

IN VITRO CYTOTOXIC EFFECTS OF METHANOL EXTRACTS OF SIX *HELICHRYSUM* TAXA USED IN TRADITIONAL MEDICINE

HALIL ERHAN EROĞLU^{1*}, ÜMIT BUDAK¹, ERGIN HAMZAOĞLU¹, AHMET AKSOY² AND SEVİL ALBAYRAK²

¹Department of Biology, Faculty of Science and Arts, Bozok University, 66200, Yozgat, Turkey.

²Department of Biology, Faculty of Science, Erciyes University, 38039, Kayseri, Turkey.

Abstract

Helichrysum Mill., (Asteraceae) species have been used in folk medicine for thousands of years in the world. The *In vitro* cytotoxic effects in human lymphocytes of 6 *Helichrysum* taxa used in Turkey folk medicine were investigated. Blood samples were obtained from healthy donors, non-smoking volunteers, which were incubated and exposed to increasing concentrations of methanol extracts of *Helichrysum* taxa (0.01, 0.05, 0.1, 0.5 and 1 mg/mL). According to results, other taxa outside *H. peshmenianum* and *H. goulandrionum* affected MN rates at high concentrations (0.5 and 1 mg/mL). However mitotic and replication indexes rates of *H. pallasii* and *H. chionophilum* did not show a cytotoxic effect.

Introduction

Helichrysum Mill., (Asteraceae) species (popularly known as immortal flower) grow wild in Anatolia. They are plants widely used as tea. They are used for the treatment of kidney stone, uro-genital and stomach pain, jaundice, diarrhea and asthma (Tabata *et al.*, 1993; Baytop, 1997). *H. pallasii* (Spreng.) Ledeb., *H. chionophilum* Boiss. & Balansa, *H. plicatum* DC. subsp. *polyphyllum* (Ledeb.) P.H.Davis & Kupicha, *H. plicatum* DC. subsp. *pseudoplicatum* (Nábělek) P.H.Davis & Kupicha, *H. peshmenianum* Erik and *H. goulandrionum* Georgiadou are aromatic plants. *H. chionophilum*, *H. peshmenianum* and *H. goulandrionum* are endemic species of the Turkish flora.

H. pallasii grows on rocky slopes and steppe at 1700-3660 m altitude (Davis & Kupicha, 1975). It is alleged to exhibit activities including antibacterial, antiviral, antifungal properties (Aslan *et al.*, 2006). *H. chionophilum* grows on rocky slopes and soils at 1300-2900 m altitude (Davis & Kupicha, 1975). It is alleged to exhibit activity including antioxidant effect (Tepe *et al.*, 2005). *H. plicatum* subsp. *polyphyllum* grows on rocky slopes, steppe and *Abies cilicica* Carr., forest at 1500-3500 m altitude. *H. plicatum* subsp. *pseudoplicatum* grows on rocky slopes at 1100-2500 m altitude (Davis & Kupicha, 1975). *H. plicatum* is alleged to exhibit activities including antibacterial, antioxidant and antidiabetic properties (Erdoğan *et al.*, 2001; Tepe *et al.*, 2005; Aslan *et al.*, 2007a; Aslan *et al.*, 2007b). *H. peshmenianum* grows on alpine steppe at almost 3000 m altitude. *H. goulandrionum* grows on *Pinus* forest at 600-900 m altitude (Davis & Kupicha, 1988). There isn't any study regarding bioactive properties of *H. peshmenianum* and *H. goulandrionum* in literature, because they are endemic species of the Turkish flora.

*Corresponding author: E-mail: herhan.eroglu@bozok.edu.tr

Tel: 00 90 354 2421021/2540

Fax: 00 90 354 2421022

The micronucleus (MN), mitotic index (MI) and replication index (RI) analysis methods are cytogenetic tests that are used both *In vivo* and *In vitro*. Micronuclei are formed as a result of chromosomal non-disjunctions after genotoxic damage and are a very sensitive index of genetic damage (Selvendiran *et al.*, 2005). The MI assay is used to characterize proliferating cells and to identify compounds that inhibit or induce mitotic progression. RI measures cell division kinetics by counting the percent of cells containing 1, 2, 3 or more nuclei per individual (Holland *et al.*, 2002).

