

In Vitro Genotoxic Effects of Four *Helichrysum* Species in Human Lymphocytes Cultures

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ABSTRACT

Helichrysum sanguineum, *Helichrysum pamphylicum*, *Helichrysum orientale*, *Helichrysum noeanum* (Asteraceae) are medicinal plants. For centuries, they have been used as tea in Turkey because of their medicinal properties. So far no scientific evidence has been found in a literature survey regarding the genotoxic effects of these plants. This work evaluated the genotoxic effects on human lymphocyte cultures induced by methanol extracts of these plants, assayed in different concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/mL). According to the results, *Helichrysum noeanum*, *Helichrysum pamphylicum* and *Helichrysum sanguineum* induced the formation of micronuclei and decreased the mitotic and replication indexes. *Helichrysum orientale* did not affect these parameters, whereas *Helichrysum noeanum*, *Helichrysum pamphylicum* and *Helichrysum sanguineum* were clearly genotoxic. They should therefore not be used freely in alternative medicine, although their antiproliferative activity may suggest antimitotic and anticarcinogenic properties. *Helichrysum orientale* could be used in alternative medicine.

Key terms: genotoxic, *Helichrysum*, micronucleus, mitotic index, replication index.

INTRODUCTION

The genus *Helichrysum* Mill, belonging to the family Asteraceae, is represented by approximately 300 species in the world. This genus is represented in Turkish flora by 26 taxa, of which 13 are endemic (Davis and Kupicha, 1975). *Helichrysum* species are aromatic plants, commonly known as everlasting, immortal flower and fadeless flower, grow wild in Anatolia and are widely used as tea. They are used for the treatment of kidney stones, urogenital disorders, stomach pain, jaundice, diarrhea and asthma (Baytop, 1997).

Basic chemical compounds of *Helichrysum sanguineum* (L.) Kostel. (Meriçli et al., 1984), *Helichrysum pamphylicum* P.H.Davis & Kupicha (Sezik and Akdemir, 1986), *Helichrysum orientale* (L.) DC. (Çubukçu and Bingöl, 1981), *Helichrysum noeanum* Boiss. (Bingöl and Çubukçu, 1984) are flavonoids. These species are responsible for the remarkable antibacterial, antiviral, antifungal (Aslan et al., 2006), antioxidant (Tepe et al., 2005) and anticancer (Topcu, 2007) properties.

The micronucleus (MN), mitotic index (MI) and replication index (RI) analysis methods are cytogenetic tests that are used both *in vivo* and *in vitro*. An MN is a small extra nucleus separated from the main one, generated during cellular division by late chromosomes or by

chromosome fragments. Because of its association with chromosomal aberrations, MN has been used since 1937 as an indicator of genotoxic exposure based on the radiation studies conducted by Brenneke and Mather (Heddle et al, 1983). The MI assay is used to characterize proliferating cells and identify compounds that inhibit or induce mitotic progression. RI measures cell division kinetics by counting the percentage of cells in first, second and third or more metaphase (Holland et al., 2002).

So far, no scientific evidence has been found in a literature survey regarding the genotoxic effects of these plants. This work evaluated the genotoxic effects of *Helichrysum sanguineum*, *Helichrysum pamphylicum*, *Helichrysum orientale*, *Helichrysum noeanum*, used in Turkey as folk medicine. *Helichrysum pamphylicum* and *Helichrysum noeanum* are endemic species of the Turkish flora.

METHODS

Plant materials

The aerial parts of *Helichrysum* species were collected from Hatay (*Helichrysum sanguineum*), Antalya (*Helichrysum pamphylicum*), Aydın (*Helichrysum orientale*) and Ankara (*Helichrysum noeanum*), Turkey, in the months of May and June, and were identified by Prof. Dr. Ergin Hamzaoğlu,

Prof. Dr. Ahmet Aksoy and Dr. Ümit Budak. Voucher specimens (Budak 2034, Hamzaoğlu 3639, Budak 2001 and Hamzaoğlu 3742) have been deposited in the Herbarium of the Department of Biology, Bozok University for future reference.

