

Antiproliferative Activity of *Hypericum perforatum*, *Achillea millefolium*, and *Aloe vera* in Interaction with the Prostatic Activity of CD82

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Abstract

Background: In recent years, prostate cancer prevails as one of the lead cancers affecting men. Currently, prostate cancer research involves the phytochemical study of plants with anti-tumour effects. This study compares the anti-tumour effects of three plant species indigenous to Iran and their interaction with cluster of differentiation (CD)-82 protein, a therapeutic target found in prostate cancer cells.

Methods: The extracts of *Hypericum perforatum*, *Achillea millefolium*, and *Aloe vera* were prepared and their toxicological, cellular and gene expression responses were evaluated in PC-3 human prostate cancer cells and normal human chondrocyte cell line C28/I2. They were exposed to different concentrations of the plants (10 mg/mL, 5 mg/mL, 1 mg/mL, 100 µg/mL, 10 µg/mL, and 1 µg/mL) at three exposure time points (24, 48, 72 hours) to determine cancer cell cytotoxicity and gene expression profiles.

Results: Half-maximal inhibitory concentration (IC₅₀) in PC-3 cells ranged from 0.6 to 8.5 mg/mL for *H. perforatum* extract, from 0.4 to 7.5 mg/mL for *A. Millefolium* extract, and from 0.2 to 8.0 mg/mL for *A. vera* extract in a time-dependent manner. *A. vera* extract caused the highest cell death levels in PC-3 cells (94%) and C28/I2 cells (57%) after 48 hours. A 1.97-, 3.00-, and 3.48-fold increase in relative gene expression of CD82 was observed for *H. perforatum*, *A. millefolium*, and *A. vera* extracts, respectively

Conclusions: *A. vera* and *A. millefolium* extracts are a selective inhibitor of prostate cancer cells and a potent activator of CD82 expression.

Keywords: CD82, Gene expression, Herbal medicine, Prostate cancer.

Introduction

Prostate cancer is one of the leading causes of mortality amongst men in several developed nations, and across the globe. Several therapeutic approaches have been proposed to treat cancer, for example, chemotherapy. In most cases, however, the non-selective and non-specific toxicity of chemotherapy drugs can result in the destruction of healthy tissue. Therefore, scientific efforts are shifting towards alternative therapies that use plant extracts as an alternative to fighting cancer. Testing anti-tumour candidates and screening for plant-

based extracts is very important prior to conducting clinical evaluation (3).

Hypericum perforatum L., known as St. John's Wort, belongs to the Hypericaceae species (4) and has been found to have anti-tumour properties due to the presence of complex compounds, namely hypericin, α -terpineol, β -carotene, caffeic acid, isoquercitrin, kaempferol, gallic acid, limonene, rutin, and vanillic acid (5). Our recent finding supports its antiproliferative activity against different cancer cells (5, 6). *H. perforatum*

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functions as a serotonin-reuptake inhibitor and serotonin antagonist in prostate tumors. Using the PC-3 human prostate adenocarcinoma cell line, its methanolic extract could successfully prevent cellular growth by 80% (7).

Achillea millefolium is another traditional plant, known as yarrow from the Asteraceae family that is widely distributed amongst Europe, Asia, North Africa and North America (8, 9). Several studies highlight the anti-tumour effects of *A. millefolium* extracts in human cancer cell lines (10, 11). It has been postulated that phenolic acid derivatives, and flavones are involved in its bioactivity (11).

Aloe vera (*Aloe barbadensis*), a popular herbal compound with an extensive medicinal history for several ailments, is known to have preventive and therapeutic impacts against a range of cancers (12-14). Lectin, aloin, barbaloin, aloe-emodin, and aloesin are some of its bioactive components substantiated for chemoprevention, immune potentiating properties, antimutagenic activity, anti-proliferation, apoptosis-inducing effects, antioxidant actions, and anti-metastatic functions (12, 13).

