Instruments, Melville, NY, USA) until the cell layer was dispersed. Then, a pre-warmed complete growth medium (3 mL) was added to the flasks followed by repeated gentle pipetting to split the cell clumps and inactivate the trypsin. After that, the cells were transferred to a 15 mL tube and centrifuged at 1500 rpm for 5 to 10 min. At this stage, the cell pellet was resuspended in a pre-warmed complete growth medium. Approximately 0.5–1 × 106 cells were sub-cultured into new 25 cm2 flasks that contained 8 mL of fresh medium. Morphological changes characteristic of cell death were observed using an inverted light microscope (Nikon Instruments, Melville, NY, USA).

Determination of Cell Viability and Proliferative Activity

The effect of the *C. cartilaginea* fruit extract on the growth of the prostate cancer 22RV1 cell line was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) calorimetric assay that measured the activity of mitochondrial dehydrogenase in living cells, which can convert pale yellow soluble MTT into an insoluble purple formazan product. Cells were grown at different densities (5000 cells/100 µL and 10,000 cells/100 µL) in 96-well microtiter plates (PermanoxTM, city) at 37 °C with 5% (v/v) CO2 overnight. Cells were treated with varying concentrations of *C. cartilaginea* fruit extract (0.5, 5, 10, 15, and 20 μg/mL). The culture plates were incubated at 37 °C with 5% (v/v) CO2 for 24, 48, and 72 h. Controls containing cells and media were included as positive controls.
After 24, 48, and 72 h of incubation, 10 µL of MTT (3 mg/mL) solution was added to each well and incubated at 37 °C with 5% (v/v) CO2. After 4 h of incubation, the supernatants were discarded. Subsequently, dimethyl sulfoxide (DMSO) (100 µL)

was added to each well and vigorously mixed to dissolve the formazan crystals. Absorbance at 570 nm was measured with an enzyme-linked immunosorbent assay ELIZA reader (BioTek Instruments, Winooski, VT, USA). The percentage of cell viability was calculated manually using the Kamiloglu *et al*. formula [24]. The percentage of cell cytotoxicity was also calculated manually using the formula presented by Choudhury *et al*. [25].
Cells in a seeding density of 10,000 cells/well (and 5000 cells/well) were individually treated with different concentrations of *C. cartilaginea* fruit extract (0.5, 5, 10, 15, and 20 µg/mL) for 24, 48, and 72 h. Morphological changes in cells were observed 24, 48, and 72 h post-treatment.
An optimization of cell densities was performed, and the best representative concentration was found at densities of 10,000 cells/well. At a cell density of 10,000 cells/well, the number of viable cells in culture after treatment with varying concentrations of *C. cartilaginea* fruit extract over time was monitored.

Wound-Healing Scratch Assay
The effect of the *C. cartilaginea* fruit extract on the migration of the prostate cancer 22RV1 cell line was determined by using a wound-healing scratch assay [26]. Prostate cancer 22RV1 cells were grown to 75% confluency on 12-well plates and allowed to adhere at 37 °C with 5% (v/v) CO2 overnight. Consistently, shaped wounds were made using a sterile 10 µL pipette tip across each well, creating a cell-free area, based on the technique described by Cormier *et al*. [27]. Cultures were gently washed with PBS to remove loose cells. The cells were then exposed to varying concentrations of *C. cartilaginea* fruit extract (0.5, 5, 10, 15, and 20 µg/mL). Cells for the control conditions were also scratched, washed, and maintained in a culture medium supplemented with 10% FBS at 37 °C with 5% (v/v) CO2 positive control.
Immediately after the scratch, at 24, 48, and 72 hours, at least three images of the scraped area were captured using the DS-U3 digital camera controller (Nikon Instruments, USA) and imaging software (NIS-Elements version 4.0). Two independent experiments were performed, using two wells for each concentration. The same scratched area was selected for the measurements at each time of the study.

Measurement of Wound Area
The wound area was calculated by manually tracing the cell-free area in captured images of different periods using the ImageJ software (Fiji-ImageJ version 1.51 h) [28]. Firstly, the imaging software was calibrated to measure the area in microns instead of a pixel using a scale slide (Graticules LTD, Tonbridge, UK). Under normal conditions, the wound area will decrease over time, and the migration rate can be expressed as the change in the wound area over time. We measured the percentage of area reduction or wound closure. The closure percentage will increase as cells migrate over time [29]:

$$\text{Wound closure \%} = \left[\frac{\text{At} = 0\text{h} - \text{At} = \Delta\text{h}}{\text{At} = 0\text{h}}\right] \times 100\% \quad (1)$$

At=0h is the area of the wound measured immediately after scratching (t = 0 h).
At=Δh is the area of the wound measured h hours after the scratch is performed.

Statistical Analysis
The data were analyzed using GraphPad Prism version 8.2.1. (Prism 8). Significant differences between the treated groups and the control were determined by one-way ANOVA using Dunnett's multiple comparisons tests. Results are expressed as mean ± standard deviation (SD) at a significance level of $p < 0.05$.