Preparation of the methanol extracts

The aerial parts of the plant material were dried in shade at room temperature and then ground to powder in a mechanic grinder. Then the powdered plant materials (10 g) were extracted in a Soxhlet extractor with 100 mL methanol (100%) at 60°C for 6 h. After determining the yield, 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, Israel), colcemid (Sigma, Germany) and giemsa stain (Merck, Germany) were used in peripheral blood cultures. PB karyotyping medium is based on RPMI-1640 basal medium supplemented with L-glutamine, foetal bovine serum, antibiotics (gentamycin) and phytohemagglutinin.

Subjects

The study involved six donors (three male and three female) at Yozgat, Turkey, in the age group 26 ± 45 years. The selection criteria for the subjects were based on a questionnaire intended to elicit information on the subject's age, smoking habits, alcohol consumption and health condition. Individuals selected for participation were non-smokers and non-consumers of alcohol who had not been exposed to x-rays or gamma rays nor had had a viral infection.

In vitro mitotic index assay

After obtaining approval from Local Ethic Committee, heparinized blood samples (0.4 mL) of six healthy donors were placed in sterile culture tubes containing 5 mL of PB karyotyping medium. Then, *Helichrysum* extract preparations 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 shaking gently, the culture tubes were incubated in a slanted position at 37°C for 72 h. After 70 h of incubation, a 0.1 mL colcemid solution (10 µg/mL) was added to each culture tube and mixed by shaking gently. After 72 h of incubation, cells were harvested by centrifugation,

by hypotonic treatment (0.075 M KCl) and fixing in a fresh fixative solution (methanol:acetic acid, 3:1). The fixation step was repeated three times. Slides were air-dried and stained with Giemsa (Ozkul et al., 2005). MI was calculated as the proportion of metaphase for 2000 cells for each donor and concentration.

In vitro micronucleus assay

For MN analysis, the peripheral lymphocytes were incubated at 37 °C for 72 h. The cells were treated with *Helichrysum* extract preparations at 0.01, 0.05, 0.1, 0.5 and 1 mg/mL concentrations. Cytochalasin B (Sigma) was added at 44 h of incubation at a final concentration of 5 µg/mL to block cytokinesis. At the end of incubation at 37 °C, cells were harvested by centrifugation. The MN staining was performed according to Ozkul et al. (2005).

The slides were scored by a single observer. About 500 cells were examined at 600 check magnification from each slide and when MN cells were located they were examined under 1000 magnification. The criteria suggested by Scarpato and Migliore (1996) for recognizing MN was followed.

The extent and progression of nuclear division were measured by counting the number of cells with one, two, three and four or more nuclei in a total of 500 cells per sample (Figure 1). RI was calculated among 500 cells per culture, according to the following formula: $RI = (1 \times M1 + 2 \times M2 + 3 \times M3) / 500$, where M1, M2 and M3 are the number of cells in first metaphase, second metaphase and third or more metaphase, respectively (Holland et al., 2002).

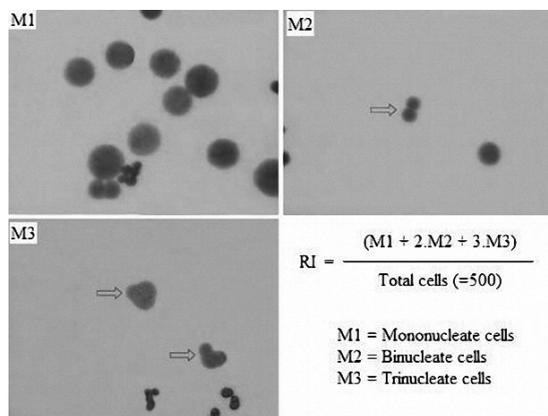


Figure 1: The general appearance of mononucleate, binucleate and trinucleate cells.