CD82, known as KAI-1, is a tetraspanin membrane protein and serves to inhibit metastasis in human cancers. Indeed, its expression is suppressed in many malignant cells (15, 16). This gene is located on chromosome 11p, and published studies have revealed its involvement in the dissemination of prostate tumor cells (17, 18). Moreover, it is involved in extensive physiological processes, such as detachment, motility/invasion, and cell survival (16). In this study, the potential of *H. perforatum*, *A. millefolium*, and *A. vera* to selectively inhibit cancer cell growth and interfere with CD82 function was investigated in PC-3 and C28/I2 cell lines.

Materials and methods

Preparation of plant extracts

H. perforatum seeds, aerial parts of *A. millefolium*, and leaves of *A. vera* were collected from National Botanical Garden of Iran (NBGI).

All three plants were compared with those species in the Medical Plant Farm, Jahad Daneshgah, Islamic Republic of Iran. Their names have been checked with www.theplantlist.org.

As for *H. perforatum*, 180 g powder of the plant material was extracted with 100 mL methanol. Following 24 hours, the mixture was filtered and then evaporated under vacuum. Thereafter, 10 mg of *H. perforatum* extract was put into a flask, and 70 mL of methanol was added in a shaking water bath at 60 °C.

The aerial parts of *A. millefolium* were initially rinsed and dried before boiling in water for 10 minutes. The extract was incubated for 30 minutes at room temperature and then passed through a filter. A 10 mg/mL of the extract was solved in 5 mL of de-ionized water in a shaking water bath at 60 °C.

The whole leaves of *A. vera* were cut into thin pieces and dried at shade and room temperature. After grinding in a mixer, distilled water was added, and the resultant mixture underwent centrifugation at 1000 ×g, followed by filtration. A 10 mg of *A. vera* extract was solved in 5 mL of de-ionized water at ambient temperature. The final solutions of each plant at a concentration of 2 mg/mL were prepared in RPMI medium prior to the next use.

Determination of phenolic content

The Folin–Ciocalteu reagent assay was used to measure the total phenolic content (19). Briefly, 2 N Folin–Ciocalteu reagent was mixed with 0.5 mL of EOs. After five minutes, 2 mL of 75 g/L sodium carbonate was added to the mixture. Afterward, absorbance was determined at 760 nm.

Cell culture

There were two cell lines in this study obtained from the Pasteur Institute of Iran: human prostate cancer cell line PC-3 and normal human chondrocyte cell line C28/I2. They were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, 2 g/L bicarbonate, 100 U/mL penicillin, and 100 µg/L streptomycin at 37 °C in a 5% CO₂ humidified atmosphere.

Determination of cell viability

The tetrazolium (MTT) test was employed to calculate cell viability. Briefly, PC-3 and C28/I2 cells (20,000 cells/mL) were cultured in 96-well

plates (BD Biosciences, USA), each well of which contained 200 μ L of supplemented RPMI medium. They were incubated for 24 hours before treating with the different concentrations of the plant extracts at 1 μ g/mL, 10 μ g/mL, 100 μ g/mL, 1 mg/mL, 5 mg/mL, and 100 mg/mL for 24, 48, 72 hours. Thereafter, 5 mg/mL MTT (Sigma, USA) solution was added to the wells, and the cells underwent an extra incubation for 4 hours at 37 °C. Following the addition of 100 μ L of dimethyl sulfoxide (DMSO; Sigma, USA), the optical density was recorded at 540 nm.

Estimation of IC₅₀ values

The IC₅₀ values were determined by ELISA and estimated from concentration–cell viability curves through linear regression. Measurements were conducted at six concentrations (1, 10 and 100 μ g/mL; 1, 5 and 100 mg/mL).

