Cytogenetic effects of Helichrysum arenarium in human lymphocytes cultures

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Abstract: Helichrysum Mill., belonging to the family Asteraceae, is commonly known as “everlasting”. Helichrysum species have been used as herbal tea for centuries in Turkey against gallbladder disorders because of their bile regulatory and diuretic properties. However, there are few reports in the literature regarding the genotoxic effects of these plants. In the present study, the genotoxic effects induced by aqueous (decoction) and methanol extracts of Helichrysum arenarium (L.) Moench subsp. rubicundum (K.Koch) P.H.Davis & Kupicha, H. arenarium (L.) Moench subsp. aucheri (Boiss.) P.H.Davis & Kupicha, and H. arenarium (L.) Moench subsp. erzincanicum P.H.Davis & Kupicha on human lymphocyte cultures at 0.01, 0.05, 0.1, 0.5, and 1 mg/mL concentrations were evaluated. Three different parameters (micronucleus, mitotic, and replication indexes) were used. H. arenarium subsp. erzincanicum induced the formation of micronuclei and decreased the mitotic and replication indexes as well. H. arenarium subsp. rubicundum and H. arenarium subsp. aucheri did not affect these parameters. Our results clearly indicate that H. arenarium subsp. erzincanicum has genotoxic effects. Therefore, this plant should not be used freely in alternative medicine although its antiproliferative activity may suggest antimitotic and anticarcinogenic properties. H. arenarium subsp. rubicundum and H. arenarium subsp. aucheri, on the other hand, do not have genotoxic effects. Thus, they can be used freely in alternative medicine. Further studies are needed to determine the effects of the main bioactive components isolated from H. arenarium on the micronucleus, mitotic index, and replication index.

Key words: Alternative medicine, genotoxic, Helichrysum arenarium, medicinal plant, micronucleus test

İnsan lenfosit kültürlerinde Helichrysum arenarium’un sitogenetik etkileri

Özet: Asteraceae familyasında yer alan Helichrysum Mill. cinsi genellikle “ölmez çiçek” olarak bilinir. Helichrysum türleri safra düzenleyici ve idrar söktürücü özelliklerinden dolayı Türkiye’de yüzylar boyunca safra kesesi düzenizliklerinde bitkisel çay olarak kullanılmaktadır. Bununla birlikte bu bitkilerin genotoksik etkileri ile ilgili olarak literatürde çok az rapor vardır. Bu çalışmada, Helichrysum arenarium (L.) Moench subsp. rubicundum (K.Koch) P.H.Davis & Kupicha, H. arenarium (L.) Moench subsp. aucheri (Boiss.) P.H.Davis & Kupicha, ve H. arenarium (L.) Moench subsp. erzincanicum P.H.Davis & Kupicha’nın 0.01, 0.05, 0.1, 0.5 ve 1 mg/mL’lik konsantrasyonlardaki su ve metanol ekstraktları ile indüklenen insan lenfosit kültürlerindeki genotoksik etkileri değerlendirildi. Üç farklı parametre (makronükleus, mitotik indeks ve replikasyon indeksleri) kullanıldı. H. arenarium subsp. erzincanicum mitotik ve replikasyon indekslerini azalttı ve makronükleus formasyonunu indükledi. H. arenarium subsp. rubicundum ve H. arenarium subsp. aucheri bu parametreleri etkilemedi. Sonuçlarımız açığa göstermektedir ki H. arenarium subsp. erzincanicum genotoksik etkileri sahiptir. Bu tür antiproliferatif aktivitesi nedeniyle antimitotik ve antikarsinojenik özellikler gösterebilmesine rağmen...
Introduction

*Helichrysum arenarium* (L.) Moench (Asteraceae) (popularly known as everlasting, immortal flower or fadeless flower) grows wild in Anatolia and is widely used as herbal tea. It is used for the treatment of kidney stones, uro-genital disorders, stomach pain, jaundice, diarrhea, and asthma (1,2).