Statistical analysis

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

RESULTS

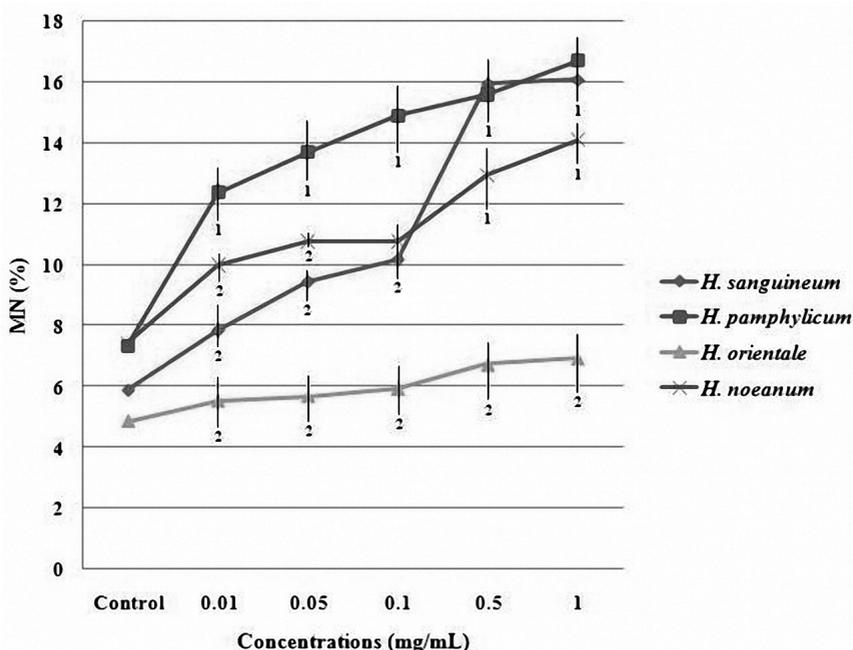
Micronucleus

The results of MN test are given in Figure 2. The mean values of the MN rates of added different concentrations of plant extracts were between 7.83 ± 3.72 and 16.06 ± 3.84 for *Helichrysum sanguineum*, 12.33 ± 1.47 and 16.66 ± 1.53 for *Helichrysum pamphylicum*, 5.50 ± 3.02 and 6.90 ± 3.17 for *Helichrysum orientale*, 9.96 ± 1.85 and 14.06 ± 2.57 for *Helichrysum noeanum*. MN rates of 1 mg/mL added to plant extracts are 1.5-3 times more than that of in the control. When MN formation was analyzed after

treatment with different concentrations of methanol extract of *Helichrysum sanguineum* (0.5 mg/mL), *Helichrysum pamphylicum* (0.01 mg/mL), *Helichrysum noeanum* (0.5 mg/mL), significant changes in the percentage of MN were detected ($p < 0.01$). *Helichrysum orientale* did not induce any change in MN frequencies ($p = 0.01$).

Mitotic index

The results of MI test are given in Table I. The mean values of the MI rates of added different concentrations of plant extracts were between 4.78 ± 1.26 and 0.21 ± 0.08 for *Helichrysum sanguineum*, 1.75 ± 1.07 and 0.01 ± 0.04 for *Helichrysum pamphylicum*, 5.05 ± 2.79 and 5.11 ± 2.43 for *Helichrysum orientale*, 3.87 ± 0.73 and 1.14 ± 0.42 for *Helichrysum noeanum*. When potential genotoxicity of the extracts on lymphocyte cultures was analyzed through MI evaluation, significant decreases were found for *Helichrysum sanguineum* (0.05 mg/mL), *Helichrysum pamphylicum* (0.01 mg/mL), *Helichrysum noeanum* (0.01 mg/mL) ($p < 0.01$). A genotoxic effect was not observed for *Helichrysum orientale* ($p = 0.01$).