Analysis of CD82 gene expression

The cells were exposed to the plant extracts at twice dose of IC₅₀ as followed: PC-3 + *H. perforatum* (S1); PC-3 + *A. millefolium* (S2); PC-3 + *A. vera* (S3); C28/I2 + *H. perforatum* (S4); C28/I2 + *A. millefolium* (S5); C28/I2 + *A. vera* (S6); PC-3 (S7); C28/I2 (S8). The RNA was extracted from the cell lines by Trizol reagent (Invitrogen, USA), and spectroscopy was utilized to photometrically detect the purity and concentration of the RNA samples by a NanoPhotometer® (Implen GmbH, Munich, Germany). Total RNA was reversed transcribed by cDNA synthesis kit (Roche Applied Science, Germany). Determination of CD82 expression was carried out by quantitative real-time polymerase chain reaction (PCR) using a SYBR Green assay (Applied Biosystems, USA). In this study, these primers were used: CD82 forward, 5'-GCTCATTCGAGACTACA ACAGC-3' and reverse, 5'-GTGACCTCAGGGCGAT TCA-3'; GAPDH forward, 5'-GGATGCTGGAGGTCTGCGAGGAAC 3' and reverse, 5'-GAGAGGAAGCGTGTGAGGCGGTAG 3'.

Statistical analysis

All measurements were performed in three replicates. The Kolmogorov–Smirnov tests were

performed to ensure normal data distribution. Given normal distribution, student's t-test was initially conducted to determine any significant difference at P-values <0.05 (SPSS 19.0 software Package, IBM Inc., Chicago IL, USA). If normal distribution evaded, the non-parametric equivalent was used.

Results

The yield was 13.94 ± 2.63 %wt/wt for the methanol extract of *H. perforatum*, 29.76 ± 2.21 %wt/wt for the aqueous extract of *A. millefolium*, and 3.41 ± 0.69 %wt/wt for the aqueous extract of *A. vera*. The ordering of the extracts in terms of total phenol content from highest to lowest was *H. perforatum* (95.28 ± 7.54 mg GAEs/g extract), *A. millefolium* (39.67 ± 1.05 mg GAEs/g extract), and *A. vera* (0.106 ± 0.007 mg GAEs/g extract).

PC-3 cells were incubated with six different concentrations of the herbal extracts for 24, 48, and 72 hours. Their effects on cell viability are presented in Figure 1. The treatment of PC-3 cells with *H. perforatum* considerably reduced growth in a dose- and time-dependent manner ($p < 0.05$). Concentrations of *H. perforatum* below 1 mg/mL carried no significant effect after 24 hours ($p > 0.05$), whereas a marked decline of at least 30% was found at 1, 5, and 10 mg/mL as compared with the control ($p < 0.05$). The 48- and 72-hour post-exposure to *H. perforatum* caused notable reductions in the cell counts at 100 μ g/mL, 1, 5, and 10 mg/mL ($p < 0.05$). As for *A. millefolium*, Figure 1B shows a dose- and time-dependent decrease in the number of the treated cells (Fig. 1B). The concentrations of *A. millefolium* extract at 1, 5, and 10 mg/mL resulted in statistically significant growth inhibitions after 24 hours as opposed to the control ($p < 0.05$). Longer exposure of the cells to *A. millefolium* extract lowered the minimum concentration associated with substantial changes in the cell count to 100 μ g/mL for 48 hours and 10 μ g/mL for 72 hours ($p < 0.05$). As can be seen in Figure 1C, significant declines in the cell growth started with 1 mg/mL (33%), 100 μ g/mL (40%), and 10 μ g/mL (38%) after 24-, 48-, and 72-hour treatment with *A. vera* extract ($p < 0.05$) (Fig. 1C).

Figure 2 displays IC₅₀ for each herbal extract at different time points (Fig. 2). It was figured out that IC₅₀ changed at different exposure time points,

and *H. perforatum* extract showed the highest values. With changing the cellular model from PC-3 cells to C28/I2 cells, different herbal extracts induced selective cell death in different cell lines. For example, 82% of PC-3 cells underwent death upon 48-hour treatment with *H. perforatum* extract at 2.720 mg/mL (~ twice dose of IC₅₀), whereas C28/I2 cells remained viable up to 65% at similar conditions (Fig. 3). The mRNA gene expression of CD82 is drawn in Figure 4. The significant elevation of CD82 expression was observed

following exposure of PC-3 cells to *A. vera* and *A. millefolium* extracts as compared with the control. A 1.97-, 3.00-, and 3.48-fold increase in the relative gene expression of CD82 was determined for *H. perforatum*, *A. millefolium*, and *A. vera* extracts, respectively. Moreover, it was found that CD82 expression levels in the normal cells considerably reduced in reference to the cancerous cells ($p < 0.05$) (Fig. 4).

