



***Achillea vermicularis* a medicinal plant from Iranian Traditional Medicine induces apoptosis in MCF-7 cells**

M. Hamzeloo-Moghadam¹, A. Khalaj^{2,3*}, M. Malekmohammadi⁴, M. Mosaddegh^{1,2}

¹Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

²Department of Traditional Pharmacy, School of Traditional Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

³Department of Traditional Medicine, Faculty of Medicine, Alborz University of Medical Sciences, Karaj, Iran.

⁴Department of Plant Sciences, School of Biology, College of Sciences, University of Tehran, Tehran, Iran.

Abstract

Background and objectives: *Achillea* is an ancient medicinal herb. The genus comprises about 100 species which are mostly distributed in northern hemisphere and some have been investigated for different biological activities. There are also several reports in Iranian Traditional Medicine (ITM) texts about its anti-tumor effect. The cytotoxic activity of the methanol extract of *Achillea vermicularis* Trin. has been reported in previous studies against different tumor cell lines. Based on these reports, the species has been further investigated for apoptosis induction ability. **Methods:** The apoptosis induction ability has been evaluated through activated caspase 3 investigation in intact MCF-7 cells *in vitro*. **Results:** The assay demonstrated signs of caspase 3 activation in MCF-7 cells. **Conclusion:** *Achillea vermicularis* is suggested for further mechanistic evaluations in apoptosis studies.

Keywords: apoptosis, cancer, caspase 3, cytotoxicity, Iranian Traditional Medicine

Introduction

The genus *Achillea* consists of more than 100 species which are mostly distributed in northern hemisphere [1]. This genus has been noted medically from antiquity and the healing properties have been listed by Dioscorides [2] and Pliny [3]. There are some biological activity reports about different species of the genus. The famous member, *Achillea millefolium*, is known as yarrow or milfoil. It is used as antibacterial, anti-inflammatory, antiedemic, vermifuge and

expectorant in herbal therapy [4]. Another species, *A. ptarmica*, which is known as sneezewort is used as astringent, analgesic and hemostat [4]. *A. eriophora* essential oil has shown considerable activity against some pathogenic microbial antifungal strains [5]. *A. alexandri-regis* has demonstrated cytotoxic activity *in vitro* against HeLa cells [6]. The present study focuses on *Achillea vermicularis* Trin. which has been evaluated in previous

studies and has exhibited cytotoxic activity against MCF-7 and A-549 cell lines with IC₅₀ values below 100 µg/mL [7]. The species has been mentioned in Iranian Traditional Medicine (ITM) for its ability to dissolve tumors and a former study has tried to somehow relate its claimed use in ITM with modern cancer researches [8]. In the present study, we have tried to find out whether apoptosis induction was responsible for the cytotoxicity of the extract. The emphasis on apoptosis induction ability was considered because so many people, including the patients and their families, are affected by cancer all over the world [9] and in many cases cancer cells would resist to chemotherapy [10] which makes the problem worse. Some acquired biological hallmarks during tumor multistep development make the cancer cells prone to resist against therapy. One of these hallmarks has been described as programmed cell death or apoptosis [11]. The essential role of apoptosis as long as tumor progression and chemotherapy resistance has been well defined [12] thus; apoptosis induction is a key therapeutic approach [13]. In other words, apoptosis induction is the main mechanism of a cancer chemotherapeutic agent for inducing cytotoxic effect [14].

There are different methods to indicate apoptosis induction [15]. In the present study we employed caspase 3 assay. Caspases are normally presented in the inactive form (zymogens) in the cytosol but they are capable of turning into active forms. The significant member of this family in the execution phase of apoptosis which can activate other caspases is caspase 3 [16].

Nowadays, so many common and distinctive natural products are known as cancer chemotherapeutic agents [17]. Special role of plants kingdom by introducing the taxanes, vinblastin, epipodophyllotoxins and some other drugs is seriously notable [18] and natural sources are providing more expectations about finding novel lead compounds [19].

Based on previous reports of *Achillea vermicularis* cytotoxic activity [7], the apoptotic induction ability of the methanol extract of this

medicinal plant has been evaluated against MCF-7 cell line using caspase-3 assay.

