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Effect of *Achillea wilhelmsii* extract on expression of the human telomerase reverse transcriptase mRNA in the PC3 prostate cancer cell line

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Abstract. Evidence has indicated that human telomerase reverse transcriptase (hTERT) was overexpressed in prostate cancer (PCa). *Achillea wilhelmsii* (AW) is a plant that has been traditionally used for its medicinal properties. The aim of current study was to evaluate the effects of AW extract on a PCa cell line. The cytotoxic activity of the hydroalcoholic extract of AW was studied on the PCa PC3 cell line using MTT assay. Flow cytometry was used to evaluate the effects of the extract on the apoptosis. The expression of hTERT mRNA was analyzed by the reverse transcription-quantitative polymerase chain reaction method. The ELISA method was used to measure the levels of telomerase enzyme. The hydroalcoholic AW extract demonstrated the appropriate inhibitory effect in 150 μg/ml concentration (IC_{50}) on PC3 cell line following 48 h treatment. Treatment of the PC3 cells with AW resulted in a significant increase in early and late apoptotic cells and a decrease in live cells (P<0.001), in a dose-dependent manner. Moreover, the early apoptotic cells were significantly higher than late apoptotic cells. The hTERT mRNA expression was decreased following 24 h treatment of AW extract, although it was not different between 2, 4, 8 and 12 h treatments or 24, 48 and 72 h treatments. In addition, the hTERT concentration was significantly decreased following 24 h treatment of AW extract with the marginal P-value. There was no significant difference regarding hTERT concentration between 2, 4, 8 and 12 h treatments or 24, 48 and 72 h treatments. The hydroalcoholic extract of AW induced potent antiproliferative and apoptotic effects in PC3 cell line, which could be explainable by its high potency to inhibit expression of the prominent oncogene hTERT in PCa. Therefore, targeting telomerase represents a promising strategy for PCa therapy, and AW may have considerable potential for development as a novel natural anticancer agent.

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Introduction

Cancer is an international public health problem, has been considered as a hyper-proliferative disorder. Cancer contains of irreversible cell change, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis (1). More than 7 million deaths per year have been reported worldwide and it is predicted that the new cancer cases will reach 15 million every year by 2020. Prostate cancer (PCa) as the most commonly diagnosed urologic malignancy is the second most common cause of cancer death in men in developed countries (2). Androgen ablation is the most common and successful treatment for progressive PCa. However, presence of androgen-independent cells increase problems with PCa treatment (3). Hence, studies have attempted to replace the new targets for treatment of PCa including telomerase activity (4,5).

Telomerase is one of the most important targets of therapeutic intervention in various cancers (6). Telomeres are conserved, repetitive and large non-coding sequences located at the ends of eukaryotic chromosomes these sequences responsible for the protection of the genomic DNA integrity (7). During each round of cell division, DNA polymerase is not able to replicate the 5' ends of chromosomes in normal somatic cells; consequently, the telomeres are progressively shortened. When the telomeres reach a critically short length, cells leave the cell cycle and DNA damage responses such as apoptosis and replicative senescence are initiated. Hence, a limited number of passages could be seen in normal somatic cells due to their telomere length (7,8).

In most tumor cells, telomerase is responsible for telomere maintenance. Telomerase is a RNA dependent DNA polymerase, synthesize TTAGGG tandem repeats to the 3' end of the telomere on the lagging strand and confers cellular immortality. In this mechanism, telomerase is preventing telomere shortening and permitting continued proliferation (9). Telomerase contains two main subunits: The catalytic subunit called human telomerase reverse transcriptase (hTERT), which synthesizes the addition of TTAGGG tandem repeats to the 3' end; and the telomerase RNA template subunit, the second subunit is an RNA template complementary to the 3' end which used as primer for telomere synthesize (10).

Although continuing improvement in treatment of cancer is observed, more patients suffer from cancer treatment-related symptoms including fatigue, paresthesias and dysesthesias, chronic pain, anorexia, insomnia, limbs edema and constipation (11,12).