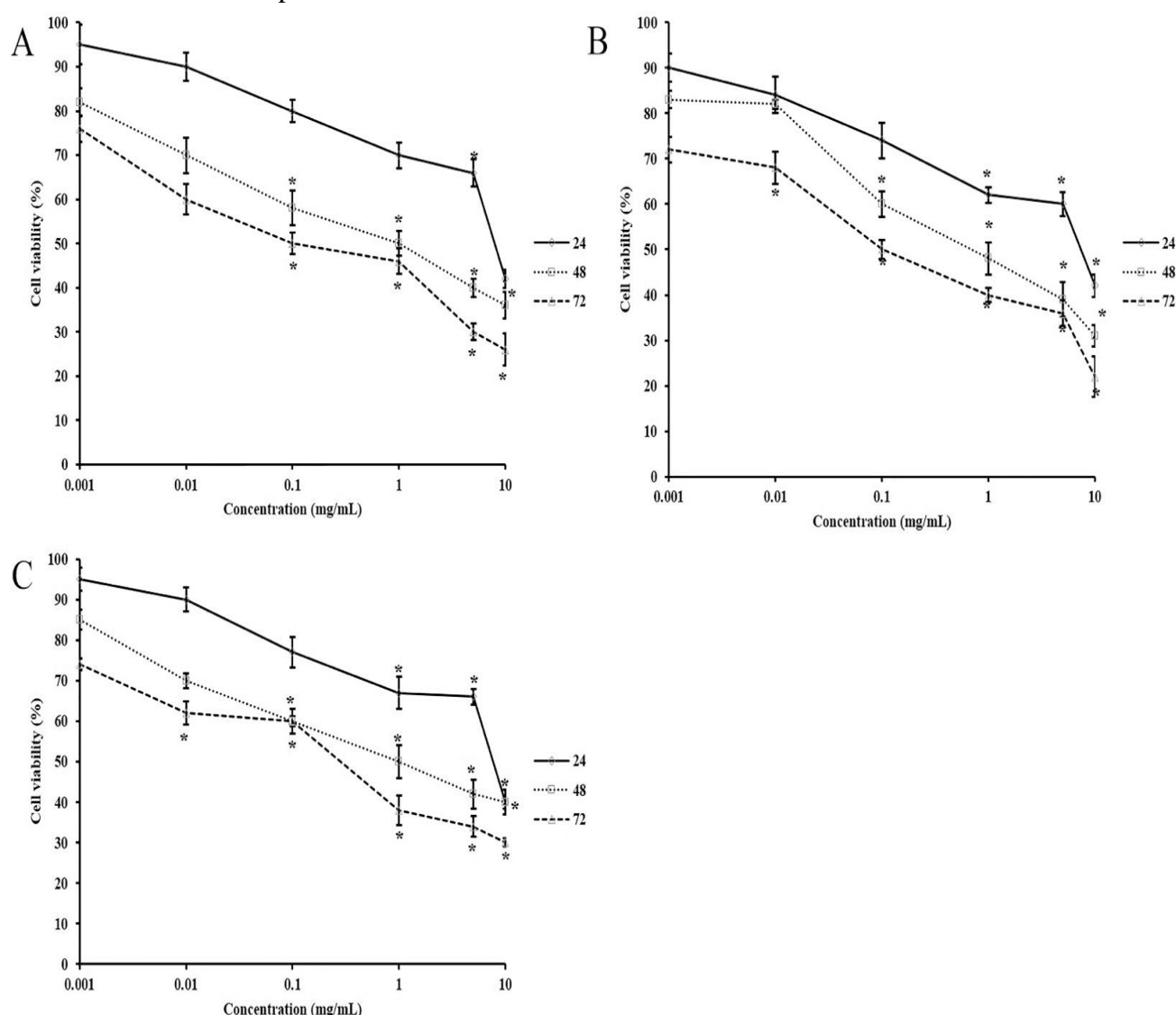


Fig. 1. The effect of *H. perforatum* (A), *A. millefolium* (B), and *A. vera* (C) extracts on the growth of PC-30 cells at different exposure time points (* $p < 0.05$).

