Research in Molecular Medicine

Original Article

2015, Vol: 3, Issue: 1, Pages: 12- 17

DOI: 10.7508/rmm.2015.01.003

# Synergistic Anti-tumoral Effect of *Achillea millefolium* Combined with Bleomycin on Prostate Cancer Cells

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**Received: 21 Nov 2014**

**Revised: 5 Dec 2014**

**Accepted: 18 Dec 2014**

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## Abstract

**Background:** The aim of this study was to investigate the effect of methanolic extract of *Achillea millefolium* L. (MEA) on the antiproliferative activity of bleomycin on human prostate cancer and normal skin cells.

**Materials and Methods:** Human prostate cancer cell (DU-145) and human non-malignant fibroblast cell (HFFF2) were treated with MEA at various concentrations (20, 100, 500, 1000 and 2000 µg/ml), using bleomycin alone or combined with MEA. Also, the effect of MEA and bleomycin on cell viability was evaluated. Free radical scavenging property was determined for this herbal extract.

**Results:** The combination of MEA and bleomycin significantly increased the inhibition of cell growth in cancer cell. The extract significantly enhanced cytotoxicity induced by bleomycin showing 60% and 49% survival rate at doses of 1000 and 2000 µg/ml, respectively. In bleomycin-treated cells the survival rate reached 85%. MEA did not exhibit any cytotoxicity on HFFF2 cells.

**Conclusion:** This study found that the methanolic extract of *Achillea millefolium* enhanced the cell toxicity induced by bleomycin in the prostate cancer cell without any significant toxicity on normal cells.

**Keywords:** *Achillea millefolium*; Prostate cancer; Antiproliferative; Bleomycin

*Please cite this article as:* Nazm Bojnordi M, Ghasemi Hamidabadi H. Spermatogonial Stem Cells: A New Pluripotent Source for Repairment in Regenerative Medicine. Res Mol Med. 2015; 3 (1): 12-17.

## Introduction

Bleomycin (BLM) is an anticancer agent used to treat several types of cancer such as lymphoma, brain, melanoma, and testicular cancers (1-4). Bleomycin is able to generate reactive oxygen species (ROS) which attack macromolecules such as DNA leading to genome instability. DNA breaks are resulting in apoptosis and cell damage, also cause carcinogenic and clastogenic effects. Bleomycin induces DNA damage with similar effect to ionizing radiation; it is namely a radiomimetic agent (5, 6). BLM causes toxicity on skin and lungs through oxidative stress and inflammations (7-9). Antioxidants might be expected to protect normal cells against BLM toxicity (6, 10).

Although BLM is effective for treatment of cancer cell, the use of BLM is limited due to developing resistance in some tumor cells and also BLM-toxicity in normal tissues. However, BLM at higher dose has more killing effect on tumor cell and causes more side effects on normal tissues (8, 11, 12). It is interesting to find an agent to increase BLM-toxicity in cancer cells, without any side effects on normal cells. *Achillea millefolium* L., belonging to the compositae family, is an aromatic perennial herb which is native to Iran (13, 14). In Iranian traditional medicine several species of *Achillea*, generally called Bumadaran in Persian have been used as a wound healer, anti-inflammatory and antispasmodic (15). Chemical compositions of *Achillea* species showed these plants contain flavonoids such as quercetin, luteolin, apigenin, cynaroside and cosmosiin, and terpenoids, lignans, amino acid derivatives, fatty acids and alkamides, quinic acid derivatives and nitrogen containing compounds (16). Several pharmacological and biological properties are reported for *A. millefolium* including anti-viral,

antimutagenic, and antispasmodic (17-19). The extracts of *A. millefolium* showed protective effects against toxicity induced by oxidative stress and inflammation in some tissues such as gastroprotective and hepatoprotective activities (20, 21). The aqueous extract of *A. millefolium* attenuated inflammatory responses and demyelinating lesions in experimental autoimmune encephalomyelitis (22). The essential oil of this plant suppresses the inflammatory responses of lipopolysaccharides-stimulated macrophages. It also decreases levels of cellular nitric oxide and superoxide anion production, lipid peroxidation and glutathione concentrations (23). In another research, the screening of bioactive agents from higher plants for anti-tumor activity showed significant cytotoxicity of methanolic extract of *A. millefolium* against human tumor cell lines in vitro (24). Based on the above findings and antioxidant and cytotoxic potential of *A. millefolium*, the present study aimed to evaluate the effects of methanolic extract of *Achillea millefolium* (MEA) on anti-proliferative effects induced by bleomycin in human non-malignant fibroblast and prostate cancer cells.