In traditional medicine, natural and herbal products have been considered as a main way for treatment of different diseases including infections and malignant diseases for a long time (13). In addition, several studies indicated that various herbal plants might have the anti-cancer effect by cell differentiation induction, inhibition of telomerase activity, induction of apoptosis in cancer cells and improving the immune system (13-15). Although it is believed that natural and herbal medicine have no side effects and lower dependency, different herbs could be toxic mainly in higher doses and long usage (16).

The *Achillea* genus (*Asteraceae*) with >130 species has been spread throughout the world, including Asia and Europe (17). In Iran, nineteen species of this genus have been recognized and approved for their essential oil (18). *Achillea* genus is widely used in traditional medicine (17,18). A previous study demonstrated that the *Achillea* species extracts exhibit antimicrobial, antioxidant and cytotoxic activities (19). *Achillea wilhelmsii* (AW) is a member of the *Achillea* genus, which is widely found in different regions of Iran. The effects of AW have been the topic of several studies, these effects may be attributed to high flavonoids and sesquiterpene lactones content (20,21). Evidences have reported that the extract and oil of *Achillea* species present cytotoxic effects against several cancer cell lines (19,22) and a recent study revealed cytotoxic activities of the hydroalcoholic extract of AW. on Breast cancer cell lines (22).

Although several research papers have evaluated the effects of AW extract on some cancers (19,22), there is no published report on the effects of AW extract in PCa cell lines. Therefore, in the current study, the effect of AW extract on apoptosis and expression of mRNA-hTERT gene was investigated in the PCa PC3 cell line.

Materials and methods

Plant material. AW C. Koch was collected from the Taftan area in Sistan and Baluchistan Province, Iran in spring 2015. The specimen was identified by one of the members of Research Institute of the University of Sistan and Baluchistan (Dr Ali Shahraki). The stem and leaf of AW were air dried in dark room and then powdered. Then, a solution (70% alcohol) of the AW was extracted using a Soxhlet extractor.

Chemicals and regents. RPMI-1640, fetal bovine serum (FBS), phosphate-buffered saline (PBS), streptomycin and Penicillin, trypsin, EDTA, trypan blue, were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). An Annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from BioVision, Inc. (San Francisco, CA, USA) and a hTERT colorimetric assay kit was procured from ZellBio GmbH (Ulm, Germany). All the other materials were of analytical grade and purchased locally.

Cell culture. PCa cell line PC3 was purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). PC3 cells were cultured at a density between 0.5-1.0x10^6 cells/ml in RPMI-1640 medium, containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin in a CO_2 incubator at 37˚C in a moistened atmosphere of 5% CO_2 and 95% air. When the PC3 cells reached logarithmic growth phase, the cells were sub-cultured and the experiments were performed immediately on the sub-cultured cells.

Cell treatments. For treatment, the powder of AW was dissolved in DMSO (HPLC grade) and kept frozen in the -20˚C. The cells were seeded into sterile 96-well microplates at a density of 5x10^3 cells/well. The cells were incubated overnight (37˚C, 5% CO_2 and 95% air) for adhering the cells to button plates. Then the medium was removed, and different concentrations of extract were added.

MTT assay. The anti-proliferative activity of the AW was assessed using MTT assay as previously described. Cells were plated onto 96-well plates at 5x10^3 cells/well in 0.1 ml RPMI medium. Following 24 h of incubation, cells were treated with 0, 12.5, 25, 50, 100, 150 and 200 μg/ml AW extract for 24, 48 and 72 h. Then, 20 μl MTT were added to each well and incubated at 37˚C for 4 h. Then the culture medium was discarded and finally, 200 μl DMSO was mixed with the cells for solubilization of Formazan crystals and incubated in a dark place for 2 h at room temperature.