Results and Discussion

Effect of the C. cartilaginea Fruit Extract on the Cellular Proliferation of the 22RV1 Cell Line
The cytotoxicity effect of the *C. cartilaginea* fruit extract on the cellular proliferation of 10,000 cells/well of prostate cancer 22RV1 cell line, which was evaluated by the MTT assay, is presented in **Table 1**. At 24 h post-treatment, despite the increased resistance of the cells to *C. cartilaginea* fruit extract, there was a slight reduction in the number of viable cells in multi-well plates in parallel with the increase in C. cartilaginea fruit extract dose compared to untreated cells. As the concentrations of *C. cartilaginea* fruit extract increase from 0.5 to 20 µg/mL, the mean ± SD decreases. The percentage of viable cells decreases from 100% for the untreated cells to 63.86% for the treated cells with 20 µg/mL *C. cartilaginea* fruit extract. There was a dose-dependent decrease in the percentage of viable cells compared to the control (inverse relationship between cell cytotoxicity and concentration). Cell cytotoxicity increases with the increased concentration of the extract, starting from 4.66% to 36.14% for the concentration of 20 µg/mL *C. cartilaginea* fruit extract **(Table 1, Figure 1)**. There was a significant correlation between the concentrations and cytotoxicity. As the incubation period increased to 48 h and 72 h, the number of viable cells in the control increased and the reduction in the number of viable cells in the treated wells became evident. Starting from the controls, the mean ± SD decreases with increasing the concentration of *C. cartilaginea* fruit extract. Furthermore, the percentage of viable cells decreases from 100% for the untreated cells to 17.54% for treated cells, and the cell cytotoxicity rises to 82.46 **(Table 1, Figure 1)**. There were no significant differences in the mean ± SD of 0.5 µg/mL of *C. cartilaginea* fruit extract compared to controls. There was a significant correlation between the concentration of the *C. cartilaginea* fruit

extract and cell viability. The cytotoxicity evaluation of the *C. cartilaginea* fruit extract against a prostate cancer 22RV1 cell line for incubation periods of 24, 48, and 72 h is shown in **Figure 1**.

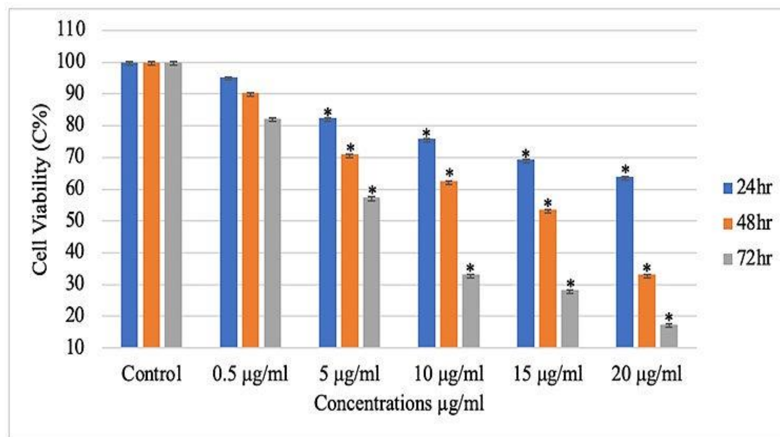

Figure 1. Effect of the *C. cartilaginea* Fruit Extract on the Cell Viability 24hr, 48hr, and 72hr post-treatment, at a cell density of 10,000 cells/ well. Bars represent the mean ± SD. * is a significant difference at the level of p < 0.05.

Table 1. Effect of the *C. cartilaginea* fruit extract on the cell viability 24, 48, 72hr post-treatment, at a cell density of 10,000 cells/ well.

24-hour post-treatment				
Concentrations	**Mean ± SD**	**C%**	**Cell Cytotoxicity %**	**Adj. *P*-Val**
Control	0.4637±0.5087	100	-	-
0.5 µg/ml	0.4421±0.03840	95.34	4.66	0.6667
5 µg/ml	0.3818±0.03367	82.34	17.66	* 0.0003
10 µg/ml	0.3522±0.02575	75.95	24.05	* <0.0001
15 µg/ml	0.3212±0.03203	69.27	30.73	* <0.0001
20 µg/ml	0.2961±0.04991	63.86	36.14	* <0.0001
48-hour post-treatment				
Concentrations	**Mean ± SD**	**C%**	**Cell Cytotoxicity %**	**Adj. *P*-Val**
Control	0.6857±0.09010	100	-	-
0.5 µg/ml	0.6192±0.1304	90.30	9.70	0.2969
5 µg/ml	0.4863±0.07583	70.92	29.08	* <0.0001
10 µg/ml	0.4293±0.06511	62.61	37.39	* <0.0001
15 µg/ml	0.3679±0.05027	53.65	46.35	* <0.0001
20 µg/ml	0.2272±0.04441	33.13	66.87	* <0.0001
72-hour post-treatment				
Concentrations	**Mean ± SD**	**C%**	**Cell Cytotoxicity %**	**Adj. *P*-Val**
Control	0.9101±0.1257	100	-	-
0.5 µg/ml	0.7492±0.2089	82.32	17.68	0.1568
5 µg/ml	0.5214±0.3170	57.29	42.71	* <0.0001
10 µg/ml	0.3010±0.02190	33.07	66.93	* <0.0001
15 µg/ml	0.2578±0.01255	28.33	71.67	* <0.0001
20 µg/ml	0.1596±0.03138	17.54	82.46	* <0.0001