## Materials and methods

### *Chemicals*

Bleomycin (Lyoble, India) were dissolved in sterile water at stock solution and diluted with RPMI 1640 medium. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MTT) was purchased from Sigma (USA). Other solvents were from Merck Company (Germany).

### *Plant material and extraction*

The aerial parts of *Achillea millefolium* were collected collected from Kalat Naderi, Khorasan-e-Razavi province, Iran at 1586 m above sea level, during the flowering stage in June 2012. A voucher specimen (44246) was deposited at the herbarium of the Research Center for Plant Sciences, Ferdowsi University of Mashhad, Mashhad, Iran. Dried aerial parts of the plant were powdered and extracted with methanol by maceration at room temperature. The extract was evaporated using a rotary evaporator (Heidolph, Germany) and dried by a freeze dryer (Zirbus, Germany).

### *Cell culture*

Human prostate cancer (DU-145) and human skin fibroblast (HFFF2) cells were obtained from the Pasture Institute of Iran and cultured at 37$^{O}$C and 5% $CO_2$ in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin–streptomycin (Gibco). Experiments on cells were performed in the exponential growth phase. MEA was dissolved in ethanol and diluted with culture medium.

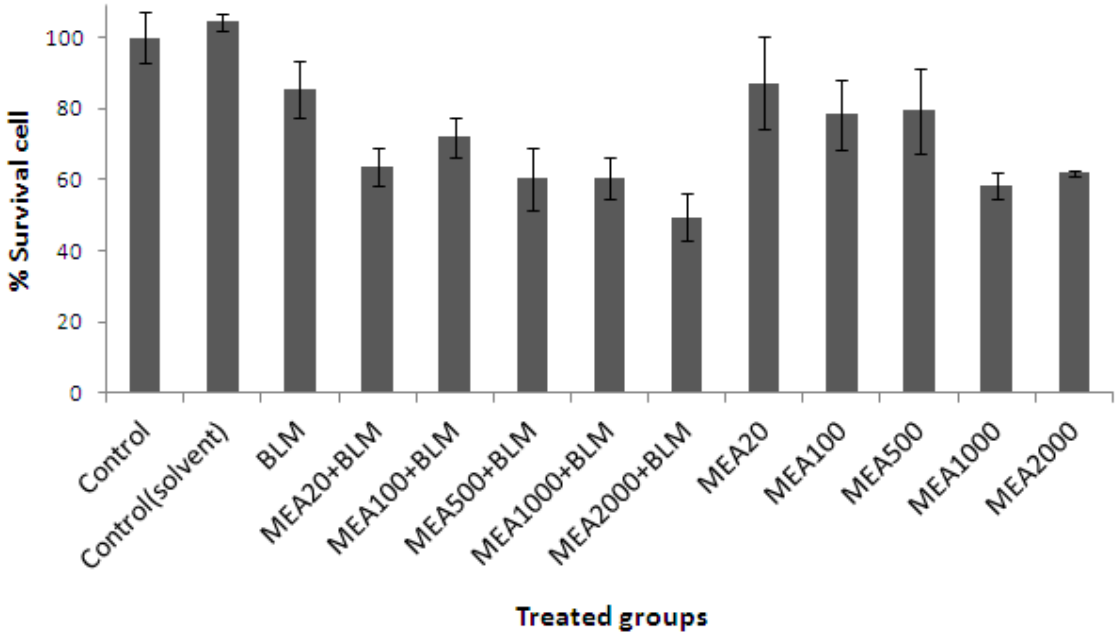


**Figure 1.** Anti-proliferative effects of methanolic extract of *Achillea millefolium* (MEA) (20, 100, 500, 1000 and 2000 µg/ml) with bleomycin (BLM, 10 µg/mL) on prostate cancer cell (DU-145). The absorbance corresponding to that of untreated control cells was assumed as 100% cell viability.

*Cell anti-proliferation assay*

MTT assay was used for cell proliferation to quantify the metabolic activity and to cleave tetrazolium salts (25, 26). DU-145 and HFFF2 cells (10,000) were seeded in 96-well plates. After 24 h incubation, the cells were treated with various concentrations of MEA (20, 100, 500, 1000 and 2000 µg/ml) and incubated for 2 h at 37$^{O}$C and 5% $CO_2$. Also, RPMI 1640 culture medium with ethanol (same concentration with extract solvent) was set as negative control. After incubation, BLM was added at10 µg/mL to each well. At 48 hours after culture, 20 µL MTT (5 mg/mL in phosphate buffer saline) was added to each well, and culturing was continued for 4 hours. Then, culture supernatant was discarded and replaced by 150 µL isopropanol (0.1% HCl), and the cell plates were shaken for 30 minutes. Finally, the absorbance of every culture well was read on an ELISA Reader (Bioteck, USA). The absorbance corresponding to that of untreated control cells was assumed as 100% cell viability. The percentage of cell survival was calculated as follows: % survival = (mean experimental absorbance/mean control absorbance) ×100.