Absorbance of cells in each well was measured at 570 nm by a microplate reader (Anthos 2020; Biochrom Ltd., Cambridge, UK). All experiments were conducted independently three times. The OD treated/OD control was considered as the cell viability percentage.

Cell apoptosis. Cell apoptosis was measured using Annexin V-Cy5 and propidium iodide (PI) staining and analyzed by flow cytometry (18). 1x10^5 cells per well were seeded in six well plates and incubated overnight, and then treated with 0, 50, 100, 150 and 200 μg/ml of HAWE for 48 h.

All cells were washed two times with cold PBS. Subsequently, the washed PC3 cells were stained in a 250 μl 1X binding buffer, 2.5 μl Annexin V/FITC, and 2.5 μl PI. Then, the stained cells were inculcated in a dark place for 15 min at room temperature.

Cell cycle analysis was conducted using a flow cytometer (Partec CyFlow Space, Sysmex Partec GmbH, Görlitz, Germany) with FloMax version 2.7 software. The distribution

of cells in different cell cycle phases including living, early apoptosis, late apoptosis and necrosis phases was assessed.

mRNA expression analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). PC3 cells ($2x10^5$ cells/well) were cultured in a six-well plates and incubated at 37˚C in a moistened air of 5% CO_2 overnight. Cells were then treated for 2, 4, 8, 12, 24, 48 and 72 h with a 150 μg/ml concentration of AW extract. Total RNA was isolated using RNx (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol (17). Isolated RNA was treated with RNase-free DNase I (Promega Corporation, Madison, WI, USA), and the concentration and purity of the isolated RNA were determined.

First strand cDNA was synthesized from the cells' extracted RNA by using the RevertAid First Strand cDNA Synthesis kit (cat. no. K1621; Thermo Fisher Scientific, Inc.) based on the manufacturer's protocol.

The expression levels of hTERT were detected using the SYBR ExScript RT-qPCR kit (Takara Bio, Inc., Otsu, Japan). PCR amplification was performed with Master PCR kit (CinnaGen, Tehran, Iran). PCR amplification for hTERT and β-actin mRNA consisted of 35 cycles (95˚C for 45 sec, 56˚C for 50 sec and 72˚C for 50 sec). To identify hTERT mRNA the following primers were used: forward primer, 5'-CGGAAG AGTGTCTGGAGCAA-3', reverse primer, 5'-GGATGAAGC GGAGTCTGGA-3' (23). To identify β-actin mRNA following primers were used: forward primer: 5'-AGAAAATCT GGCACCACACC-3', reverse primer: 5'-GGAAGGAAG GCTGGAAGAGT-3'.

hTERT concentration assay. The hTERT concentration was measured by ELISA assay kit (cat. no. ZB-10934B-H9648; ZellBio GmbH) according to manufacturer's protocol.

Statistical analysis. The SPSS software package (version 23.0; IBM SPSS, Armonk, NY, USA) was used for statistical analysis. Each experiment was performed at least three times for all data. All the data were represented as mean ± standard deviation, and the nonparametric analysis of variance test, with post hoc Tukey test, was used for statistical analysis between the groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxicity activity. Significant inhibitory effect of AW extract has been seen in 150 μg/ml concentration (IC_{50}) on PC3 cell line after treatment for 48 h. In addition, the inhibitory effect of AW extract was in dose- and time-dependent manner (Fig. 1). The half maximal inhibitory concentration (IC_{50}) was defined as the AW concentration value which inhibits the viability of PC3 cells in culture by 50% compared to the untreated cells (control).

Cell apoptosis. The percentage of cells in Live (Annexin V-/PI-), early apoptosis (Annexin V+/PI-), late apoptosis and dead (Annexin V+/PI+) phases are presented in Fig. 2. Following a 48 h treatment of the PC3 cells with AW extract, a significant increase in early and late apoptotic cells and a decrease in live cells was observed in a dose-dependent manner. Moreover, the early apoptotic cells were significantly higher than late apoptotic cells (Fig. 3).