Data are expressed as mean±SD, C%: cell viability, and Adj. *P*-Val: Adjusted. *P*-value, SD: standard deviation, **P*<0.05

Evaluation of Morphological Changes Upon Treatment with C. cartilaginea Fruit Extracts

The morphological alteration of prostate cancer 22RV1 cell lines after exposure to different concentrations of *C. cartilaginea* fruit extract (0.5, 5, 10, 15, and 20 µg/mL) over time was observed under an inverted light microscope at 20× magnification. The microscopic observations revealed that the *C. cartilaginea* fruit extract had a significant effect on cell death, and the mortality rate correspondingly increased with the increase in concentration. After 24 h of incubation, cells exhibited typical proliferation in the absence of C. cartilaginea fruit extracts. Cells presented intact morphology, uniform size, clear outline, and vigorous growth, all of which grew adherently. No contact inhibition was observed between cells, indicating that cells were in a very healthy state **(Figure 2A; 24 h)**. Treated cells with 0.5 µg/mL *C. cartilaginea* fruit extract displayed characteristic features similar to untreated cells. There were no noticeable changes in cell morphology **(Figure 2B; 24 h)**. Cells treated with

10, 15, and 20 µg/mL *C. cartilaginea* fruit extracts were heterogeneous in appearance, with some cells demonstrating a tight adherence to the tissue culture plate and others remaining less adherent and round. The reduction in the size of the cells could be a feature of early necrosis **(Figure 2D-2F; 24 h)**.

After 48 h of incubation, cells under control conditions appeared healthy and produced up to ~70% confluency **(Figure 2A; 48 h)**. The cells look normal and have a bright color, and clear boundaries, as well as branching and spreading on the surface of the tissue culture plate **(Figure 2A; 48 h)**. For treated cells with 0.5 and 5 µg/mL *C. cartilaginea* fruit extracts, there was no major presence of cell shrinkage, rounding, and partial detachment **(Figure 2B and 2C; 48 h)**. Few cells exhibited morphological changes and their shape changed to circular **(Figure 2B and 2C; 48 h)**. On the other hand, the cells indicated the most prominent effects as they began to leach from the tissue culture plate and became flat and shrunken after incubation with 10 µg/mL *C. cartilaginea* fruit extracts **(Figure 2D; 48 h)**. After exposure to 15 µg/mL *C. cartilaginea* fruit extracts for 48 h, necrosis becomes evident. The majority of cells experienced a loss of their original morphology and had a swollen appearance **(Figure 2E; 48 h)**. Treating cells with 20 µg/mL *C. cartilaginea* fruit extracts displayed clearer changes in cell morphology comparable to those observed in cells treated with 15 µg/mL *C. cartilaginea* fruit extracts **(Figure 2F; 48 h)**.

At 72 h of incubation with *C. cartilaginea* fruit extracts, cells under control conditions appeared healthy and produced up to ~90% confluency. The cells appear to be over con-fluent, branching and spreading on the surface of the tissue culture plate since cells compete for the rest of the nutrients to survive **(Figure 2A; 72 h)**. At a concentration of 0.5 µg/mL *C. cartilaginea* fruit extracts, some cells had characteristic features of cell shrinkage and partial detachment **(Figure 2B; 72 h)**. The cells demonstrated morphological changes compared to the control. Cell shrinkage and losses were adherent to the plate after incubation with 5 µg/mL *C. cartilaginea* fruit extracts **(Figure 2C; 72 h)**. At a concentration of 10 µg/mL *C. cartilaginea* fruit extracts, dead cells leached from the culture plate and filled the media, revealing an interruption in the extracellular matrix, as well as inhibition of cell-to-cell contact **(Figure 2D; 72 h)**. Although wells were treated with 15 and 20 µg/mL *C. cartilaginea* fruit extracts, marked by the complete circularization of almost all cells and cloudy appearance **(Figure 2E and 2F; 72 h)**. The cells exhibited significant morphological changes, such as a decrease in cellular volume, rounding, and shrinkage. Cells dramatically decreased in number, with almost no presence of viable cells **(Figure 2E and 2F; 72 h)**.