The present study examined the *In vitro* cytotoxic effects of the six *Helichrysum* taxa viz., *H. pallasii*, *H. chionophilum*, *H. plicatum* subsp. *polyphyllum*, *H. plicatum* subsp. *pseudoplicatum*, *H. peshmenianum* and *H. goulandrionum* used in alternative medicine.

Materials and Methods

Collection of plant material: Vernacular names and collection information of the six plant species which are individually numbered are listed below:

1. *H. pallasii* (Immortal flower) - Bayburt: between Bayburt and Aşkale, Kop mountain, at 2450-2500 m altitude (40° 00.755 N 40° 29.212 E), 18.VII.2006, Hamzaoğlu 4322, Aksoy & Budak.
2. *H. chionophilum* (Immortal flower) - Kayseri: between Arslantaş plateau and Tufanbeyli, 2nd km at 2030 m altitude (38° 22.459 N 36° 11.224 E), 14.VIII.2007, Budak 2289, Hamzaoğlu & Aksoy.
3. *H. plicatum* subsp. *polyphyllum* (Immortal flower, Gold flower, Plateau flower, Bee flower, Fadeless flower) - Adana: between Tufanbeyli and Bakırdağ, Gebzeli passage at 1950 m altitude (38° 12.125 N 35° 59.891 E), 07.VII.2007, Hamzaoğlu 4769, Aksoy & Budak.
4. *H. plicatum* subsp. *pseudoplicatum* (Immortal flower, Gold flower, Plateau flower, Bee flower, Fadeless flower) - Bitlis: exit of Bitlis to Tatvan at 1180 m altitude (38° 26.323 N 42° 08.114 E), 31.VII.2007, Budak 2223, Hamzaoğlu & Aksoy.
5. *H. peshmenianum* (Immortal flower, Yellow flower) - Niğde: Ulukışla, Yazığöl plateau at 2700 m altitude (37° 22.685 N 34° 27.323 E), 17.VII.2007, Budak 2177, Hamzaoğlu & Aksoy.
6. *H. goulandrionum* (Immortal flower, Yellow flower) - Kahramanmaraş: Kazıma bağları at 915 m altitude (37° 37.340 N 36° 50.304 E), 07.VII.2007, Hamzaoğlu 4761, Aksoy & Budak.

Ergin Hamzaoğlu, Ahmet Aksoy and Ümit Budak authenticated the plant material. Voucher specimens have been deposited at the Herbarium of the Department of Biology, Bozok University, Yozgat, Turkey.

Preparation methanol extracts: Dried plants at room temperature were ground to powder with a grinder. Then the powdered plant materials (10 g) were extracted in a Soxhlet extractor with 100 mL methanol (MeOH) at 60°C for 6 h. The extracts were filtered and concentrated to dryness under reduced pressure at 40°C with a rotary evaporator. Finally, the extracts were kept at +4°C until tested.

Chemicals: Peripheral blood (PB) karyotyping medium (Biological Industries), colcemid (Sigma) and giemsa stain (Merck) were used in peripheral blood culture. PB karyotyping Medium is based on RPMI-1640 basal medium supplemented with L-glutamine, foetal bovine serum, antibiotics (gentamycin) and phytohemagglutinin (PHA-M).

Human lymphocyte cultures and cell harvesting: After obtaining approval from Local Ethic Committee, heparinized blood samples (0.4 mL), obtained from six healthy donors, were placed in sterile culture tubes containing 5 mL of PB karyotyping medium. Then, methanol extracts were added to obtain the five final concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/mL). After mixing the contents of each culture tube by gently inverting them a few times, the culture tubes were incubated at a slanted position at 37°C for 72 h. After a culture period of 70 h, 0.1 mL colcemid solution (10 µg/mL) was added to each culture tube and mixed by shaking gently. After incubation of 72 h, cells were harvested by centrifugation, given hypotonic treatment (0.075 M KCl) and fixed in fresh fixative solution (methanol:acetic acid, 3:1). This fixation step was repeated three times. Slides were air-dried and stained with giemsa (Ozkul *et al.*, 2005).