*Measurement of free radical scavenging activity*

The free radical-scavenging capacity of the methanolic extract was determined as bleaching of the stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) (27).

Different concentrations of the extract (0.05 to 0.8 mg/ml in ethanol: water) were added, at 2 ml, to 2 ml ethanol solution of DPPH. After 15 min at room temperature, the absorbance was recorded by UV/Visible spectrophotometer (GENWAY, UK) at 517 nm. The experiment was performed in triplicate, and butylated hydroxytoluene (BHT) was used as a standard antioxidant agent. Percent scavenging was calculated using the formula ((mean control absorbance- mean test absorbance)/mean control absorbance)*100.

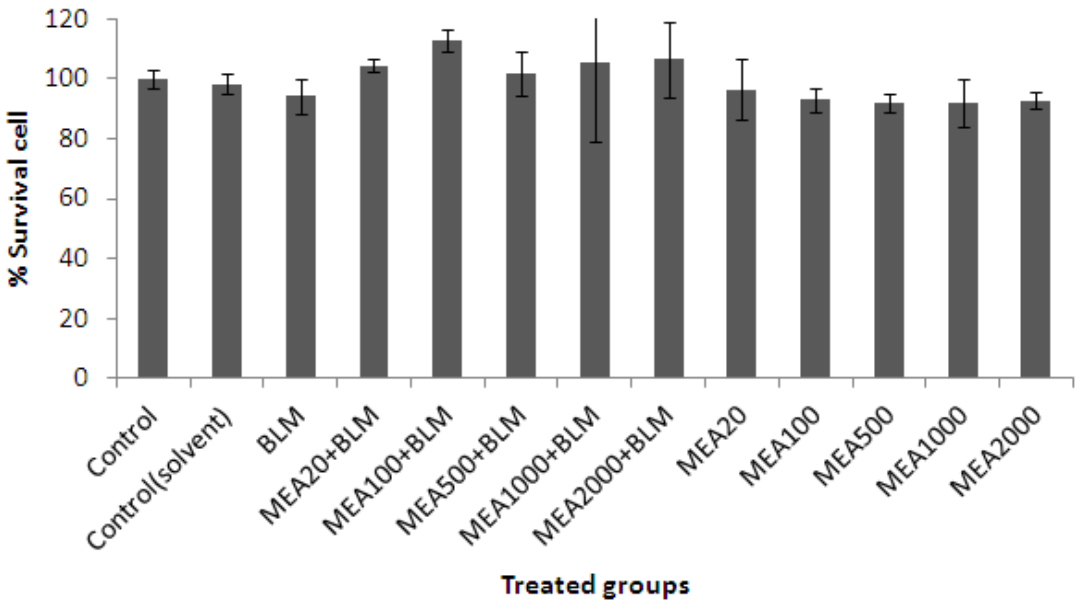


**Figure 2.** Anti-proliferative effects of methanolic extract of *Achillea millefolium* (MEA) (20, 100, 500, 1000 and 2000 µg/ml) on with bleomycin (BLM, 10 µg/mL) on human normal skin cell (HFFF2).

**Statistical analysis**

Data were presented as mean ± standard deviation (SD) of three independent experiments. Data were compared and the differences were considered significant if the p value was <0.05.

**Results**

*Antiproliferation*

Cell viability of DU-145 and HFFF2 cells treated with various concentration of MEA in combination with BLM or alone was determined by MTT assay. DU-145 cells were treated with various concentrations (20, 100, 500, 1000 and 2000 µg/ml) of the methanolic extract for 48 h. MEA alone inhibited cancer cell growth at 100, 500, 1000 and 2000 µg/ml ($p<0.05$).

BLM significantly inhibited growth of DU-145 cells; however, the extract in combination with BLM exhibited a dose-dependent manner in growth inhibitory on prostate cancer cells (Figure 1).

BLM exhibited 85±8% survival rate in DU-145 cells, while the combination of MEA and BLM showed 63±5%, 72±5%, 60±9%, 60±6%, and 49±6% survivals at concentrations 20, 100, 500, 1000 and 2000 µg/ml in DU-145 cells, respectively. HFFF2 cell was selected as a normal human skin fibroblast for assessment of any cytotoxicity induced by BLM in combination with MEA. BLM did not show any cell toxicity on HFFF2 cells at same concentration which was used for DU-145. Antiproliferation on HFFF2 cells was not observed at various concentrations of the extract alone. Also, no enhanced cytotoxicity was observed from the extract in combination with BLM on HFFF2 (Figure 2).