ANOVA: 1 ($p < 0.01$)

ANOVA: 2 ($p = 0.01$)

Figure 2: Micronucleus changes of *Helichrysum sanguineum*, *Helichrysum pamphylicum*, *Helichrysum orientale* and *Helichrysum noeanum*. *Helichrysum sanguineum*, *Helichrysum pamphylicum* and *Helichrysum noeanum* induced MN in human lymphocytes. These increases were dose-dependent. When MN formation was analyzed after treatment with different concentrations of methanol extract of *Helichrysum sanguineum* (0.5 mg/mL), *Helichrysum pamphylicum* (0.01 mg/mL), *Helichrysum noeanum* (0.5 mg/mL), significant changes in the percentage of MN were detected ($p < 0.01$). *Helichrysum orientale* did not induce any change in MN frequencies ($p = 0.01$).

Table I

Mitotic index (%) (mean \pm SDs) in human lymphocyte cultures exposed to extracts of *Helichrysum sanguineum*, *Helichrysum pamphylicum*, *Helichrysum orientale* and *Helichrysum noeanum*

Species	Concentrations (mg/mL)	Total counted cells	Total number: dividing cells	Mean \pm SDs(%)
<i>Helichrysum sanguineum</i>	Control	12000	576	4.80 \pm 1.27
	0.01	12000	574	4.78 \pm 1.26
	0.05	12000	362	3.01 \pm 1.55 *
	0.1	12000	133	1.10 \pm 0.43 *
	0.5	12000	37	0.30 \pm 0.05 *
	1	12000	26	0.21 \pm 0.08 *
<i>Helichrysum pamphylicum</i>	Control	12000	763	6.35 \pm 2.71
	0.01	12000	211	1.75 \pm 1.07 *
	0.05	12000	175	1.45 \pm 0.99 *
	0.1	12000	76	0.63 \pm 0.53 *
	0.5	12000	15	0.12 \pm 0.10 *
	1	12000	2	0.01 \pm 0.04 *
<i>Helichrysum orientale</i>	Control	12000	676	5.63 \pm 2.51
	0.01	12000	607	5.05 \pm 2.79
	0.05	12000	640	5.33 \pm 2.84
	0.1	12000	637	5.30 \pm 2.78
	0.5	12000	629	5.24 \pm 2.46
	1	12000	614	5.11 \pm 2.43
<i>Helichrysum noeanum</i>	Control	12000	749	6.24 \pm 2.40
	0.01	12000	465	3.87 \pm 0.73 *
	0.05	12000	255	2.12 \pm 0.89 *
	0.1	12000	168	1.40 \pm 0.55 *
	0.5	12000	149	1.24 \pm 0.58 *
	1	12000	137	1.14 \pm 0.42 *

ANOVA: * $p < 0.01$ (significantly different from control)

Replication index

RI results are parallel to MI results. Increasing extract concentrations have caused a decreasing rate of RI. When RI was studied, no modifications could

be detected for *Helichrysum orientale* ($p = 0.01$). As shown in Table II, changes in RI reflecting genotoxic effects were observed for *Helichrysum sanguineum* (0.05 mg/mL), *Helichrysum pamphylicum* (0.01 mg/mL) and *Helichrysum noeanum* (0.1 mg/mL) ($p < 0.01$).

Table II

Replication index (mean \pm SDs) in human lymphocyte cultures exposed to extracts of *Helichrysum sanguineum*, *Helichrysum pamphylicum*, *Helichrysum orientale* and *Helichrysum noeanum*

Species	Concentrations of plant extracts (mg/mL)					
	Control	0.01	0.05	0.1	0.5	1
<i>H. sanguineum</i>	1.416 \pm 0.127	1.327 \pm 0.104	1.248 \pm 0.059 *	1.219 \pm 0.059 *	1.168 \pm 0.050 *	1.144 \pm 0.054 *
<i>H. pamphylicum</i>	1.495 \pm 0.103	1.337 \pm 0.086 *	1.334 \pm 0.064 *	1.220 \pm 0.075 *	1.148 \pm 0.037 *	1.071 \pm 0.021 *
<i>H. orientale</i>	1.534 \pm 0.112	1.481 \pm 0.139	1.449 \pm 0.108	1.469 \pm 0.157	1.404 \pm 0.133	1.400 \pm 0.129
<i>H. noeanum</i>	1.368 \pm 0.085	1.352 \pm 0.083	1.275 \pm 0.067	1.224 \pm 0.063 *	1.226 \pm 0.058 *	1.112 \pm 0.035 *