Staining and examination of micronucleus: The method used by Ozkul *et al.*, (2005) for staining of MN was followed. The slides were randomized and scored by a single observer. From each slide, about 500 cells were examined under 600 magnification, and when MN cells were located they were examined under 1000 magnification. The criteria suggested by Scarpato & Migliore (1996) for recognizing micronuclei were followed. Dead or degenerating cells were excluded from evaluation. Nuclear blobbing (MN-like structure connected with the main nucleus with a bridge) were not considered. Only micronuclei equal to or smaller than one-fifth of the main nucleus were assumed to have resulted from chromosome breakage and were considered. Multimicronucleated cells were also scored but not included in the evaluation of MN frequency.

Examination of mitotic index: MI was calculated as the proportion of metaphase for 2000 cells in each donor and concentration.

Examination of replication index: A total of 500 cells were scored for the determination of the RI. RI was calculated by use of the following formula: $RI = (1 \times M1 + 2 \times M2 + 3 \times M3) / 500$, where M1, M2 and M3 are the cells containing 1, 2, 3 or more nuclei (Eke & Çelik, 2008).

Statistical analysis: The computer software program SPSS 10.0 was used to analyze the data. The statistical significance of the effects of *Helichrysum* taxa on MN, MI and RI was tested by repeated measures of analysis of variance (ANOVA) and differences between groups were determined by the least significant differences (LSD) test with $p < 0.05$ and $p < 0.01$ considered significant.

Results

Micronucleus (MN) test: The results of MN test are given in Table 1. When MN formation was analyzed after treatment with different concentrations of methanol extracts of *H. pallasii*, *H. chionophilum*, *H. plicatum* subsp. *polyphyllum*, *H. plicatum* subsp. *pseudoplicatum*, significant changes in the percentage of MN were detected (0.5 mg/mL; $p < 0.05$ and $p < 0.01$). *H. peshmenianum* and *H. goulandrionum* did not induce any change in MN frequencies ($p > 0.05$ and $p > 0.01$).

Table 1. Induction of Micronuclei (MN) (%) in human lymphocyte cultures exposed to methanol extracts of *H. pallasii*, *H. chionophilum*, *H. plicatum* subsp. *polyphyllum*, *H. plicatum* subsp. *pseudoplicatum*, *H. peshmenianum*, *H. goulandrionum*

Taxa	Concentrations (mg/mL)					
	Control	0.01	0.05	0.1	0.5	1
<i>H. pallasii</i>	1.30 ± 0.58	1.73 ± 0.70	1.93 ± 0.61	2.23 ± 1.11	3.86 ± 0.86**	3.96 ± 0.89**
<i>H. chionophilum</i>	1.36 ± 1.14	1.86 ± 1.29	2.36 ± 1.13	2.63 ± 1.00	3.80 ± 0.92**	3.90 ± 0.95**
<i>H. plicatum</i> subsp. <i>polyphyllum</i>	1.53 ± 1.06	1.73 ± 1.18	1.70 ± 1.19	1.90 ± 1.30	3.06 ± 1.25*	3.30 ± 1.60*
<i>H. plicatum</i> subsp. <i>pseudoplicatum</i>	0.86 ± 0.71	1.33 ± 0.67	1.43 ± 0.87	2.26 ± 1.10	3.63 ± 1.64**	3.83 ± 1.71**
<i>H. peshmenianum</i>	1.93 ± 0.46	1.73 ± 0.45	1.63 ± 0.51	1.63 ± 0.36	1.86 ± 0.35	2.03 ± 0.51
<i>H. goulandrionum</i>	1.63 ± 0.52	1.63 ± 0.73	1.70 ± 0.76	2.16 ± 1.19	2.43 ± 1.09	2.60 ± 1.03

* =Significantly different from control ($p < 0.05$)

** = Significantly different from control ($p < 0.05$) and ($p < 0.01$)

X: Mean

SD: Standard deviation

Mitotic index (MI): When the potential cytotoxicity of the extracts on lymphocyte cultures was analyzed through MI evaluation, a significant decrease was found for *H. plicatum* subsp. *polyphyllum*, *H. plicatum* subsp. *pseudoplicatum*, *H. goulandrionum* (0.5 mg/mL; $p < 0.05$ and $p < 0.01$) and a significant increase was found for *H. peshmenianum* (0.05 mg/mL; $p < 0.05$ and $p < 0.01$). A cytotoxic effect was not observed for *H. pallasii*, *H. chionophilum* ($p > 0.05$ and $p > 0.01$) (Table 2).