Experimental

Plant material

Achillea vermicularis aerial parts were collected from Ardabil province, Iran (2012). The species was authenticated by botanists of the Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. A voucher specimen (3478-TMRC) was deposited at the TMRC Herbarium for future reference. The plant was dried in shade and ground.

Extraction

Ten grams of the dried powdered *Achillea vermicularis* was macerated with methanol at room temperature for 24 h (thrice). The methanol extract was further concentrated using a rotary evaporator at 40 °C and the concentrated dried extract was kept in 2-8 °C for future assays.

Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), Fetal Bovine Serum (FBS), (Gibco, Auckland, New Zealand), penicillin-streptomycin, MTT ([3-(4, 5-dimethylthiazol-2-yl) -2, 4-diphenyl tetrazolium bromide]), PBS (Sigma, St. Louis, MO, USA) and DMSO (dimethyl sulfoxide) (Merck, Hohenbrunn, Germany), were used in the apoptosis evaluation and methanol (Mojalali, Iran), was used for plant extraction. The NucView™ 488 Caspase 3 Assay Kit for Live Cells (Biotium, Hayward, CA, USA) was used in apoptosis assays. 5-FU (Sigma, St. Louis, MO, USA) was used as the positive control.

Cell line

MCF-7 (human breast adenocarcinoma) cells were obtained from the Pasteur Institute, Tehran, Iran. This cell line was cultured in DMEM with 5% FBS to maintain the desired growth in a humidified incubator at 37 °C in an atmosphere of 5% CO₂. It was further treated with 1% penicillin-streptomycin.

Sample preparation

Serial concentrations were prepared from a stock solution (10 mg/mL) of the methanol extract in DMSO. The final concentration for probable apoptosis induction was about 80% of reported IC_{50} amount for MCF-7 cells in previous studies which was 34.8 $\mu\text{g/mL}$ (final concentration of DMSO was 1%) [7].

Apoptosis induction

The apoptosis ability of *Achillea vermicularis* extract has been evaluated by observing active caspase 3 in the test medium [20]. MCF-7 cells were seeded in 96-well plates at 6×10^3 . After 24 h, the medium was replaced with fresh medium containing 25 $\mu\text{g/mL}$ of the extract to, expectedly, induce apoptosis. After 20 h of exposure, the medium was replaced with 200 μL fresh medium. Then 3 μL of the NucView™ 488 Caspase 3 Assay Kit for Live Cells was added to the well which was considered qualified by

observing the light microscope field and evaluating the cells morphologically. The cells were incubated for 5 min in a dark place at room temperature. The results were evaluated using an inverted florescent microscope (HUND) and by a digital camera (Canon 600D). The green florescence demonstrated the presence of activated caspase 3 enzyme in the cytosol. This, subsequently, would be the indicator of the activated apoptotic cascade. The excitation/emission wavelengths were 450/490 nm, respectively. 5-FU was utilized as the positive control.

Results and Discussion

Figure 1 exhibits the possible apoptosis induction ability of *Achillea vermicularis* methanol extract by observing activated caspase 3 in live cells. Figure 2 demonstrates the same probable induction for 5-FU as the positive control.

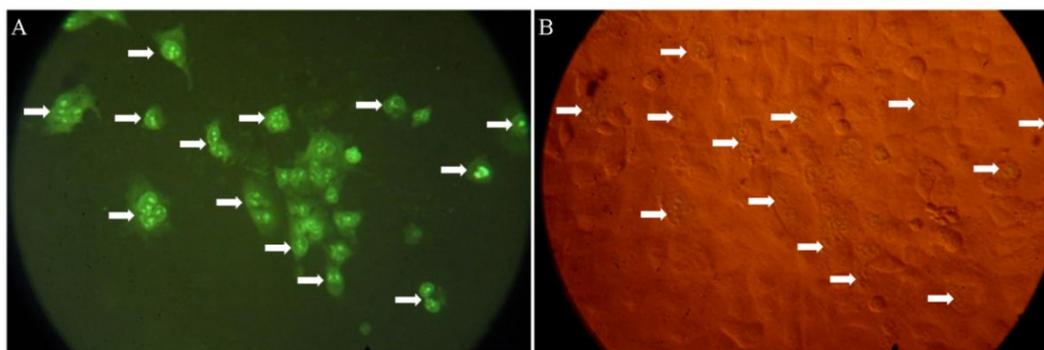


Figure 1. MCF-7 cells treated with 25 $\mu\text{g/mL}$ of *Achillea vermicularis* methanol extract. A remarks fluorescent and B remarks the light microscopic view of the same field. The arrows point to the affected cells.