*H. arenarium* is an aromatic plant. It grows on dry calcareous or sandy soils, steppes, and banks at altitudes from 250 to 3200 m. There are 3 subspecies of *H. arenarium* in Turkey: *H. arenarium* subsp. *rubicundum* (K.Koch) P.H.Davis & Kupicha, *H. arenarium* subsp. *aucheri* (Boiss.) P.H.Davis & Kupicha, and *H. arenarium* subsp. *erzincanicum* P.H.Davis & Kupicha (3).

*H. arenarium* exhibits various biological properties including antioxidant, hepatoprotective, cholangenic, choloretic (4-6), antibacterial, antiviral, and antifungal (7) activities. Furthermore, this species is a source of many active compounds such as flavonoids, phenolics, polyphenols (4,6,8), flavones (9), essential oils (10), polysaccharides (11), glycosides (12), and coumarins (13).

The micronucleus (MN), mitotic index (MI), and replication index (RI) analysis methods are cytogenetic tests that are used both in vivo and in vitro. The in vitro MN test was first used in genotoxicity in the 1970s. A long experience of this system was first obtained from early studies on human lymphocytes (14,15). Today the in vitro MN assay in human lymphocytes is widely used in biomonitoring studies and for detecting clastogens and aneugens (15). The MI depends on 2 factors: first the proportion of the cell population that participates in the whole cycle of interphase leading to division; second the relative lengths of interphase and recognizable mitotic stages (18). The RI measures cell division kinetics by counting the percentage of cells in the first, second, and third or more metaphases (17).

So far no scientific evidence has been found in the literature regarding the genotoxic effects of *H. arenarium*. This study evaluated the genotoxic effects of the subspecies of *H. arenarium* in Turkey.

Materials and methods

Plant material

The aerial parts of *H. arenarium* were collected from Erzurum (*H. arenarium* subsp. *rubicundum*), Kırşehir (*H. arenarium* subsp. *aucheri*), and Erzincan (*H. arenarium* subsp. *erzincanicum*) in Turkey from June to July and were identified by Prof. Dr. Ergin Hamzaoğlu, Prof. Dr. Ahmet Aksoy, and Dr. Ümit Budak. The voucher specimens (Hamzaoğlu 4984, Budak 2184, and Hamzaoğlu 4650) have been deposited in the Herbarium of the Department of Biology, Bozok University, for future reference.

Preparation of the extracts

The aqueous extracts (AEs) (decoction) were prepared by boiling the air-dried aerial parts of the plants, ground using a mechanical mill in water (4%, w/v) at 100 °C for 5 min in the case of decoction. The preparations were sterilized through a filter and stored at 4 °C.

The plants dried at room temperature were ground down to powder using a grinder. The powdered plant
materials (10 g) were extracted in a Soxhlet extractor with 100 mL of methanol (MeOH) (100%) at 60 °C for 6 h. The extracts were filtered and concentrated to dryness under reduced pressure at 40 °C in a rotary evaporator. The methanol extracts (ME) 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 was based on RPMI-1640 basal medium supplemented with L-glutamine, fetal bovine serum, antibiotics (gentamycin), and phytohemagglutinin.

**In vitro mitotic index assay**

Heparinized blood samples (0.4 mL), which were obtained from 6 healthy donors with the permission of Yozgat State Hospital, were placed in sterile culture tubes containing 5 mL of PB karyotyping medium. Then AE and ME were added to obtain 5 final concentrations (0.01, 0.05, 0.1, 0.5, and 1 mg/mL). However, AE and ME were not added to the tubes of control groups. The contents of each tube were mixed by gentle shaking, and then the tubes were incubated in a slanted position at 37 °C for 72 h. After 70 h of incubation, 0.1 mL of colcemid solution (10 μg/mL) was added to each tube and the contents were mixed again by shaking the tubes gently. At the end of the incubation, the tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded. The pellet was resuspended using 10 mL of hypotonic solution (0.075 M KCl) and the tubes were incubated at 37 °C for a further 4 min. The tubes were centrifuged again at 2000 rpm for 4 min and the supernatant was discarded. Following this, the pellet was resuspended using 10 mL of fresh fixative solution (methanol:acetic acid, 3:1). The tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded. This procedure was repeated 3 times. The pellet was resuspended and 0.5-1 mL of fresh cold fixative solution was added to the tubes. Then 3 or 4 drops of cell suspension were placed on cold wet glass slides. The slides were air dried and stained with 5% Giemsa. The MI was calculated as the proportion of metaphase for 2000 cells in each donor and concentration.