Plant-induced Changes in CD82 Expression

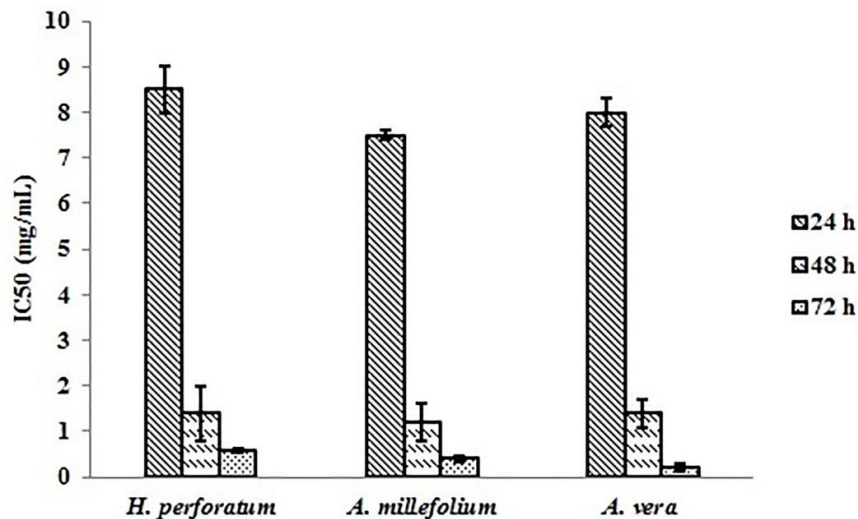


Fig. 2. The IC₅₀ values of *H. perforatum*, *A. millefolium*, and *A. vera* extracts at different exposure time points.

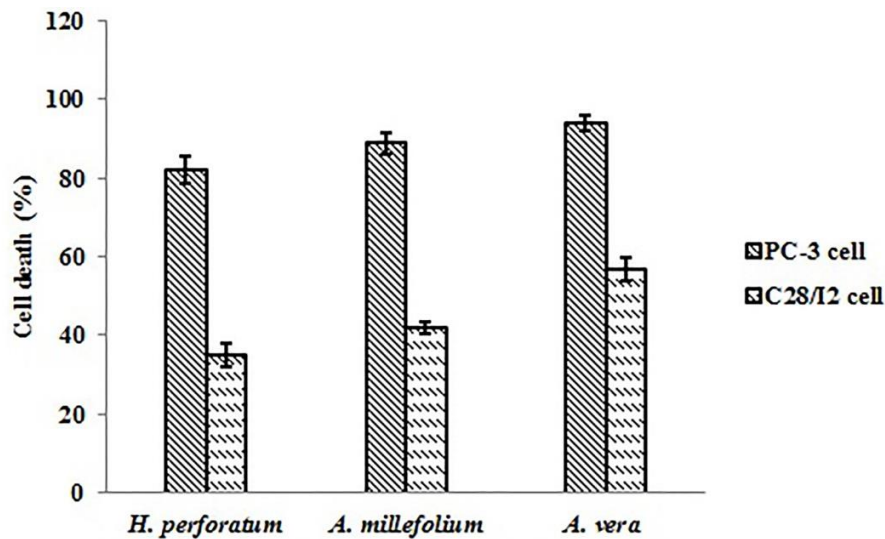


Fig. 3. The cytotoxicity of *H. perforatum*, *A. millefolium*, and *A. vera* extracts against PC-30 and C28/I2 cell lines.