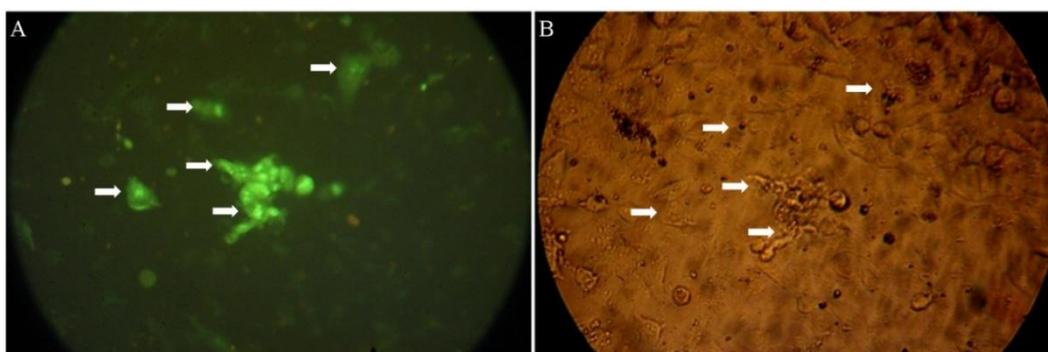


Figure 2. MCF-7 cells treated with 0.5 $\mu\text{g/mL}$ of 5-FU. A remarks fluorescent and B remarks the light microscopic view of the same field. The arrows point to the affected cells.

The term apoptosis was introduced in 1972 which represented distinct morphologic changes such as cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation that could be observed by light microscope during apoptosis induction [21]. This could distinguish the affected cells among regular ones. In the present study along with the morphologic changes such as condensed chromatin which could be observed in the light field microscopy, some more specific aspects of apoptosis induction by the activated caspase-3 assay was noticed.

Evaluating activated caspase 3 is a proper indicator of apoptosis induction [22]. In the present study, activated cytosolic caspase 3 cleaved the NucView™ 488 Caspase-3 substrate and released the specific nucleus dye which stained the DNA. The NucView 488 DNA dye was attached to the caspase-3 substrate peptide sequence DEVD.

In this case, it couldn't bind to the DNA and stayed non-fluorescent. The time the substrate crossed the plasma membrane to enter the cytoplasm, it was cleaved by caspase-3 and the high-affinity DNA dye was released, which could migrate to the cell nucleus to stain the nucleus with bright green fluorescence which confirms apoptosis induction.

Both the fluorescent shining nuclei of the apoptotic cells and the morphologic changes of nucleus such as chromatin condensation were observed during apoptosis by fluorescent microscopy. These changes were demonstrated in figure 1 by brilliant fluorescence caused by activated caspase 3 in the condensed nuclei of MCF-7 cells and the light microscopic field view certified the apoptotic process by obvious shrunk cytoplasm.

There are strong statements about anti-tumor properties of *Achillea vermicularis* in ITM. It is claimed that the species could smooth or dissolve the tumors that were formed in testis, uterus, anus, viscera and liver [7]. The cytotoxic activity, as demonstrated in previous research [7], along with the apoptosis observations in the present study, could provide a promising sign toward

usefulness of this traditional medication and rise probabilities about finding new lead compound clues for cancer research through surveying traditional knowledge.

Acknowledgments

We would like to express our especial appreciation for late Dr. Farzane Naghibi's selfless efforts, unsparing support and effective guidance in this project. The results were based on a PhD thesis of Traditional Pharmacy (Amir Khalaj) granted by the Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (grant No.142).