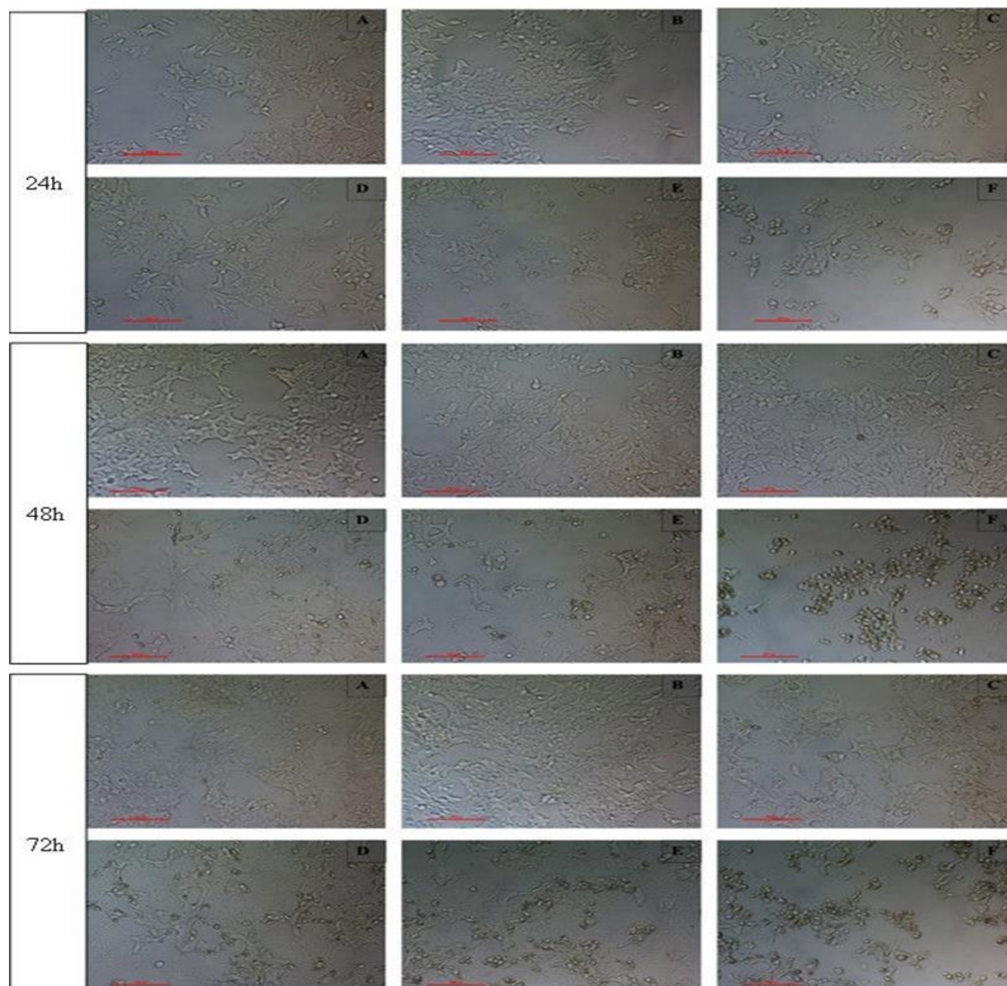

Figure 2. Morphological Changes Observation 24h, 48h, and 72hr post-treatment, **A.** A control untreated cells, **B.** 0.5 µg/ml, **C.** 5 µg/ml, **D.** 10 µg/ml, **E.** 15 µg/ml, **F.** 20 µg/ml, (magnification 20x). Bar = 500 mm

The Effect of C. cartilaginea Fruit Extracts on 22RV1 Cell Line Migration at 24, 48, and 72hr Post-treatment.
Scratched prostate cancer 22RV1 cells culture were pre-treated with varying concentrations of *C. cartilaginea* fruit extract to determine the percentage of reduction in the area of the scratch borders. All the cells were grown to 70 % confluency to ensure that imaging software can precisely identify the boundaries of the scratch. Immediately after the scratch, at 24, 48, and 72 hours, the total scratched areas of the control (untreated cells) and treated cells were measured **(Figures 3 and 4, Table 2)**. At zero hours, the initial total scratch area of untreated cells was 315688.3 µm with mean±SD of 47.40±99.2 µm. As time passes on to 72hr, the total area of the scratch area closed to 84422.9 µm with mean±SD of 105.24±125.5 µm. The initial total scratch area for treated cells with 0.5 µg/ml *C. cartilaginea* fruit extracts was 305253.3 µm with a mean±SD of 105.52±125.6 µm. Over time, the total area of the scratch reached 259266.4 µm with a mean±SD of 92.77±122.7 µm. At a concentration of 5 µg/ml of *C. cartilaginea* fruit extracts, the initial total scratch area was 230996.8 µm with mean±SD of 108.02±126.0 µm. And the total scratch area closes over time but at a lower rate to 204002.5 µm and mean±SD of 100.11±124.5 µm. When the cells were treated with 10 µg/ml *C. cartilaginea* fruit extracts, at zero hours, the total wound area was, 300977.6 µm with mean±SD of 94.97±123.3 µm. As the incubation period increases, the total area of the scratch opens up. Ultimately, at 72 hours of incubation, the total area opened to 391211.8 µm with a mean±SD of 65.82±111.6 µm. At zero hours, the total scratch area in treated cultures with 15 µg/ml *C. cartilaginea* fruit extracts, was 320546.0 µm with a mean±SD of 73.73±115.6 µm. There was an increment in the total area overtime to 410022.7 µm with mean±SD of 61.36±109.0 µm. The number of dead cells in the cell-free area was also noticed. The cell density across the scratch seems to be lessened and we had to withdraw the media to be able to capture an image of the scratch area **(Figure 4)**. At a concentration of 20 µg/ml, the initial total scratch area was 388649.9 µm with a mean±SD of 82.07±119.1 µm. As the incubation period passes on, the total area of the scratch widely opened up, the number of dead cells increases, and most of the cells appear to die rather than move across the scratch area. Also, we had to withdraw the media to be able to capture the image of the scratch area. This observation could be seen clearly after 72hr of incubation with a total area of 475422.7 µm and mean±SD of 73.81±115.6 µm **(Figures 3 and 4, Table 2)**.