*Antioxidant activity*

Methanolic extract of *A. millefolium* exhibited potent antioxidant activity. Scavenging effects of MEA on DPPH radicals increased by increasing the concentrations. Percentage of radical inhibition by the extract was calculated 84%, 89% and 94% at 0.2, 0.4 and 0.8 mg/ml, respectively (Figure 3).

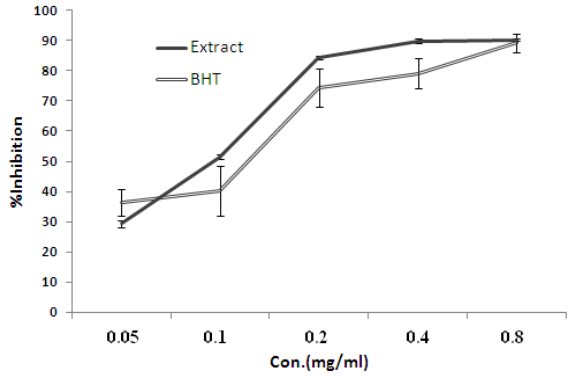


**Figure 3.** Scavenging effect of different concentrations of methanolic extract of *Achillea millefolium* (extract) and Butylated hydroxytoluene (BHT) on the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical at 517 nm.

## Discussion

In this study, we showed the beneficial effect of *Achillea millefolium* in the growth suppression of prostate cancer cells and suggested the potential uses of phytochemicals in combination with bleomycin in treatment of some cancers. MEA exhibited a synergistic effect in combination with BLM by factors a range of 1.2 and 1.7 on prostate cancer cell killing. Furthermore, MEA did not show any chemosensitive effect on non-malignant fibroblast cells treated with BLM. It is a promising result that is focusing the combination of *A. millefolium* with bleomycin; it is possible to improve the effectiveness of cancer treatments and minimizing toxicity on normal cells. Oxidative stress and inflammations are contributed in DNA damages and cause chromosome instability and cell death induced by BLM in cells (28-30). *A. millefolium* as a medicinal plant has numerous biological activities mainly through antioxidant activity and anti-inflammatory effect. Our results showed MEA has potent antioxidant activity with free radical scavenging property. MEA alone inhibited proliferation of prostate cancer cells, so this effect might be synergetic with BLM on inhibition of prostate cancer cell growth. Different skeletal types of sesquiterpene lactones especially eudesmanolide and guaianolides have been reported from the genus *Achillea*. Achillinin A, as a guaianolide isolated from the methanolic extract of *A. millefolium*, exhibited potential antiproliferative activities toward five human lung tumor cell lines that was stronger than that of cisplatin (31). In another research *A. millefolium* exhibited anti-hepatoma activity on human liver-cancer cell lines (32). *A. millefolium* contains flavonoid casticin, a potent cytotoxic compound, which exhibited cell growth arrest in G2/M and in apoptotic death and resulted in cell killing (33). Casticin downregulated cell survival proteins and induced death receptor 5 (DR5) mediated by ROS in colon cancer cells (34). Casticin is a P-glycoprotein inhibitor, which contributes in increasing the anti-tumor effects of drugs (33, 35). Tozyo et al isolated sesquiterpenoids, achimillic acids A, B and C which were active against mouse P-388 leukemia cells in vivo (36). These findings demonstrated the chemical compounds present in *A. millefolium* are responsible for anti-tumor activity. However, *A. millefolium* enhanced cell toxicity induced by BLM in prostate cancer; it did not show any BLM-sensitive effect on normal human fibroblast cells. These results showed that *A. millefolium* probably affect with different mechanisms for antiproliferation on normal and cancerous cells.

## Conclusion

The present study shows that the methanolic extract of *A. millefolium*, could enhance the antiproliferation of bleomycin on prostate cancer cells (DU-145). It was also shown that *A. millefolium* did not cause any growth inhibition on normal human skin cells HFFF2. As evidenced from previous results, cytotoxic flavonoids such as casticin and sesquiterpenoids may be responsible for observed anti-tumor effects of *A. millefolium* but the mechanisms remain to be elucidated.

## Acknowledgments

This study was supported by a grant from Mazandaran University of Medical Sciences, Sari, Iran. This research was the subject of a Pharm D. thesis of Negar Hamzkanlu as a student in Mazandaran

University of Medical Sciences.

### Conflict of interest

The authors declare no potential conflict of interest with respect to the authorship, and/or publication of this study.

## Authors' Contributions

ShS and HSJ designed, supervised of the study, and prepared the manuscript; HN and ZN carried out the experiments.

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