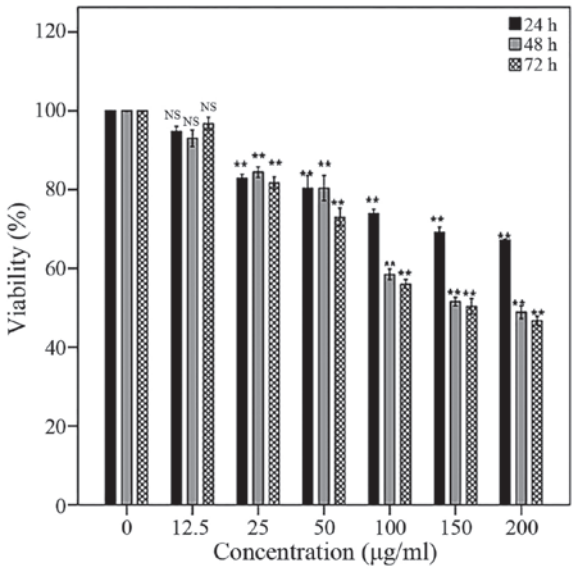

Figure 1. Cytotoxic effects of 0, 50, 100, 150 and 200 μg/ml *Achillea wilhelmsii* extract analyzed by MTT assay after 24, 48 and 72 h on PC3 cells. Changes occurred in a dose dependent and time dependent manner. The data were represented as mean ± standard deviation. **P<0.001 vs. untreated control groups. NS, not significant.

hTERT expression. The hTERT mRNA expression levels were not different compared to control group after 2, 4, 8 and 12 h. However, the hTERT mRNA expression levels were significantly decreased after 24, 48 and 72 h treatment with 150 µg/ml concentration of AW extract compared to control. There were no significant differences regarding to hTERT mRNA expression levels between 24, 48 and 72 h of treatment (Fig. 4).

hTERT level. The hTERT levels have been presented in Fig. 5. Following treatment for 2, 4, 8 or 12 h, the hTERT levels were not different compared to control. The hTERT level was significantly decreased following 24 h treatment with marginal P-value (P=0.043). However, the hTERT levels were significantly declined in 48 and 72 h following treatment (P<0.001). There were no significant differences between 24, 48 and 72 h treatments (Fig. 5).

Discussion

PCa is the second reason of cancer-related deaths after lung cancer in men. Androgen ablation is the prominent and the most successful treatment of progressive PCa, however, after treatment the tumor cells become insensitive to androgen hormone. Therefore, new targets required for therapeutic intervention of androgen-independent PCa. Therefore, telomerase could be a suitable and main target for therapeutic intervention in this cancer (2,3). Most normal cells typically lack telomerase activity, but this enzyme expressed in >85% of all human cancer cells (23,24). Higher telomerase levels are identified in the most of PCas, but, not in normal or benign prostatic hyperplasia tissues (6). Existing data suggest that hTERT expression