Table 2. The Change in the Wound Area Overtime

Concentrations	Total Area (µm)	mean±SD
Control (0hr)	315688.3	47.40±99.2
Control (24hr)	127933.8	99.50±124.4
Control (48hr)	89097.1	106.56±125.8
Control (72hr)	84422.9	105.24±125.5
0.5 µg/ml (0hr)	305253.3	105.52±125.6
0.5 µg/ml (24hr)	289319.5	99.78±124.5
0.5 µg/ml (48hr)	260139.9	97.80±124.0
0.5 µg/ml (72hr)	259266.4	92.77±122.7
5 µg/ml (0hr)	230996.8	108.02±126.0
5 µg/ml (24hr)	213715.9	103.02±125.1
5 µg/ml (48hr)	212547.4	101.09±124.7
5 µg/ml (72hr)	204002.5	100.11±124.5
10 µg/ml (0hr)	300977.6	94.97±123.3
10 µg/ml (24hr)	324596.2	73.18±115.3
10 µg/ml (48hr)	346573.9	63.93±110.5
10 µg/ml (72hr)	391211.8	65.82±111.6
15 µg/ml (0hr)	320546.0	73.73±115.6
15 µg/ml (24hr)	350285.5	54.75±104.7
15 µg/ml (48hr)	381066.4	57.88±106.8
15 µg/ml (72hr)	410022.7	61.36±109.0
20 µg/ml(0hr)	388649.9	82.07±119.1
20 µg/ml(24hr)	420741.5	67.13±112.3
20 µg/ml(48hr)	456377.8	56.68±106.0
20 µg/ml(72hr)	475422.7	73.81±115.6

Data are expressed as the total area in microns (µm) and mean±SD, SD: standard deviation.