3.3. Replication index (RI): When RI was studied, no modifications could be detected for *H. chionophilum* ($p > 0.05$ and $p > 0.01$). As shown in Table 3, changes in RI reflecting cytotoxic effects were observed for *H. pallasii*, *H. plicatum* subsp. *polyphyllum* (0.5 mg/mL; $p < 0.05$ and $p < 0.01$), *H. plicatum* subsp. *pseudoplicatum*, *H. goulandrionum* (0.5 mg/mL; $p < 0.05$) and *H. peshmenianum* (0.05 mg/mL; $p < 0.05$ and $p < 0.01$).

Discussion

Helichrysum species have been used in folk medicine for thousands of years in the world (especially *H. plicatum* in Turkey). There are few reports in literature about the genotoxic, cytotoxic and mutagenic effects of *Helichrysum* species. There is not any report about cytotoxic effects of *Helichrysum* taxa used this study. For this reason, it is important to determine cytotoxic effects of these taxa.

There are many factors affecting the MN frequency in lymphocytes: age, gender, smoking and alcohol consumption, viral infection, X and gamma ray exposures (Müller, 1996). In this study, donors neither smoking nor drinking alcohol were included. They were not exposed to X and gamma ray and had no viral infection. An increase in MN may result from interactions of a great variety of cytotoxic and genotoxic agents with DNA. MN is an extremely valuable and highly relevant endpoint for the detection of potential carcinogens. Our results show an increase in the percentage of MN (Table 1), suggesting a strong interaction between extracts of *H. pallasii*, *H. chionophilum*, *H. plicatum* subsp. *polyphyllum*, *H. plicatum* subsp. *pseudoplicatum* and DNA, which could be responsible for the observed cytotoxicity. There are no significant effects on MN frequency of *H. peshmenianum* and *H. goulandrionum*.

MI and RI are used as indicators of adequate cell proliferation biomarkers. MI measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be considered as cellular death or delay in the cell proliferation kinetics (Rojas *et al.*, 1993). MI frequencies and RI values decreased with increasing extracts of *H. pallasii*, *H. plicatum* subsp. *polyphyllum*, *H. plicatum* subsp. *pseudoplicatum*, *H. goulandrionum*. This state can explain with two different mechanisms: cellular death and decreasing of cell divisions. The results point to cytotoxic as well as antiproliferative effects and suggest that extracts of these species exhibit cytotoxic as well as antimitotic and possibly anticarcinogenic properties. A negative correlation was observed between MN induction and cell proliferation; the higher the MN frequency detected in exposed individuals, the lower the values of nuclear division progression expressed as RI. This may mean that cells with greater chromosomal damage may die before cell division or may be less capable to enter this phase (Santos-Mello *et al.*, 1974). Multiple MN as the result of the loss of large part of the genome impairs or even prevents cell division (Nath & Ong, 1990).

Table 2. Mitotic index (MI) (%) in human lymphocyte cultures exposed to methanol extracts of *H. pallasii*, *H. chionophilum*, *H. plicatum* subsp. *polyphyllum*, *H. plicatum* subsp. *pseudoplicatum*, *H. peshmenianum*, *H. goulandrionum*.