**In vitro micronucleus assay**

For the MN analysis, the peripheral lymphocytes were incubated at 37 °C for 72 h. The cells were treated with AE and ME at concentrations of 0.01, 0.05, 0.1, 0.5, and 1 mg/mL. Cytochalasin B (Sigma) was added at 44 h of the incubation at a final concentration of 5 μg/mL to block cytokinesis. At the end of the incubation at 37 °C, the cells were harvested by centrifugation. The MN staining was performed according to Eroğlu et al. (19).

The slides were scored by a single observer. Five hundred cells were examined at 600× magnification from each slide and when MN cells were located they were examined at 1000× magnification (20). The criteria suggested by Scarpato and Migliore (21) for recognizing the MN were adopted.

The RI was calculated for 500 cells per culture according to the following formula: $RI = \left(1 \times M_1 + 2 \times M_2 + 3 \times M_3\right)/500$, where $M_1$, $M_2$, and $M_3$ stand for the number of cells in the first metaphase, second metaphase and third or more metaphases, respectively (17,22).

**Statistical analysis**

SPSS 10.0 was used to analyze the data. The statistical significance of the effects of *H. arenarium* subspecies on the MN, MI, and RI was assessed using repeated-measures analysis of variance (ANOVA) and the differences between groups were determined by the least significant differences (LSD) test. The differences were considered significant (P < 0.05 and P < 0.01).

**Results**

**Micronucleus**

The results of the MN test are given in Table 1. When the MN formation was analyzed after the treatment of *H. arenarium* subsp. *erzincanicum* with different concentrations of AE and ME, significant changes were detected in the percentage of MN for AE (0.5 mg/mL: P < 0.05; 1 mg/mL: P < 0.01) but not for ME. AE and ME of *H. arenarium* subsp. *rubicundum* and *H. arenarium* subsp. *aucheri* did not induce any changes in MN frequencies.
Mitotic index

When the genotoxic potentials of the extracts in lymphocyte cultures were analyzed through MI evaluation, a significant decrease was found for *H. arenarium* subsp. *rubicundum* (AE-1 mg/mL: $P < 0.05$) and *H. arenarium* subsp. *erzincanicum* (AE-0.5 mg/mL: $P < 0.05$; 1 mg/mL: $P < 0.01$) (ME-1 mg/mL: $P < 0.01$). No genotoxic effects were observed for AE and ME of *H. arenarium* subsp. *aucheri* (Table 2).

Replication index

When the RI was analyzed, no modifications were detected for *H. arenarium* subsp. *rubicundum* and *H. arenarium* subsp. *aucheri*. As shown in Table 3, changes were observed in the RI reflecting the genotoxic effects for AE (0.1 mg/mL: $P < 0.05$; 0.5 and 1 mg/mL: $P < 0.01$) and ME (0.5 and 1 mg/mL: $P < 0.01$) of *H. arenarium* subsp. *erzincanicum*.

Discussion

*Helichrysum* species have been used in folk medicine for thousands of years. Particularly the species *H. arenarium*, *H. plicatum* DC., and *H. stoechas* (L.) Moench are used in Turkey for this purpose. There are few reports in the literature regarding the genotoxic, cytotoxic, and mutagenic effects of *Helichrysum* species. Eroğlu et al. (19) reported the inhibitory effects of *H. stoechas* subsp. *barrelieri*, *H. armenium* DC. subsp. *armenium*, *H. armenium* DC. subsp. *araxinum* (Kirp.) Takht., *H. plicatum* subsp. *plicatum*, *H. compactum* Boiss., and *H. artvinense* P. H. Davis & Kupicha on the MI and RI, indicating that these taxa can have genotoxic effects. The increasing effects of *H. stoechas* subsp. *barrelieri*, *H. armenium* subsp. *armenium*, *H. armenium* subsp. *araxinum*, *H. chasmolyticicum* P. H. Davis, *H. plicatum* subsp. *plicatum*, *H. compactum*, and *H. artvinense* on the MN rates showed that these taxa can have genotoxic and carcinogenic effects. Reid et al. (23) evaluated the mutagenic and antimutagenic effects of South African plants (*H. herbaceum* Sweet., *H. nudifolium* Less., *H. ruderale* Hilliard. & B. L. Burtt, *H. rugulosum* Less., *H. simillimum* DC., *H. umbraculigerum* Less.) and reported that *H. simillimum*, *H. herbaceum*, and *H.
Table 2. Mitotic index (%) (mean ± standard deviation) in human lymphocyte cultures exposed to the extracts of Helichrysum arenarium subsp. rubicundum, Helichrysum arenarium subsp. aucheri, and Helichrysum arenarium subsp. erzincanicum.