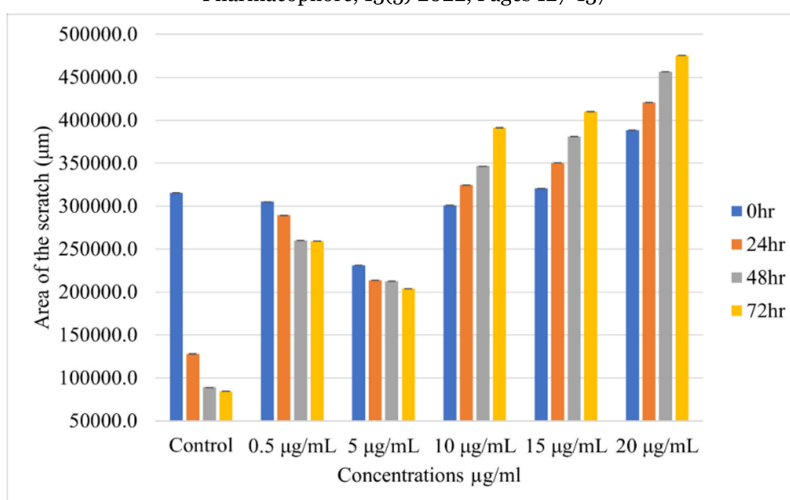

Figure 3. The Change in the Wound Area Overtime

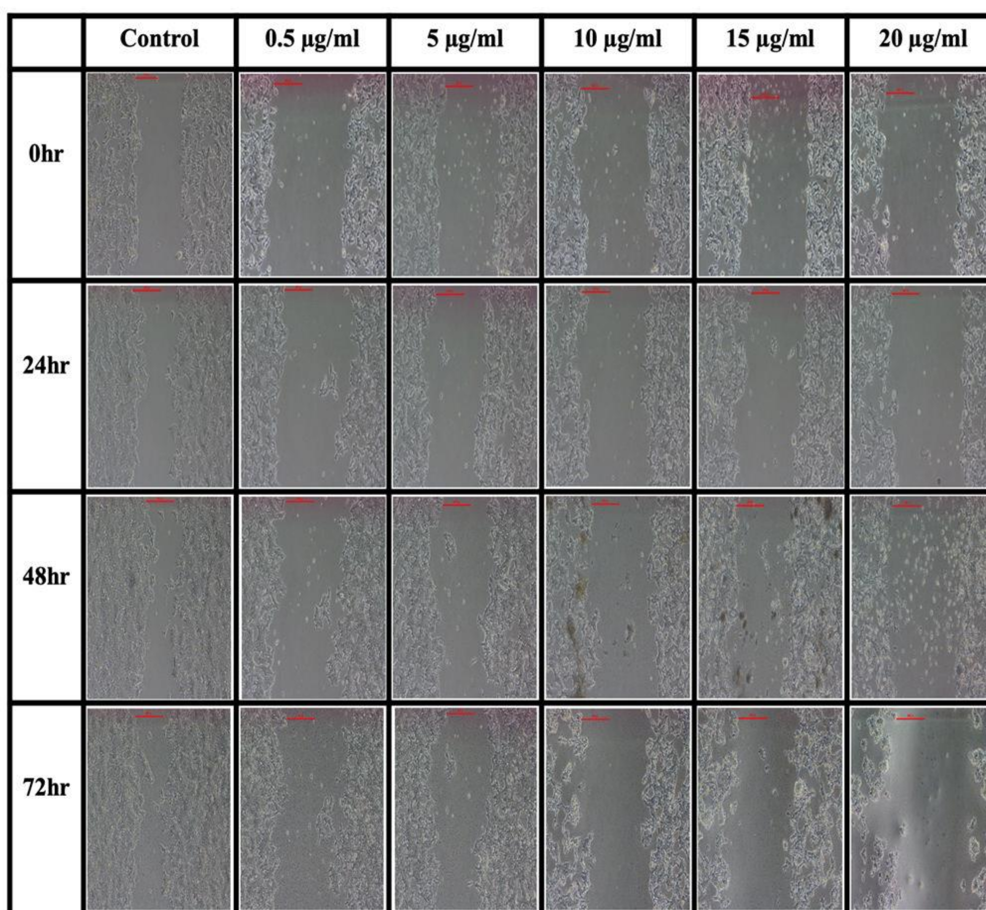

Figure 4. Effect of the *c. cartilaginea* fruit extract on prostate cancer 22RV1 cells migration. The pictures demonstrate the wound-healing assay at 0, 24, 48, and 72 hours of exposure to 0.5, 5, 10, 15, and 20 µg/ml *c. cartilaginea* fruit extract. The control indicates untreated cells. The magnification is 10x.

Cell migration in the scratch borders was evaluated by calculating the percentage of area reduction. The change in the closure percentage over time for the control and treated cells with different concentrations of *C. cartilaginea* fruit extract was estimated. As time passes, the percentage of wound closure increases from 59.47 to 73.26 %. At a concentration of 0.5 µg/ml, the percentage of wound closure also rises but at a lower rate from 5.22 to 15.07 %. At 24hr post-treatment, the wound closure percentage for the concentration of 5 µg/ml was 7.48 %. With time, the percentage increased to 11.69 %. In treated cultures with 10 µg/ml, the percentage of wound closure decreases compared with the untreated cells from -7.85 to -29.98 %. While the wound closure percentage for treated cells with 15 µg/ml was -9.28% after 24hr of incubation and decreased to -27.91 % at 72hr post-treatment. At a concentration of 20 µg/ml, the wound closure percentage was -8.26 %. Over time, the percentage dropped to -22.33% **(Figure 5)**.

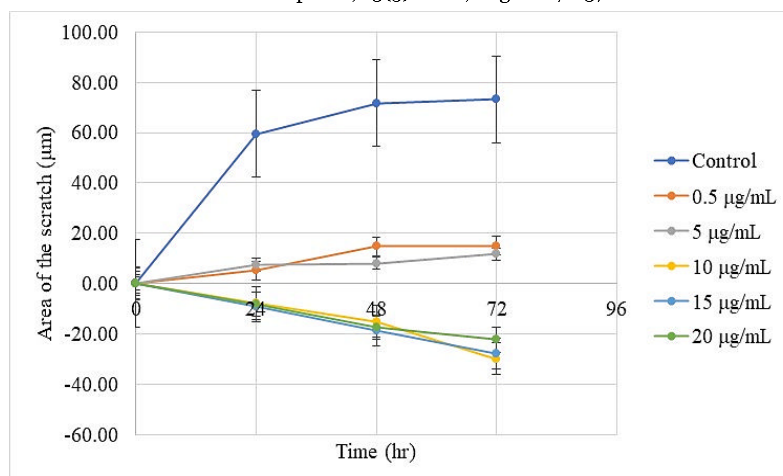

Figure 5. Wound Closure Percentage Overtime

Phenolics and flavonoids are well-known to have anticancer activity on various cancer cells and induce apoptosis [30]. Methanolic extract of *C. Cartilaginea* fruit extract contains a steroid, carbohydrate, alkaloid, saponins, cardiac glycosides, flavonoids, and phenolics [14, 31]. The fruit of *C. cartilaginea* extract was examined to characterize the phytochemical profiles of the plant to gain a better insight into the optimal consumption levels which could provide pharmacological significance concentrations in body fluids and tissues. Here, the anticancer activity of the ethanolic extract of *C. Cartilaginea* fruit along with apoptotic machinery has been evaluated against the 22RV1 human prostate cancer cell line. Our *C. Cartilaginea* fruit extract may seem to possess an effect against PCa affecting cell migratory capacity as the total scratch area widely opened and the numbers of dead cells increased up to ~ 70%.