Taxa	Concentrations (mg/mL)					
	Control	0.01	0.05	0.1	0.5	1
<i>H. pallasii</i>	2.85 ± 1.18	2.68 ± 1.22	2.42 ± 1.05	2.20 ± 1.00	2.00 ± 1.01	1.82 ± 1.14
<i>H. chionophilum</i>	2.75 ± 0.86	2.75 ± 0.83	2.87 ± 0.91	3.06 ± 0.77	3.41 ± 0.70	3.70 ± 0.77
<i>H. plicatum</i> subsp. <i>polyphyllum</i>	5.84 ± 1.21	5.88 ± 1.16	5.83 ± 1.09	5.27 ± 1.22	2.05 ± 0.35**	1.85 ± 0.17**
<i>H. plicatum</i> subsp. <i>pseudoplicatum</i>	3.40 ± 1.33	3.13 ± 1.60	2.91 ± 1.50	2.19 ± 1.46	1.05 ± 0.71**	0.95 ± 0.56**
<i>H. peshmenianum</i>	0.85 ± 0.36	1.00 ± 0.45	2.22 ± 0.93**	2.60 ± 0.91**	3.08 ± 0.87**	3.10 ± 1.08**
<i>H. goulandrionum</i>	4.39 ± 2.37	4.46 ± 2.68	3.91 ± 2.35	2.67 ± 1.60	1.26 ± 0.70**	1.16 ± 0.66**

* = Significantly different from control ($p < 0.05$)

** = Significantly different from control ($p < 0.05$) and ($p < 0.01$)

X: Mean

SD: Standard deviation

Table 3. Replication index (RI) in human lymphocyte cultures exposed to methanol extracts of *H. pallasii*, *H. chionophilum*, *H. plicatum* subsp. *polyphyllum*, *H. plicatum* subsp. *pseudoplicatum*, *H. peshmenianum*, *H. goulandrionum*.

Taxa	Concentrations (mg/mL)					
	Control	0.01	0.05	0.1	0.5	1
<i>H. pallasii</i>	1.459 ± 0.199	1.403 ± 0.186	1.410 ± 0.206	1.290 ± 0.141	1.184 ± 0.103**	1.163 ± 0.104**
<i>H. chionophilum</i>	1.182 ± 0.107	1.183 ± 0.101	1.184 ± 0.106	1.196 ± 0.103	1.262 ± 0.114	1.276 ± 0.112
<i>H. plicatum</i> subsp. <i>polyphyllum</i>	1.343 ± 0.086	1.345 ± 0.106	1.331 ± 0.099	1.330 ± 0.104	1.128 ± 0.056**	1.107 ± 0.047**
<i>H. plicatum</i> subsp. <i>pseudoplicatum</i>	1.287 ± 0.177	1.281 ± 0.170	1.261 ± 0.180	1.255 ± 0.167	1.096 ± 0.071*	1.087 ± 0.069*
<i>H. peshmenianum</i>	1.063 ± 0.044	1.077 ± 0.040	1.215 ± 0.097**	1.231 ± 0.100**	1.231 ± 0.103**	1.236 ± 0.104**
<i>H. goulandrionum</i>	1.249 ± 0.117	1.242 ± 0.137	1.251 ± 0.156	1.225 ± 0.118	1.110 ± 0.055*	1.101 ± 0.064*

* = Significantly different from control ($p < 0.05$)

** = Significantly different from control ($p < 0.05$) and ($p < 0.01$)

X: Mean

SD: Standard deviation

RI = (1xM1 + 2xM2 + 3xM3)/500

M1: The number of cells in first metaphase

M2: The number of cells in second metaphase

M3: The number of cells in third or more metaphase

In the present study, we found that *H. plicatum* subsp. *polyphyllum* and *H. plicatum* subsp. *pseudoplicatum* using in alternative medicine induced MN, decreased MI and RI in human lymphocytes. In our opinion, they should not be used in high quantities in the general population because of their genotoxic and cytotoxic properties. They cause indeed chromosomal damage (an increase in MN) and cell death (decrease in MI and RI). The decrease in cell proliferation may indicate that these taxa may also act as an antimitotic and anticarcinogenic agent. Further studies are needed to determine the effects of main bioactive components isolated from these six taxa on MN, MI and RI.

Acknowledgements

This study was supported with research projects (FBD-07-281 and FBT-06-82) by the Scientific Research Projects Fund of Erciyes University. The authors wish to thank Emine Ölmez for sending blood samples used in this study.