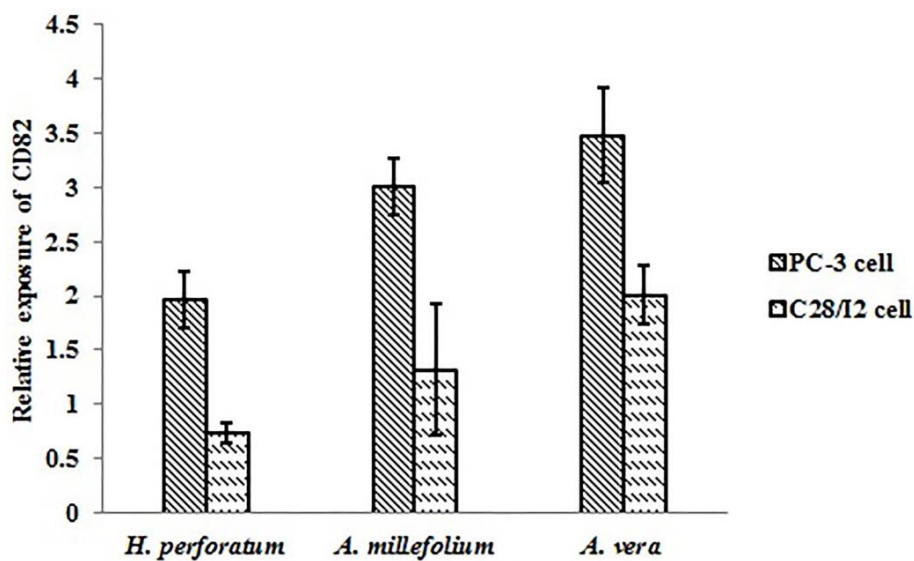


Fig. 4. The relative expression of CD82 after the administration of *H. perforatum*, *A. millefolium*, and *A. vera* extracts to PC-30 and C28/I2 cell lines (* $p < 0.05$).

Discussion

The extraction yield and phenolic content of *H. perforatum* were 13.94 ± 2.63 %wt/wt and 95.28 ± 7.54 mg GAEs/g extract, respectively. There have been three published papers about the methanolic extract of *H. perforatum* outside of Iran. Stamenković et al. prepared the methanol extract from *H. perforatum* collected from Leskovac in July with a yield of 20.16%. Upon re-extraction, the yield increased to 27.13% (20). Spiridon et al. with *H. perforatum* from Iasi, Romania developed a methanolic extract containing 50 mg GAEs/g extract (21). Another study by Öztürk et al. obtained the methanol extract using *H. perforatum* collected in Eskişehir, Turkey, in June. They found a yield of 39.6 %w/w and total phenol content of 319.34 ± 2.46 mg GAEs/g extract (22), which were inconsistent with our findings.

The extraction yield and phenolic content of *A. millefolium* were 29.76 ± 2.21 %wt/wt and 39.67 ± 1.05 mg GAEs/g extract, respectively. There has been three published papers about the aqueous extract of *A. millefolium*; two of which has been reported from Iran. Nouredini and Rasta obtained an aqueous extract from a plant found in Kashan, Iran. Their study yielded 36% phenolic content in their aqueous extract (23). Further, Eghdami and Sadeghi investigated the chemical composition and biological properties of *A. millefolium* extract obtained from Nowbaran, a district located in Saveh County in Markazi Province, Iran. The resultant extract had a phenolic content of 48.4 ± 2.7 mg GAEs/g extract (24). *A. millefolium* from a different region, Rhodope Mountains (Bulgaria), was used as an aqueous extract. In this study, the yield of the dried extract was 20 %w/w (25).