ANOVA: * $p < 0.01$ (significantly different from control)

DISCUSSION

Helichrysum species have been used in folk medicine for thousands of years throughout world. There are a lot of studies interested in properties of *Helichrysum* species and the use folk medicine derived from them. Some members of this genus have cholagogue and choleric activity and stimulate the secretion of gastric juice (Litvinenko et al., 1992). Studies regarding *Helichrysum* species used in this study are summarized. The literature indicates the antibacterial, antiviral, antifungal (Aslan et al., 2006), antioxidant (Tepe et al., 2005) and anticancer (Topcu, 2007) effects of *Helichrysum sanguineum*, *Helichrysum pamphylicum*, *Helichrysum orientale* and *Helichrysum noeanum*. The major components of these species are flavonoids (Bingöl and Çubukçu, 1984; Çubukçu and Bingöl, 1981; Meriçli et al., 1984; Sezik and Akdemir, 1986). The genotoxic effects of *Helichrysum sanguineum*, *Helichrysum pamphylicum* and *Helichrysum noeanum* may result from flavonoids. However, there are few reports about the cytotoxic, genotoxic and mutagenic effects of *Helichrysum* species and no information is available about the genotoxic effects of the *Helichrysum* species used in this study. For this reason, it is important to determine the genotoxic effects of these species.

There are many factors affecting the MN frequency in lymphocytes: age, gender, smoking and alcohol consumption, viral infection and X and gamma ray exposure (Müller, 1996). In this study, donors included only non-smokers and non-consumers of alcohol who had not been exposed to X and gamma rays, nor had had a viral infection. An increase in MN may result from interactions of a great variety of cytotoxic and genotoxic agents with chromosomal damage. 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 (Fig. 2), suggesting a strong interaction between extracts of *Helichrysum sanguineum*, *Helichrysum pamphylicum*, *Helichrysum noeanum* and chromosomal damage, which could be responsible for the observed genotoxicity. However, there are no significant effects of *Helichrysum orientale* on MN frequency.

MI and RI are used as indicators of adequate cell proliferation biomarkers. We detected a positive correlation between MI and RI; namely the lower the MI rates detected in exposed individuals, the lower the values of nuclear division progression expressed as RI. The decreases of MI and RI rates and the positive correlation between MI and RI could result from a cell cycle delay and cytotoxic effects of *Helichrysum* species. MI measures the proportion of cells in the

M-phase of the cell cycle and its inhibition could be considered as delay in the cell proliferation kinetics (Rojas et al., 1993). Except for *Helichrysum orientale*, there are major differences among MI and RI rates of other *Helichrysum* species.

The present results also show that RI values were significantly lower in extract concentrations than in controls. This state can be explained with mitotic delay, which, by permitting the repair of genotoxic lesions or cells with greater chromosomal damage, may die before cell division (Santos-Mello et al., 1974). Multiple MN as the result of the loss of large part of the chromosome impairs or even prevents the cell division (Nath and Ong, 1990).

In the present study, we found that extracts of four *Helichrysum* species used in alternative medicine induced MN decreased MI and RI in human lymphocytes. We are of the opinion that *Helichrysum sanguineum*, *Helichrysum pamphylicum* and *Helichrysum noeanum* should not be used in high quantities in the general population because of their genotoxic properties (especially *Helichrysum pamphylicum*). Indeed this plant extracts could cause chromosomal damage (an increase in MN) and mitotic delay (decrease in MI and RI). According to our results, *Helichrysum orientale* is not genotoxic and thus it can be freely used in alternative medicine. The decrease in cell proliferation may indicate that *Helichrysum sanguineum*, *Helichrysum pamphylicum* and *Helichrysum noeanum* may also act as antimitotic and anticarcinogenic agents. In contrast, increasing MN rates showed that they could have a carcinogenic effect at high concentrations. Furthermore, there are a lot of compounds in the contents of *Helichrysum* species. The use of single constituents would allow more precise interpretation of data and the added effect of the numerous compounds will be lost. It is therefore likely that the mixture of *Helichrysum* compounds may result in synergistic effects. A further study will be needed to determine the effects of different compounds isolated from *Helichrysum* species and evaluate the synergistic effects on MI, RI and MN. Besides, this study should support with reports about the metabolisms of extracts of these species and others genotoxic studies in mammals.