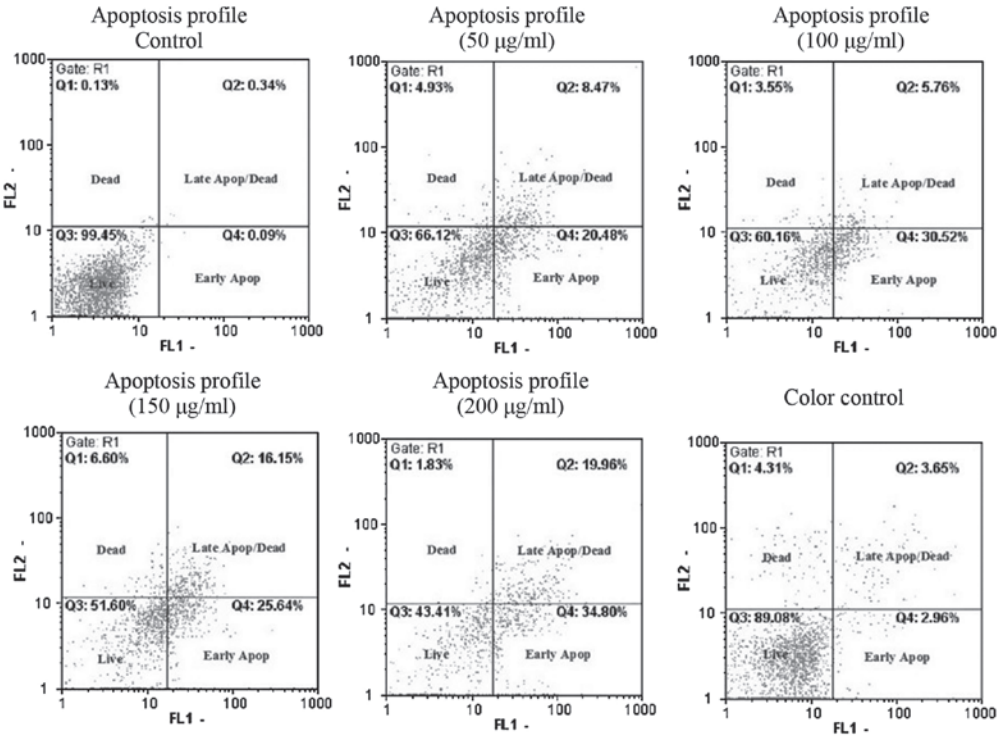

Figure 2. Apoptosis of PC3 cells was induced by *Achillea wilhelmsii* extract. Early apoptotic cells are presented in the lower-right quadrant of the scatter plot, and live cells are in the lower-left quadrant. Dead cells are presented in the upper-left and late apoptotic/dead cells in the upper-right quadrant of the scatter plot.

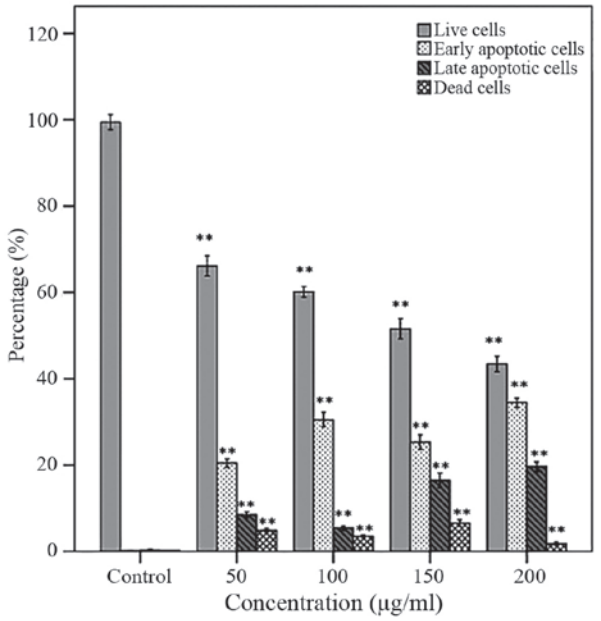

Figure 3. Flow cytometric analysis of apoptosis in PC3 cells using Annexin V/PI staining. Following 48 h treatment with 0, 50, 100, 150 and 200 μg/ml *Achillea wilhelmsii* extract, there was a significant increase in early and late apoptotic cells and a decrease in live cells. The data were represented as mean ± standard deviation. **P<0.001 vs. untreated control groups.