The extraction yield and phenolic content of *A. vera* were 3.41 ± 0.69 %wt/wt and 0.106 ± 0.007 mg GAEs/g extract, respectively. There have been five published papers about the aqueous extract of *A. vera*, however, no study has been performed on the plant species indigenous to Iran, yet. Regarding the aqueous extract yield of *A. vera*, previous evidence reports that 1.2% from Boksburg, South Africa (26), 5.4% from Kogi State (27), Nigeria, and 11.5% from Assam, India (28). Heş et al. showed a total phenol content of 17.85 mg GAE/g dry matter for its aqueous extract (29). Kammoun et al. tested the aqueous extract obtained from a plant indigenous to

Kairouan, Tunisia. The extraction yield and phenolic content were 66.67% and 2.072 ± 0.002 mg GAE/g of extract, respectively (30). Such differences found for each extract with the mentioned studies could be due to various collection times, environmental conditions, and geographic locations.

In this work, the administration of *H. perforatum* extract negatively affected PC-3 cells in a concentration and time dependent manner. This anti-proliferative effect in prostate cancer was corroborated by Martarelli et al., whose *in vitro* study showed an IC₅₀ of 0.42 mg/mL after a 3-day treatment (7). Consistently, the IC₅₀ in the present study was 0.60 mg/mL at 72 hours. The inhibitory impact of *H. perforatum* extract against prostate cancer cells was confirmed by Toros et al., as well (31). In another study by Colasanti et al., it was found that hypericin, one of the biologically active compounds of *H. perforatum*, induced phototoxic effects on PC-3 (32).

In contrast, exposure to *A. millefolium* extract led to considerable declines of PC-3 cells in a time and concentration dependent fashion. Comparing the methanolic extract of *H. perforatum* with aqueous extract of *A. millefolium*, it was found that the latter carried more destructive influences on PC-3 cells at the same exposure time. The pertinent literature reveals the antimicrobial, antioxidant, antitumor, and cytotoxic activities of the *Achillea* species (33, 34). *A. wilhelmsii*, a member of the *Achillea* genus, is widely distributed in Iran, has been found to have beneficial effects on breast cancer cell lines (34). The methanolic extract of *A. millefolium* elevated the antiproliferative activity induced by bleomycin in the prostate cancer cell (DU-145) without any significant toxicity on normal cells (human normal skin cell; HFFF2) (35). The growth of various human cancer cell lines, including PC-3 cells, was inhibited by the methanol and chloroform extracts of *A. millefolium* obtained from the root, stem, and floral parts (36). Only one study shows the antitumor potential of the aqueous extract of *A. millefolium* (37).

The treatment of PC-3 cells with *A. vera* extract produced detrimental effects on cellular growth in a

time and concentration dependent fashion by affecting CD82 function. A number of laboratory and clinical studies support the anticancer properties of *A. vera* (38-40). In a study on PC-3 cells, aloe emodin, an ingredient in *A. vera*, appeared to repress the growth of prostate cancer cells that act through the mammalian target, rapamycin complex 2 (41).

In reference to *H. perforatum* extract, *A. millefolium* and *A. vera* extracts demonstrated increasing potency and selective anti-proliferative activities by promoting CD82 function in both physiological and disease conditions. Also, it can be concluded that *A. vera* and *A. millefolium* extracts act as a potent activator of CD82 expression in human prostate cancer cells. Studies find that phosphoinositide 3-kinase (PI3K) activation, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) loss are principally involved in prostate tumorigenesis. These two changes stimulate Akt (also known as protein kinase B or PKB), which has implications for cell survival and cell

growth. Phosphorylation of Akt at Ser473 plays role in the complete stimulation of mammalian target of rapamycin 2 (mTOR2).

Additionally, mTOR2 is responsible for transforming human prostate epithelial cells deficient in PTEN causing tumor formation (42, 43). Liu et al. observed that Akt is up-regulated in PC-3 cells (41). In contrast, it has been previously reported that KAI1/CD82 attenuated the metastatic phenotype of H1299 lung carcinoma cells via the down-regulation of Rac1 expression (Rho guanosine triphosphatase; GTPase) through the PI3K/Akt/mTOR pathway (44). Currently, there is no evidence regarding the effect of KAI1/CD82 on the PI3K/Akt/mTOR pathway in prostate cancer cells warranting future investigation.

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