<table>
<thead>
<tr>
<th>Concentrations of Plant Extracts (mg/mL)</th>
<th>H. arenarium subsp. rubicundum</th>
<th>H. arenarium subsp. aucheri</th>
<th>H. arenarium subsp. erzincanicum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decoction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control *</td>
<td>2.41 ± 0.92</td>
<td>4.73 ± 1.69</td>
<td>2.88 ± 1.32</td>
</tr>
<tr>
<td>0.01</td>
<td>1.98 ± 1.30</td>
<td>3.15 ± 1.35</td>
<td>2.20 ± 0.80</td>
</tr>
<tr>
<td>0.05</td>
<td>2.04 ± 1.06</td>
<td>3.78 ± 1.59</td>
<td>1.89 ± 0.76</td>
</tr>
<tr>
<td>0.1</td>
<td>1.83 ± 0.94</td>
<td>3.50 ± 1.71</td>
<td>1.95 ± 0.83</td>
</tr>
<tr>
<td>0.5</td>
<td>1.70 ± 1.17</td>
<td>4.03 ± 1.26</td>
<td>1.58 ± 0.67 *</td>
</tr>
<tr>
<td>1</td>
<td>1.10 ± 0.70 *</td>
<td>3.57 ± 1.81</td>
<td>1.36 ± 0.44 **</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control *</td>
<td>2.57 ± 1.00</td>
<td>2.65 ± 0.99</td>
<td>4.09 ± 1.92</td>
</tr>
<tr>
<td>0.01</td>
<td>2.41 ± 0.95</td>
<td>2.49 ± 1.04</td>
<td>3.78 ± 1.91</td>
</tr>
<tr>
<td>0.05</td>
<td>2.35 ± 0.90</td>
<td>2.35 ± 1.03</td>
<td>3.82 ± 1.91</td>
</tr>
<tr>
<td>0.1</td>
<td>2.21 ± 0.85</td>
<td>2.31 ± 0.87</td>
<td>3.73 ± 1.96</td>
</tr>
<tr>
<td>0.5</td>
<td>1.90 ± 0.74</td>
<td>2.01 ± 0.86</td>
<td>2.53 ± 1.77</td>
</tr>
<tr>
<td>1</td>
<td>1.81 ± 0.70</td>
<td>1.89 ± 0.77</td>
<td>0.92 ± 0.78 **</td>
</tr>
</tbody>
</table>

* not AE or ME
** – significantly different from control (P < 0.01)

Table 3. Replication index (mean ± standard deviation) in human lymphocyte cultures exposed to the extracts of Helichrysum arenarium subsp. rubicundum, Helichrysum arenarium subsp. aucheri, and Helichrysum arenarium subsp. erzincanicum.