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REFERENCES

- ASLAN M, ÖZÇELİK B, ORHAN I, KARAOĞLU T, SEZİK E (2006) Screening of antibacterial, antifungal and antiviral properties of the selected Turkish *Helichrysum* species. *Planta Med* 72:997-997.
- BAYTOP T (1997) Türkçe Bitki Adları Sözlüğü. Türk Dil Kurumu Yayınları, Ankara, p:220.
- BİNGÖL S, ÇUBUKÇU B (1984) Flavonoids of *Helichrysum noeanum* Boiss. (Asteraceae). *Sci Pharm* 52:127-130.
- ÇUBUKÇU B, BİNGÖL S (1981) A further investigation on the flavonoids of *Helichrysum orientale*. *J Fac Pharm İstanbul Univ* 7:86-90.
- DAVIS PH, KUPICHA FK (1975) *Helichrysum* Gaertn. In: DAVIS PH (eds) Flora of Turkey and the East Aegean Islands, part 5. Edinburgh: Edinburgh University Press. pp:80-97.
- HEDDLE JA, HITE M, KIRKHART B, MAVOURNIN K, MACGREGOR JT, NEWELL GW, SALAMONE MF (1983) The induction of micronuclei as a measure of genotoxicity. A report of the US Environmental Protection Agency Gene Tox. Program. *Mutat Res* 123:61-118.
- HOLLAND N, DURAMAD P, ROTHMAN N, FIGGS L, BLAIR A, HUBBARD A, SMITH M (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.
- LITVINENKO VI, POPOVA P, POPOVA NV, BUBENCHIKOVA VN (1992) Medicinal plants and preparations derived from them. *Farmaseotychnyi Zhurnal* 53:83-84.
- MERİÇLİ AH, ÇUBUKÇU B, DORTUNÇ T (1984) Flavonoids and anthocyanins of *Helichrysum sanguineum*. *Fitoterapia* 55:112-115.
- MÜLLER WU (1996) Micronuclei: A biological indicator of radiation damage. *Mutat Res* 366:163-169.
- NATH CJ, ONG T (1990) Micronuclei assay in cytokinesis-blocked binucleated and conventional mononucleated methods in human peripheral lymphocytes. *Teratogen Carcin Mut* 10:273-279.
- OZKUL Y, SILICI S, EROĞLU E (2005) The anticarcinogenic effect of propolis in human lymphocytes culture. *Phytomedicine* 12:742-747.
- ROJAS E, HERRERA LA, SORDO M, GONSEBATT ME, MONTERO R, RODRIGUEZ R, OSTROSKYWEGMAN P (1993) Mitotic index and cell proliferation kinetics for the identification of antineoplastic activity. *Anti-Cancer Drug* 4:637-640.
- SANTOSMELLO R, KWAN D, NORMAN A (1974) Chromosome aberrations and T-cell survival in human lymphocytes. *Radiat Res* 60:482-488.
- SCARPATO R, MIGLIORE L (1996) Comparison of spontaneous structural chromosome aberration frequency in 48 h-cultured human lymphocytes mitotically arrested by different colcemid treatments. *Mutat Res* 361:35-39.
- SEZİK E, AKDEMİR Z (1986) Flavonoids of *Helichrysum pamphylicum* Davis&Kupicha. *Acta Pharm Turc* 28:141-144.
- TEPE B, SOKMEN M, AKPULAT HA, SOKMEN A (2005) *In vitro* antioxidant activities of the methanol extracts of four *Helichrysum* species from Turkey. *Food Chem* 90:685-689.
- TOPCU Z (2007) DNA Topoisomerases as the targets of anticancer drugs. *Acta Biochem Pol (Suppl)* 5:56-56.