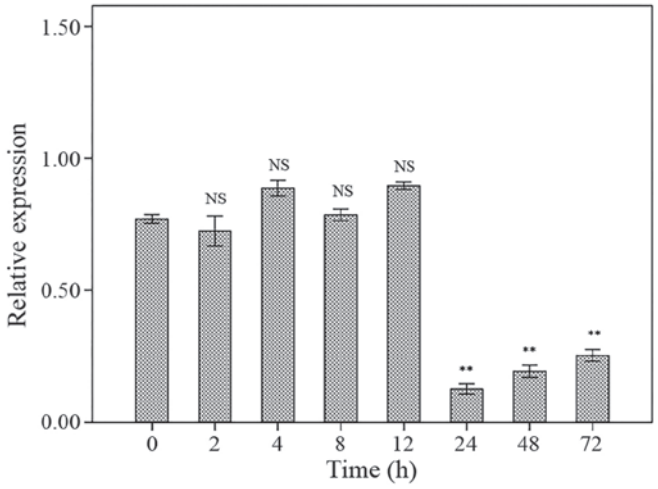

Figure 4. Relative mRNA expression levels of human telomerase reverse transcriptase following treatment of PC3 cells with 150 μg/ml concentration of Achillea wilhelmsii extract for 2, 4, 8, 12, 24, 48 and 72 h, performed by reverse transcription-quantitative polymerase chain reaction method. The data were represented as mean ± standard deviation. **P<0.001 vs. untreated control groups. NS, not significant.

and telomerase activity are positively regulated by androgenic stimuli in androgen-dependent PC (ADPC) cells. Evidence demonstrated that AR regulates the EGR1 expression that in turn controls positively the hTERT expression in CRPC cells. Thus, AR exerts an inhibitory effect on hTERT expression and telomerase activity by modulating EGR1 levels in CRPC cells (25).

Expression and reactivation of telomerase has also been described as an important feature of PCa. Telomerase activity was found in up to 100% of analyzed PCa cases (26). Interestingly, high expression of telomerase components does not always result in mandatory telomerase activity (27). In addition, hTERT expression was significantly associated with the aggressive behavior of prostate tumors (28). Previously, promising *in vitro*

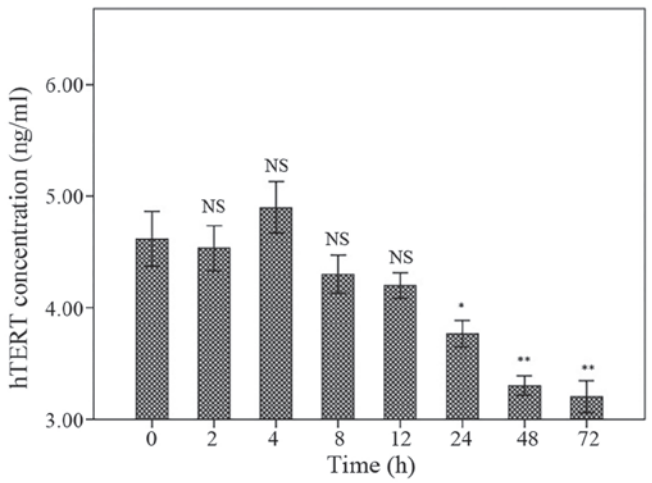

Figure 5. The hTERT concentration following treatment of PC3 cells with 150 µg/ml concentration of Achillea wilhelmsii extract for 2, 4, 8, 12, 24, 48 and 72 h, which performed by ELISA. The data were represented as mean ± standard deviation. *P<0.01, **P<0.001 vs. untreated control groups. hTERT, human telomerase reverse transcriptase; NS, not significant.

data were published identifying telomerase as a main target of an anti-androgen therapy in PCa, and the effectiveness of boron derivatives as a telomerase inhibitors (29,30). These data suggested telomerase inhibition as a reasonable therapeutic approach for the treatment of PCa, though, the molecular and cellular pathways involved in telomerase reactivation in PCa are still not clear. Expression of TERT and the telomerase activity were regulated by androgen receptor signaling, whereas exogenous expression of AR surprisingly led to inhibition of TERT transcription in PCa cells (31,32).

According to this fact that some PCa cells become insensitive to AR treatment, in the current study, the authors investigated the effects of the herbal extract of AW on apoptosis and hTERT expression in an insensitive prostatic cancer cell line to AR treatment.