References

- Aslan, M., B. Özçelik, I. Orhan, T. Karaoglu and E. Sezik. 2006. Screening of antibacterial, antifungal and antiviral properties of the selected Turkish *Helichrysum* species. *Planta Med.*, 72: 997-997.
- Aslan, M., A. Demir, B. Mercaoglu, A. Aykac and E. Yesilada. 2007a. Antimicrobial effect of *Helichrysum plicatum* DC. subsp. *plicatum* extracts on *Escherichia coli* O157:H7. *Planta Med.*, 73(9): 889-889.
- Aslan, M., D.D. Orhan, N. Orhan, E. Sezik and E. Yeşilada. 2007b. *In vivo* antidiabetic and antioxidant potential of *Helichrysum plicatum* ssp. *plicatum* capitulums in streptozotocin-induced diabetic rats. *J Ethnopharmacol.*, 109(1): 54-59.
- Baytop, T. 1997. *Türkçe Bitki Adları Sözlüğü*. Atatürk Kültür, Dil ve Tarih Kurumu. Türk Dil Kurumu Yayınları, Ankara, p. 220.
- Davis, P.H. and F.K. Kupicha. 1975. *Helichrysum* Gaertn. In: *Flora of Turkey and the East Aegean Islands* 5. (Ed.): Davis, P.H. Edinburgh University Press, Edinburgh, pp. 80-97.
- Davis, P.H. and F.K. Kupicha. 1988. *Helichrysum* Gaertn. In: *Flora of Turkey and the East Aegean Islands* 10 (Supp.1). (Eds.): P.H. Davis, R.R. Mill & Kit Tan, Edinburgh University Press, Edinburgh, pp. 159-160.
- Eke, D. and A. Çelik. 2008. Genotoxicity of thimerosal in cultured human lymphocytes with and without metabolic activation sister chromatid exchange analysis proliferation index and mitotic index. *Toxicol In vitro.*, 22(4): 927-934.
- Erdoğrul, Ö.T., E. Çakiroğlu and S. Karaman. 2001. Antibacterial activities of *Helichrysum plicatum* subsp. *plicatum* extracts. *The Sciences*, 1(3): 176-178.
- Holland, N., P. Duramad, N. Rothman, L. Figgs, A. Blair, A. Hubbard and M. Smith. 2002. Micronucleus frequency and proliferation in human lymphocytes after exposure to herbicide 2,4-dichlorophenoxyacetic acid *In vitro* and *In vivo*. *Mutat Res.*, 521: 165-178.
- Müller, W.U. 1996. Micronuclei: A biological indicator of radiation damage. *Mutat Res.*, 366: 163-169.
- Nath, C.J. and T. Ong. 1990. Micronuclei assay in cytokinesis-blocked binucleated and conventional mononucleated methods in human peripheral lymphocytes. *Teratogen Carcin Mutagen.*, 10: 273-279.
- Ozkul, Y., S. Silici and E. Eroğlu. 2005. The anticarcinogenic effect of propolis in human lymphocytes culture. *Phytomedicine*, 12: 742-747.
- Rojas, E., L.A. Herrera, M. Sordo, M.E. Gonsebatt, R. Montero, R. Rodriguez and P. Ostrosky-Wegman. 1993. Mitotic index and cell proliferation kinetics for the identification of antineoplastic activity. *Anti-Cancer Drug.*, 4: 637-640.

- Santos-Mello, R., D. Kwan and A. Norman. 1974. Chromosome aberrations and T-cell survival in human lymphocytes. *Radiat Res.*, 60: 482-488.
- Scarpato, R. and L. Migliore. 1996. Comparison of spontaneous structural chromosome aberration frequency in 48 h-cultured human lymphocytes mitotically arrested by different colchemid treatments. *Mutat Res.*, 361: 35-39.
- Selvendiran, K., R. Padmavathi, V. Magesh and D. Sakthisekaran. 2005. Preliminary study on inhibition of genotoxicity by piperine in mice. *Fitoterapia*, 76(3-4): 296-300.
- Tabata, M., G. Honda, E. Sezik and E. Yeşilada. 1993. A report on traditional medicine and medicinal plants in Turkey (1990, 1991). Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto.
- Tepe, B., M. Sokmen, H.A. Akpulat and A. Sokmen. 2005. *In vitro* antioxidant activities of the methanol extracts of four *Helichrysum* species from Turkey. *Food Chem.*, 90: 685-689.

(Received for publication 20 June 2008)