In our study, we found that there was a difference between cell densities and viability. However, the cell viability does not follow one rhythm. MTT assay relies on a mitochondrial reductase to convert the tetrazole to formazan depending on the number of viable cells [32]. We anticipated that this observation may be due to natural variations of cellular metabolism or because slightly more cells were seeded in the wells due to small pipetting errors. In a concentration and time-dependent manner, *C. cartilaginea* fruit extract induces inhibition of the growth of 22RV1 prostate cancer cells. As the dose of *C. cartilaginea* fruit extract increase with increasing time, the prostate cancer cells' cytotoxicity increase. The difference between the prostate cancer cell line 22RV1 control and treated cells with 10, 15, and 20 µg/ml concentration of *C. cartilaginea* fruit extract was significant (<0.0001). Our result was novel since previous studies concentrated on other species of the Capparidaceae family. However, similar results were demonstrated by Khodaei *et al*. who showed that caper induced significant cytotoxicity in colon cancer (HT-29) cells followed by mitochondrial membrane potential collapse, reactive oxygen species overproduction, NAD-dependent deacetylase sirtuin-3 (Sirt3) activity alteration, and cell death. Cells were treated with different concentrations of the extracts containing 1, 5, and 10 mg/ml and were added to each well for 24, 48, and 72hr [33]. Furthermore, Khodaei *et al*. used the MTT assay for evaluating the effect of caper methanol extract on the proliferation of HT-29 cells. After 24, 48, and 72hr of treatment, the antiproliferative effect was evaluated and they represented the maximum cytotoxic effect of caper extract reported at 0.1 mg/mL. They suggested that the variation could be due to extracting type and different geographical regions [33]. Likewise, our results presented comparable results to Khodaei *et al*. as a significant reduction in viability was observed upon 24hr exposure of prostate cancer 22RV1 cells to *C. cartilaginea* fruit extract at 5 µg/ml ($P<0.0003$) and 10, 15, and 20 µg/ml ($P<0.0001$). Furthermore, a significant reduction in cell viability was also noticed after 48hr exposure of prostate cancer 22RV1 cells to C. cartilaginea fruit extract at these concentrations 5, 10, 15, and 20 µg/ml ($P<0.0001$). The reduction observed was significant ($P<0.0001$) after 72hr treatment with *C. cartilaginea* fruit extract.

C. cartilaginea's oil contained methyl isothiocyanate, isopropyl isothiocyanate, isobutyl isothiocyanate, ethyl isothiocyanate, and butane, 1-isothiocyanate [31, 34]. Crowley *et al*. studied the anticancer properties of dietary isothiocyanate (allyl-isothiocyanate, benzyl- isothiocyanate, and phenylethyl- isothiocyanate) and synthetic (phenylbutyl- isothiocyanate and phenylhexyl- isothiocyanate) on prostate carcinoma cells in vitro. They assessed the effect of isothiocyanate on PCa viability and cell cytotoxicity. Treatment of androgen-dependent (22RV1) and androgen-independent (DU145) cell lines with isothiocyanates outcomes in a dose-dependent reduction of cell viability. Rising concentrations (0-15 µM) of isothiocyanate for 24 and 48hr were used for carcinoma cells. MTT assay was used to determine the ability of these isothiocyanates to decrease cellular viability [35]. Upon 24hr exposure of prostate cancer 22RV1 cells to allyl-isothiocyanate, a significant reduction in viability was observed at 10 µM ($P<0.05$) and 15 µM ($P<0.0001$). The 48hr exposure resulted in a further significant reduction in cell viability at these concentrations ($P<0.0001$) in contrast to the control alone. Natural isothiocyanate treated (5 µM) DU145 cells, demonstrated no significant reduction of migration as compared to the control. On the other hand, benzyl-isothiocyanate, allyl- isothiocyanate, and phenylbutyl- isothiocyanate decreased the motility of prostate cancer 22RV1 cell ($p<0.05$) with a greater than twofold in contrast to control. The long-chain isothiocyanate phenylethyl- isothiocyanate, did not show inhibition on cellular migration in either prostate cancer cell line. The reduction observed was not significant ($P>0.05$) after 72hr treatment with natural isothiocyanate (allyl- isothiocyanate, benzyl- isothiocyanate, and phenylethyl- isothiocyanate)

of the 22RV1 cell line. However, the treatment of DU145 prostate carcinoma cells with natural isothiocyanate caused a more than 40% reduction compared to the control. Synthetic isothiocyanate exposure of both prostate carcinoma models did not inhibit the invasive capacity of these cells (P>0.05) [35]. Interestingly, in agreement with Crowley1 *et al*. result, we observed a significant reduction in prostate cancer 22RV1 cells viability upon 24hr exposure to C. cartilaginea fruit extract at 5 µg/ml (P<0.0003) and 10, 15, and 20 µg/ml (P<0.0001). Furthermore, a significant reduction in prostate cancer 22RV1 cell viability was also noticed after 48hr exposure to C. cartilaginea fruit extract at these concentrations 5, 10, 15, and 20 µg/ml (P<0.0001). The reduction observed was significant (P<0.0001) after 72hr treatment with C. cartilaginea fruit extract. Moharram *et al*. examined the cytotoxic effect of the methanolic extracts of C. cartilaginea leaves, stem, and twigs against the human lung carcinoma (A549) cell line. The results showed that the twigs have a higher cytotoxic effect with a concentration of 57.5 µg/ml, followed by the stem extract with a concentration of 240 µg/ml [34].