PC3 is a PCa cell line that is established from bone metastasis of grade IV of PCa. This cell line has potential metastatic activity and do not respond to androgens, glucocorticoids or fibroblast growth factors. This cell line is useful in investigating the therapeutic interventions in progressive prostatic cancer cells (33).

In the current study, the apoptotic effect of AW extract on the PC3 cell line was assessed and the effects of this plant on hTERT expression and concentration were determined.

The findings demonstrated the appropriate inhibitory effect of AW extract in 150 μg/ml concentration (IC_{50}) on PC3 cell line following AW treatment for 48 h. Proliferation of the PC3 cells was significantly inhibited in a dose- and time-dependent manner. A significant increase in early and late apoptotic cells and a decrease in live cells in a dose-dependent manner was observed following treatment with AW extract for 48 h. Moreover, the early apoptotic cells were significantly higher than late apoptotic cells. The relative mRNA expression was decreased following 24 h treatment of AW extract compared to control, however it was not different between 2, 4, 8 and 12 h treatments or 24, 48 and 72 h treatments. In addition, the hTERT concentration was significantly decreased after 24 h treatment of AW extract with marginal P-value. There was no significant difference regarding to hTERT concentration between 2, 4, 8 and 12 h treatments or 24, 48 and 72 h treatments.

The apoptotic effects of AW species extracts have been investigated in various cancer cell lines. The flavonoid casticin derived from *Achillea millefolium*, demonstrated an important effect in cancer therapy (34). The anticancer effects of the various species of *Achillea* have been reported in different cancer cell lines (35,36). The cytotoxic and pro- apoptotic effects of methanol and water extracts of *Achillea teretifolia* on DU145 and PC3 PCa cell lines were considered in a recent study (37).

However, studies on the apoptotic effects of AW extract are rare. In a study, the cytotoxic effects of essential oils extracted from leaves of AW C. Koch have been described in several cancer cell lines including PC3 (38). In a similar study, the anti-proliferative and apoptosis-inducing potential of hydroalcoholic AW C. Koch extract were investigated on MCF-7 and MDA-Mb-468 human breast carcinoma cell lines. Consistent to results of current study, the apoptosis-inducing potential of the hydroalcoholic AW extract was indicated, however they observed this effect with lower concentration of AW extract compared with the present authors' results on the PC3 cell line (25 vs. 150 μg/ml) (22).

In addition, there was no published report on the effects of not only AW C. Koch extract but also other species of *Achillea* on the hTERT mRNA expression or concentration in different cancer cell lines. However, several studies investigated the effect of other herbal extracts on telomerase expression and concentration in PCa cell lines. Fruit extract of *Gleditsia sinensis* exhibited anti-cancer and telomerase inhibition effects on ESCC (esophageal squamous cell carcinoma) cell line (39). In human lung carcinoma cell line A549, aqueous extract of the *Platycodon grandiflorum* root represents the apoptotic events with the reduced telomerase activity and downregulation of Bcl2 expression (40).

The effect of hydroalcoholic extract of *Melissa officinalis* on human lung, breast and PCa cell lines demonstrated the potent anti-proliferative activity of this extract via parallel downregulation of VEGF-A and hTERT (41).

The investigation on the pristimerin effects on PCa LNCaP and PC3 cell lines exhibited that this quinonemethide triterpenoid inhibited the hTERT mRNA expression and suppressed the native and phosphorylated hTERT protein and hTERT telomerase activity (42).

In another study, the effects of PC-SPES, herbal formulation for PCa, on LNCaP, PC-3 and DU 145 PCa cell lines have been studied and the finding presented unchanged levels of telomerase in each of the lines (43).

In conclusion, the current findings demonstrated that the herbal extract of AW has an apoptotic effect on PC3 PCa cell line. In addition, the herbal extract of AW can inhibit hTERT mRNA expression. Further studies with each component of AW are required in various cancer cell lines to identify the active substance of this plant on telomerase expression and activity.

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