<table>
<thead>
<tr>
<th>Concentrations of Plant Extracts (mg/mL)</th>
<th>H. arenarium subsp. rubicundum</th>
<th>H. arenarium subsp. aucheri</th>
<th>H. arenarium subsp. erzincanicum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decoction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control *</td>
<td>1.270 ± 0.078</td>
<td>1.253 ± 0.060</td>
<td>1.354 ± 0.033</td>
</tr>
<tr>
<td>0.01</td>
<td>1.238 ± 0.081</td>
<td>1.238 ± 0.070</td>
<td>1.332 ± 0.047</td>
</tr>
<tr>
<td>0.05</td>
<td>1.236 ± 0.098</td>
<td>1.226 ± 0.067</td>
<td>1.337 ± 0.036</td>
</tr>
<tr>
<td>0.1</td>
<td>1.227 ± 0.100</td>
<td>1.231 ± 0.068</td>
<td>1.291 ± 0.036 *</td>
</tr>
<tr>
<td>0.5</td>
<td>1.218 ± 0.099</td>
<td>1.181 ± 0.063</td>
<td>1.125 ± 0.037 **</td>
</tr>
<tr>
<td>1</td>
<td>1.205 ± 0.100</td>
<td>1.176 ± 0.069</td>
<td>1.088 ± 0.045 **</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control *</td>
<td>1.379 ± 0.187</td>
<td>1.431 ± 0.281</td>
<td>1.379 ± 0.164</td>
</tr>
<tr>
<td>0.01</td>
<td>1.347 ± 0.190</td>
<td>1.412 ± 0.248</td>
<td>1.319 ± 0.169</td>
</tr>
<tr>
<td>0.05</td>
<td>1.327 ± 0.213</td>
<td>1.380 ± 0.253</td>
<td>1.324 ± 0.195</td>
</tr>
<tr>
<td>0.1</td>
<td>1.293 ± 0.189</td>
<td>1.363 ± 0.258</td>
<td>1.271 ± 0.124</td>
</tr>
<tr>
<td>0.5</td>
<td>1.285 ± 0.208</td>
<td>1.336 ± 0.235</td>
<td>1.118 ± 0.071 **</td>
</tr>
<tr>
<td>1</td>
<td>1.275 ± 0.202</td>
<td>1.333 ± 0.235</td>
<td>1.091 ± 0.069 **</td>
</tr>
</tbody>
</table>

* not AE or ME
** – significantly different from control (P < 0.01)
rugulosum has mutagenic effects. Moreover, it was reported in several other studies that *H. italicum* (Roth) D.Don does not have genotoxic effects (24-26). Elgorashi and van Staden (27) also reported that the methanol extracts of *H. simillimum* have mutagenic effects. Therefore, it is important to determine the genotoxic effects of *H. arenarium*. In the present study, 3 different parameters (MN, MI, and RI) were used to determine the genotoxic effects of AE and ME of *H. arenarium* subspecies.

There are many factors affecting the MN frequency in lymphocytes: age, gender, smoking, alcohol consumption, viral infections, and exposure to X-ray and gamma-ray (28). The donors chosen for this study did not smoke or consume alcohol. They had not been exposed to X-ray and gamma-ray and they did not have any viral infections. An increase in the MN may result from the interaction of a great variety of cytotoxic and genotoxic agents with chromosomal damage. The MN is an extremely valuable and a highly relevant endpoint for the detection of potential carcinogens. The results of this study show an increase in the percentage of MN (Table 1), suggesting a strong interaction between the extracts of *H. arenarium* subsp. erzincanicum and chromosomal damage. However, *H. arenarium* subsp. rubicundum and *H. arenarium* subsp. achari did not induce any changes in the MN frequencies.

In the present study, we found that *H. arenarium* subsp. erzincanicum induced the formation of MN, and decreased the MI and RI in human lymphocytes. *H. arenarium* subsp. rubicundum and *H. arenarium* subsp. achari, on the other hand, did not have any effects on these parameters. This is a firm indication that *H. arenarium* subsp. erzincanicum has genotoxic effects. Consequently, we are of the opinion that this species should not be used in high quantities by the public because of its genotoxic and cytotoxic properties. Indeed these plant extracts could cause chromosomal damage (an increase in the MN) and mitotic delay (a decrease in the MI and RI). The decrease in cell proliferation may indicate that *H. arenarium* subsp. erzincanicum also acts as an antimitotic and antineoplastic agent. It should therefore not be used freely in alternative medicine although its antiproliferative activity may suggest antimutagenic and antineoplastic properties. Additionally, these results show that *H. arenarium* subsp. rubicundum and *H. arenarium* subsp. achari have no genotoxic effects. Thus they can be used freely in alternative medicine. Further studies should be carried out to determine the effects of main bioactive components isolated from *H. arenarium* on the MN, MI, and RI.

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