Accumulative studies have similarly focused on the cytotoxic effect of a species belonging to the Capparidaceae family on different types of cancer cell lines. Mansour *et al*. assessed the hydroethanolic extract of C. spinosa L. for cytotoxic effect against the human epithelial cervical cancer cell line (HeLa). The extract exhibited an inhibition effect on cell growth in a dose-dependent manner [36]. Whereas Moghadamnia *et al*. studied the effects of C. spinosa L. extract on breast carcinoma (MCF-7), human osteosarcoma (Saos), HeLa, and Fibroblast cancer cell lines. In comparison with normal cells, the effectual dose was 250 µg/ml 72 hr post-treatment [37]. Kulisic-Bilusic *et al*. inspected the effect of C. spinosa L. aqueous infusion and essential oil on the proliferation of colon cancer (HT-29) cell lines for 15 and 72hr. At a concentration of 0.1 g/l, aqueous infusion exhibited a higher antiproliferative effect on HT-29 cells than the essential oil after 15 and 72hr [38]. Likewise, the effect of C. spinosa L. ethanolic extract in a dose-dependent manner was investigated by Sheikh *et al*. on the liver cancer (PLC/PRF/5) cell line. The growth inhibitory effect of the ethanolic extract was the most at a concentration of 5000 µg/ml [39]. Karamallah *et al*. used the MCF-7 cell line to estimate the cytotoxic effect of C. spinosa L. extract. The cells were treated for 24, 48, and 72hr with the different concentrations of the extract (12.5, 25, 50, 100, and 200 mg/ml). The evaluation showed that 70 mg/ml was the effective concentration [40]. On reflection, the cytotoxicity effect of the extracts on cellular proliferation may be due to extracting type and different geographical regions of the plant beside the part used from the plant. Taking into consideration that the cell line type, seeding densities, incubation periods, and the concentration used also play a role in the variety of outcomes.

In the present study, we evaluated the morphological changes in prostate cancer 22RV1 cell lines upon treatment with different concentrations of *C. cartilaginea* fruit extracts (0.5, 5, 10, 15, and 20 µg/ml) over time under an inverted light microscope. Reduction in the size of the cells treated with 10, 15, and 20 µg/ml for 24,48, and 72hr was noticed. Treated cells became flat and shrunken with the appearance of early necrosis features, along with dramatic morphological and circularization of almost all cells was obvious toward 72hr. However, Sheikh *et al*. observed the effects of different concentrations of C. spinosa L. ethanolic extract (150, 310, 620, 1250, 2500, and 5000 µg/ml) on morphological characteristics of the PLC/PRF/5 liver cancer cell line. The result showed that the morphology of the cells treated with 1250 µg/ml for 48hr changed dramatically, exhibiting hallmark features of apoptosis including cell shrinkage with an irregular shape, reduction of cell volume, plasma membrane blebbing, nucleus pigmentation, and apoptotic bodies [39]. Whereas, Zhang *et al*. evaluated the morphological changes in DU145 cells after exposure to three flavonoids (genistein, luteolin, and quercetin) using a normal inverted light microscope. Treated cells with different concentrations of the three flavonoids (20, 40, 80, and 100 µM) for 24hr displayed a morphological characteristic of cell swelling and damage to the plasma membrane [40]. For all that, the microscopic observations revealed that the *C. cartilaginea* fruit extract had an outstanding effect on cell death, and the mortality increased correspondingly with the concentration increment. The changes in the cell morphology may be due to interruption of the extracellular matrix, as well as inhibition of cell-to-cell contact.

The effect of *C. cartilaginea* fruit extract on the migration of the prostate cancer 22RV1 cell line was determined by a wound-healing scratch assay in a dose and time-dependent manner. A similar study evaluated the effect of quercetin on the migration of prostate cancer (PC-3) cell lines. The observation revealed that the migration rate of PC-3 cells was decreased upon quercetin treatment [41]. Yang *et al*. also studied the effect of quercetin on PC-3 cell migration. Their results of the wound healing assay demonstrated that the migratory rate of PC-3 cells was significantly decreased after treatment with quercetin (P<0.05) [42]. Correspondingly, our extract significantly reduces the migration rate of prostate cancer 22RV1 cells. Not to mention that the number of dead cells in the cell-free area was noticed at a higher concentration and the cell density across the scratch decreased with time and the total scratch area of untreated cells was sealed.

Conclusion

As cancer is one of the main causes of death worldwide, the progression of which may be a consequence of defects in apoptotic machinery, so we proposed that agents such as medicinal plants, which can modulate apoptosis in human PCa cells may be valuable in the therapy of PCa. Phytochemicals are playing an important role currently to treat various diseases and have been proven to significantly treat cancer. From the literature it was stated that *C. cartilaginea* are rich in flavonoids such as kaempferol-3-O-rutinoside, quercetin-3-O-rutinoside, quercetin-7-O-rutinoside, and quer-cetin-3-glucoside-7-O-rhamnoside. Phytoestrogens and flavonoids have concerned a lot of scientists, due to their putative health-promoting activities possibly resulting from their antioxidant effects. Still, its overall biological impact remains controversial, generally because of incomplete information about its bioavailability, endogenous dynamics, and the relative contribution of different types of

conjugates in humans and animals. Our study showed that *C. cartilaginea* fruit extract significantly reduces the migration rate of prostate cancer 22RV1 cells and reduces its wound healing properties. However, further investigation is needed to identify its exact mechanism of action before introducing it as a new anticancer medication. Our study has some limitations. One of the limitations is that we didn't use a normal cell line for comparison but we have positive control as the control group for *C. cartilaginea* fruit extract. Also, the fruit is seasonal, and by the time the fruits arrived at the lab, many fruits are rotten. In future studies, we suggest the use of other parts of the *C. cartilaginea* plant such as the stem, roots, and leaves on human normal prostate and prostate cancer cells. Also, study the effect of the *C. cartilaginea* fruit extract on the identified genes expression level.

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