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

References

- [1] Zhang Q, Lu Z, Ren T, Ge Y, Zheng Y, Yao D, He X, Gu Y, Shi Q, Huo C. chemical composition of *Achillea alpina*. *Chem Nat Compd*. 2014; 50(3): 534-536.
- [2] Beck LY. *De Materia Medica*. Hildeshiem: Olms-Weidmann, 2005.
- [3] Duke James A. *Handbook of Medicinal Herbs*, 2nd ed. Boca Raton: Taylor & Francis, 2010.
- [4] Ghasemi Y, Khalaj A, Mohagheghzadeh A, Khosravi A. Composition and *in vitro* antimicrobial activity of the essential oil of *Achillea eriophora*. *Chem Nat Compd*. 2008; 44(5): 663-665.
- [5] Cho WCS, Ed. *Materia Medica for Various Cancers*. London: Springer, 2011.
- [6] Kundaković T, Stanjković T, Juranić Z, Kovacević N. Cytotoxic and antioxidant activity of *Achillea alexandri-regis*. *Pharmazie*. 2005; 60(4): 319-320.
- [7] Naghibi F, Khalaj A, Mosaddegh M, Malekmohamadi M, Hamzeloo-Moghadam M. Cytotoxic activity evaluation of some

- medicinal plants, selected from Iranian Traditional Medicine pharmacopoeia to treat cancer and related disorders. *J Ethnopharmacol.* 2014; 155(1): 230-239.
- [8] Naghibi F, Khalaj A, Hamzeloo-Moghadam M. Cancer terminologies used in the medieval texts to the early modern Iranian Traditional Medicine (ITM). *Res Hist Med.* 2014; 3(2): 63-72.
- [9] Aggarwal BB, Danda D, Gupta S, Gehlot P. Models for prevention and treatment of cancer: problems vs promises. *Biochem Pharmacol.* 2009; 78(9): 1083-1094.
- [10] Lanzotti V, Tagliatela-Scafati O, Fattorusso E, Di Pietro A. *plant compounds and derivatives as inhibitors of cancer cell multidrug resistance*, in *Bioactive compounds from Natural Sources*. Tringali C, Ed. Boca Raton: CRC press, 2011.
- [11] Hanahan D, Weinberg RA. Hallmarks of Cancer: the next generation. *Cell.* 2011; 144(5): 646-674.
- [12] Cotter TG. Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer.* 2009; 9(7): 501-507.
- [13] Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer.* 2008; 8(2): 121-132.
- [14] Werner JM, Steinfelder HJ. A microscopic technique to study kinetics and concentration–response of drug-induced caspase-3 activation on a single cell level. *J Pharmacol Toxicol.* 2008; 57(2): 131-137.
- [15] LeBlanc AC, Ed. *Apoptosis techniques and protocols*. Totowa: Humana Press Inc, 2002.
- [16] Logue SE, Martin SJ. *Mammalian caspase activation pathways in apoptosis and inflammation*. In: O'Brien T, Linton SD, Eds. *Design of caspase inhibitors as potential clinical agents*. Boca Raton: CRC Press, 2009.
- [17] Cragg GM, Kingston DGI, Newman DJ. *Anticancer Agents from Natural Products*. Boca Raton: Taylor & Francis, 2005.
- [18] Brunton LL, Chabner BA, Knollmann BC. *Goodman and Gilman's manual of pharmacology and therapeutics*. New York: McGraw-Hill Professional, 2008.
- [19] Cragg GM, Newman DJ. Natural products: A continuing source of novel drug leads. *BBA-Bioenergetics.* 2013; 1830(6): 3670-3695.
- [20] Teng H, Zhang ZG, Wang L, Zhang RL, Zhang L, Morris D, Gregg SR, Wu Z, Jiang A, Lu M, Zlokovic BV, Chopp M. Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. *J Cereb Blood Flow Metab.* 2008; 28(4): 764-771.
- [21] Vázquez-Nin GH, Escobar ML, De Felici M, Echeverría OM, Klingner FG. *Cell Death in Mammalian Ovary*. London: Springer 2011.
- [22] Porter AG, Jänicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* 1999; 6(2): 99-104.